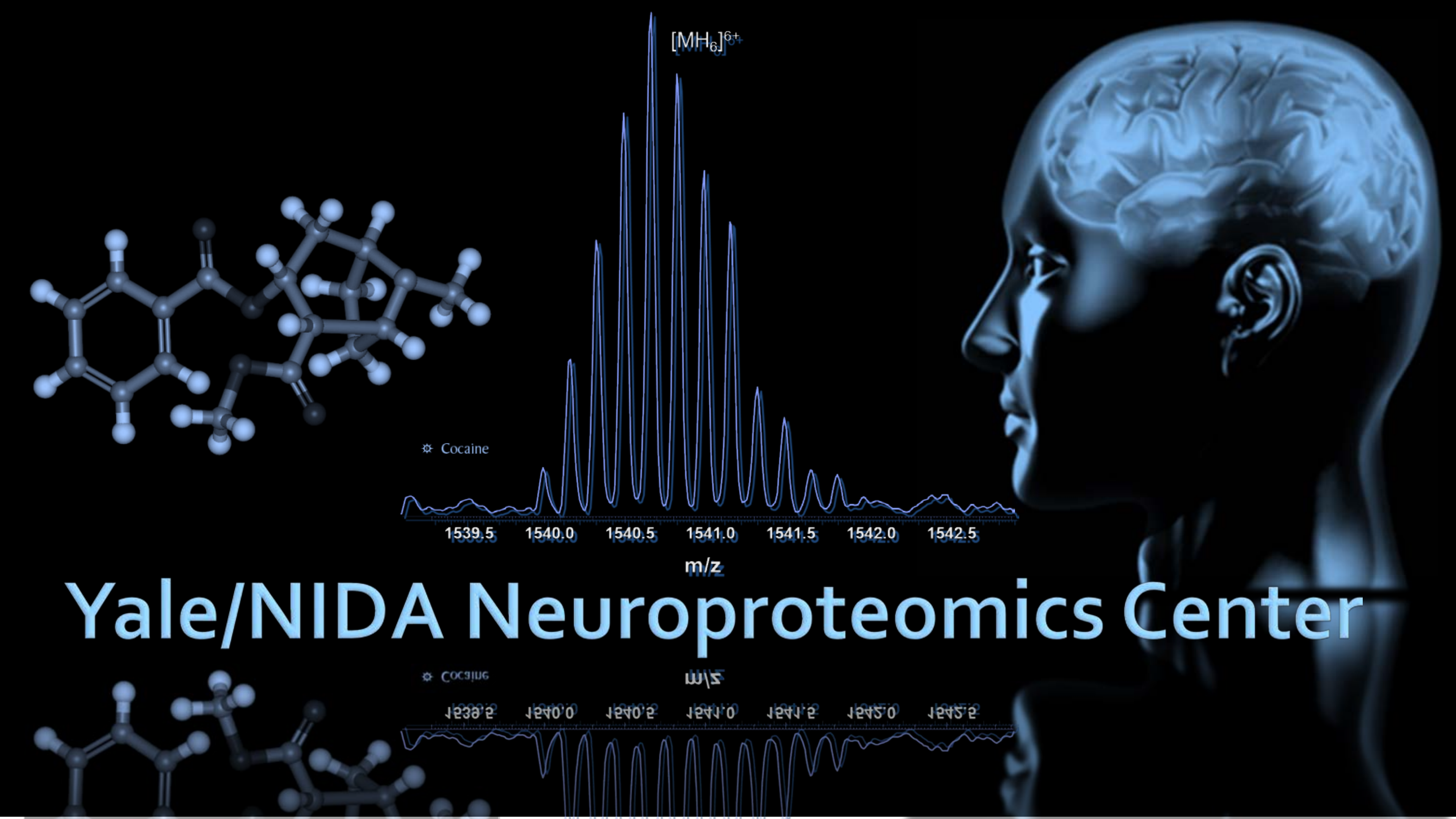


# Yale/NIDA Neuroproteomics Center

## Targeted Proteomics

Christopher M. Colangelo

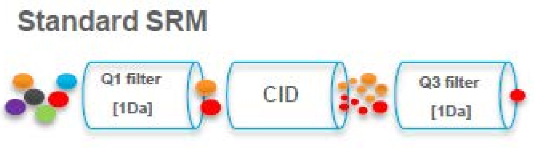
# Growth since 2009

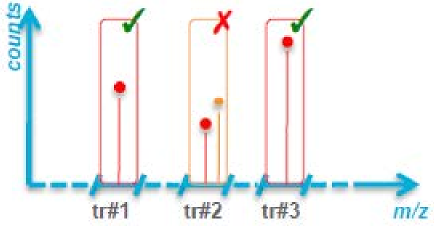
2010 – AB SCIEX 5500 QTRAP
1 S10 RR0296707-01, PI: C. Colangelo

2011 - AB SCIEX TripleTOF 5600
YSM+CTSA funding, 2012, R. Sherwin

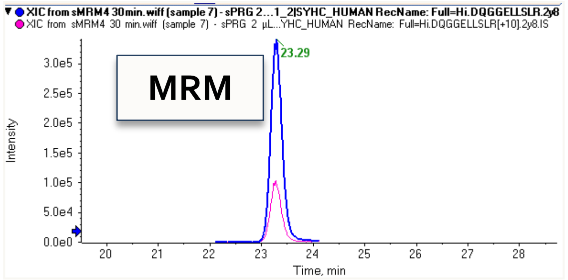
2013- AB SCIEX 6500 QTRAP
(Pending) S10, PI: C. Colangelo

# Two Modes of Accurate Peptide Quantitation MRM and SWATH

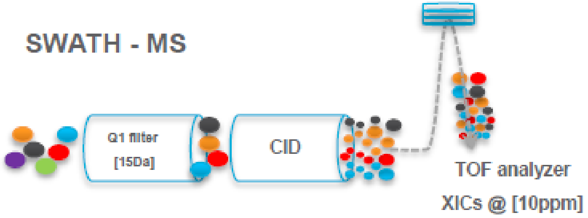


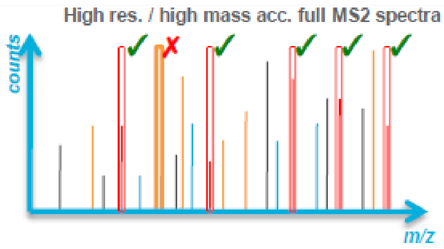


QTRAP® 5500 System

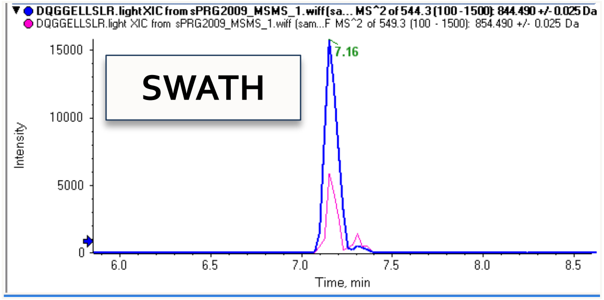


▼ ● XIC from sMRM4 30min.wiff (sample 7) - sPRG 2…1_2|SYHC_HUMAN RecName: Full=Hi.DQGGELLSLR.2y8
● XIC from sMRM4 30 min.wiff (sample 7) - sPRG 2 µL…YHC_HUMAN RecName: Full=Hi.DQGGELLSLR[+10].2y8.IS





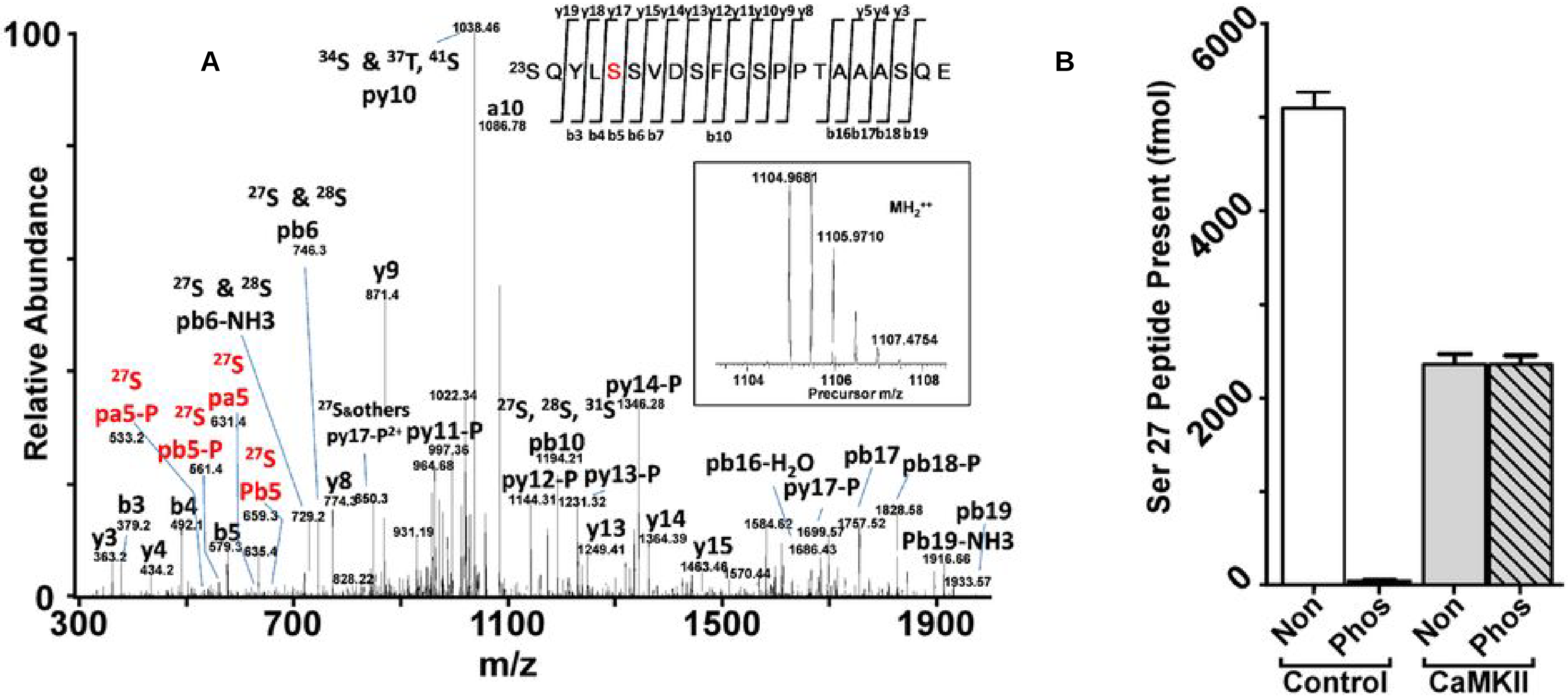
TripleTOF™ 5600 System



▼ ● DQGGELLSLR.light XIC from sPRG2009_MSMS_1.wiff (sa… MS^2 of 544.3 (100 - 1500): 844.490 +/- 0.025 Da
● DQGGELLSLR.light XIC from sPRG2009_MSMS_1.wiff (sam…F MS^2 of 549.3 (100 - 1500): 854.490 +/- 0.025 Da

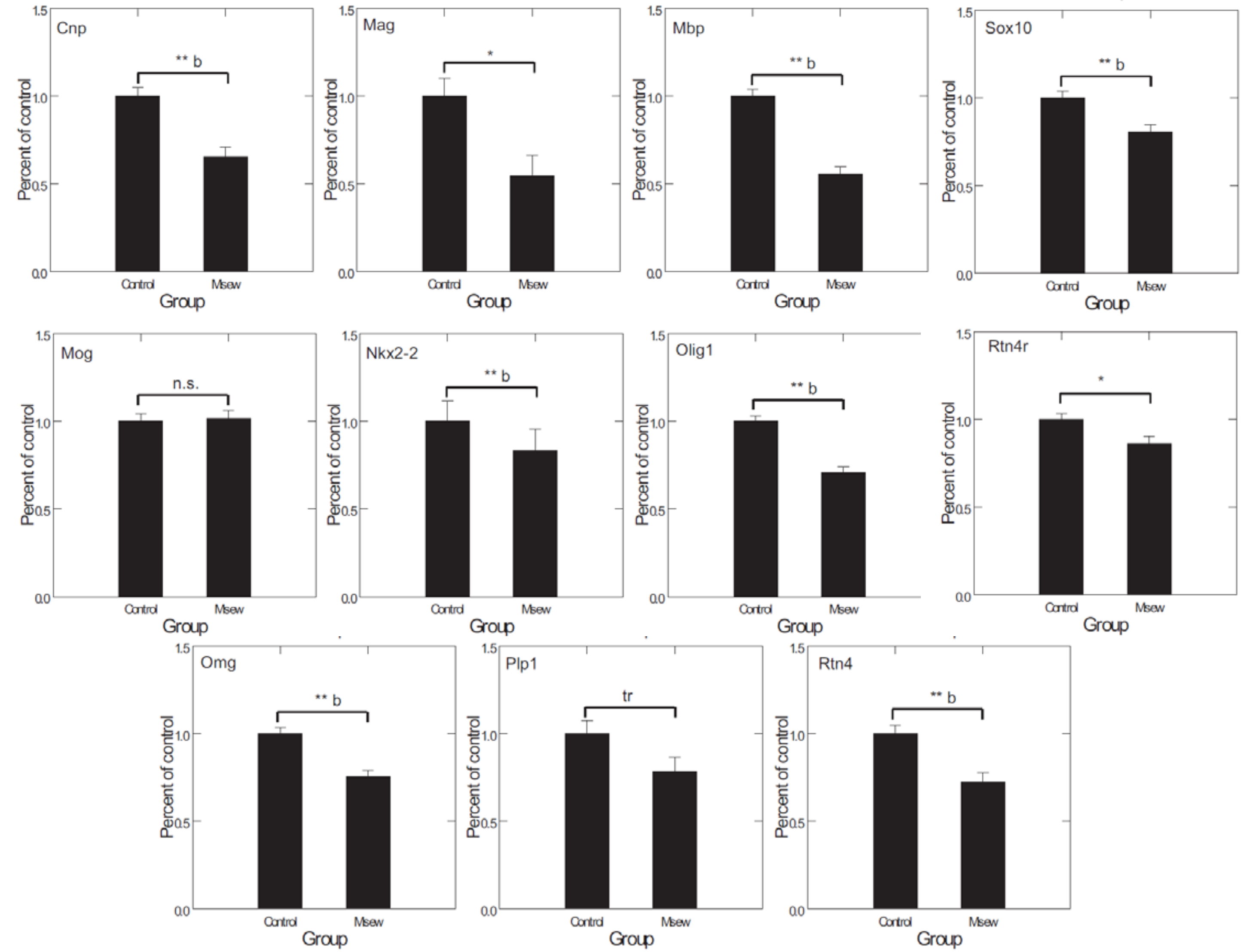
*Courtesy of Ludovic Gillet, ETH*

# MRM analysis of FosB phosphorylated in vitro by CaMKII reveals that Ser27 is a potent CaMKII substrate

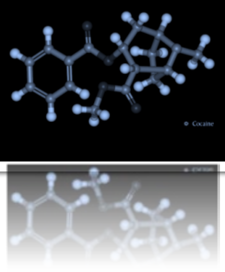


Robison A.J et. al. (2013) Behavioral and Structural Responses to Chronic Cocaine Require a Feedforward Loop Involving ΔFosB and Calcium/Calmodulin-Dependent Protein Kinase II in the Nucleus Accumbens Shell. J. Neurosci., 33(10)4295-4307

# MRM Confirmation on MESW Mice Model

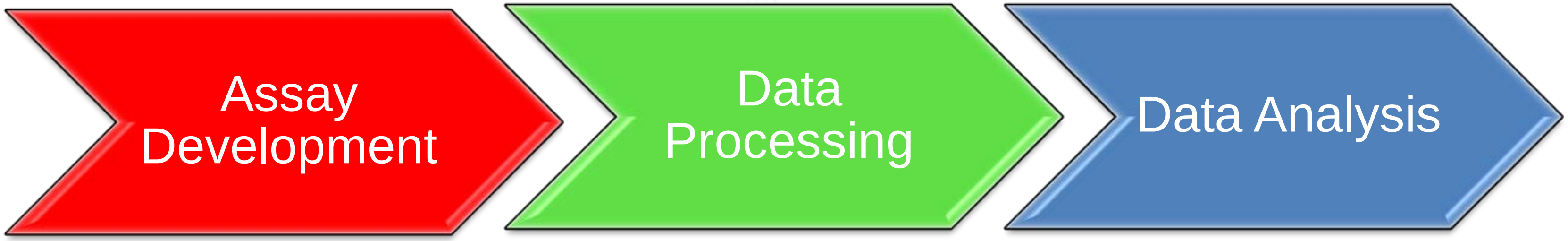


Bordner, K.A. et. al. (2011) Functional genomic and proteomic analysis reveals disruption of myelin related genes and translation in a mouse model of early life neglect. Front Psychiatry. 2:18.



# Yale Targeted Proteomics Pipeline

(MRM and MS/MS$^{all}$ with SWATH Acquisition (SWATH))



**Assay Development**

Yale Protein Expression Database (YPED)

1 Protein Identification
2. Spectral Library
3. MRM Transitions
4. Data Collection
sMRM/SWATH

**Data Processing**

Multiquant – MRM
Skyline - MRM
Peakview – SWATH
Skyline - SWATH

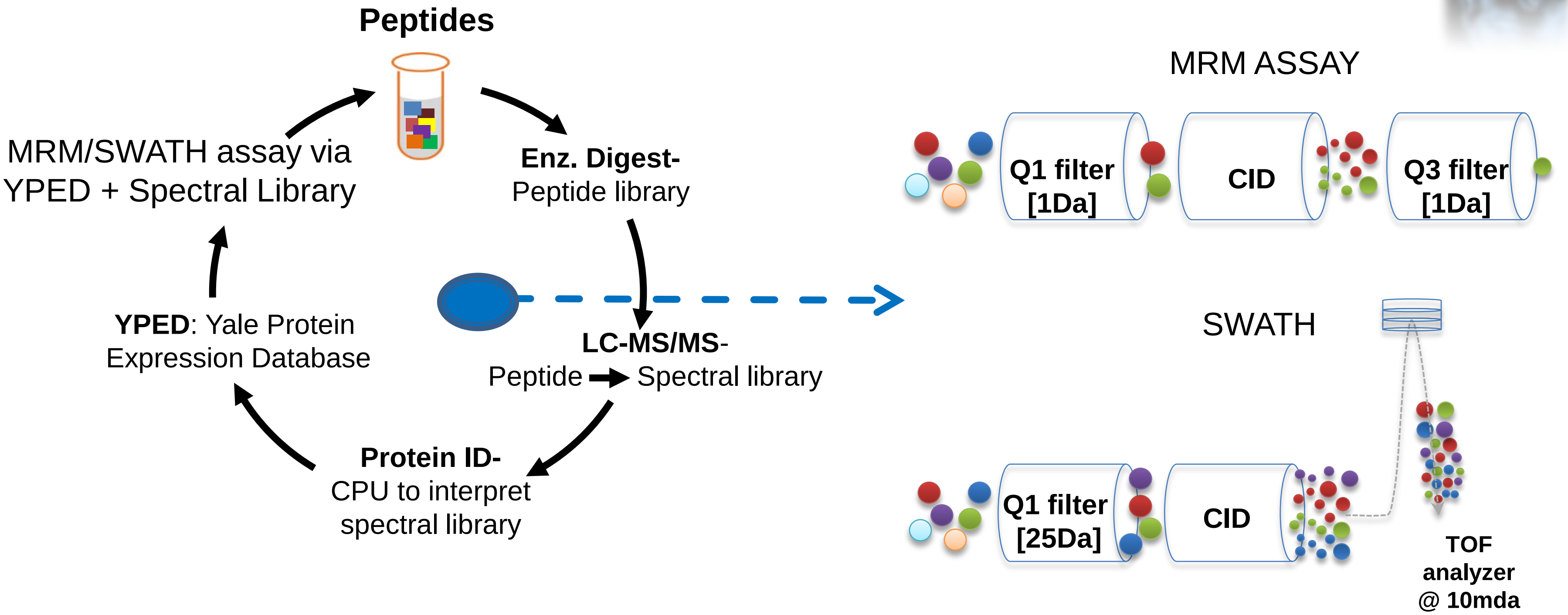
1. Peak Integration
2. Data Metrics
3. Confidence Values

**Data Analysis**

R-scripts and Matlab

1 Normalization
2. Cluster Dendrograms
3. PCA analysis
4. Correlation plots
5. Fold change analysis

# MRM and SWATH Assay Development



**Peptides**

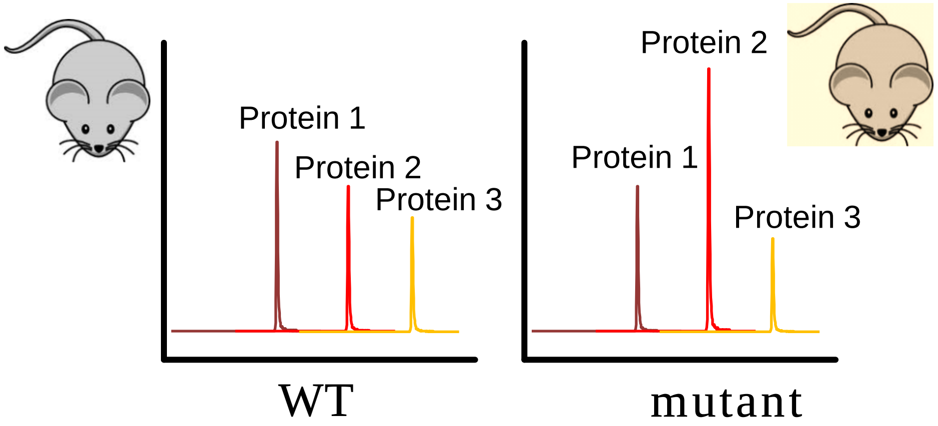
**Enz. Digest-** Peptide library

**LC-MS/MS-** Peptide → Spectral library

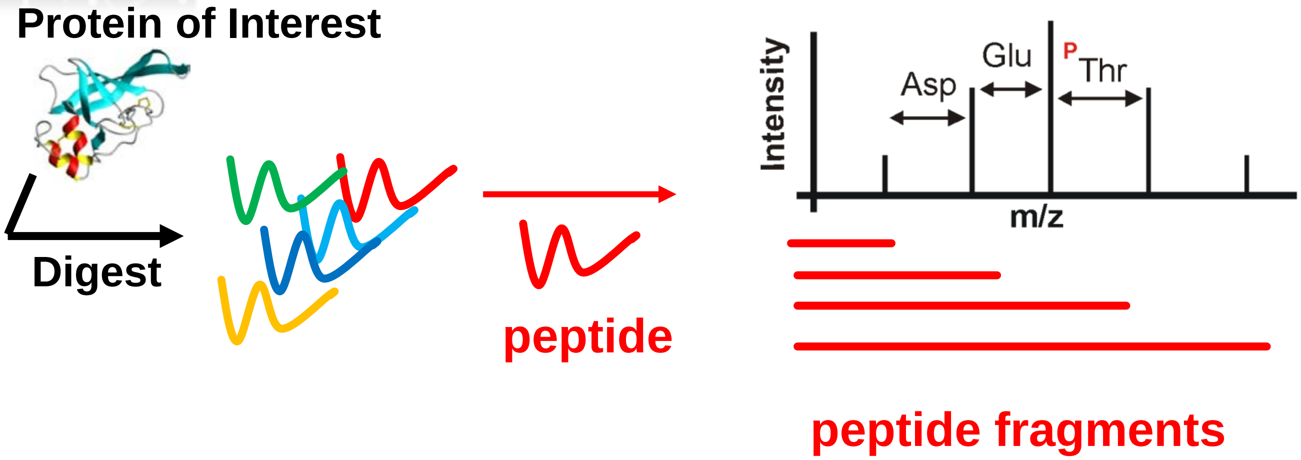
**Protein ID-** CPU to interpret spectral library

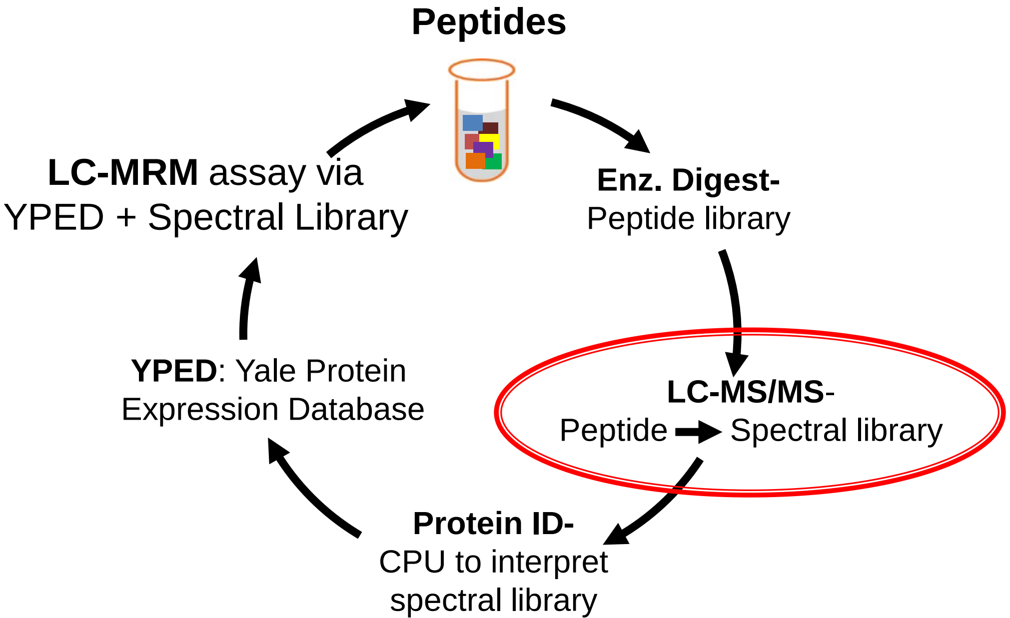
**YPED**: Yale Protein Expression Database

MRM/SWATH assay via YPED + Spectral Library



# Choosing the Best Peptide Spectra





**Peptides**

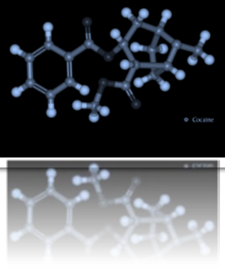
**Enz. Digest-**
Peptide library

**LC-MS/MS-**
Peptide → Spectral library

**Protein ID-**
CPU to interpret
spectral library

**YPED**: Yale Protein
Expression Database

**LC-MRM** assay via
YPED + Spectral Library



# YPED Spectral Library

| | |
|---|---|
| Users | 1,313 |
| Datasets | 15,142 |
| Unique LC-MS Proteins | 626,695 |
| Distinct LC-MS Peptide Identifications | 3,008,435 |

| Organism | Blast Protein Count | Blast Peptide Count |
|---|---|---|
| E.Coli | 3,970 | 41,760 |
| Yeast | 5,684 | 54,948 |
| Rat | 11,962 | 122,322 |
| Mouse | 20,059 | 214,905 |
| Human | 20,843 | 243,749 |

# Initial Project Goals for LC-MRM

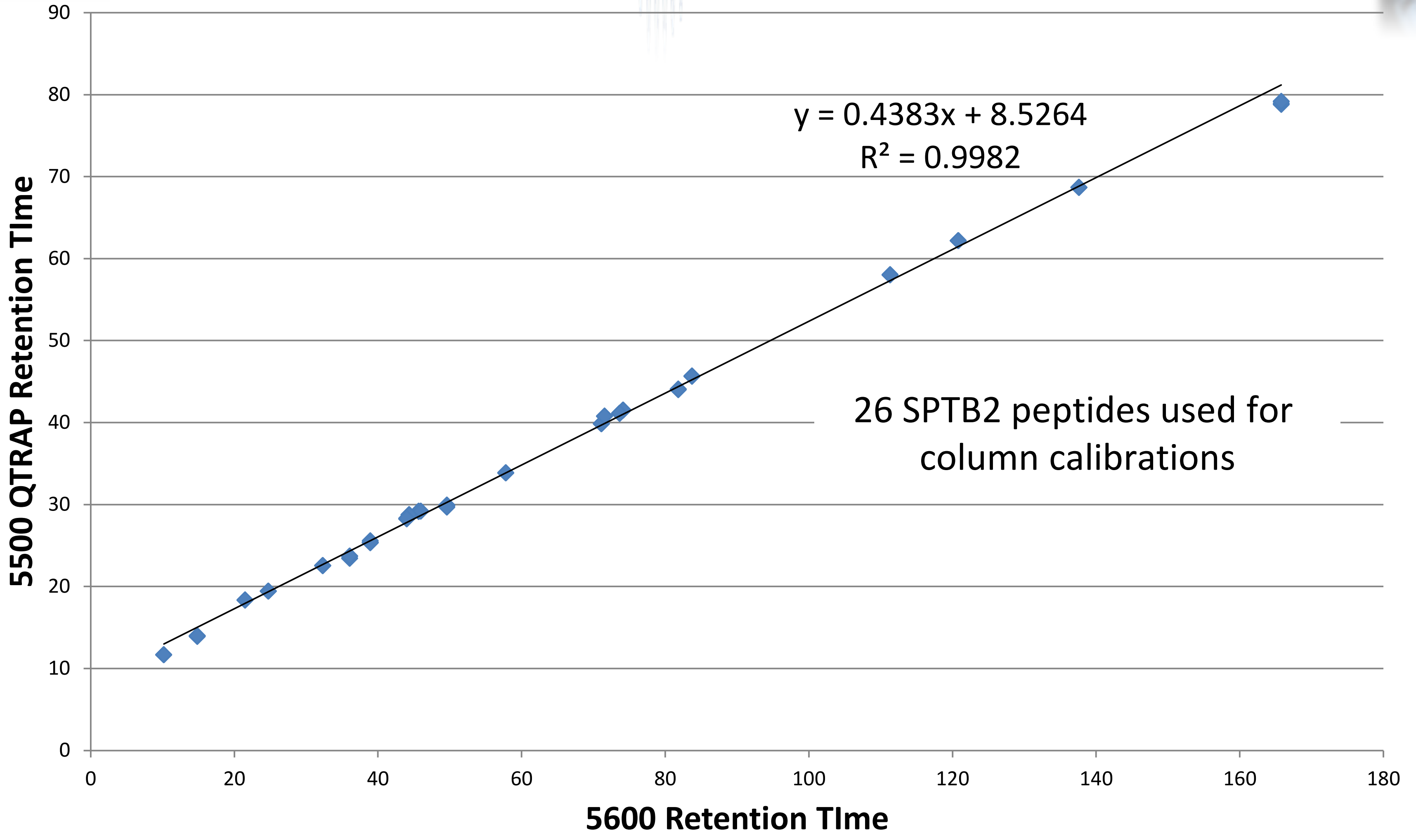
Develop a robust, routine, 90 minute, scheduled LC-MRM assays to relatively quantify 100 proteins with 3 peptides per protein and 5 transitions/peptide (1500 data points).

**A typical LC-MRM assay details**

**Protein sample that will be used in "production mode"**

| | Total Number |
|---|---|
| **Proteins** | 100 |
| **Peptides/protein** | 3 |
| **Transitions/peptide** | 5 |
| **LC-MRM Replicates** | 3 |
| **Total data points** | 4500 |

# Discovery to MRM Column Calibration



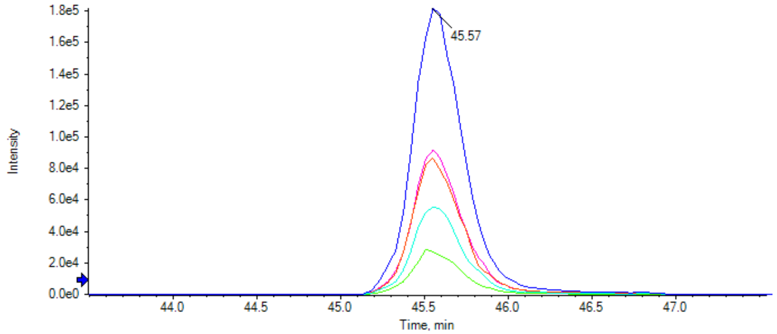
26 SPTB2 peptides used for column calibrations

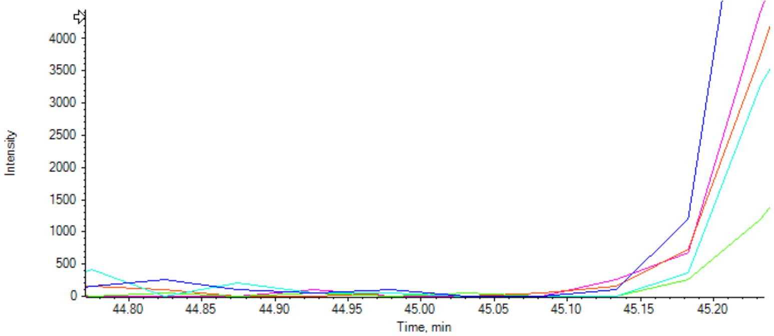
# Improvements to Scheduled MRM

- Benefits
  - Tolerant to shifts in RT
  - Improved cycle and dwell times
  - Reduces irrelevant data (improves data quality)
  - Improves data quality for low S:N compounds
- Features
  - **Variable windows**
  - **Window extension (timeslip)**
  - **MRM triggered MRM (xMRM)**
  - Group triggered IDA
  - Dwell weighting

# Scheduled vs. Triggered MRM

## Normal Scheduled MRM

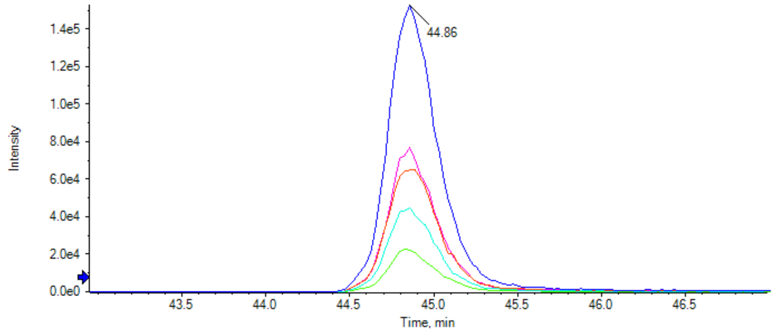

XIC from 5500Q11-1880.wiff (sample 1) - Sakaue_PSDCorte...WSR_2_KCC2A_MOUSE, Gaussian smoothed, Gaussian smoothed

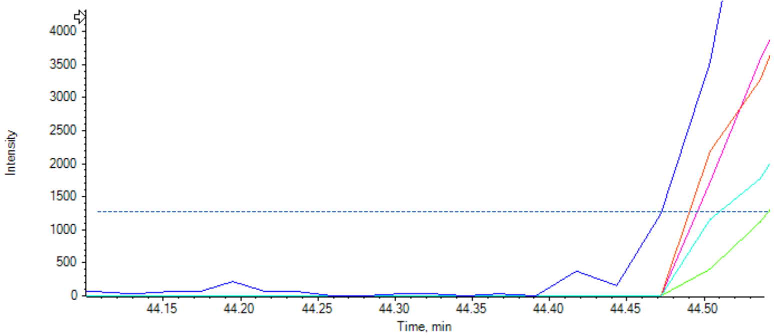

XIC from 5500Q11-1880.wiff (sample 1) - Sakaue_PSDCortex..., +MRM (1442 transitions): y(7)_FYFENLWSR_2_KCC2A_MOUSE

dwell time = 19.23 msec

## Scheduled MRM triggered MRM (xMRM)

- secondary MRM are only monitored when the primary MRM goes above a threshold


XIC from 5500Q11-1850.wiff (sample 1) - Sakaue cortex PS...WSR_2_KCC2A_MOUSE, Gaussian smoothed, Gaussian smoothed


XIC from 5500Q11-1850.wiff (sample 1) - Sakaue cortex PS...2, +MRM (1442 transitions): y(7)_FYFENLWSR_2_KCC2A_MOUSE

dwell time = 32.25 msec

# Rat Brain Post Synaptic Density

Total of 112 proteins
3 peptides/proteins
5 transitions/peptide
Total number of transitions are 1697

Protein List

ADT1 AT1A3 ATPA ATPB BAIP2 BASP1 BEGIN BSN CI172 CLH CMC1
CMTD1 CNTN1 COX5A CTNA2 CTRO DLG2 DLG3 DLG4 DLGP1 DLGP2 DLGP3
DLGP4 DPYL2 DYN1 ERC2 FLOT2 GD1L1 GDAP1 GLPK GNAZ HSP7C
HXK1 IMMT IQEC1 IQEC2 KCC2A KCC2B KCC2D KCC2G KCD16 KIF2A LIPA2 LRRC7
M2OM MBP MPCP MTCH1 MYH10 MYO5A MYPR NDUA7 NDUA8 NDUA9 NDUAC
NDUS1 NDUS2 NDUS3 NDUS7 NDUS8 NDUV1 NDUV2 NEUM NFL NFM NMDE2 NMDZ1
NRX1A NSF ODO1 ODP2 ODPB OPA1 PHB PHB2 PKP4 PLEC PP1A QCR1 QCR2
RAB35 RIMS1 RL9 RPGF4 SEPT8 SFXN3 SHAN2 SHAN3 SHSA7 SPTA2 SPTB2 SRCN1
STX1A STX1B STXB1 SYN2 SYNPO SYT1 TBA1B TBB2C TBB3 TBB5 TENR TOM40
TOM70 VA0D1 VAPA VATB2 VDAC1 VDAC2 VISL1 VPP1

# Rat Brain Cortex

PSD 1 PSD2 PSD3 PSD4 PSD5 PSD6

↓ Targeted or Data-Independent Analysis

**MRM Data Collection**

Calibrate Retention Time and Schedule Transitions

Data Acquisition

1. MRM
2. sMRM
3. triggered MRM

**Data Analysis**

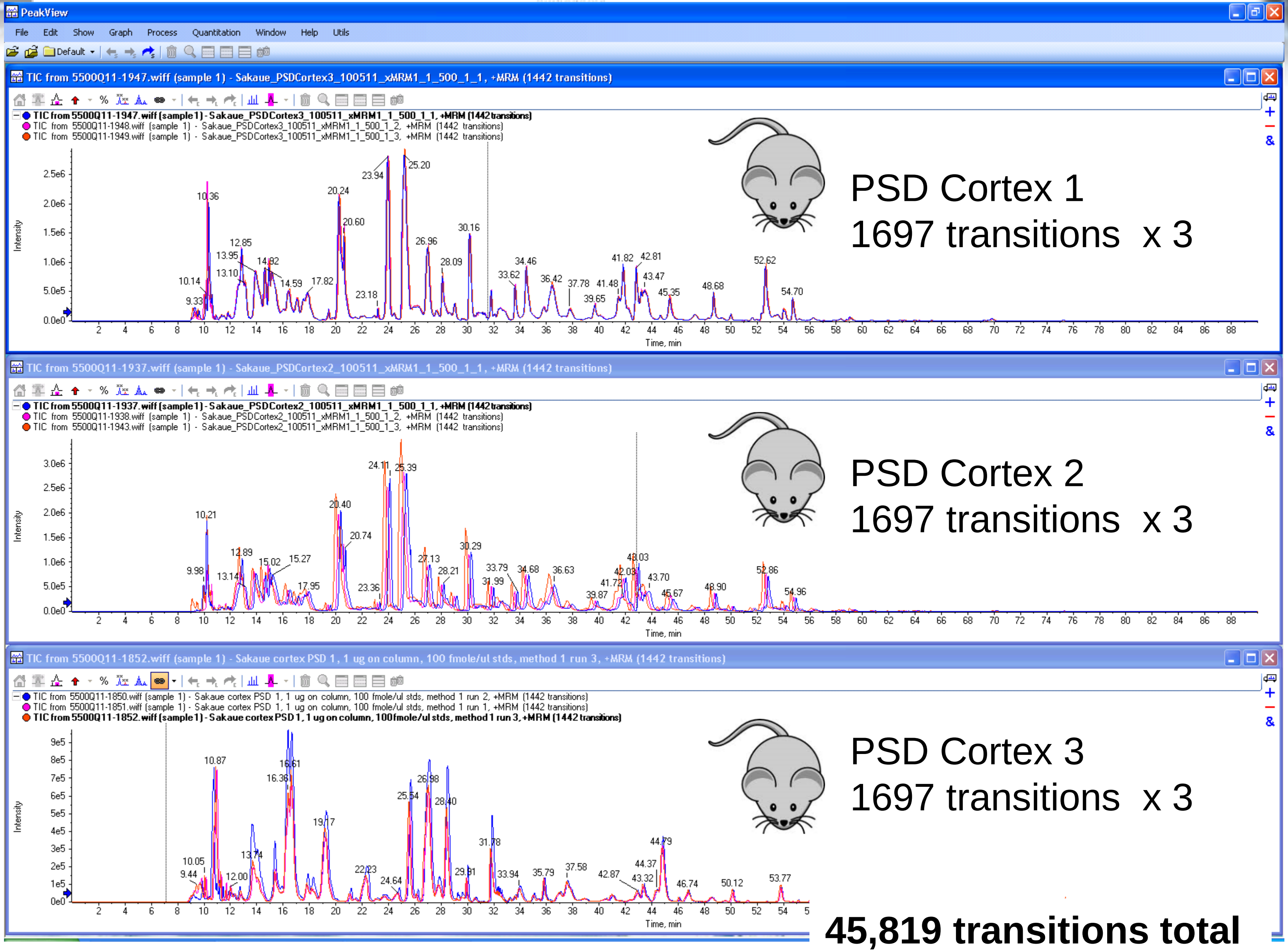
Peak Picking and Integration

Quality Assessment Analysis

Fold change analysis and Statistical Inference

Data Repository

# TIC of PSD Cortex sample 1,2,3 each in triplicate



TIC from 5500Q11-1947.wiff (sample 1) - Sakaue_PSDCortex3_100511_xMRM1_1_500_1_1, +MRM (1442 transitions)

● **TIC from 5500Q11-1947.wiff (sample 1) - Sakaue_PSDCortex3_100511_xMRM1_1_500_1_1, +MRM (1442 transitions)**
● TIC from 5500Q11-1948.wiff (sample 1) - Sakaue_PSDCortex3_100511_xMRM1_1_500_1_2, +MRM (1442 transitions)
● TIC from 5500Q11-1949.wiff (sample 1) - Sakaue_PSDCortex3_100511_xMRM1_1_500_1_3, +MRM (1442 transitions)

PSD Cortex 1
1697 transitions x 3

TIC from 5500Q11-1937.wiff (sample 1) - Sakaue_PSDCortex2_100511_xMRM1_1_500_1_1, +MRM (1442 transitions)

● **TIC from 5500Q11-1937.wiff (sample 1) - Sakaue_PSDCortex2_100511_xMRM1_1_500_1_1, +MRM (1442 transitions)**
● TIC from 5500Q11-1938.wiff (sample 1) - Sakaue_PSDCortex2_100511_xMRM1_1_500_1_2, +MRM (1442 transitions)
● TIC from 5500Q11-1943.wiff (sample 1) - Sakaue_PSDCortex2_100511_xMRM1_1_500_1_3, +MRM (1442 transitions)

PSD Cortex 2
1697 transitions x 3

TIC from 5500Q11-1852.wiff (sample 1) - Sakaue cortex PSD 1, 1 ug on column, 100 fmole/ul stds, method 1 run 3, +MRM (1442 transitions)

● TIC from 5500Q11-1850.wiff (sample 1) - Sakaue cortex PSD 1, 1 ug on column, 100 fmole/ul stds, method 1 run 2, +MRM (1442 transitions)
● TIC from 5500Q11-1851.wiff (sample 1) - Sakaue cortex PSD 1, 1 ug on column, 100 fmole/ul stds, method 1 run 1, +MRM (1442 transitions)
● **TIC from 5500Q11-1852.wiff (sample 1) - Sakaue cortex PSD 1, 1 ug on column, 100 fmole/ul stds, method 1 run 3, +MRM (1442 transitions)**

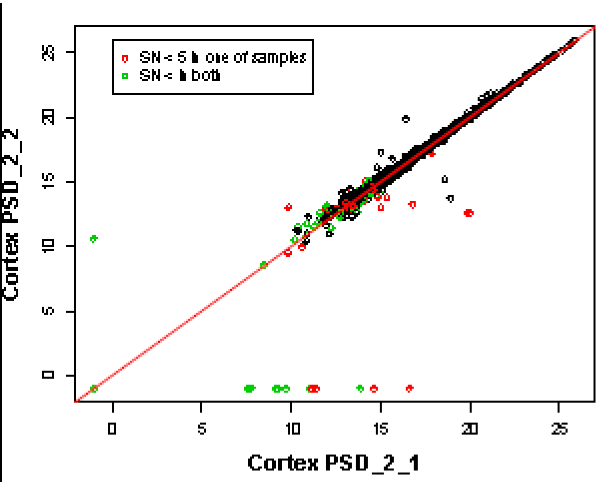
PSD Cortex 3
1697 transitions x 3

**45,819 transitions total**

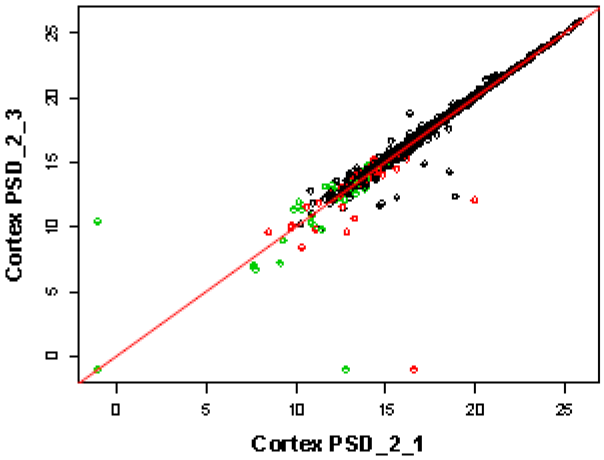
# Scatter Plots and Venn diagrams

## PSD Cortex 2

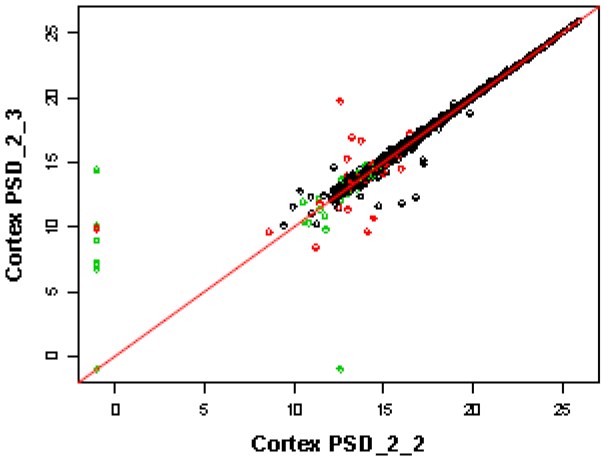
Log2 (peak area), cor =0.93



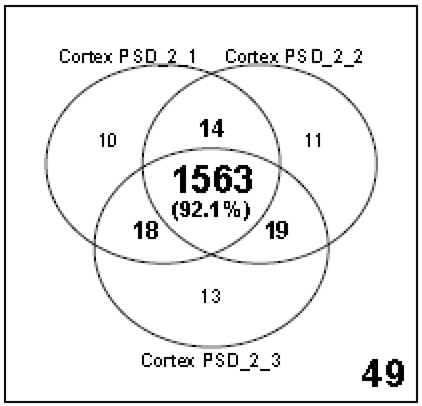
Log2 (peak area), cor =0.97



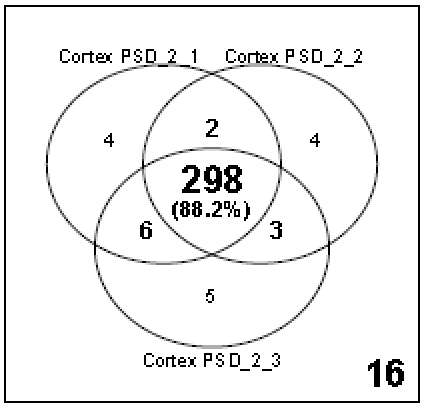
Log2 (peak area), cor =0.94



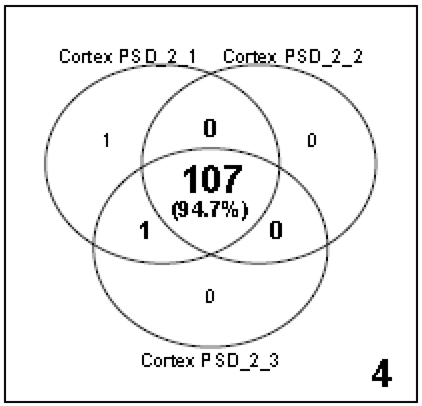
transition-level



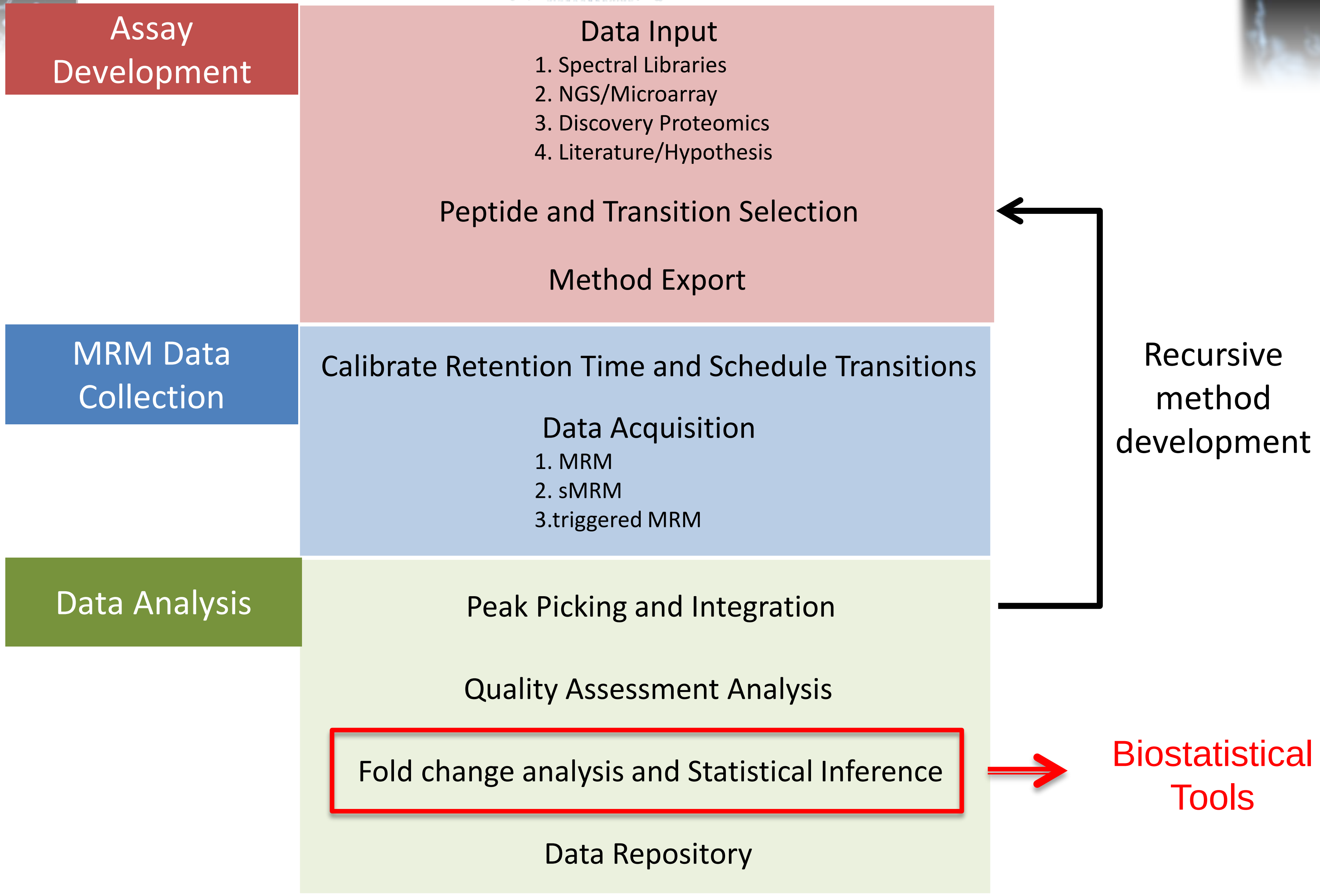
peptide-level



protein-level



# Targeted Proteomics Workflow



**Assay Development**

Data Input
1. Spectral Libraries
2. NGS/Microarray
3. Discovery Proteomics
4. Literature/Hypothesis

Peptide and Transition Selection

Method Export

**MRM Data Collection**

Calibrate Retention Time and Schedule Transitions

Data Acquisition
1. MRM
2. sMRM
3.triggered MRM

**Data Analysis**

Peak Picking and Integration

Quality Assessment Analysis

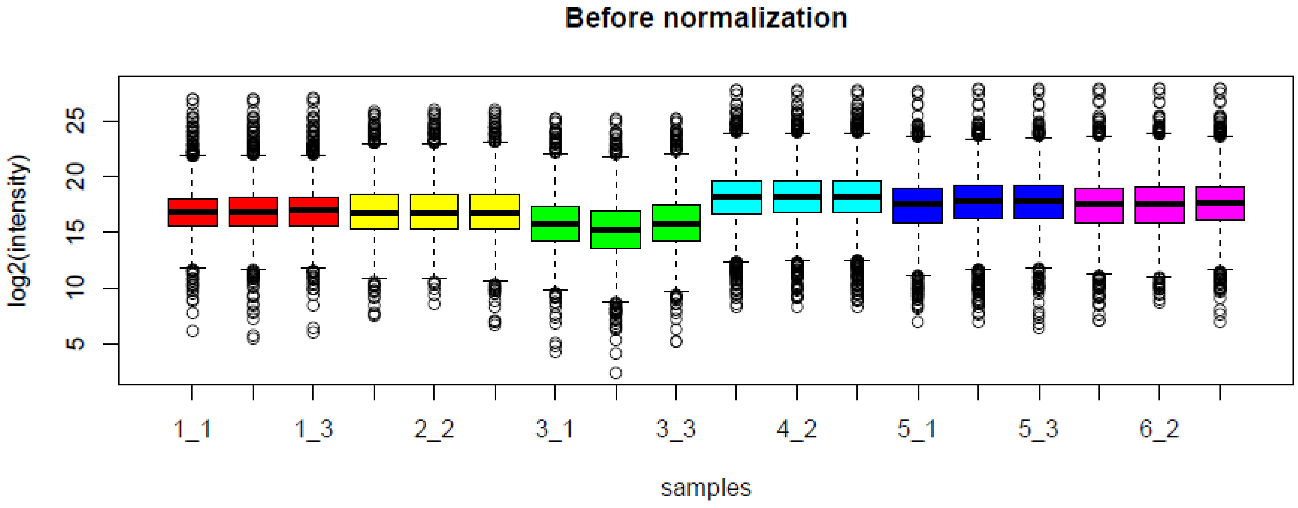
Fold change analysis and Statistical Inference

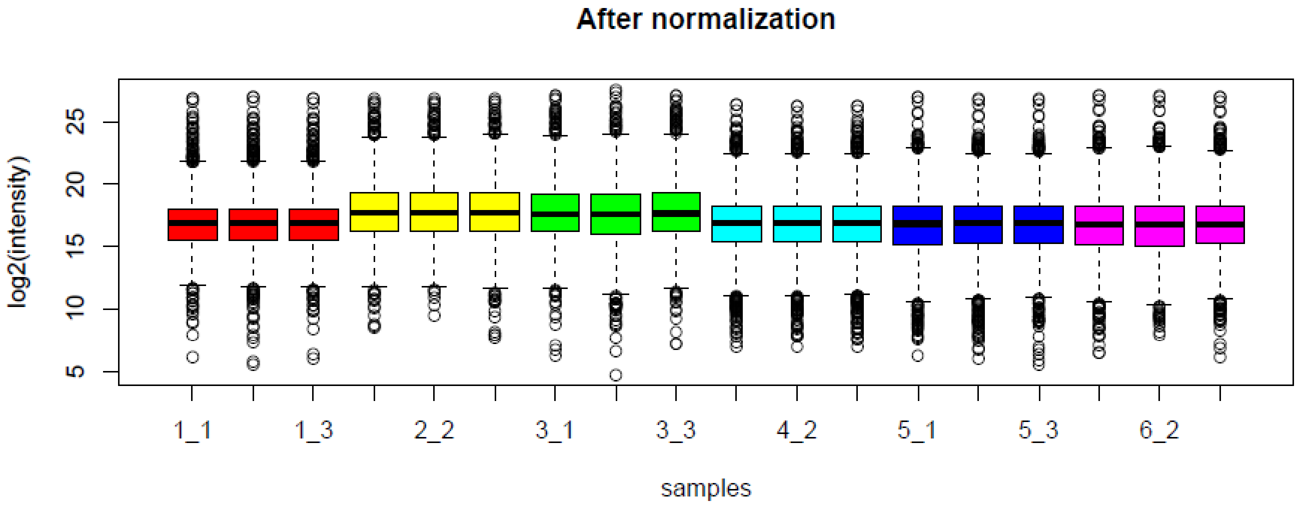
Data Repository

Recursive method development

Biostatistical Tools

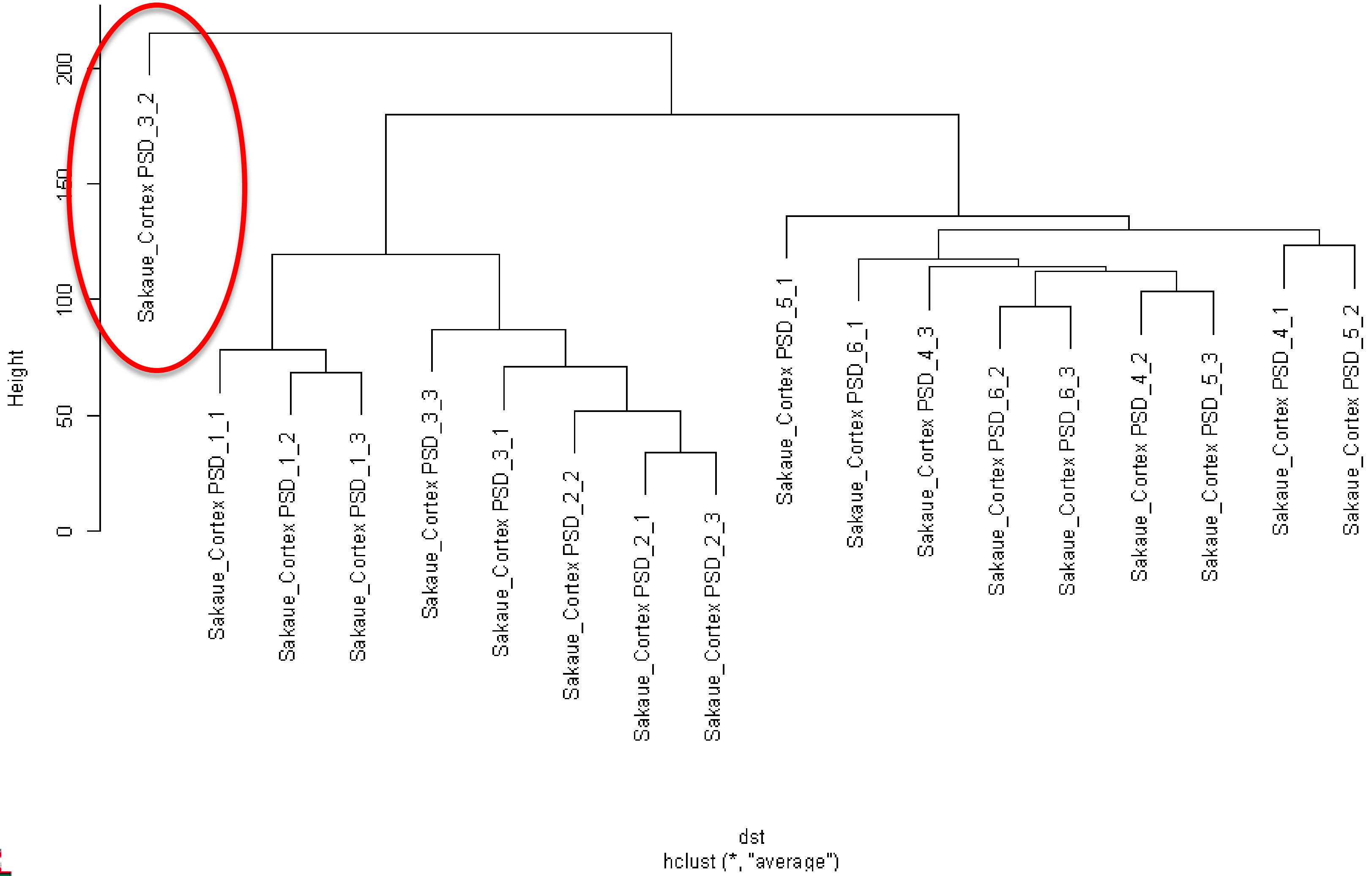
# Trimmed Median


Before normalization

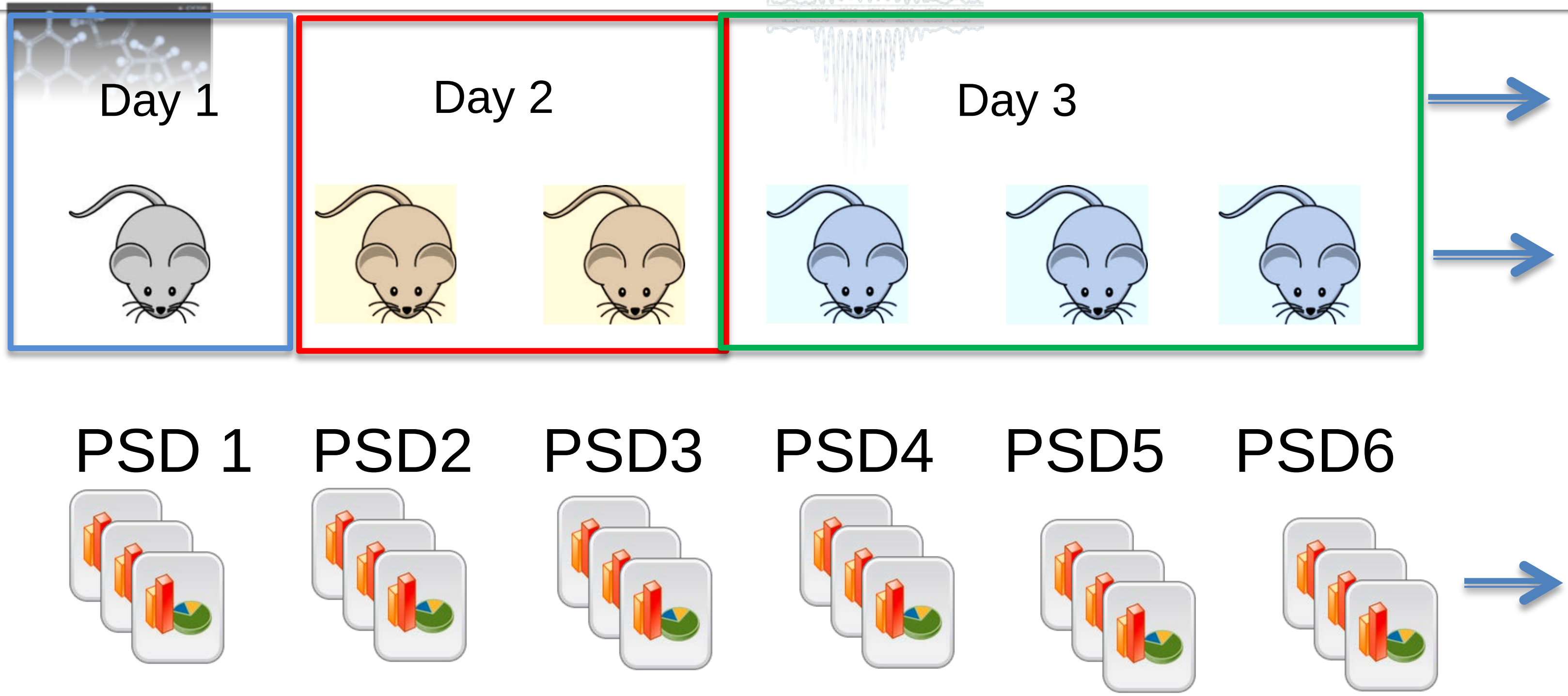

After normalization

Trimmed median adjustment makes the median “expect mitochondrial proteins” equal across samples. The box plot shows the overall distribution, including the mitochondrial proteins.

# Hierarchical Clustering



# Statistical Inference on Fold Change for Rat Brain Cortex



Day 1

Day 2

Day 3

→ Day Effect

→ Biological Variation

PSD 1 PSD2 PSD3 PSD4 PSD5 PSD6

→ Technical Variation

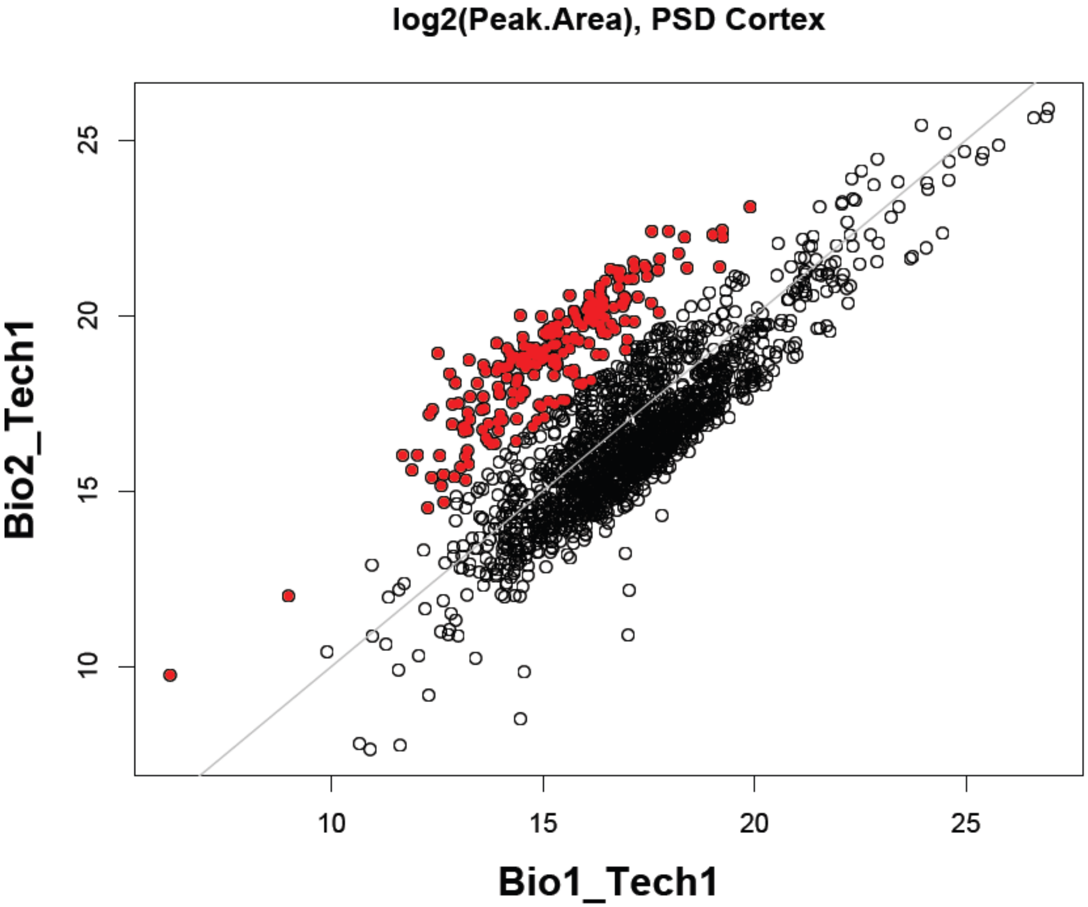
Day 1 vs. Day 2

Day 2 vs. Day 3

Evidence: Day 1 and Day 3 Rat were Ultracentrifuged immediately

Day 2 Rats were left on ice for 5 hours prior to spinning in Ultracentrifuge

# Mitochondrial Proteins are > 4-fold greater in PSD Cortex 2 vs. PSD Cortex 1

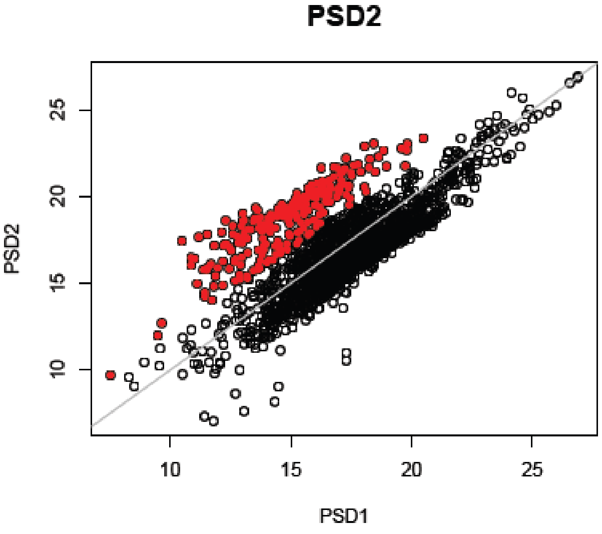


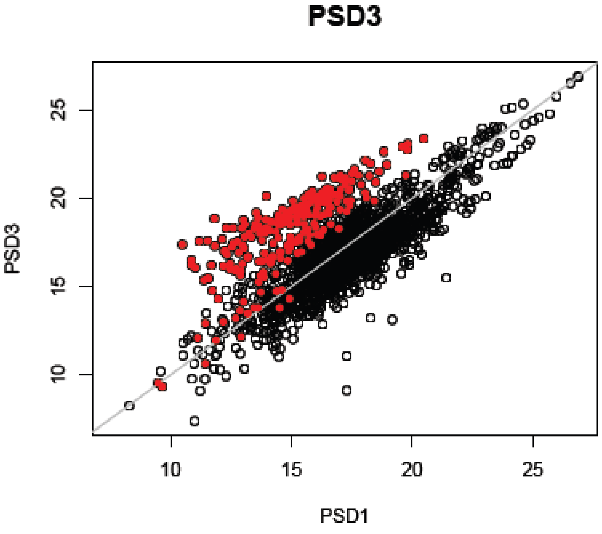
| Protein | Transitions |
|---|---|
| NDUA9 | 15 |
| NDUS3 | 15 |
| NDUS8 | 15 |
| QCR1 | 15 |
| QCR2 | 15 |
| NDUA7 | 14 |
| NDUA8 | 14 |
| NDUS1 | 14 |
| NDUS2 | 14 |
| NDUV1 | 14 |
| NDUV2 | 14 |
| COX5A | 13 |
| NDUAC | 10 |
| SFXN3 | 10 |
| M2OM | 9 |
| NDUS7 | 8 |

Grey line is 45 degree, diagonal line.
Red dots indicate 218 transitions with over 4 fold-up in the second biological replicates

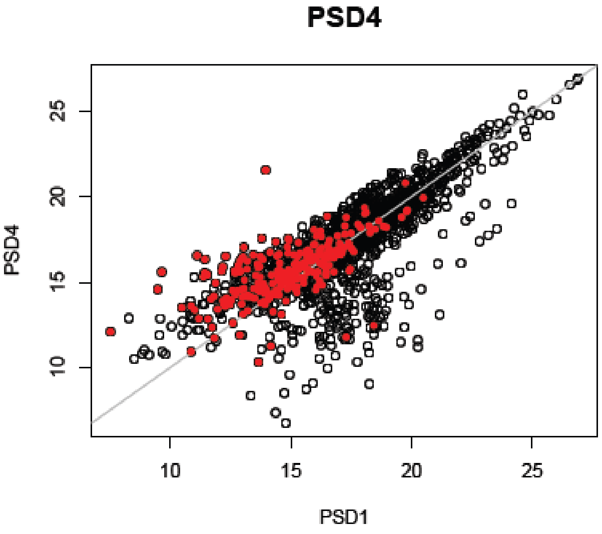
This could be due to sample preparation, but also could be due to mitochondrial fragmentation which has been previous linked to (Knott et. al Nature Reviews 2008)

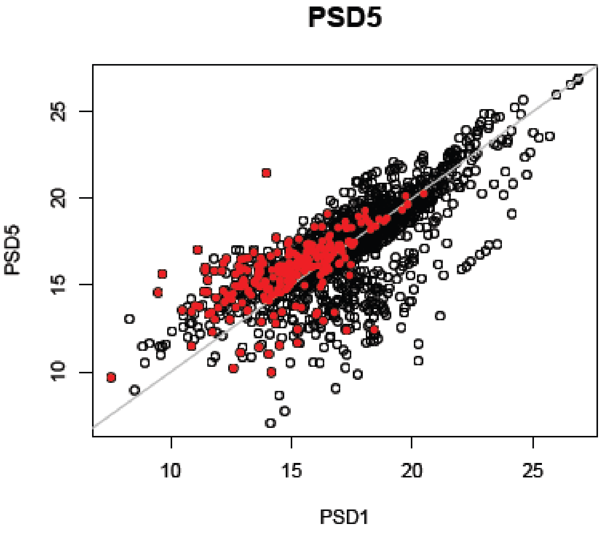
# Mitochondrial Proteins are > 4-fold greater in both PSD Cortex 2 and 3 compared to PSD Cortex 1

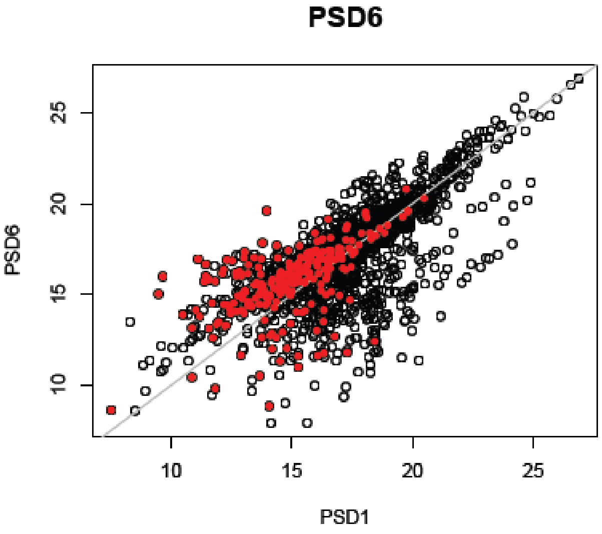




Red dots indicate 218 transitions with over 4 fold-up in the second and third biological replicates

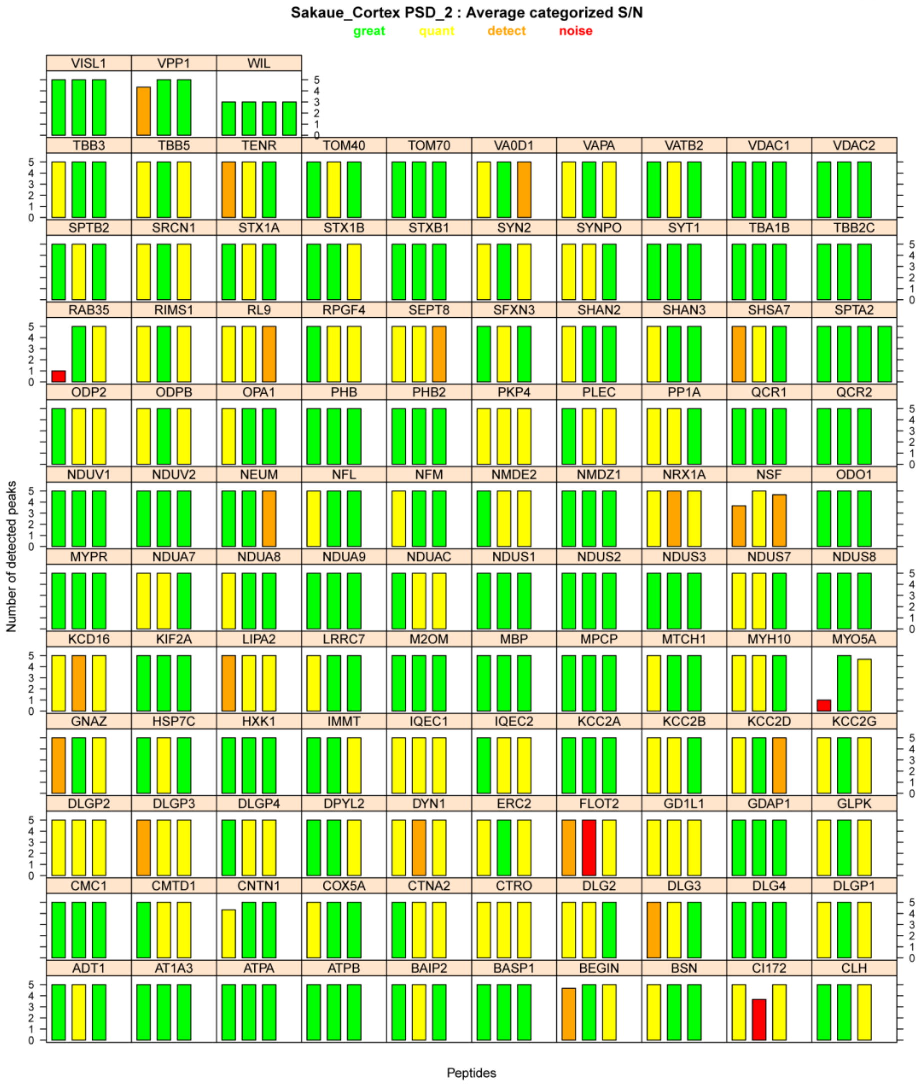






# Bar Charts for Data Visualization

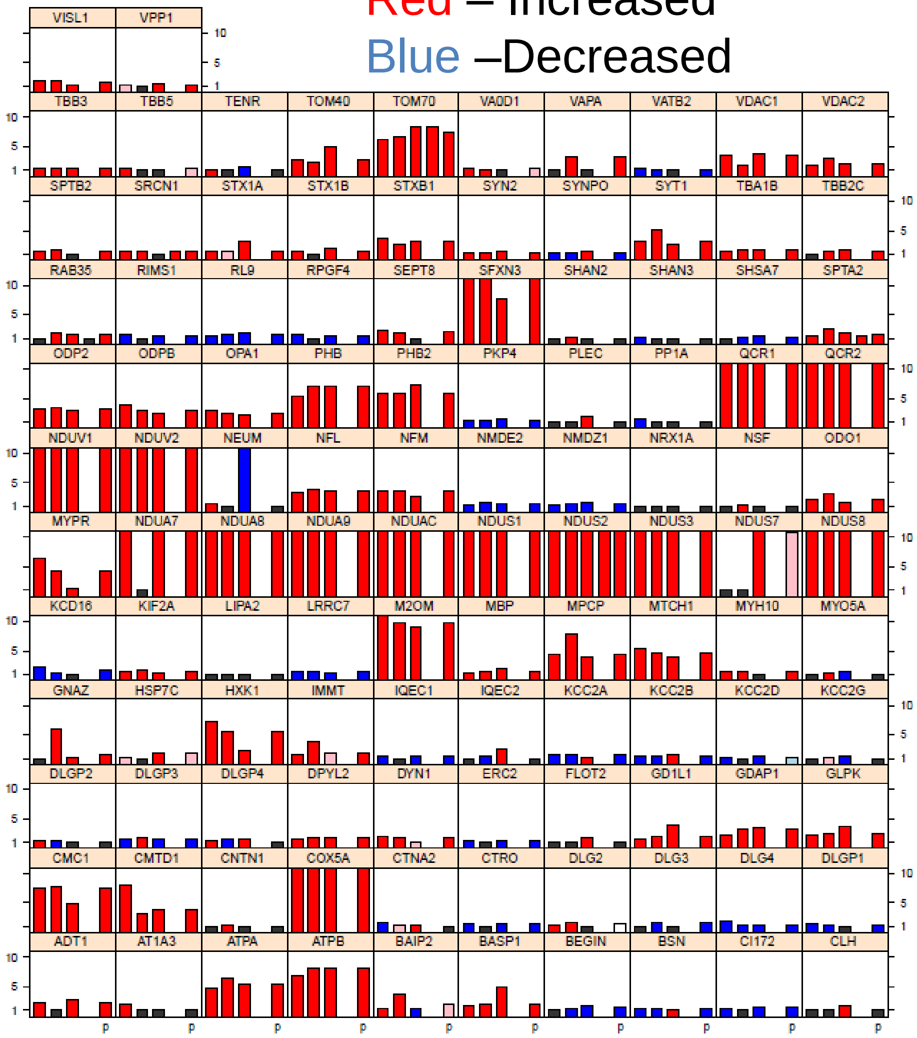
## Signal/Noise



## Fold-Change

Red – Increased

Blue –Decreased



# SWATH Data Extraction

Input


ProteinPilot™

Protein Identification and Labeled Quantitation

AB SCIEX

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MATRIX SCIENCE

Yale Protein Expression Database (YPED)

Ion Library Text format


PeakView™

Qualitative Processing, Review, and Comparison Software

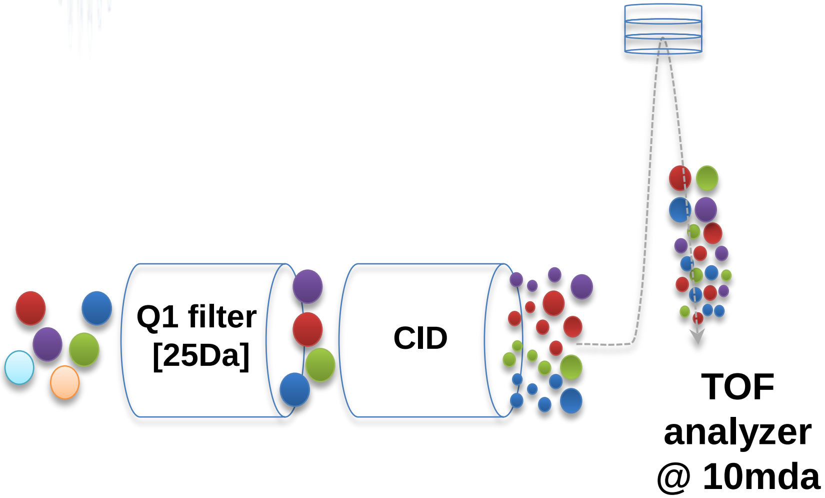
AB SCIEX

©2011 AB SCIEX. All rights reserved.

Skyline

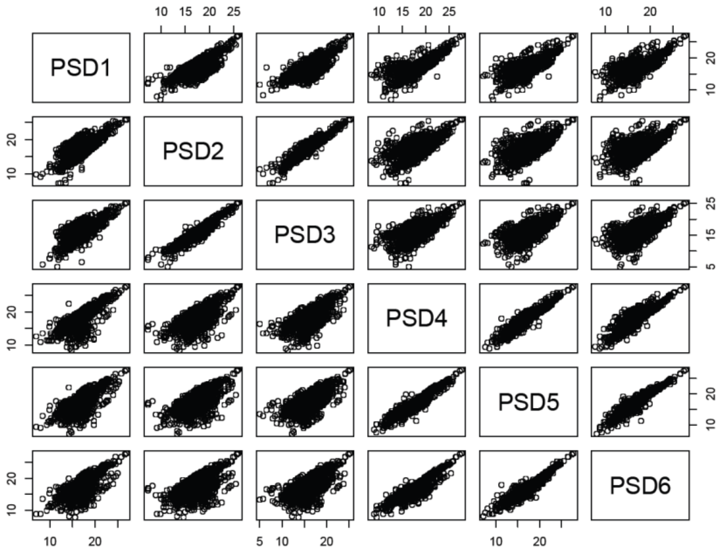
Text export of peak areas

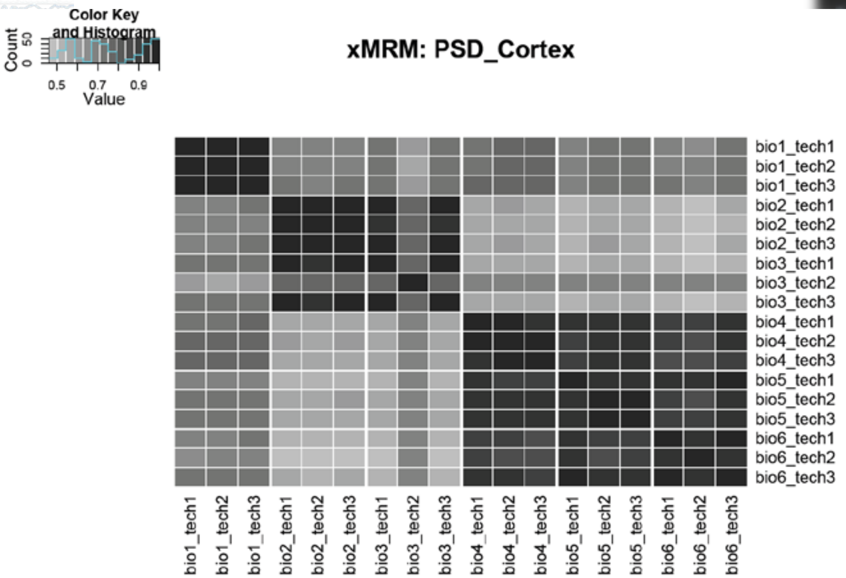
Output



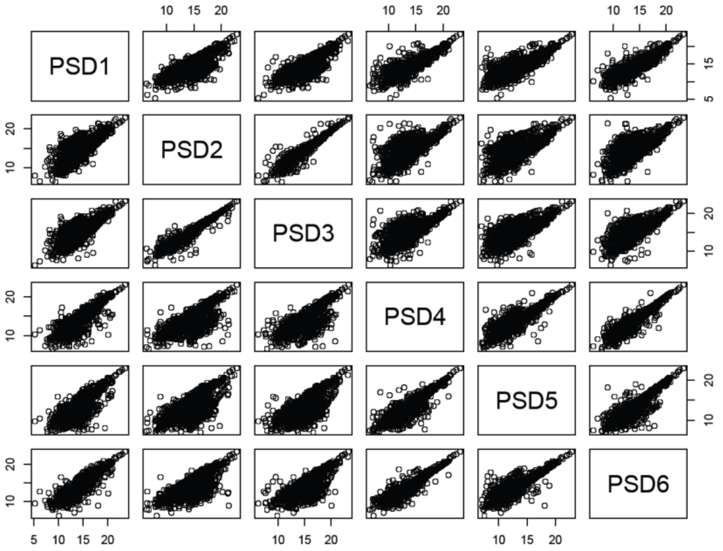
# xMRM and SWATH Correlation between Biological Replicates

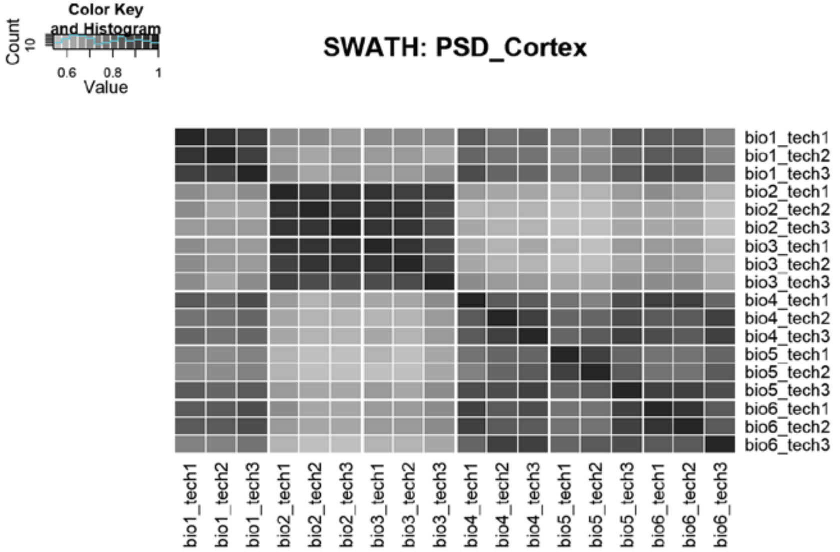
xMRM PSD



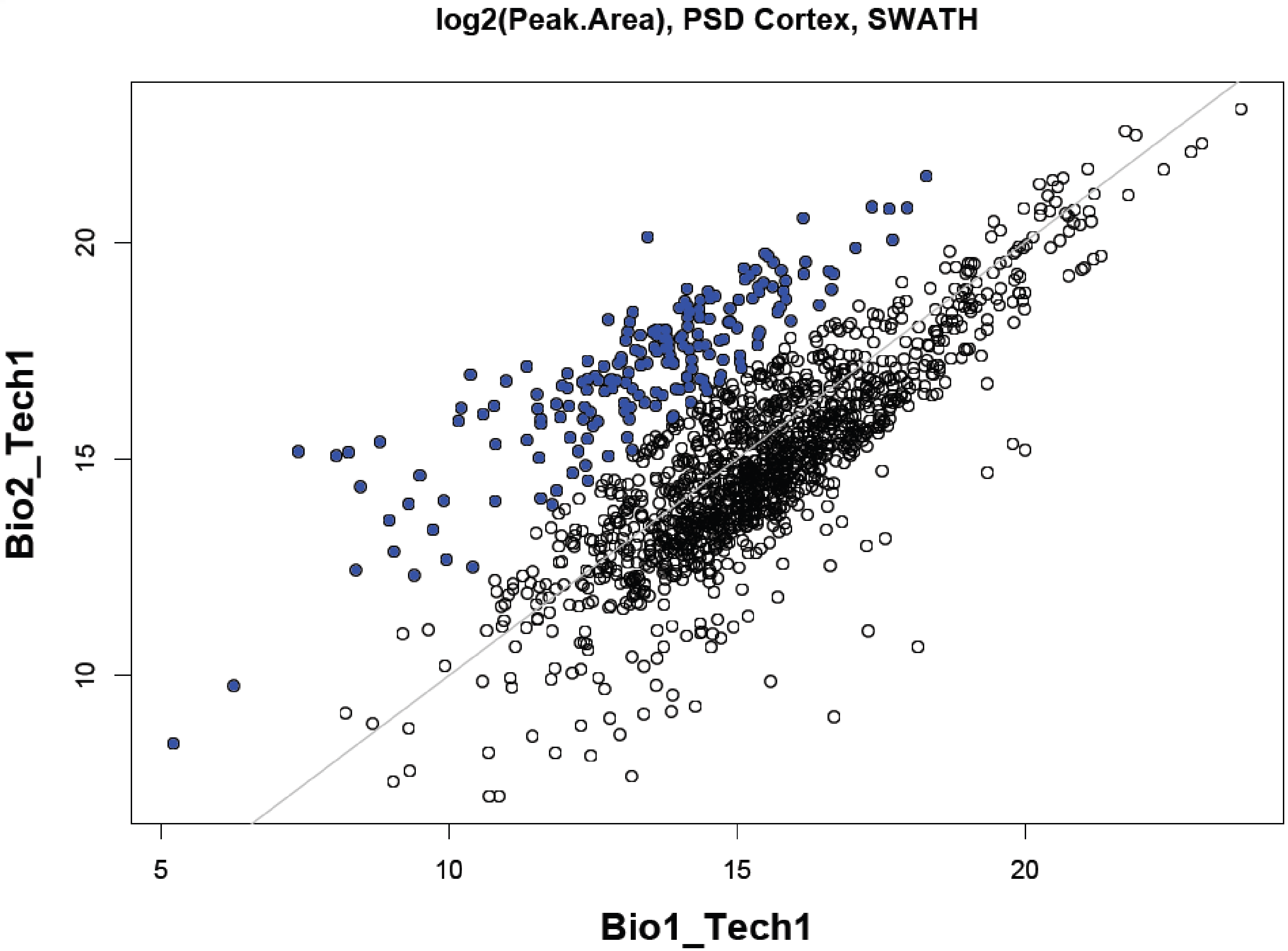


SWATH PSD



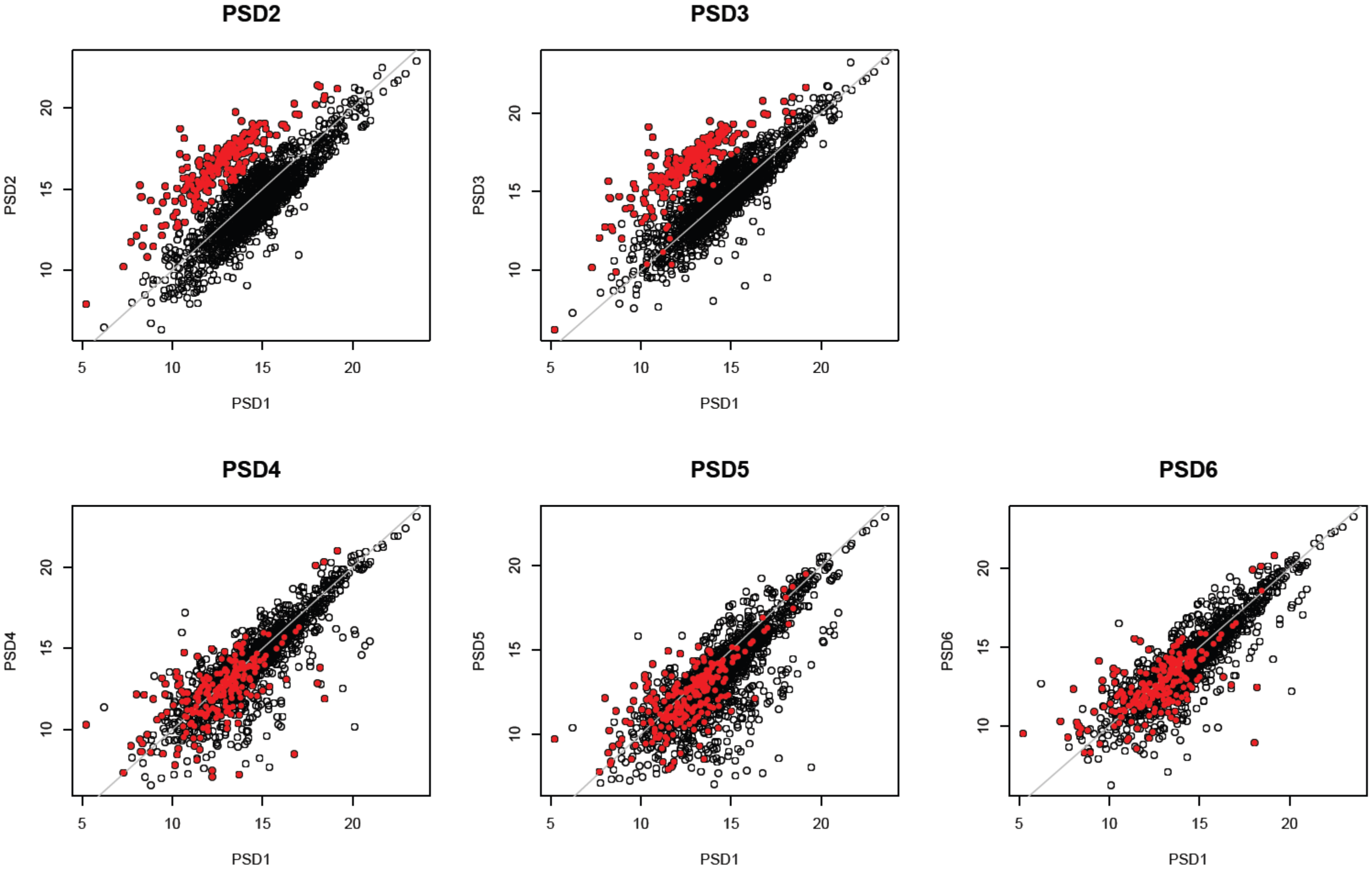


# Mitochondrial Proteins are also > 4-fold greater in PSD Cortex 2 vs. PSD Cortex 1



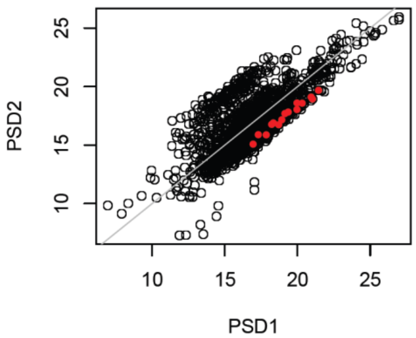
| **Protein** | **Transitions** |
|---|---|
| NDUA9 | 14 |
| COX5A | 13 |
| NDUV2 | 13 |
| M2OM | 12 |
| NDUA8 | 12 |
| NDUAC | 12 |
| NDUS1 | 12 |
| NDUS3 | 12 |
| NDUV1 | 11 |
| NDUS7 | 10 |
| QCR1 | 10 |
| QCR2 | 10 |
| NDUS8 | 9 |
| NDUS2 | 8 |
| NDUA7 | 7 |

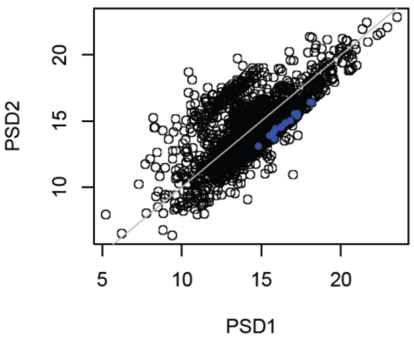
# SWATH - Mitochondrial Proteins are > 4-fold greater in both PSD Cortex 2 and 3 compared to PSD Cortex 1

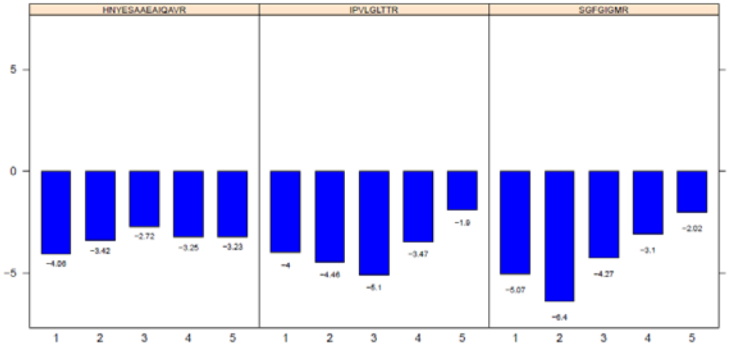


# Decreased Proteins in both xMRM and SWATH

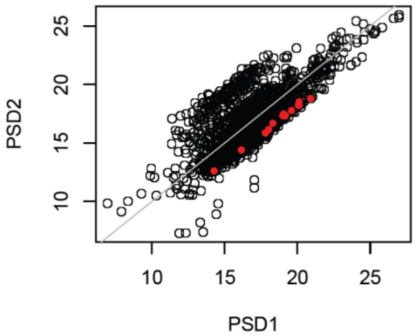
NMDZ1_MOUSE

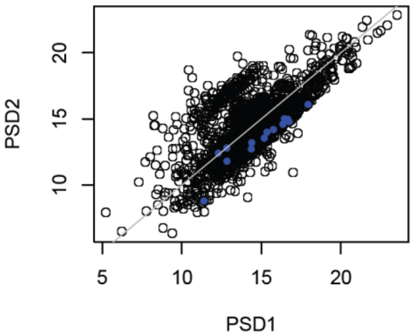

**sMRM, PSD 1 vs. PSD2**

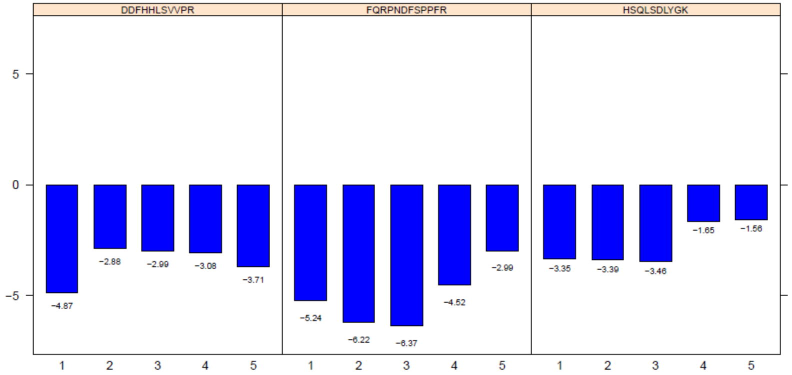

**SWATH, PSD1 vs. PSD2**



NMDE2_MOUSE


**sMRM, PSD 1 vs. PSD2**


**SWATH, PSD1 vs. PSD2**



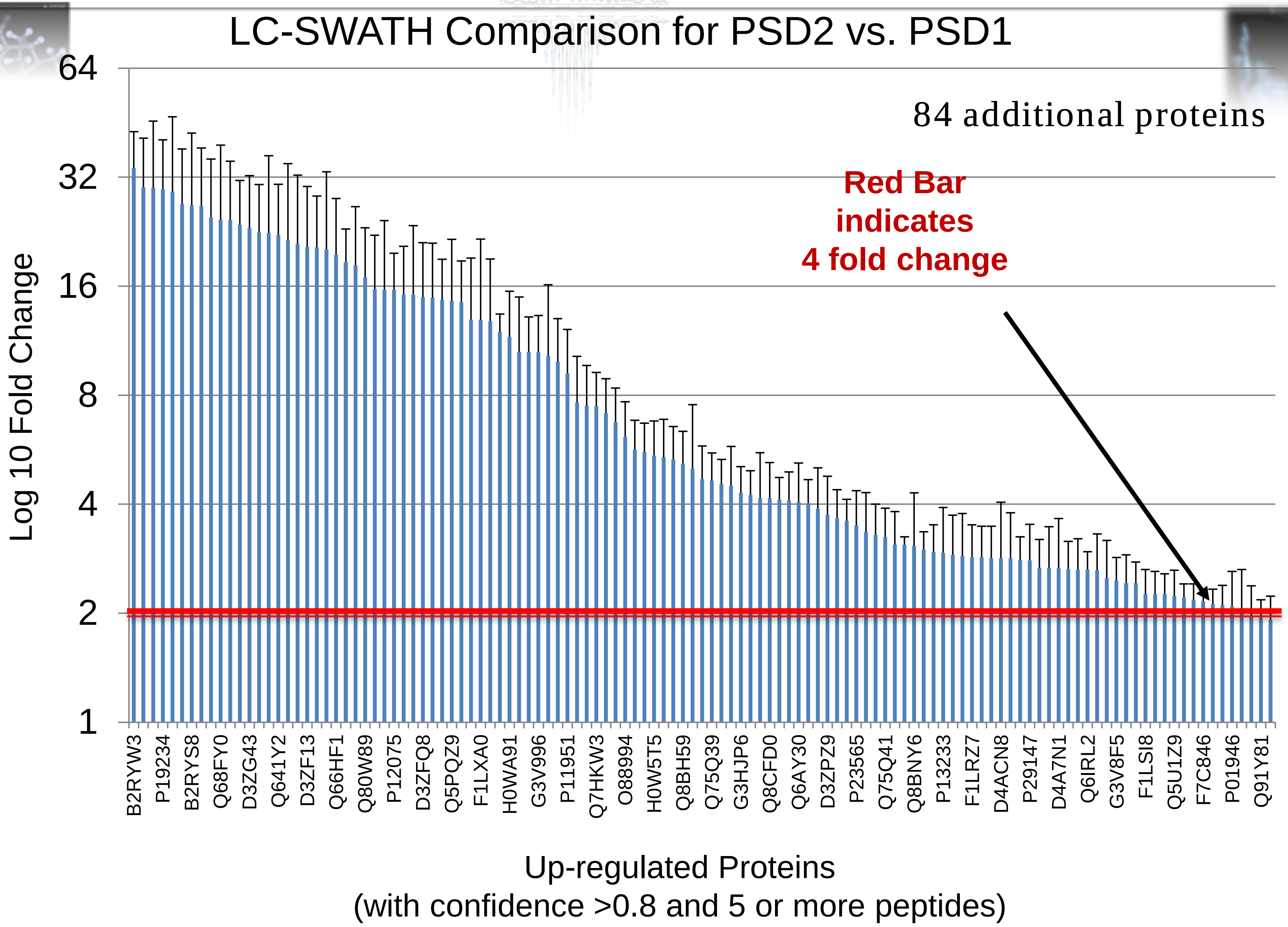
# xMRM vs SWATH peak area

Shift in the plot is the 3-3.5 fold sensitivity between the two systems

Correlation Between SWATH and sMRM is 0.90

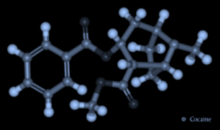
Red line is 45 degree, diagonal line

# Extraction of 1200 PSD Proteins from SWATH



84 additional proteins

**Red Bar indicates 4 fold change**



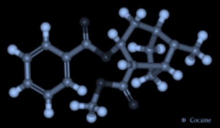
# Conclusions and Current NIDA Projects

## Conclusions

- Developed a pipeline to translate discovery data, collect, assess, and quantitate both SWATH and MRM data.
- Fold-change Correlation between SWATH and MRM in our Rat PSD Cortex Samples and virtually identical fold-change measurements in our samples (0.90 correlation)
- For our Rat PSD Cortex Comparison we were able to quantitating 84 additional up-regulated proteins by expanding our assay with SWATH

## NIDA Research Projects

- Small Scale Targeted MRM – Biederer (SynCam), Nairn (LFC)
- Global Targeted Proteome Assays – Nairn (PSD and Mitochondria), Hemby (PSD)
- SWATH Assays – Nairn (Mitochondria), Eipper (Kal-7 Phosphorylation), Chandra (CSF)



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