Presentation 1 Highlights (and Lowlights)

- Solid oral speaking style
- Background and functional studies-described well
- Significance of manuscripts-average (lit. search abilities)
- Driving the importance of MS for breakthrough-poor
- Description of MS setup and Data analysis – Very poor
- Just a reminder…this is a mass spectrometry course!
- Second presentation-Quantitative mass spectrometry or post-translational modification mapping by mass spectrometry
Lecture Layout

- Introduction to proteomics
- LC/MS/MS methodologies
- Multidimensional methodologies
- Recent advancements
Introduction

• The proteome
  – *Protein* complement of a genome

• Variable
  – In different cell and tissue types in same organism
  – In different growth and developmental stages of organism

• Dynamic
  – Depends on response of genome to environmental factors
    » Disease state
    » Drug treatment
    » Growth conditions
    » Stress
Why Study Proteomics?

- Less than 2% of our total disease load are monogenic diseases.

- There is very low correlation between mRNA abundance and protein level.

- Gene products are modified by:
  - complex gene interactions
  - cellular events
  - environmental influences
  - post-translational modifications
Proteomics applications

- Studies of basic cell function and molecular organisation
- The discovery of novel drug targets
- The discovery of novel biologically active molecules and drugs
- Subtype individuals to predict response to therapy
- The discovery of molecular markers for diagnosis and monitoring disease
Applications of Proteomics

Oncology
Detection of differences between healthy & tumor cells
Response of cells to drug treatment

Developmental Biology
Monitor changes in the cell at the molecular level as cells proliferate & differentiate

Environmental Biology
Monitor changes in cells and tissues in response to environmental stresses

Pharmaceutical Arena
Monitor changes in cells and tissues in response to drugs
Different strategies for proteome purification and protein separation prior to identification by Mass Spectrometry

- **Cell fractionation** for separation of organelles and complex protein mixtures including hydrophobic membrane proteins.
- **Separation of individual proteins by 2-D Electrophoresis**
- **Multidimensional chromatography.**
Biological question defines the technique(s) to use
What needs to be separated?
(single vs complex protein mixture)
Identification of Novel Signaling Complexes

Affinity purification and SDS/PAGE

↓

In gel Trypsin Digestion

↓

Zip Tip cleanup (desalting)

↓

MALDI-Linear - internal and external calibration
- peptide mass fingerprint

↓

LC/MS/MS using Q-ion trap

Search Database

Tentative matches

Protein identified

Analyze data manually
And by database searching

Functional analysis
LC/MS/MS

• Powerful discovery tool for Proteins of Unknown Function (PUFs)

• Not Suitable for studying entire proteome (by itself)
Traditional Proteomic Strategy

• Core technology
  – 2D-PAGE
    • High resolution protein separation and display
  – Mass spectrometry
    • Protein identification
The Problem

- 2D Gels can routinely be used to separate ~1000 spots, yet cells express 1000’s-10000’s of proteins.

- Approaches to improve protein coverage:
  - Separation on the basis on differential compartmentalization/solubilization
  - Narrow range IEF strips for focusing on particular pl ranges.
  - Detection method specific for segment of proteome (antibodies, radioactivity, fluorescent dyes...etc)
2D Gel Technology

• Other Limitations
  – Many problem proteins
    • Large (do not migrate into 2D gel)
    • small (run off bottom of 2D gel)
    • acidic (do not focus)
    • basic (streak due to electroendoosmosis)
    • Membrane (hydrophobic) (aggregate and precipitate)
    • Abundant proteins predominate detection

• 2D Liquid Chromatography as alternative has emerged
Multidimensional Separation Approaches for Proteomics

* “Bottom-Up”
  - digest complex protein sample and fractionate peptides by 2D HPLC
  - identify proteins by mass spectrometry
  - excellent for determining protein *identities*, but limited for characterization of *molecular forms* of intact proteins

* “Top-Down”
  - Fractionate and analyze mass of intact proteins
  - query protein databases to identify proteins (usually digest/tandem MS/MS)
  - excellent for determining *molecular forms* of intact proteins, but limited in the *number of proteins identified*
Combining the “Top-Down” and “Bottom-Up” Approaches

Ion Exchange Fractionation

Tryptic Digest

2D LC/MS/MS

Intact Mass Analysis

FTICR
mw=26444.688

mass
MudPIT-
Multidimensional Protein Identification Technology
(Washburn and Yates)

- Two dimensional liquid Chromatography
  - Separate crude protein mixture based on charge and hydrophobicity.
- Couple to mass spectrometer for ID.
MudPIT

1) Load Acidified Digest
2) Equilibration
3) Salt Step
4) Wash
5) RP Gradient
6) Go to #2

50 μm Split Capillary

50 μm Fused Silica Capillary from HPLC

100 μm Packed Capillary Column

PEEK Micro Cross

Gold Lead for Electrical Contact with Liquid (1.8 kV)

Capillary Opening into Mass Spectrometer

Large-scale analysis of the yeast proteome by multidimensional protein identification technology

MudPIT was applied to the proteome of the *Saccharomyces cerevisiae* strain BJ5460 grown to mid-log phase and yielded the largest proteome analysis to date.

A total of 1,484 proteins were detected and identified. Categorization of these hits demonstrated the ability of this technology to detect and identify proteins rarely seen in proteome analysis, including low-abundance proteins like transcription factors and protein kinases. Also, >100 proteins were identified as being transmembrane proteins.
Large-scale analysis of the yeast proteome by multidimensional protein identification technology

Limitations
➢ Co-eluting peptides
➢ Inadequate resolution of complex mixture
➢ Long run times needed

Improvements
➢ Quaternary pump switching system
Schematic for 2D Switching system

loading solvent
30 μL/min

gradient
200 nL/min

SCX
1 mm i.d. x 50 mm

Nano-Precolumn C18 RP
300 μm i.d. x 1 mm

Nano column C18
75 μm i.d. x 15 cm

UltiMate™

waste

SWITCHOS™
Column setups for 2D Switching system

SCX column: 0.500 cm x 150 mm SCX
Precolumn: 0.3 x 5 mm PepMap
Nano column: 0.075 x 150 mm C18

Switchos: 30 µl/min 0.1% formic acid
FAMOS: 50 µl loop NH₄OAc salt injections (either 20ul or 50ul)
Ultimate: RP gradient is started 10 min after salt injections
Gradient: 200nL/min 2-2.5 hours 95% H₂O- 30% H₂O (balance Acn)

A variety of columns with different sizes can be easily incorporated
Molecular constituents of the postsynaptic density fraction revealed by proteomic analysis using multidimensional liquid chromatography-tandem mass spectrometry

Yoshiyuki Yoshimura,* Yoshio Yamauchi,† Takashi Shinkawa,‡ Masato Taoka,‡ Hitomi Donai,* Nobuhiro Takahashi,†§ Toshiaki Isobe†‡ and Takashi Yamauchi*

PSD-subcellular organelle found in neurons
Function in synaptic remodeling
Previous separate proteomic attempts have identified ~90 proteins in PSD
Comprehensive knowledge of the proteins present in the PSD would provide novel clues to the mechanism of important brain functions such as learning and memory
Method

PSD was purified from fractionation of rat forebrain homogenates

Equal amounts of purified PSD was analyzed by two-dimensional gel electrophoresis (3 days) and two dimensional LC/MS/MS (16 hrs)

2D GE followed by LC/MS/MS identified ~80 proteins

2D LC/MS/MS identified 492 unique proteins including numerous low abundant signaling proteins (kinases, phosphatases etc) as well as many transmembrane proteins
A proteomics approach to understanding protein ubiquitination

Junmin Peng¹, Daniel Schwartz¹,², Joshua E Elias¹,², Carson C Thoreen¹,², Dongmei Cheng², Gerald Marsischky³, Jeroen Roelofs¹, Daniel Finley¹ & Steven P Gygi¹,²
Study identified 1075 yeast proteins modified by Ub
Including mapping 110 precise Ub sites in 72 proteins
Many low abundant regulatory proteins identified
Future work will include proteosome inhibitors to capture rapid turnover Ub proteins
Recent Advancements

- Semi continuous ion exchange gradients
- Monolithic column supports
Semi continuous gradients

[Diagram showing a flow process involving SCX Column, Injector, Capillary Pump, Enrichment Column 1, Enrichment Column 2, Nanoflow LC Pump, Nanobore Column, Ion Trap, and Waste.]
Semi continuous gradients
Semi continuous gradients
Monolithic design results in dramatic pressure drop allowing for increased flow rates and decrease run times when using SCX monolithic columns.

Monolithic C18 columns also have lower back pressure.
Conclusions

One-dimensional LC-MS/MS
* Useful for analyzing single protein
* Fully automated
* Reproducible retention times

Two-Dimensional LC-MS/MS
* Increases peak capacity and number of proteins i.d.
* Requires sophisticated instrumentation
* Automated reproducible separations possible
* Nonselective - all classes of proteins recovered