UAB *University of Alabama at Birmingham*
School of Medicine

Quantitative Proteomics; Are We There Yet?

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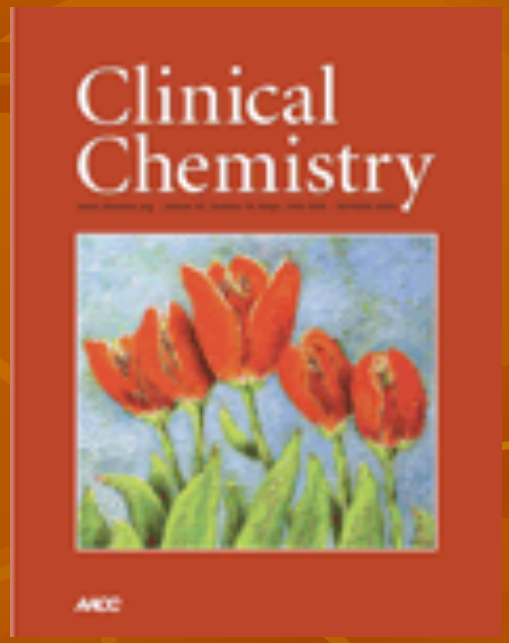
Inspiration of Today's Subject……



Protein Quantification by Mass Spectrometry: Is It Ready for Prime Time? Diamandis *Clin Chem.*2009; 55: 1427-1430

Diamandis EP, Hanash S, Lopez M, Carr S, Petricoin EF 3rd.

Why do you think mass spectrometry– based methods for measuring proteins are not yet in widespread use in clinical laboratories?

Samir Hanash: The instrumentation available in clinical laboratories generally has features particularly designed to meet the work flow and performance requirements applicable to a clinical laboratory, together with standard operating procedures. Proteomic analysis by mass spectrometry is currently applied primarily for discovery and does not meet these requirements for routine clinical assays. At best, it would have to be considered a "specialized assay platform," available at a limited number of laboratories.

Mary Lopez: There exists the misconception that MS-based assays are difficult and require very experienced operators. The rapid evolution of this technology has made its operation no more complicated than the operation of clinical analyzers. There is also a perception that MS-based assays are expensive. With higher throughput, and the ability to multiplex assays, the cost per assay is not much different than for ELISAs or other routine assays. Lastly, there is a natural reluctance of users to adopt new methods that are as of yet perceived to be "unproven." Related to this point is the misconception that MS-based assays are not robust or reproducible.

When & How did protein discovery MS get started?

Structural identification, neuronal synthesis, and role in male copulation of myomodulin-A of Lymnaea: a study involving direct peptide profiling of nervous tissue by mass spectrometry.

Li KW, van Golen FA, van Minnen J, van Veelen PA, van der Greef J, Geraerts WP.
Brain Res Mol Brain Res. 1994 Sep;25(3-4):355-8.

A rapid protein profiling system that speeds study of cancer and other diseases.

Rubin RB, Merchant M.
Am Clin Lab. 2000 Sep-Oct;19(8):28-9.

Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks.
Goodacre R, Timmins EM, Burton R, Kaderbhai N, Woodward AM, Kell DB, Rooney PJ.
Microbiology. 1998 May;144 (Pt 5):1157-70.

Computer matching of pyrolysis chromatograms of pathogenic microorganisms.
Menger FM, Epstein GA, Goldberg DA, Reiner E.
Anal Chem. 1972 Feb;44(2):423-4.Links

Chemometric classification of some european wines using pyrolysis mass spectrometry

Luca Montanarella *, Maria Rosa Bassani, Olivier Bréas

Commission of the European Communities, Joint Research Centre, Environment Institute, 21020 Ispra, Italy

Application of LC/MS to proteomics studies: current status and future prospects

Guodong Chen and Birendra N. Pramanika

Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

Available online 26 February 2009.

Liquid chromatography/mass spectrometry (LC/MS) has become a powerful technology in proteomics studies in drug discovery, including target protein characterization and discovery of biomarkers. This review article will describe current LC/MS approaches in protein characterization, including a bottom-up method for protein identification and quantitative proteomics. We will discuss the investigation of protein post-translational modifications such as glycosylation (glycoproteomics) and phosphorylation (phosphoproteomics) using LC/MS. Future trends in LC/MS with respect to proteomics studies will also be illustrated.

Quantitative strategies to fuel the merger of discovery and hypothesis-driven shotgun proteomics

Kelli G. Kline, Greg L. Finney and Christine C.Wu

The ultimate goal of most shotgun proteomic pipelines is the discovery of novel biomarkers to direct the development of quantitative diagnostics for the detection and treatment of disease. Differential comparisons of biological samples identify candidate peptides that can serve as proxys of candidate proteins. While these discovery approaches are robust and fairly comprehensive, they have relatively low throughput. When merged with targeted mass spectrometry, this pipeline can fuel hypothesis-driven studies and the development of novel diagnostics and therapeutics.

Quantitative strategies

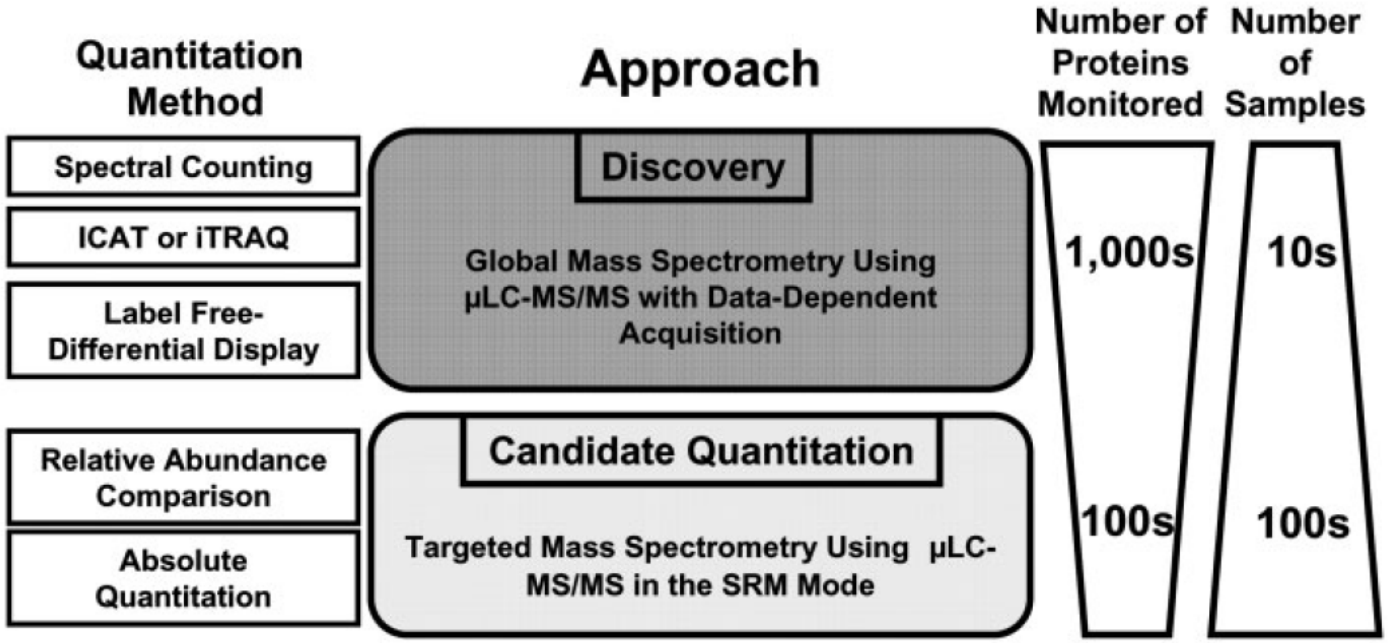


Figure I: Overview of discovery and candidate quantitation approaches.

Kline, et. al

Questions to ask before you begin……..

- Discovery (unknown) vs Directed (known)
- Non- labeled vs chemical or metabolic labeled
- Proteomics vs. Small molecules, or Oligonucleotides
- By mass spectrometry vs spectroscopy

1. Identification of a problem to be addressed
2. What sample set to address the problem
3. Fractionation approaches to get at what may be of interest
4. Digested or non-digested proteins
5. MALDI or ESI
6. Spectral Preprocessing
7. Quantification
8. Protein ID's
9. Statistics
10. Informatics
11. Confirmation
12. Validation

Overviews of Discovery Approaches

- Imaging MS – Human Pancreatic Samples
- MALDI-TOF Profiling Biological Fluids (with Top-Down Directed ID approach)– Human Pancreatic Samples
- Quantitative Proteomics (SILAC) – cell lines treated with N-Acetyl Glucosamine
- Non-Tagged Peptidomics – Prostate Tissue

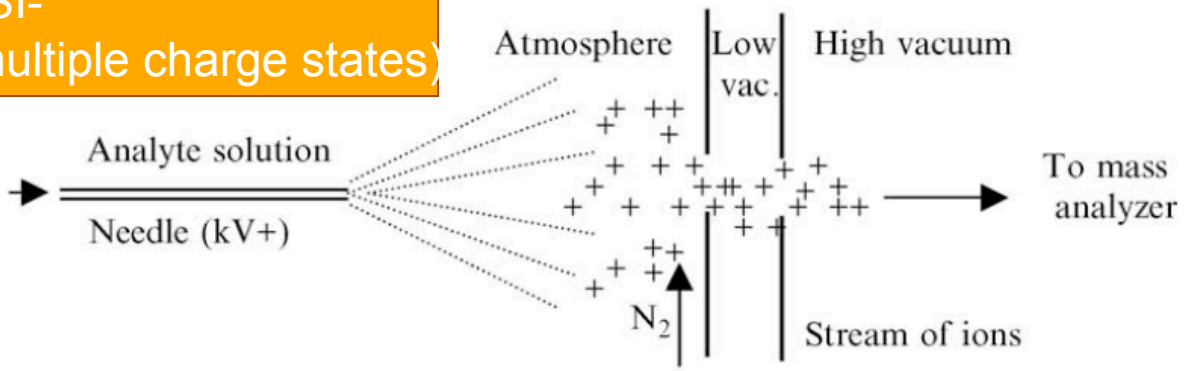
- Summation – Overview of the talk.

MS Profiling

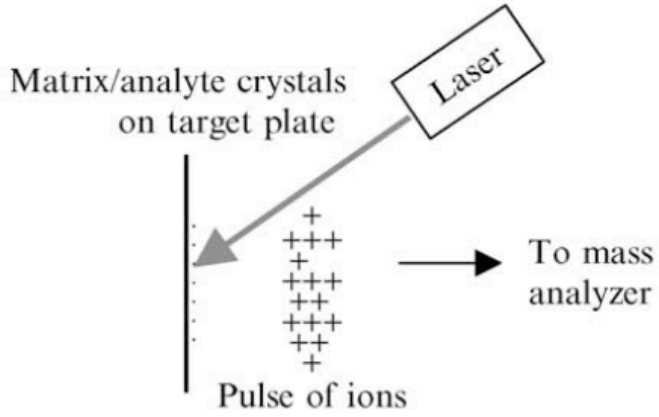
- MALDI-ToF (HTP)
  - Profiling of Biological Fluids
  - Direct Tissue Profiling
- ESI-Trap (LTP)
  - Peptide based profiling; 100-1000 proteins studied in single experiment from protein digests.
- Together – Very Powerful tools for BMD!

Soft Ionization Techniques

ESI-
(multiple charge states)

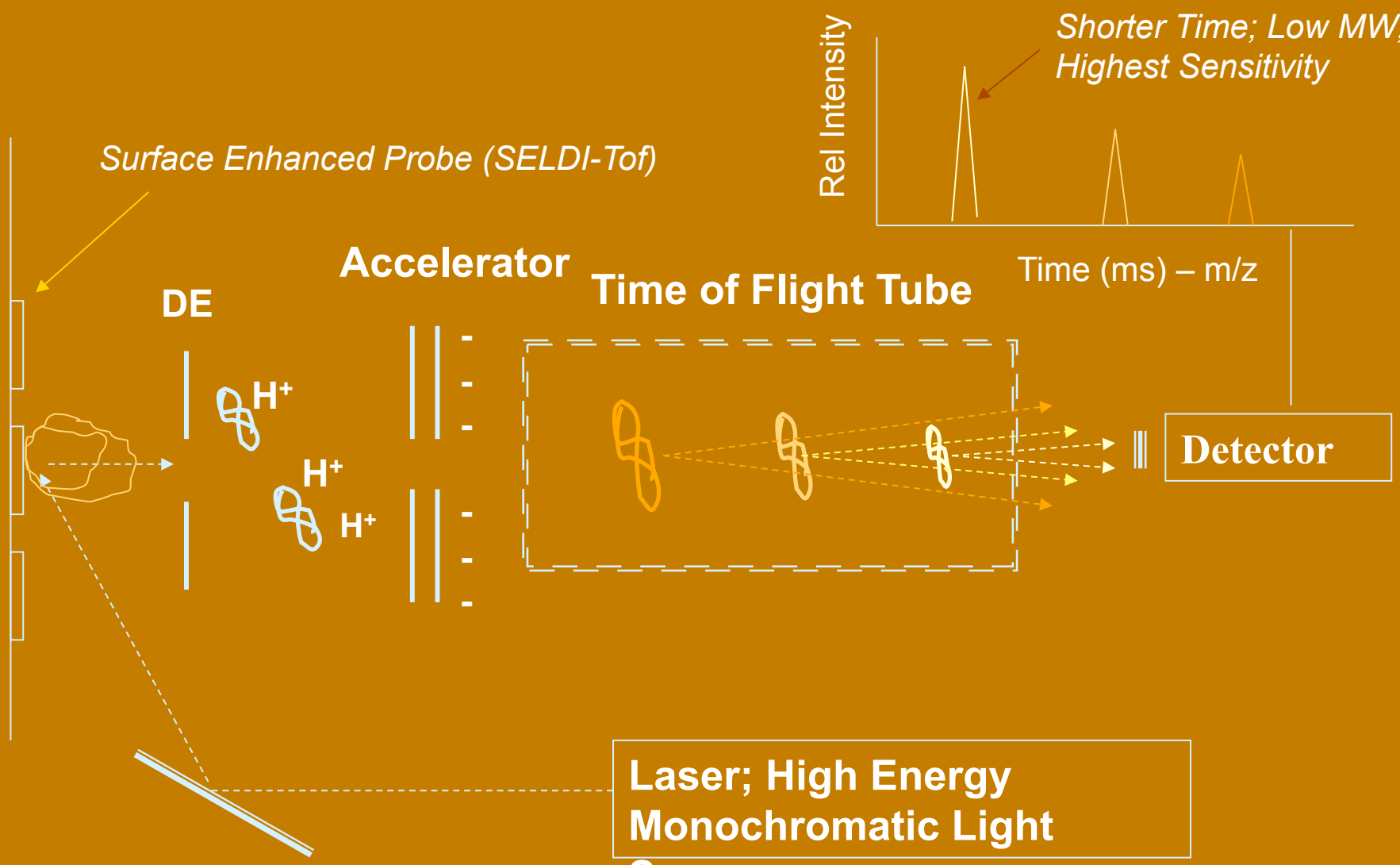


MALDI-
(singly charged ions)



Baldwin M.A., Mass spectrometers for the analysis of biomolecules, Methods in Enzymology, Vol 402

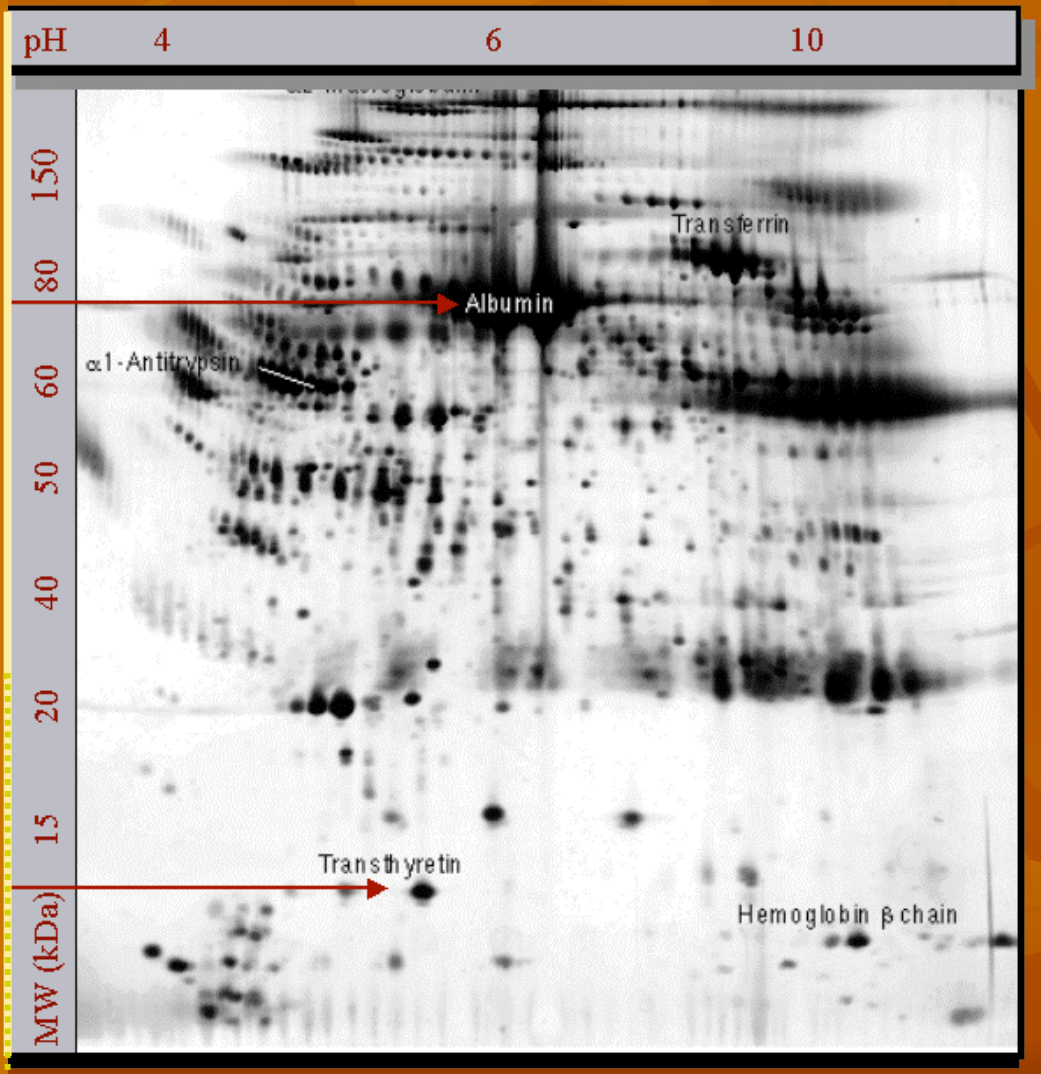
MALDI-Tof Mass Spectrometry



Why Use MALDI-ToF for Proteomics Discovery?

Primarily LMW Proteins!

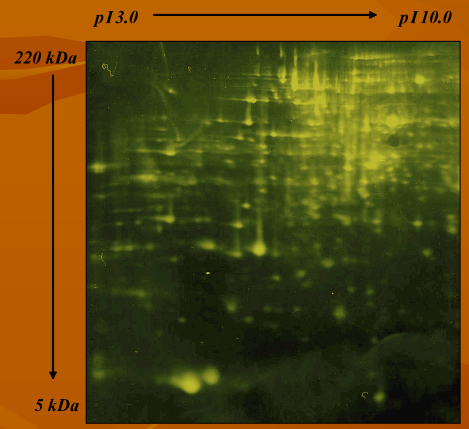
2D PAGE, ID by MALDI-Tof



General Theme: Mass Spectrometry Driven Discovery & Directed Quantitative Proteomics

(non-Tagged/ SILAC/ AQUA)

(SILAC)
1D or 2D PAGE



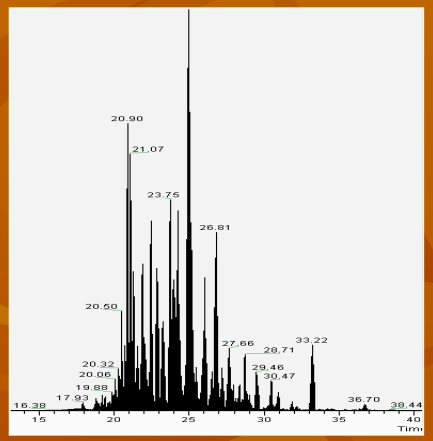
HTP-MALDI-Tof/Tof
(200 spots per night)

IP (PTM or Protein Directed)

Systems Biology
(i.e. Cytoscape)
Cellular Location
Molecular Process
Biological Function

Characterization
(Relative/ Absolute Quantification)

(SILAC/ AQUA)
In-Solution Digest

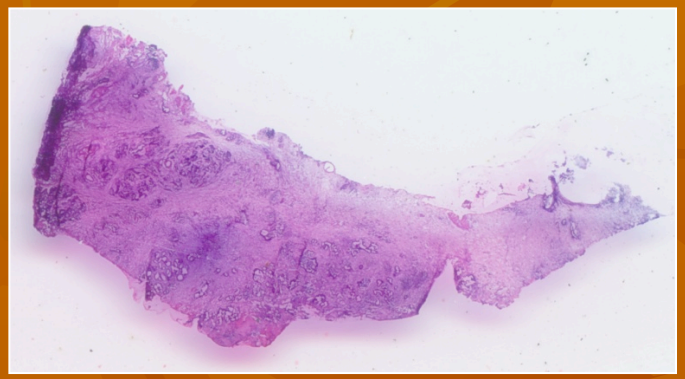


Nano-LC-MS(MS)^2-CID/ETD
(~6000/spectra/hr)

Example; Human Pancreatic Cancer Specimens

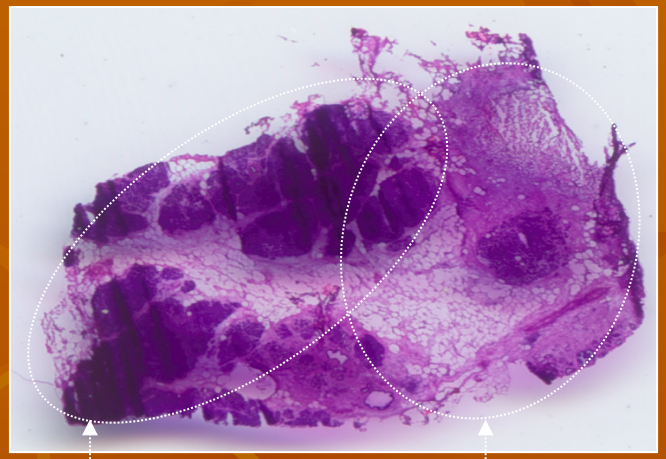
Tissue Profiling/ Imaging by MALDI-MS

Tissue Specimens Studied by Imaging/ Profiling MALDI-MS



Cancer

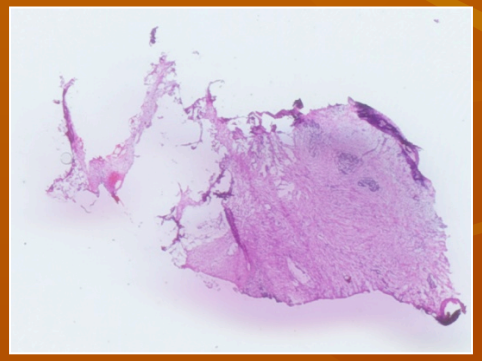
Normal



Normal

Cancer

Fibrosis (intermittent)



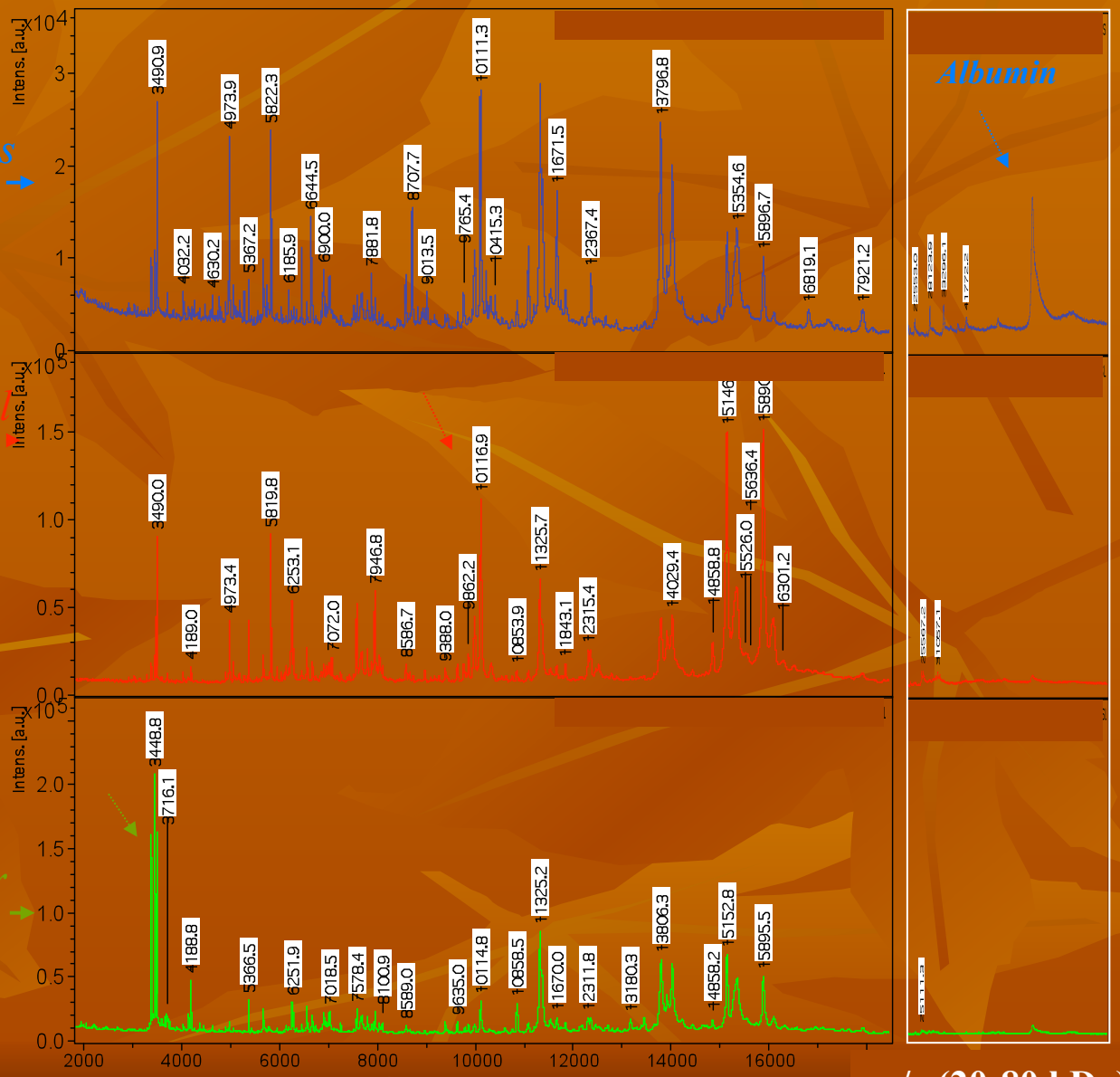
Fibrosis (Pancreatitis)

Averaged MALDI-TOF Spectra (Representing Each Tissue Type)

Fibrosis

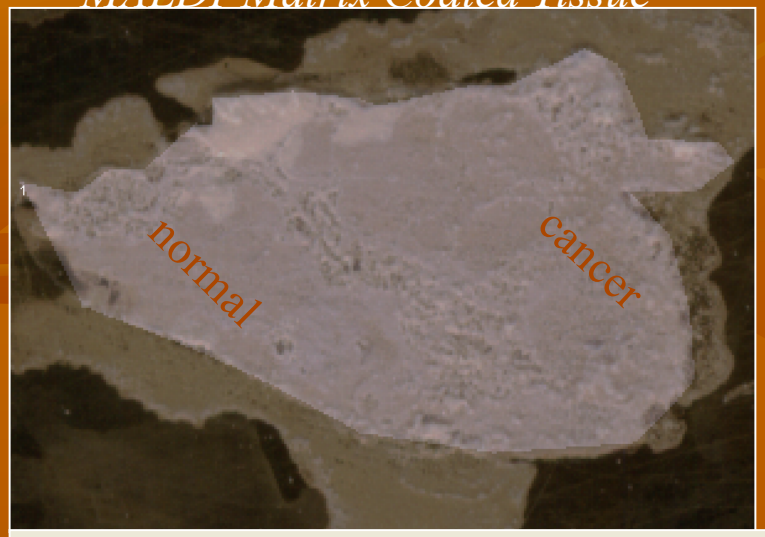
Normal

Cancer

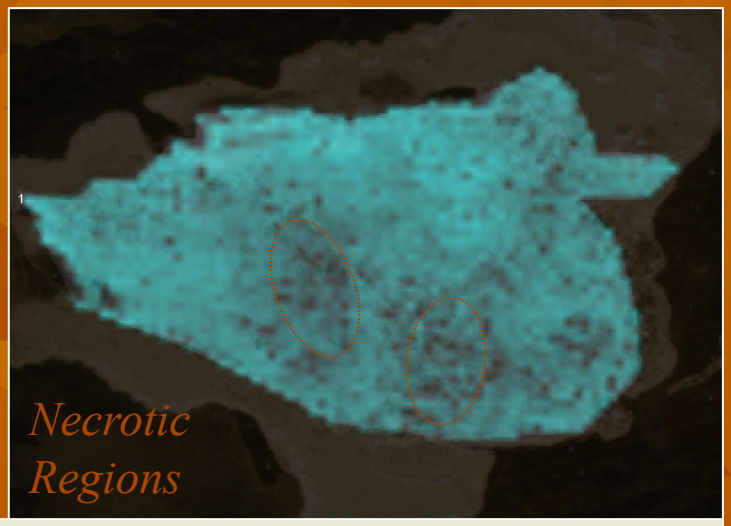


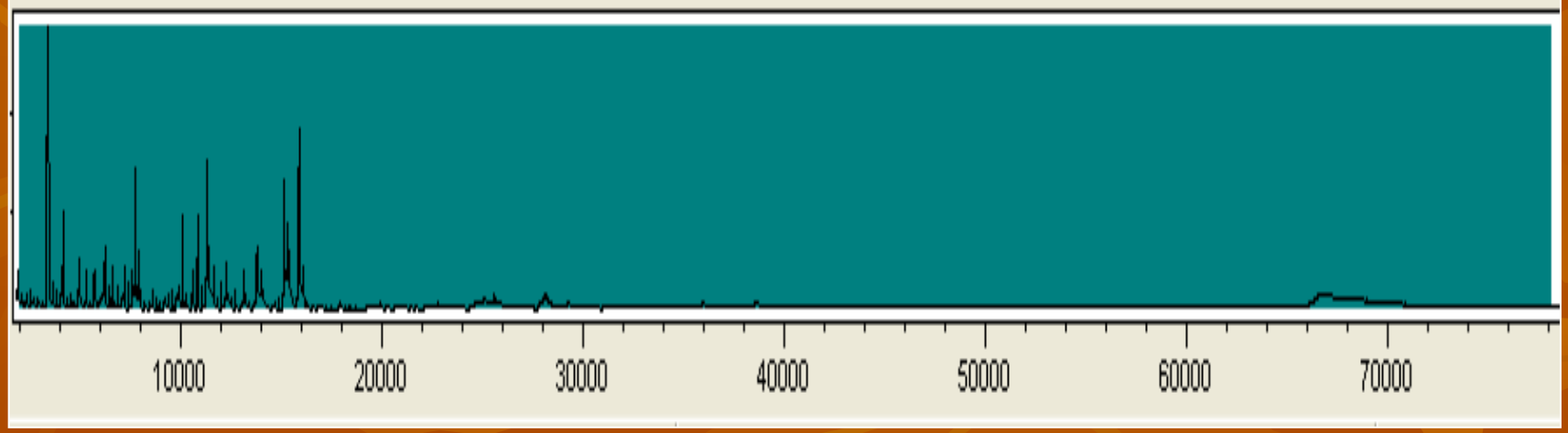
MALDI-MS IMAGING on Tissue of "Mixed" Phenotype

Photo Image
MALDI-Matrix Coated Tissue

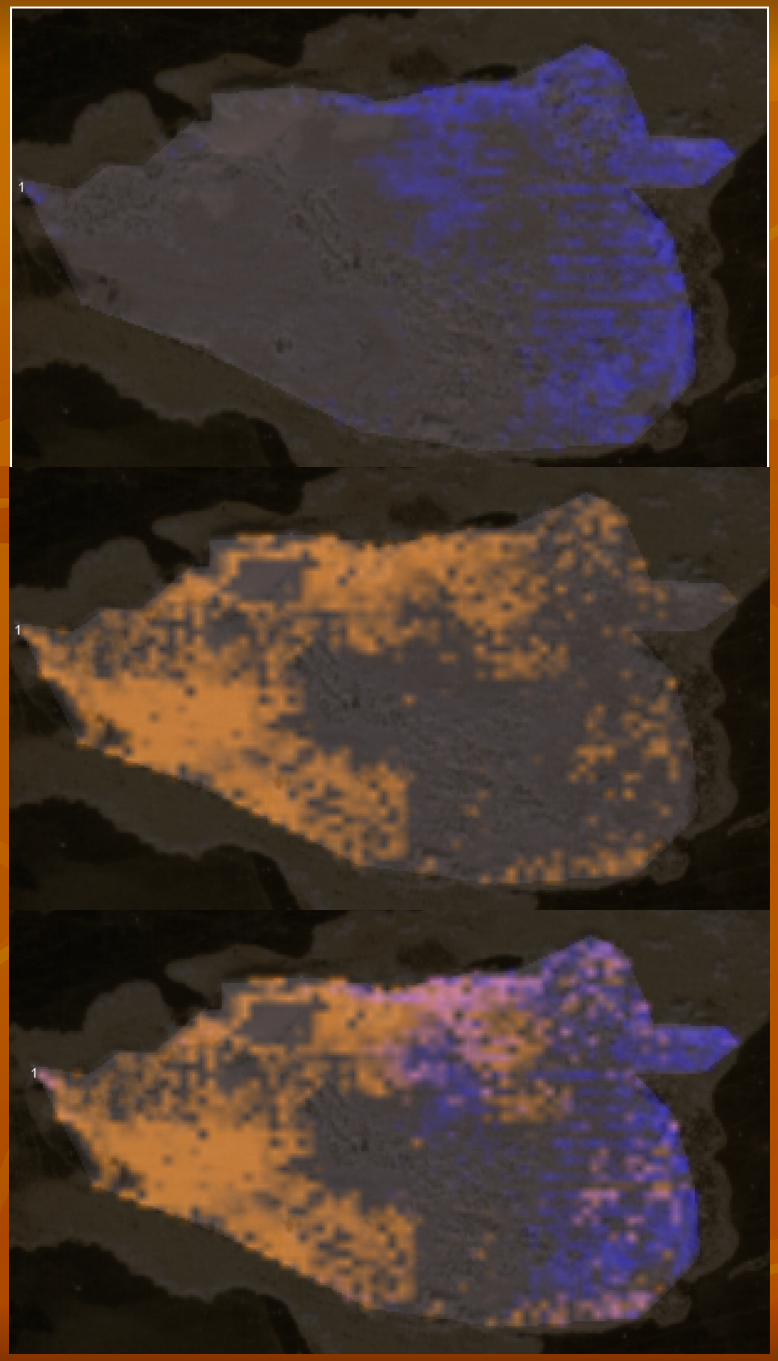


MALDI-MS Image
(Combined Signals)





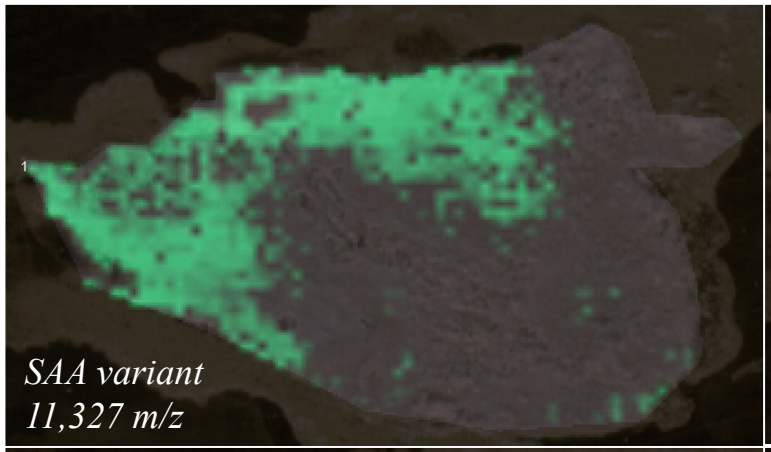
M/Z (Average Spectra for Entire Tissue Sample)

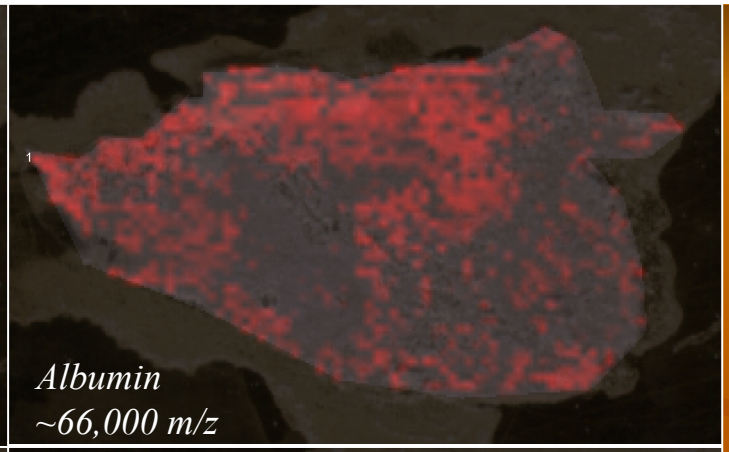


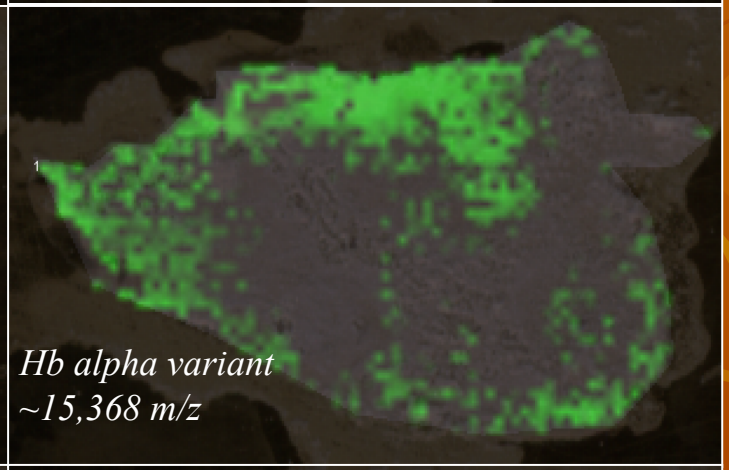
Defensin II
Increased in Cancer
3374 m/z (Blue)

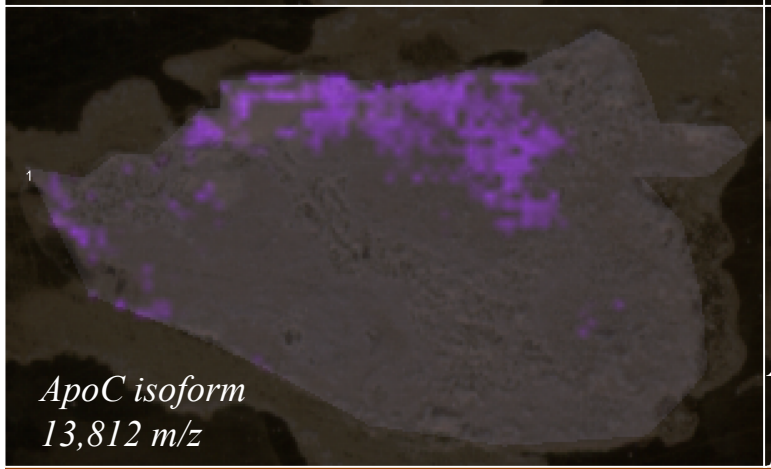
Uncharacterized protein
Lost in Cancer
10117 m/z (Orange)

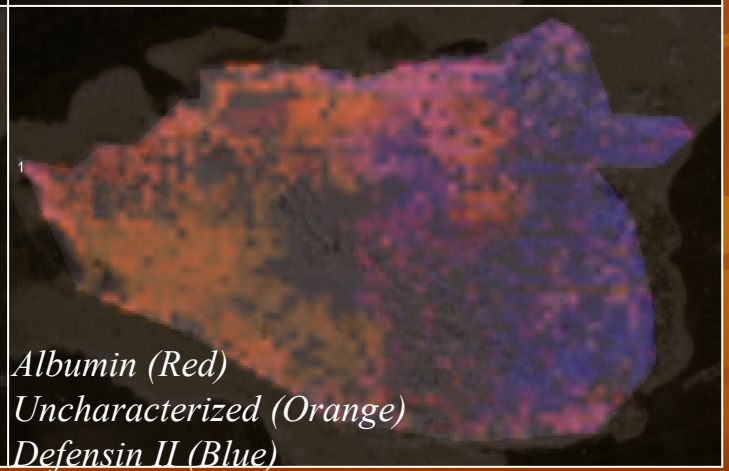
Combined







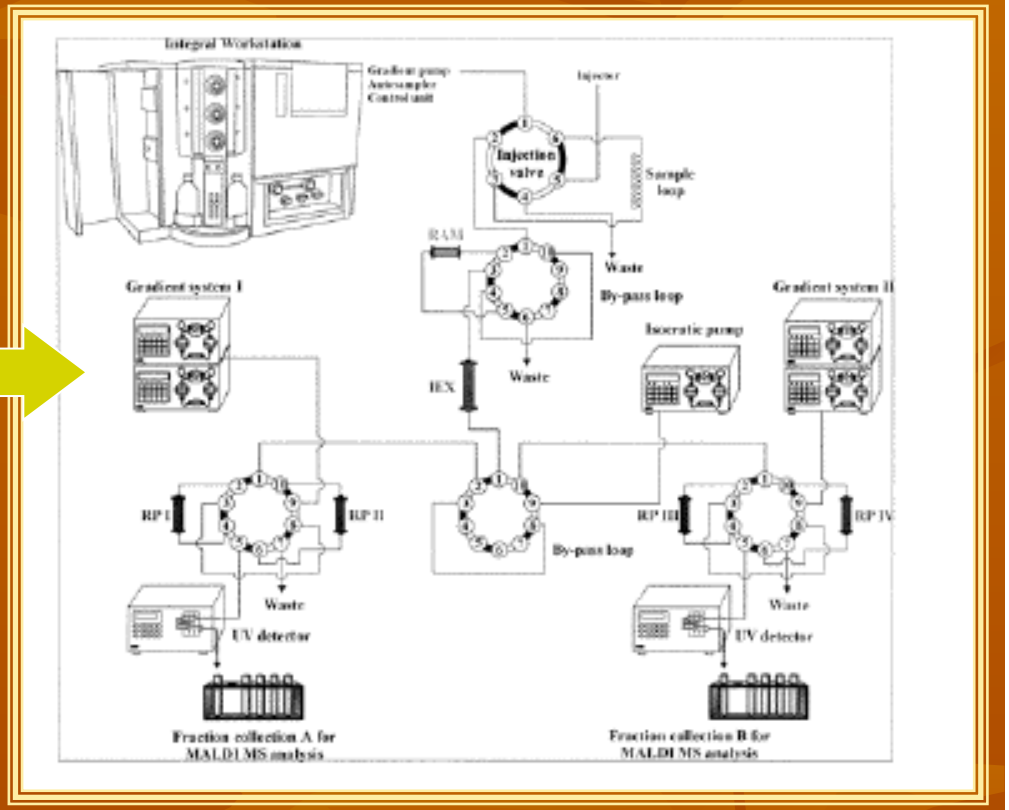




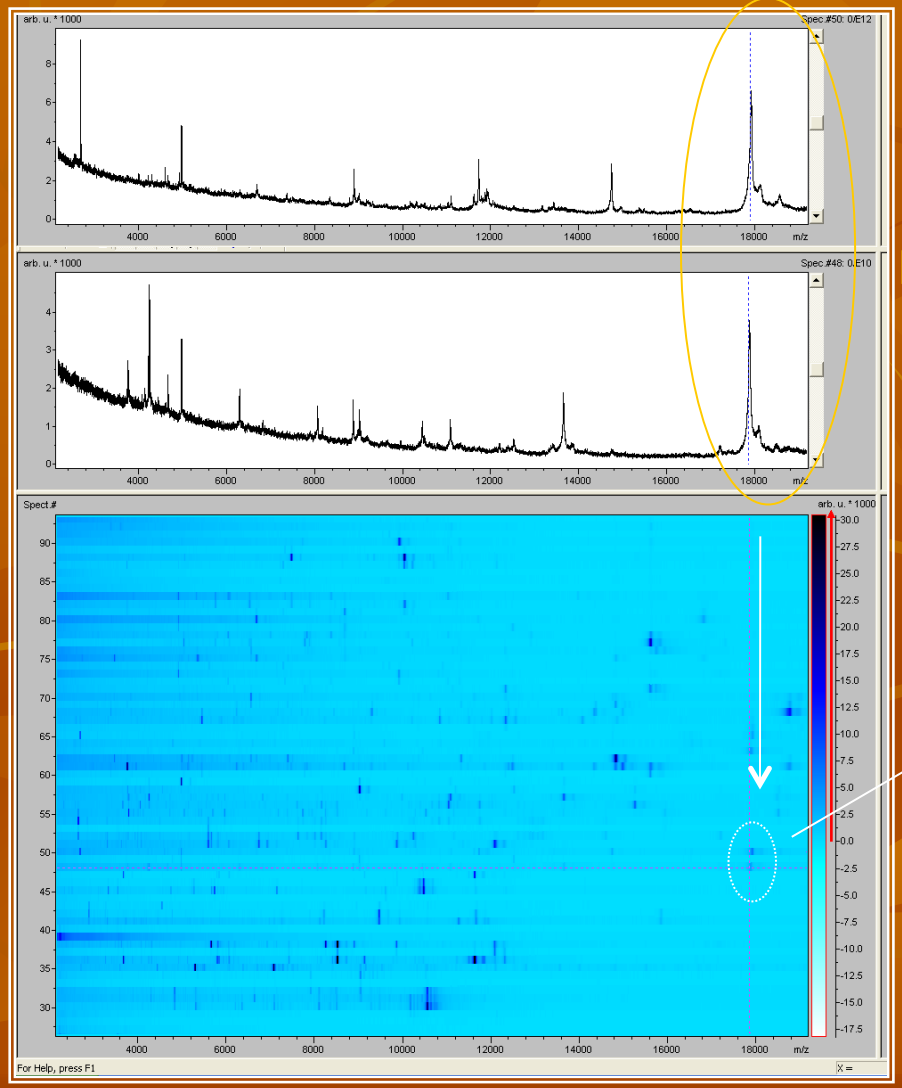
Various Potential Markers

3374 (defensin 2)
3446 (defensin 1)
3500 (TA-NA88A)
5748 (insulin)
7014 (USH1C-BP1)
11327 (SAA isoform)
13812 (ApoC isoform)
13878 (Thioredoxin)
14031(Histon H2)
14180 (Ribonuclease 8)
15368 (Histone)

How do We ID These Proteins?
TDD using Separation in Multidimension!



LCMALDI using WARP-LC

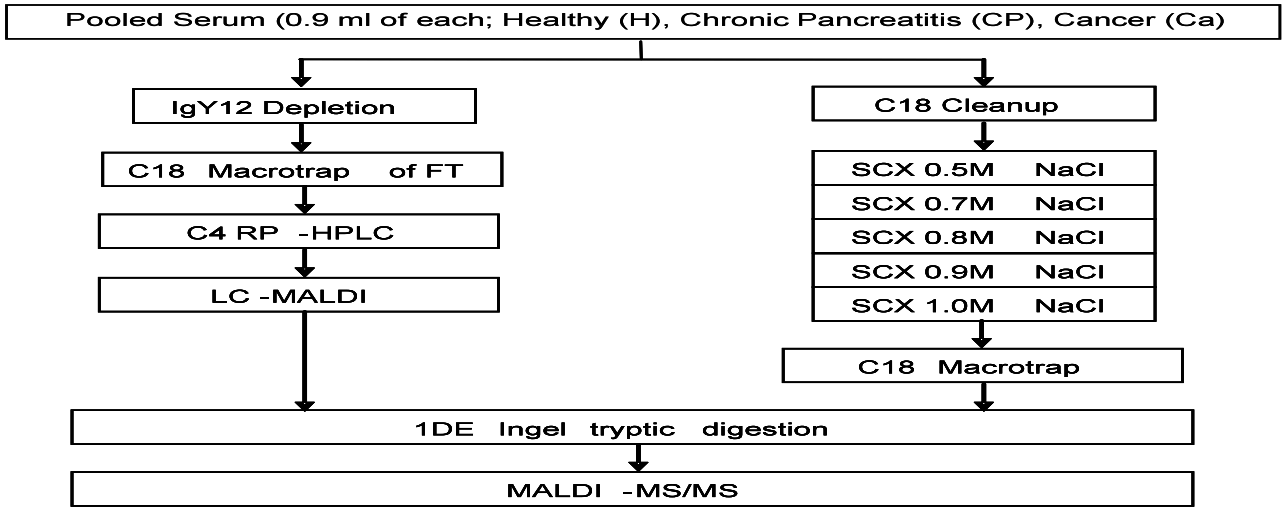


Retention Time

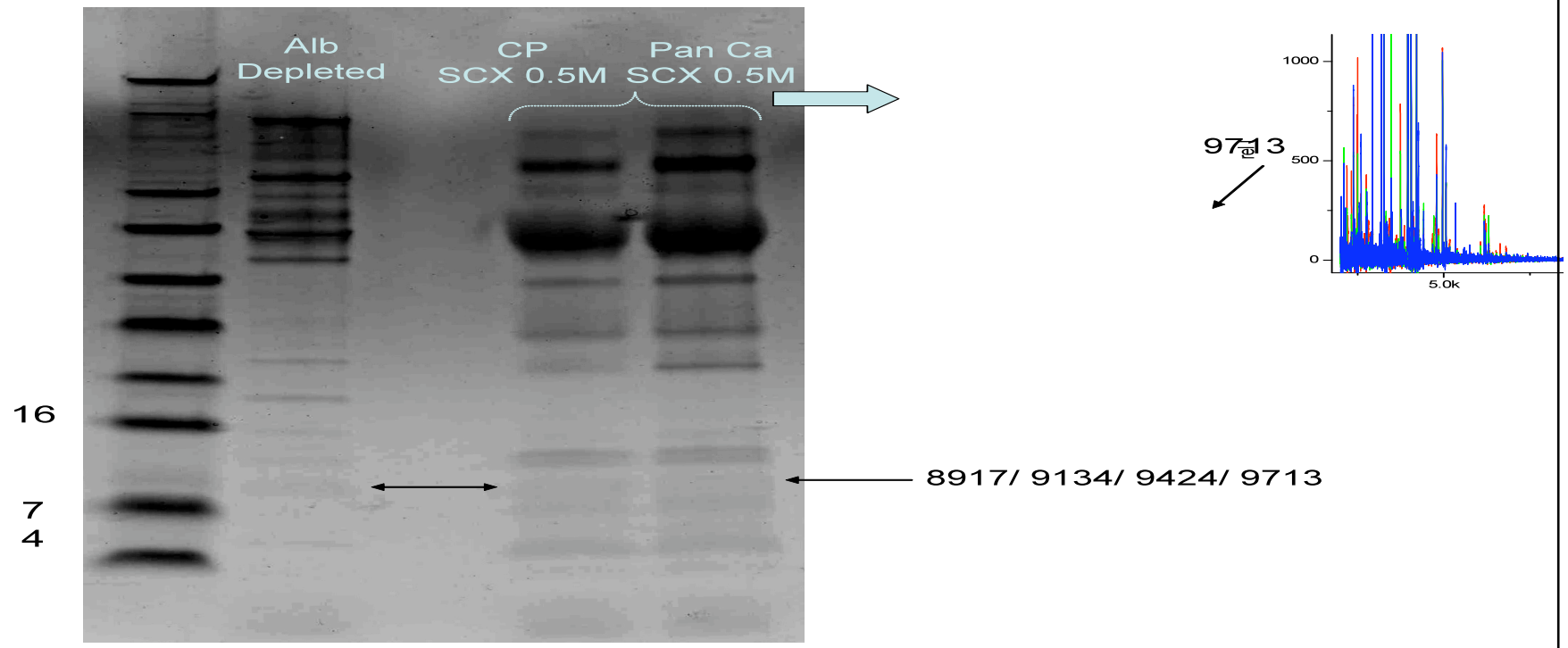
Two peaks Identified by IMS to be of Interest!

Found in Fr's # 43 & 45
Shift by 42 Da
N-Acetyl?

4*a*. Top-Down-Directed Protein ID (Workflow)



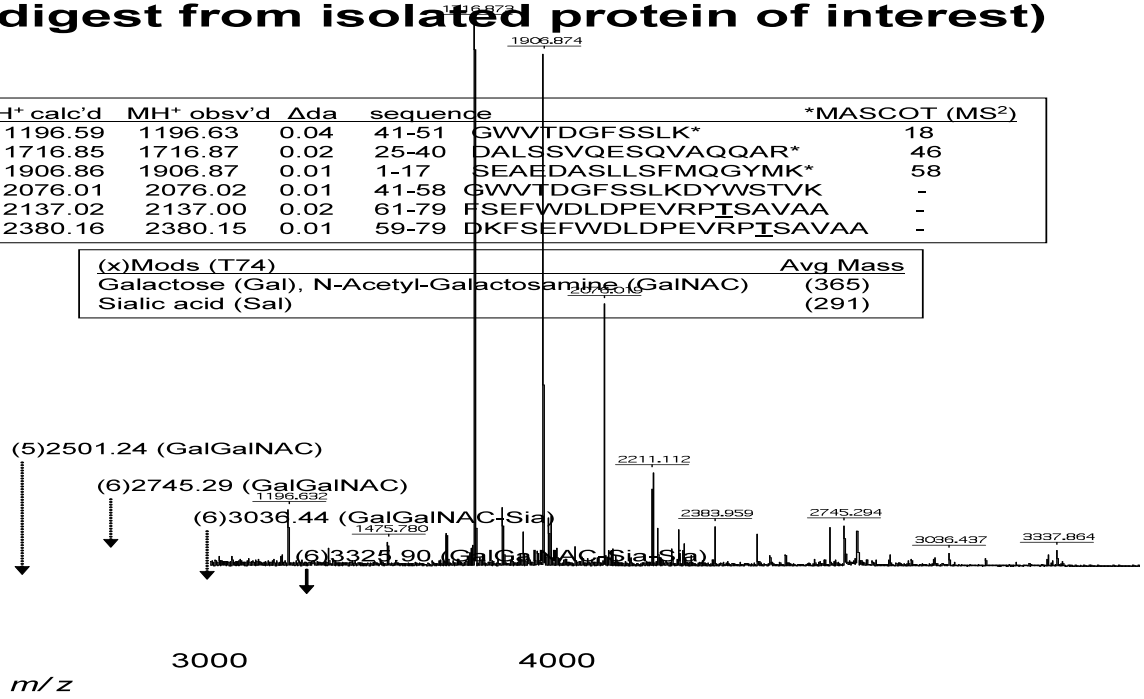
4*b*. 1D PAGE, Post LC-MALDI Selected Fractions



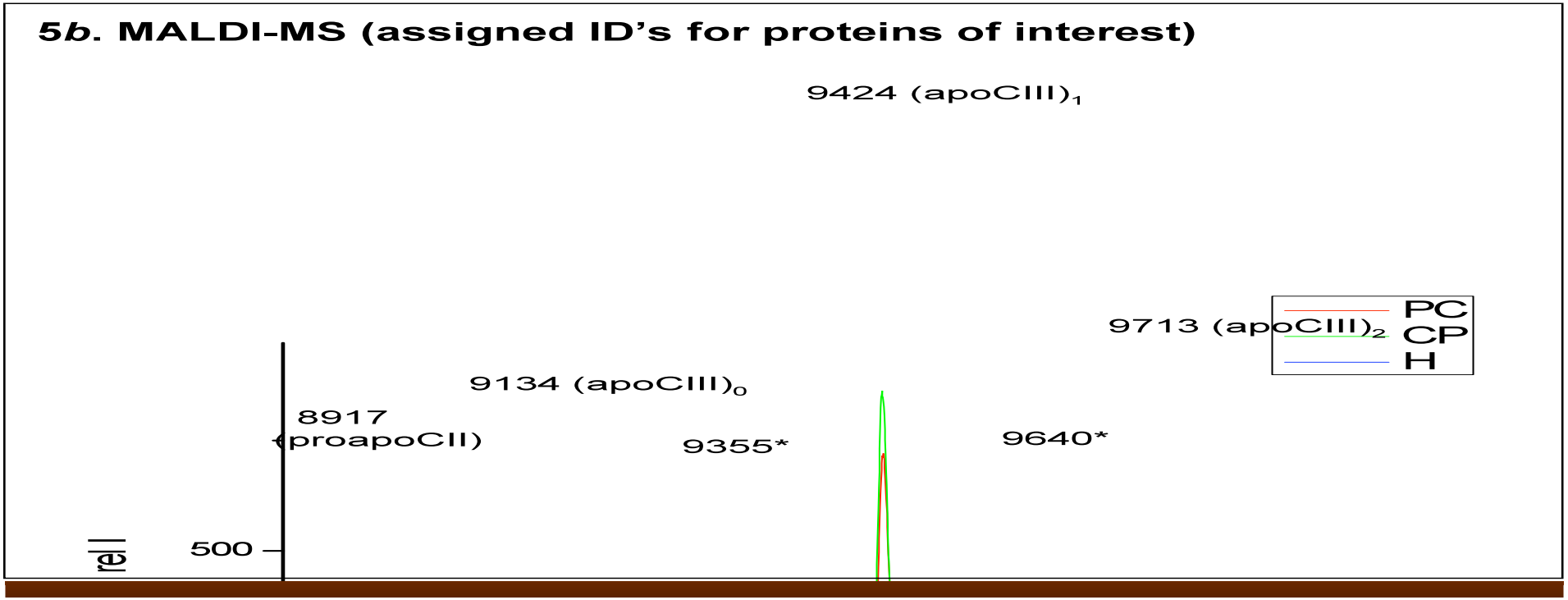
5*a*. MALDI-MS PMF (tryptic digest from isolated protein of interest)

MH⁺ calc'd	MH⁺ obsv'd	Δda	sequence		*MASCOT (MS²)
1) 1196.59	1196.63	0.04	41-51	GWVTDGFSSLK*	18
2) 1716.85	1716.87	0.02	25-40	DALSSVQESQVAQQAR*	46
3) 1906.86	1906.87	0.01	1-17	SEAEDASLLSFMQGYMK*	58
4) 2076.01	2076.02	0.01	41-58	GWVTDGFSSLKDYWSTVK	-
5) 2137.02	2137.00	0.02	61-79	FSEFWDLDPEVRPTSAVAA	-
6) 2380.16	2380.15	0.01	59-79	DKFSEFWDLDPEVRPTSAVAA	-

(x)Mods (T74)	Avg Mass
Galactose (Gal), N-Acetyl-Galactosamine (GalNAC)	(365)
Sialic acid (Sal)	(291)

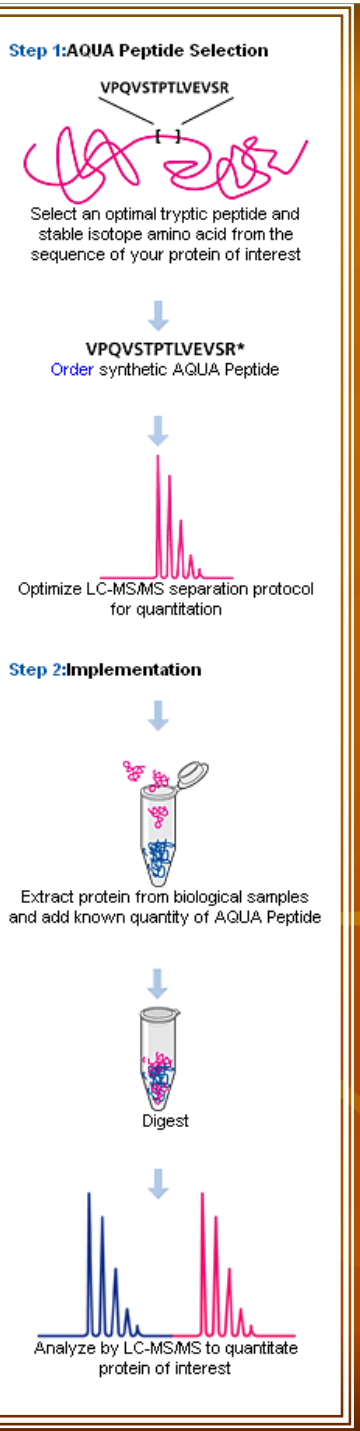


5*b*. MALDI-MS (assigned ID's for proteins of interest)



What to do With these Markers?

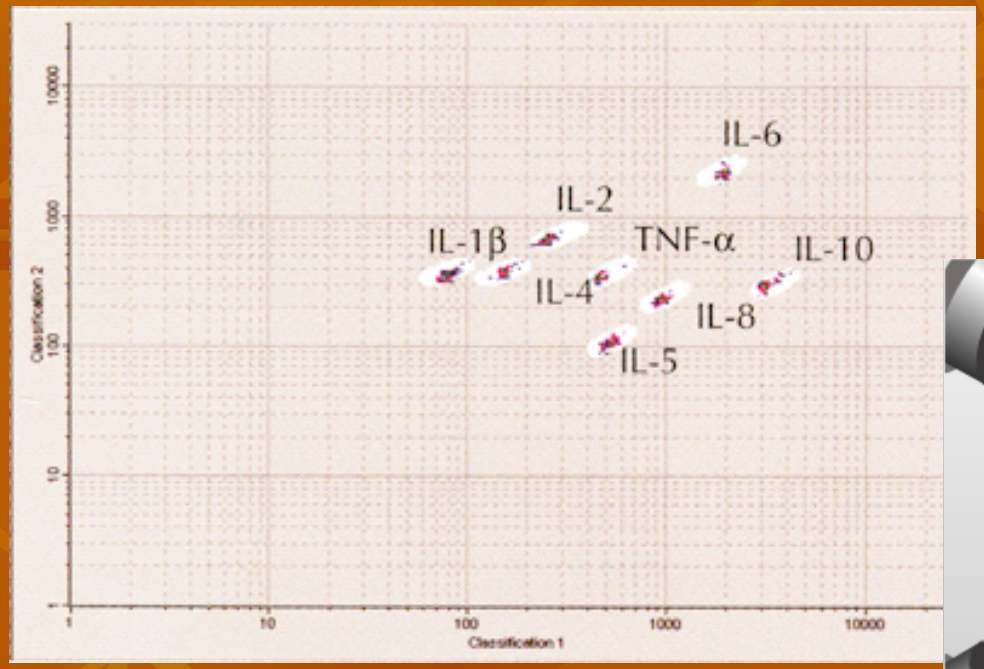
- First and foremost validation using immuno-directed or quantitative MS techniques must also be carried out.
- Mechanistic studies, i.e. knocking out a gene found to be involved in the disease process (LOF).
- This can be combined with global or directed stable isotope label studies in cell culture (i.e. SILAC, ITRAQ, ICAT).

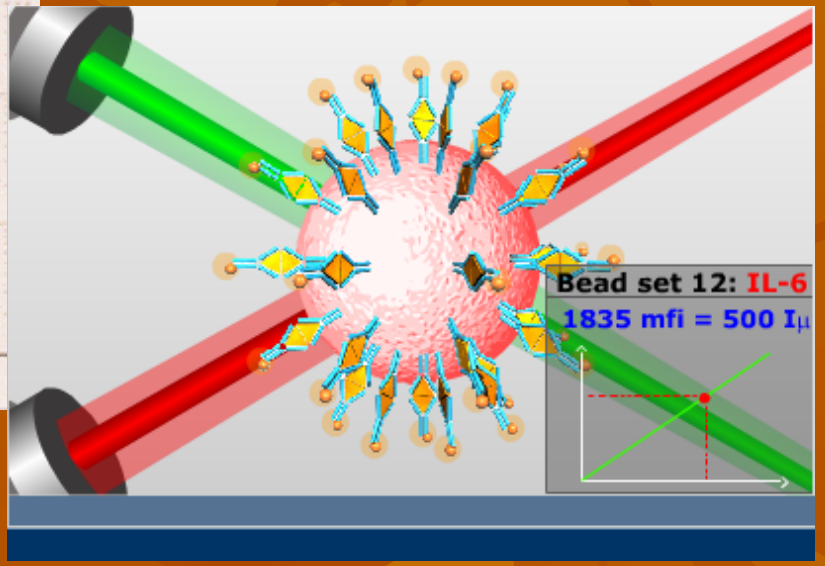


Absolute Quantification of Abundance *(AQUA)*

1) Select "best" peptide for analysis!
2) Purchase synthetic heavy labeled (^{13}C) equivalent.
3) Add known amount of std peptide(s) to samples to be tested.
4) Run MRM study to quantify as many proteins as you want!

Ex: HTP Validation of Novel Markers with Multiplex Bead Assays (Luminex)





Multiplex Bead Assay for cytokines

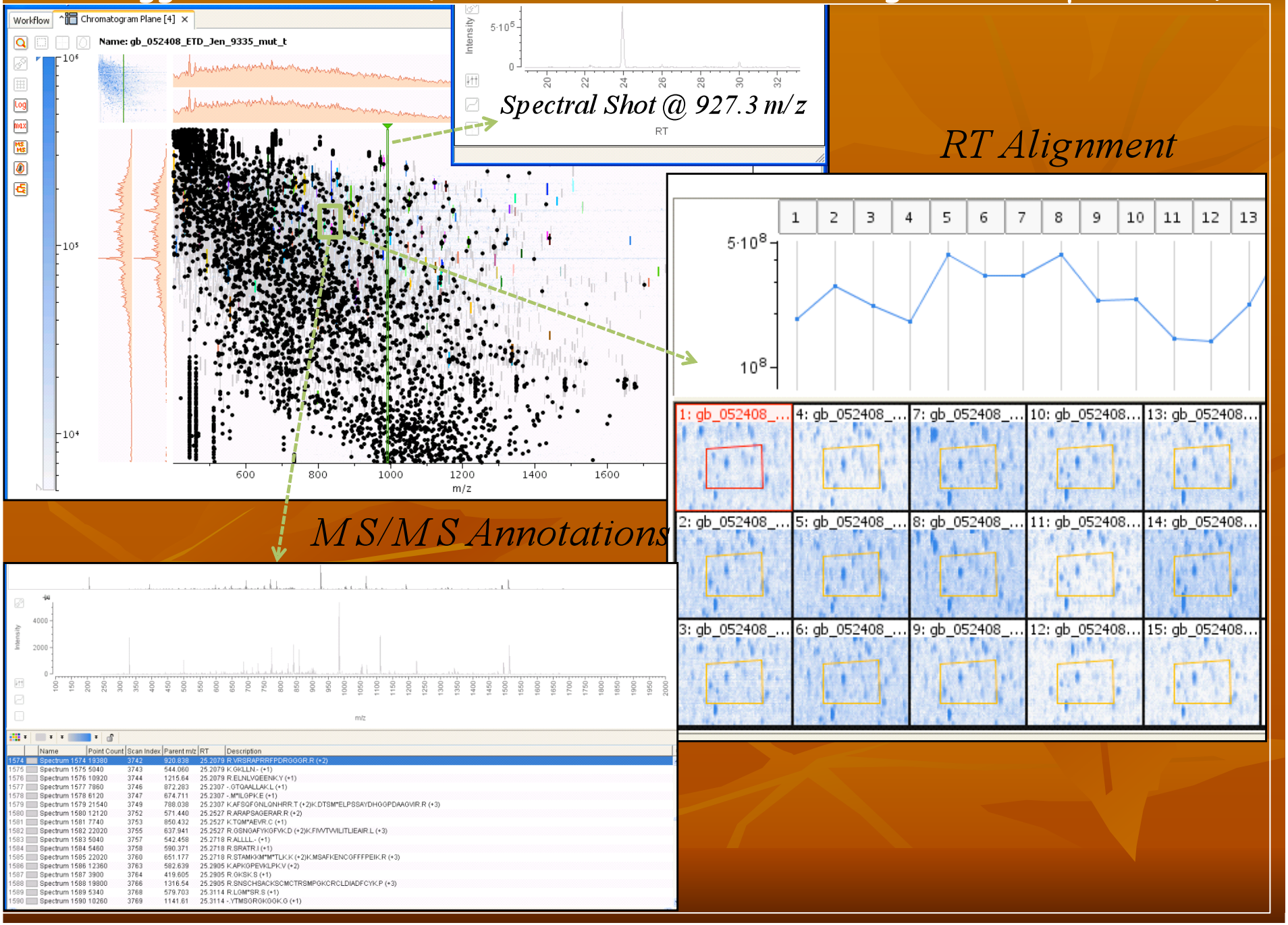
The highlighted area represent populations of fluorescent beads, distinctively labeled, and carrying capture antibodies for sandwich assay of different cytokines.
All detection antibodies carry the same fluorophore, which is read in a third channel to quantify sample cytokine concentration

Bosch I. et. al. work in progress!

Example: Prostate Cancer Markers of Progression

Non-Tagged LCMS Peptidomics

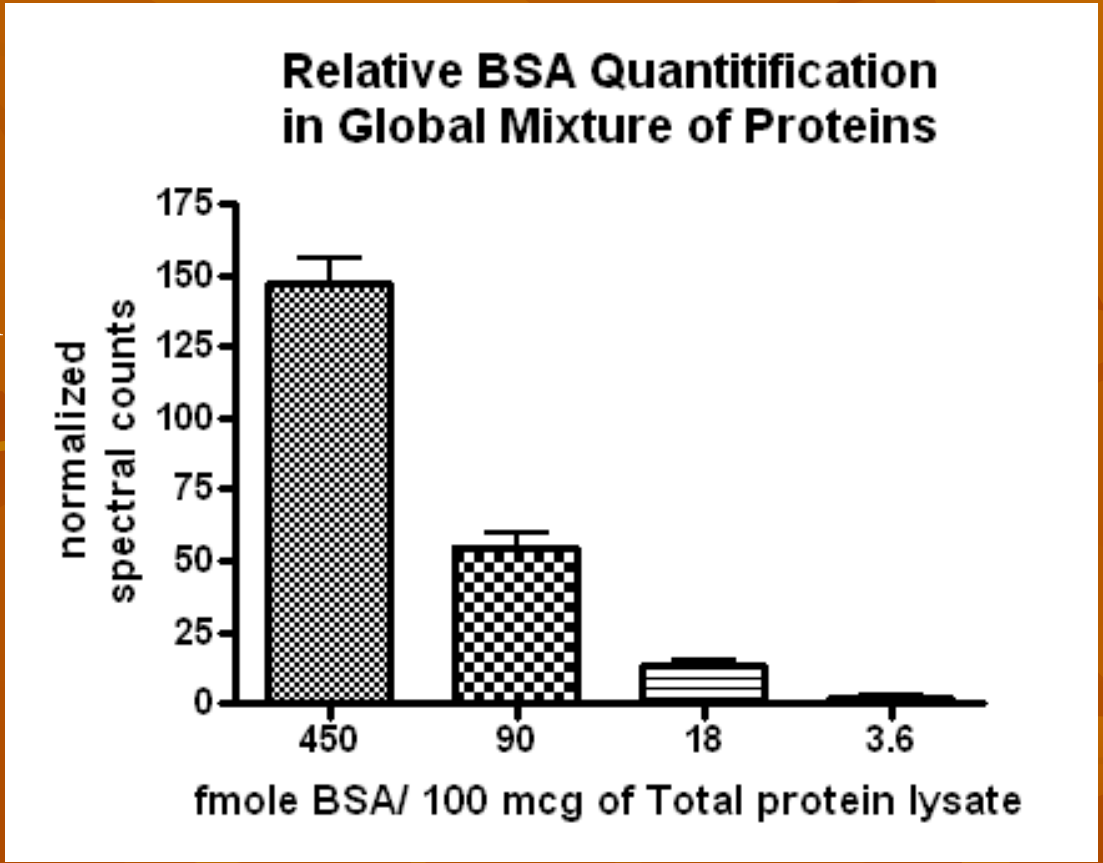
Non-Tagged Proteomics (2D Plots Rendered Following LCMS Experiment)

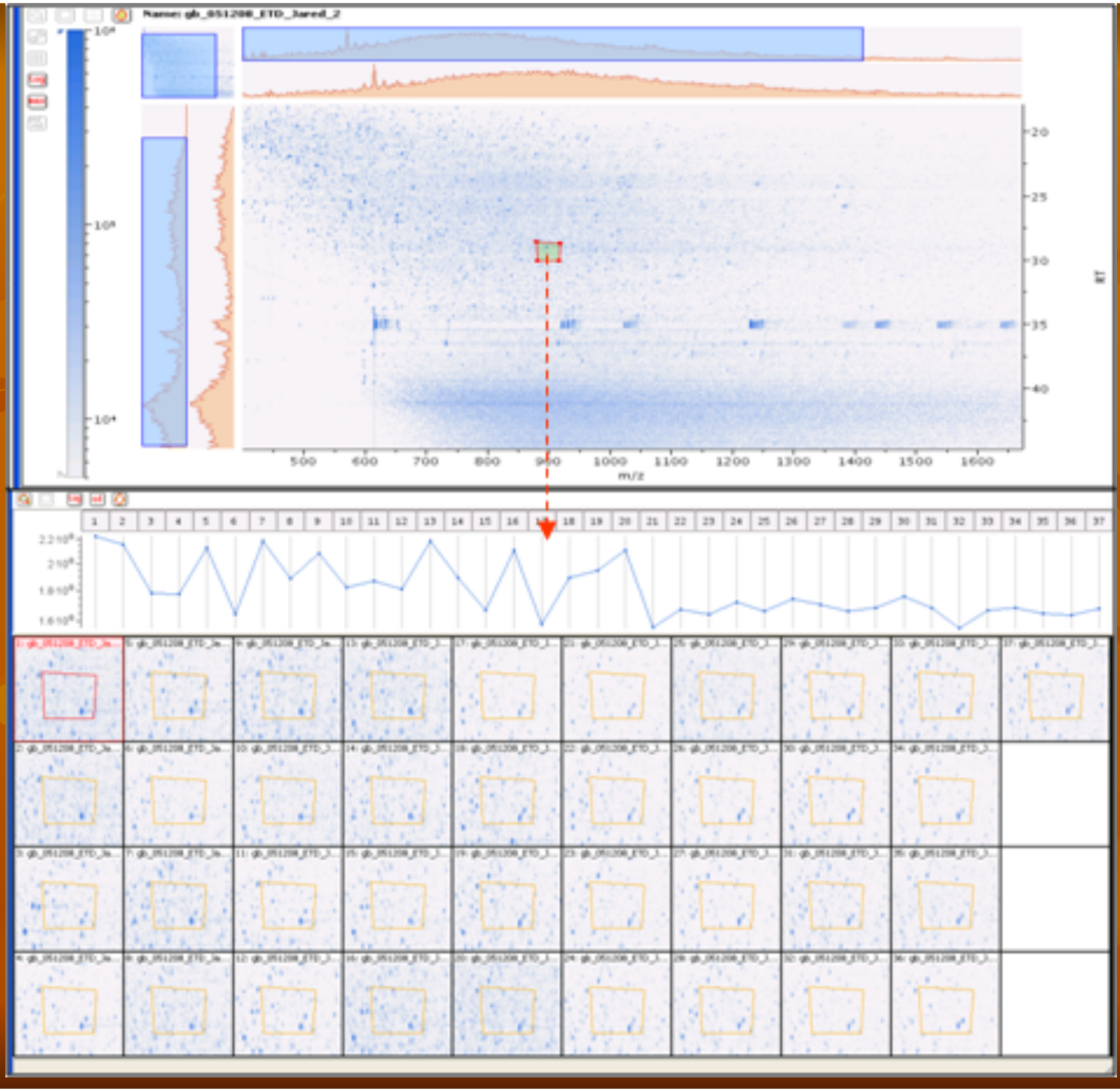


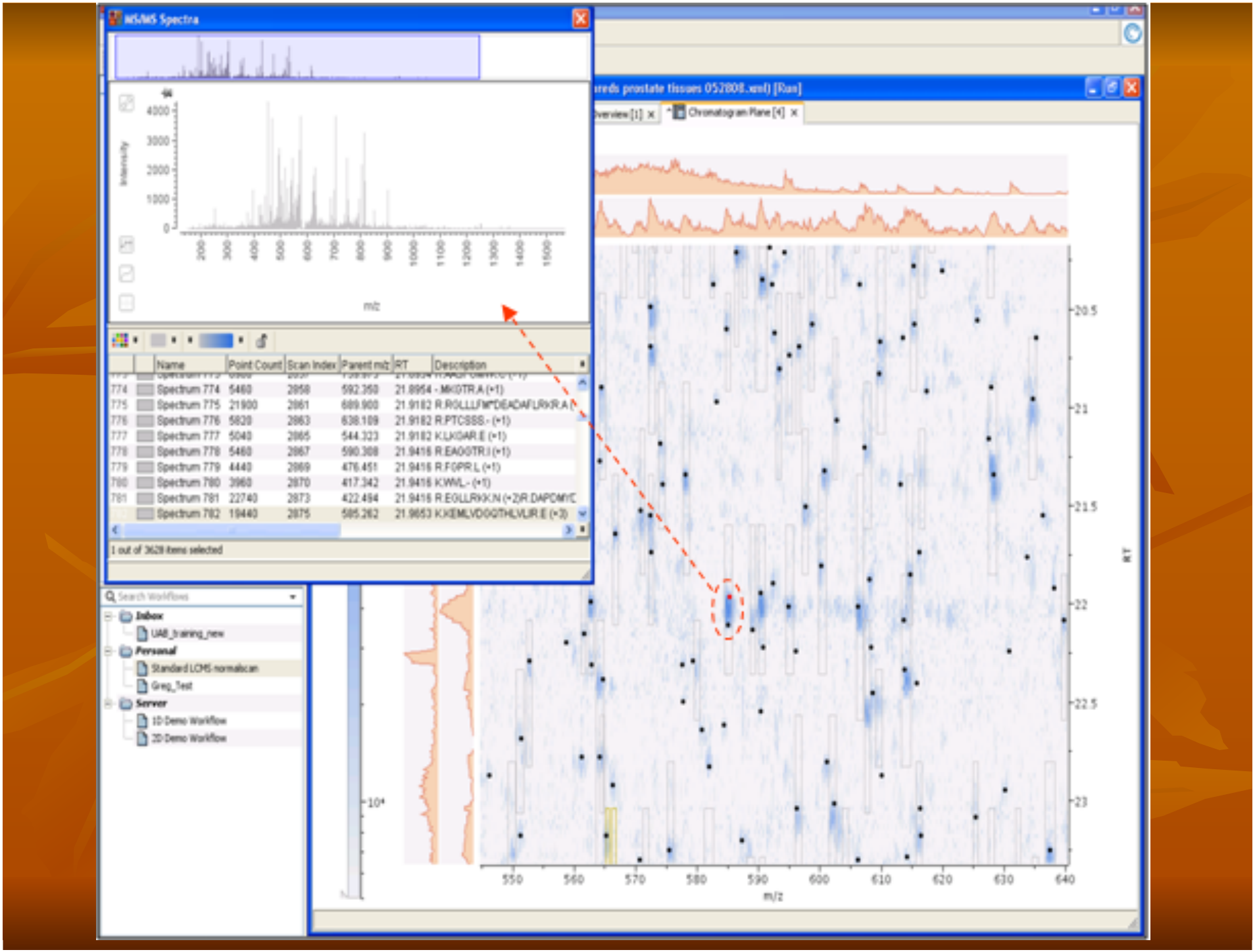
But does this work?
Similar outcomes using various total ion counting and spectral ion counting methods

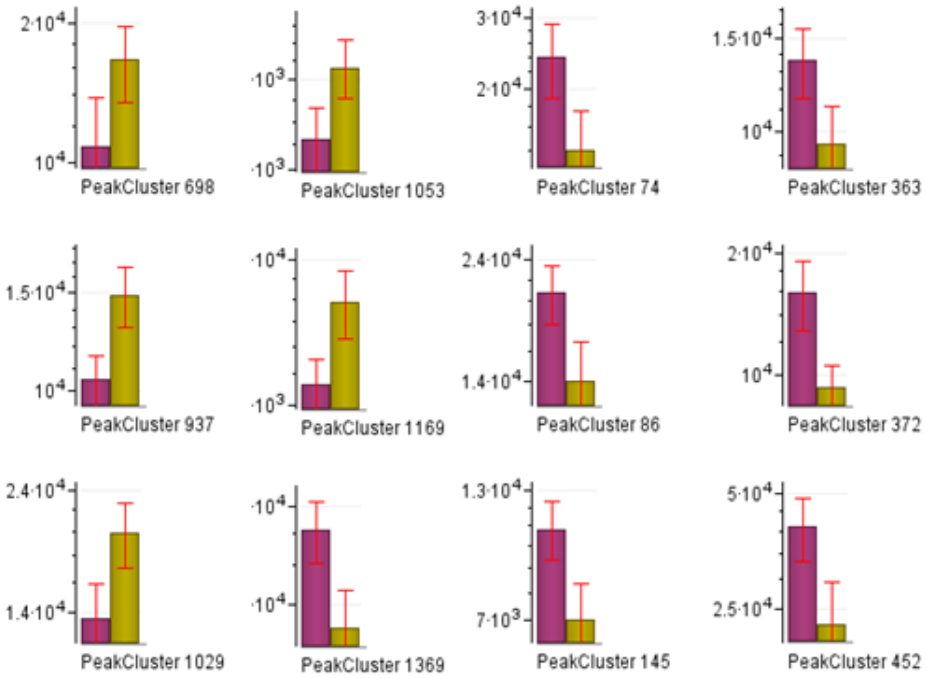
Purified BSA std doped at varied concentrations into protein isolates from rat brain protein isolates.

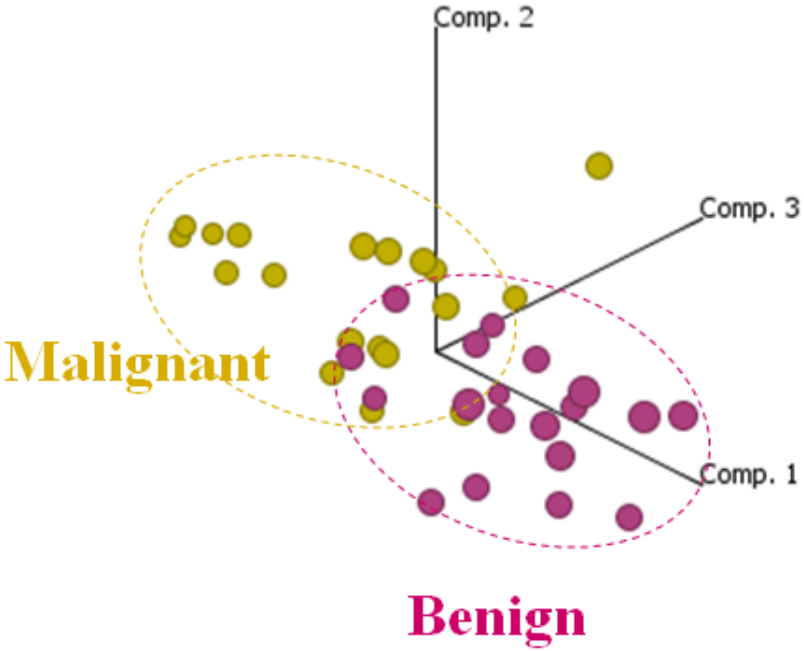
Samples were digested and run in replicates of n=5 and processed using spectral counting and total ion counting.




500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
m/z
20
25
30
35
40
RT

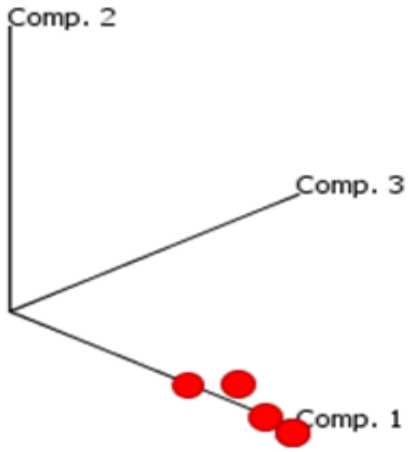

MS/MS Spectra
Intensity
m/z
Name
Point Count
Scan Index
Parent m/z
RT
Description
1 out of 3628 items selected
Chromatogram Plane [4]
RT
m/z
Search Workflows
Inbox
Personal
Server


PeakCluster 698
PeakCluster 1053
PeakCluster 74
PeakCluster 363
PeakCluster 937
PeakCluster 1169
PeakCluster 86
PeakCluster 372
PeakCluster 1029
PeakCluster 1369
PeakCluster 145
PeakCluster 452


Comp. 2
Comp. 3
Comp. 1
Malignant
Benign

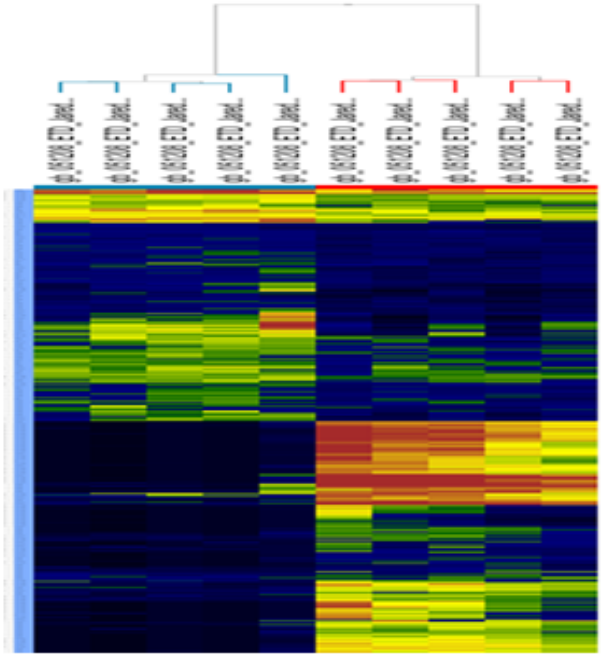
Malignant

(low grade 9)

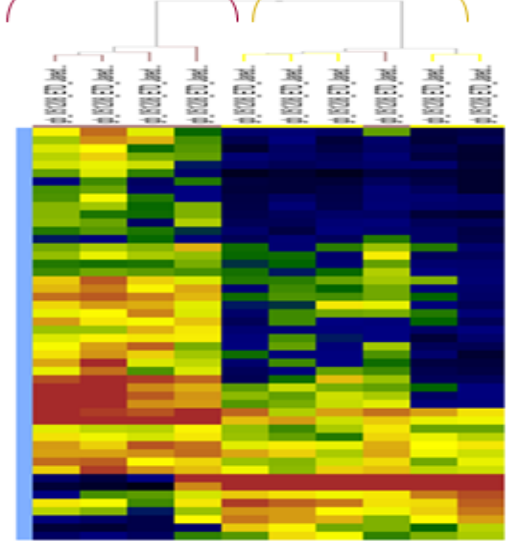


Malignant

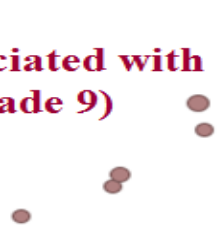
(high grade 9)

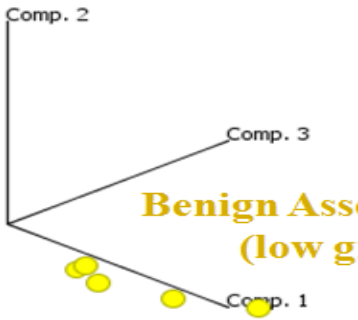


grade 9 grade 6



Benign Associated with
(high grade 9)





Benign Associated with
(low grade 6)

Protein Identified in this Study:

Diagnostic - Cancer Specific

Heat shock cognate 71 kDa protein, Prostate specific antigen, Alpha-2-HS-glycoprotein, IGHA2, Golgi phosphoprotein 2, APS protein, 60 kDa heat shock protein, RcTPM3, Macrophage migration inhibitory factor, Histone 1, Antithrombin-III, Peroxiredoxin-1, Alpha-enolase, Malate dehydrogenase, Fructose-bisphosphate aldolase, ATP synthase subunit alpha

Prognostic - Grade Specific

Malate dehydrogenase, Alpha-enolase, Tropomyosin, Filamin-A, Apolipoprotein A-I precursor (Apo-AI), Zinc-alpha-2-glycoprotein, Serotransferrin, prostate specific antigen, Lipoma-preferred partner, Prostatic acid phosphatase, APS protein, Vimentin, Calponin-1, Cytochrome c oxidase, Alpha-1-antichymotrypsin, Smooth muscle myosin, Beta-microseminoprotein, Elongation factor 1-alpha 1

Summary

- The end result, we are just touching the edge of this new technology and it all starts with a solid MS and proteomics based foundation that is tied to high level computing and ever improving software.
- A combination of instruments and applications get us much further down the road!

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Questions?