

Profiling and Imaging Mass Spectrometry

Compilation of Work from the Caprioli Lab

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Primary Focus of Today's Lecture?

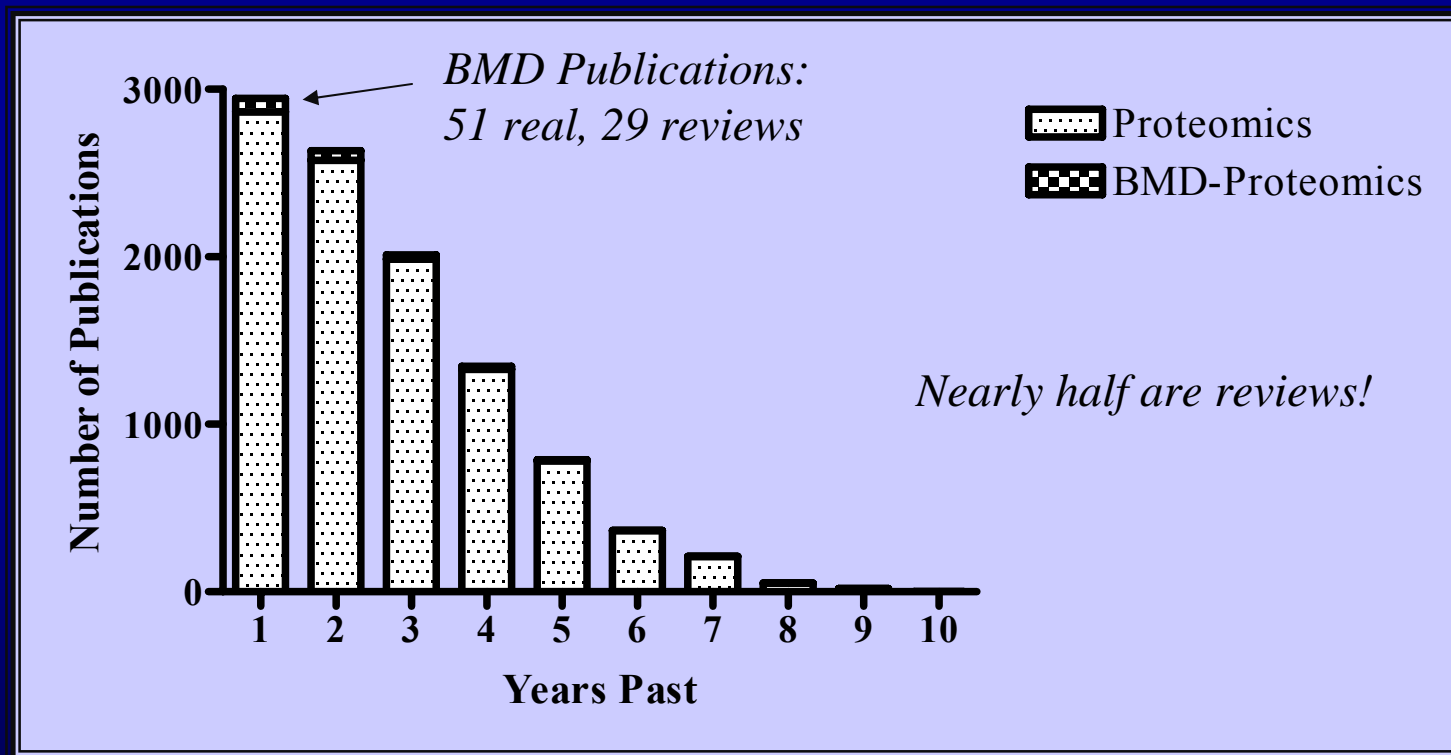
- Brief Overview of Biomarker Discovery (BMD) for Clinical Applications, Why do we do it, Why do we use MALDI-ToF?
- Understanding Advances in MALDI-ToF Driven Profiling of Tissue Sections for BMD, and the bottlenecks in this newly emerging field.
- How to produce a Mass Image from a Series of Profiles.
- What to Do with All that Data (Following the Workflow from Pre-processing to Statistical Analysis).
- How to ID those Peaks, are they really Proteins?

First off.....

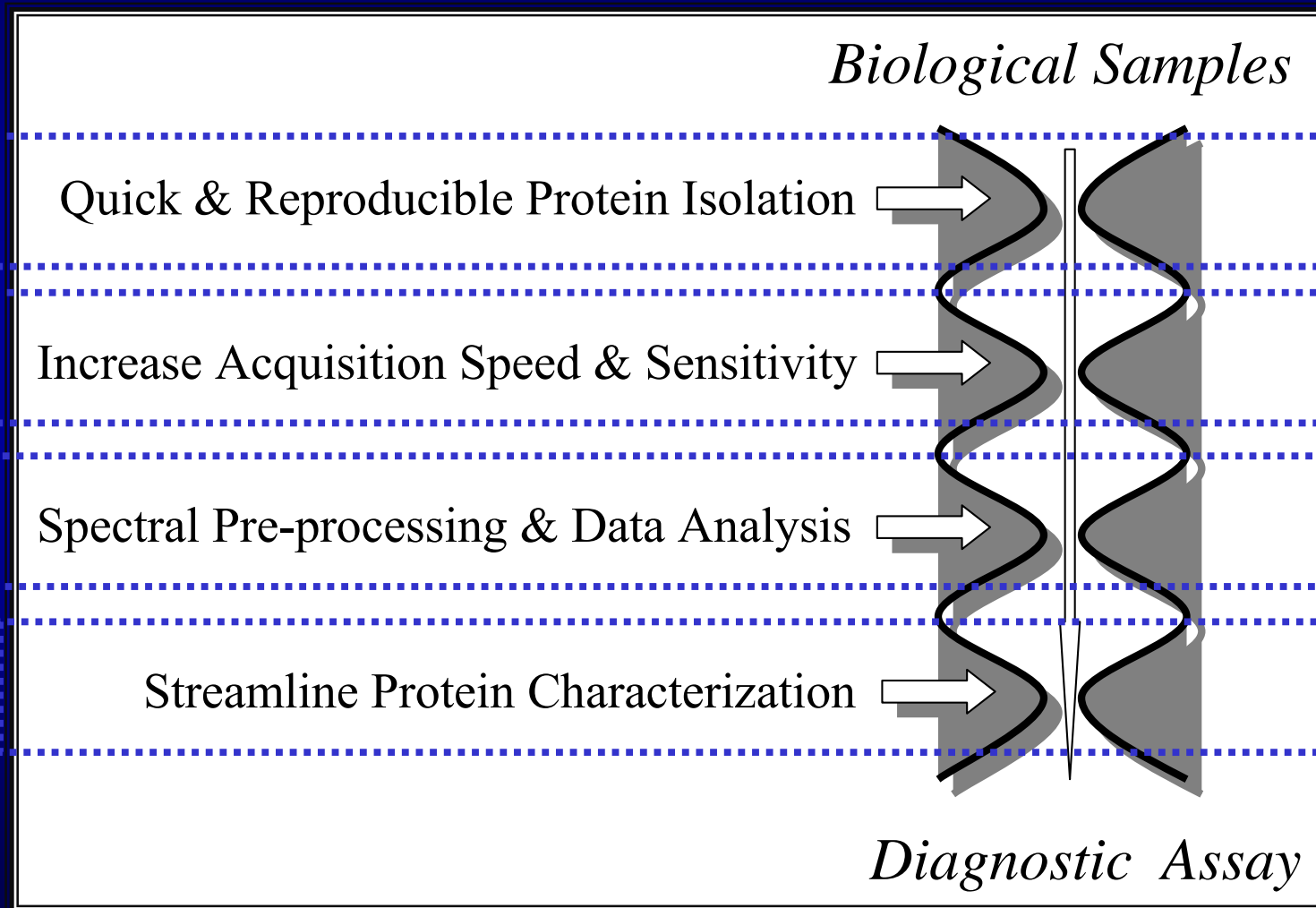
Why Do Biomarker Discovery?

- To find associations between biological components (i.e. SM, FA's, Proteins) and any clinical endpoint quickly, non-invasively, affordably.
- To non-invasively determine.....
 - Pathologic Changes (i.e. early detection of cancer)
 - Aggressiveness/ Stage of Disease
 - Predicting Rx Response
 - Drug Target Discovery
 - Mechanistic Studies (Systems Biology)
- The Potential Clinical Impact is Tremendous!!

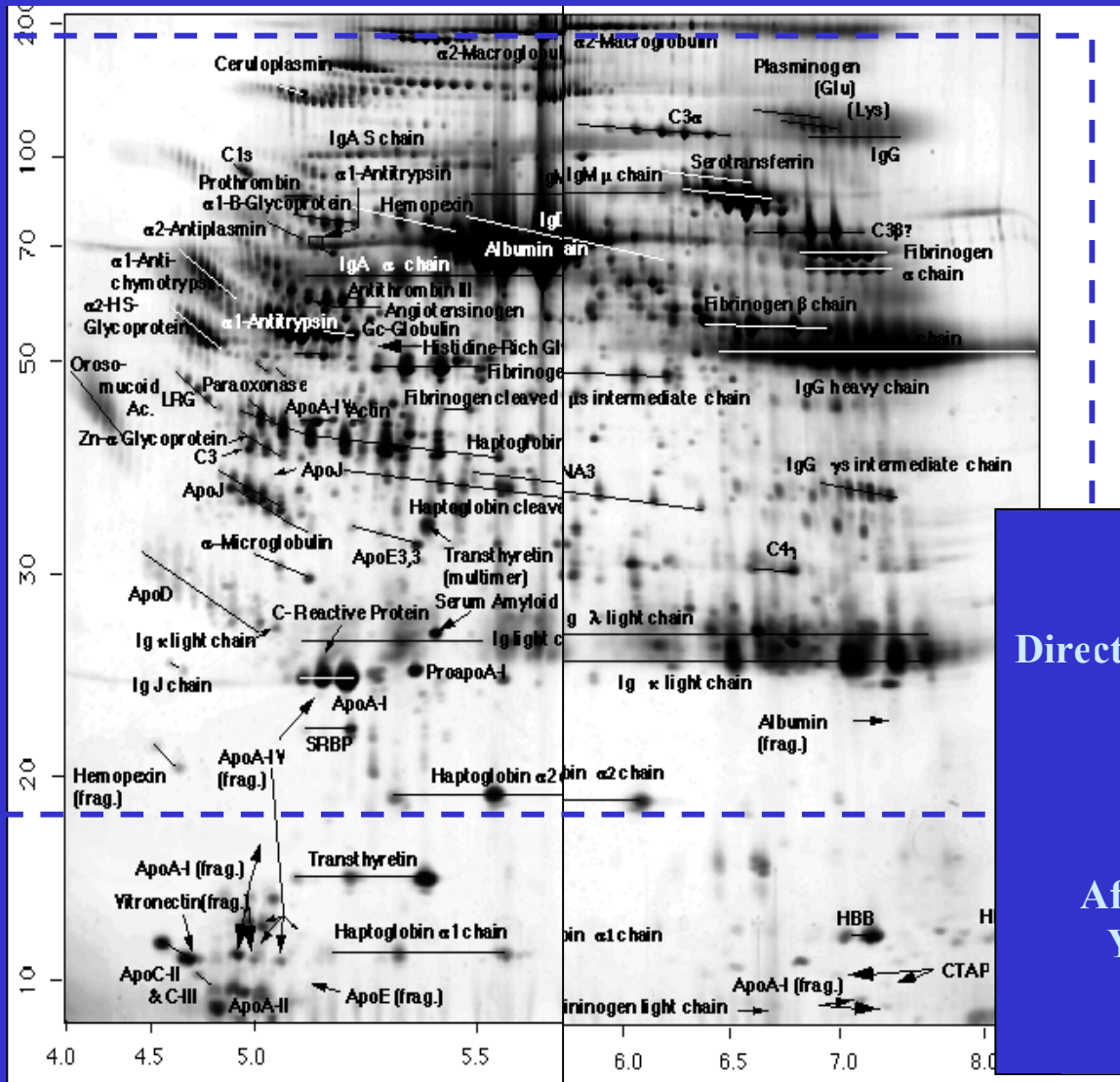
Using Proteins as an Example; *How new is BMD?*



Bottlenecks in Biomarker Discovery



Can 2D PAGE Get Us There?



Serum Proteome

Anderson, PNAS, 1977

Direct Analysis ~1000 Protein Spots



25yrs later

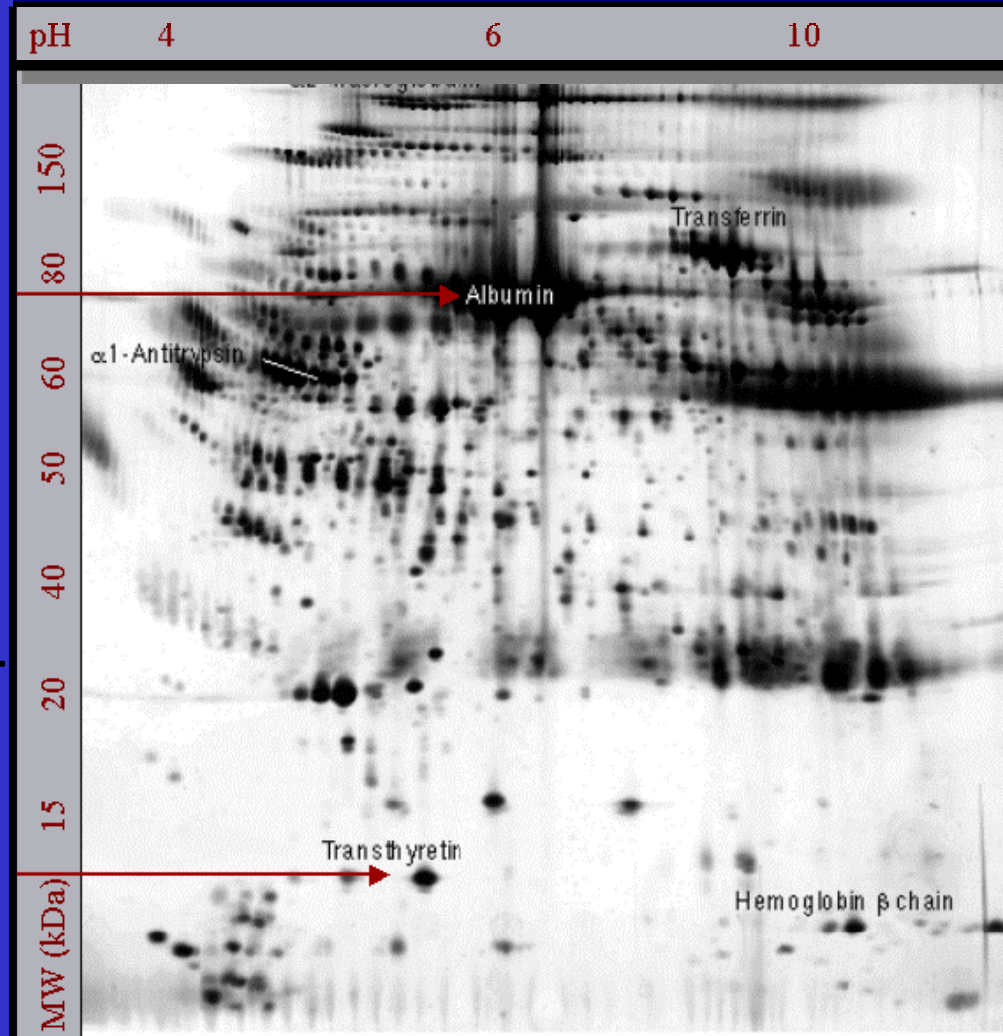
Pieper, Proteome, 2003

Affinity, SCX, Size Exclusion
Yielded 74 Fractions - 2D

3700 Proteins Spots
327 Distinct Proteins

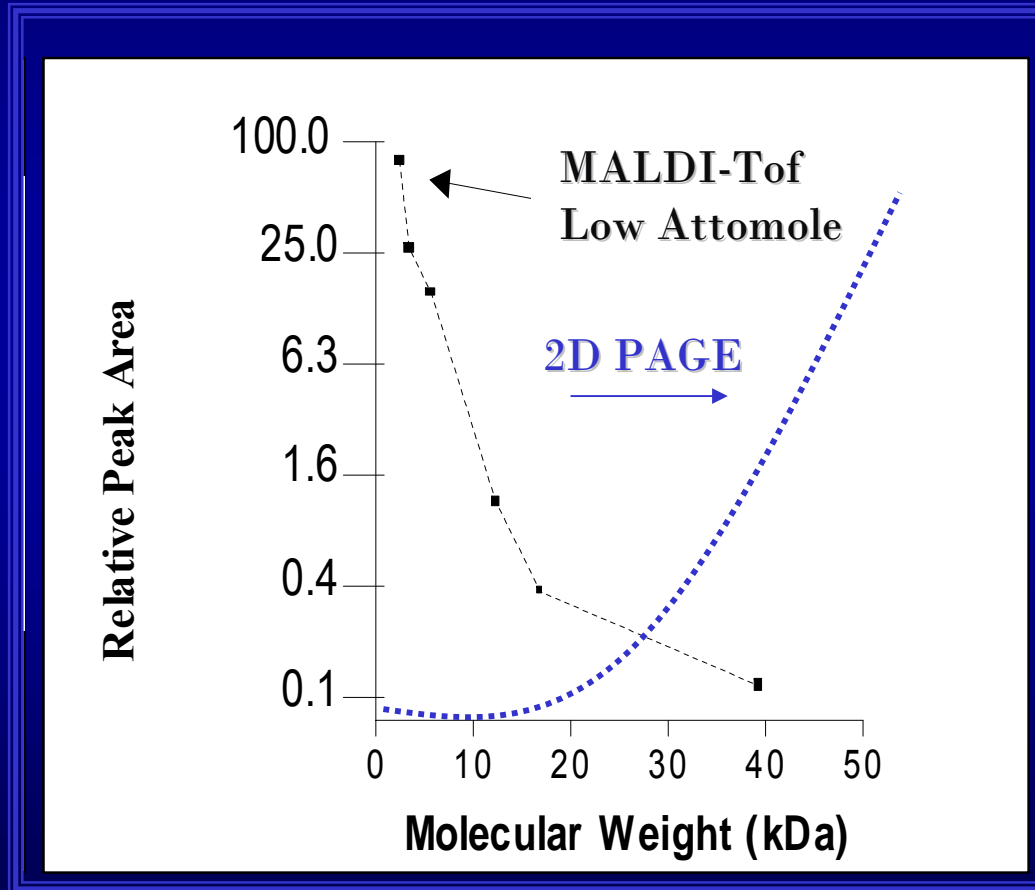
What About MALDI-ToF for Biomarker Discovery?

2D PAGE, ID by MALDI-ToF



Primarily LMW Proteins!

Sensitivity is Inversely Proportional to Mass (MALDI-ToF Example)



Taking a Closer Look at our Proposed Target

Primary Target: Many growth factors and cytokines are secreted into the plasma.

Growth factor were previously referred to substances that promote cell growth. Promote/ Inhibit: mitogenesis, chemotaxis, apoptosis, angiogenesis, differentiation.

Cytokines were simply known as proteins that exhibited immuno-modulating effects. A generic name for a diverse group humoral regulators.

Chemokines are a family of cytokines previously referred to as the SIS, SIG, SCY, PF4, and Intercrine families. **8-10 kDa**, chemotactic agents, with high homology (contain C, CC, CXC, or CX3C).

Known In Late 90's, the cytokine family was limited mainly consisted of **22 lymphokines**; **But Now**, Cytokines, including those with no names: **491**, Separate Chemokines: **345**

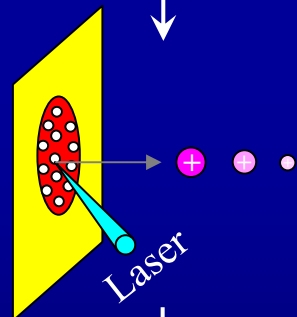
Primary Source: Cytokines Online Pathfinder Encyclopaedia
<http://www.copewithcytokines.de/>

Advantages of MALDI-ToF for Profiling Biological Soln's and Tissue Sections

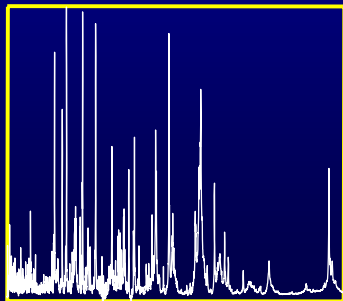
- Resistant to many impurities, robust!
- Highly sensitive in low mass range, which is just starting to be chartered.
- Direct Analysis on Tissue; “very” little protein is required for analysis.
- Analysis of crude extracts of biological fluids.
- Primarily 1+ charged proteins/ peptides (less complex spectra).
- **HTP!!**

Directed
Discovery
“Profiling”

Droplets



Protein profiles



Slice frozen tissue on
cryostat (~12 μm thick)

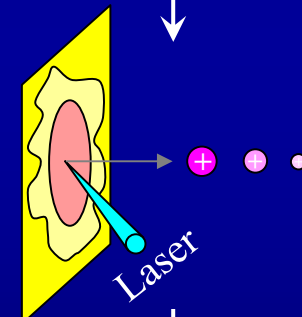
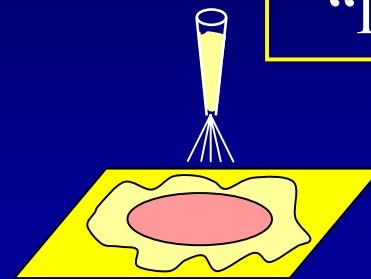
Thaw slice onto MALDI
plate, allow to dry

Apply matrix

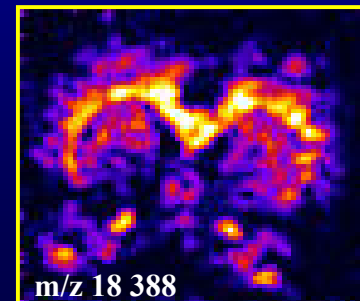
Acquire
mass spectra

Non-Directed
Discovery
“Imaging”

Spray
coating

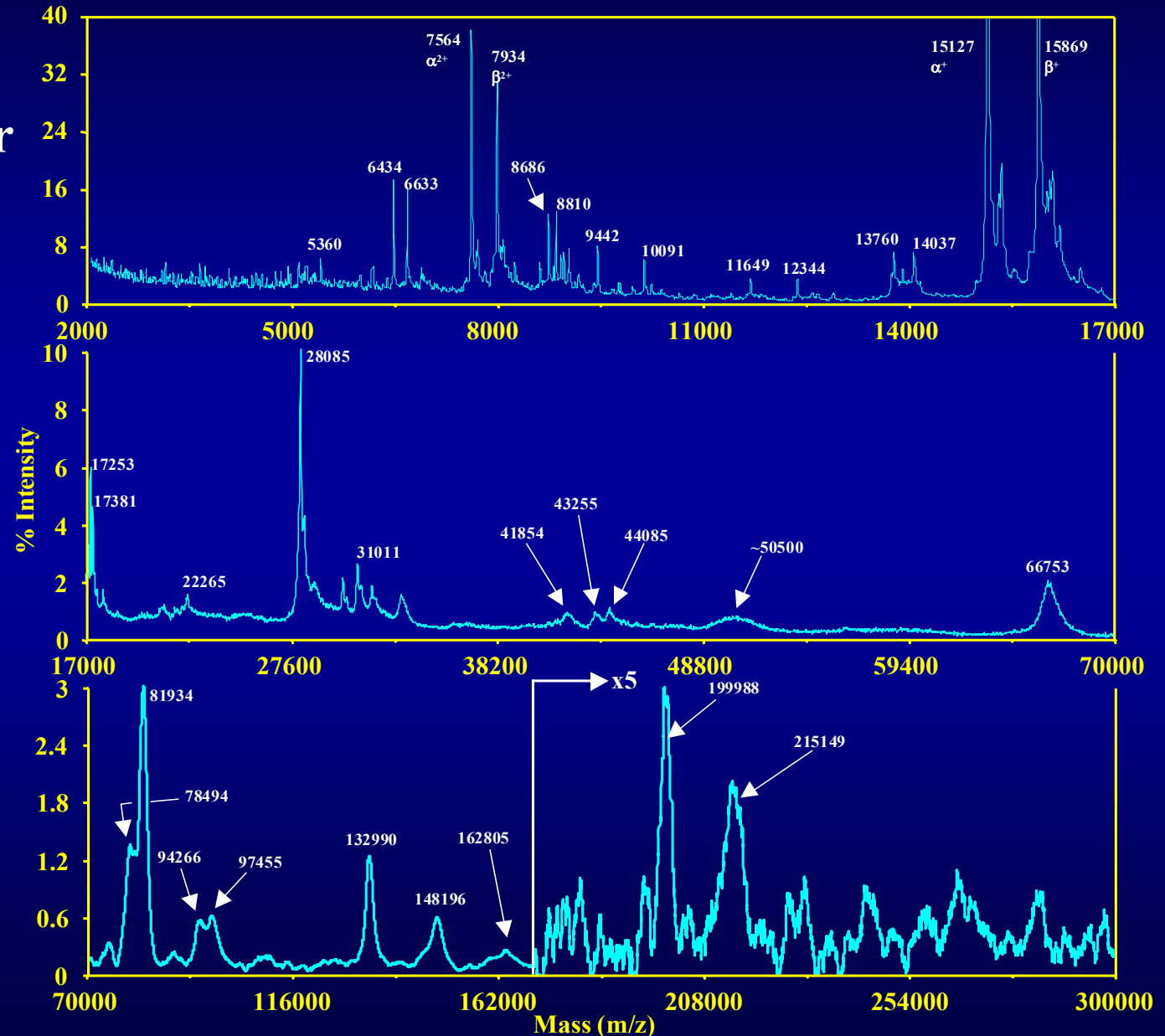
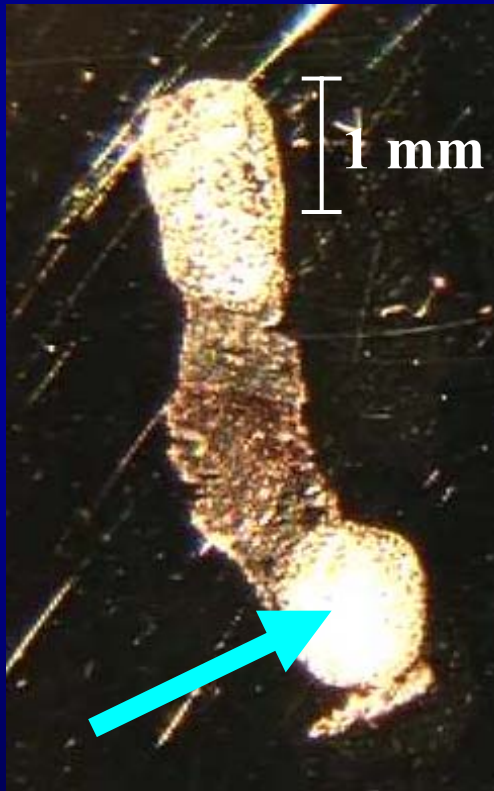


Protein images

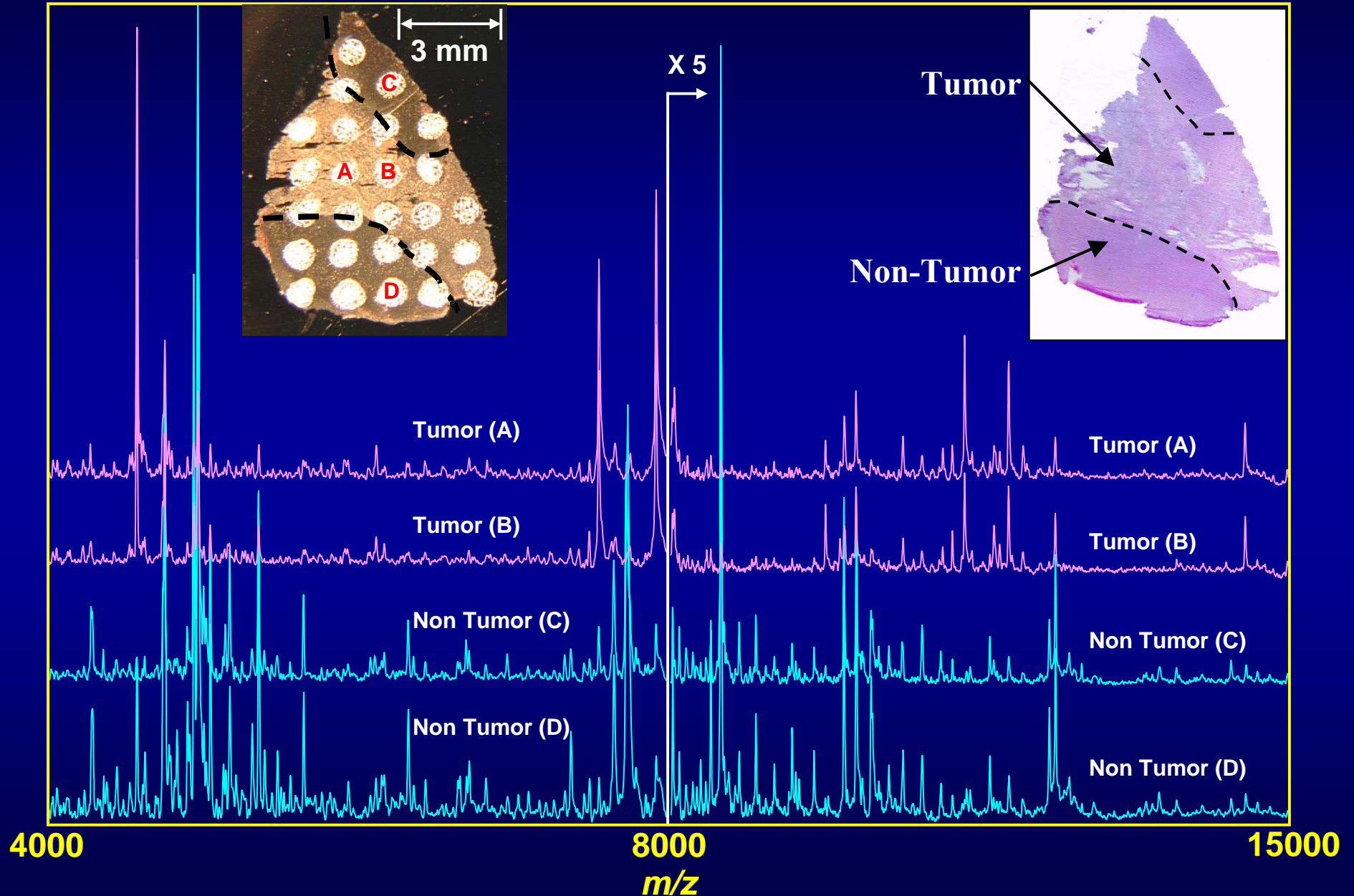


Protein Expression Profiling by MALDI-MS

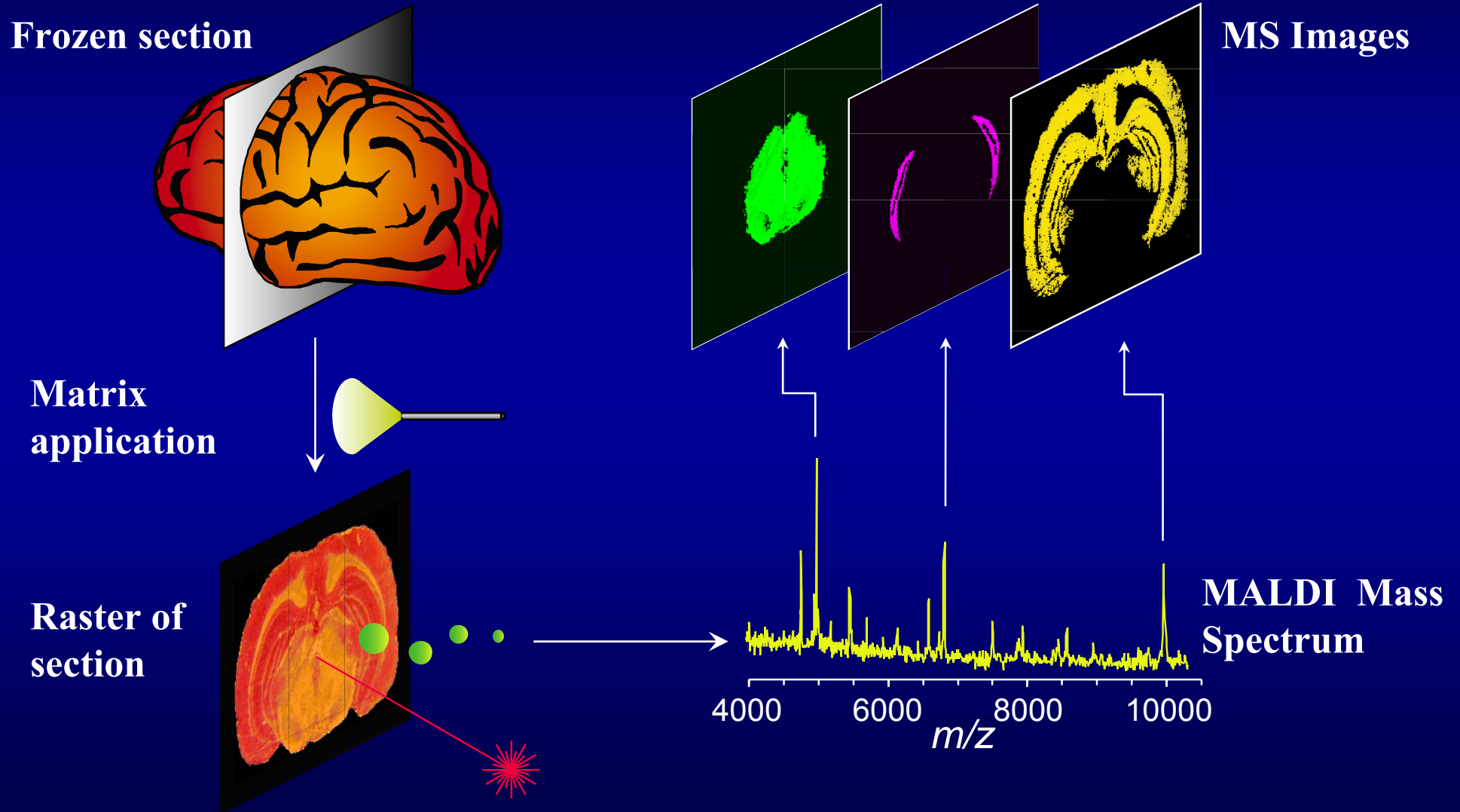
Human breast tumor
needle biopsy



Human Glioma Biopsy

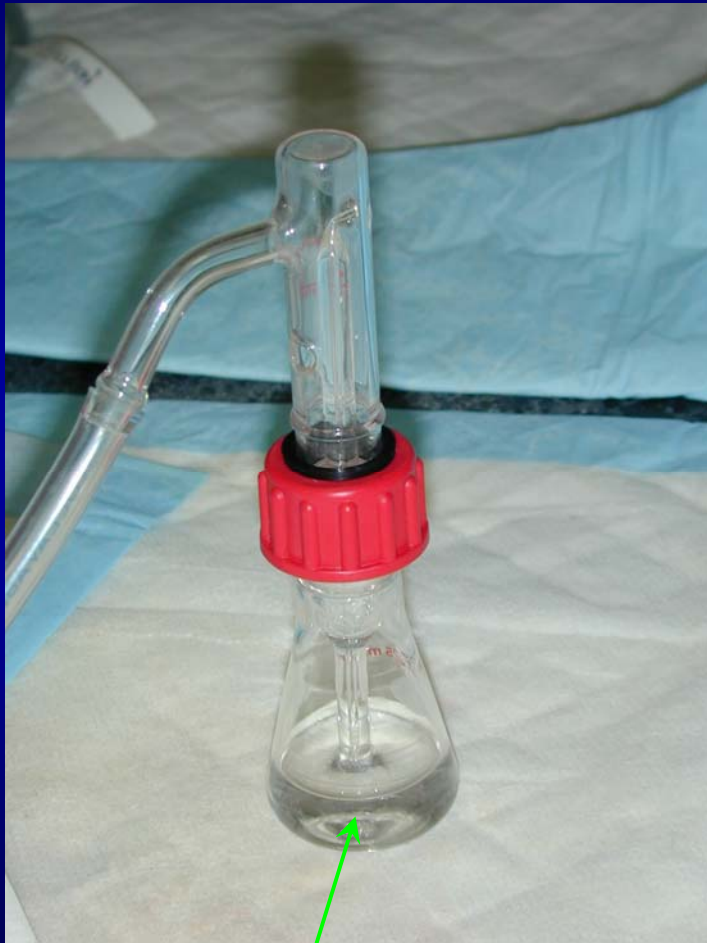


Principle of MALDI MS Imaging

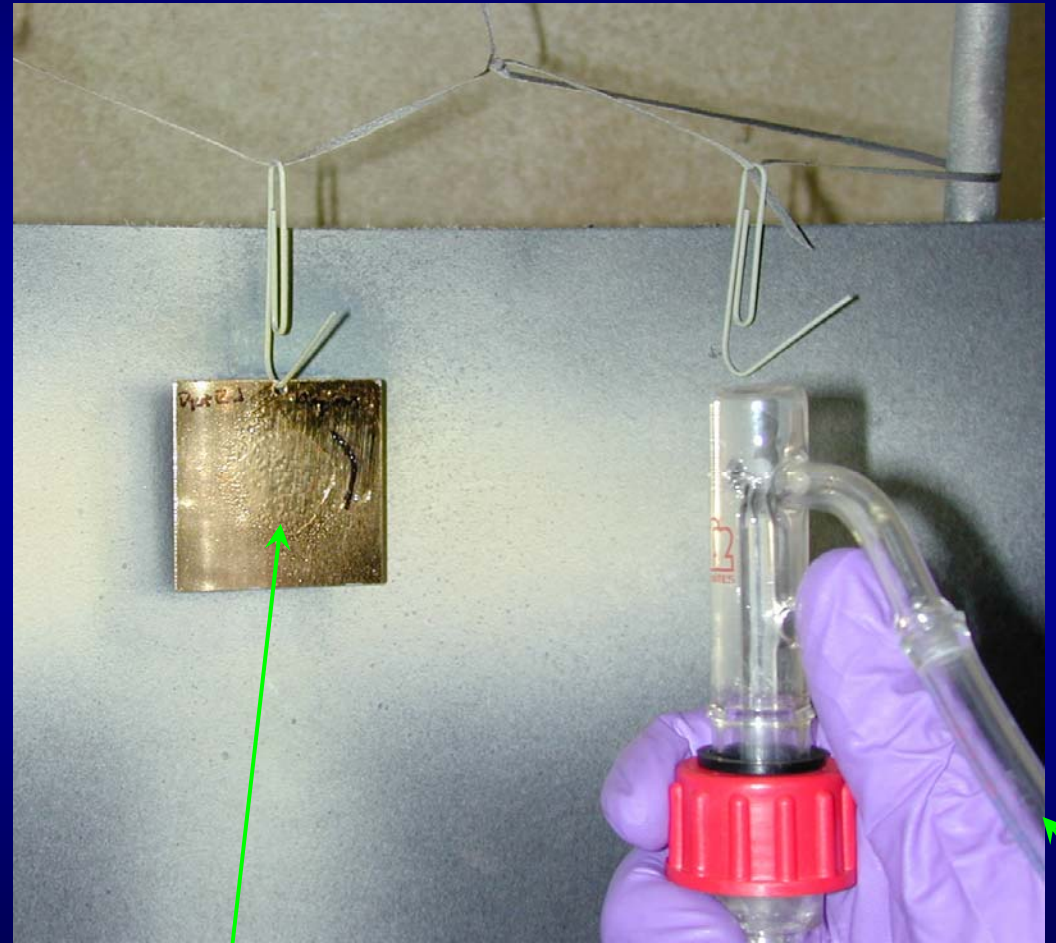


Spray Deposition of Matrix on Tissue Sections

Spray nebulizer for TLC plates



Matrix solution

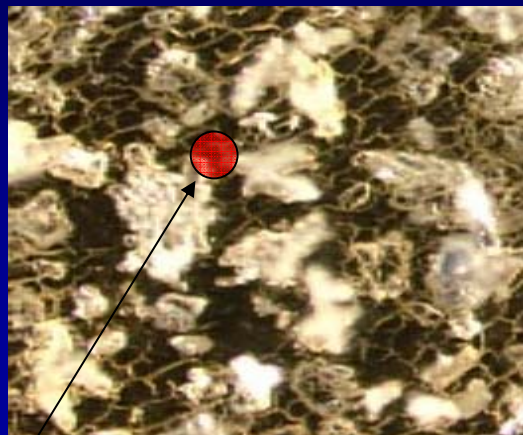


Tissue section

Nitrogen

Comparing Matrix Coatings

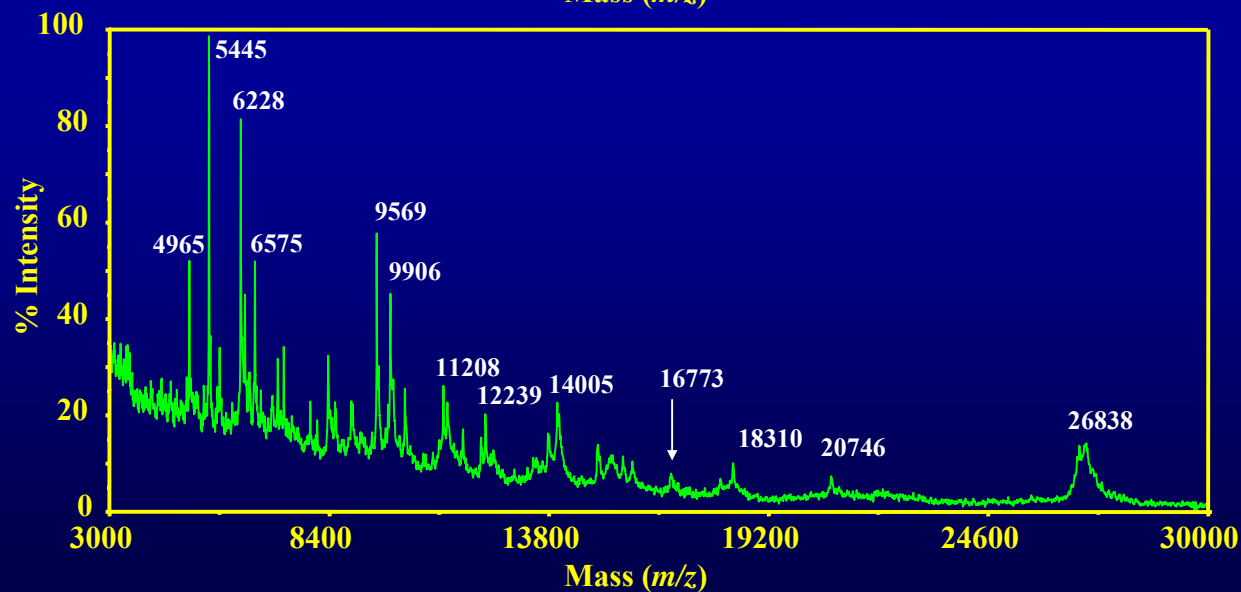
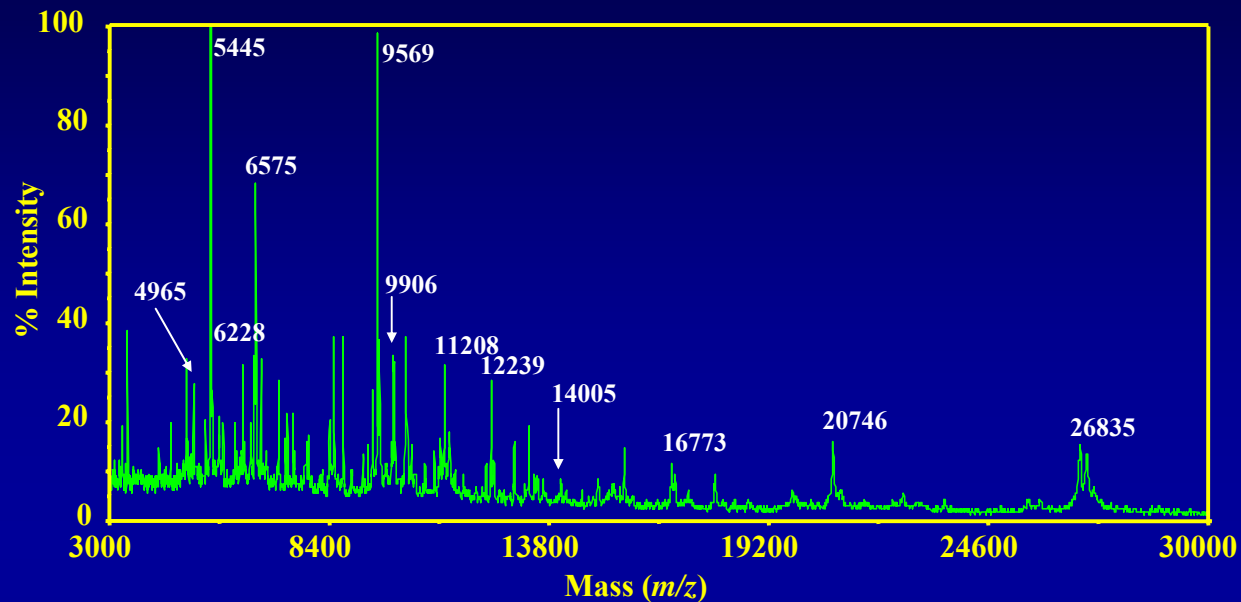
Spotted



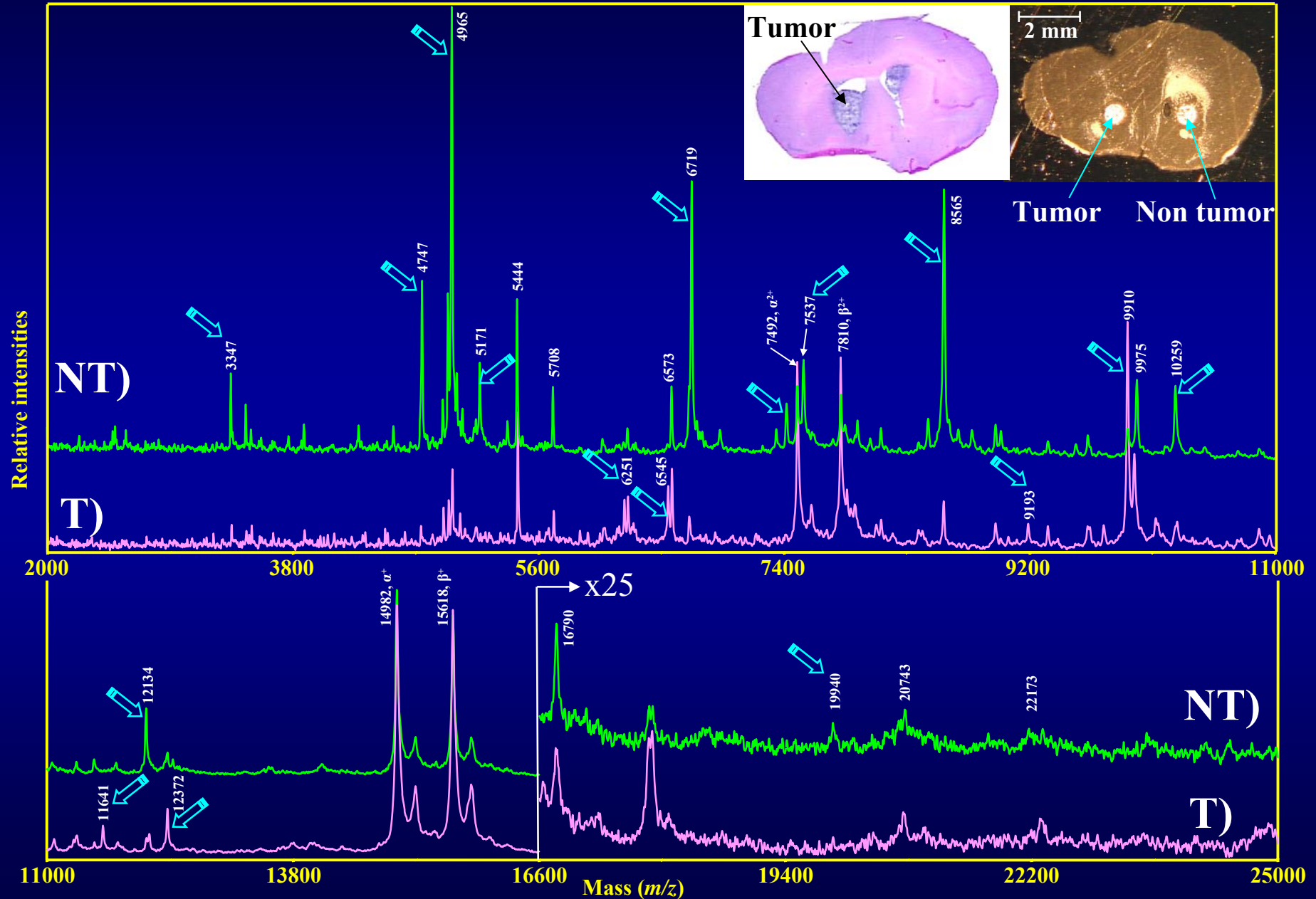
50um Laser spot

200 μm

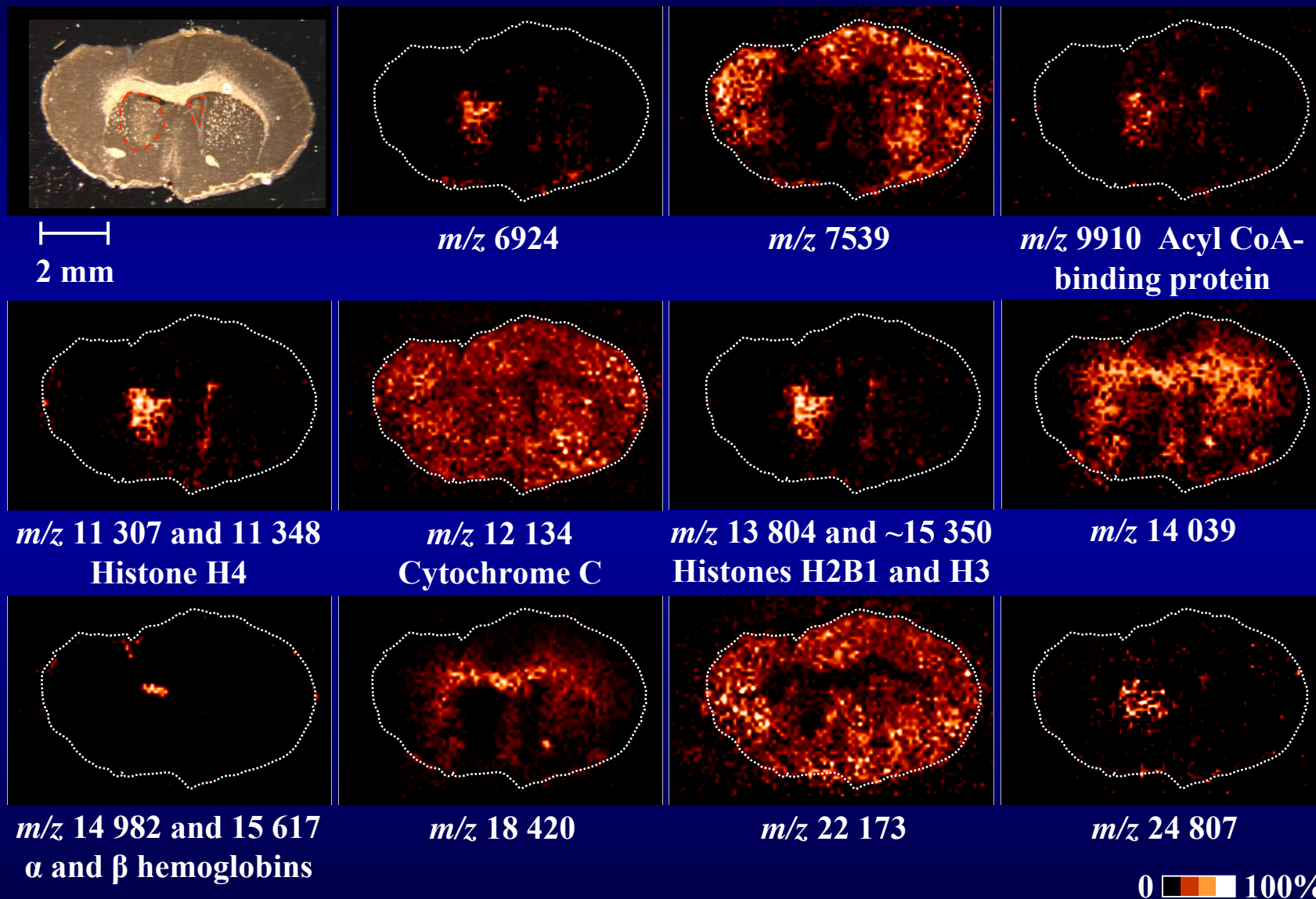
Sprayed



Glioma Mouse Model - Intracranial Injection of GL261 Cancer Cells



Glioma Mouse Model. Imaging Resolution: 100 μm



Can We Do Better?

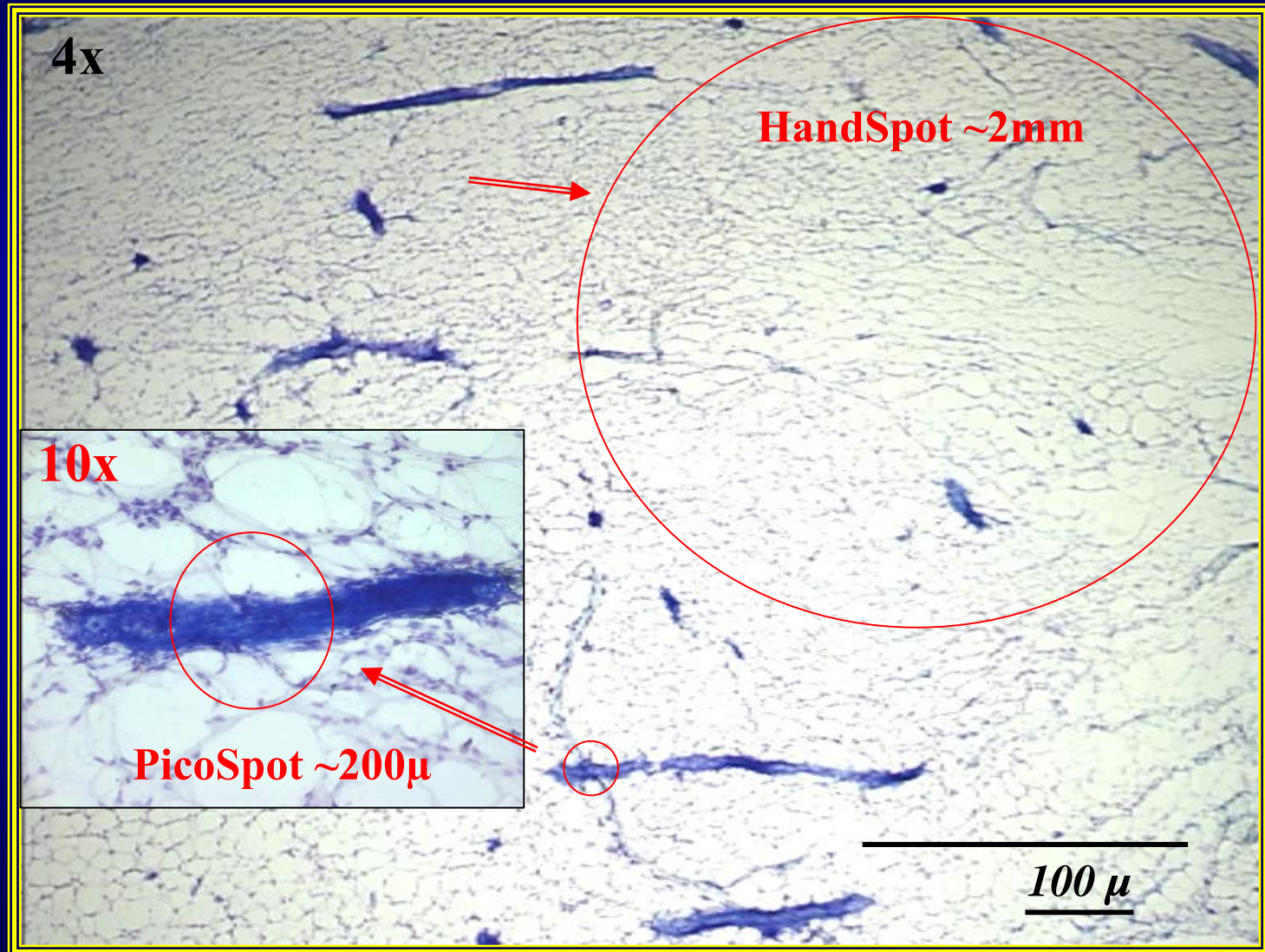
Tissue Profiling/Imaging – How Best to Apply Matrix?

Matrix Deposition Variables:

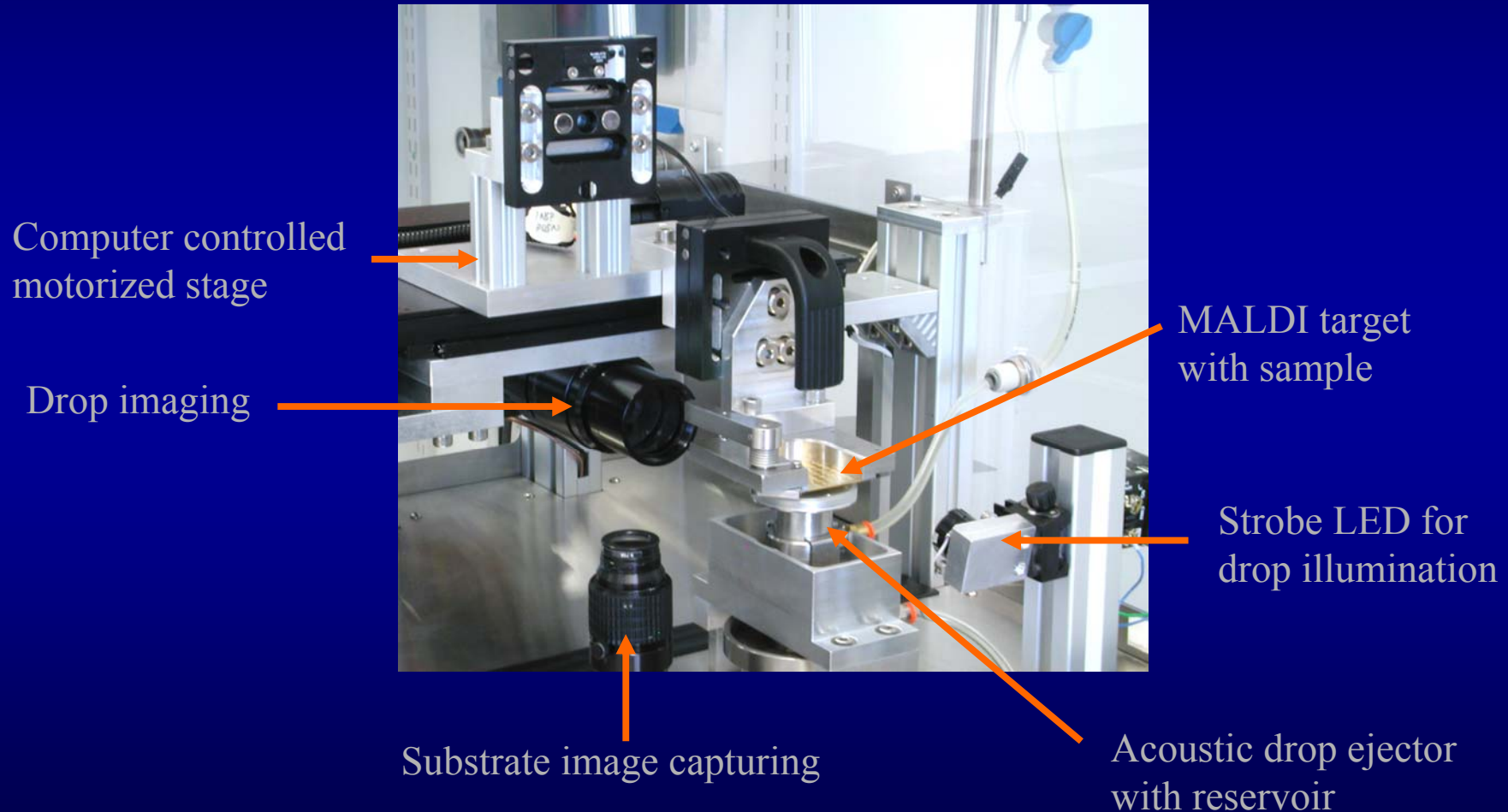
| | Time Demand | Repr. | Inherent BG | Spot Res. | Laser Dependent Spot Size |
|------------------|-------------|----------|-------------|-----------------------|---------------------------|
| Manual Spotting | seconds | variable | N | >1mm | Y |
| Robotic Spotting | minutes | good | N | ~200 μ | Y |
| Laser Capture | hours | good | Y | ~7 μ to 100 μ | N |

Current Laser Spot Size for Old STR: 25 μ x 50 μ

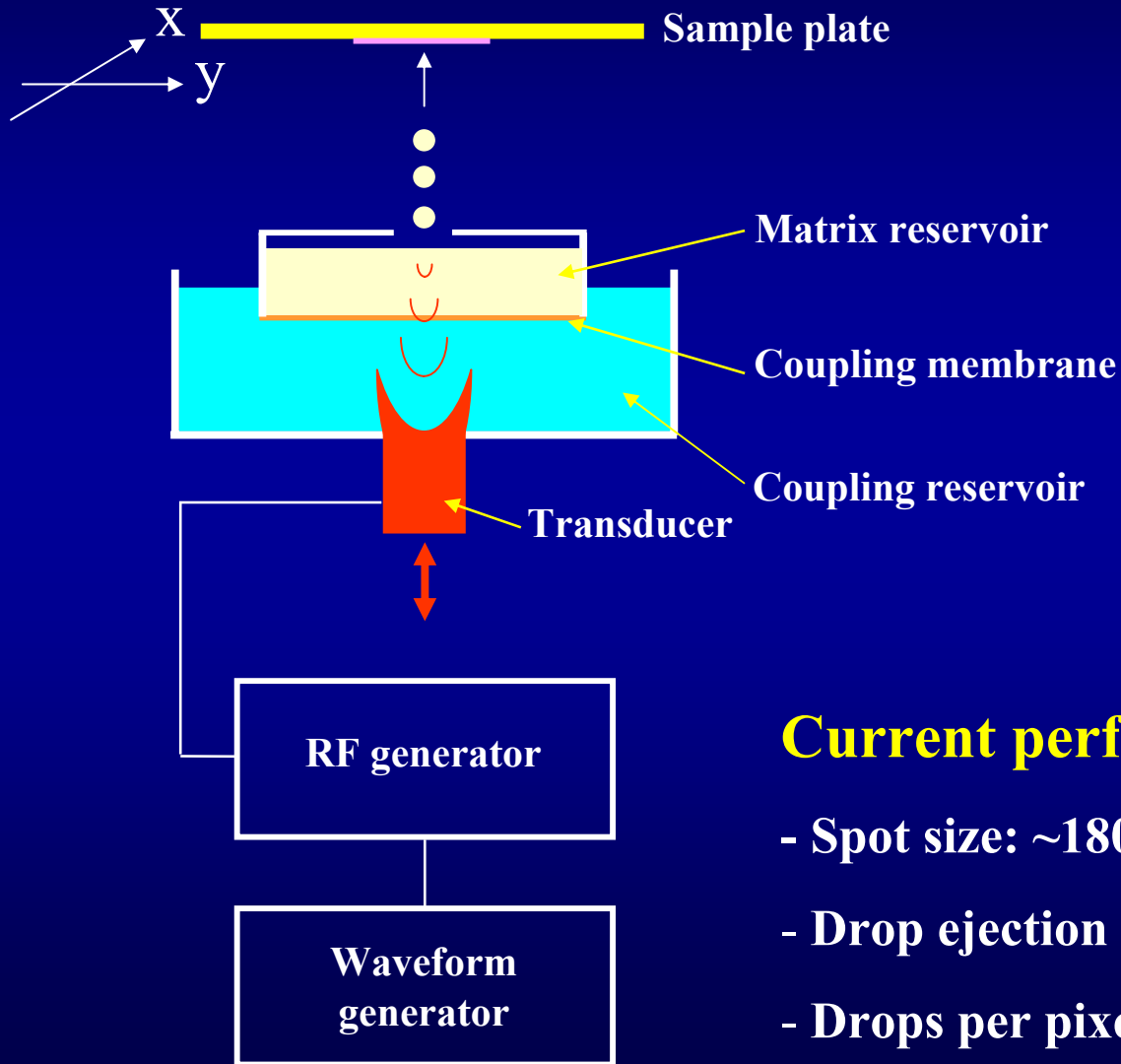
Hand Spotting Vs. Pico Spotting Vs. LCM



The Robotic Spotter



Acoustic Drop Ejection Technology



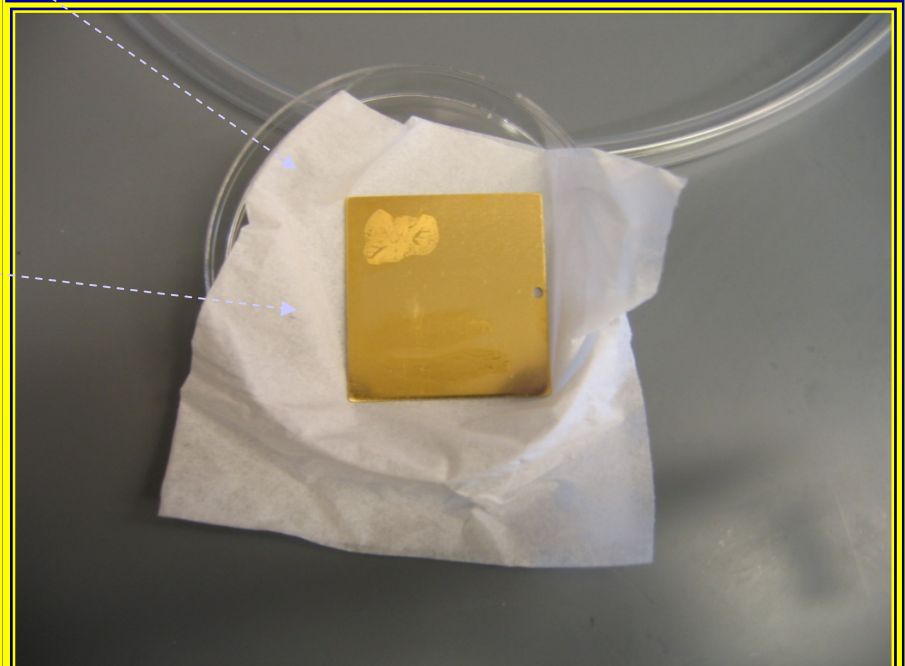
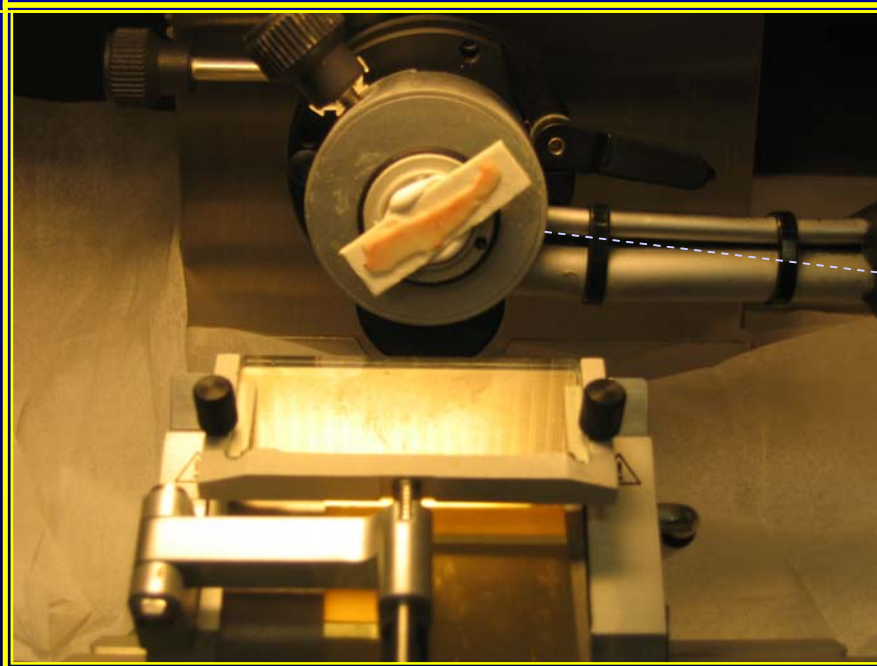
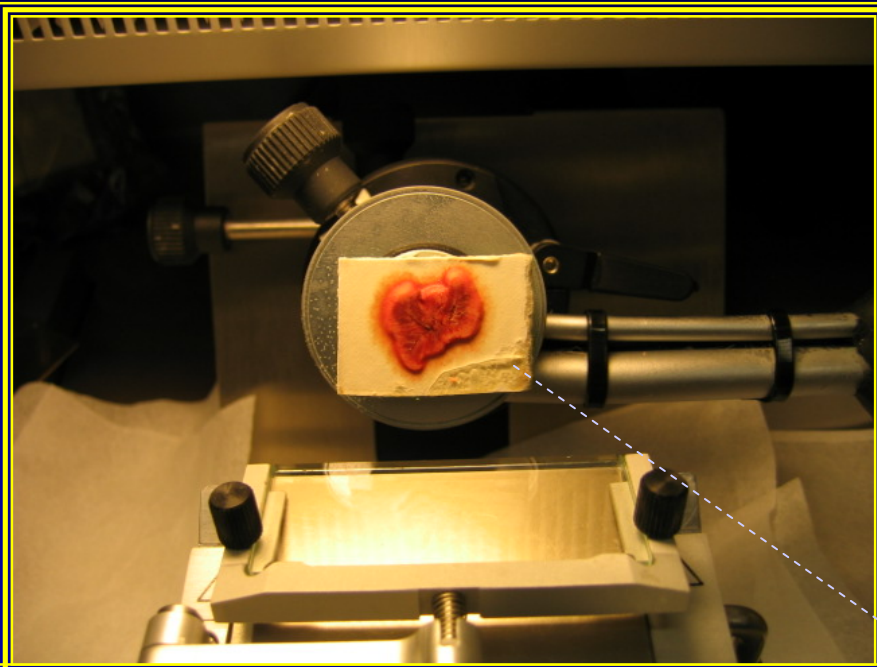
“Ejection of microdroplets”

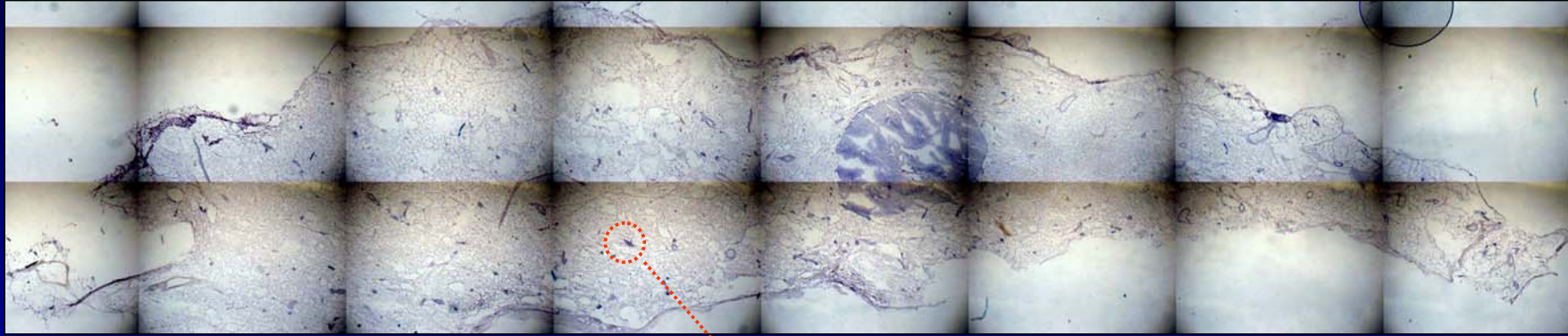


Current performances:

- Spot size: $\sim 180\text{-}200\ \mu\text{m}$
- Drop ejection rate: 10 Hz
- Drops per pixel: 60-80

Tissue Sectioning for Protein Profiling



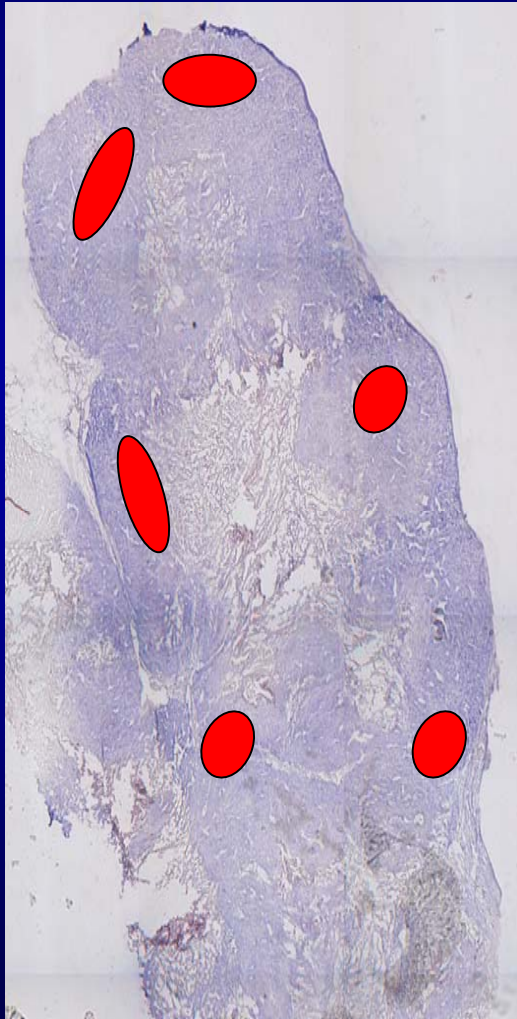
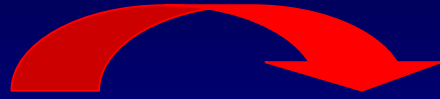


Whole Mouse Mammary Gland

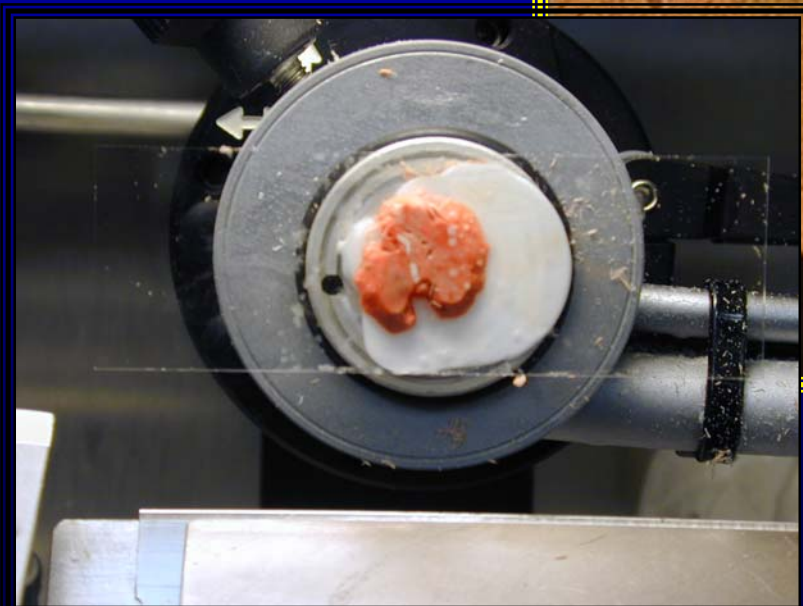
Seamless High Resolution
Imaging of Histological Slides



Histology Directed Matrix Deposition

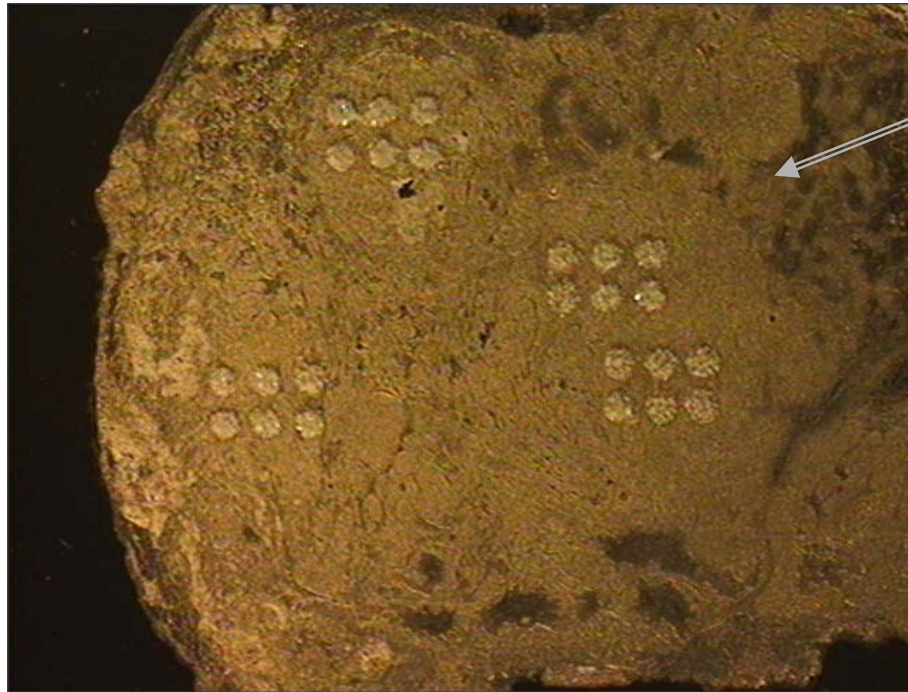


Whole Lung Sections

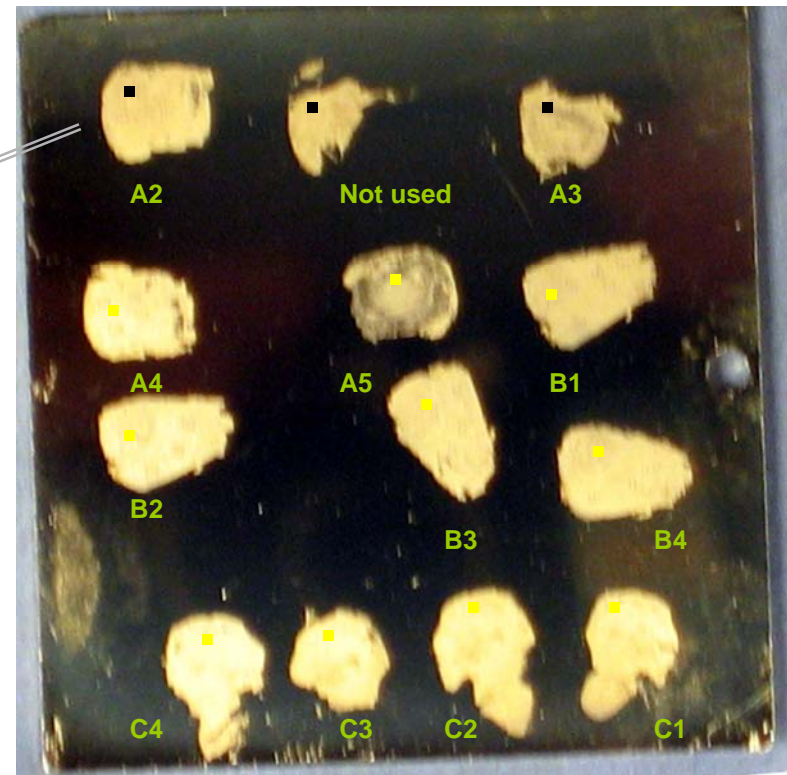


Automating – “Whole Plate Profiling”

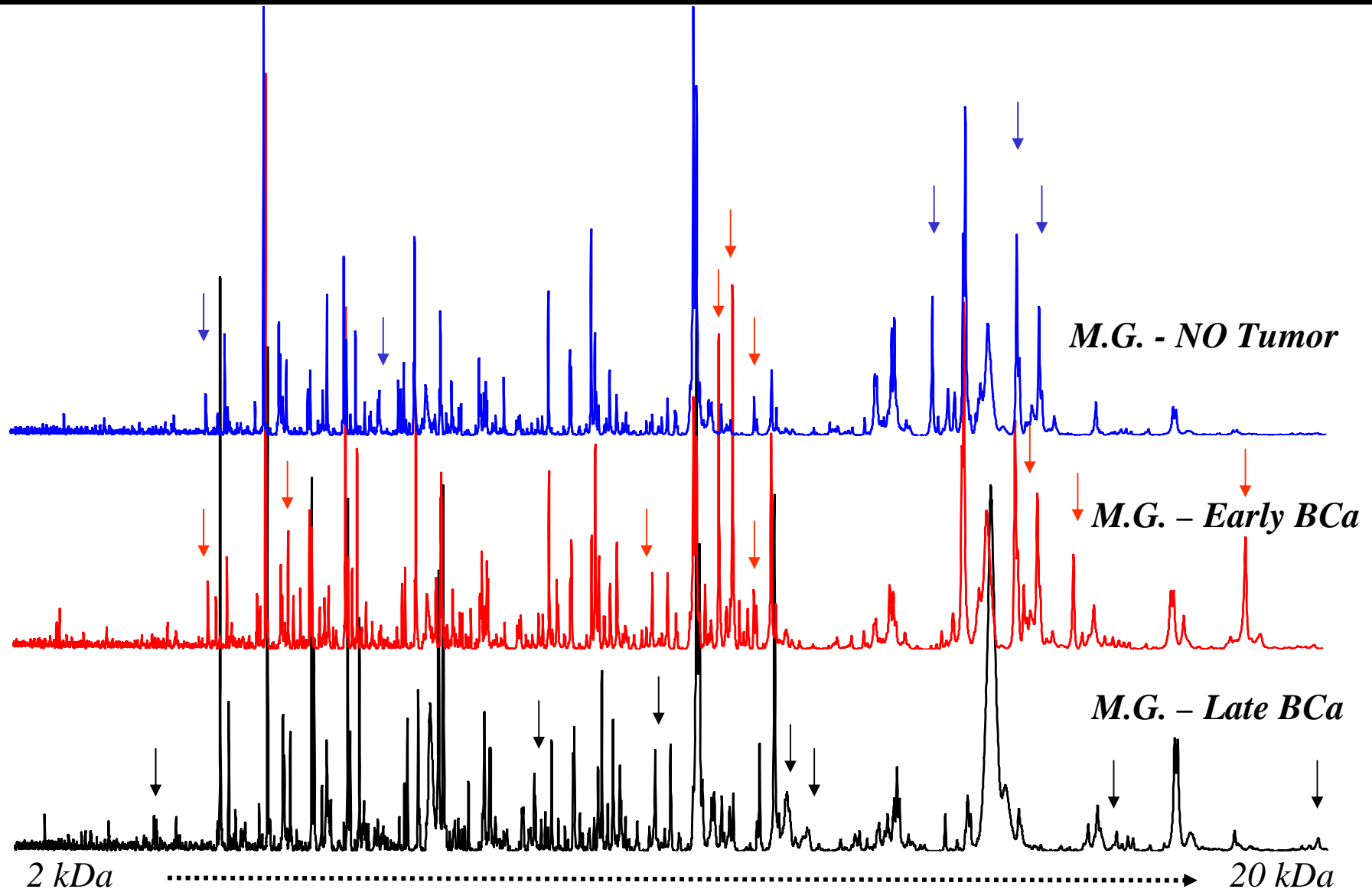
Rapid Spotting Over Entire Plate



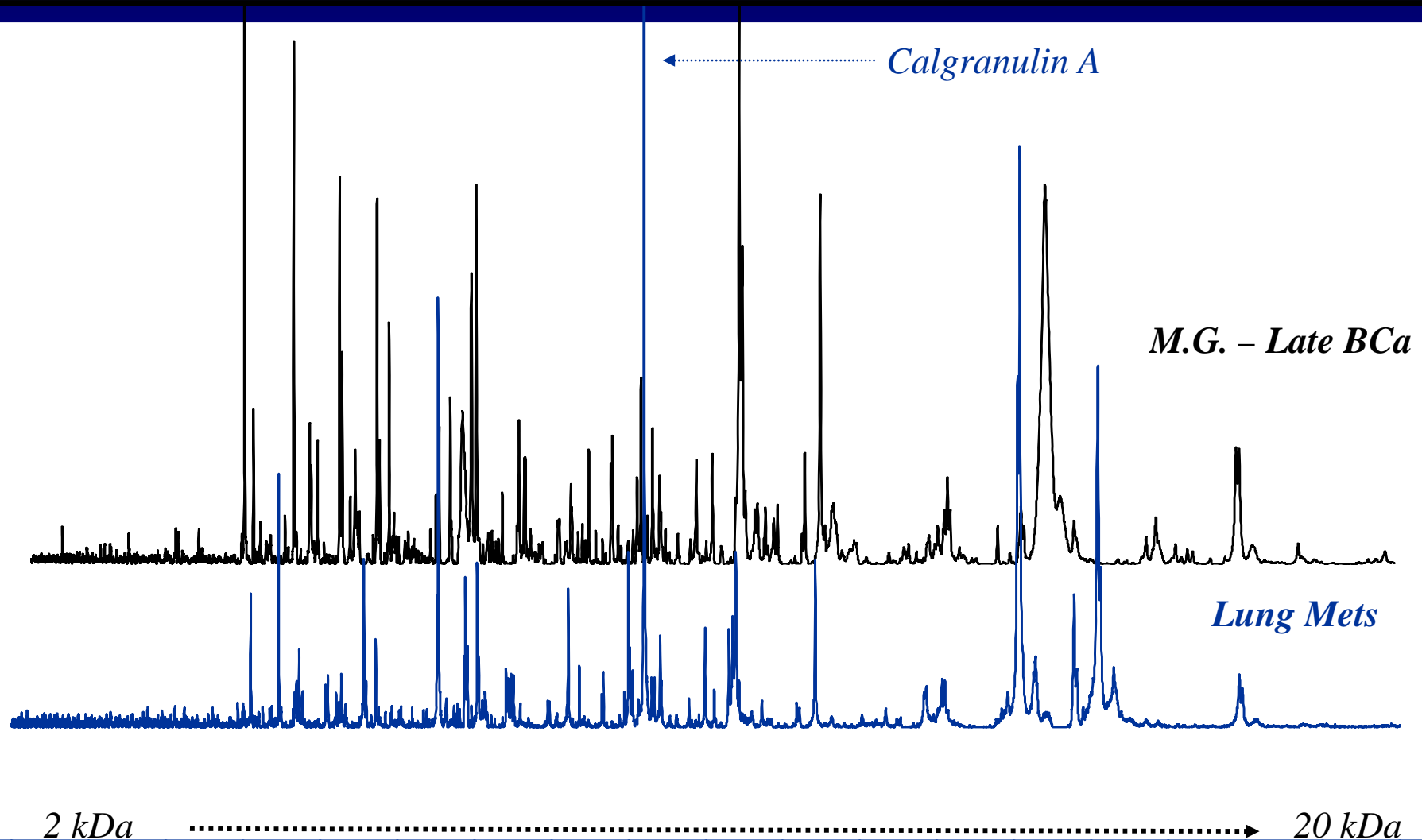
MALDI-Tof Plate



Breast Cancer Early Vs. Late Disease

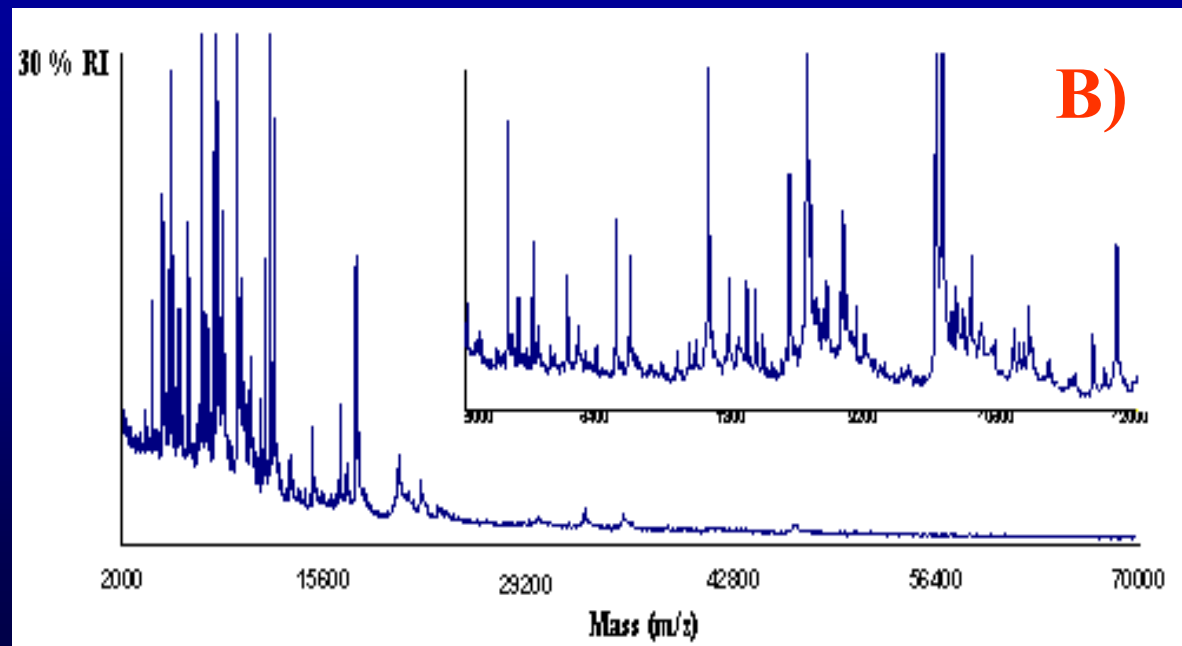
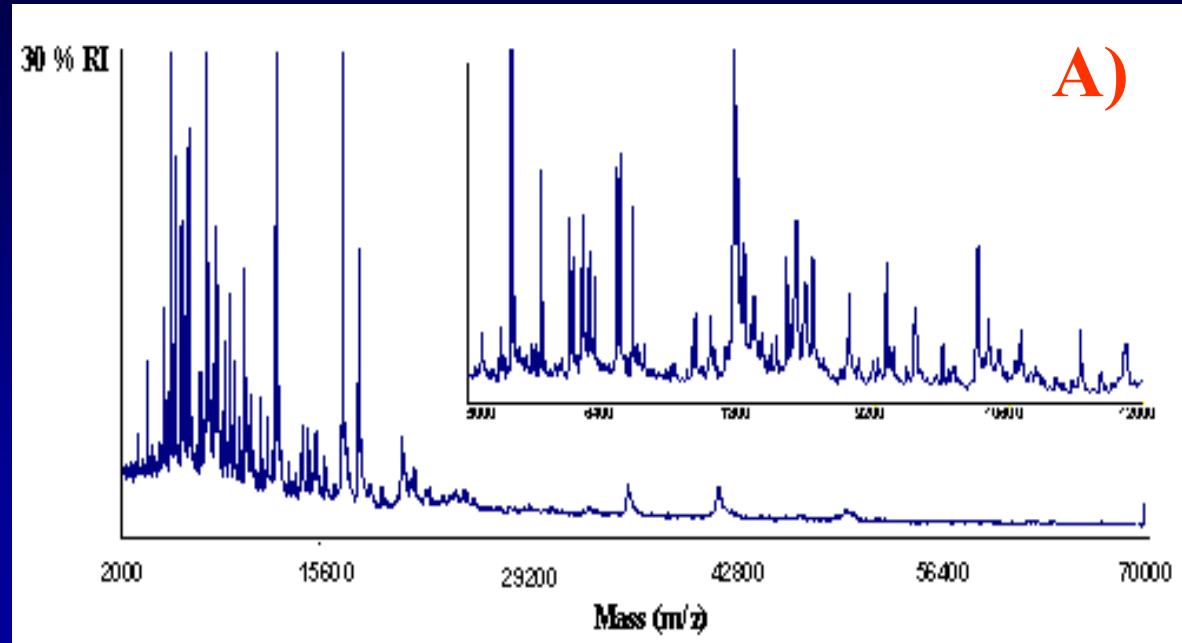
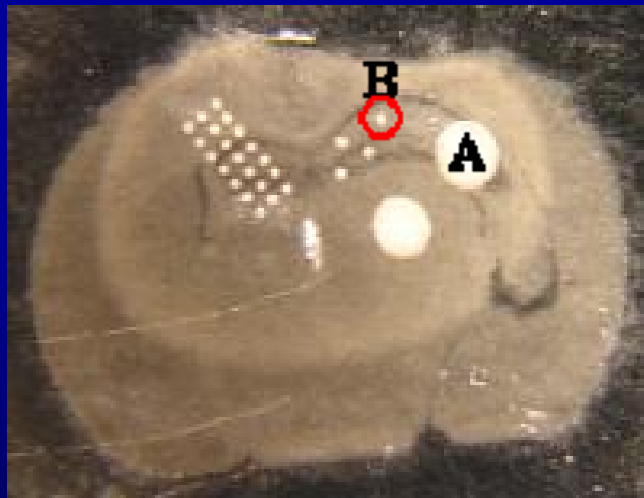


Lung Mets Vs. Late Carcinoma of the Breast

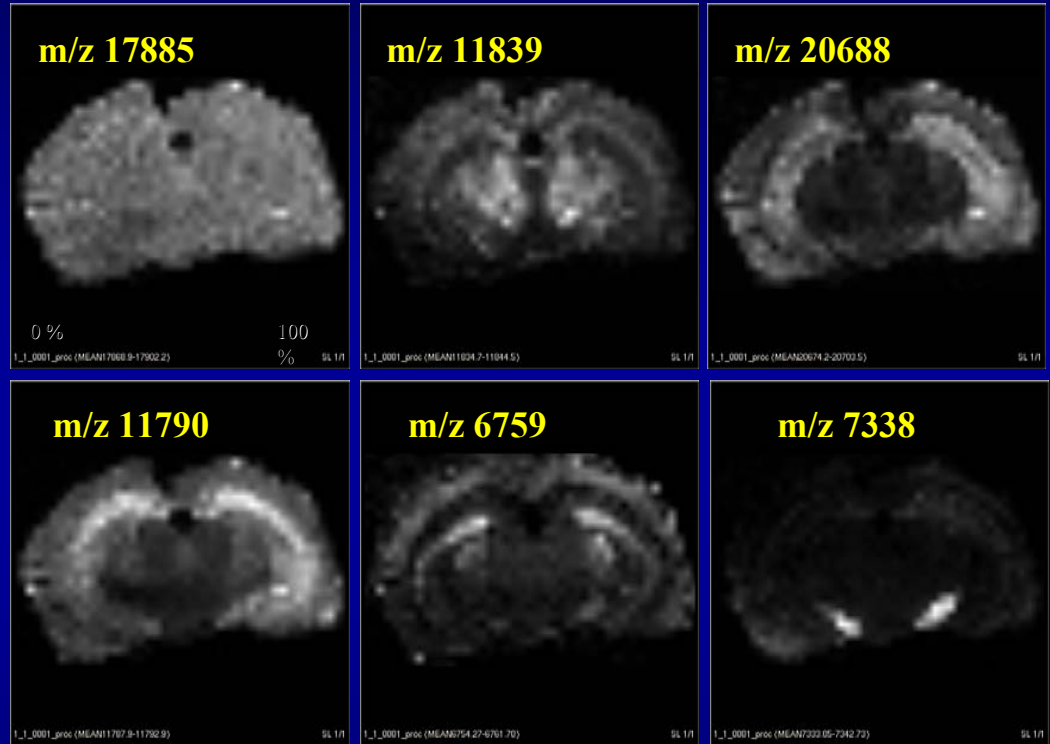
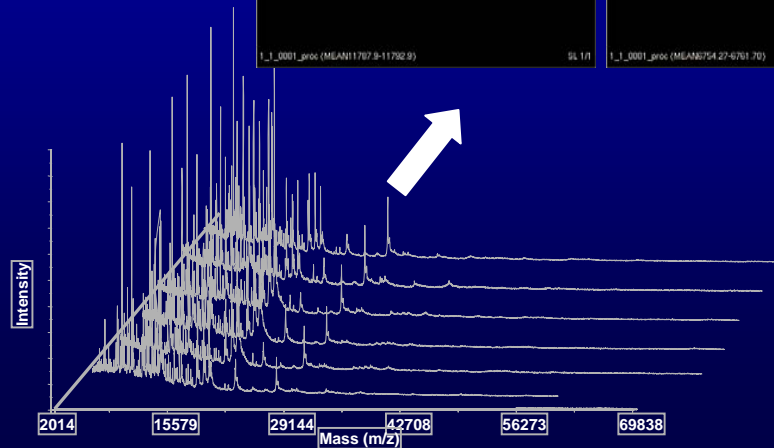
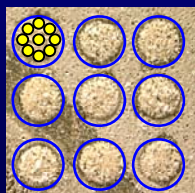
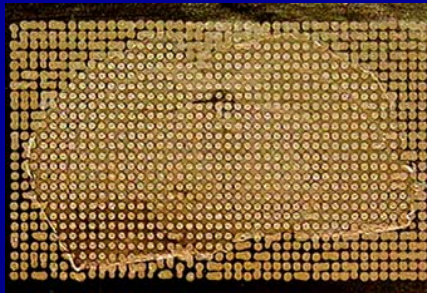
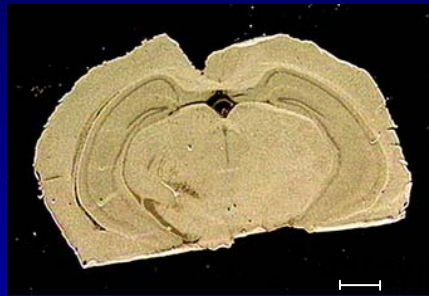


Manual Spotting Vs. The Robotic Spotting

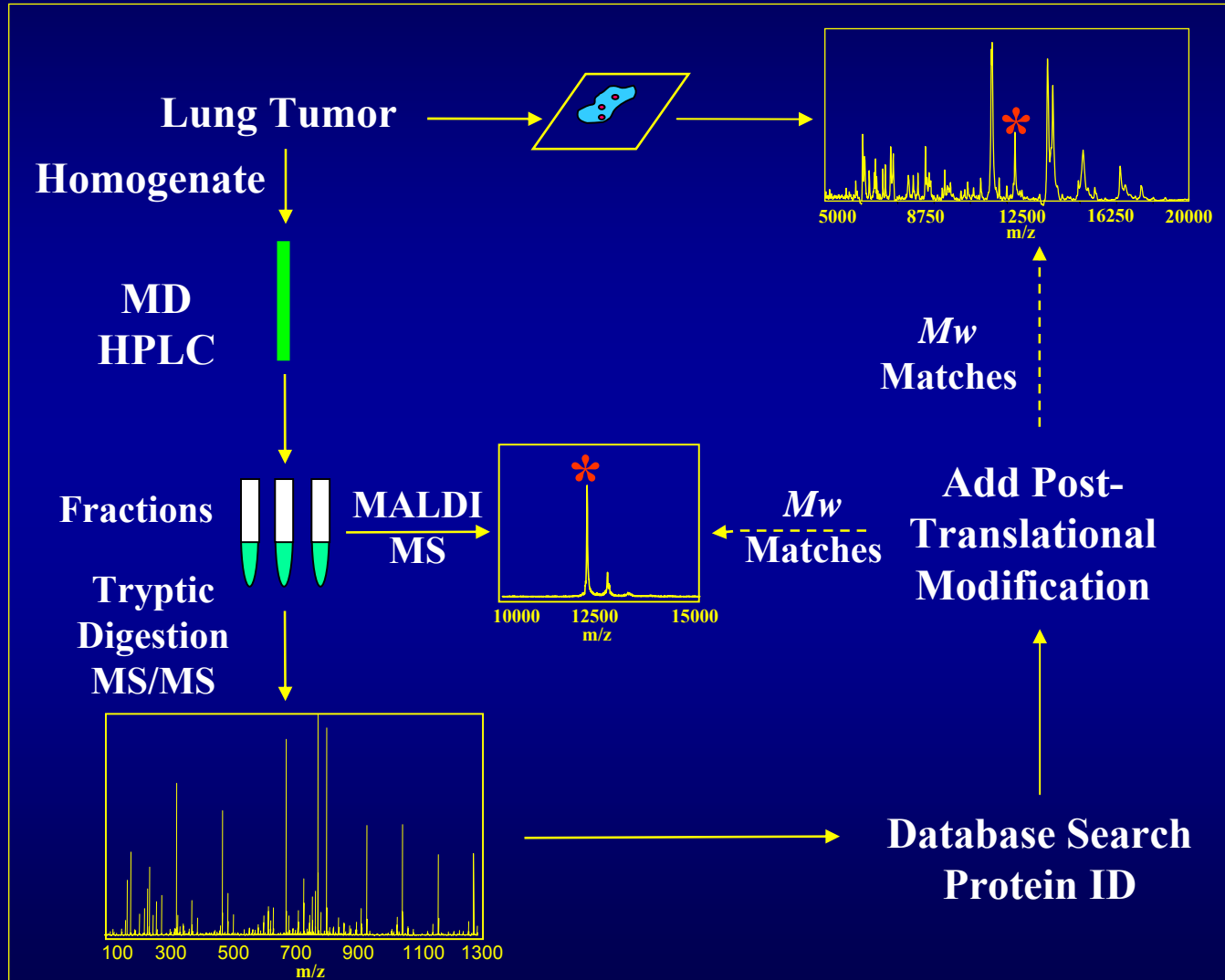
Mouse brain,
Analysis of the corpus callosum



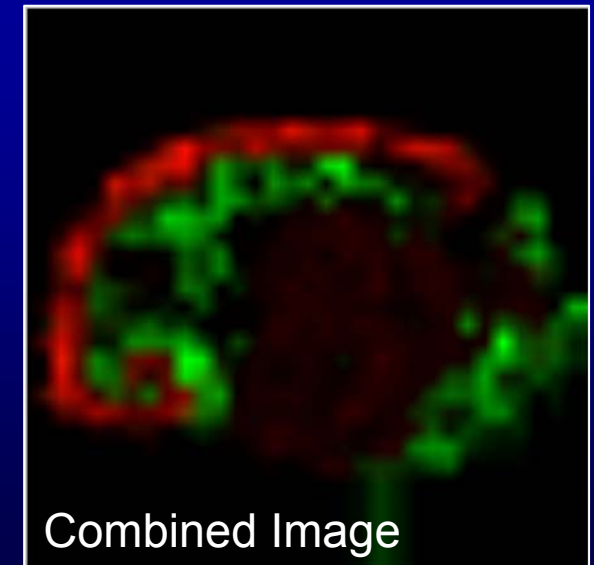
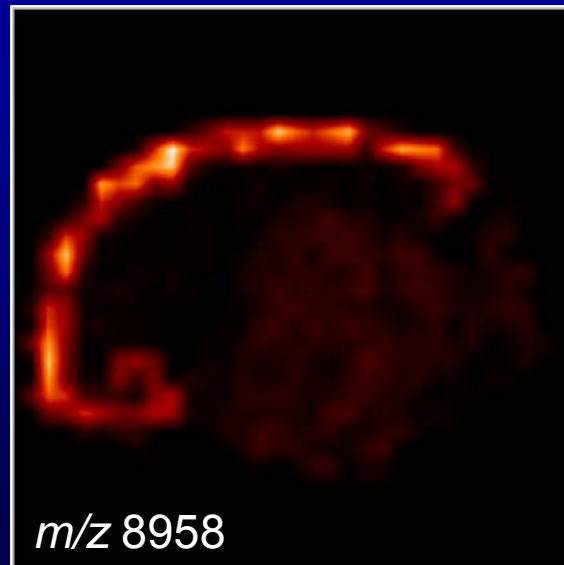
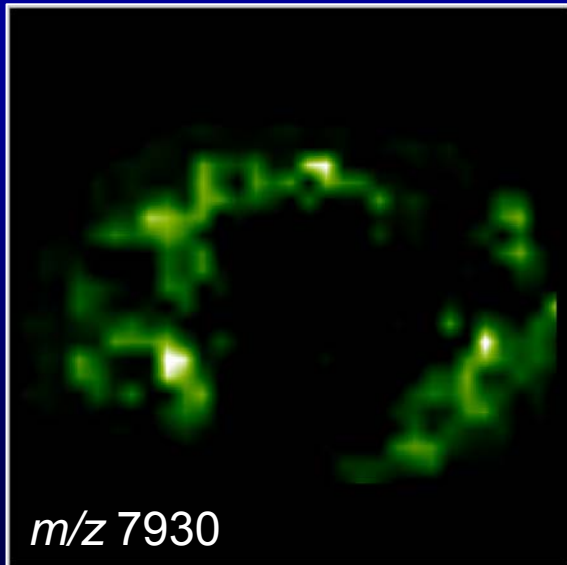
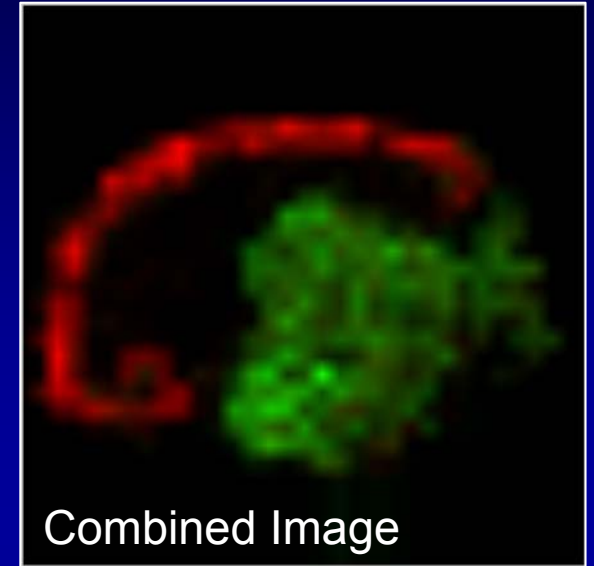
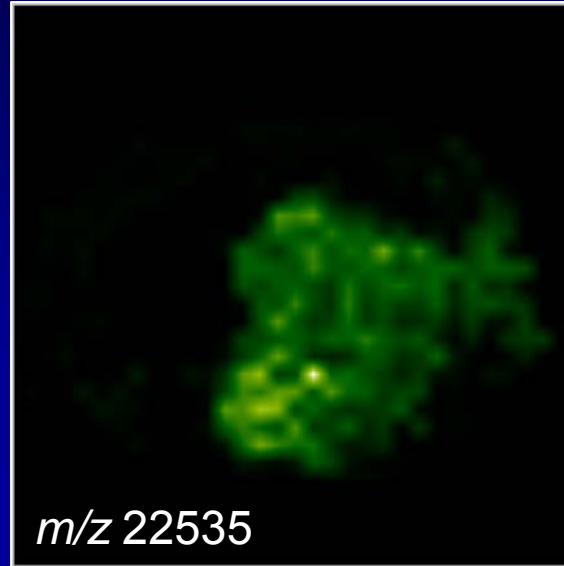
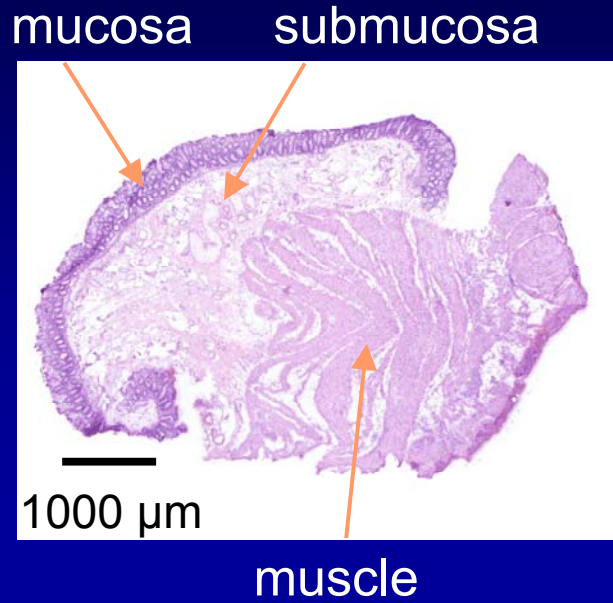
MS Imaging of a Mouse Brain Section By Robotic Spotting



Schematic Representation of Protein Marker Identification



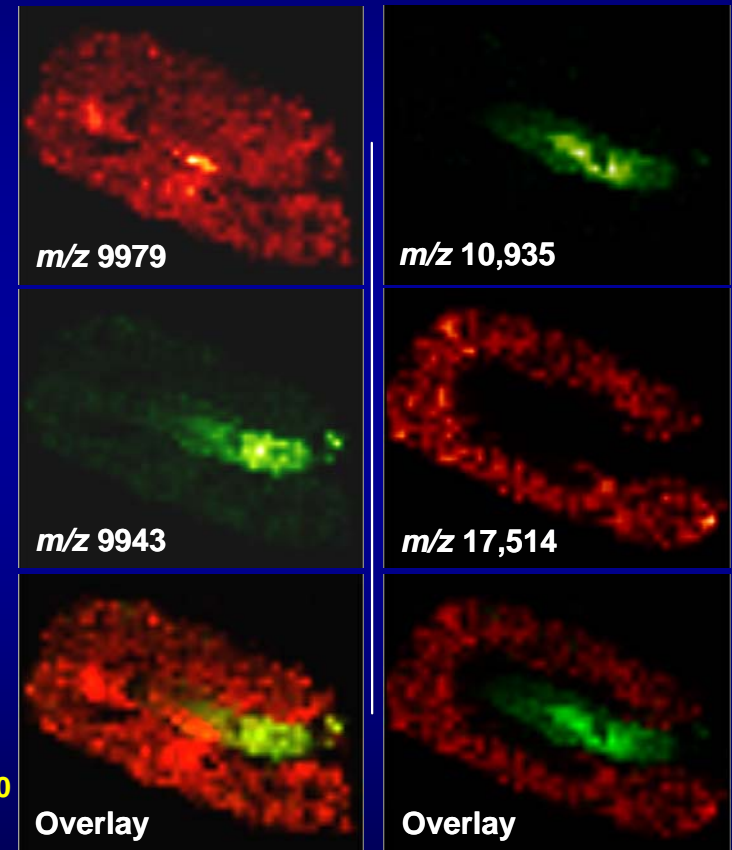
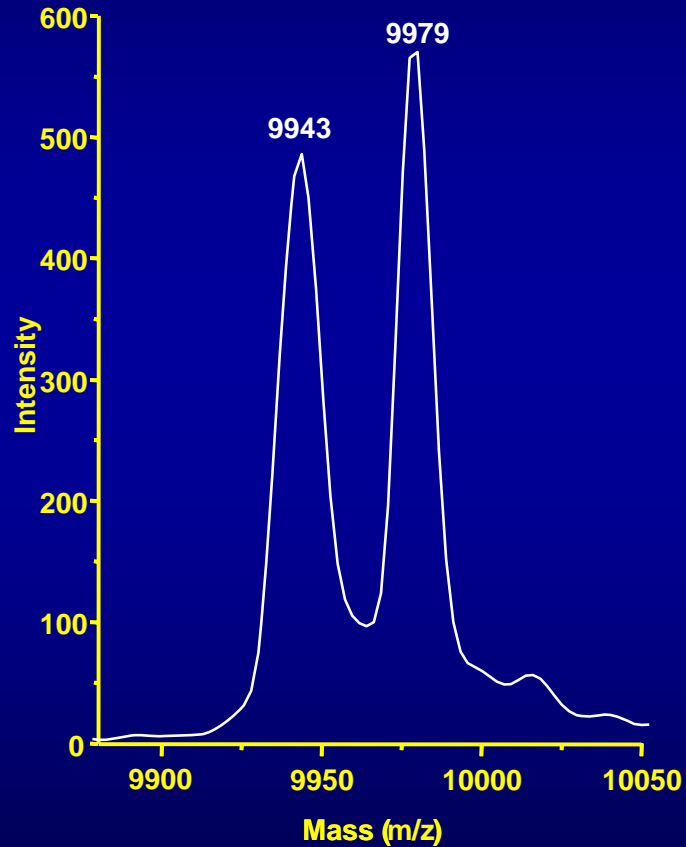
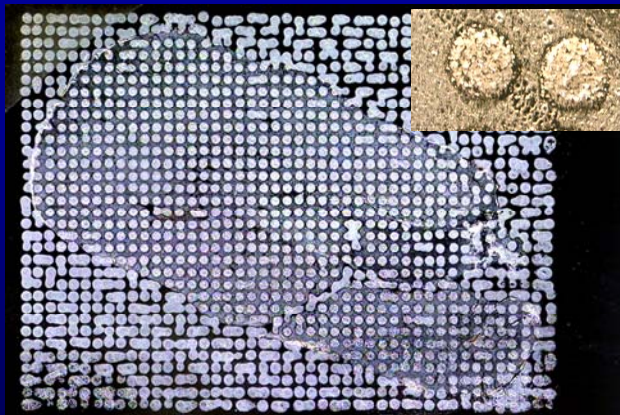
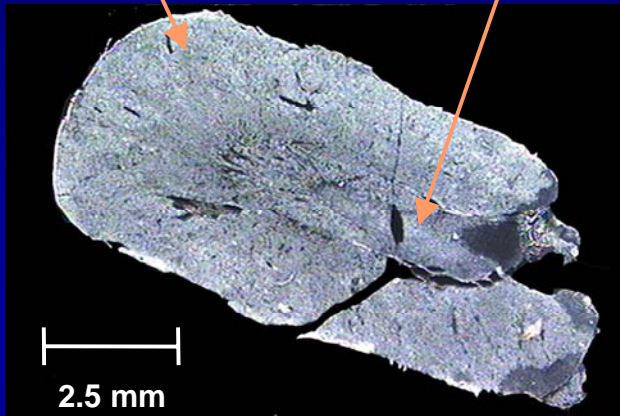
Normal Human Colon Biopsy



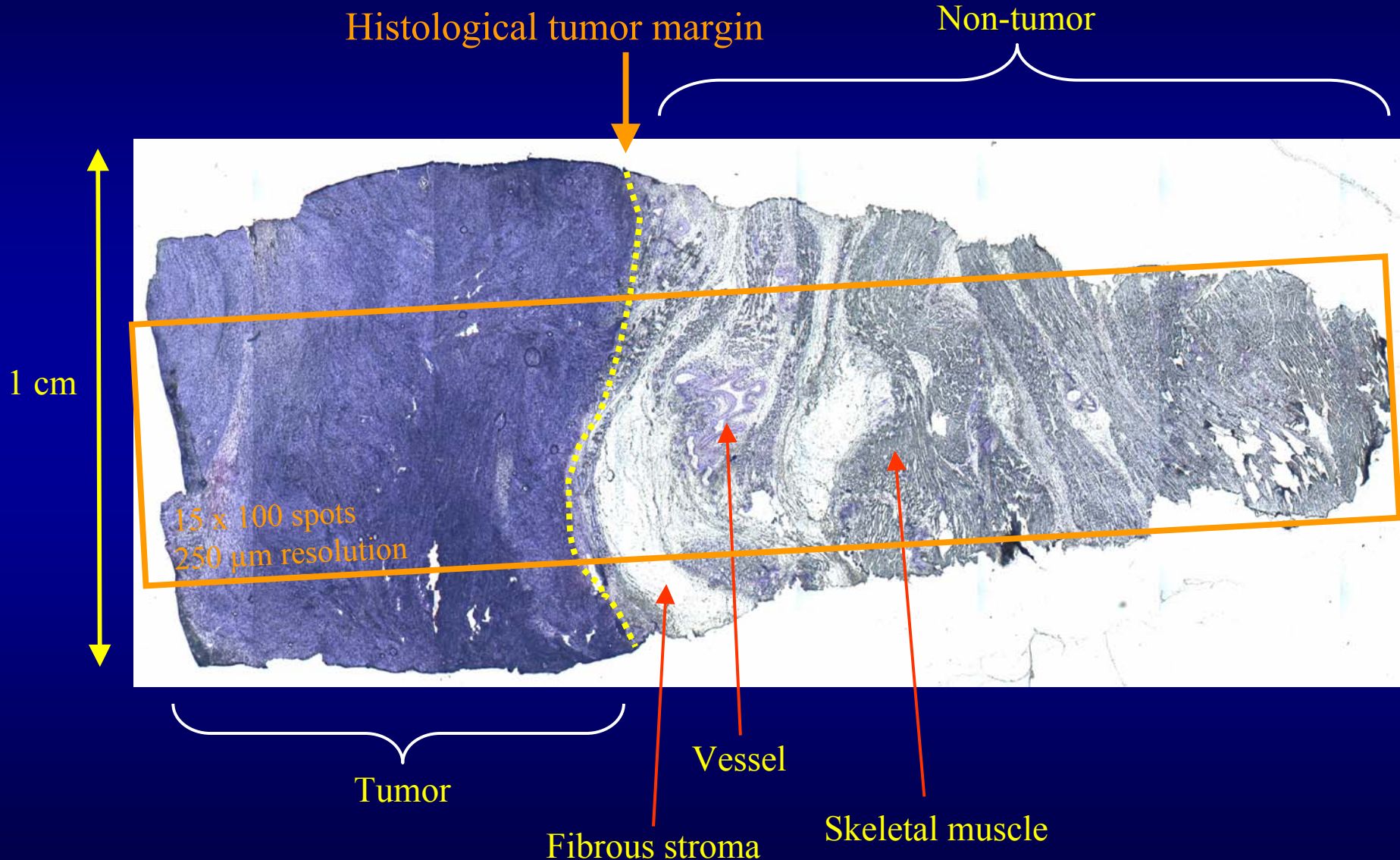
Rat Kidney Sagittal Section

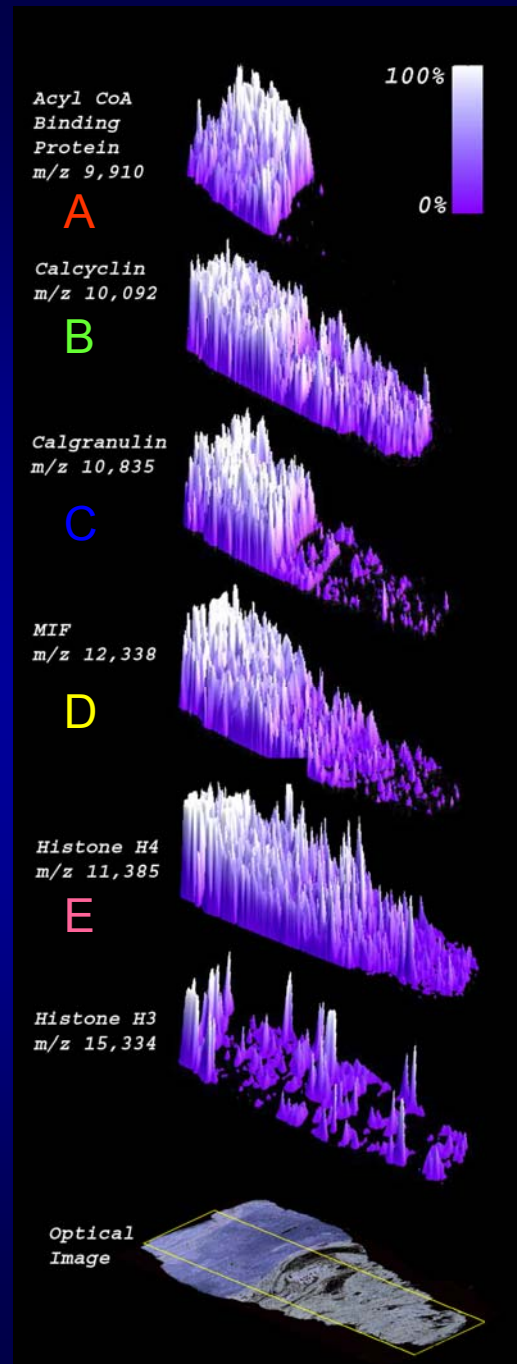
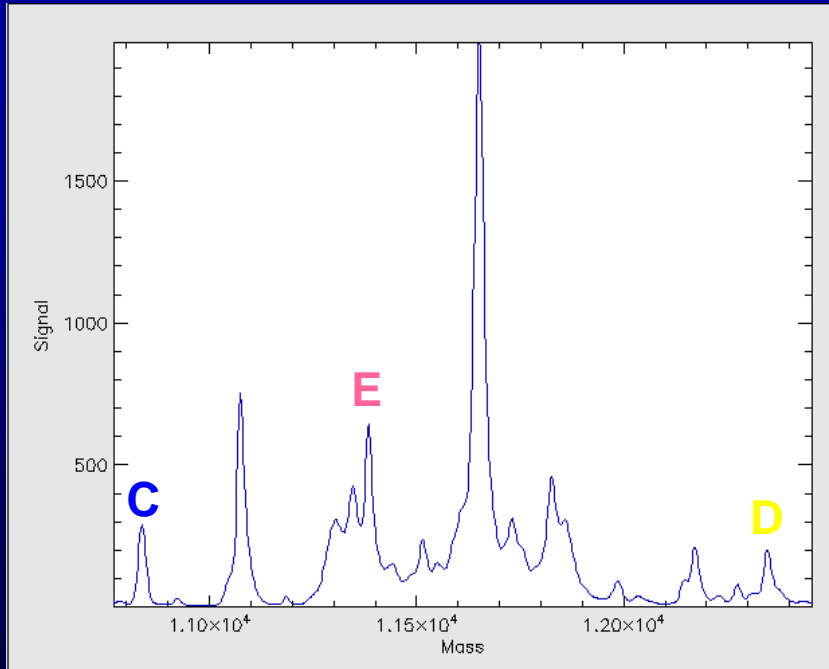
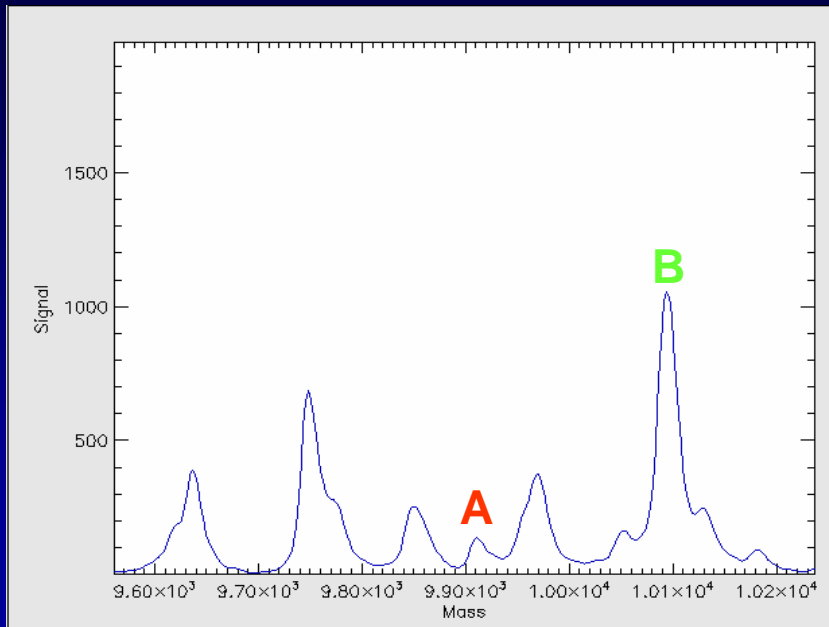
cortex

medulla

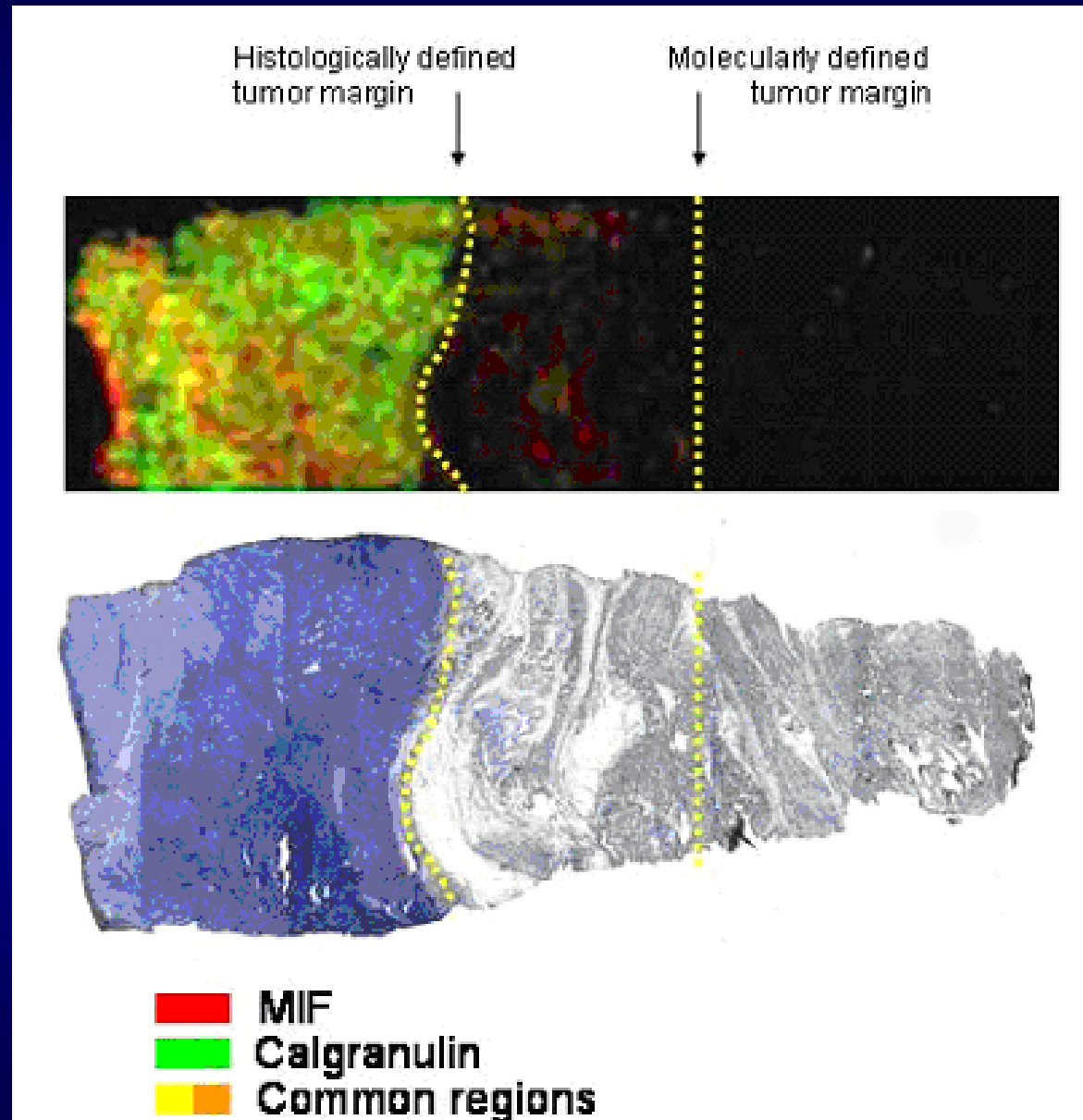


Molecular Determination of Tumor Margins





Histological vs. Molecular Assessment of the Tumor Margin:

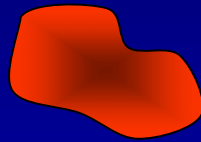


Proteins are OK.....But What About Drug Distribution/ Metabolism? Better.....Correlating Drug Effects with Protein Expression.

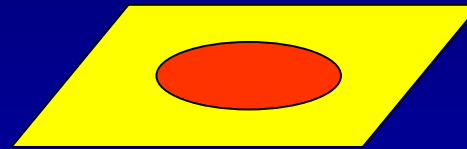


Dose animal

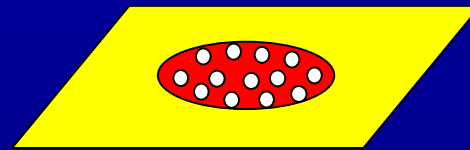
- ◆ orally
- ◆ i.v.



Remove
tissue



- ◆ Cut frozen
slice (12 μm)



- ◆ Apply
matrix



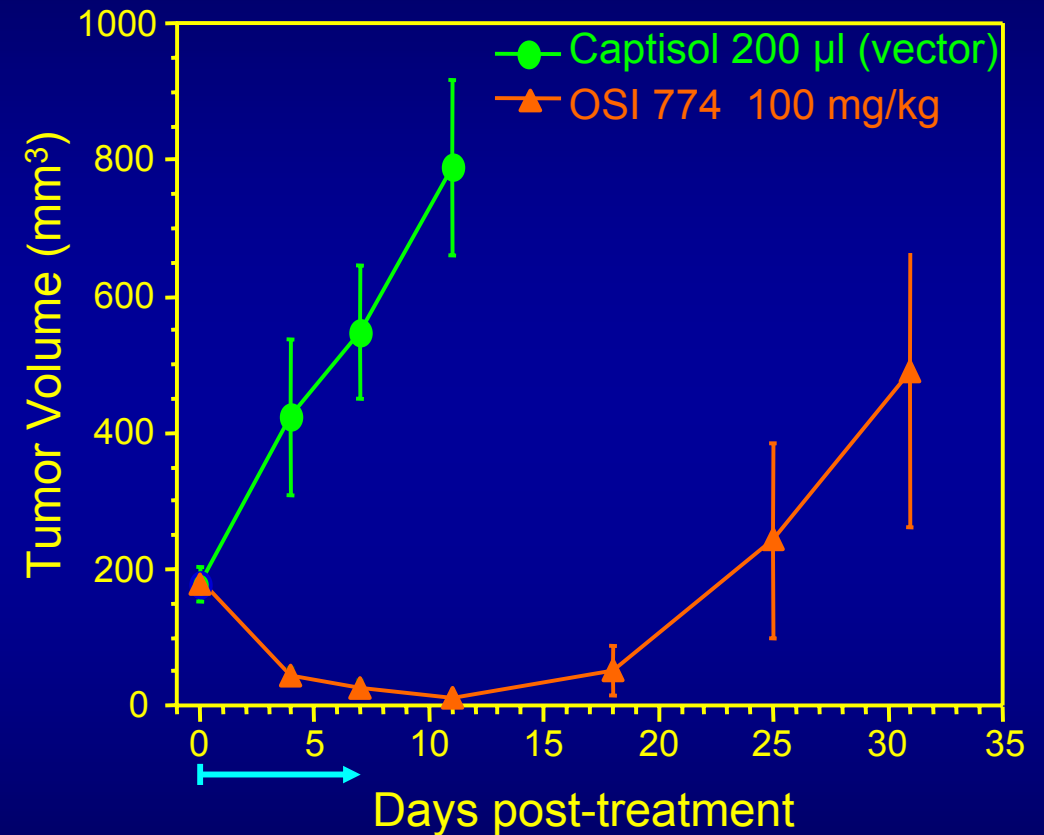
- ◆ Analyze by:
MALDI MS and
MALDI MS/MS

Reyzer ML et al, J Mass Spectrom, 38, 1081-1092 (2003)

Reyzer ML et al, Cancer Res 64, 9093-9100 (2004)

MMTV/HER2 Transgenic Mouse Mammary Tumors

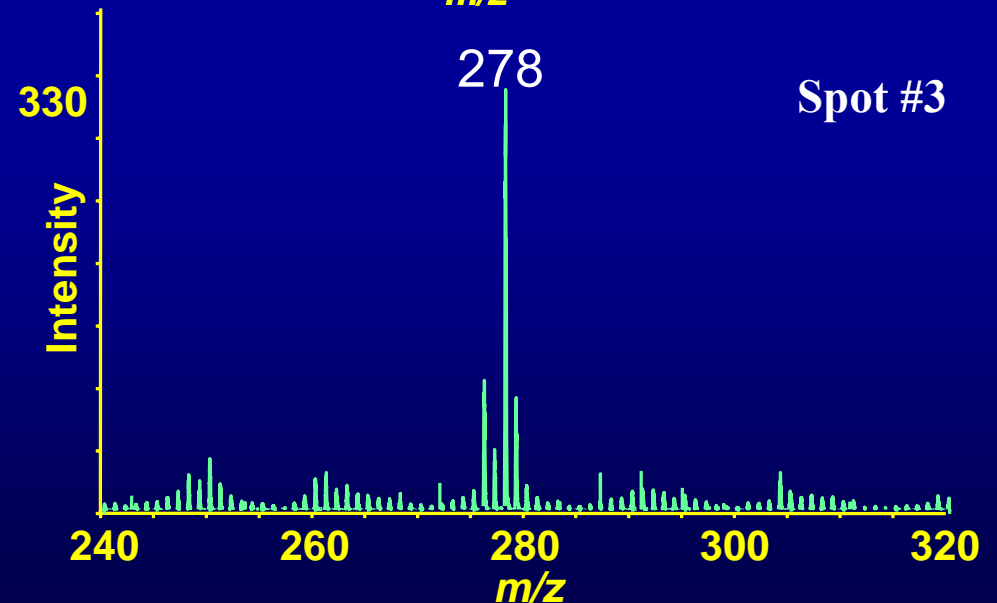
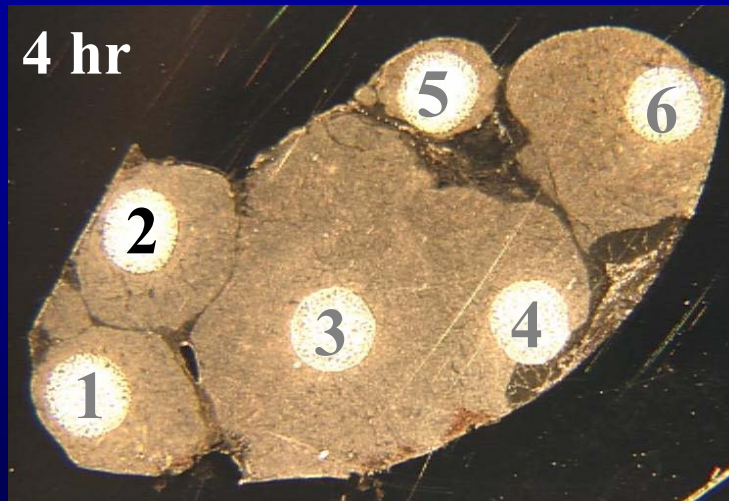
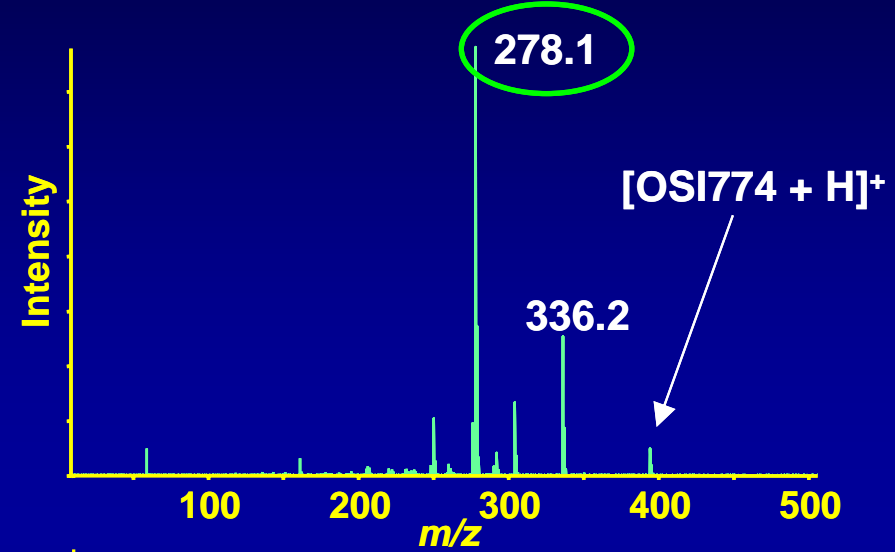
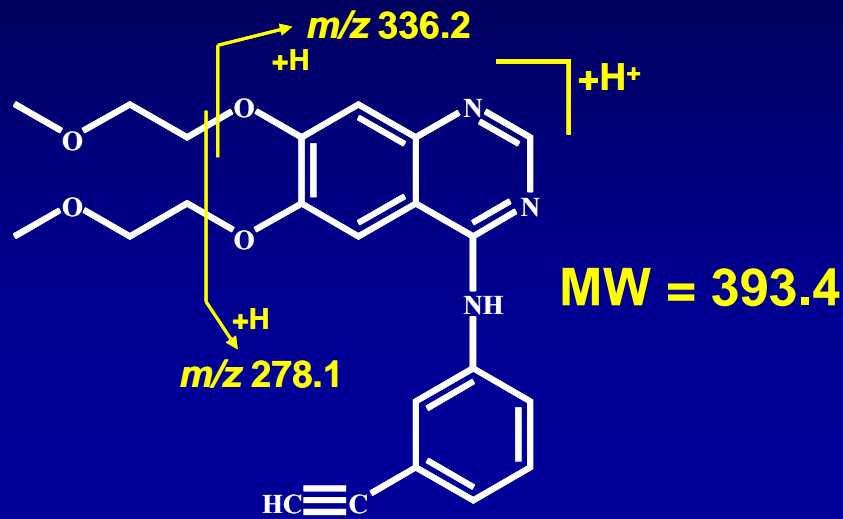
- MMTV/HER2 cells transplanted in FVB female mice.
- Tumor grown to a size of $\sim 200 \text{ mm}^3$.
- OSI 774 is an intracellular tyrosine kinase EGF receptor inhibitor.
- Administered orally for 1 week



Contributed by M. Sliwkowski (Genentech, Inc.)

MS/MS Analysis of OSI-774 in Tumor Tissue

Tumors removed after a single 100 mg/kg dose of OSI-774

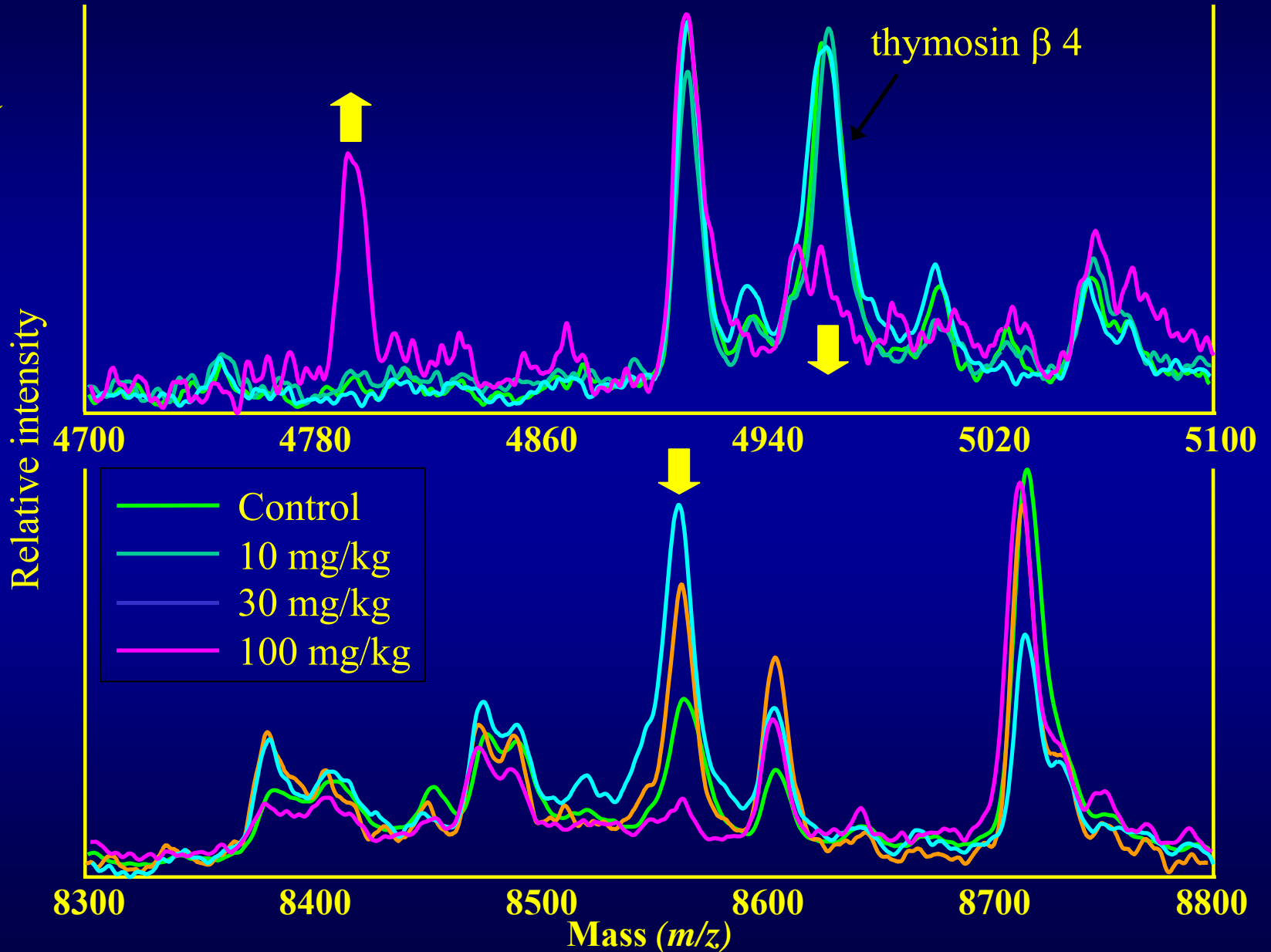


◆ Monitored CAD transition m/z 394 \rightarrow 278

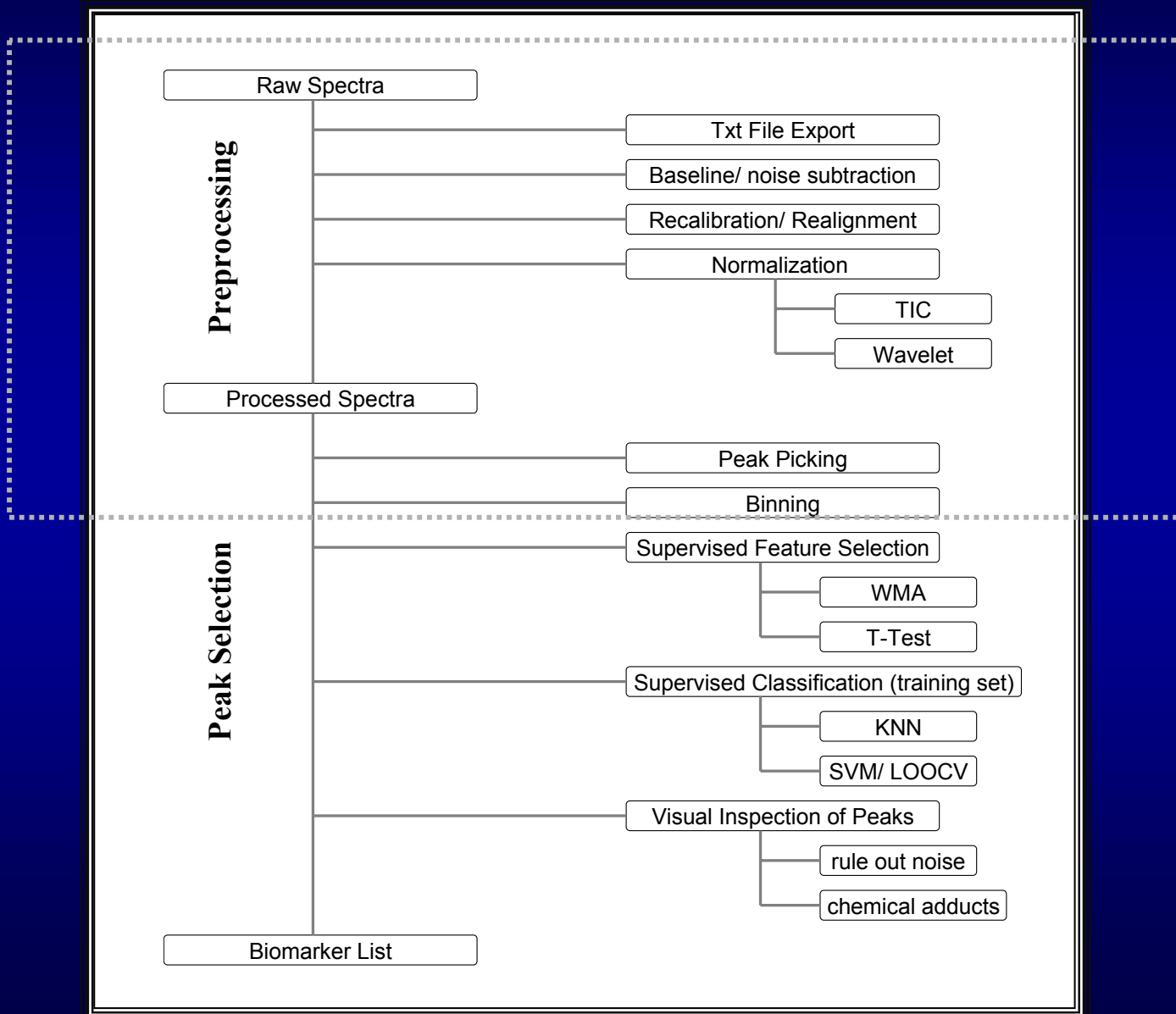
Dose Dependence of Protein Alteration (20 hr after dose)

9 control mice,
Av of 54 spectra

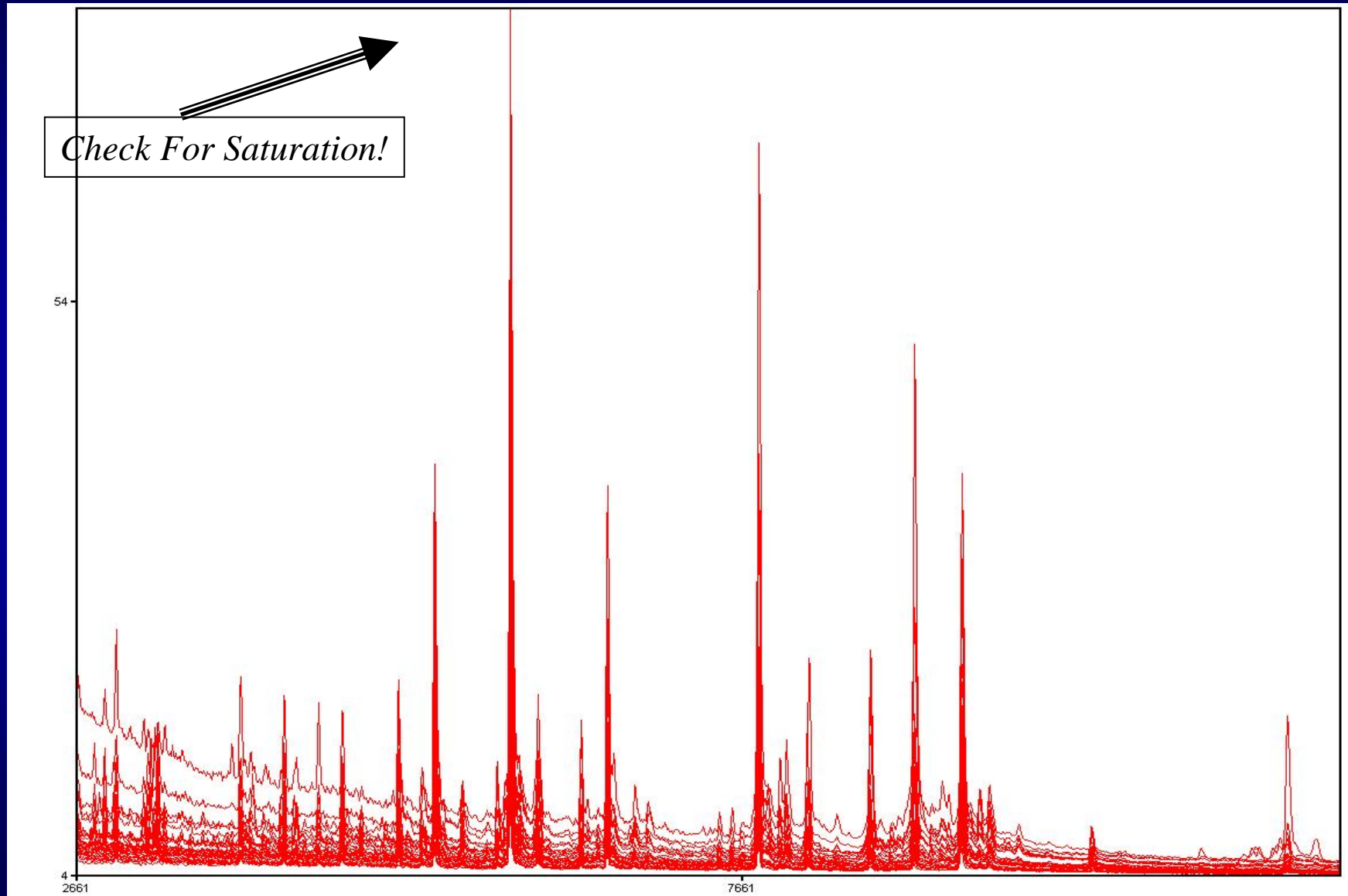
3x3 dosed mice,
Av of 9 spectra
per dose



Analysis of MALDI-ToF Data – The Workflow



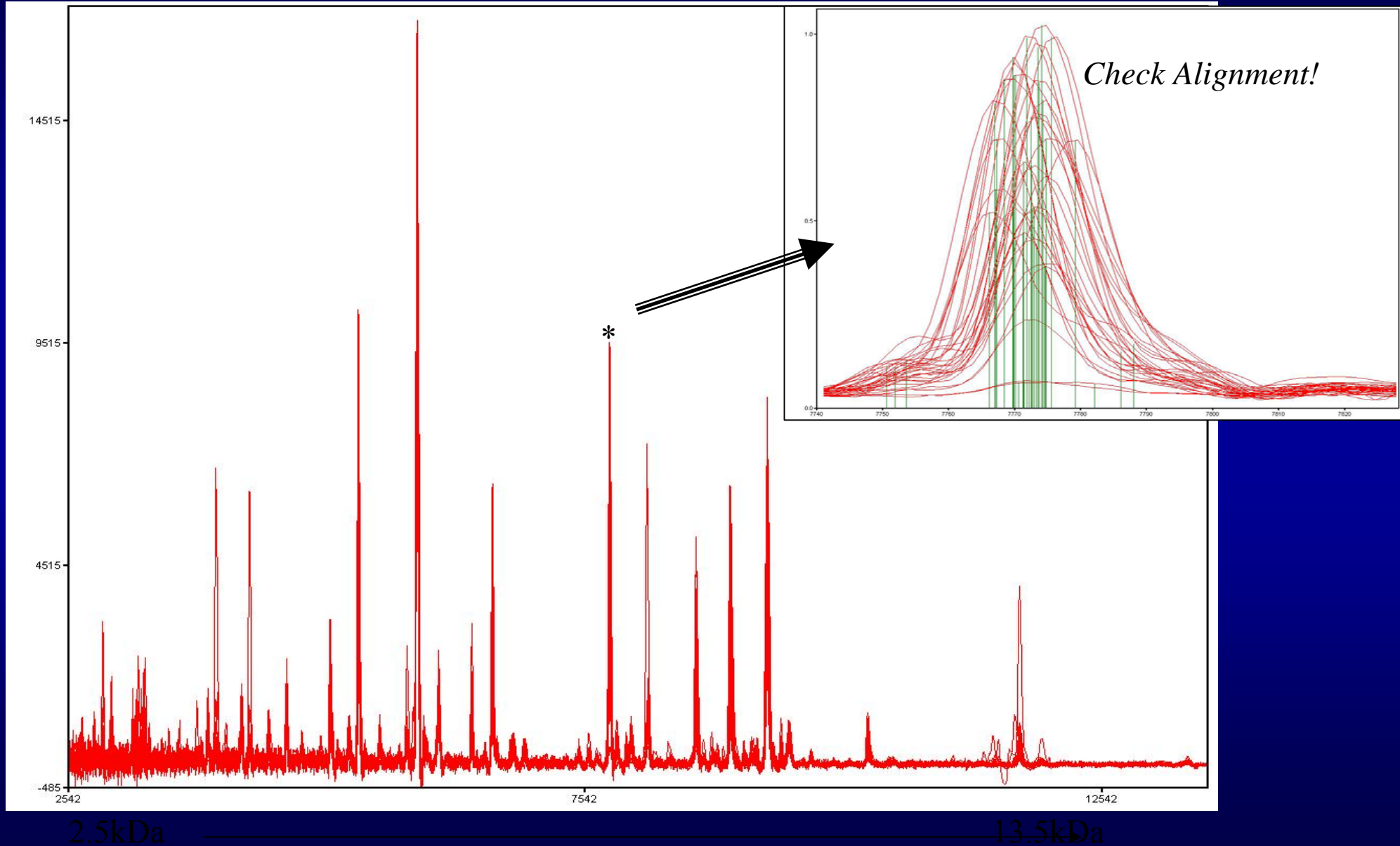
Recent Example; No Data Processing – Raw Files



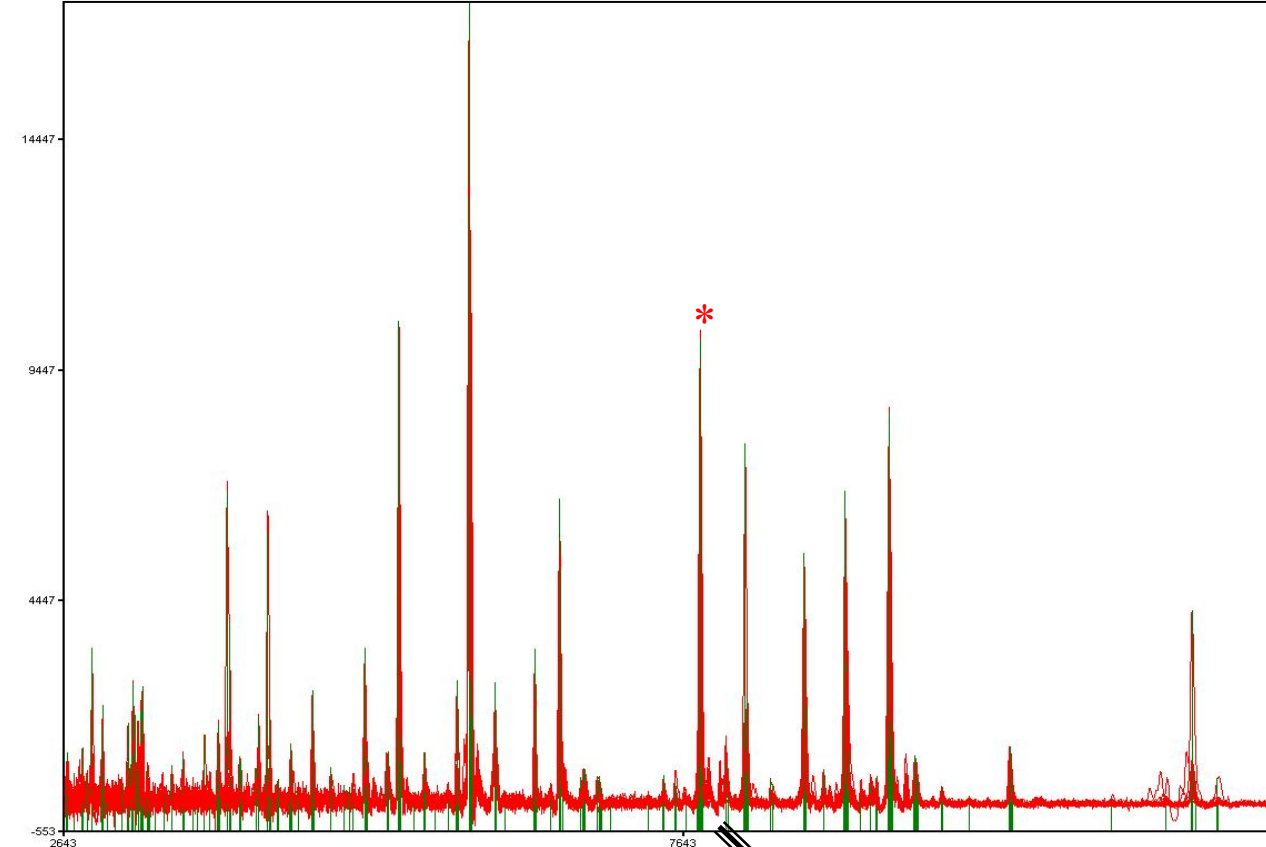
2.5kDa

13.5kDa

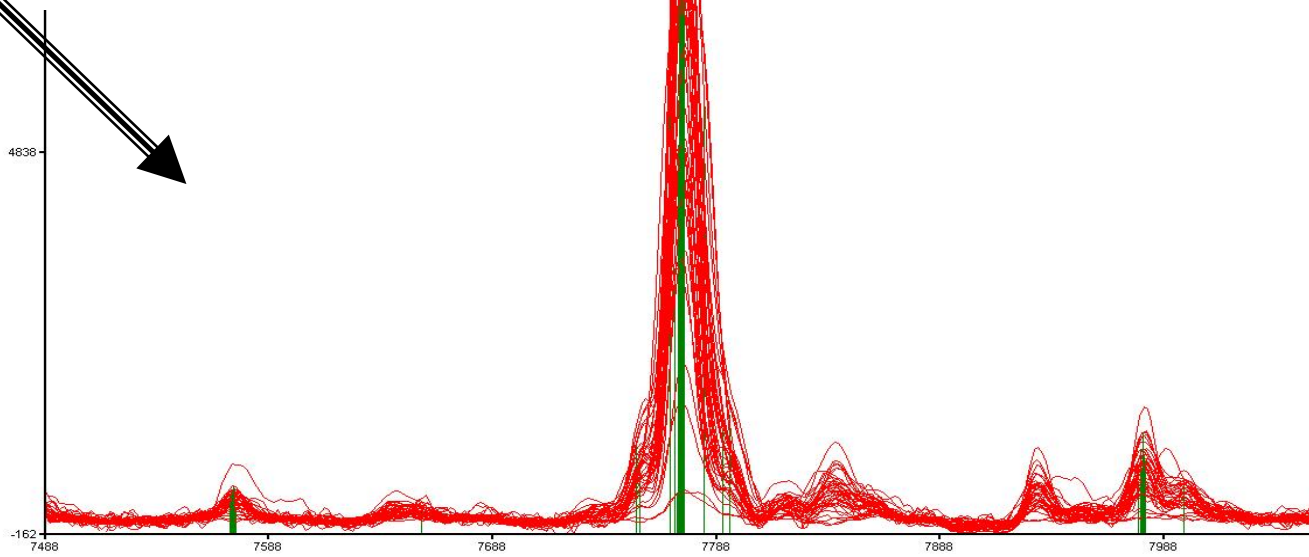
Post Baseline and Normalization – Pre Alignment



Post-BSL/Norm/Align
[No Smoothing]



*Good Alignment
Decreased Error
Smaller Bin Sizes!*

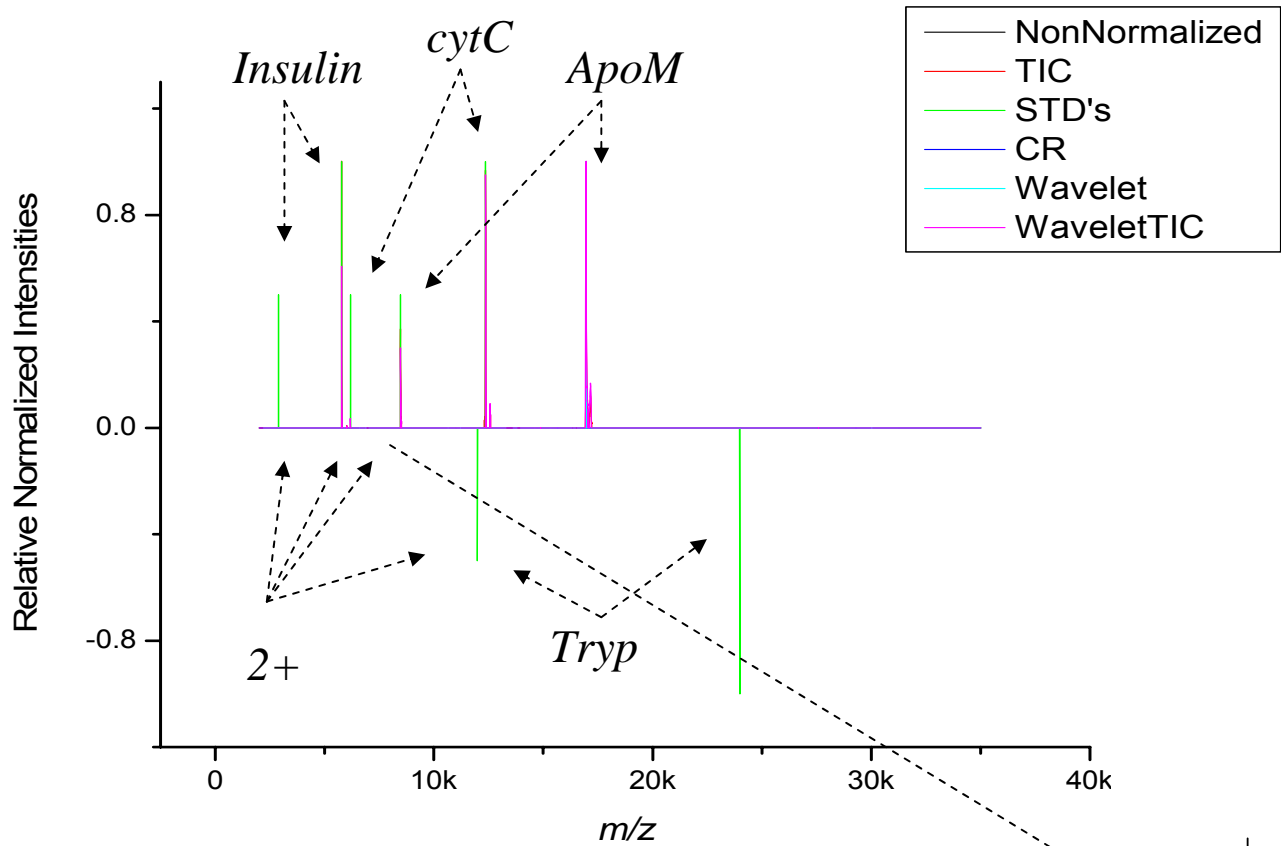


Testing the Approach: Liver Set Doped with Known Proteins 10 Spectra/ Set

1. Baseline Correct (Efeckta)
2. Smoothing (none)
3. Calibration (Efeckta)
4. Normalize/ Transform (TIC, Wavelet, Log, Ln, CR)
5. Standardization (none)
6. Peak Picking (WMA – data dependent cutoff)

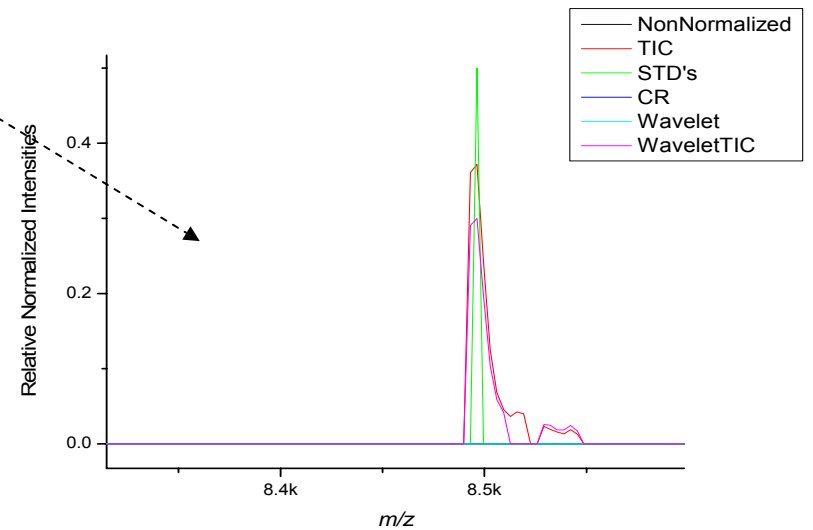
| | m/z | mix 1 | 2 | 3 | 4 |
|-------------------|---------|--------|---------|----------|---------|
| Insulin (porcine) | 5778.6 | 0.2375 | 0.11875 | 0.059375 | 0.02375 |
| Cytochrome C | 12361.2 | 0.95 | 0.475 | 0.2375 | 0.095 |
| Apomyoglobin | 16952.5 | 2.375 | 1.1875 | 0.59375 | 0.2375 |
| Trypsinogen | 23982 | 2.5 | 3.75 | 4.375 | 4.75 |

Spectra contain liver extract proteins spiked with the standard proteins listed above.
Concentrations are in pmol/uL (μM)

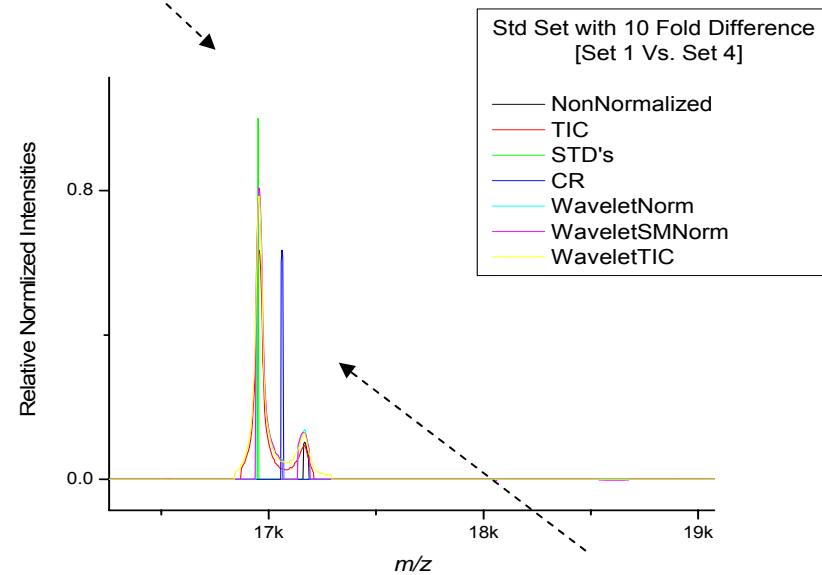
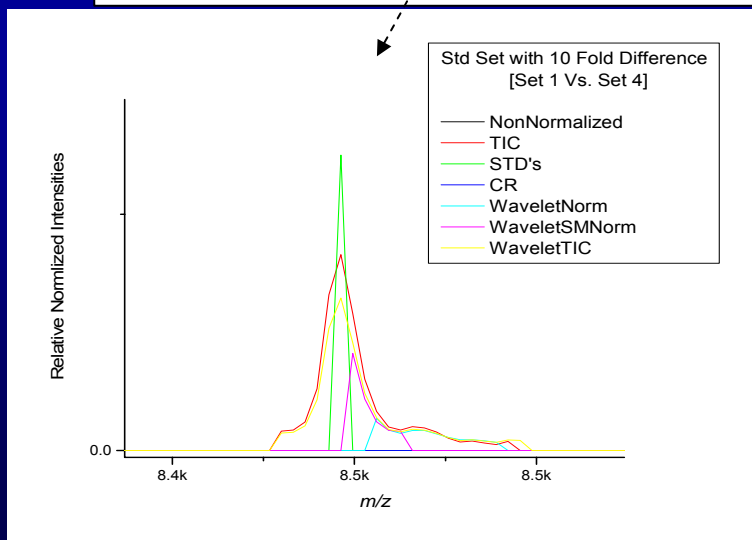
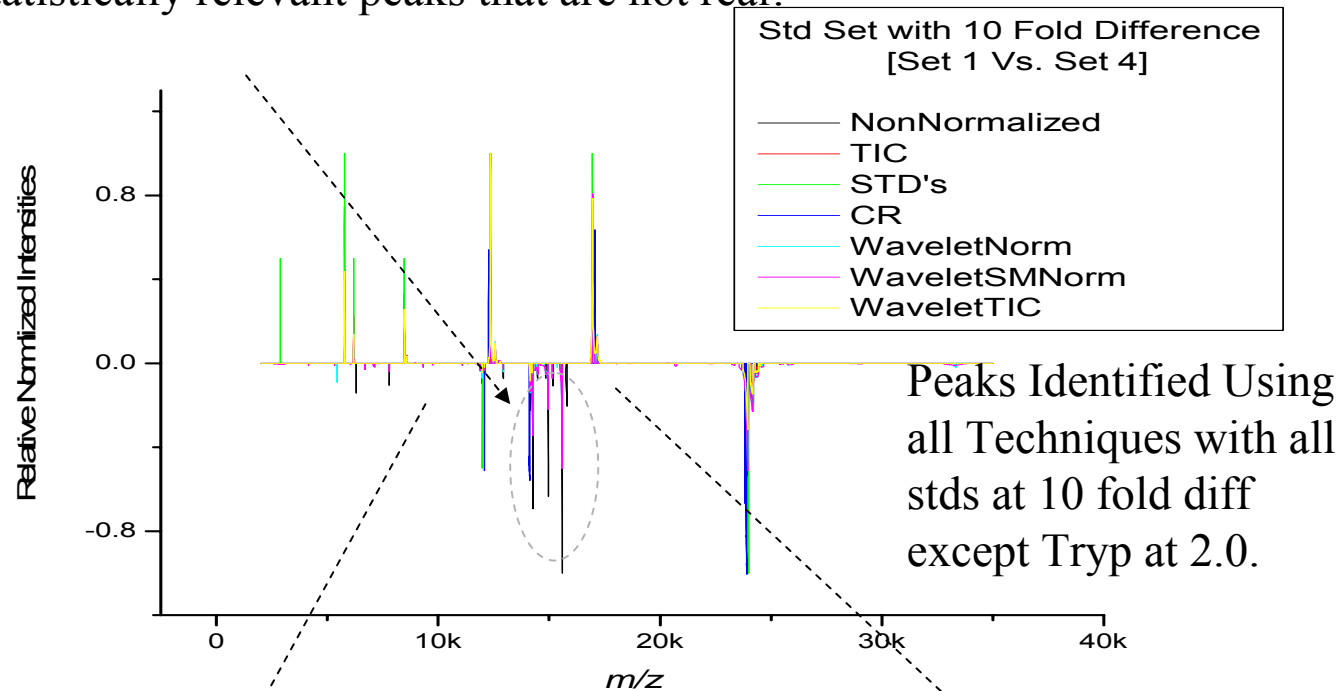


Peaks Identified Using all Techniques with all stds at 2 fold diff except Tryp at 1.3.

TIC and TIC with Wavelet work best!

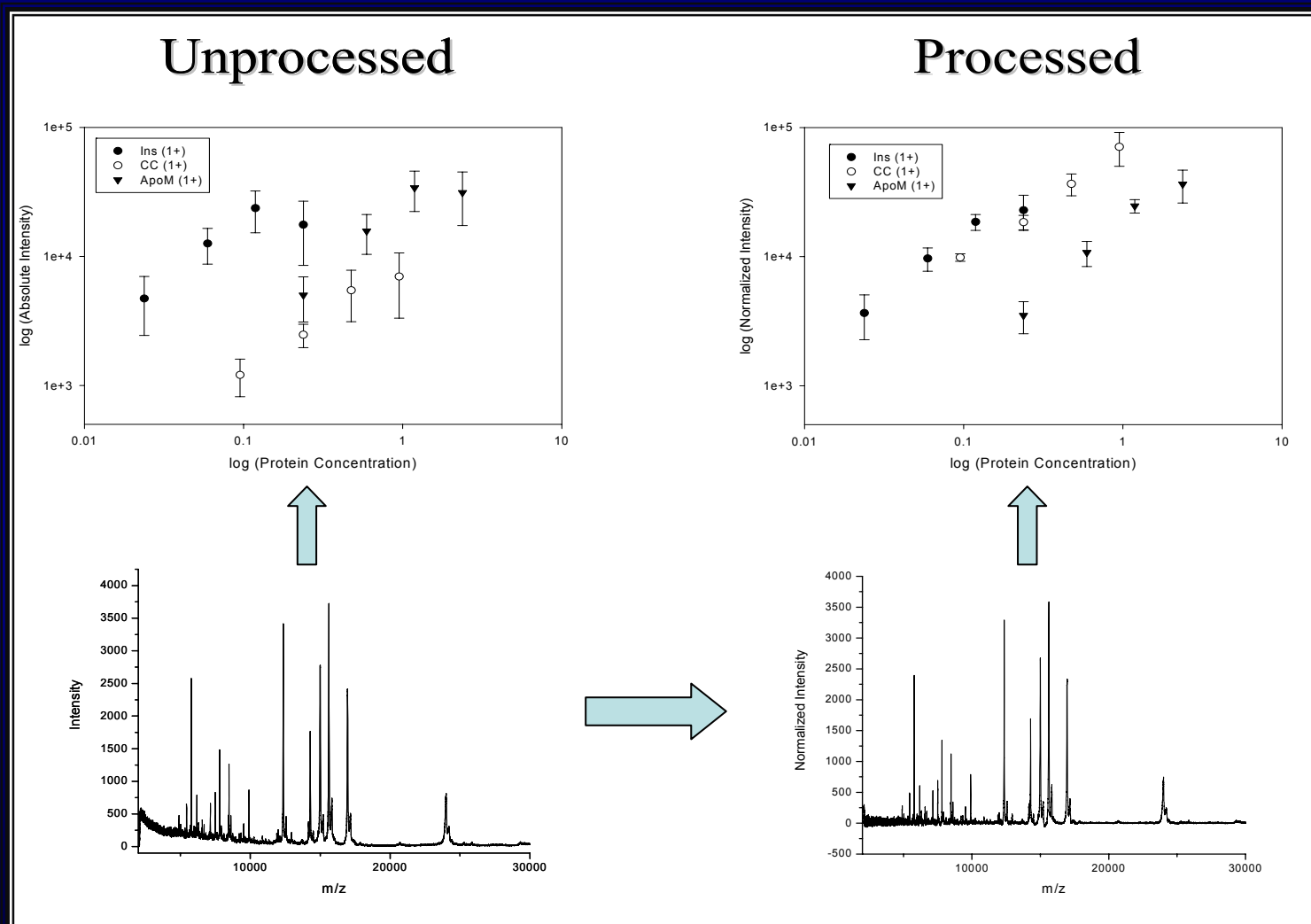


-statistically relevant peaks that are not real!

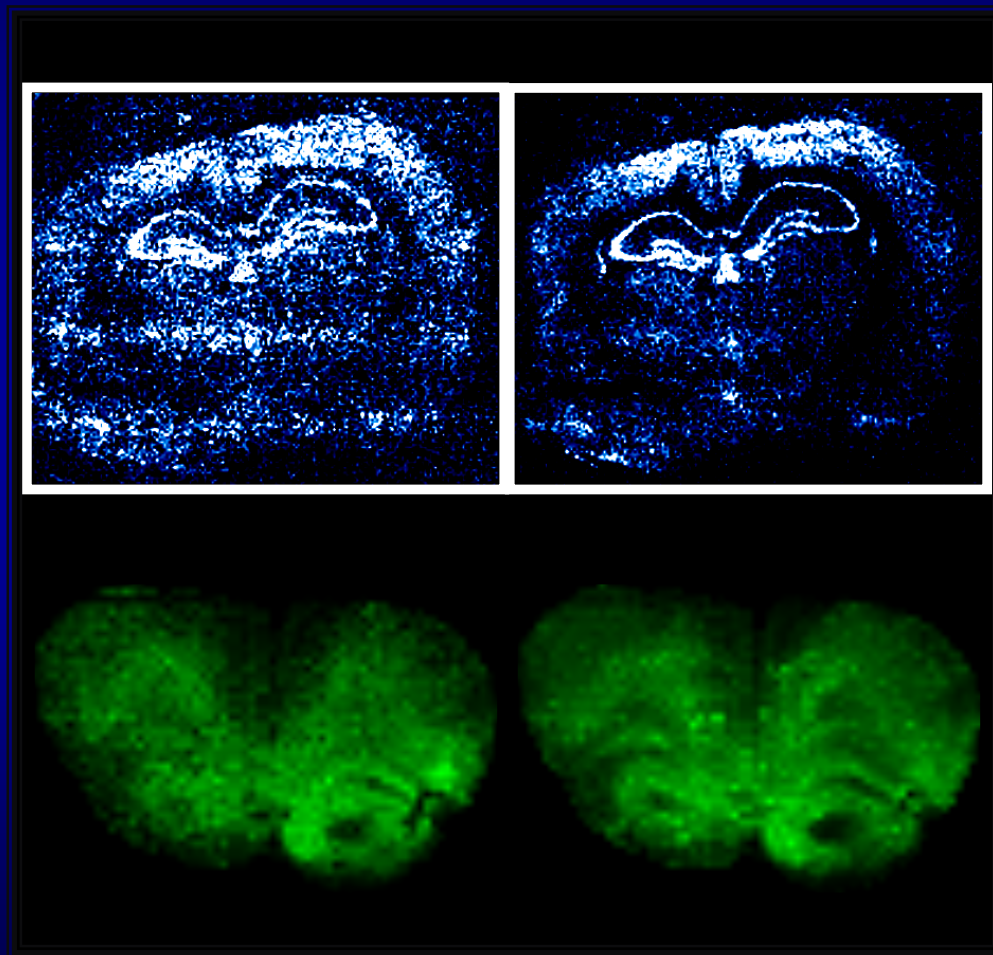


- logging techniques pick shoulders not peak asymptotes!

Spectral Processing Increases Detection Range and Sensitivity



These Techniques also Greatly Enhance MALDI Generated Images!

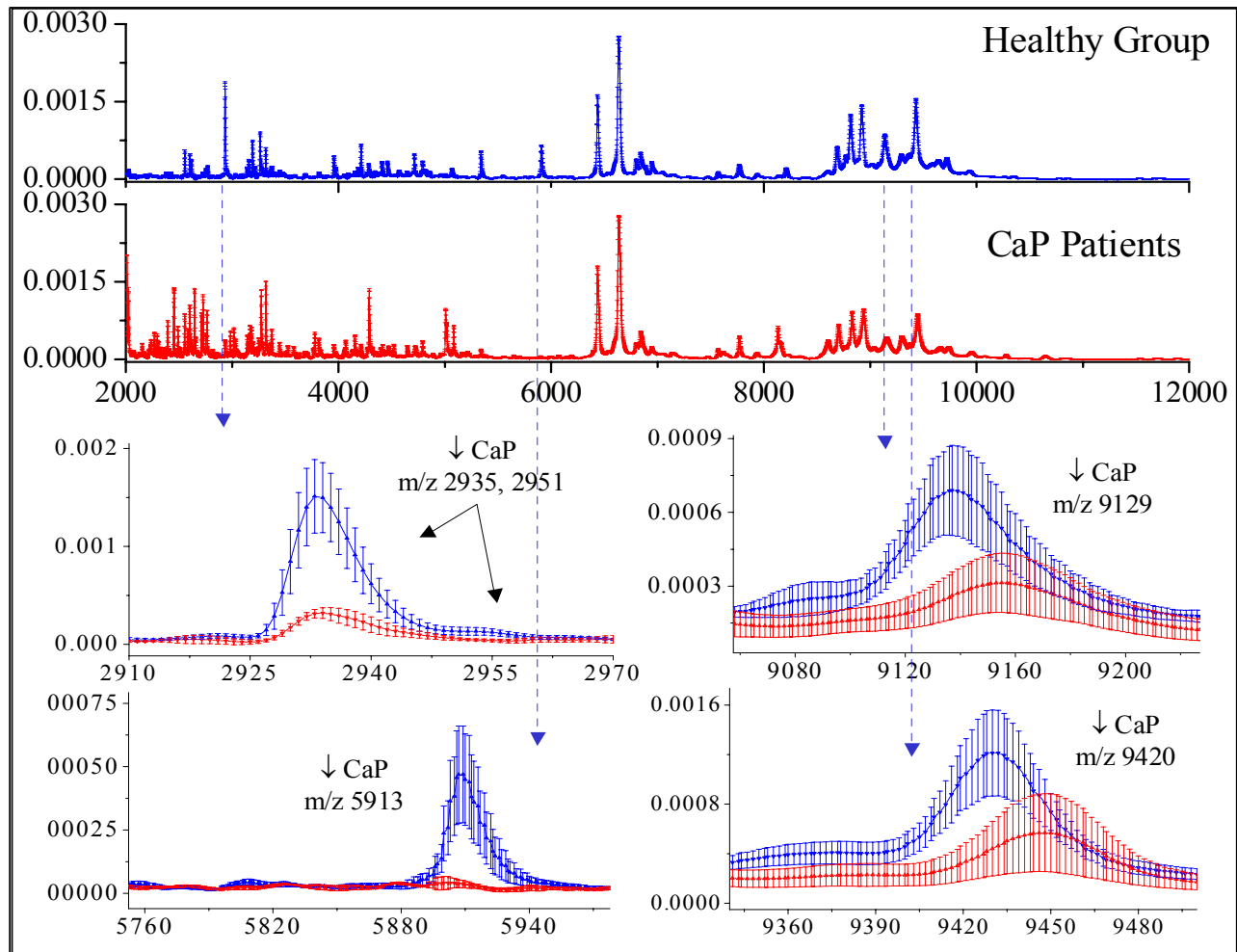


Norris J.L., Cornett, D.S., Mobley J.A., Andersson M., Caprioli R.M. *Processing MALDI Mass Spectra to Aid Biomarker Discovery and Improve Mass Spectral Image Quality*, International Journal of Mass Spectrometry, 2006 (In Print).

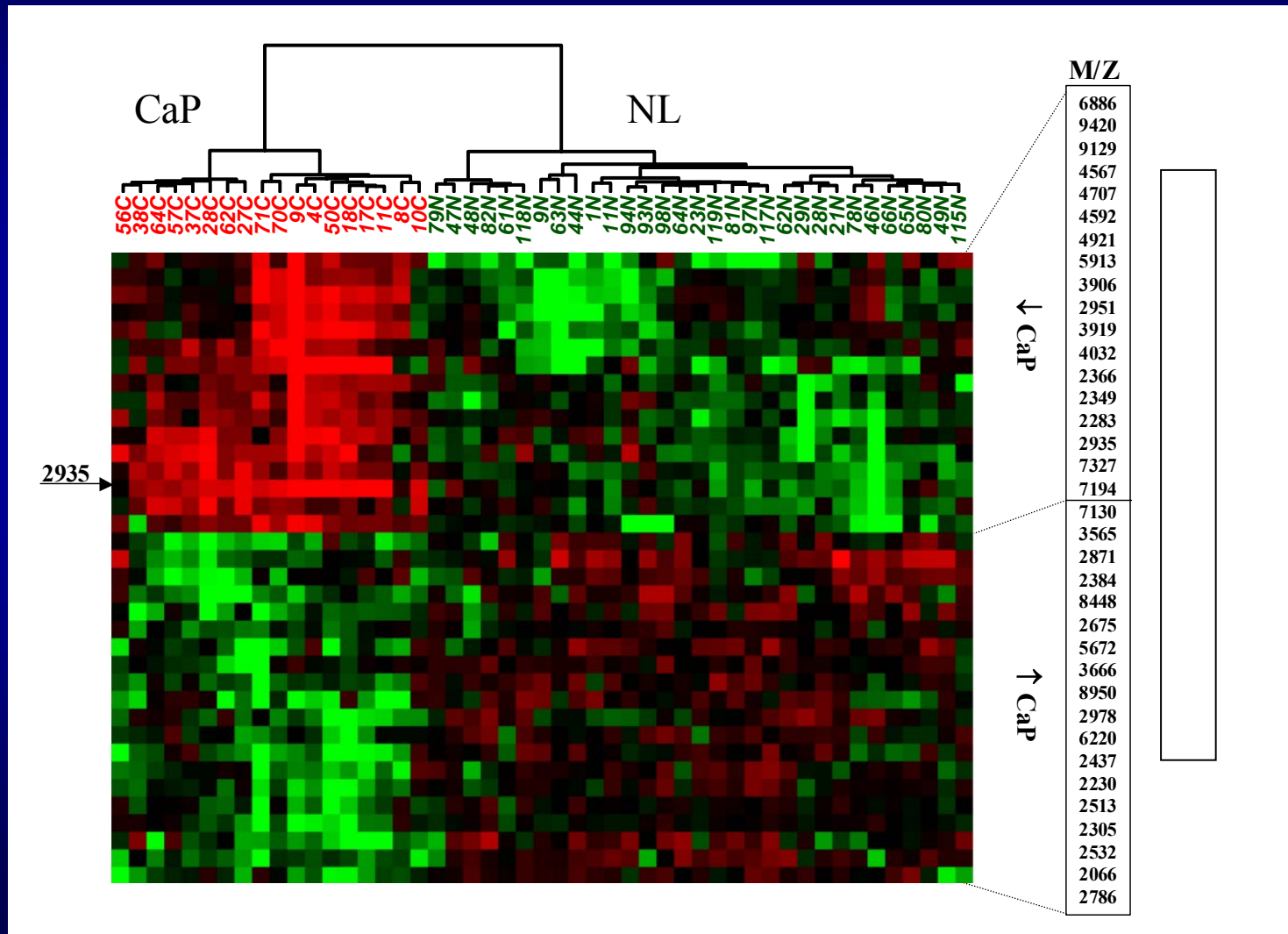
What About Statistics?

- Now we can apply these data sets to really any type of statistics that one might want to employ.
- But be careful, we have to insure that the peaks are real and not noise and not adducts of sodium, potassium, or matrix.
- This is “very” common and the mass spec person needs to be involved again at this point!

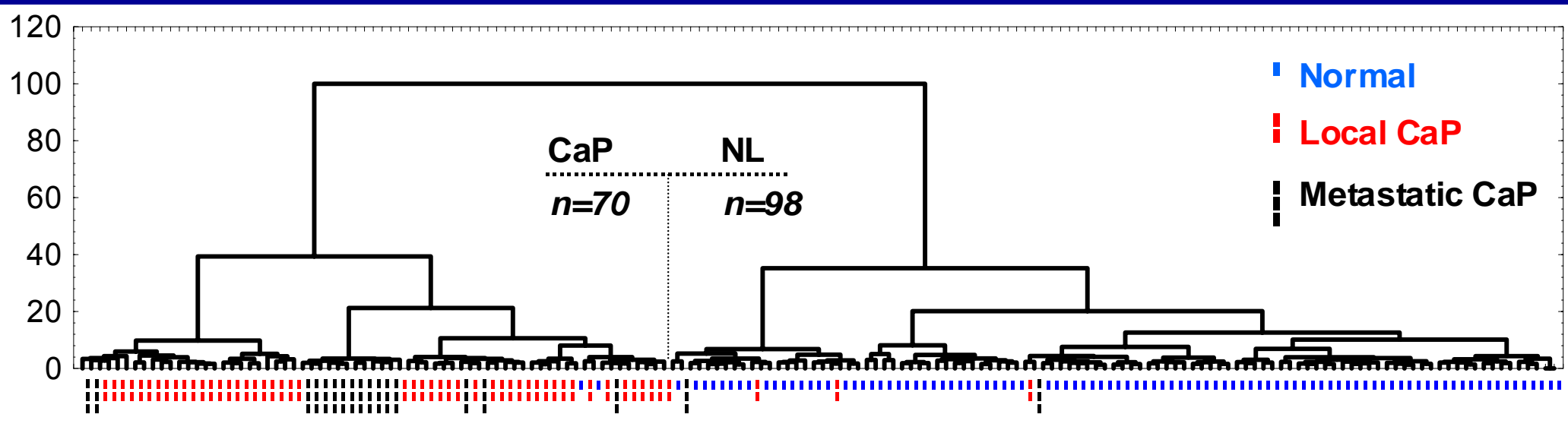
Evaluation of Peaks in Pairwise Analysis with a Std. Err Plot



Hierarchical Clustering Analysis Carried out Statistically Differing Peaks from a Training Set



Clinical Serum Profiling Experiment; Example of Hierarchical Clustering Analysis Carried out on 168 Patient Sample Set



Clinical Test Based on Specific Protein Peaks

Diagnostic Efficiency as Determined through Class Prediction
by Weighted Voting Scheme

General Stats:

| | N | Median Age | PSA > 4.0 | PSA < 4.0 |
|----------------|-----------|-------------------|---------------------|---------------------|
| Normals | 98 | 55 | 12 (4.2-7.8) | 86 |
| CaP | 70 | 64 | 62 | 8 (1.9-3.6) |

Diagnostic Stats:

| # Variables | N | Sensitivity | Specificity | PPV | NPV | Non-Predicted |
|--------------------|------------|--------------------|--------------------|-------------|-------------|----------------------|
| 280 | 168 | 94.1 % | 99.0 % | 0.99 | 0.96 | 5 |

Sensitivity; $TP / (TP + FN)$

Specificity; $TN / (TN + FP)$

PPV; $TP / (TP + FP)$

NPV; $TN / (TN + FN)$

Note: Non-predicted values were not included in final calculations

TP – true positive

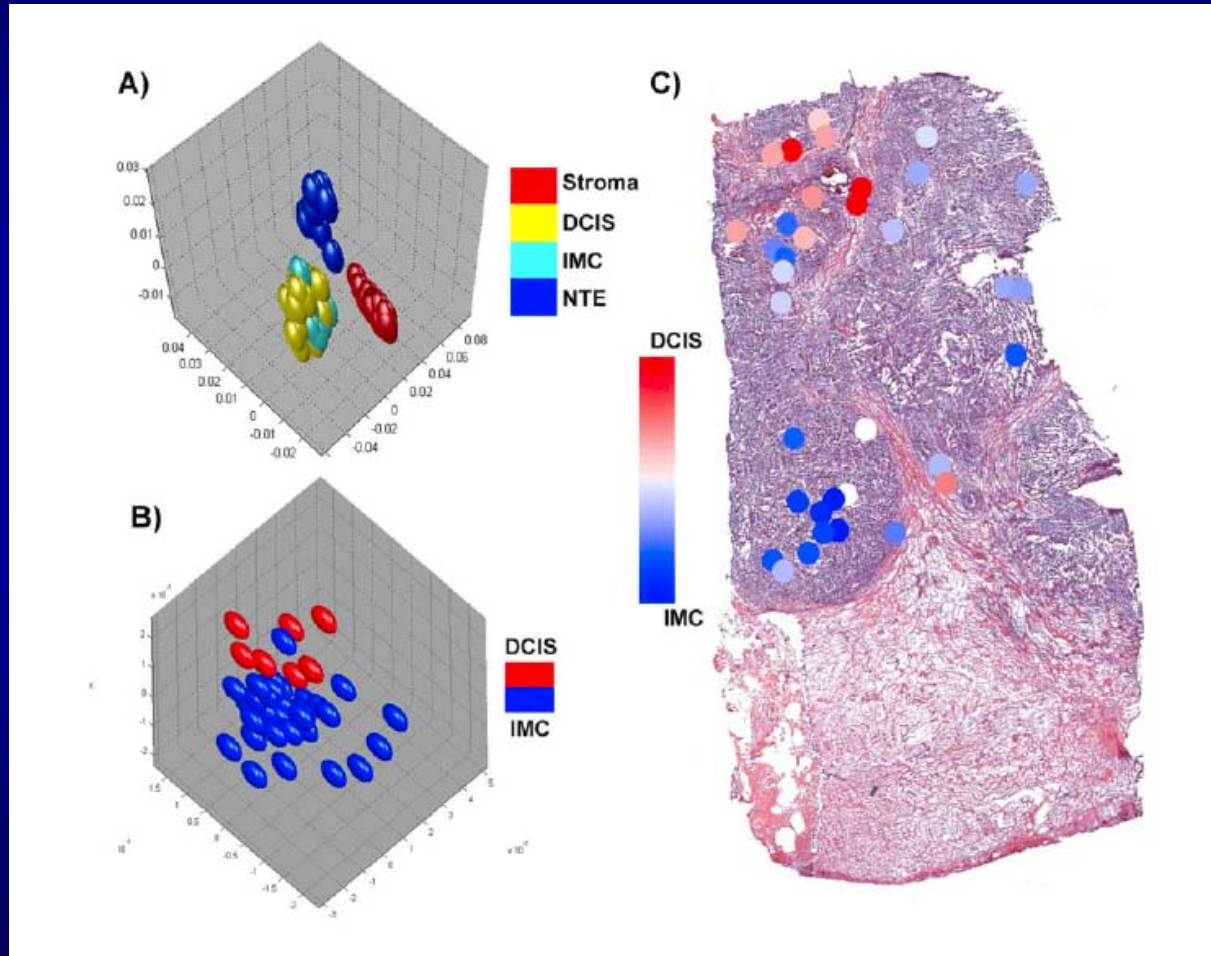
TN – true negative

FP – false positive

FN – false negative

Another Example:

Biomarker Identification and Classification was Applied to a Single Section; (May be able to differentiate DCIS from IMC??)



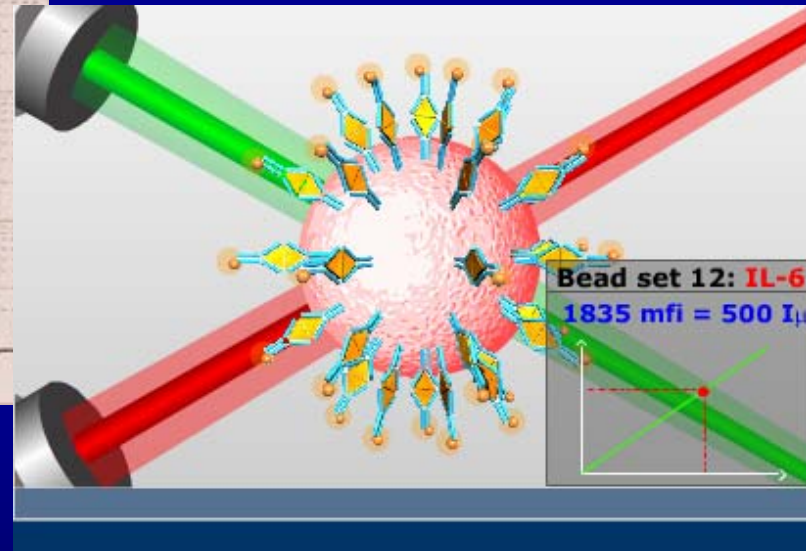
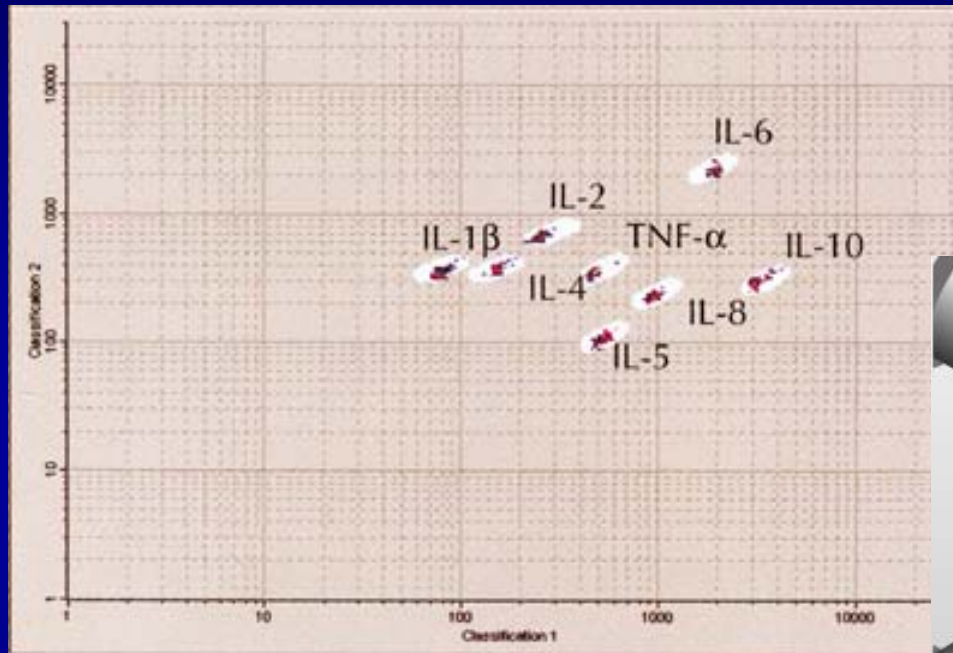
What to Do with These Peaks?

- Top Down Proteomics
- Bottom up Peptidomics
- Medium Down Approaches
- Top Down Directed

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).

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!

Quantitative Proteomics

(Mechanism Studies)

- Stable Isotope Tags:
 - ICAT (isotope coded affinity tag)
 - SILAC (stable isotope labeled AA in cell culture)
 - iTRAQ (Isotope tags for relative and absolute and quantification)
 - ^{18}O Digests (labels trypsin digested peptides at lysine and arginine)
 - Many Other Chemical Tags

Conclusions??

- Draw your own.....
- Just Another Tool in the Toolbox???
- Too Soon to Know, Let's see where it takes us!

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Questions?

