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…….Mapping a new direction

in Proteomics……..

HPLC has been so successful in routine Pharma small molecule – drug development…….

In the post-Genomic era, however, …

<u>How do you make HPLC a similar success story in routine intact protein analysis in the Bio World?</u>

- ***Separate Expressed Proteins from highly complex samples – Lysates, Subcellular Extracts, Sera, BioFluids, etc.***
- ***Protocol Driven Experimentation – Inter and intralab comparison of data from complex samples.***
- ***Visual Imaging of Complex Systems – 100's to 1000's of Proteins to be separated per sample.***
- ***Smooth Interface with MS – ESI-TOF (MW), LCMSMS (ID)***
- ***Smooth Interface with Antibody and Staining Detection Methods – Westerns, ELISIA's, etc.***

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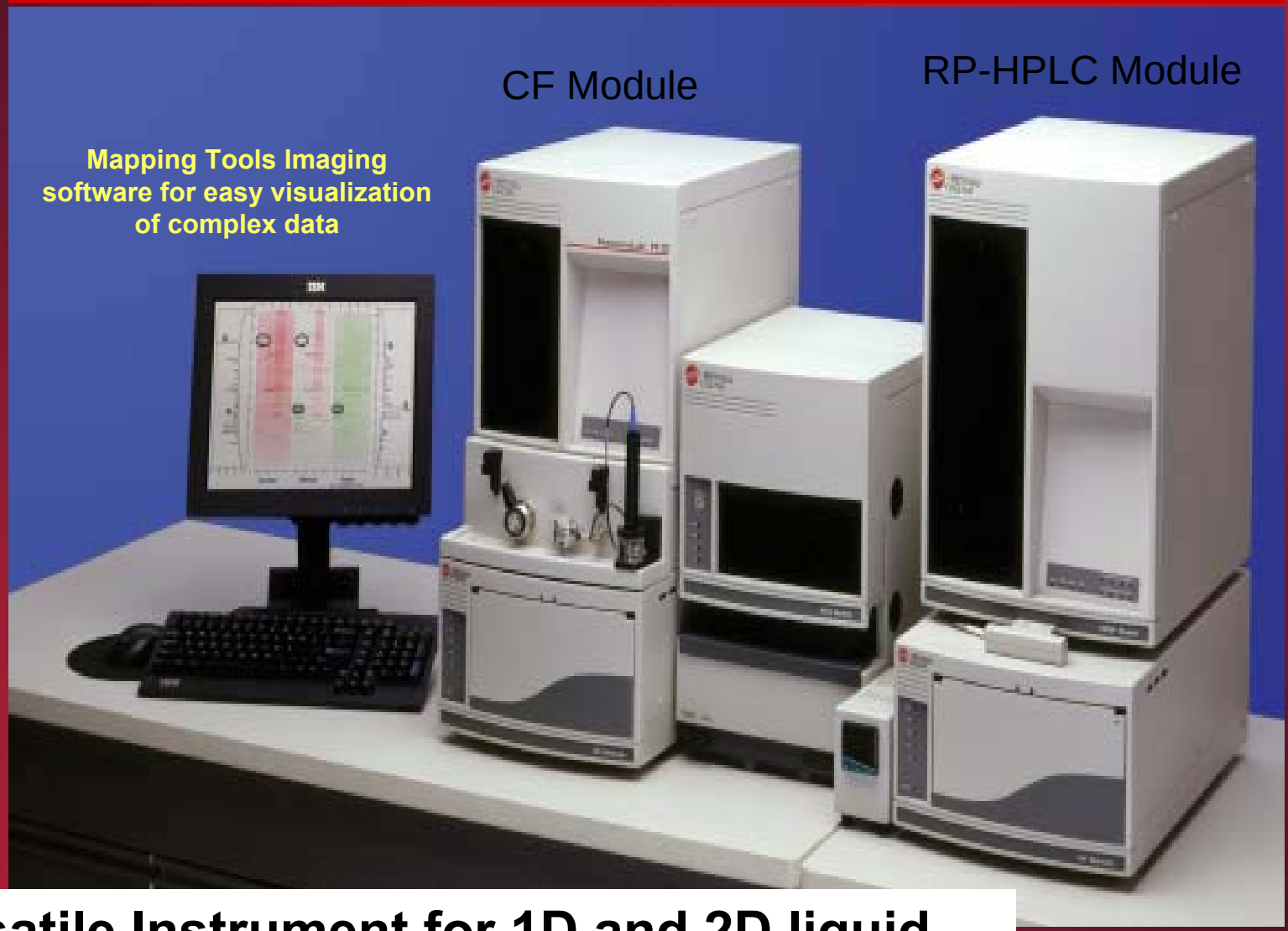
HPLC Based Strategies for Analysis of Proteins in Complex Biological Systems

1. **Using the PF2D liquid fractionation system provides strategies for protein mapping that serve as useful alternatives to 1D and 2D Gel electrophoretic based methods in Proteomics.**
    - ***NPS*® RP HPLC for 1D separations**
    - **CF – *NPS*® RP HPLC for 2D orthogonal separations**
2. **Liquid fractions generated by the PF2D instrument provide a very efficient means of mining for new and existing protein biomarkers:**
    - **Proteins are isolated "intact" for direct PTM analysis.**
    - **High degree of automation possible from sample prep to detection using antibodies and MS techniques.**
    - **1D and 2D Protein Microarrays can be easily printed for testing with a variety of probes and detection methods.**

Chromatofocusing (pI) – RP-HPLC (Hydrophobicity)

ProteomeLab® PF2D System from Beckman Coulter



Versatile Instrument for 1D and 2D liquid fractionation of complex protein samples

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ProteomeLab PF2D Flow-Scheme

1st Dimension
pI – (Chromatofocusing)

Liquid CF Fractions
Every 0.2 – 0.3 pH units

~ 20 Liquid CF Fractions
100 ug – 5 mg protein loadings

2nd Dimension
Hydrophobicity (RPHPLC)

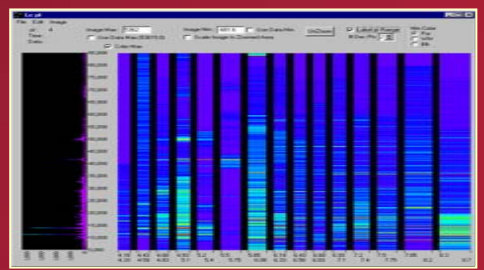
Liquid Fractions in
Multi-well plates

UV
Detector

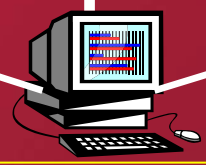
~ 1000 Liquid Protein fractions for MS analysis
or traceable "Array of Proteins" for analysis

Mass
Analyzer

2D (pI/Mass) Map

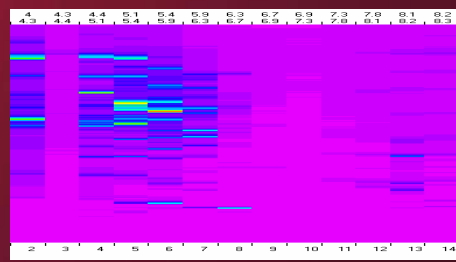


ProteoVue® Software Suite



Bioinformatics

2D (pI/Hyd) Map



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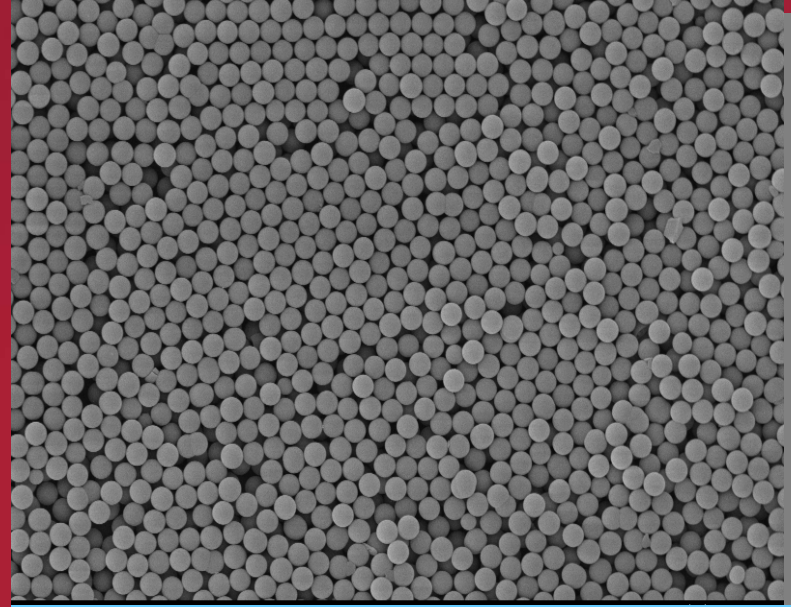
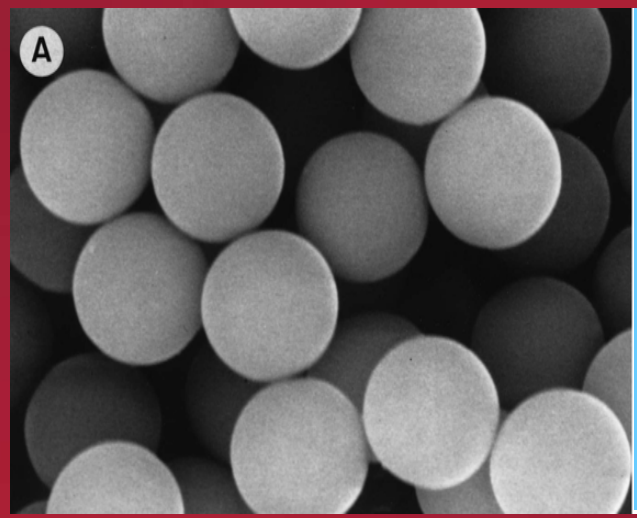
NPS® – An Ultra-Fast, high-resolution HPLC support tailored specifically for, Proteins & MS



NPS Beads SEM Mag 5000x



1.5 µ NPS Beads

The totally non-porous nature of NPS provides for fast mass transfer kinetics and high recovery of proteins at low surface carbon loads and organic MP modifiers. This allows for easy elution and recovery of highly hydrophobic proteins.

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Biological Samples Analyzed

Whole Cell Lysates

Melanoma, Breast, Colon & Ovarian Cancer Lysates; Hepatocytes, Mouse embryonic stem cells, Yeast, E. coli, Staph Bacteria, Rat Brain Tissue, PBMC's

Protein BioFluids

Secreted Proteins (conditioned media), Sera, Plasma, Amniotic Fluid, Ascites, Saliva, Urine, Various Lavages, CSF.

Misc. Protein Samples

Veterinary Vaccines, Bacterial Antigens, Bacterial spores and extracts, Plant extracts, GMO samples, Meat Product extracts, Milk/Cheese Extracts

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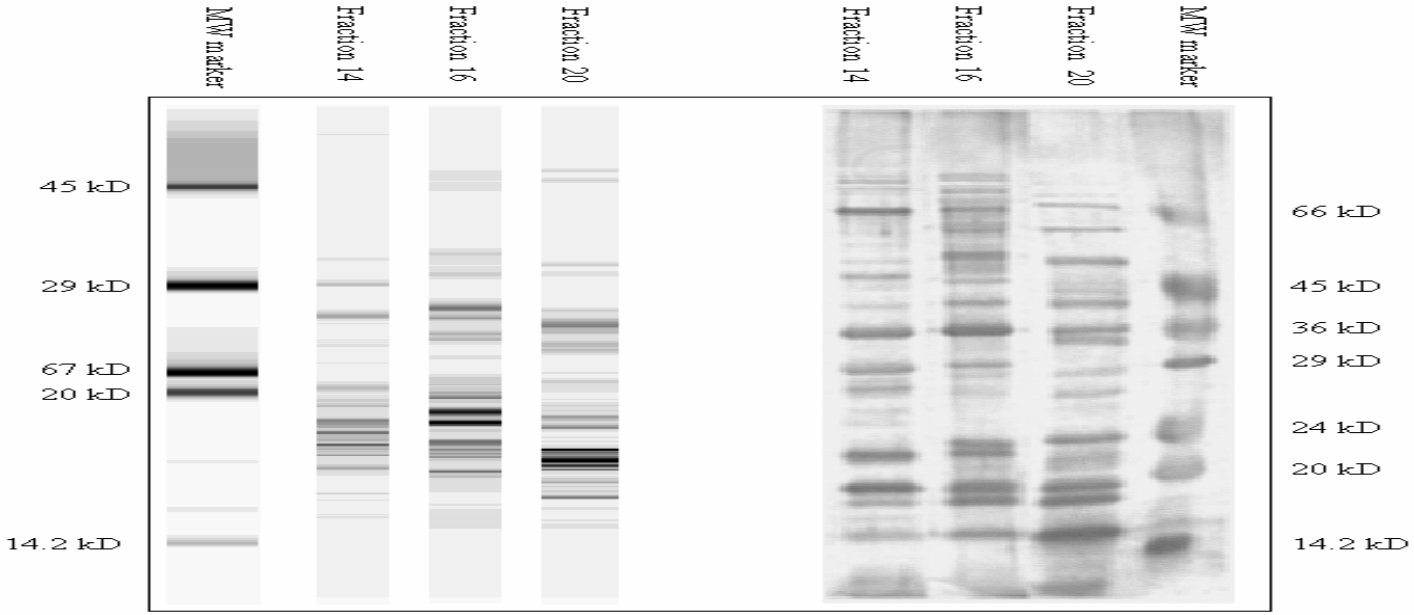
HEL Cell Lysate Fractions



NPS RP-HPLC

1D Gel

LOD UV214 = 2 – 5 ng

Wall, DB; et.al., Anal. Chem. 72(6) 2000, 1099-1111

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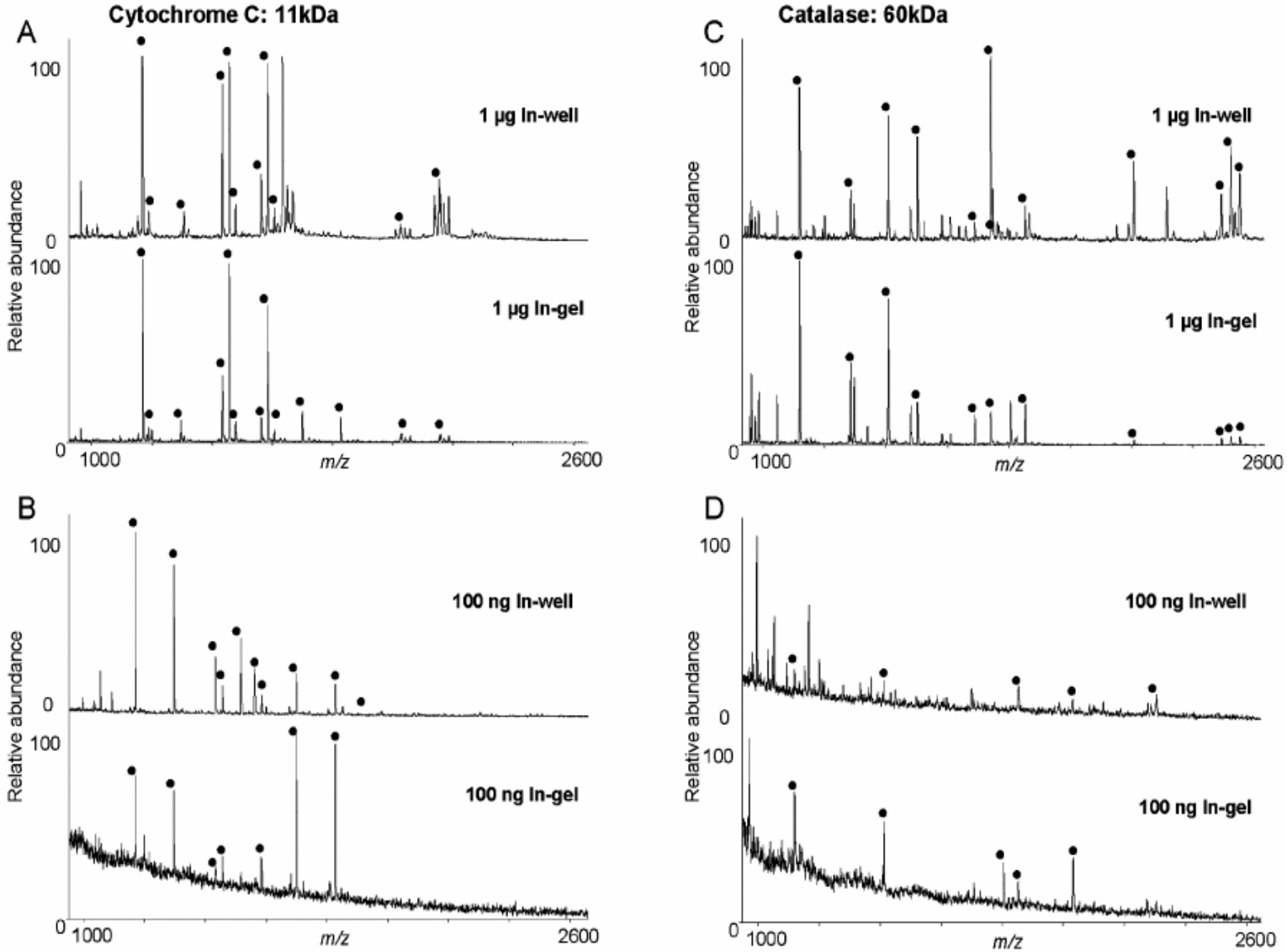
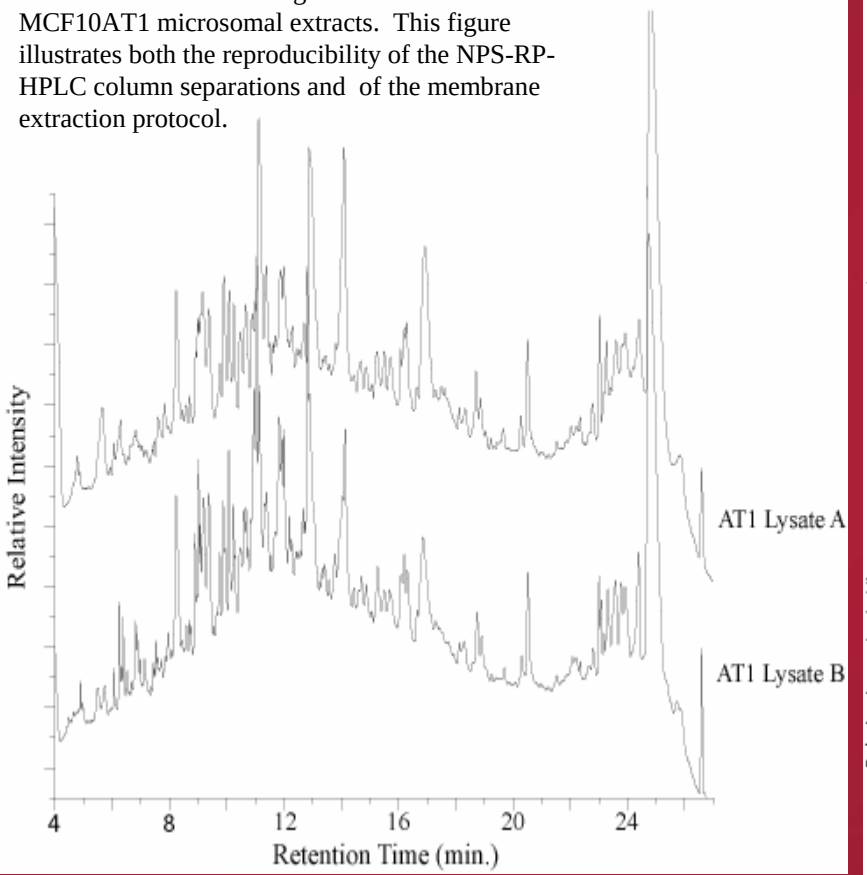


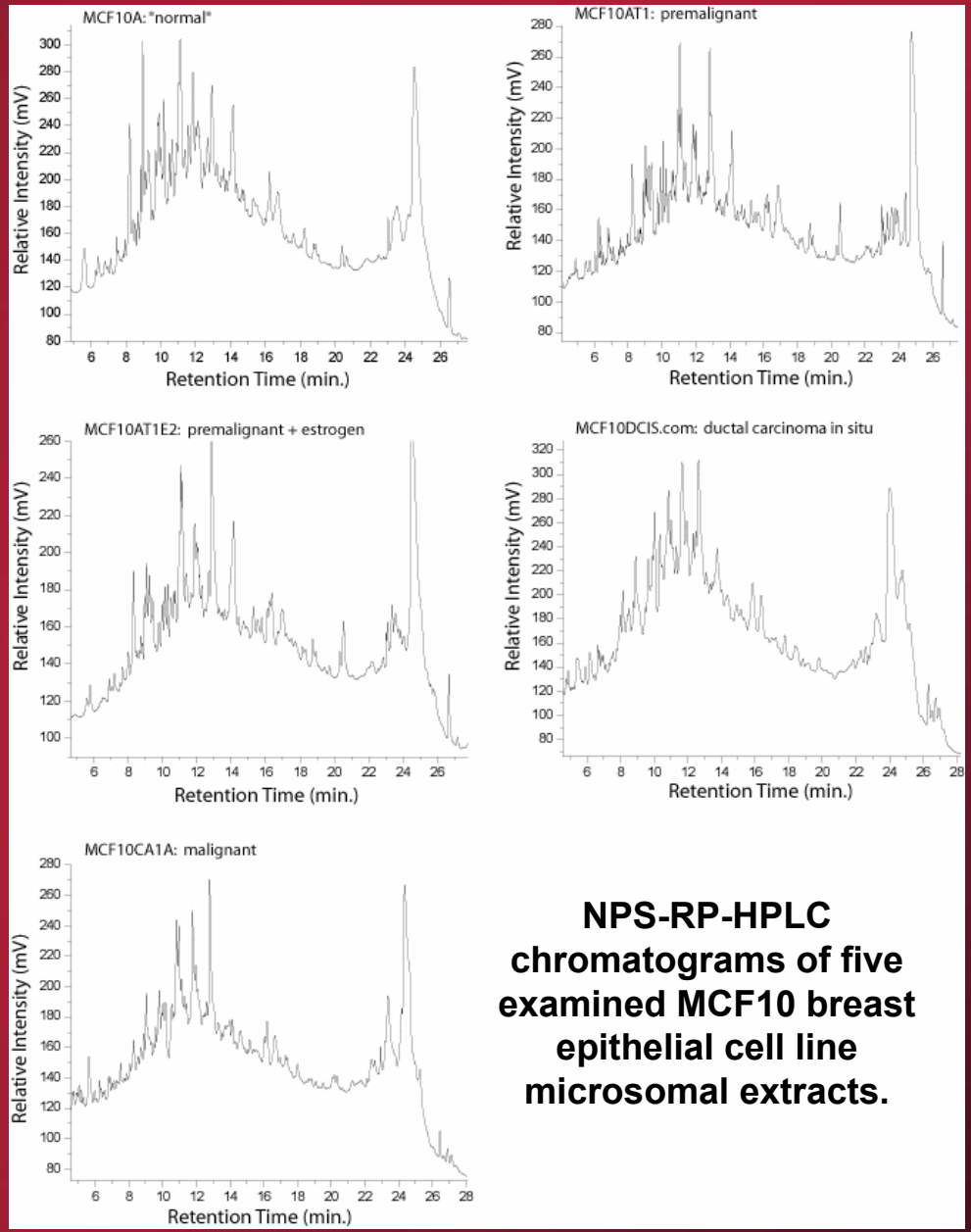
Figure 6. In-well vs in-gel digestion of separated proteins. Cytochrome *c* (A and B) and catalase (C and D) (1 μg or 100 ng) were separated from the whole protein standard by either by np-RP−HPLC or by SDS-PAGE electrophoresis. Corresponding fractions or bands were respectively in-well or in-gel digested with trypsin and resulting peptides were analyzed by MALDI-TOF−MS. Spectra corresponding to peptides from cytochrome C and catalase were aligned and peaks which matched the theoretical digest of the protein are highlighted (•) for each protein. Values correspond to results shown in Table 2.

Dauly, C., et. al., Journal of Proteome Research 2006, 5, 1688 -1700

Fast and High Resolution Analysis of Complex Protein Samples using *NPS* HPLC Columns

NPS-RP-HPLC chromatograms of two different MCF10AT1 microsomal extracts. This figure illustrates both the reproducibility of the NPS-RP-HPLC column separations and of the membrane extraction protocol.





NPS-RP-HPLC chromatograms of five examined MCF10 breast epithelial cell line microsomal extracts.

Chong BE, Lubman DM, Miller FR, Rosenspire AJ. Rapid Commun. Mass Spectrom. 1999; 13: 1808.

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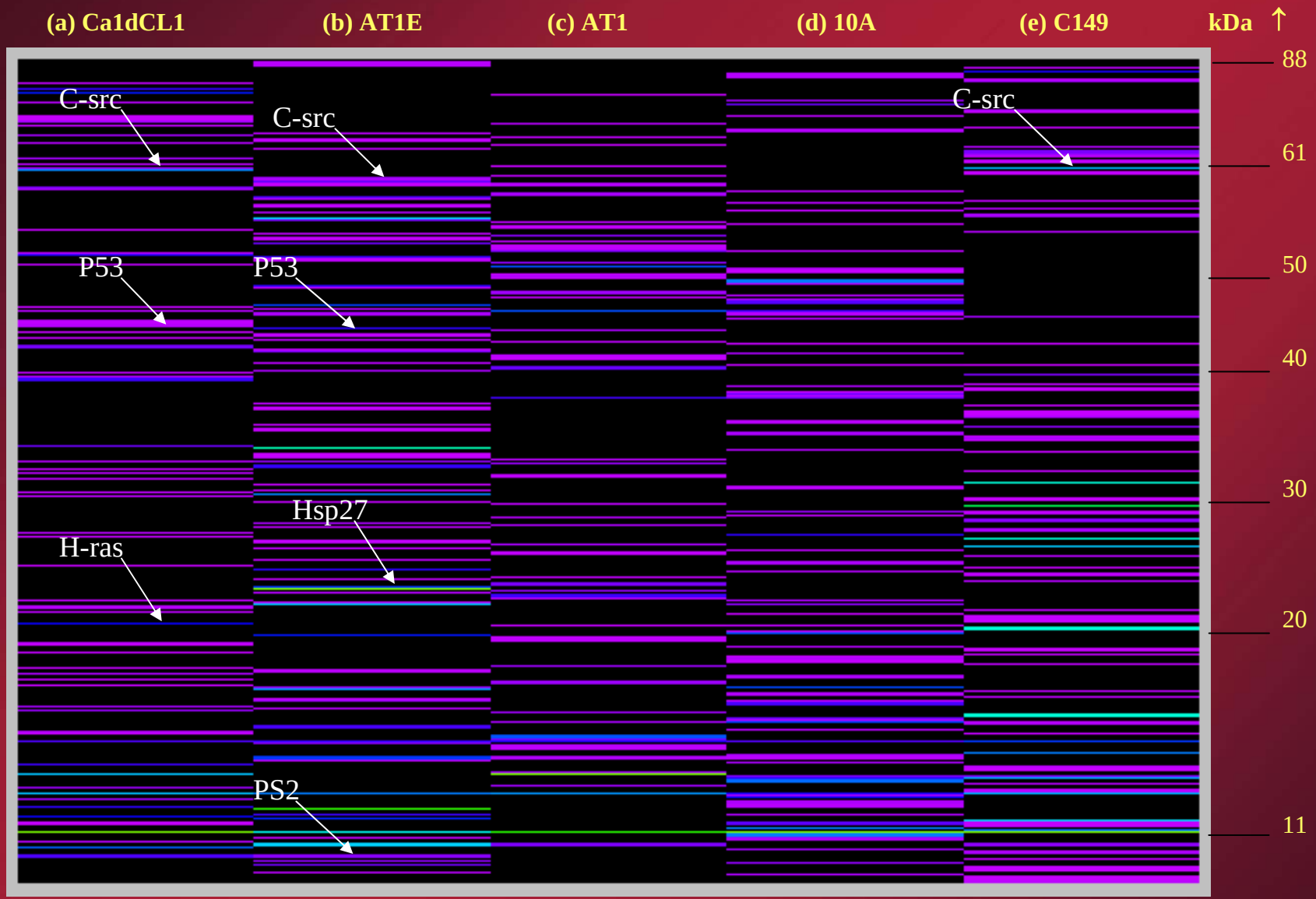
Mapping Tools Software for Imaging of Liquid Fraction Data



1D Maps of LC-ESI TOF MS Data – Direct Interface of *NPS* RP-HPLC with ESI-TOF

Chong, BE; et.al., Anal. Chem. 73(6) 2001, 1219-1227

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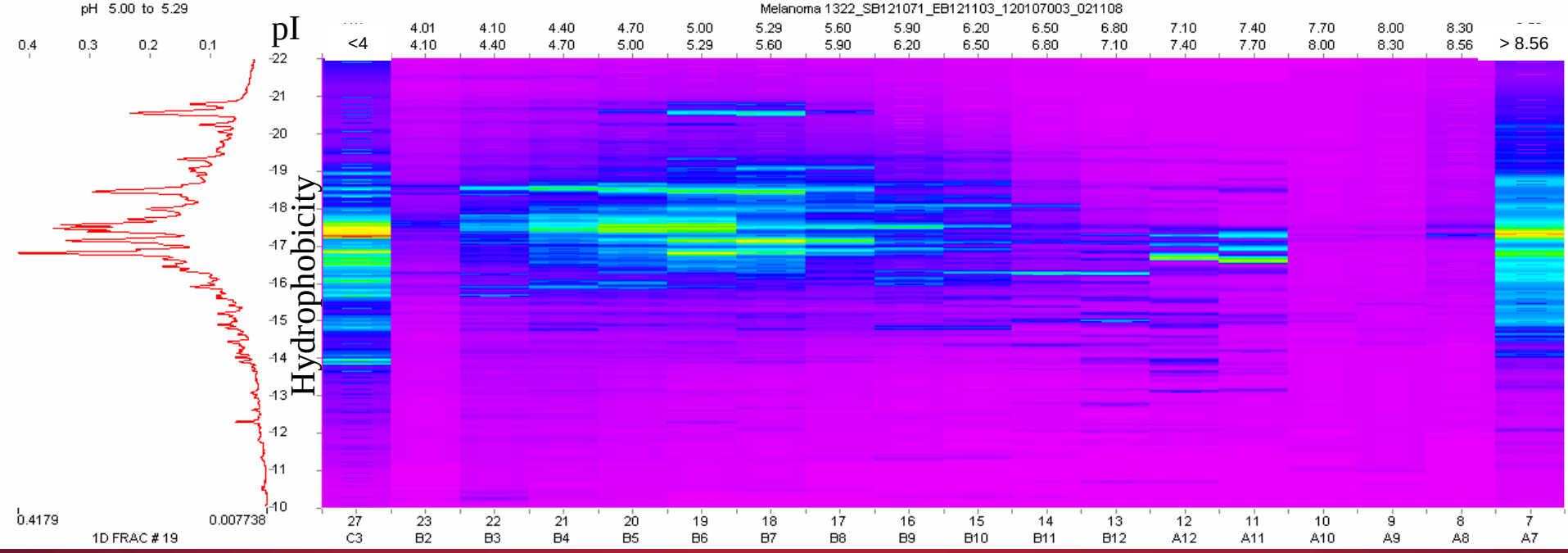
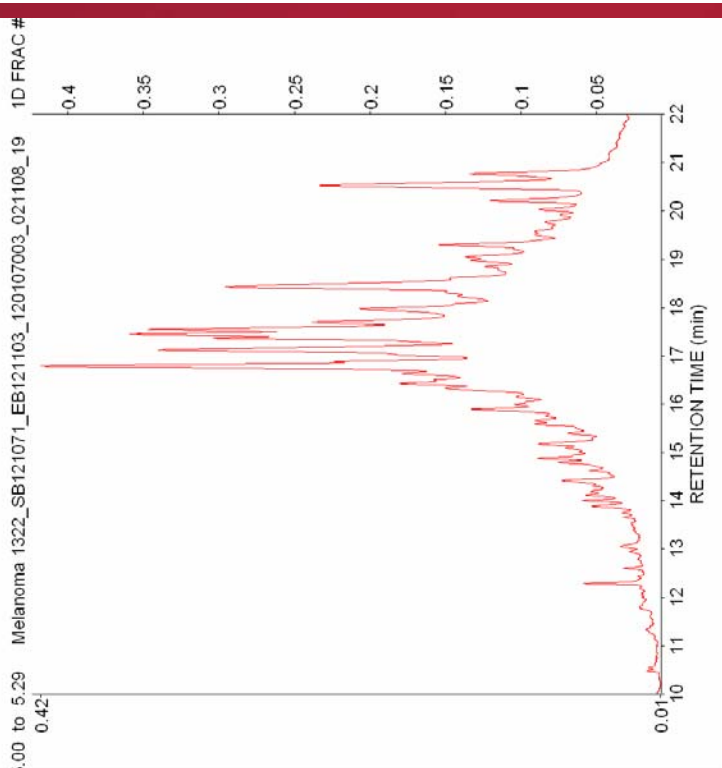
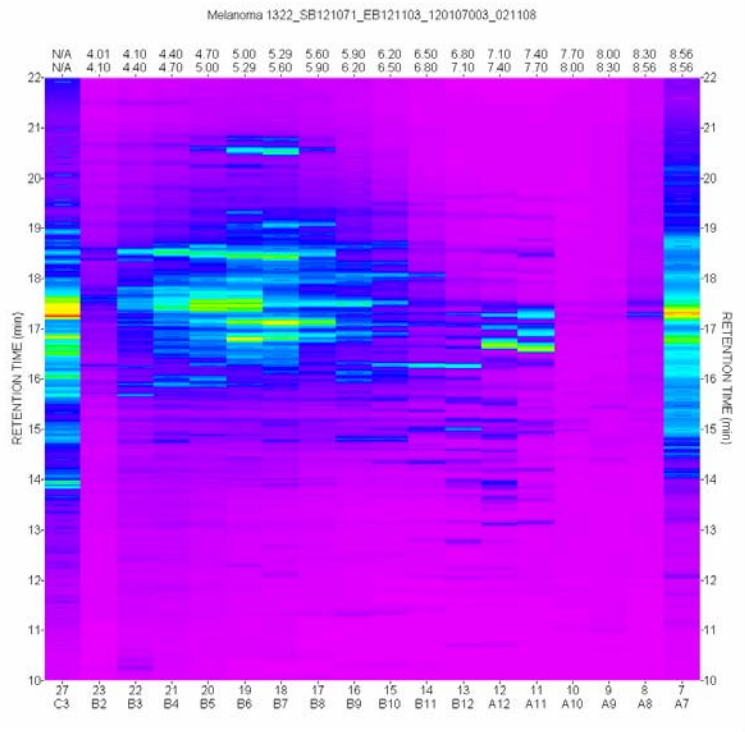
2D Maps of Melanoma Cell Lysates

66 Protein "Bands" identified for this pI fraction alone.







Differential Proteomic Analysis of Ovarian Cell Lines

Ovarian clear cell adenocarcinoma (ES2) cell line & HPV16 E6/E7 ovarian surface epithelial (OSE) cell line

Here is why Intact Protein Analysis is important! It is what HPLC brings to the table!

Maureen T. Kachman, Haixing Wang, Donald R. Schwartz, Kathleen R. Cho, and David M. Lubman, Analytical Chemistry. Vol. 74, No. 8 (2002) 1779-1791.

Yanfei Wang, Rong Wu, Kathleen R. Cho, Kerby A. Shedden , Timothy J. Barder and David M. Lubman; Mol Cell Proteomics, 2006, 5(1):43-52

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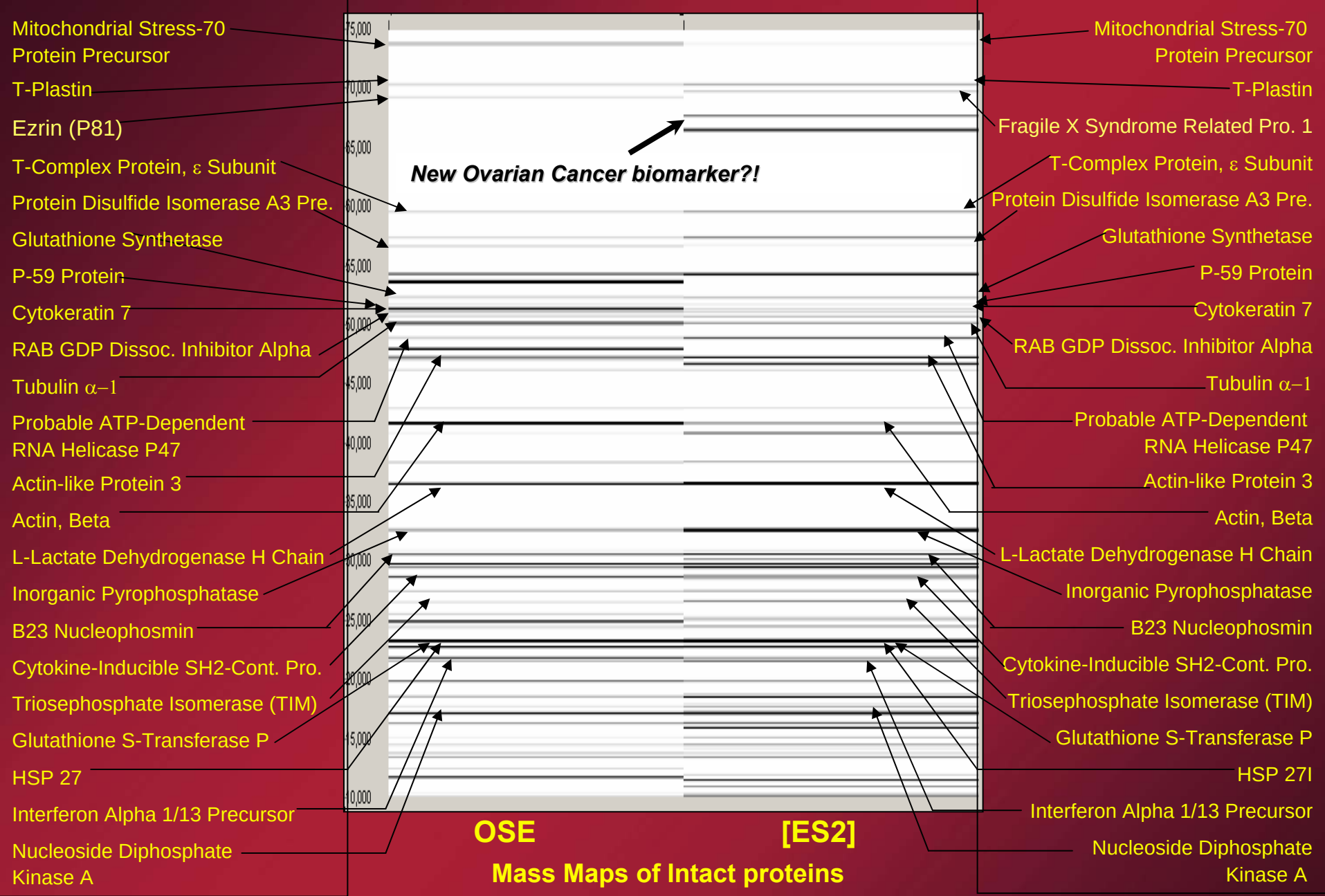

Voyager-STR MALDI TOF peptide mapping to ID proteins

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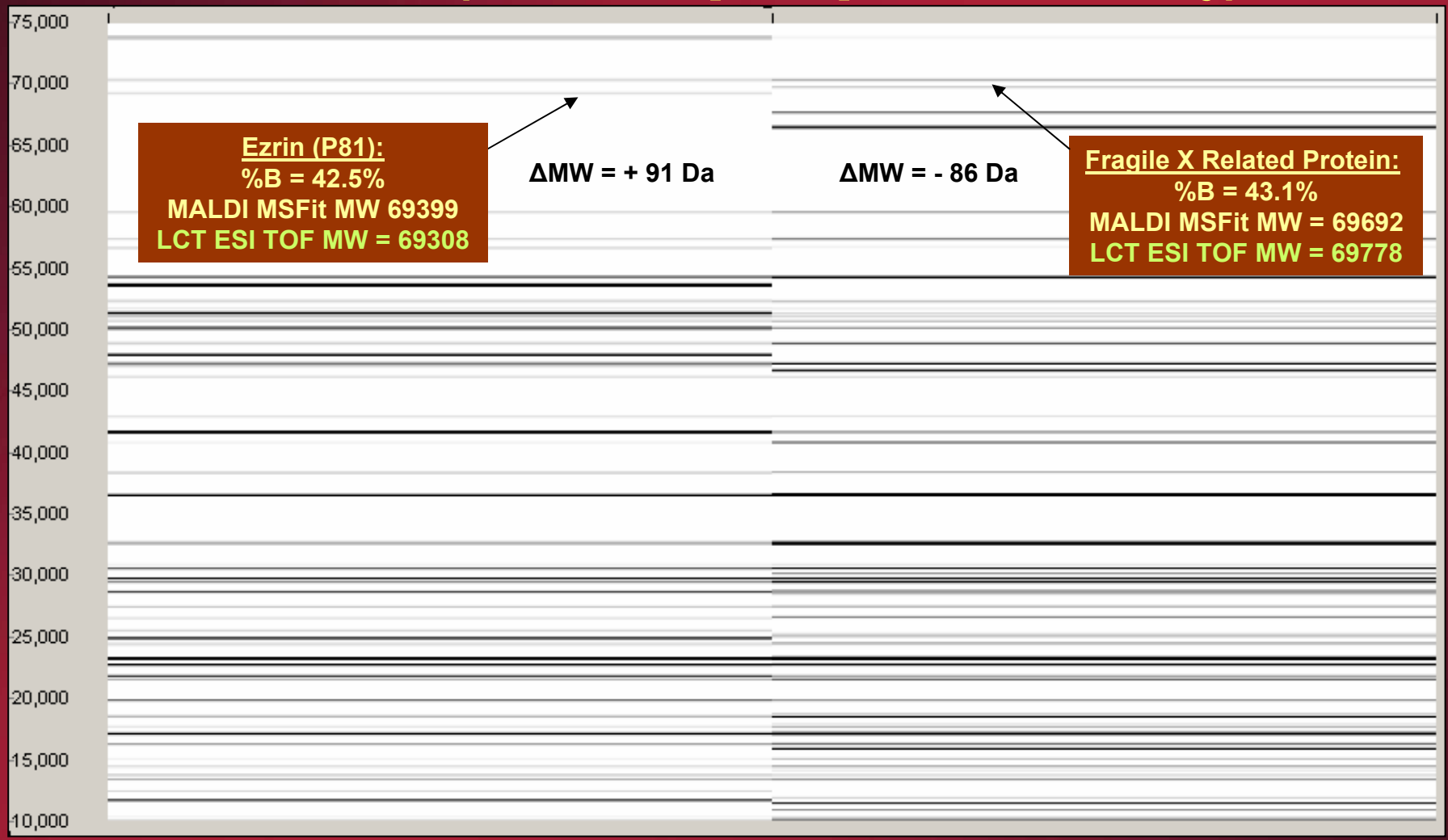
Mapping Tools Mass Map Images of pI 6.4 fraction for Ovarian Cell Lines

Database MW – Intact protein MW [Δ MW] values indicate type of PTM



Micromass LCT ESI-TOF analysis of comparative pI fraction

Kachman, M T.,et. al, Analytical Chemistry. Vol. 74, No. 8 (2002) 1779-1791.

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New Protein Biomarker Search Protocol

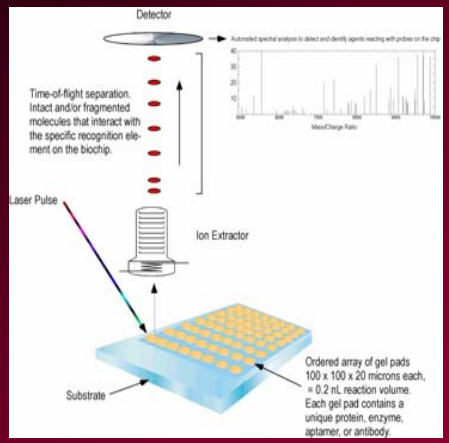
Liquid Fractionation provides direct access to the "intact" proteins reducing sample complexity and allowing for easy interface with MS Tools and Microarray Printers!

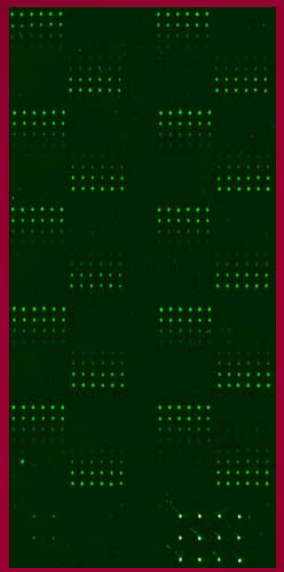
⇒ Combine MS Tools with Microarray detection methods to make a better biomarker search and selection platform!

Complementary and Independent detection methods for the same Sample (Fraction)!

Initial Biomarker Validation data at the start of the search and selection process!

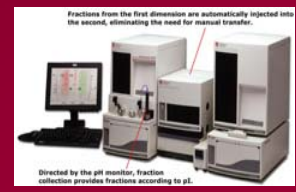
Protein identification
(On-Chip mass spectral analysis)



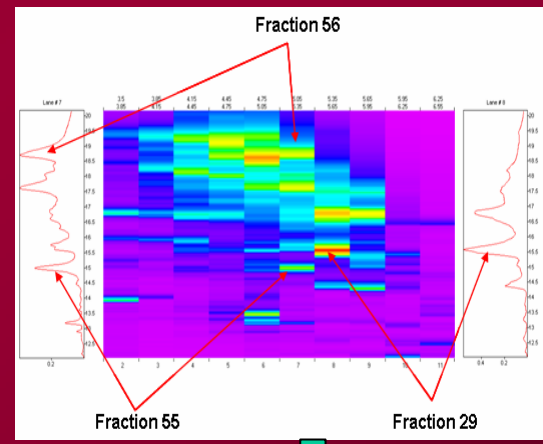


PF2D liquid-phase fractionation
(PI, hydrophobicity)

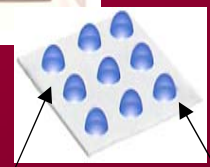
Whole cell or Sub-cellular isolate (membrane, cytosol)



Proteomic profile
(analytical)



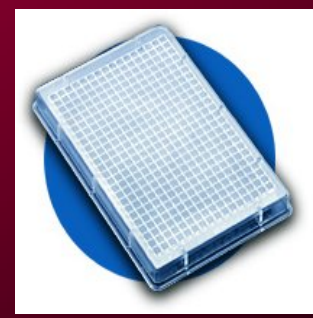
"A bottleneck in creating protein arrays, especially those which aim to be global, is the production (expression and purification) of the huge diversity of proteins which will form the array elements, including capture molecules." The European Science Foundation



Glass support

Gel drops with immobilized probes

Array fabrication



Fraction collection
(preparative)

Dan Schabacker; ANL

Patwa, T., et. al.,
Anal. Chem., 78 (2006)
6411-6421

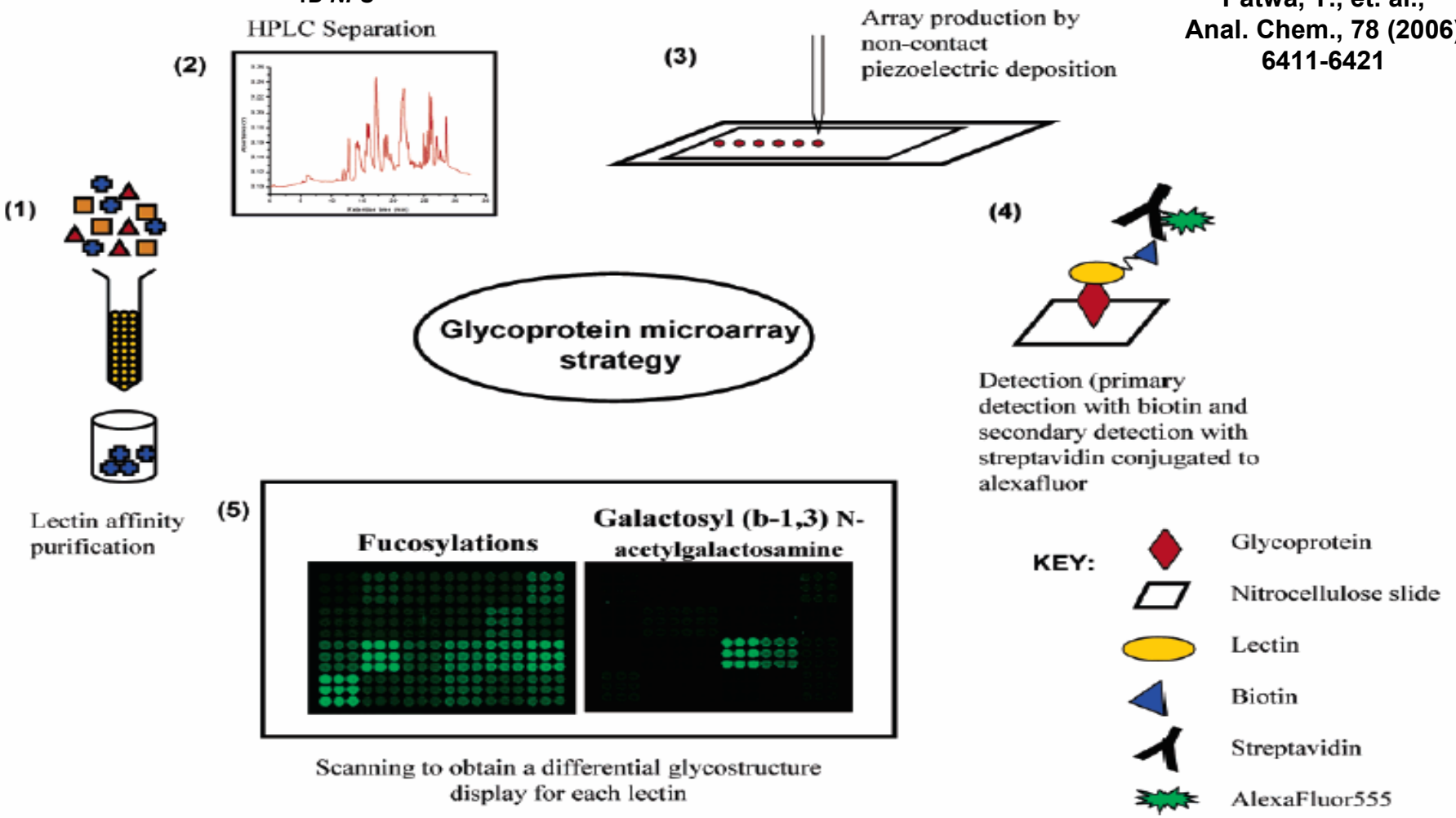


Figure 1. Proposed experimental strategy for studying serum glycoproteins. (1) Lectin purification using a general lectin column. (2) Nonporous reversed-phase HPLC separation and fraction collection. (3) Microarray production using a noncontact piezoelectric printing device. (4) Glycan detection using biotinylated lectin−streptavidin−Alexafluor555. (5) Image acquisition and spot analysis using Genepix 6.0 software.

Table 1. Biotinylated Lectins Used for Glycan Detection and Their Specificities

biotinylated lectin	glycan structure detected
concanavilin A (ConA)	α-linked mannose
Maackia amurensis II (MAL)	sialic acid in an (α-2,3) linkage
Aleuria aurantia (AAL)	fucose linked (α-1,6) to *N*-acetylglucosamine or to fucose linked (α−1,3) to *N*-acetyllactosamine
Sambucus nigra (elderberry) bark (SNA)	sialic acid attached to terminal galactose in (α−2,6), and to a lesser degree, (α-2,3), linkage
peanut agglutinin (PNA)	galactosyl (β-1,3) *N*-acetylgalactosamine

Can Monitor Type and Extent of Glycosylation with this methodology.

Zhao, J., et. al., J Proteome Res. 2007 May 6(5) 1864-74.

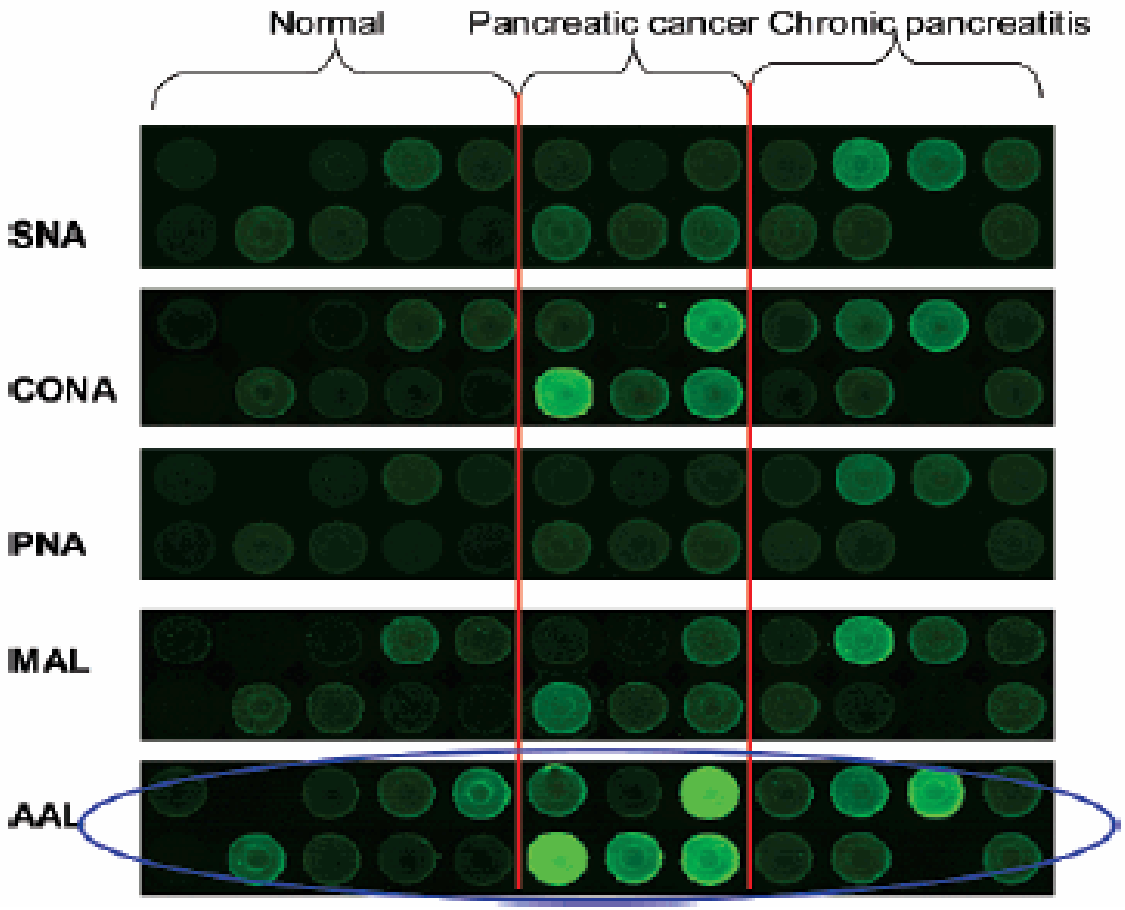


Table 1. *Z* Value of the Altered Glycosylations Detected by Five Lectins ($Z > 2$ or $Z < -2$ Corresponds to $P < 0.05$)

	AAL		MAL		SNA		ConA		PNA	
protein ID/acc #	normal	cancer	normal	cancer	normal	cancer	normal	cancer	normal	cancer
beta-2-glycoprotein 1 (P02749)		2.49		3.3		2.08		2.07	−2.47	2.13
hemopexin (P02790)		6.15		2.85		3.24		3.01		
haptoglobin-related protein (P00739)	−3.6	2.82	−2.49		−3.71	2.41	−3.08		−3.63	
serum amyloid P-component (P02743)	−4.96	4.02	−4.96	2.85	−5.28	4.11	−5.31	3.59	−5.96	3.12
clusterin (P10909)	−2.22	2.92	−2.52			2.22			−2.08	
antithrombin-III (P01008)	−3.5	3.18	−2.9	3.28	−2.93	2.58	−3.24	2.63	−3.44	2.56
kininogen-1 (P01042)	−2.69	4.31		2.39	−2.64	3.98	−3.06	3.95	−2.1	2.14
plasma protease C1 inhibitor (P05155)						−2.97				

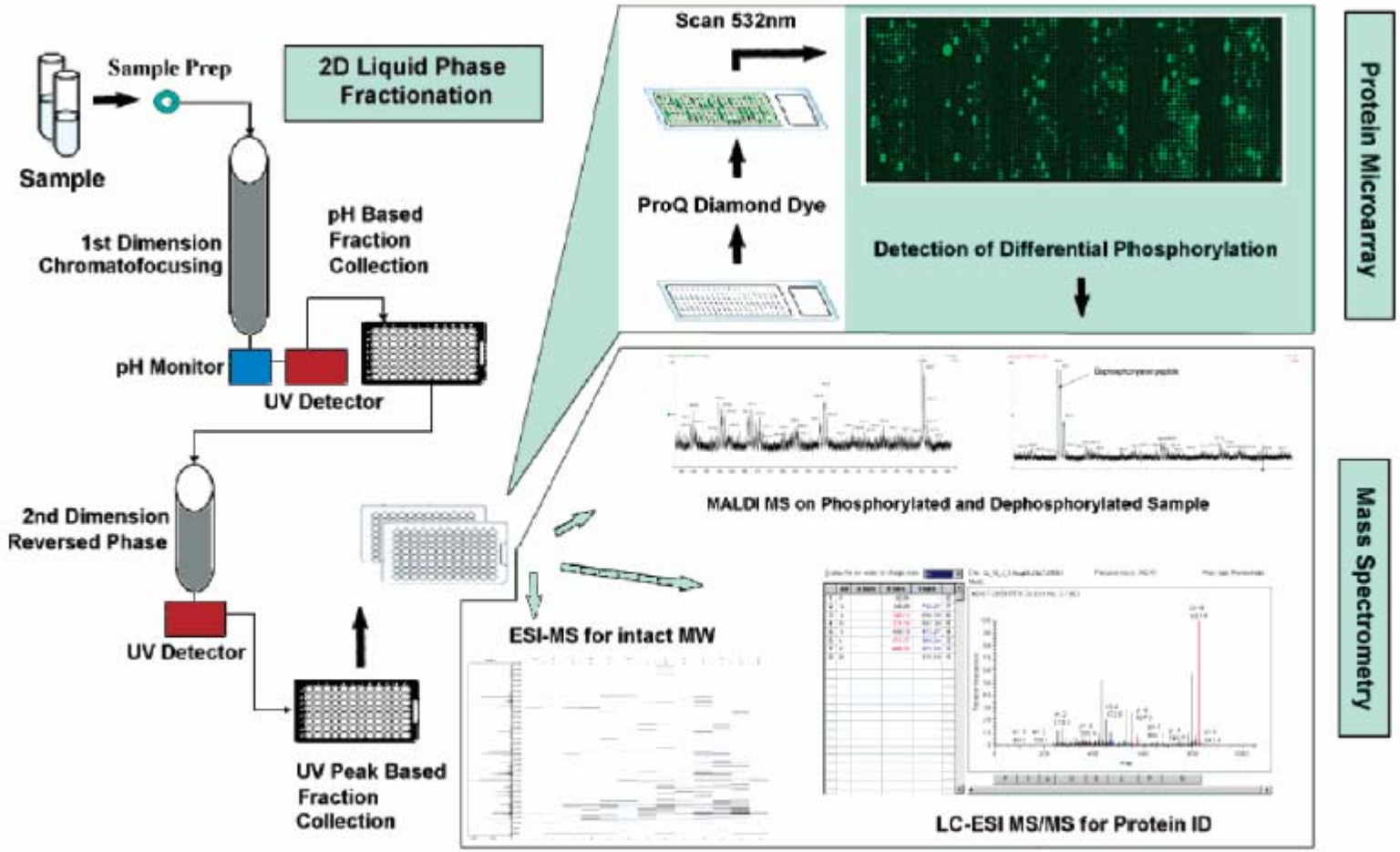


Figure 1. Flow diagram of experiment.

Differential Phosphoprotein Mapping in Cancer Cells Using Protein Microarrays Produced from 2-D Liquid Fractionation

Manoj Pal,[†] Allison Moffa,[‡] Arun Sreekumar,[§,∥] Stephen P. Ethier,[‡] Timothy J. Barder,[⊥] Arul Chinnaiyan,[§,∥] and David M. Lubman*[,†,§,∥,#]

Department of Chemistry, Department of Surgery, Department of Pathology, and Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan 48109, Barbara Ann Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201, and Eprogen, Inc., Darien, Illinois 60561

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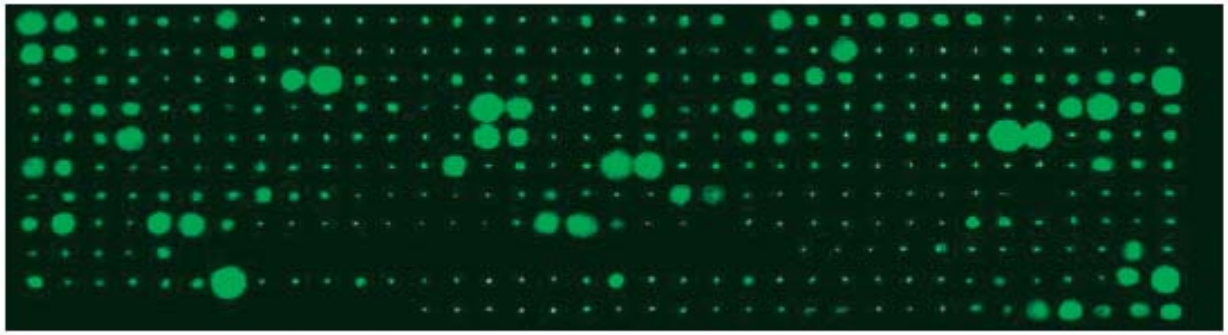


MS relevant "spots" only!

Figure 3. Microarray image showing fractions with pH 5.2–4.3 for SUM52-PE where phosphorylation is detected using ProQ Diamond dye.

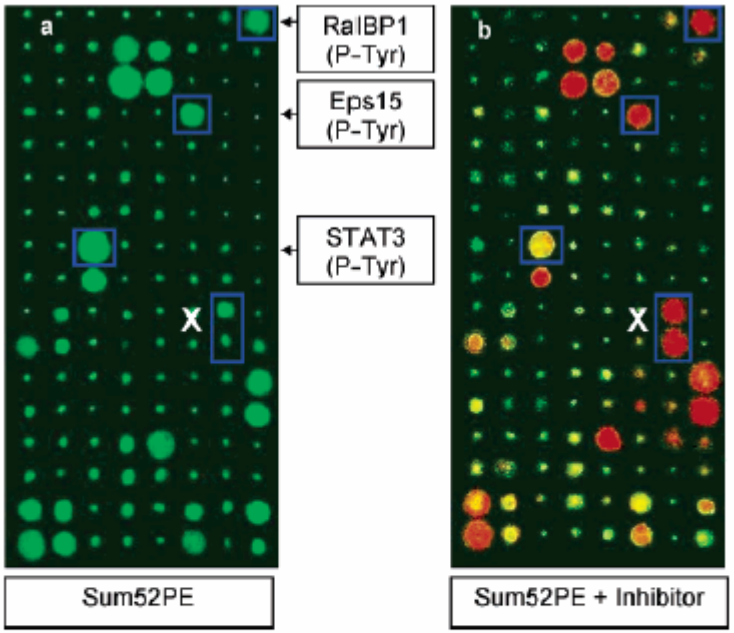


Figure 6. Slide image for pH fraction 4.6–4.3 processed with anti-phosphotyrosine antibody (b) after having been visualized with ProQ Diamond dye (a). Eps15 and RalBP1 show Tyr phosphorylation, and STAT3 shows phosphorylation on amino acids other than tyrosine. The spot marked X displays the case where the antibody binds to the protein either through nonspecific interactions or the concentration of the protein in that spot is below the sensitivity limit of ProQ Diamond dye. Spots reacting positively to the Pro-Q dye are shown in green while those that bind with the anti-PTyr antibody are shown in red.

Stain with ProQ then anti P-Try

[Global] **[Specific]**

"X"

(+) MA result

but

(–) MS result!

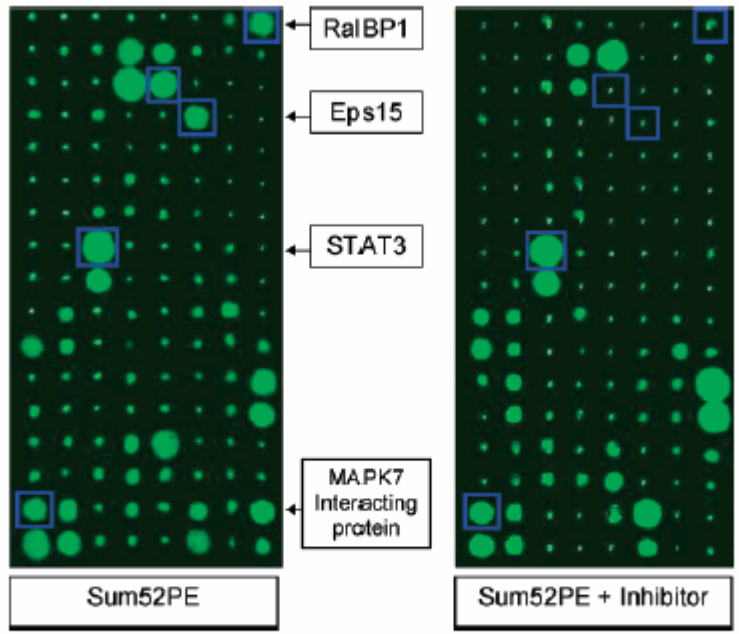


Figure 4. Microarray image showing pH fraction 4.6–4.3 for SUM52-PE before (left) and after (right) stimulation by PD.

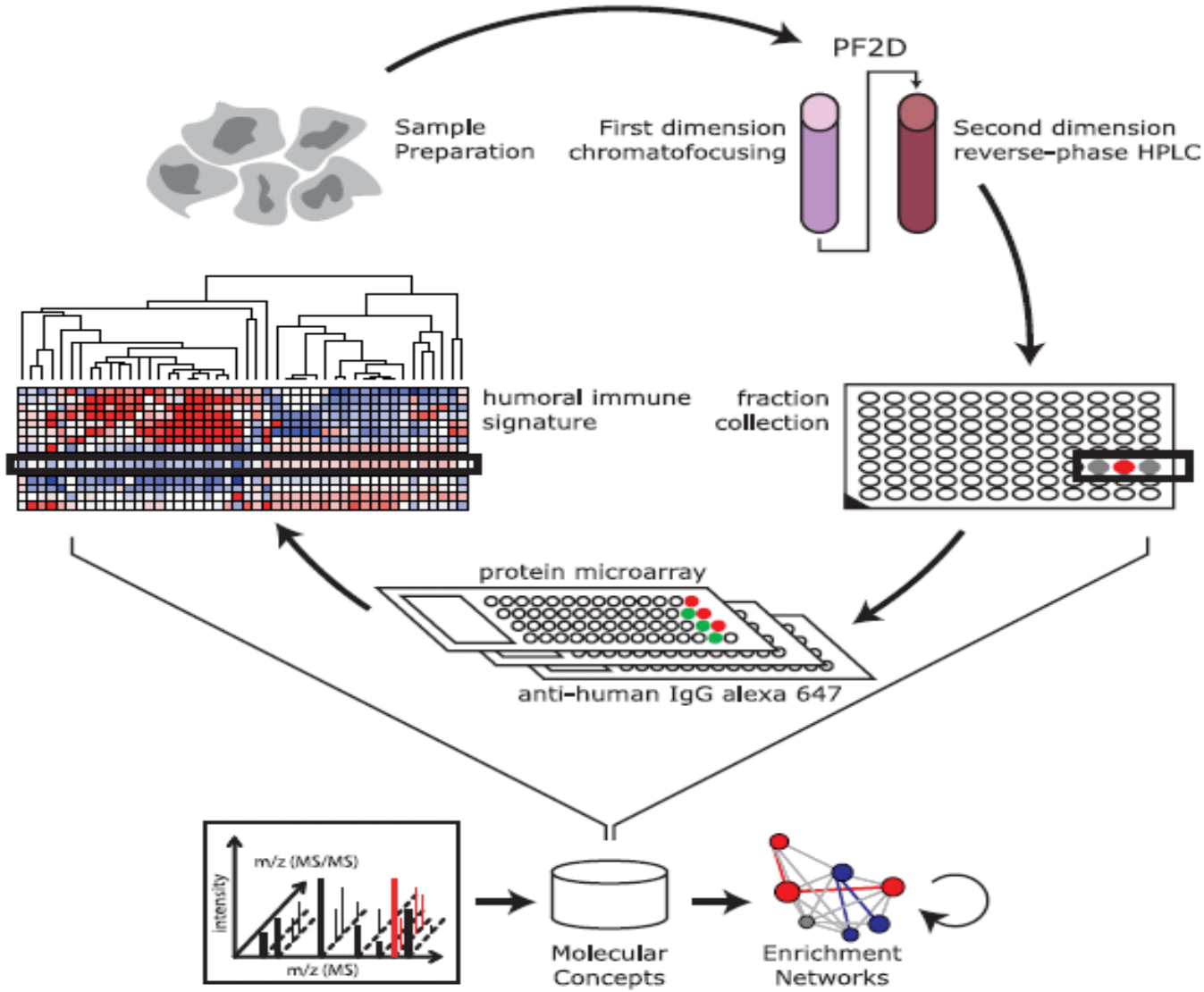
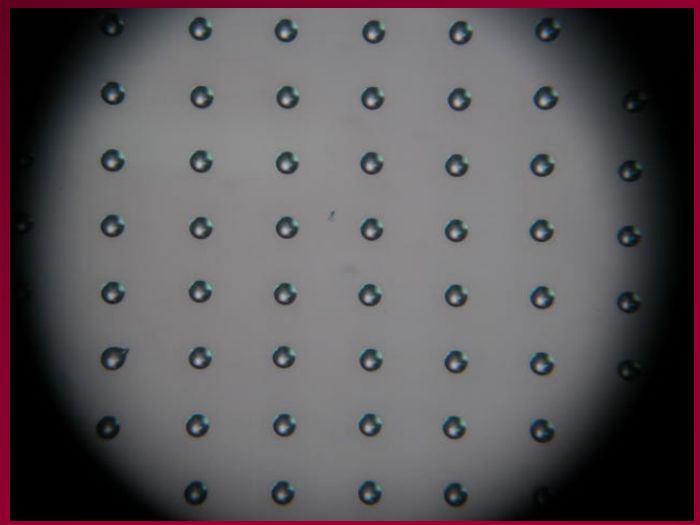


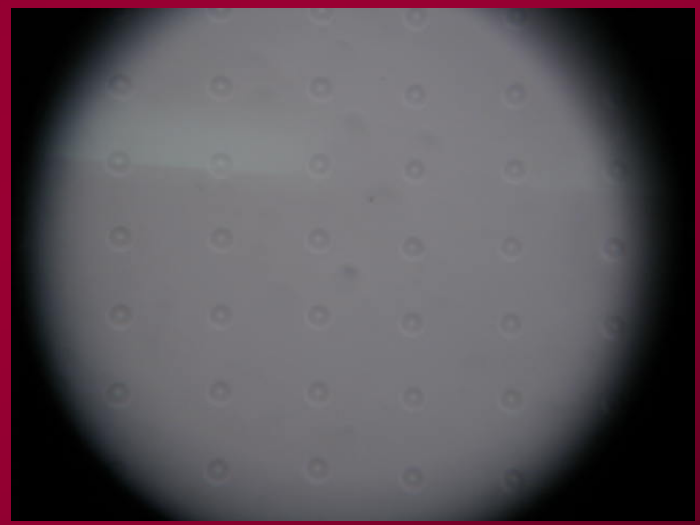
FIG. 1. **Schematic representation of the development and analysis of humoral response profiling protein microarrays.** Patient tissue extracts are procured, pooled, and subjected to two-dimensional protein fractionation, a first dimension CF followed by a second dimension RP-HPLC. Fractions are collected in 96-well plate format by peak based on UV detection with absorbance at 214 nm and subsequently printed on nitrocellulose slides. Slides are then hybridized with patient serum samples from which a humoral response profile can be generated. Fractions producing the best classification are then digested and sequenced by mass spectrometry, identified and validated with downstream protein informatics, and screened for biological involvement in a compendium of molecular concepts.

Eprogen's Novel 3D Gel Drop morphology for printing Microarrays

Hydrated (glycerol present)



Glycerol removed



Deposition with 150 micron pins results in a drop diameter of approximately 110 microns and a wet volume of 0.13-0.17 nl

Microarray Applications

A means of probing samples for "what we do not know!"

- **Humoral (Autoantibody) Response**

  Normal – Benign – Drug Treated – Diseased Patients Serum Screened using 2D Microarrays produced from Standard Cell lines and Primary Tissues .

- **Antibody Screening/Profiling**

  Tissue/BioFluid screened against known Antibodies

  Antibody screening for improved selectivities

  Host Cell protein contamination screening

- **Post Translational modification screening.**

  Phosphorylation Activity

  Glycosylation

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…….Mapping a new direction

in Proteomics……..