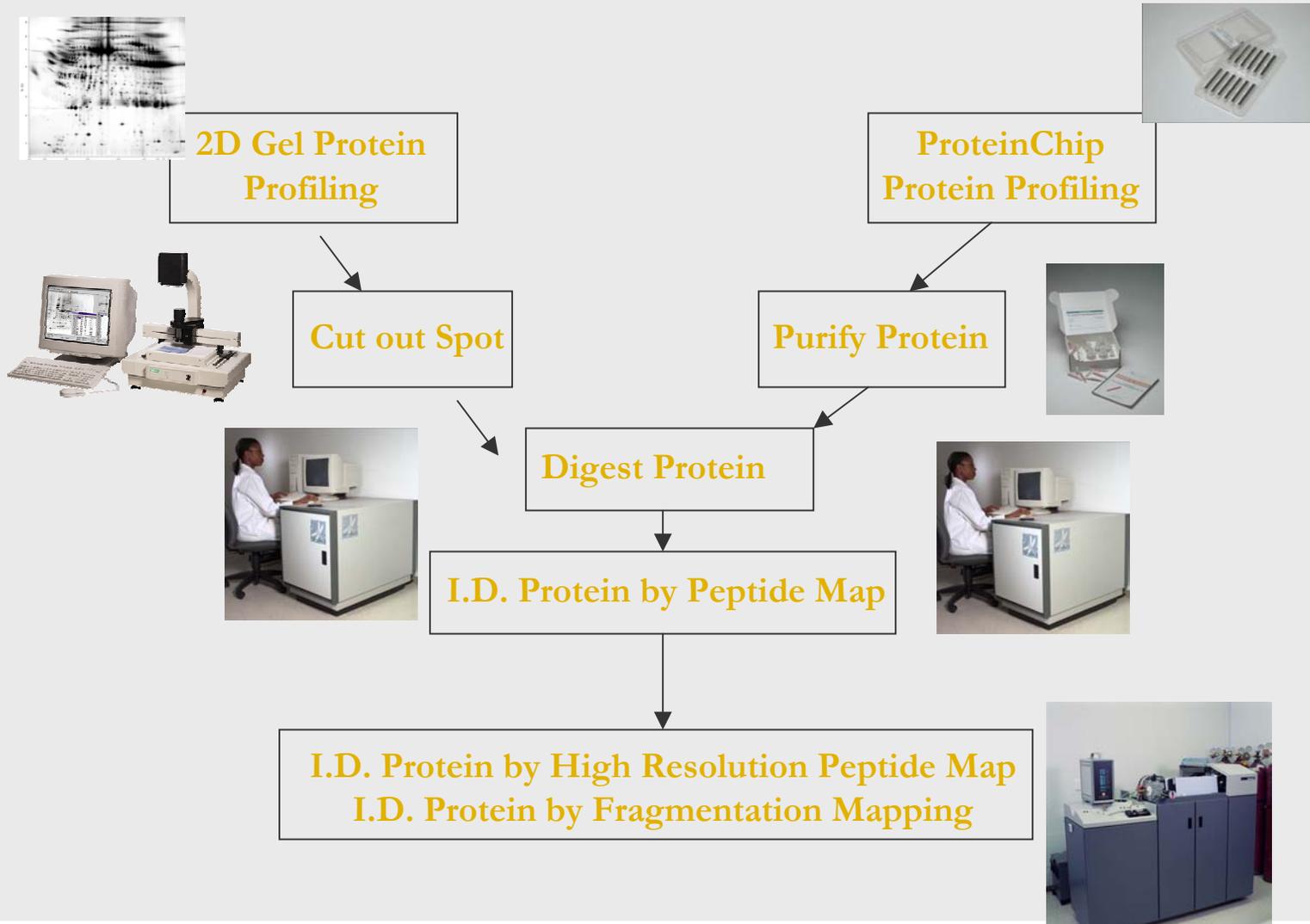


ProteinChip® Systems and Arrays



Tackling the Proteome: Strategies



ProteinChip® Applications

Protein Profiling

- Target discovery and validation
- Disease monitoring (drug efficacy studies)
- Toxicology
- Diagnostics

Protein Purification and Characterization

- Purification development and monitoring
- Peptide mapping and protein identification
- Sequencing
- Analysis of post-translational modifications
- Epitope mapping

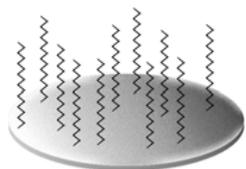
Molecular Recognition

- Immunoassay development and screening
- Receptor-ligand assays
- Protein-protein interactions
- DNA-protein interactions

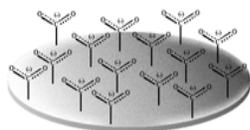
Derivatized 'Affinity' Surfaces



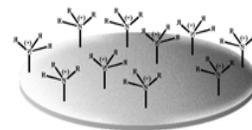
Surfaces by Chemistry:



(Reverse Phase)



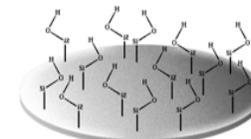
(Cation Exchange)



(Anion Exchange)

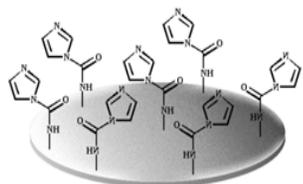


(Metal Ion)

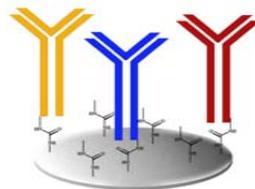


(Normal Phase)

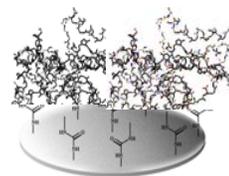
Surfaces by Biology:



(PS-10 or PS-20)



(Antibody - Antigen)



(Receptor - Ligand)

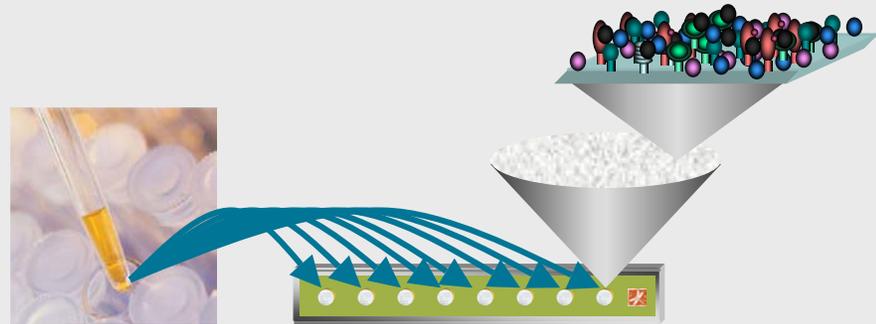


(DNA - Protein)

ProteinChip Technology: ProteinChip Array Preparation

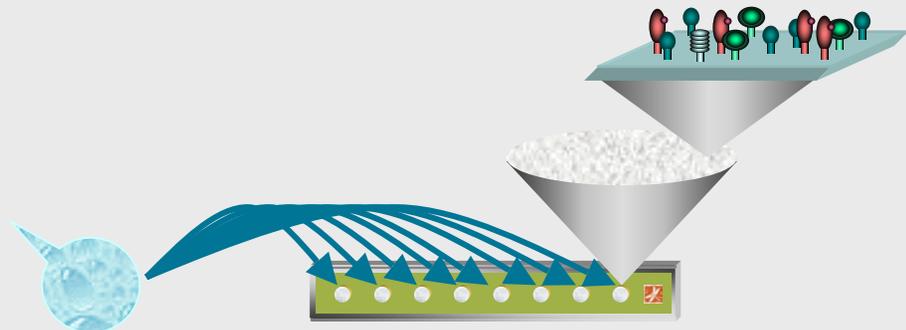
1. Apply Crude Sample

Proteins within the sample bind to chemical or biological "docking sites" on the ProteinChip surface through an affinity interaction.



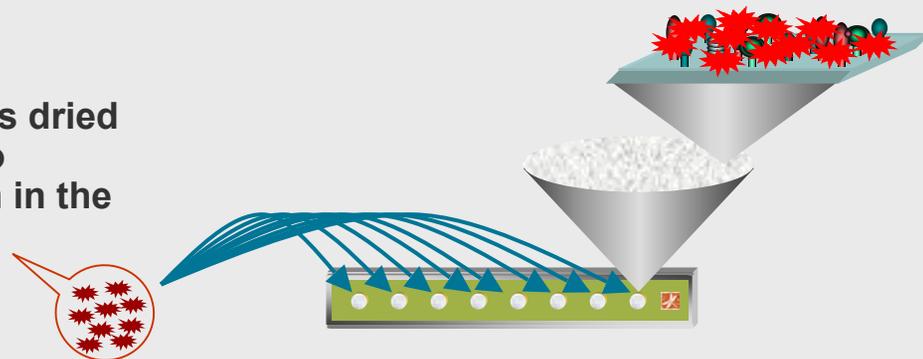
2. Wash ProteinChip Array

Proteins that bind non-specifically or buffer contaminants are washed away, eliminating sample "noise".

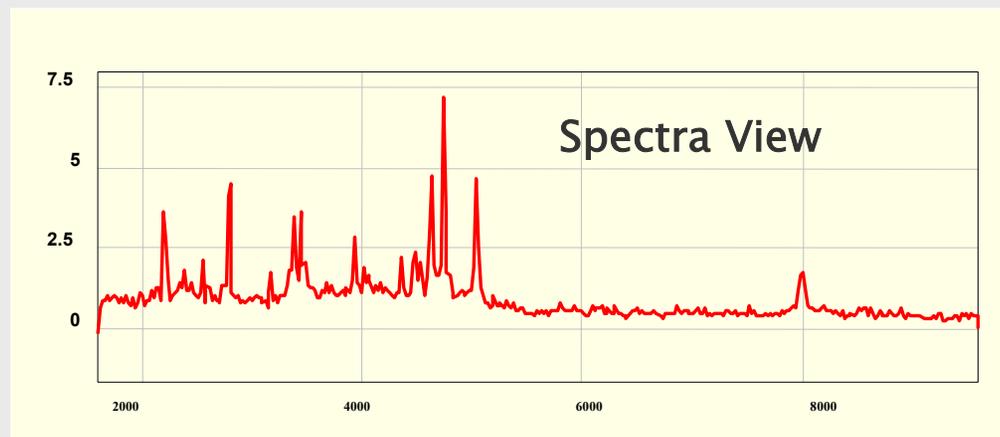
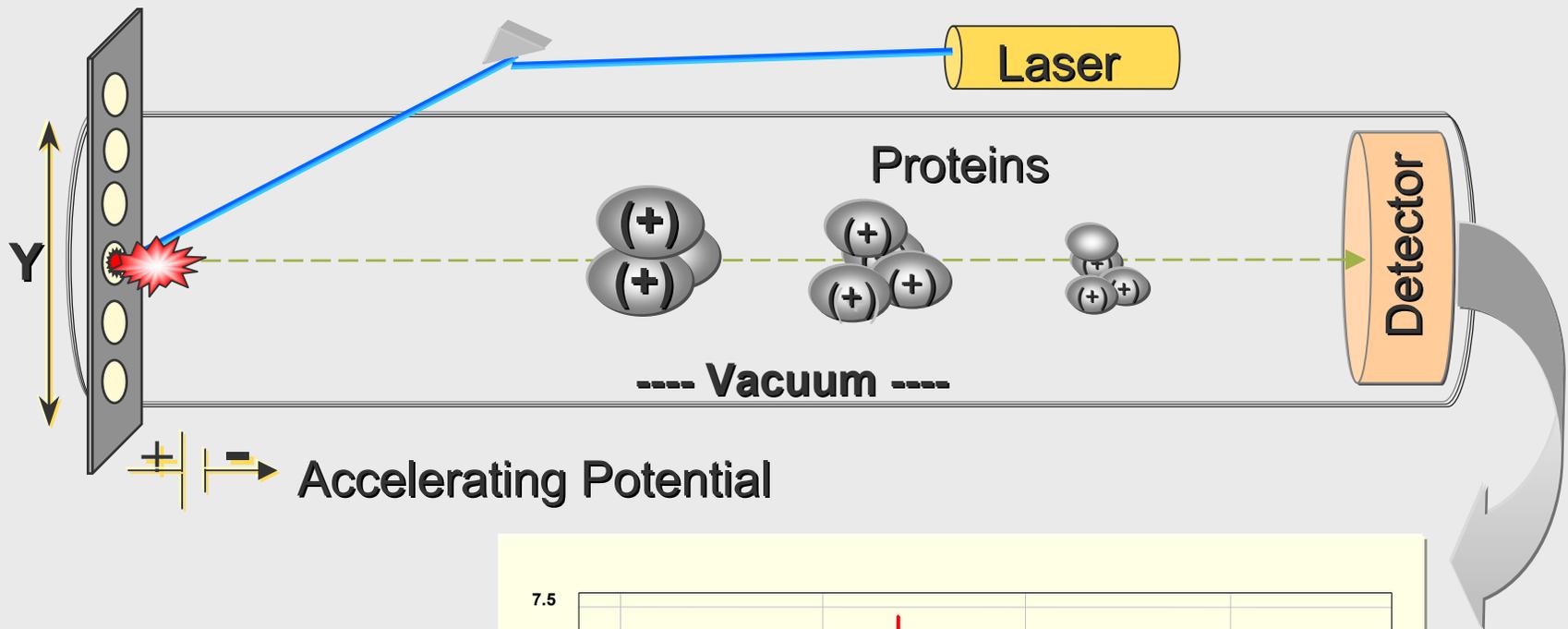


3. Add Energy Absorbing Molecules

After sample processing the chip is dried and EAM is applied to each spot to facilitate desorption and ionization in the TOF-MS.



SELDI Detection Using the ProteinChip® Reader



Surface Specificity: Crude Cell Extract

H4
(hydrophobic)

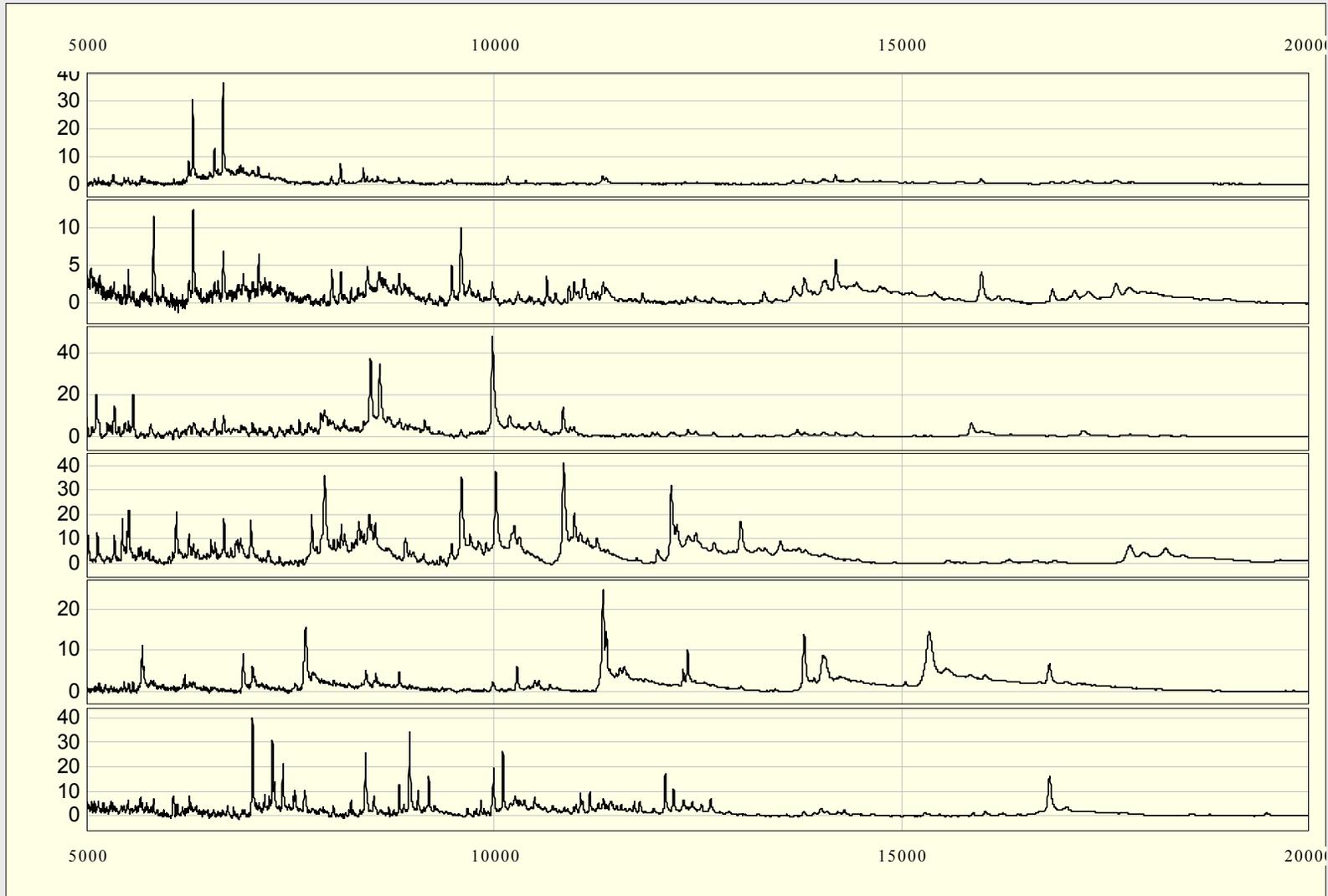
IMAC-Cu
(metal affinity)

WCX, pH 4
(cation exchange)

WCX, pH 7

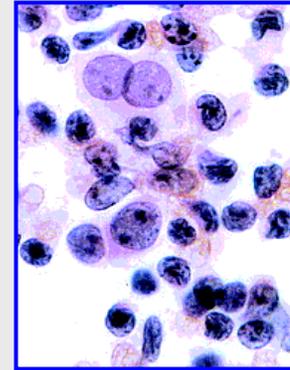
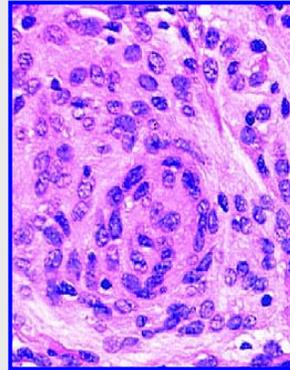
SAX, pH 9
(anion exchange)

SAX, pH 5



Tracking Markers from Tumor to Fluids

Enabling Biomarker Discovery to Predictive Diagnostics



Sample Type:

**Neoplastic
Tissue**

Cells

Body Fluids

Biomarker Concentration

High

Medium

Low

Source of patient specimen

Invasive

Minimally invasive

Optimal

**Examples Analyzed by
ProteinChip® Technology**

Biopsy

**Nipple Aspirates
Pleural Effusion**

**Serum
Plasma
Urine**

LCM

Basic Components and Supplies

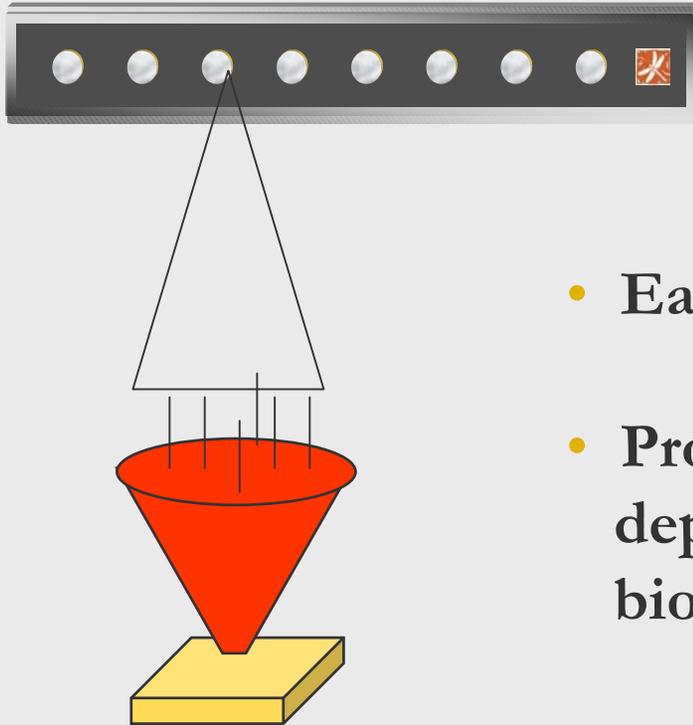
- **ProteinChip reader**
- **Computer and monitor**
- **SELDI Software (version 3.0)**
- **ProteinChip arrays**
- **Bioprocessor (for volumes of 25-350 μ l)**
- **EAMs (energy absorbing molecules) – also known as matrix in MALDI**
- **Hydrophobic PAP pen**
- **Trifluoroacetic acid (TFA), acetonitrile, high quality water**
 - **ALWAYS USE THE BEST QUALITY REAGENTS YOU CAN !!**

ProteinChip[®] Analysis

Capabilities and Advantages

- **Minimal sample prep**
- **Enrichment of lower abundance proteins (>1500 proteins from serum)**
- **Small sample size (as few as 2000 cells for LCM samples)**
- **Capable of analyzing 1000s of samples a month**
- **High sensitivity (low femtomoles)**
- **Single system for multi-panel assay**

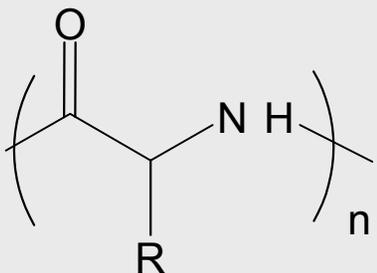
The CIPHERGEN ProteinChip® Platform



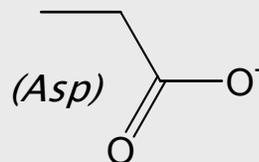
- Each spot is a chromatographic surface
- Proteins binding to the surfaces are dependent on individual protein biochemical characteristics

Proteins

- **Polymer of amino acid monomer units**
- **20 natural amino acids**
- **Primary sequence**

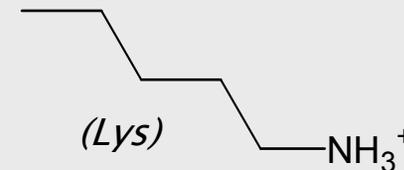


anionic



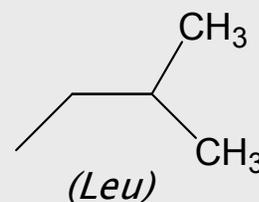
pK_a=3.9-4.0

cationic

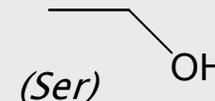


pK_a=10.4-11.1

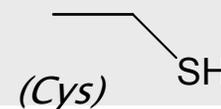
hydrophobic



hydrophilic



sulfur containing



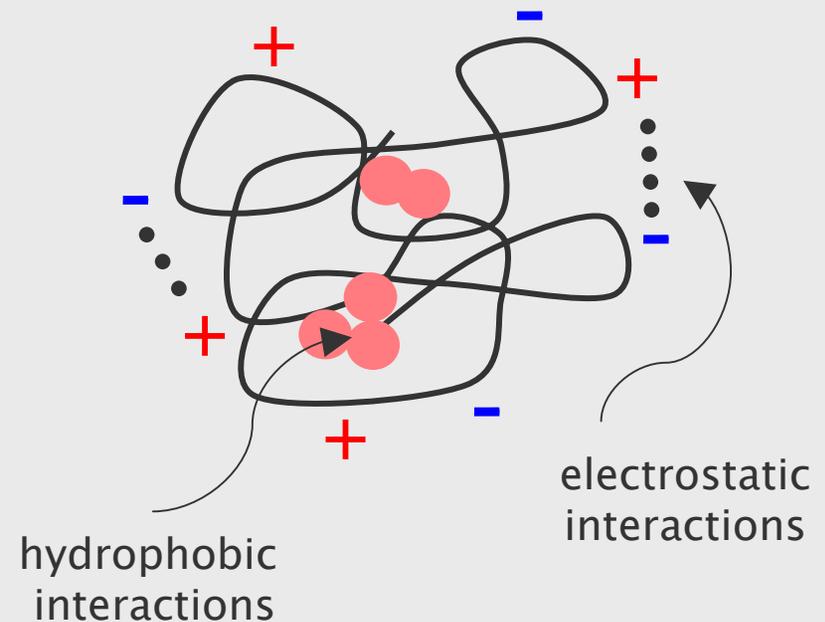
Protein Structure

■ Physical Interactions

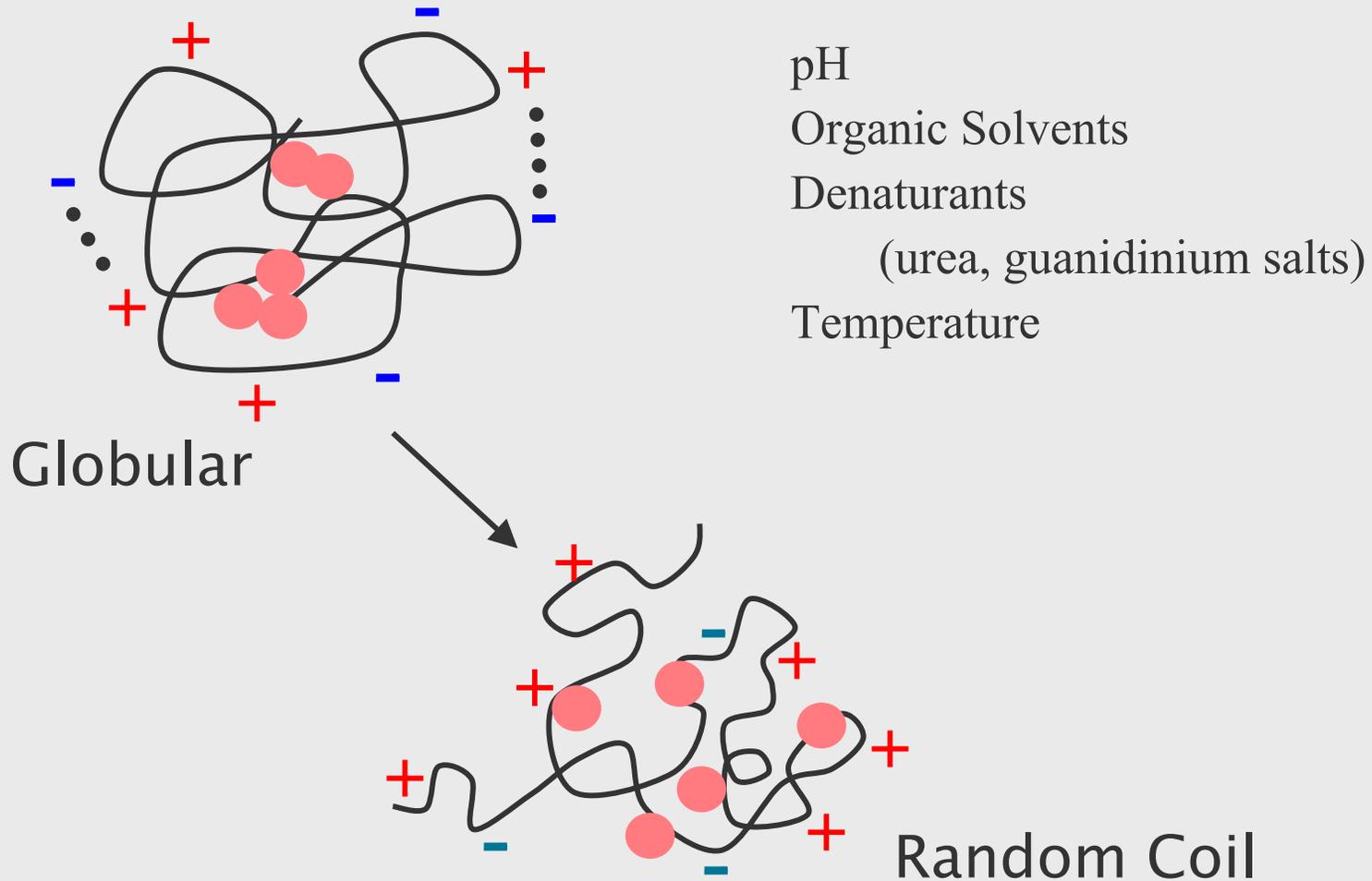
- van der Waals
- electrostatic
- dipole-dipole
- hydrogen bonds
- hydrophobic
- S-S bonds

■ Protein Folding

- secondary structure
(*α helix, β sheet*)
- tertiary structure
(*coiled coil, globular*)
- quaternary structure

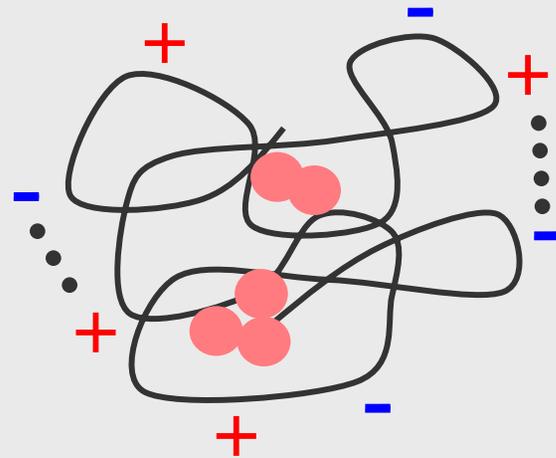


Protein Denaturation

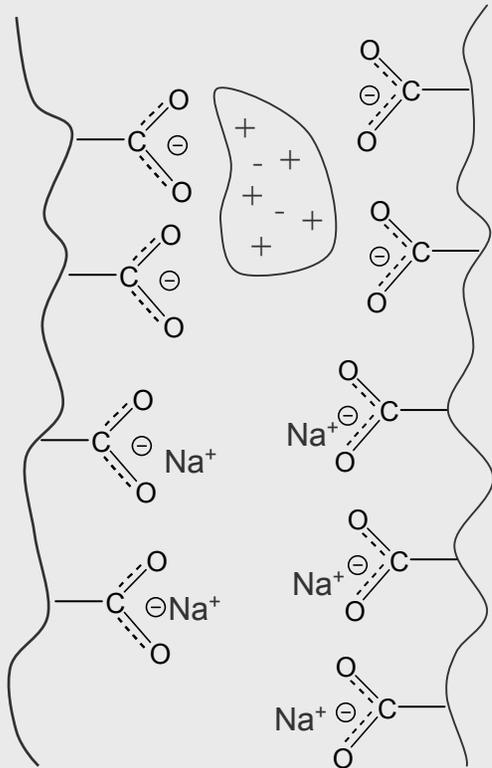


Protein Isoelectric Point (pI)

- **pI = pH at which the protein has no net charge**
- **at pI equal + and - charges**
- **above pI (basic conditions) more - charges**
- **below pI (acidic conditions) more + charges**

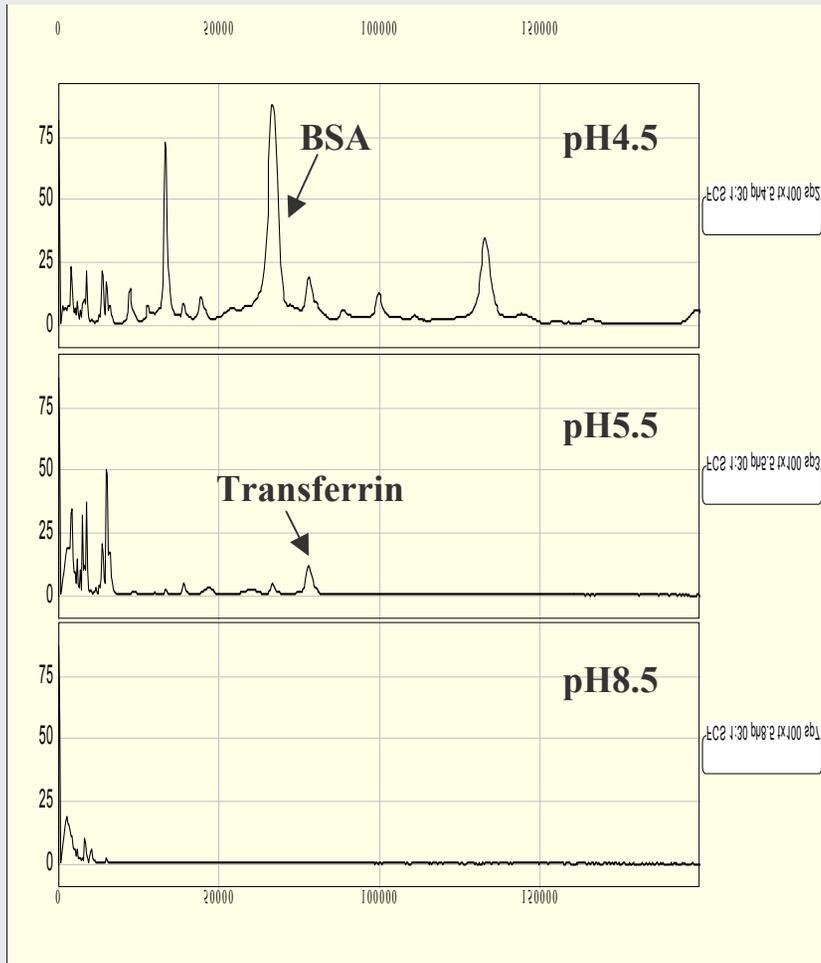


Cationic Exchanger: WCX2



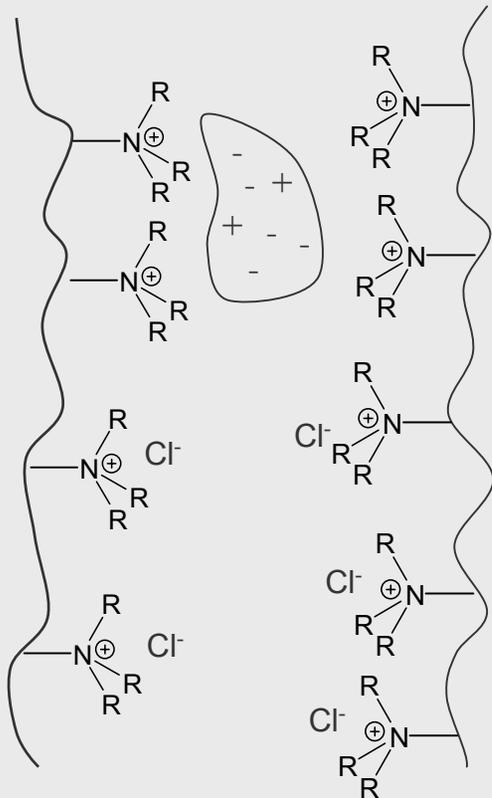
- **Anionic Surface -- Carboxylate groups**
- **Electrostatic interaction**
- **Binds through positively charged residues**
(lysine, arginine, histidine)
- **Binding conditions**
 - low pH ($\text{pH} < \text{pI} - 1$)
 - low salt
- **Decrease binding**
 - increase salt
 - increase pH

Binding of FCS to WCX2 Chip



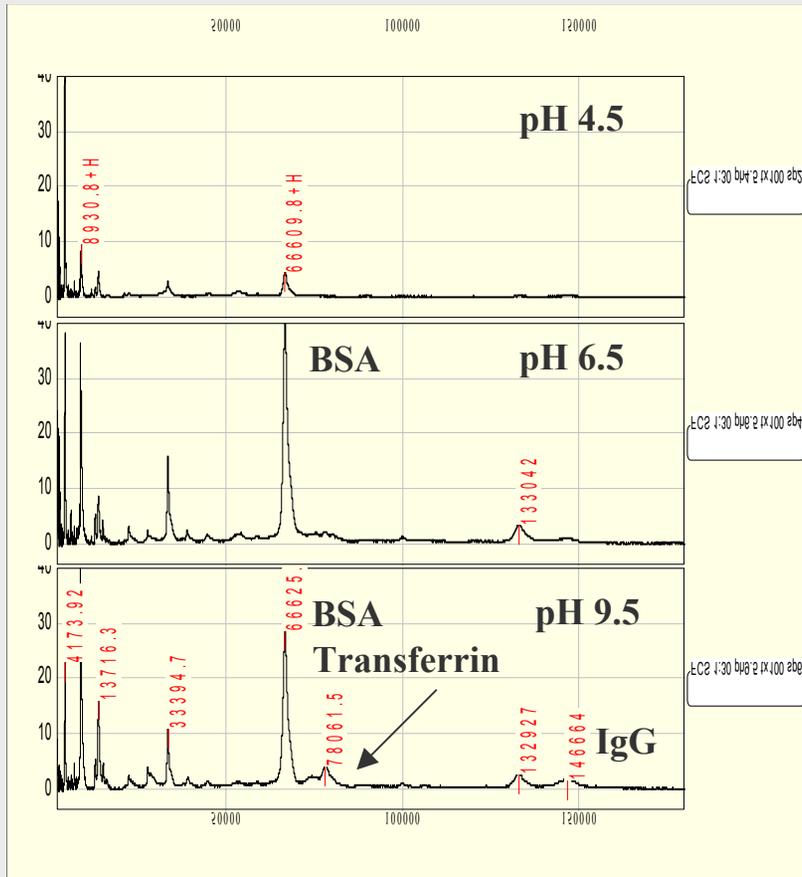
- **BSA pI ~ 5.5**
 - pH 4.5 \oplus net charge
 - pH 5.5 no net charge
 - pH 8.5 \ominus net charge
- **Transferrin pI ~ 6.5**
 - pH 4.5 \oplus net charge
 - pH 5.5 \oplus net charge
 - pH 8.5 \ominus net charge

Anionic Exchanger: SAX2



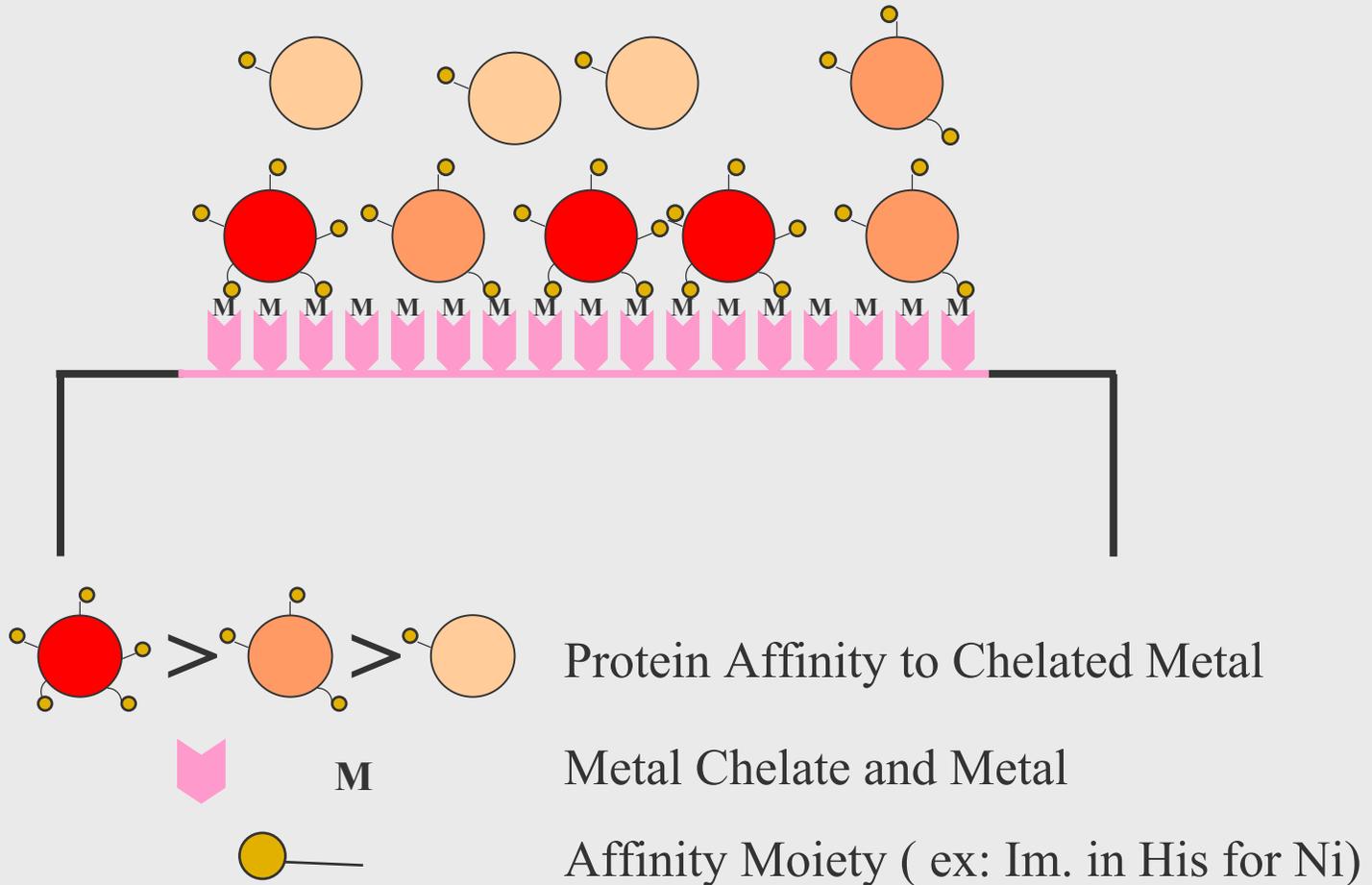
- **Cationic Surface -- Ammonium groups**
- **Electrostatic interaction**
- **Binds negatively charged proteins**
 - aspartic acid, glutamic acid residues
- **Binding conditions**
 - high pH ($\text{pH} > \text{pI} + 1$)
 - low salt
- **Decrease binding**
 - increase salt
 - decrease pH

Binding of FCS to SAX2 Chip

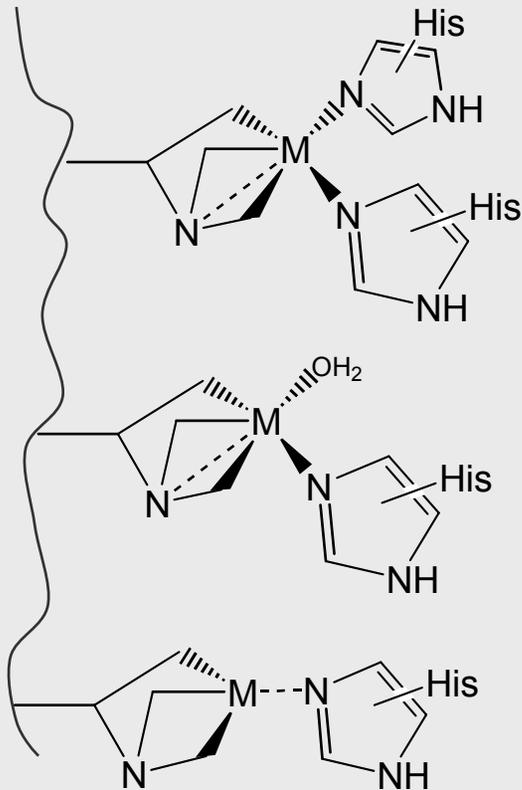


- **BSA pI ~ 5.5**
 - pH 4.5 \oplus net charge
 - pH 6.5 \ominus net charge
 - pH 9.5 \ominus net charge
- **Transferrin pI ~ 6.5**
 - pH 4.5 \oplus net charge
 - pH 6.5 no net charge
 - pH 9.5 \ominus net charge

IMAC Chip



IMAC: Immobilized Metal Affinity Capture

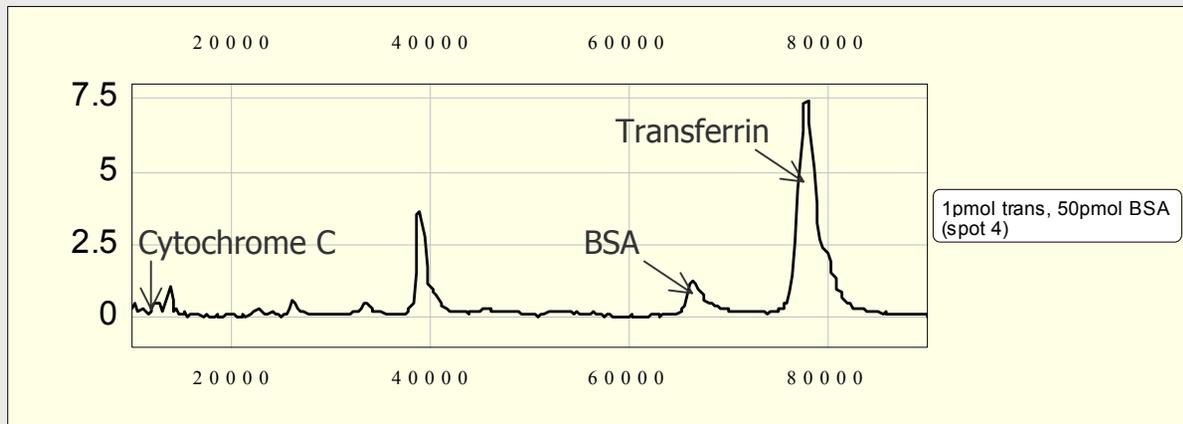


- **Binds Proteins/Peptides with**
 - Histidine
 - Tryptophan, Cysteine
 - Phosphorylated Proteins (Fe^{+3})
- **Binding conditions**
 - high salt
 - pH 6-8
- **Decrease Binding**
 - increase imidazole
 - increase glycine
 - increase phosphate (when capturing phosphorylated proteins)

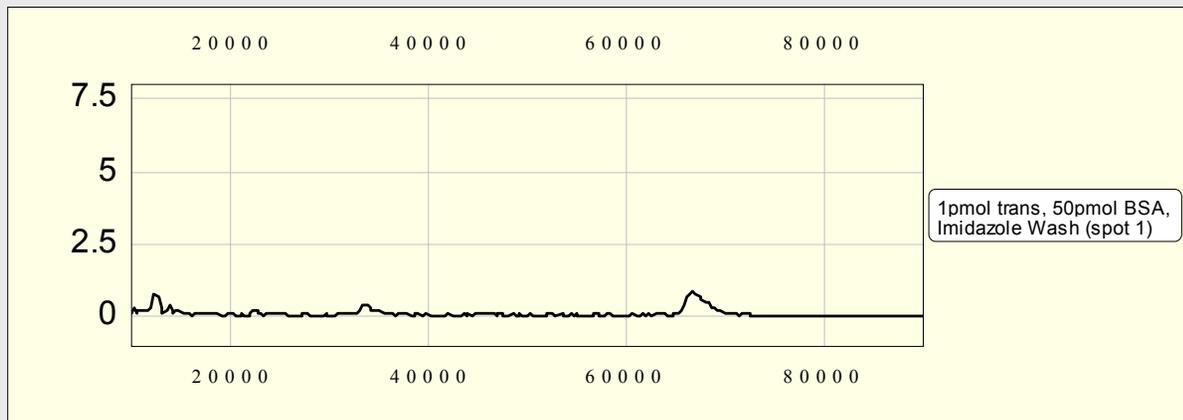
Binding to IMAC3

BSA, Transferrin, Cytochrome C

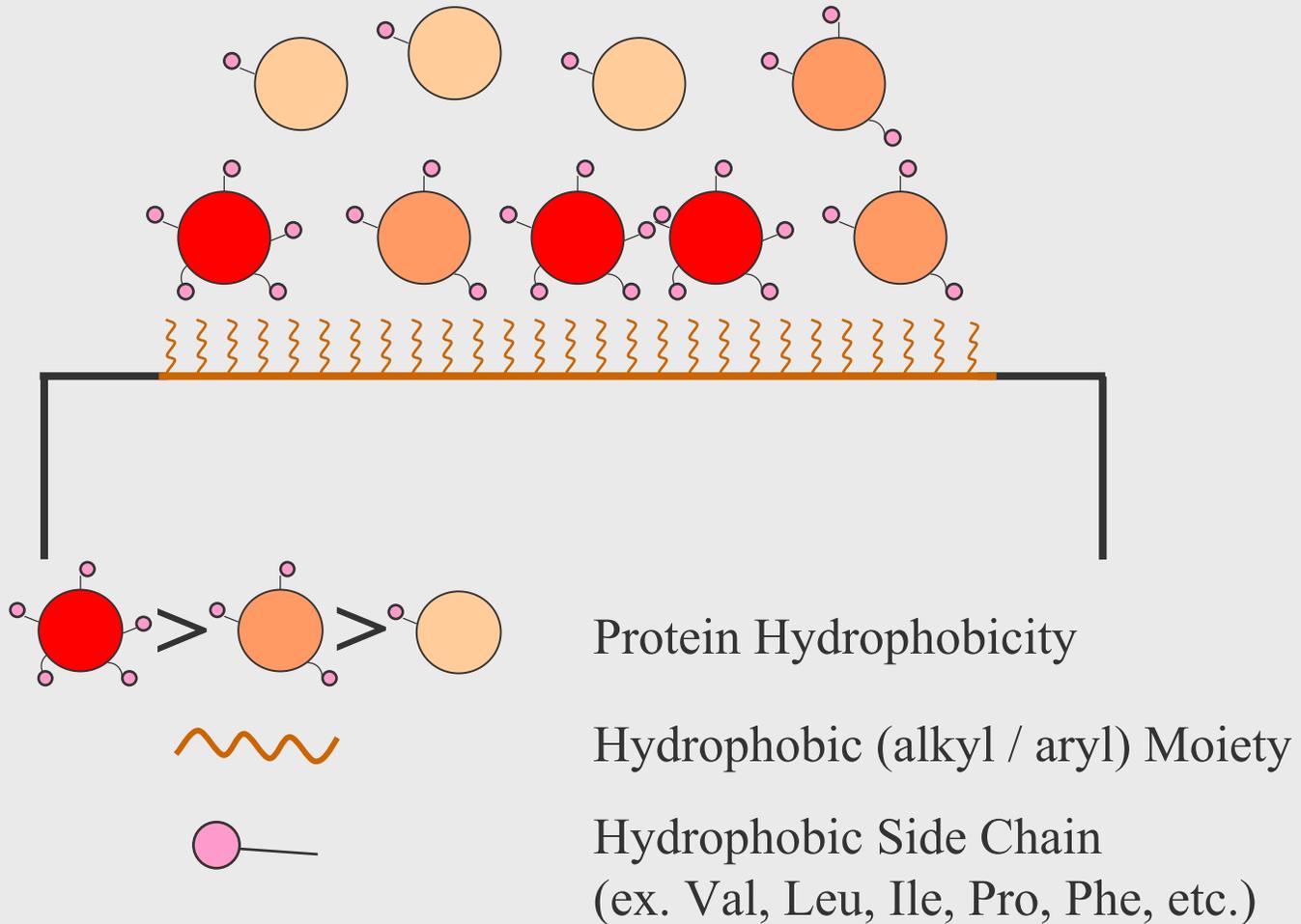
IMAC-3 Ni with regular PBS wash



IMAC-3 Ni with PBS/imidazole wash

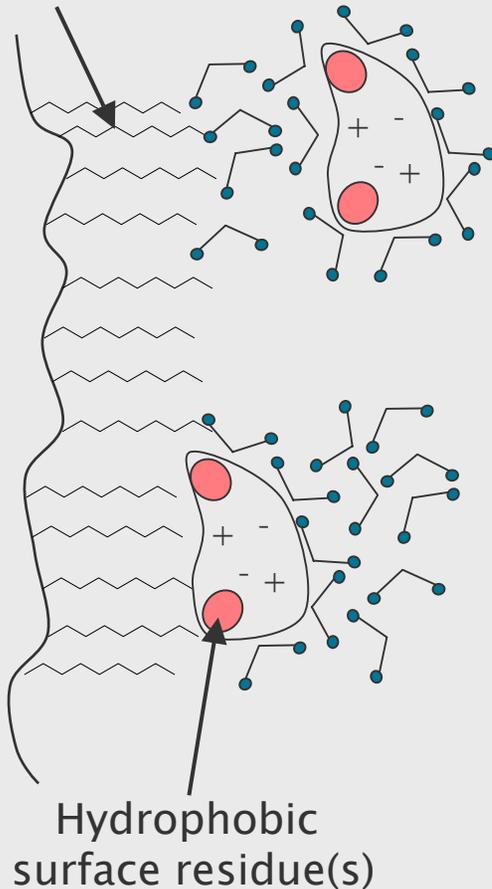


Hydrophobic Chip



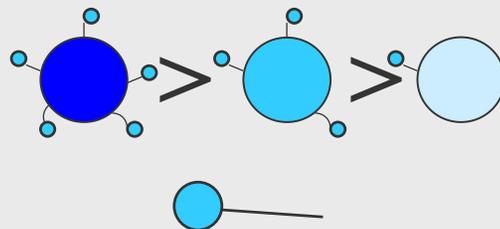
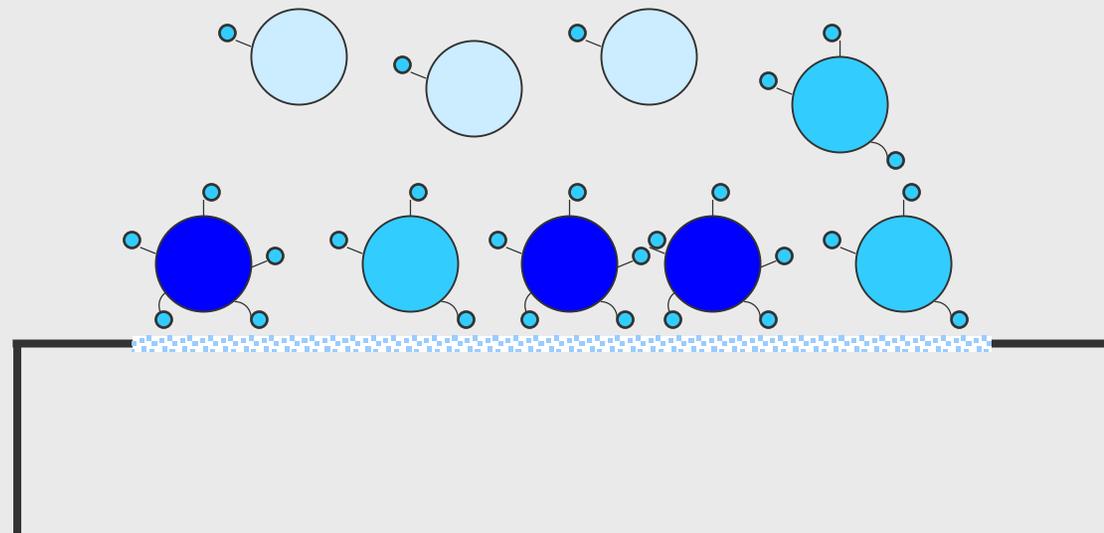
Hydrophobic

Hydrophobic groups
on chip surface



- **Hydrophobic Surface -- C16**
- **Hydrophobic interaction**
- **Binds through hydrophobic residues on protein surface**
 - Alanine, Valine, Leucine, Isoleucine, phenylalanine, tryptophan, tyrosine
- **Binding conditions**
 - high salt
 - aqueous (low organic)
- **Decrease binding**
 - decreasing salt
 - increasing organic

Normal Phase Chip

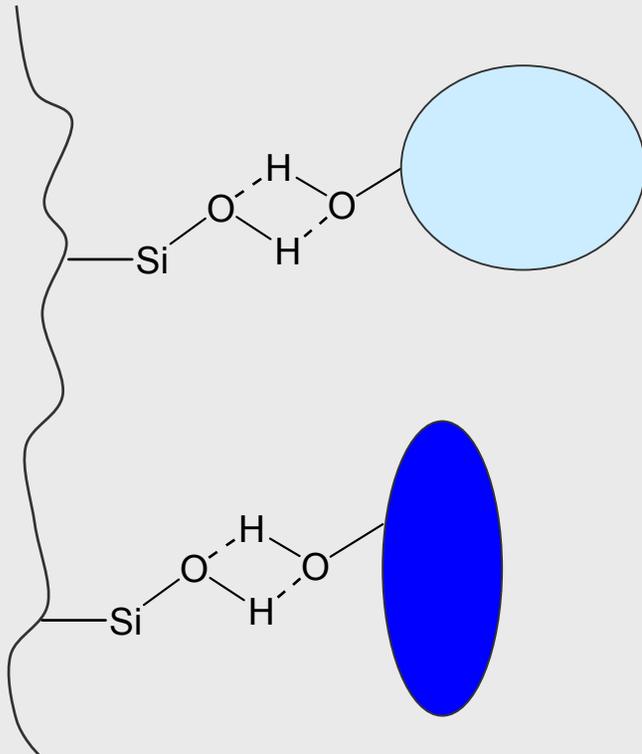


Protein Affinity to NP Surface

Affinity Moiety

(eg: serine, threonine, lysine)

Normal Phase



- **SiO₂ surface**
- **Electrostatic and dipole-dipole interactions**
- **Hydrogen bonds**
- **Binds through hydrophilic and charged residues on protein surface**
- **Binding conditions**
 - Buffer
- **Washing conditions**
 - water wash

Basic Concepts of Protein Profiling



CIPHERGEN®

Protein Expression Profiling

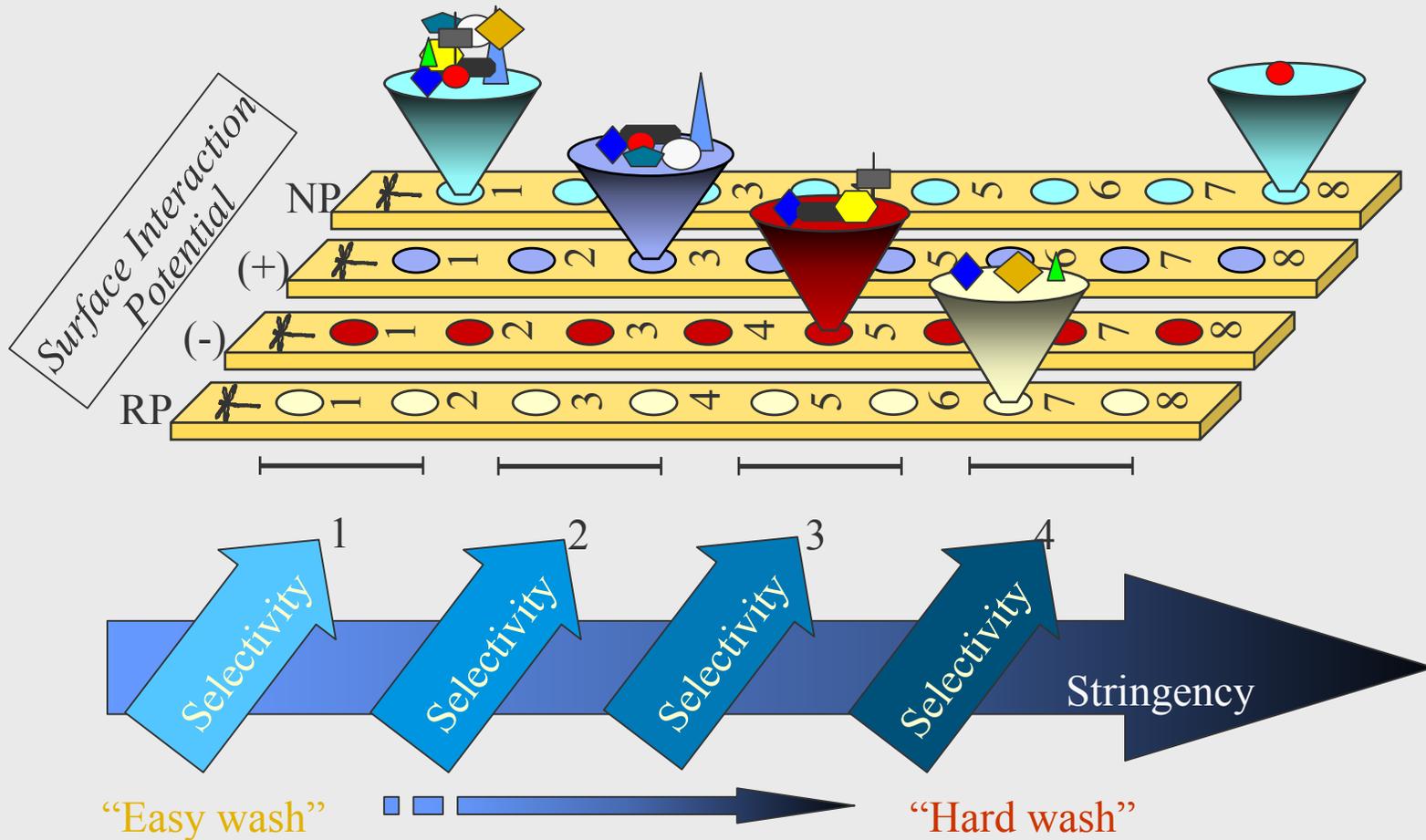
Comparison of protein expression patterns between biological samples (cells, tissues, plasma, urine, saliva, LCM caps, etc.)

- **Normal vs Diseased (Biomarker Discovery; Diagnostics)**
- **Responders vs Non-responders (Pharmacoproteomics)**
 - **Find markers that predict response to drugs (clinical stratification and outcome prediction)**
 - **Identify markers that can be used to monitor drug toxicity**

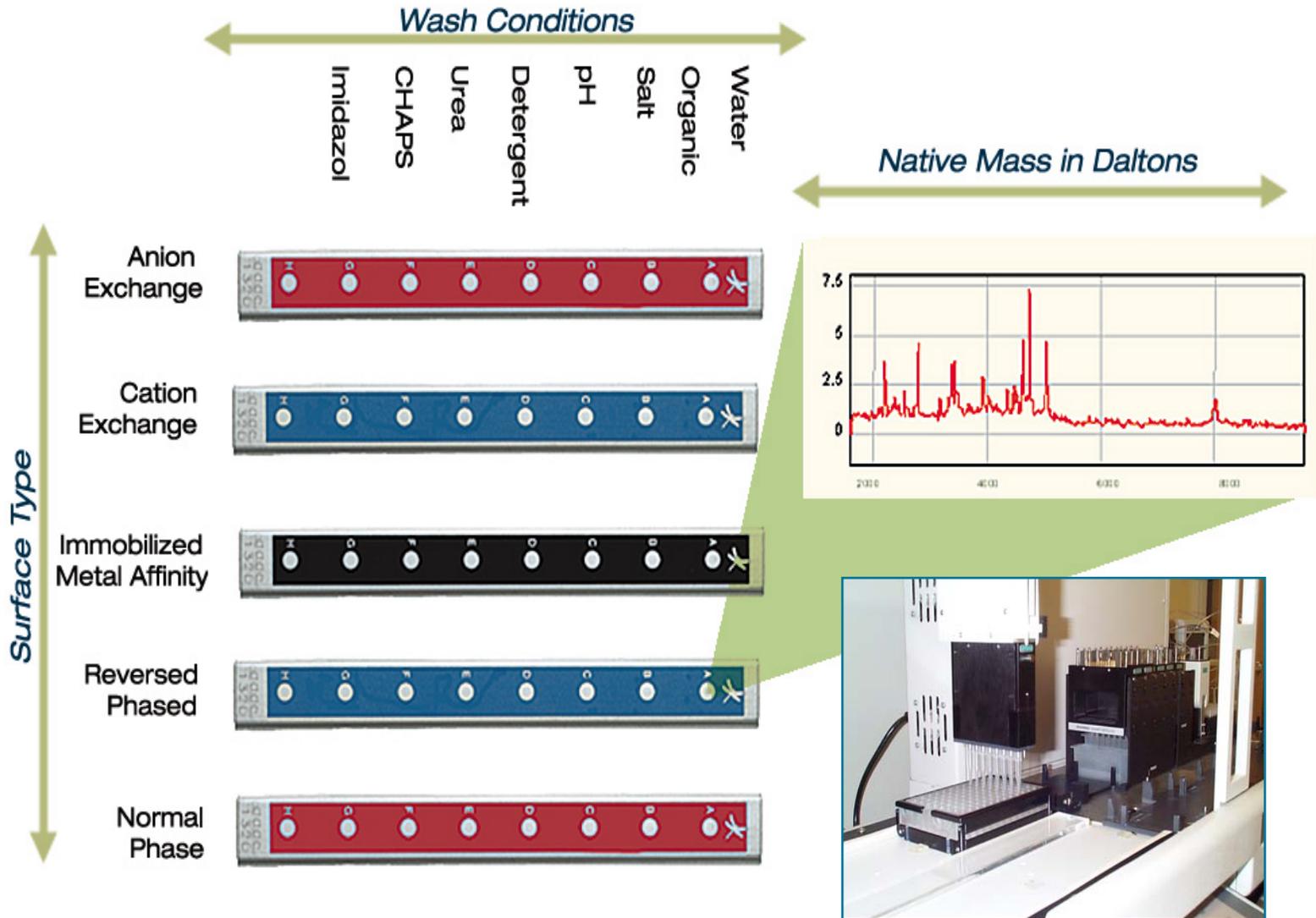
Traditional Method: 2D gel electrophoresis

SELDI Method: Uses ProteinChip® surfaces to selectively capture different protein subsets from biological samples based on protein characteristics

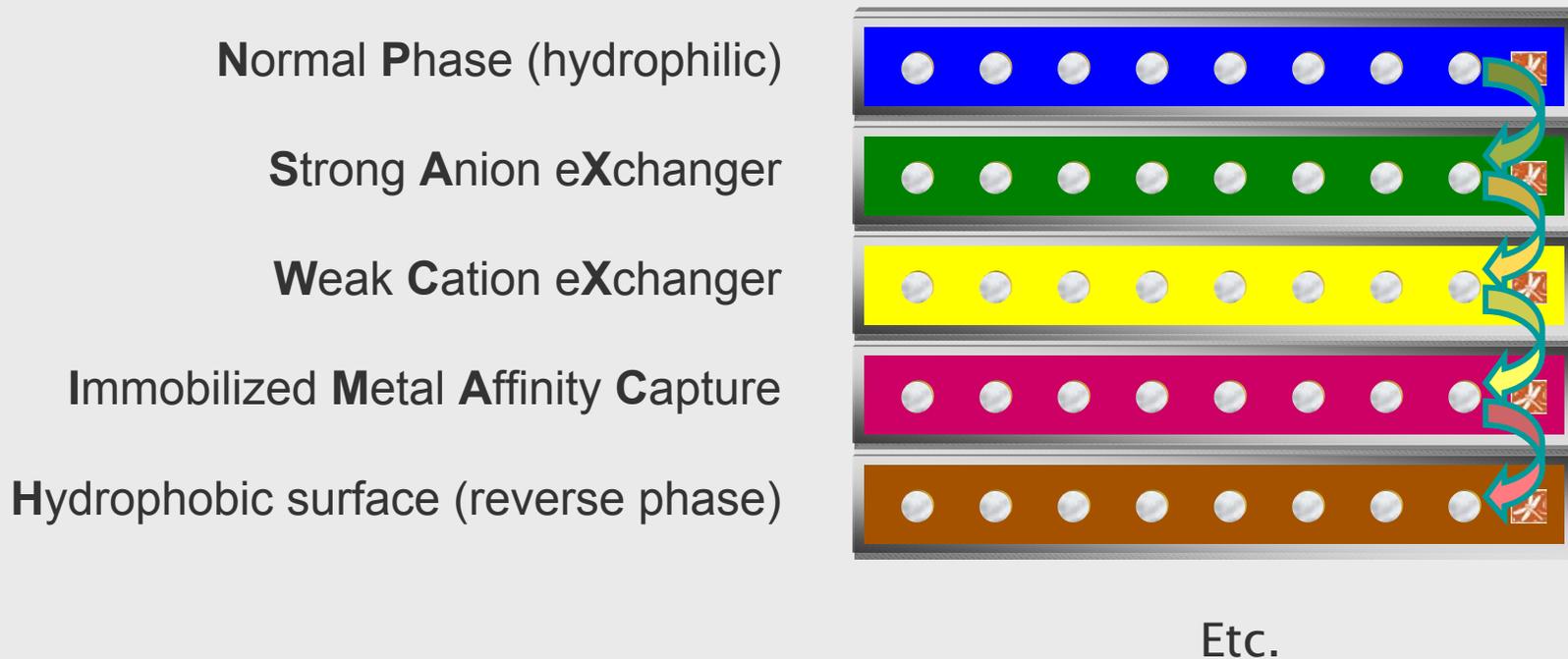
ProteinChip® Arrays: “Retentate Chromatography” Series



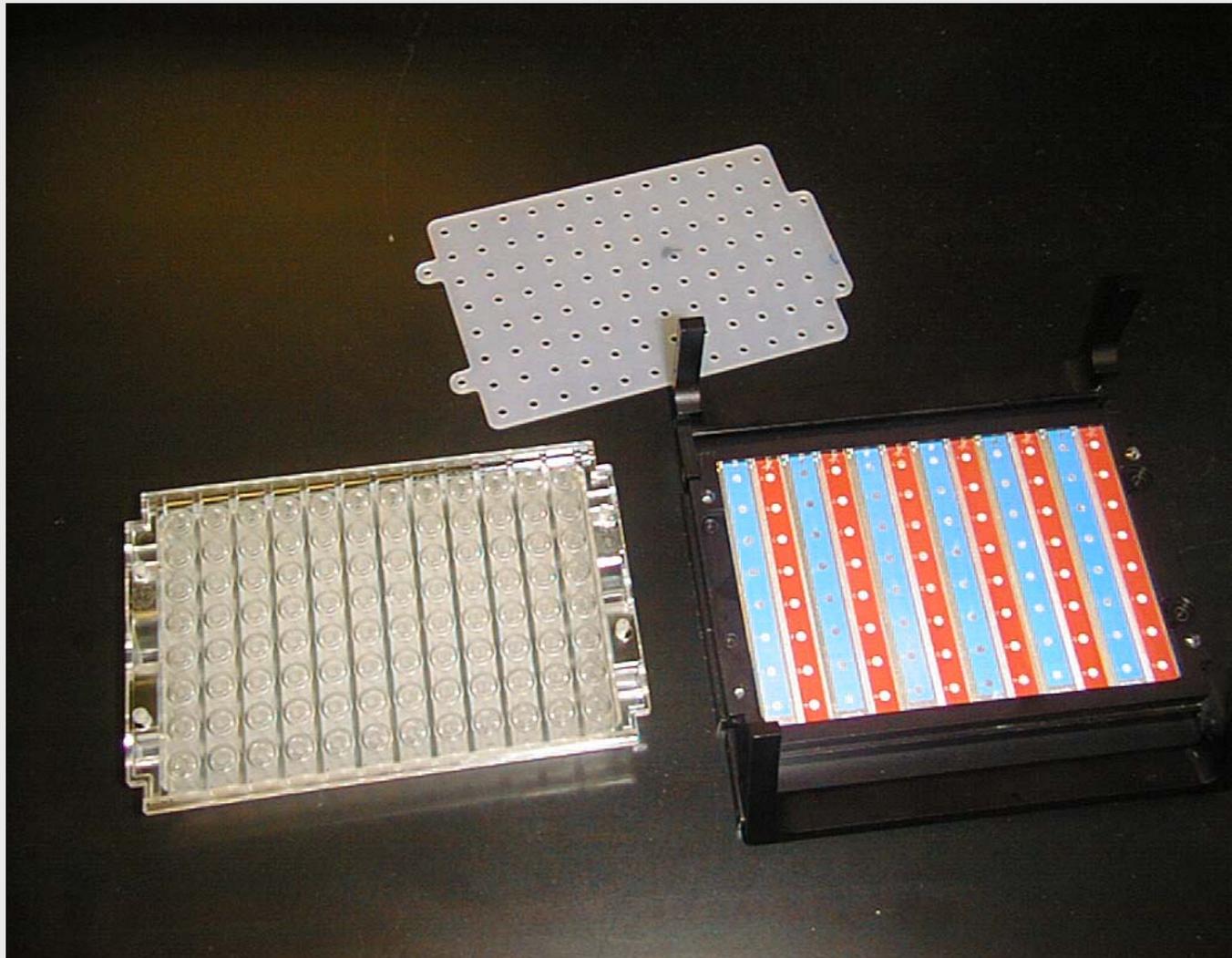
Three Dimensions of Separation



Tandem ProteinChip Profiling: mining proteins from minute biological samples using serial transfer on different ProteinChip arrays



The ProteinChip® Bioprocessor



Processing of 12 ProteinChips® simultaneously in 96 well Format to increase through-put

Biomarker Wizard

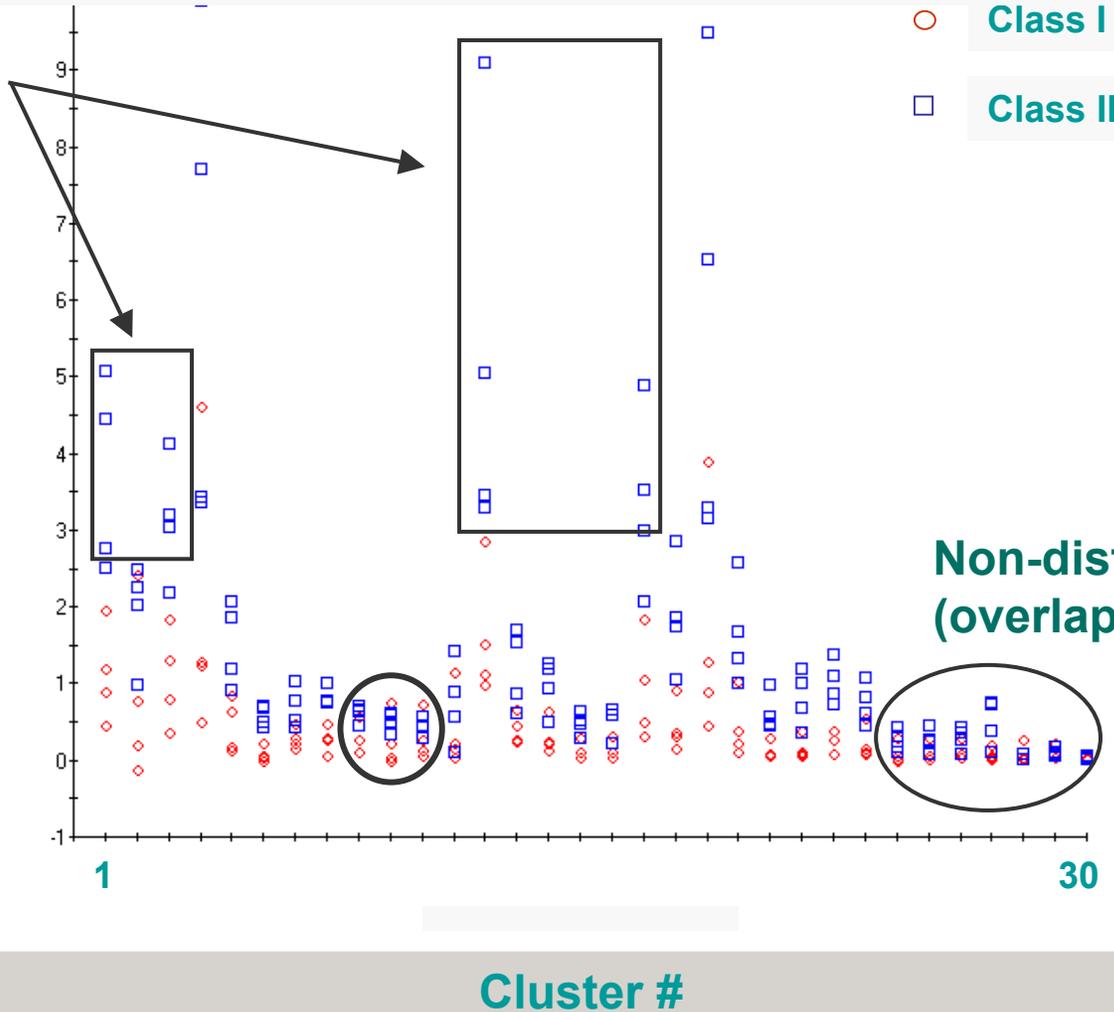
Molecular classification of cancer using multiple biomarkers:

- **Clustering**
- **Pattern recognition**

Plotting the intensity vs. cluster # (scatter plot) allows rapid visualization of potential pattern(s) to distinguish subtype I and II of cancer A

Potentially distinctive patterns

Intensity



Non-distinctive pattern (overlapping)

Protein Profiling Considerations

- Know thy sample! (What is sample, lysis conditions)
- For that matter, know thy buffers (binding and wash conditions, chip surfaces, etc.)
- Simplify! Simplify!!
 Fractionation of sample decreases complexity, increases likelihood of seeing less abundant proteins
- For meaningful data, compare apples to apples

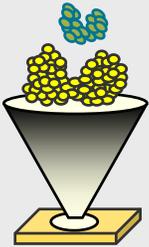
Use of ProteinChip® Technology for Studying Protein: Protein Interactions

Practical Considerations



CIPHERGEN®

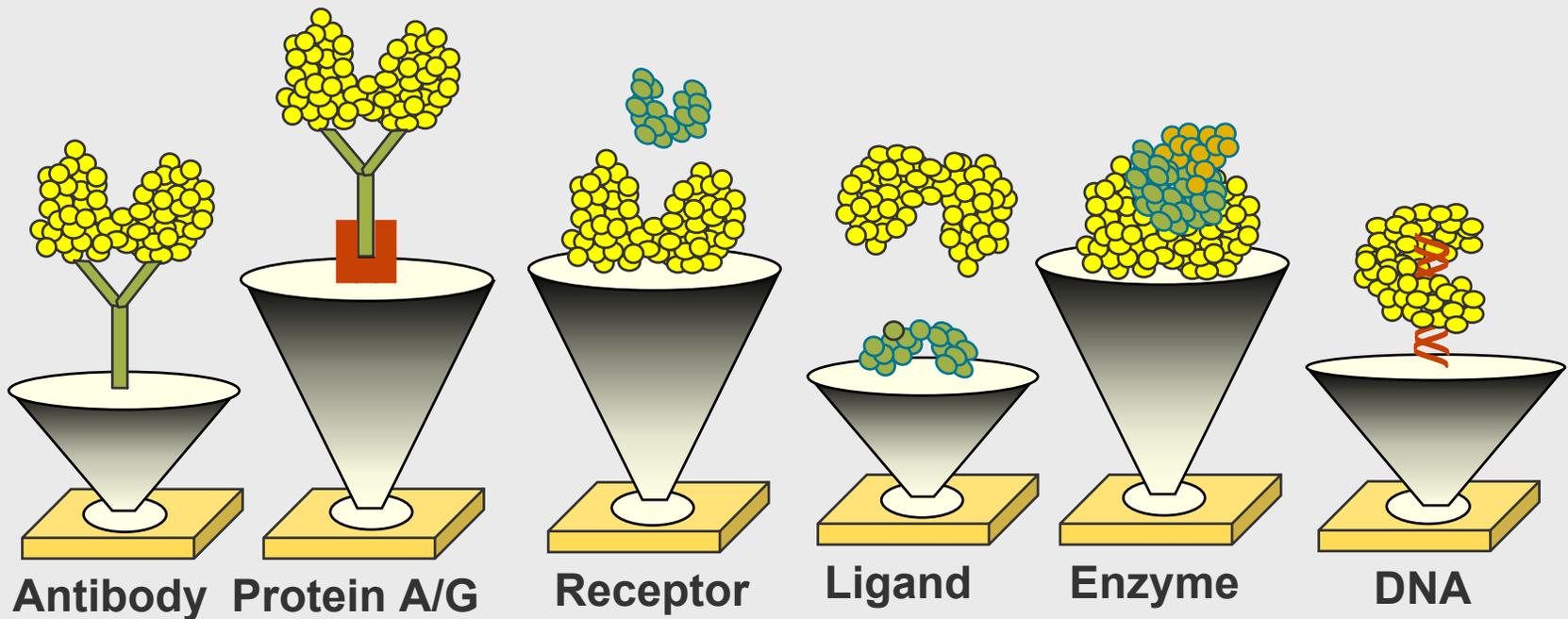
ProteinChip® Applications



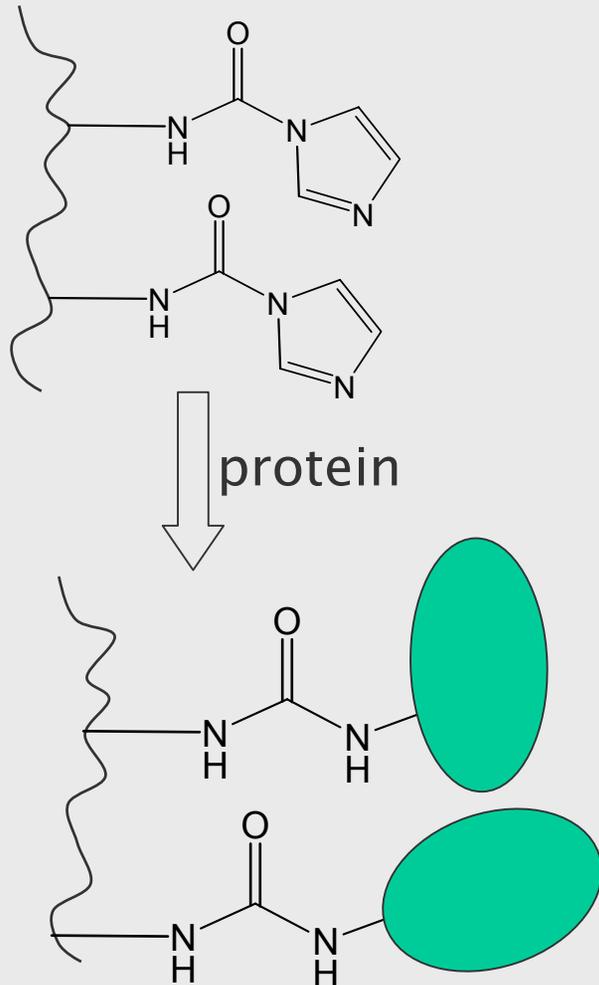
Signal Transduction/Biochemistry

- protein:protein interactions
- study Post-translational modifications
- epitope binding and mapping studies
- phosphopeptide mapping
- receptor-ligand identification
- DNA:protein binding studies

ProteinChip Arrays for Molecular Recognition Studies

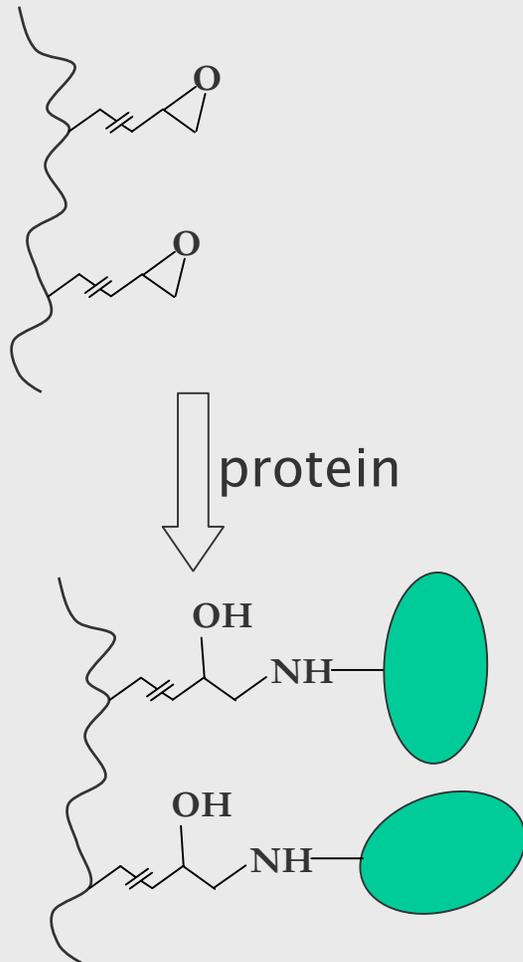


PS10: Preactivated Surface



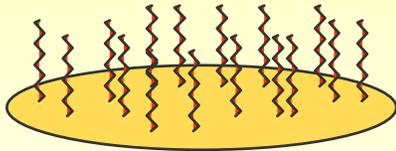
- Carbonyl Diimidazole (CDI) activated amine surface
- Covalently Binds Proteins/Peptides through amine group
- Immobilization conditions
 - pH 8–9
 - med – high salt
- Washing conditions
 - Buffer with detergent (eg PBS 7.2 with TX100)

PS20 Preactivated ProteinChip Arrays

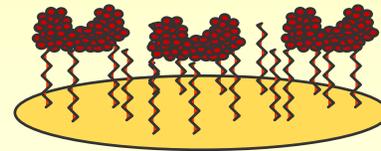


- Epoxy activated amine surface
- Covalently Binds Proteins/Peptides through amine group
- Immobilization conditions
 - pH 8-9
 - med - high salt
- Washing conditions
 - Buffer with detergent (eg PBS 7.2 with TX100)

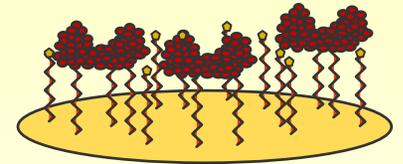
SELDI Biological ProteinChip® Array Method



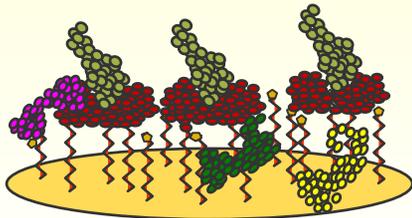
1. Preactivated ProteinChip



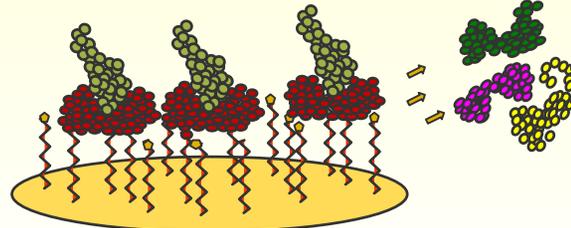
2. Bind 'Capture' Molecule



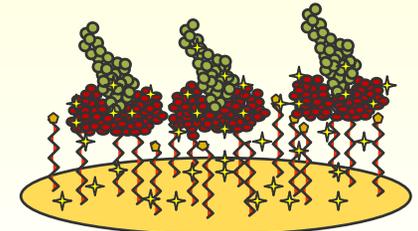
3. Block unused sites



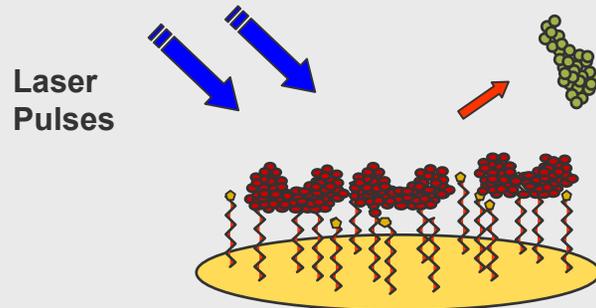
4. Analyte Capture



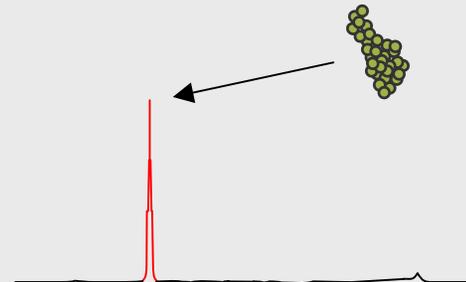
5. Wash



6. Add EAM



7. Laser Ionisation / Desorption



8. Direct Mass Detection

PS10 vs. PS20 Preactivated Arrays

PS10 Arrays

- Higher capacity
- Higher non-specific binding
- Use higher detergent (typically 0.5% Triton X-100)
- Longer washes
- Hydrophobic background

PS20 Arrays

- Lower capacity
- Lower non-specific binding
- Use lower detergent (typically 0.1% Triton X-100)
- Shorter washes
- Hydrophilic background

Best surface chemistry for a given application must be determined empirically.

Direct vs. Indirect Coupling

Protein A/G then Antibody

- Antibodies all in same orientation
- Antibody will “fly” - interference from peaks at ~75 and 150 kD
- Can use antibodies contaminated with other proteins, Tris, azide, etc.

Direct Coupling of Antibody

- Antibodies in random orientation
- Antibody does not fly – no interference
- Antibodies must be pure and free of other sources of free nitrogens

Experimental Parameters for Interaction Experiments

- Proper controls are critical!

Always use a control antibody or other control protein to identify nonspecifically bound components

- Protein to be covalently attached must be pure and free of other free nitrogens (i.e. Tris, azide, glycine)

Watch out for antibodies containing BSA or gelatin – these will need to be purified before direct coupling

Alternatively, couple protein A/G to chip and then add impure antibody

- After binding and blocking, wash vigorously with Triton X-100 to remove excess non-covalently bound protein
- Use shorter and gentler washes for samples that may have high off-rates

Antibody Capture Experiments

Important Ab Facts

- Antibodies are glycoproteins having 2 heavy and 2 light chains (~22–25 and 49–53 kDa), disulfide linked
 - Poor quality Ab have free heavy and/or light chains
 - DDT or other reducing agents during binding/washing steps will reduce some Ab
- Antigen binding site in the F_{ab} portion
- Protein G/A and F_c receptor binding sites in the F_c domain of the protein (c= constant domain)
- Polyclonal Ab preparation has extensive aa sequence heterogeneity (including aa number variation)

Evaluating Antibodies

- Antibodies that work in immunoprecipitation or ELISA are most likely to work on chip. Antibodies that only work for Western Blotting recognize only denatured protein and probably will not work for SELDI.
- QC the antibody yourself
 - Check the literature to see if Tris, azide, albumin, or gelatin are in the prep
 - Check the antibody yourself on a NP or H4 array to make sure it is intact and there are no other contaminants

Evaluating Antibodies

- If antibody contains interfering compounds consider
 - Does this antibody type bind well to proteinA/G?
 - *Couple protein A/G to chip*
 - *Purify on protein A/G beads (allows direct coupling to array)*
 - For Tris and azide contamination, dialysis will be easiest – PBS is generally a suitable buffer

Take Home Messages

- PS10, PS20 proteinchip experimental success depends on good experimental design
 - What's being covalently attached? Is it free of interfering reagents? What will serve as a good negative control?
- Interaction experiments are more often successful when capturing a low MW protein
- Always start with what is known about the interaction
- Most experiments will need to be optimized to find best binding buffer, temperature, etc

An Introduction to the ProteinChip® MS Reader



CIPHERGEN®

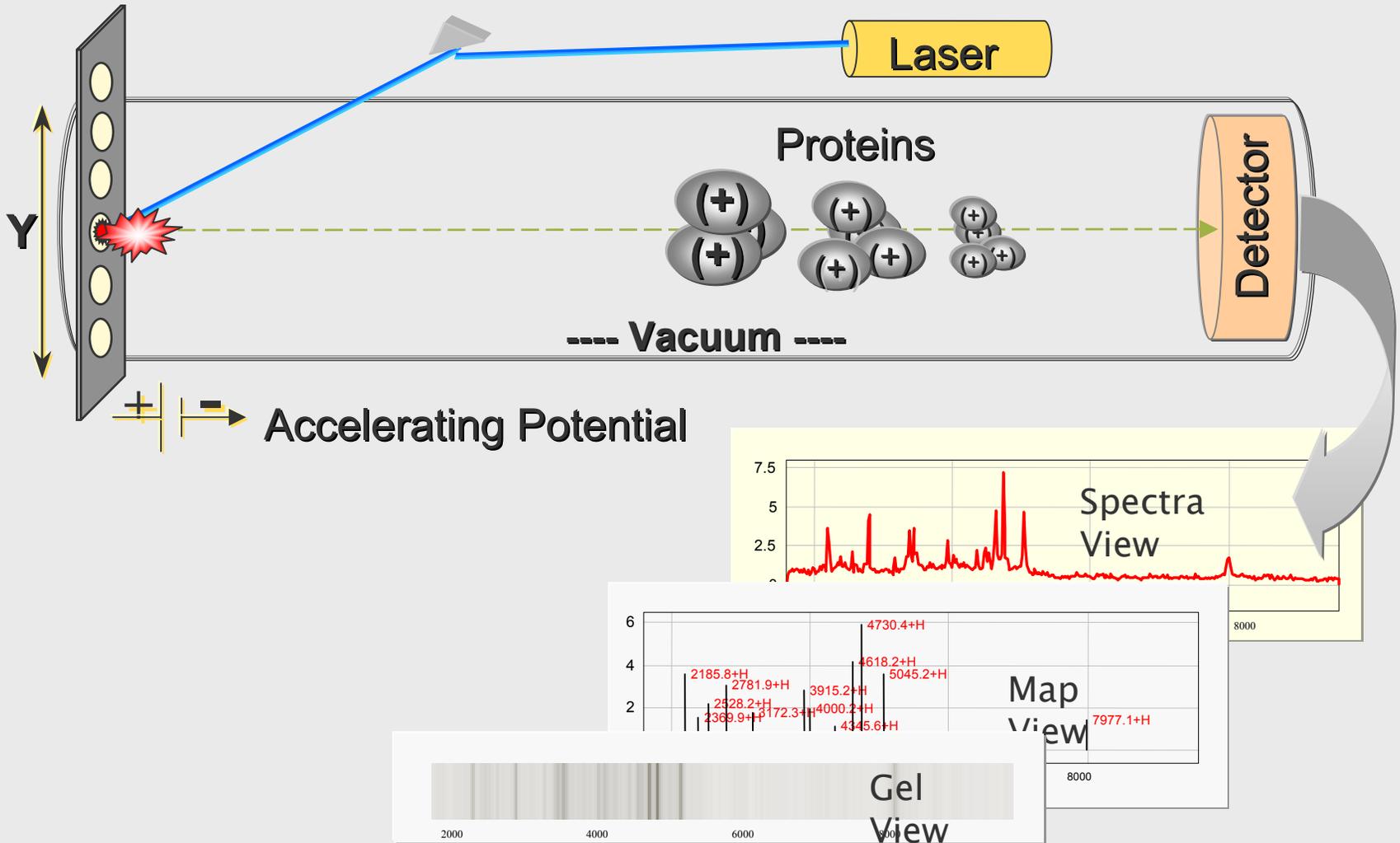
Matrix-assisted Laser Desorption/Ionization (MALDI)

- **Desorption/ionization method of non-volatile compounds in which the analyte is embedded in a solid-state matrix crystal, or suspended in a pool or stream of liquid matrix**
- **Matrix is an energy absorbing compound responsible for converting laser energy to thermal energy, which facilitates the desorption/ionization process**
- **Samples are introduced to the MS on a passive probe or a continuous-flow aerosol stream**

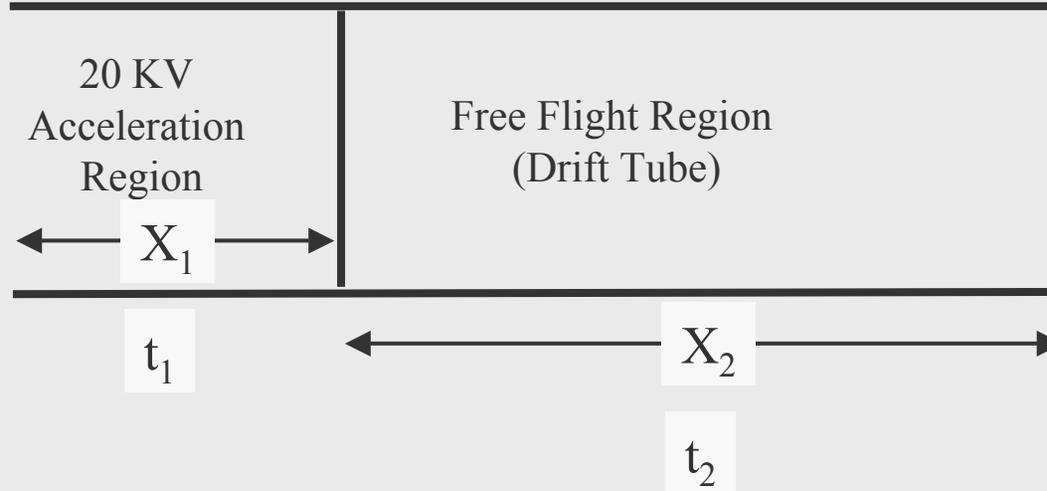
Surface Enhanced Laser Desorption/Ionization (SELDI)

- **Desorption/ionization method of non-volatile compounds in which the sample presenting surface plays an active role in the extraction, presentation, structural modification, amplification, and/or ionization of a given sample**
- **Invented by T. William Hutchens and Tai-Tung Yip**

SELDI ProteinChip® Array Reader



The Time-Of-Flight Mass Spectrometer



Time-Of-Flight Equation

$$E = 1/2 mv^2 = z \cdot V$$

$$m/z = \frac{2V}{v^2} \quad \text{where} \quad v = \frac{\Delta x}{\Delta t} = \frac{x_1 + x_2}{t_1 + t_2}$$

$$m/z = \frac{2V}{\Delta x^2} \cdot \Delta t^2$$

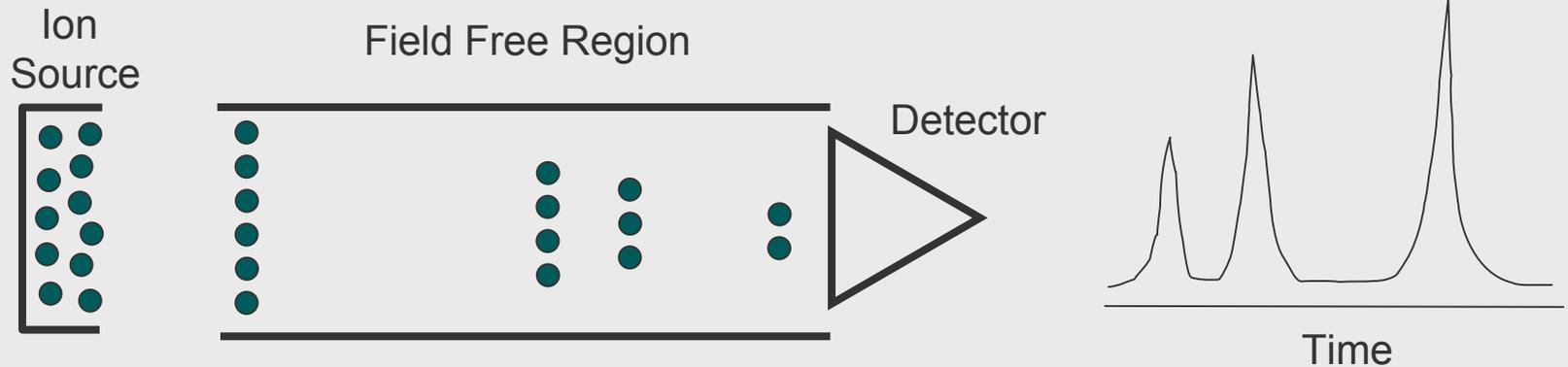
Assuming $\frac{2V}{\Delta x^2}$ is constant,

$$m/z = \mathbf{K} \cdot \Delta t^2$$

V= acceleration voltage

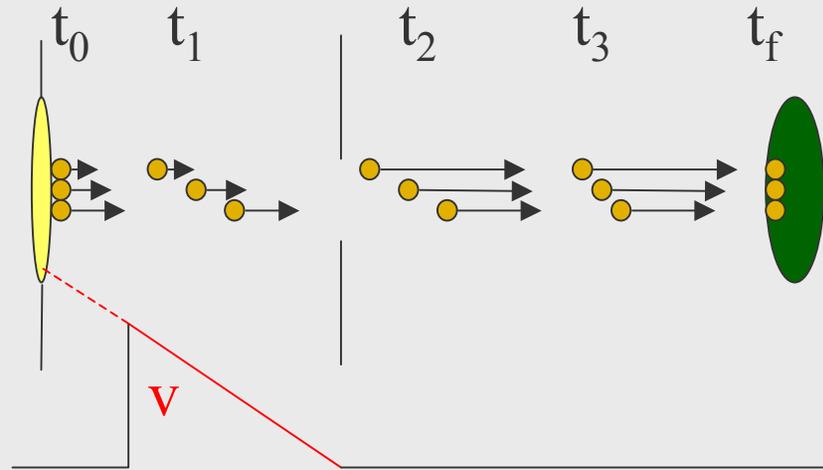
v= average final velocity

Time-Of-Flight Fundamentals



Ions leaving the source are accelerated to the same energy. Ions of different masses have different velocities, and therefore arrive at the detector at different times.

Time-Lag Focusing



- Improves Mass Resolution
 - Compensates for initial velocity distribution
 - Sharpens peaks near 'focus' mass
- Reduces Chemical Noise

PBS-II: Time-Lag Focusing (TLF)

Time-lag Focusing

Also called delayed extraction

Similar to a stacking gel on SDS-PAGE

Benefits:

Improves Mass Resolution

Compensates for initial velocity distribution

Sharpens peaks near 'focus' mass

Reduces Chemical Noise

Considerations

For accurate mass calibration, must run standards using same focus mass

For profiling, can rerun chip several times with different focus masses

Greatest improvement in resolution in low mass range (< 20 kD)

Molecular Weight Determination Accuracy

■ **System calibration**

- Well-characterized samples of known molecular weight are analyzed and their flight times used to derive the coefficients for a TOF calibration function on a specific instrument
- Calibrations are best performed using identical or similar compounds and/or matrices
- Multi-component mixtures providing multiple calibration points should be used (at least 3 points)

Molecular Weight Determination Accuracy

- **Internal standard measurement (“internal calibration”)**
 - System calibration is performed during analysis of the unknown
 - *Provides the highest degree of MW determination accuracy*
 - *Calibrants are added to unknown sample in an effort to bracket the unknown with calibration points*

- **External standard measurement (“external calibration”)**
 - System calibration is performed as a separate experiment
 - *Greater increase in mass error (about 5 fold)*
 - *Easiest to perform on unknown mixtures*

Calibration Considerations

- **For highest accuracy, keep all acquisition parameters the same**
- **More spot-to-spot variation than chip-to-chip variation, so do spot for spot calibration where necessary**
- **Recalibrate frequently, after instrument service, and whenever mass accuracy is critical to your results**
- **Peptide Range (<10 kD)**
 - Recommend doing a whole chip so you can do spot for spot calibration later
 - Usually optimize 1-7.5 kD, but can do multiples for your region of interest
- **Protein Range (>10 kD)**
 - Do multiple optimization ranges for different mass ranges (i.e. 10-30 kD, 30-60 kD)