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Sample preparation & protein enrichment for proteomics and mass spectrometry

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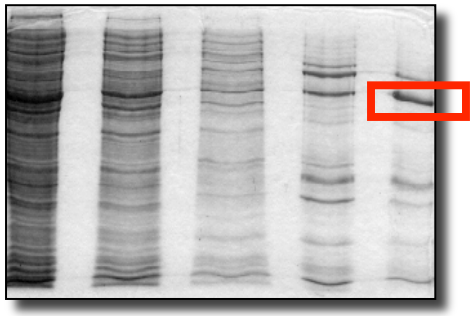
Learning objectives

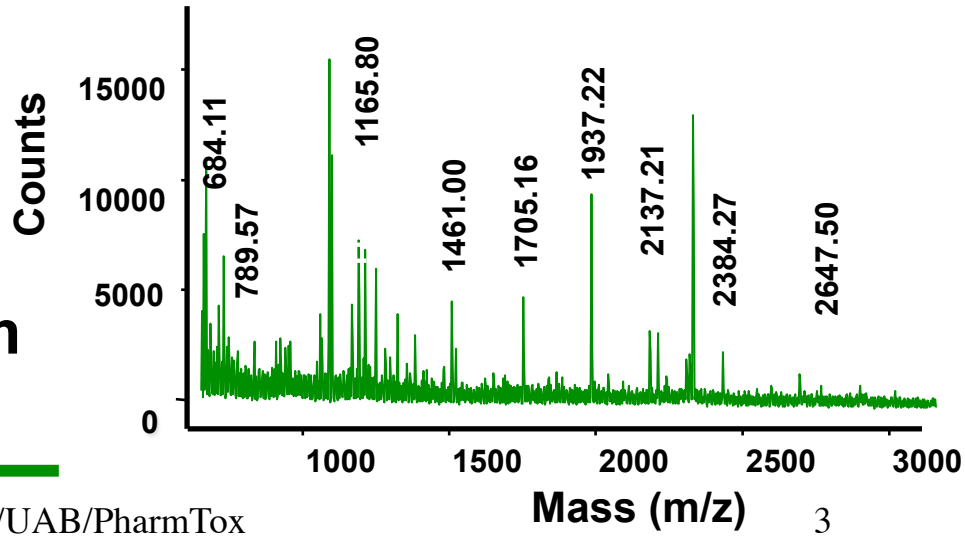
- **"Smart" proteomics:**
  - **lowest proteome complexity, highest biological specificity**
- **How do we achieve lowest proteome complexity:**
  - **Purify according to intrinsic properties**
  - **Purify according to biological properties**
  - **Enrich for subcellular compartments**
- **If the chemistry in sample preparation has been ignored, or hasn't been dealt with properly, the most "pure" proteomics sample will give lousy mass spectra.**

The basic elements of intact protein proteomics: (1) separation, (2) processing for MS analysis, (3) identification and characterization



trypsin

MALDI-TOF mass spectrometry



ID of parent polypeptide

MASCOT search engine

http://www.matrixscience.com

Handling proteins and peptides

- **Collecting and storing the sample**
  - **Use the same type of storage device for all the samples in a study…quality control!!**
  - **Some samples are sensitive to freezing**
    - **Mitochondria and other organelle fractions should be prepared using fresh tissue**
    - **Samples especially fluids and organelle preparations should be placed in same volume aliquots and only thawed one time to avoid the effects of multiple freeze-thaw cycles**
  - **Freeze fast (into liquid N_2)**
    - **Buffers such as sodium phosphate can selectively precipitate while ice and water are in equilibrium (down to -20^{o}C) - this can lead to a substantial change in pH**
    - **Similarly, thaw fast rather than slowly, then keep on ice. (thaw by running between your hands, vs letting it sit on bench while you eat lunch.**

Tissue disruption/cell lysis

- Manual and mechanical homogenization
  - Pestle and mortar, Dounce and Potter-Elvehjem homogenizers, Waring blender, Polytron
- Grinding with beads, sonication and freeze-thaw
- Osmotic shock - or not!
- Bugbuster™ for bacteria
- Detergents: CHAPS, Triton X-100, cholate and deoxycholate
- Protease inhibitors

Parameters that govern the choice of protein separation method

- Purity of protein
- Speed of purification
- Quantity of protein
- What is the question:most important
  - Discovering a new protein/proteome
  - Identifying protein-protein interactions
  - Identifying potential modifications of known proteins

Properties of polypeptides that enable separation from each other

- **Intrinsic properties**
  - **Size—number of amino acids**
  - **Net Charge**
- **Biological/functional properties**
  - **Intracellular location**
  - **Enzyme activity**
  - **Undergoes oligomerization**
  - **Undergoes modification**

Some protein separation methods are both preparative and analytical

- **2D electrophoresis**
  - **Resolves polypeptides dissociated from each other**
  - **Good for isoform detection/quantitation, PTM changes detection/quantitation**
- **2D blue native electrophoresis:**
  - **Resolves complexes containing intact proteins**
  - **Can be quantified by MS, or by blotting for specific proteins**

Separating proteins by size

- For purification: Gel filtration chromatography
- Analytic: SDS-PAGE

Separating proteins by charge

- **Purification: Ion exchange chromatography**
- **Analytic: isoelectric focussing**
- **But may use SDS-PAGE to monitor purification**

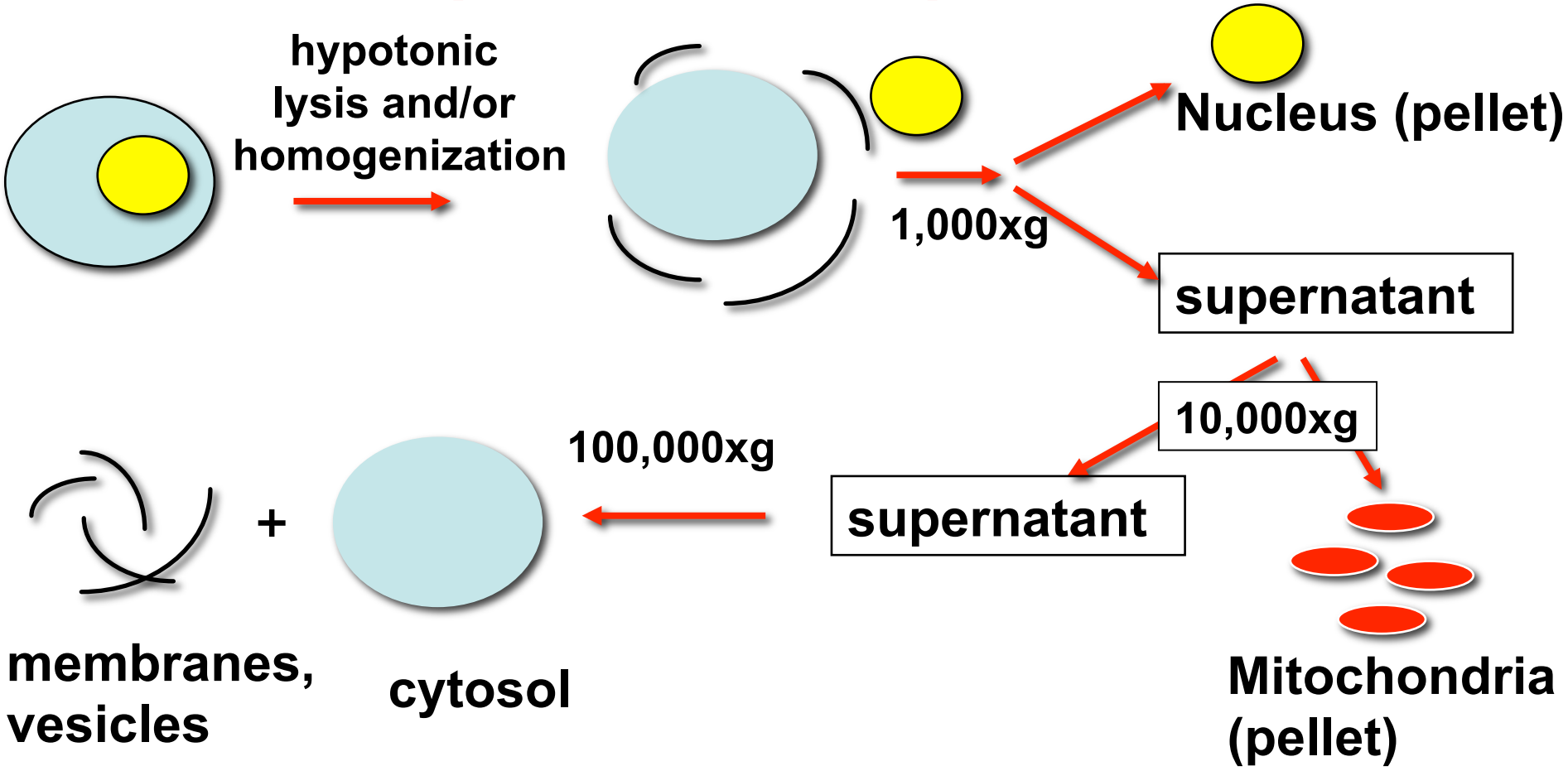
Protein functional properties that can be basis for purification

- **Oligomerization:**
  - **Each cytoskeletal component undergoes reversible oligomerization from its monomers**
- **Intracellular location:**
  - **Change in**
  - **Increase in same location**
- **Protein-protein interaction**

Wide dynamic range for proteins in most cells: rationale for protein enrichment

- **A gel that is overloaded with respect to the abundant proteins, may still have only *barely detectable or nondetectable* amounts of a low abundance protein**
  - **If you can't see it, you don't know it's there;**
  - **Even if software detects and quantitates it, you can't do MS of it, because there isn't enough protein.**
- **No one stain will detect 9 orders of magnitude differences in abundance of proteins.**
- **In MS experiments, bypassing gels, the greater the complexity of the peptide mixture, the lower the chance of detecting very low abundance proteins/ peptides.**

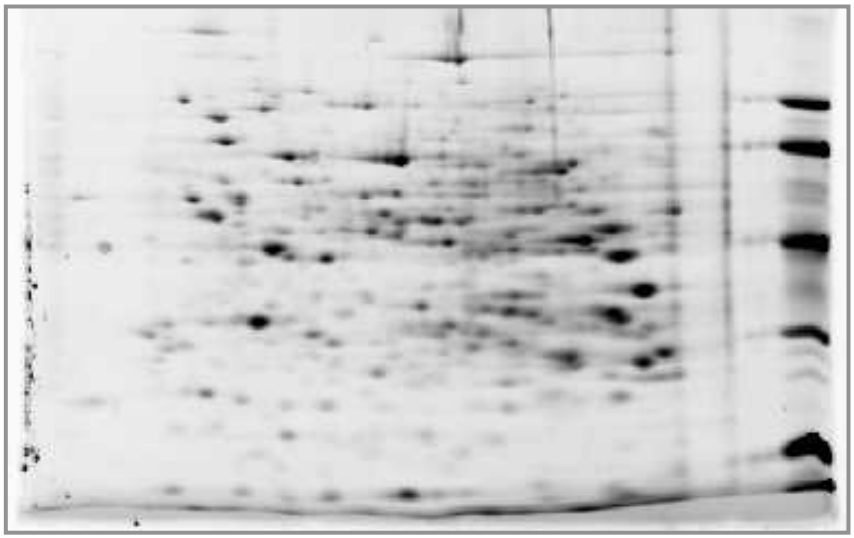
Use “old” information and “conventional” approaches like differential centrifugation to enhance proteomics experiments.



Analysis of mitochondrial proteins enhanced by purifying that subproteome

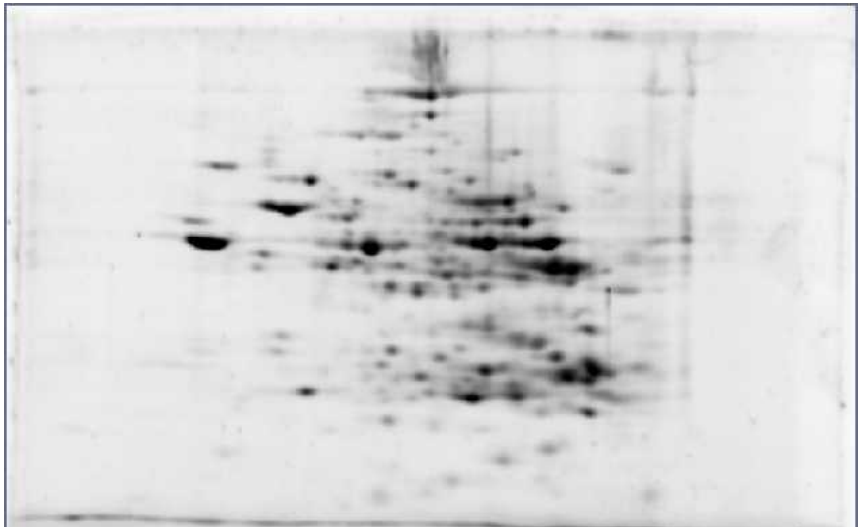
Whole Rat Liver

3 10



Rat Liver Mitochondria

3 10



2D gel analysis using the Invitrogen ZOOM system

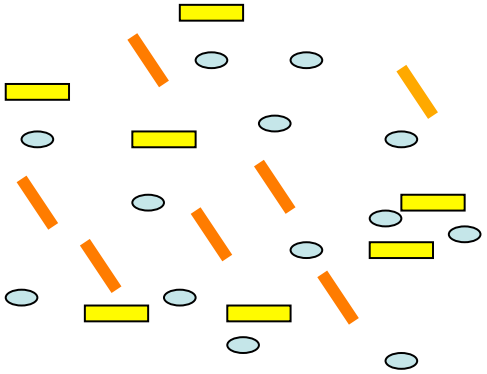
(Courtesy of Shannon Bailey Lab – Whitney Theis and Kelly Andringa)

The good news:
Several subcellular proteomes have been "catalogued."

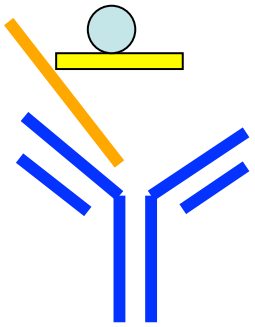
Compartment	# polypeptides in SWISSPROT as of 2000
Mitochondria (1000/cell)	269-----5% of total
Lysosome (400/cell)	50-----1% of total
Peroxisome	35-----0.6%
ER and Golgi apparatus	157-----3%
Nuclei (5% cell volume)	964-----17%
Others (cytosol, membrane)	4228---75%
	total: 5703

(Jung et al. [2000] Electrophoresis)
Note date: this is old!!! But the principle is the same; homework for class, find current numbers, please.

Antibodies can reduce the complexity of the proteome, as well as enhance biological specificity, by 10,000-fold



A cell lysate: 6,739 polypeptides



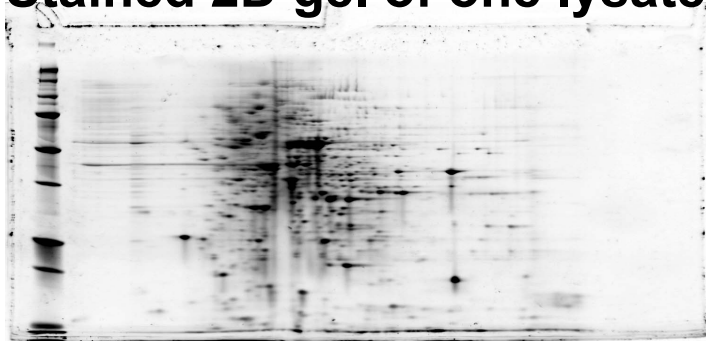
An immune complex of 1-10 polypeptides (why might there be more than one polypeptide?)

Which sample would you rather deal with on a 1D or 2D gel?

2D Western blot + Mass spectrometry identified colitogenic antigens from a particular bacterium

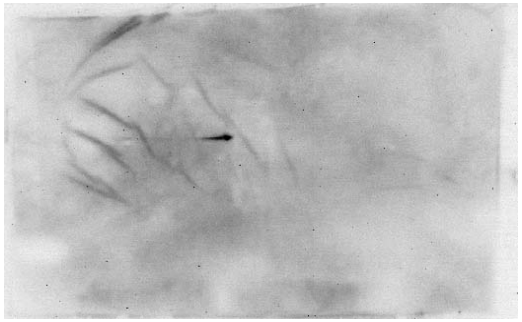
Western Blot

Stained 2D gel of one lysate



Bacterial lysates probed with mouse serum

Duplicate 2D gel Western blotted with the same serum

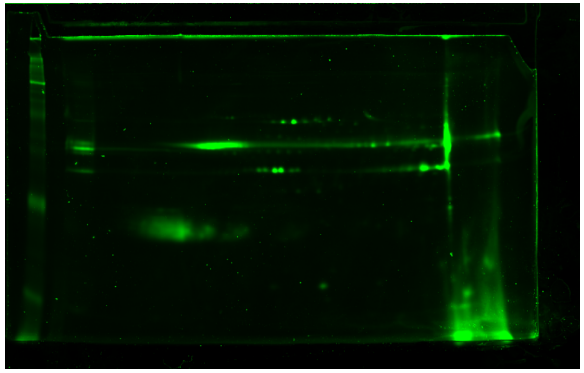


MS

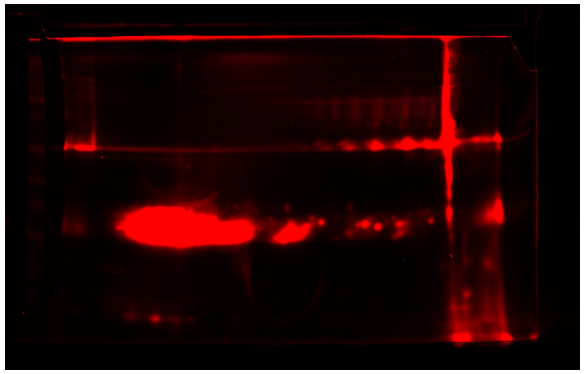
gi|29338205
Mass: 45415
MOWSE Score: 100
Putative identification:
tetratricopeptide repeat family protein [Bacteroides thetaiotaomicron VPI-5482]

(Elson et al., unpublished data)

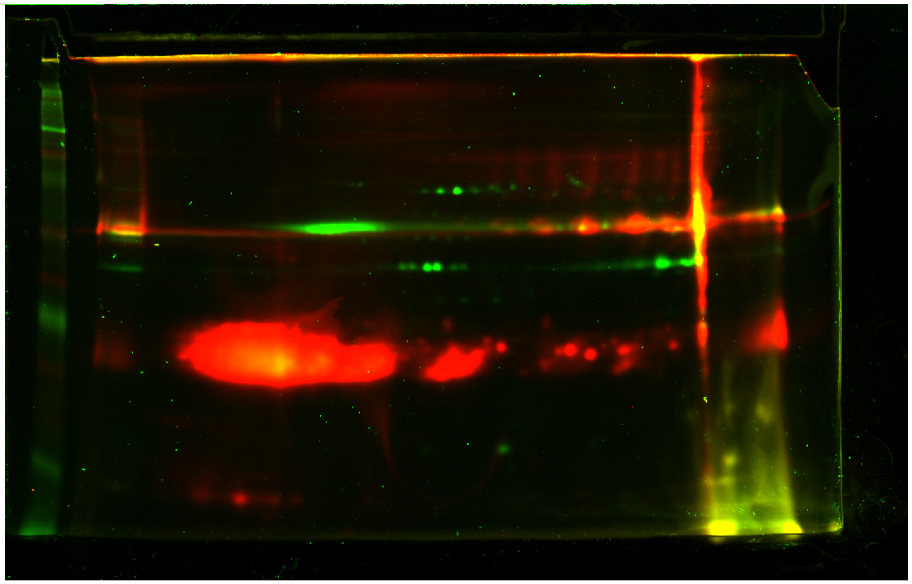
2D DIGE of immunoprecipitates to determine tubulin-binding proteins



A



B



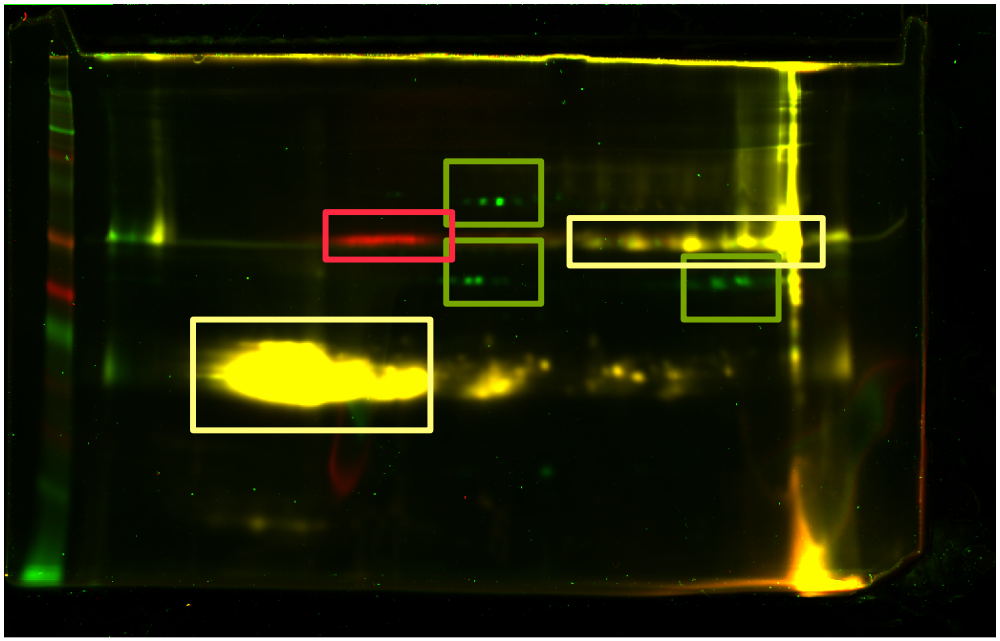
Overlay of A and B images

GREEN (A): starting tubulin and MAPs

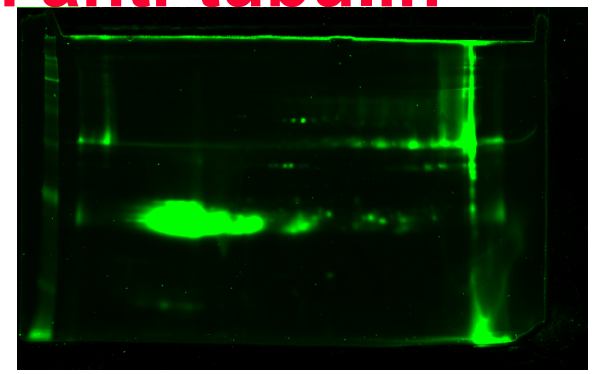
RED (B): antibody alone, 8 ug

Conclusion: tubulin, MAPs and antibody resolved from each other, without having to deplete of antibody

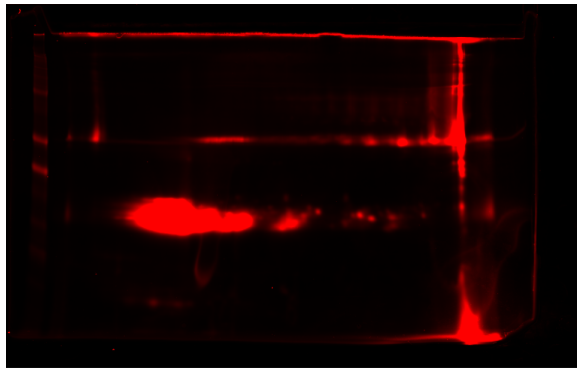
Complexity in initial 2D analysis of tubulin immunoprecipitates: Something in the MAPs co-precipitates with anti-tubulin



A and B overlay



A



B

GREEN (A): pellet of anti-tubulin + MAPs, no tubulin (commercial preparation);

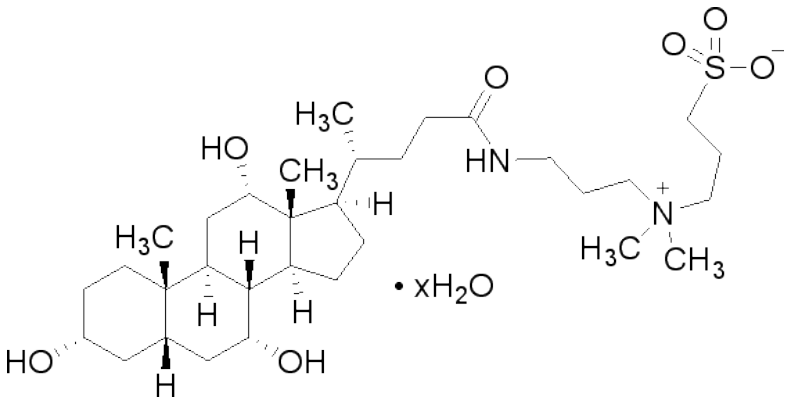
RED (B): pellet of anti-tubulin + tubulin, no MAPs;

YELLOW: proteins common in both pellets; IDEALLY, there should be no green

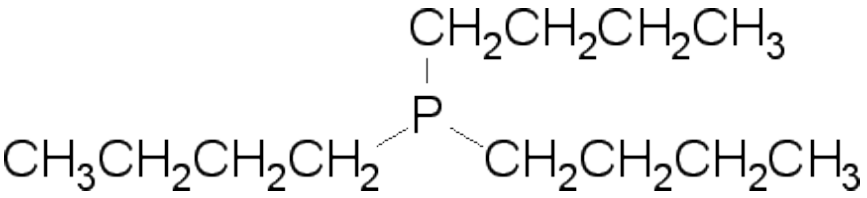
Affinity purification: Antibodies are available for many protein posttranslational modifications

- Phosphorylation
- Glycosylation
- Oxidative modifications:
  - Protein carbonyls
  - Reactive aldehyde adduct formation: 4HNE
- Keep in mind that these modifications each involve mass changes, thus can be detected directly by MS.

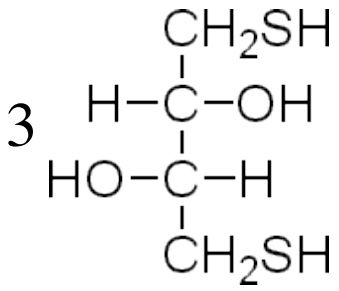
Structures important in protein electrophoresis to know



1



2



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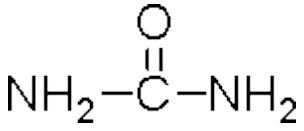

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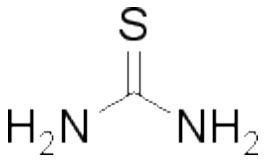

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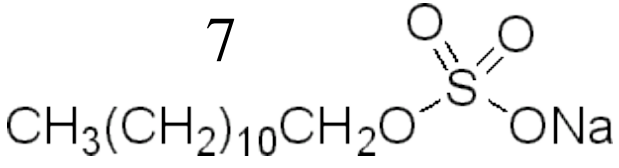
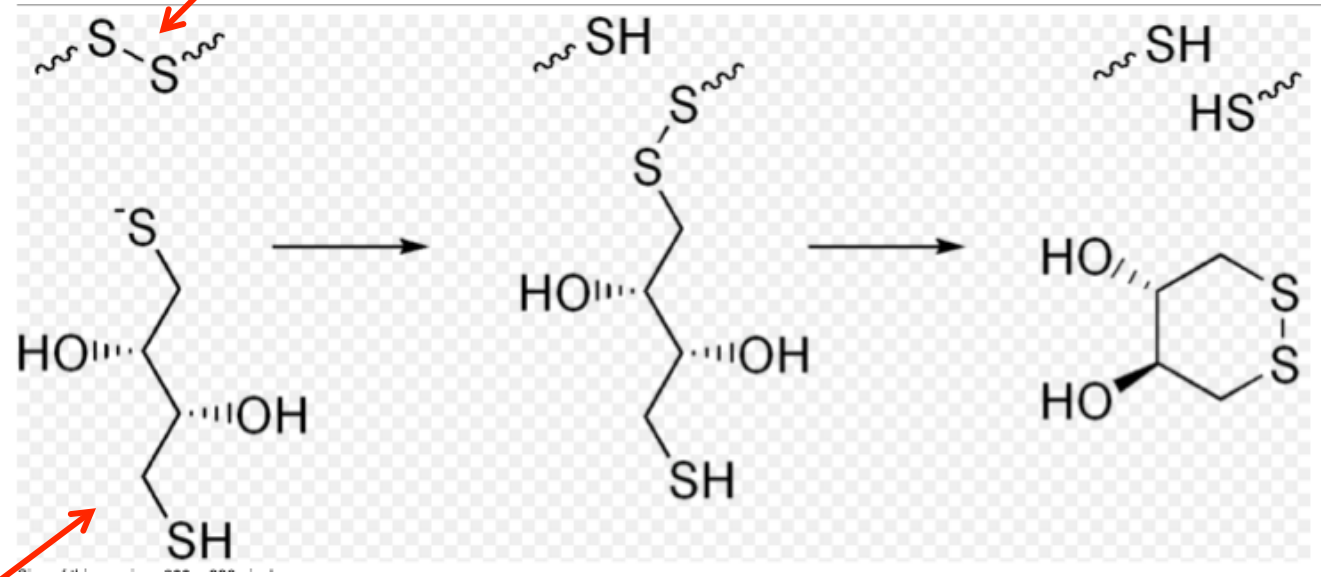
7



The action of a "reductant"

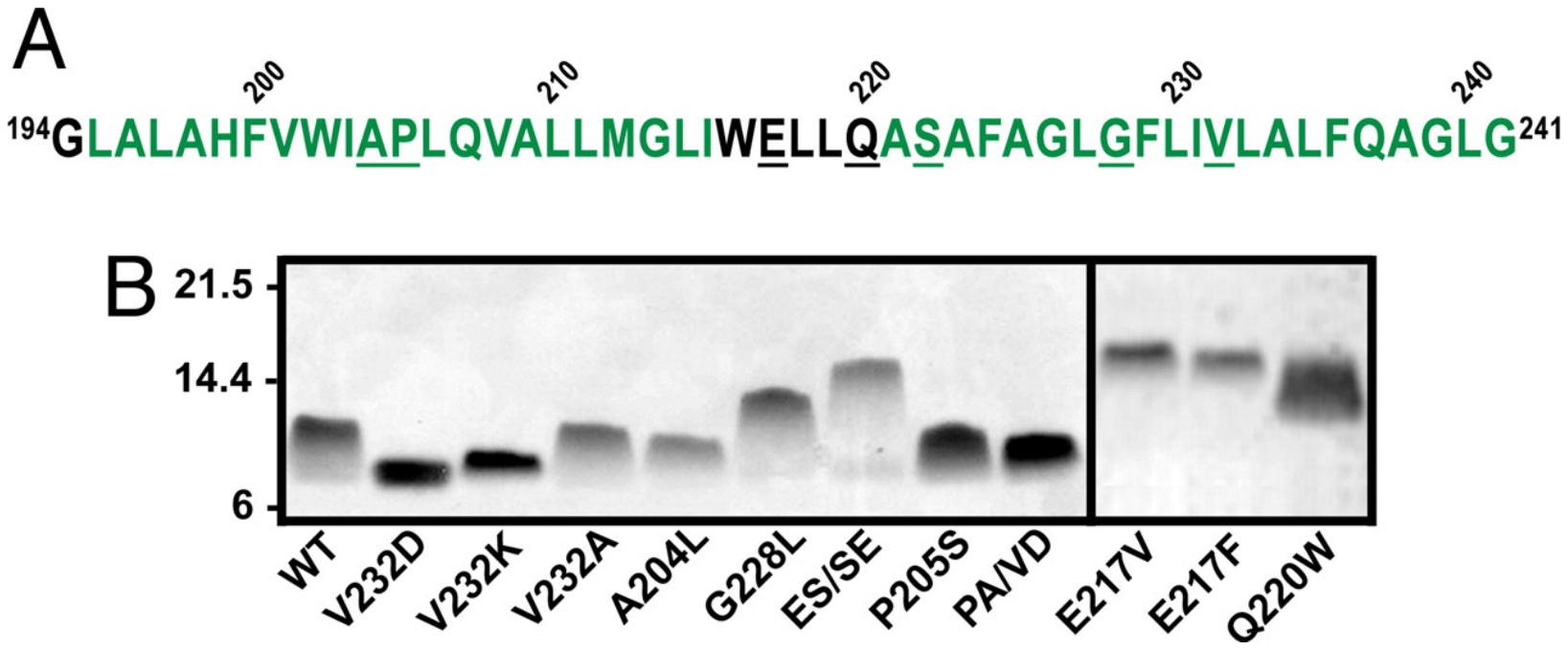
A disulfide bond



"the reductant"

(believe it or not, from Wikipedia, 2011)

The interplay of protein structure and SDS: Single mutations in a hairpin sequence affect mobility of an intact polypeptide on SDS-PAGE.



(Taken from Rath et al., PNAS 2009)

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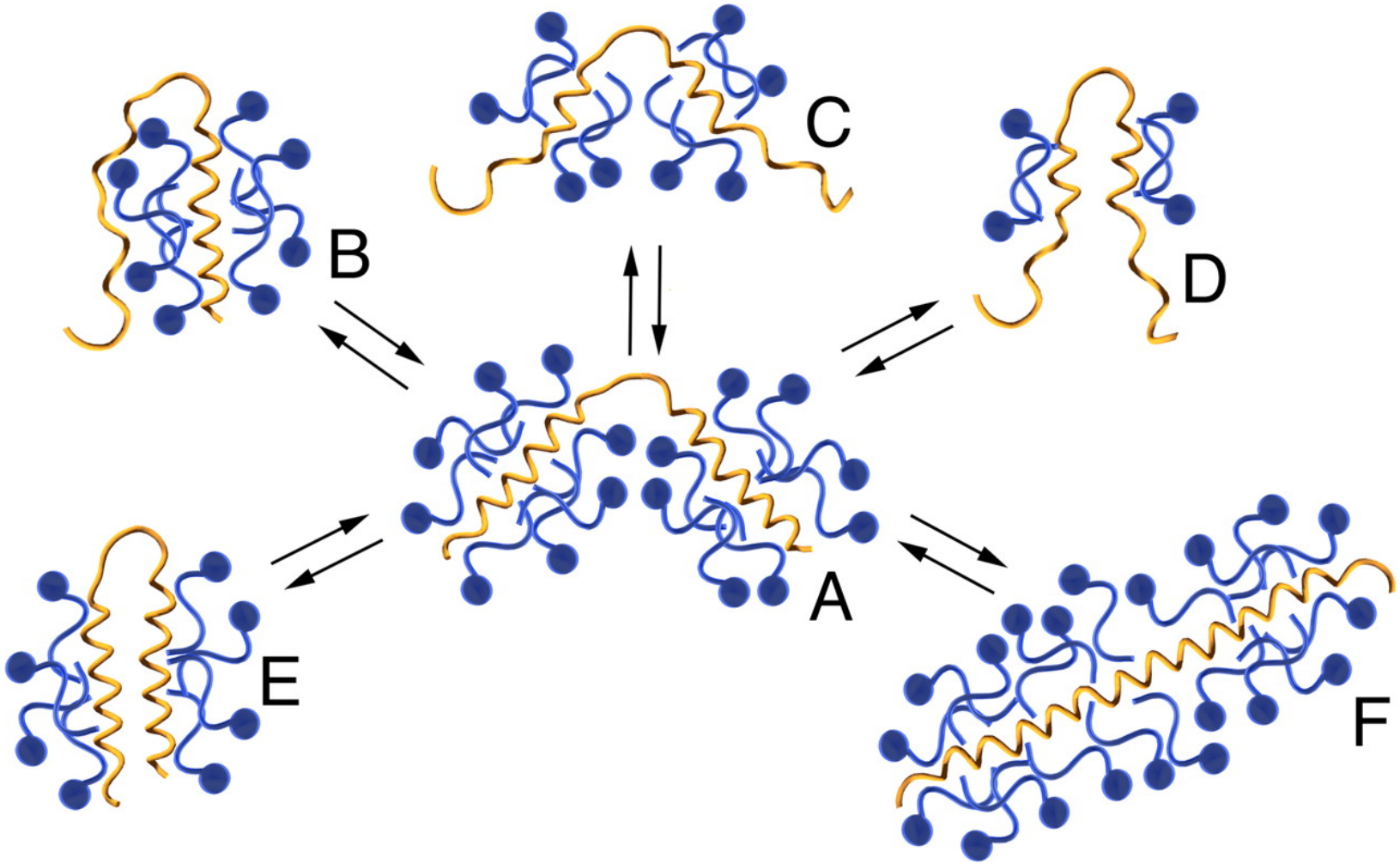
Relationship between hairpin conformation and detergent binding.



(Taken from Rath et al., PNAS 2009)

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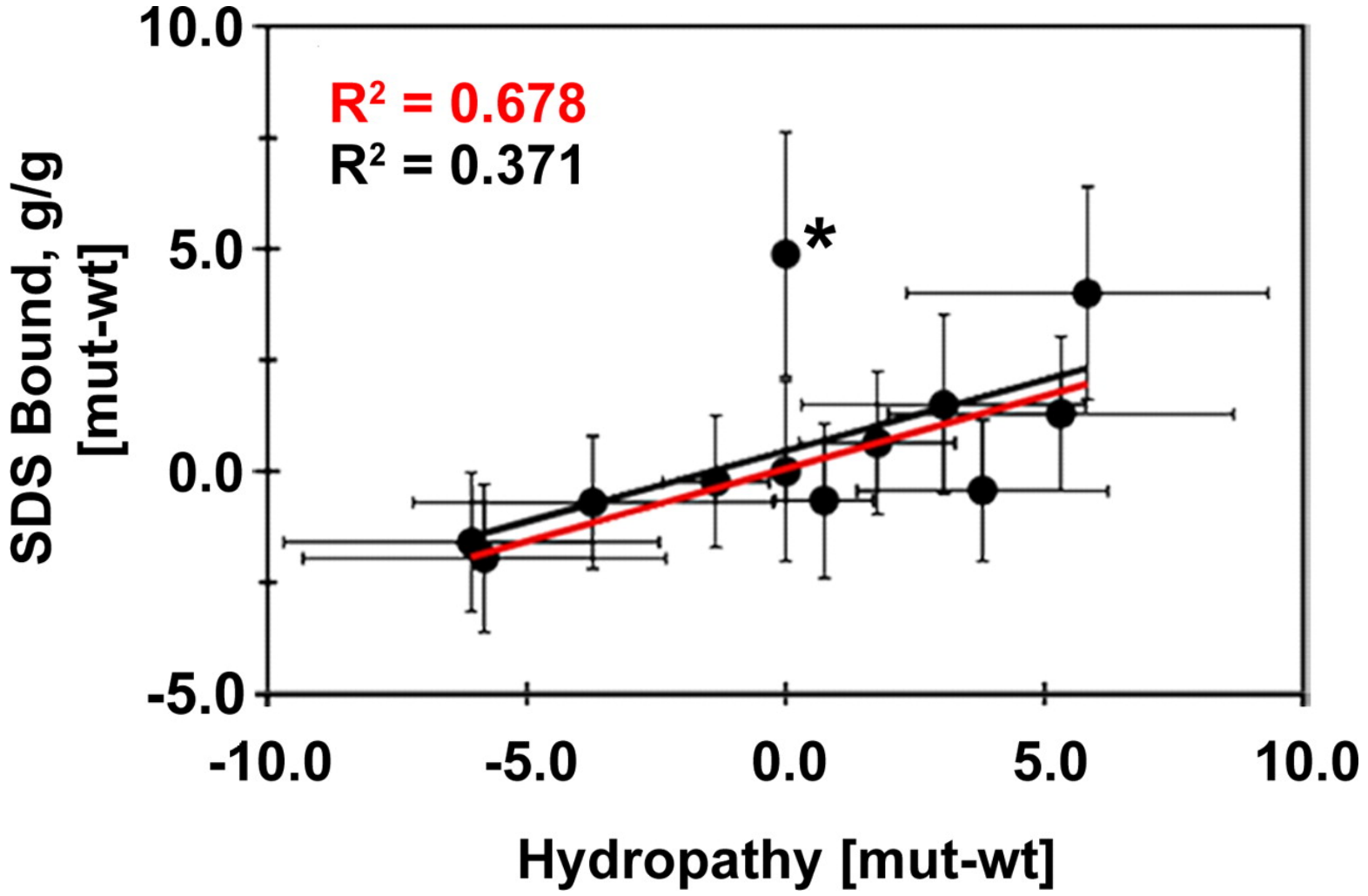
Correlation of SDS binding and hydropathy.



(Taken from Rath et al., PNAS 2009)

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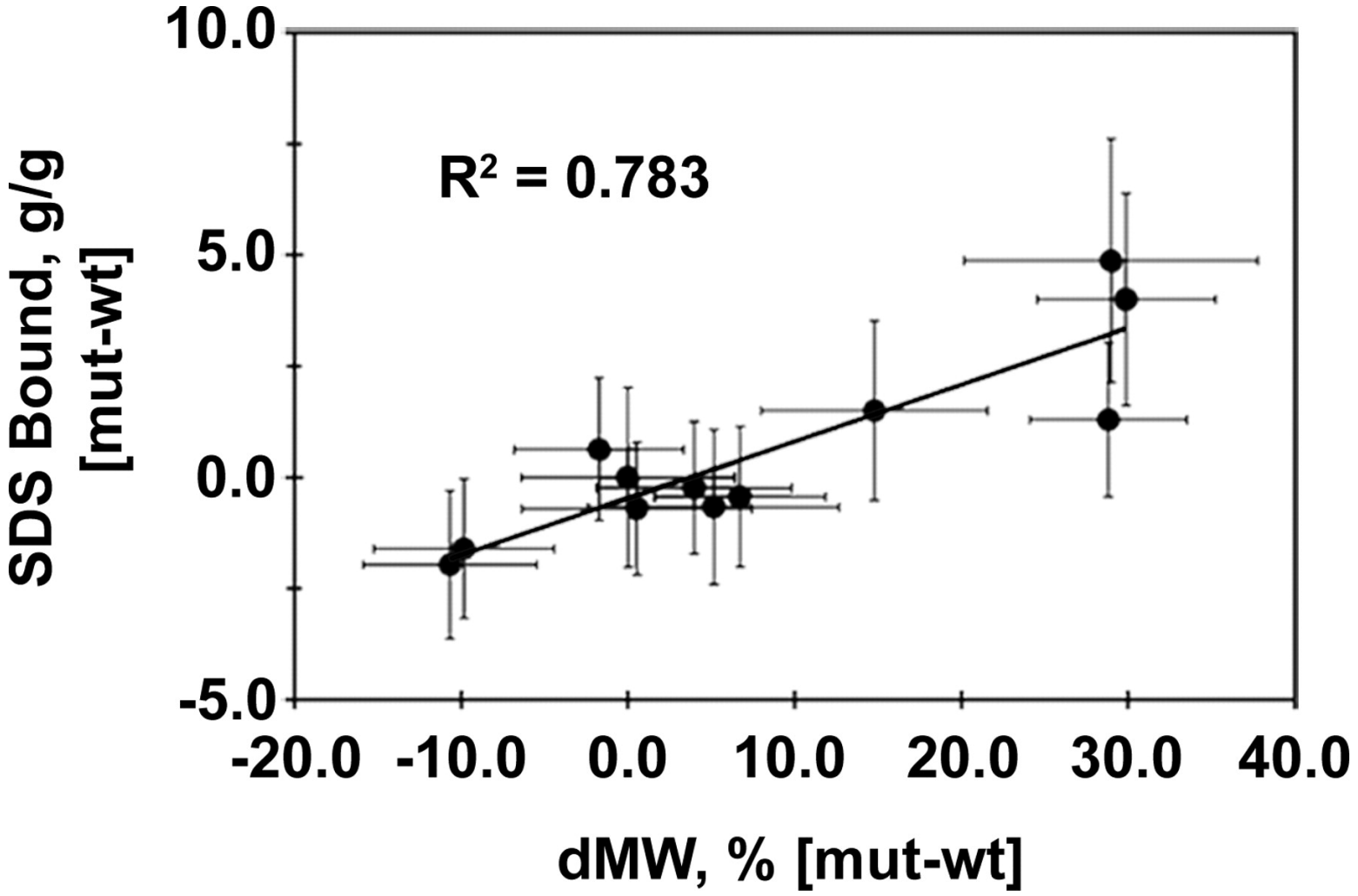
SDS-binding and mobility on SDS gels.



(Taken from Rath et al., PNAS 2009)

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Why do we care about SDS binding and minute differences in protein structure:

- **Western blot analysis can detect a polypeptide at an unexpected mw; doesn't mean its mass is different necessarily in the mutant or diseased tissue;**

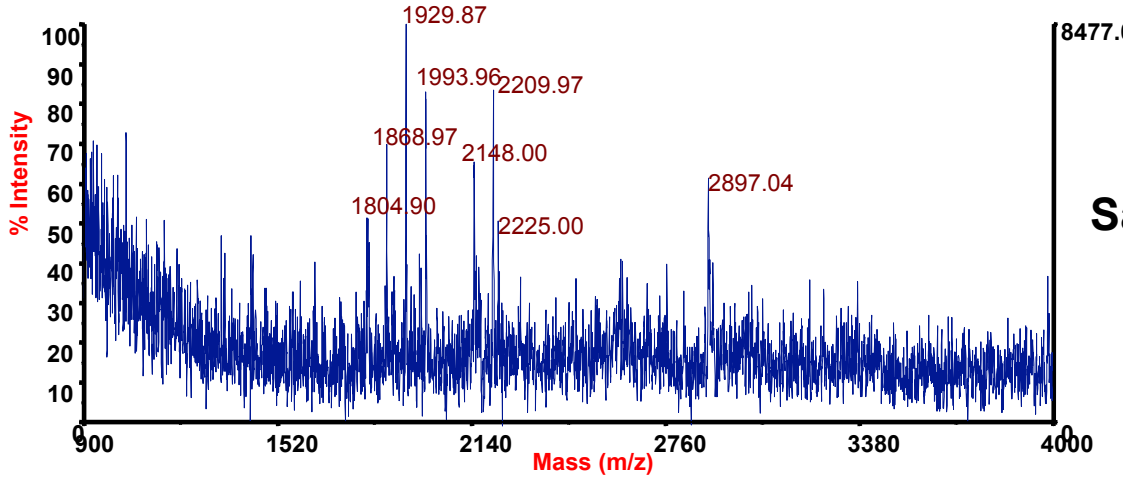
- **Western blot detection of multiple bands may reflect structural or conformational heterogeneity in that one protein in the sample, not cross-reactivity with other proteins.**

- **Cannot presume a polypeptide will be at or near its predicted or WT mw.**

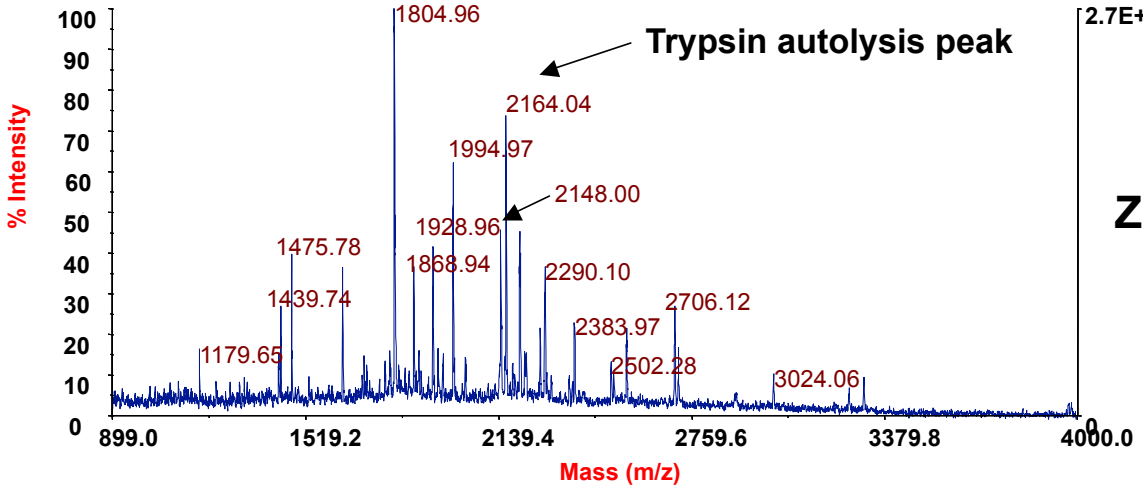
Wide dynamic range for proteins in most cells: rationale for protein enrichment

- **A gel that is overloaded with respect to the abundant proteins, may still have only *barely detectable or nondetectable* amounts of a low abundance protein**
  - **If you can't see it, you don't know it's there;**
  - **Even if software detects and quantitates it, you can't do MS of it, because there isn't enough protein.**
- **No one stain will detect 9 orders of magnitude differences in abundance of proteins.**
- **In MS experiments, bypassing gels, the greater the complexity of the peptide mixture, the lower the chance of detecting very low abundance proteins/ peptides.**

Benefit of removing salt from tryptic digest



Salt Contamination



ZipTip (desalting)

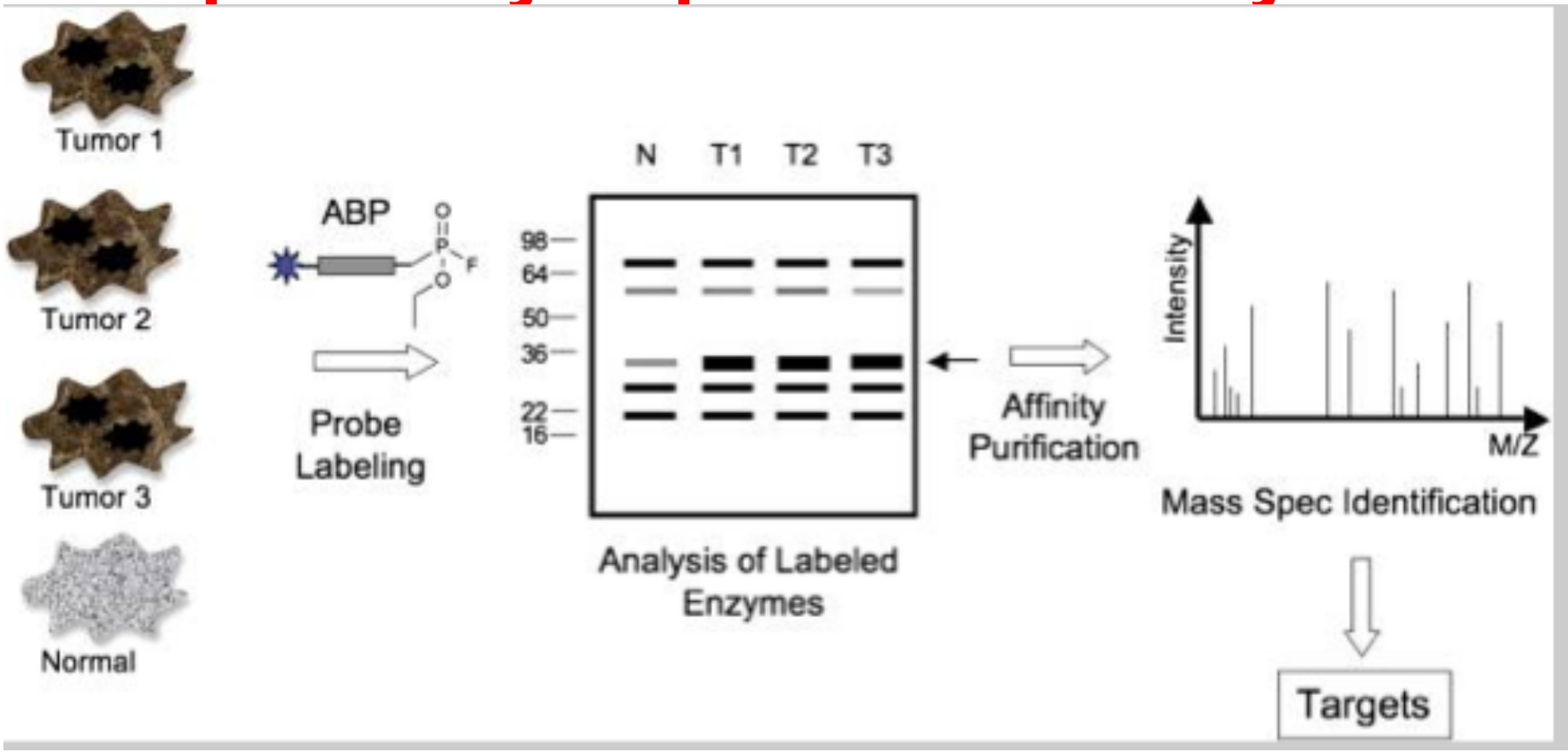
Preparing proteins for peptide mass fingerprinting (1)

- **The mass spectrometry procedure has to be preceded by high resolution protein chromatography steps……the LC part of LC-MS.**
- **Or, it can be a sample with a few intense bands of interest - e.g., a recombinantly expressed protein in bacteria lysate.**
- **SDS-PAGE may be the best thing you can do for your protein preparation…it gets rid of lots of potential contaminants, and at the same time concentrates the proteins into "bands."**

Preparing proteins for peptide mass fingerprinting (2)

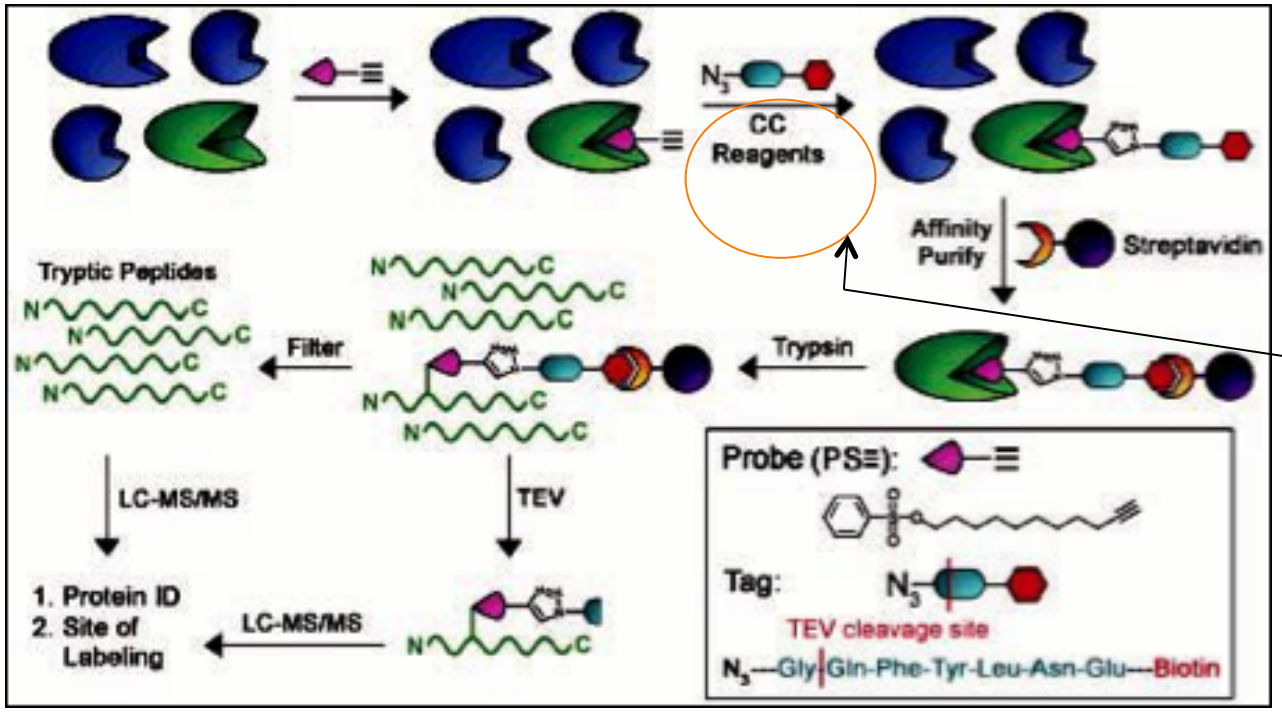
- **Once the protein is precipitated in the SDS gel matrix, electrolytes/salts and the SDS are largely removed by washing the gel pieces with 50% aqueous acetonitrile containing 25 mM NH_4HCO_3 buffer, pH 8**
- **The gel is dehydrated and rehydrated in 25 mM NH_4HCO_3 buffer, pH 8 to which trypsin added for overnight digestion.**
- **The resulting peptides are extracted with 50% aqueous acetonitrile containing 25 mM NH_4HCO_3 buffer, pH 8 - the extract is evaporated, and brought up in MS-compatible buffer/solvent.**

Activity-based protein profiling (ABPP) to increase biological specificity of proteomic anslysis



(Taken from Bogyo, PNAS, Feb, 2010)

Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP): reducing complexity and increasing biological specificity at the same time



CC reagents:
Click chemistry is at the heart of this

(Weerapana, Speers, Cravatt, Nature Protocols, 2007)

Take home messages regarding protein purification

- **Reduce complexity of proteome**
  - **by conventional protein separation approaches (size exclusion, ion exchange, reverse phase).**
  - **Let biology work for you**
    - **Enrich for subcellular compartment**
    - **Affinity chromatography indicated by response to ligand**
    - **Oligomerization, protein-protein interactions**
- **Choice of purification/separation approach governed by**
  - **Abundance of sample**
  - **Abundance (if known) of protein in question**
  - **Question being asked**
  - **What technologies you can access readily**
    - **LAST BUT NOT LEAST: What you can afford**

Take home points, part II

- What proteomics technology gives back is like any other analytical approach: the quality is as good as what you put in;
- Be mindful of distinguishing between low abundance proteins versus low level contaminants;
- Keep in mind "conventional" approaches like Western blotting to validate proteomic results;
- Purify, purify, purify before running any proteomic experiment.
- Formulating a hypothesis forces you to incorporate biological information that can enhance proteomic analysis.
- The "end result" in proteomics is just a beginning:
  I. Some changes are causal to the disease/phenotype;
  II. Some are "real" but not causal;
  III. Some could be response of the cells/tissues TO the disease, not causing the disease.

Suggested readings

- **Cañas et al., 2007. "*Trends in sample preparation for classical and second generation proteomics*" Journal of Chromatography A, vol. 1153, pp 235-258.**

- **Ishihama Y, Rappsilber J, Mann M. Modular stop and go extraction tips with stacked disks for parallel and multidimensional peptide fractionation in proteomics. *J Proteome Res*. 2006 Apr;5(4):988-94.**

- **Durbin KR, Tran JC, Zamdborg L, Sweet SMM, Catherman AD, Lee JE, Li M, Kellie JF, Kelleher NL, Intact mass detection, interpretation, and visualization to automate Top-Down proteomics on a large scale. *PROTEOMICS* Special Issue: Focus on Top-Down Proteomics 10 (20): 3589–3597, 2010.**