CORSO DI LAUREA IN BIOLOGIA PER LA SOSTENIBILITÀ

BIOCHIMICA APPLICATA (6 CFU)

LEZIONE 10

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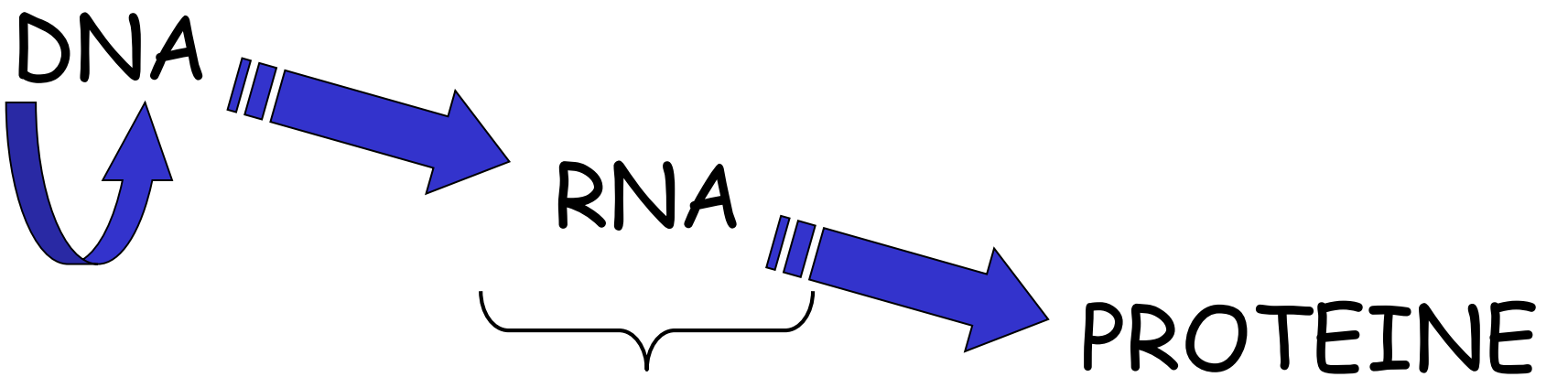
Determinazione del proteoma

- ✓Definizione
- ✓Separazione bidimensionale
- ✓Rivelazione
- ✓Analisi

Proteomica

Il concetto di proteoma è stato coniato nel 1995 dall'australiano Mark Wilkins, che voleva indicare l'intero equivalente in proteine di un genoma.

Un proteoma è la rappresentazione quantitativa dell'intero pattern di espressione proteica di una cellula, organismo o fluido corporeo in condizioni esattamente definite.



GENOMICA

Mantenimento e trasmissione informazione

L'informazione di base di un organismo è uguale per tutte le sue cellule

TRASCRITTOMICA

Trasferimento informazione

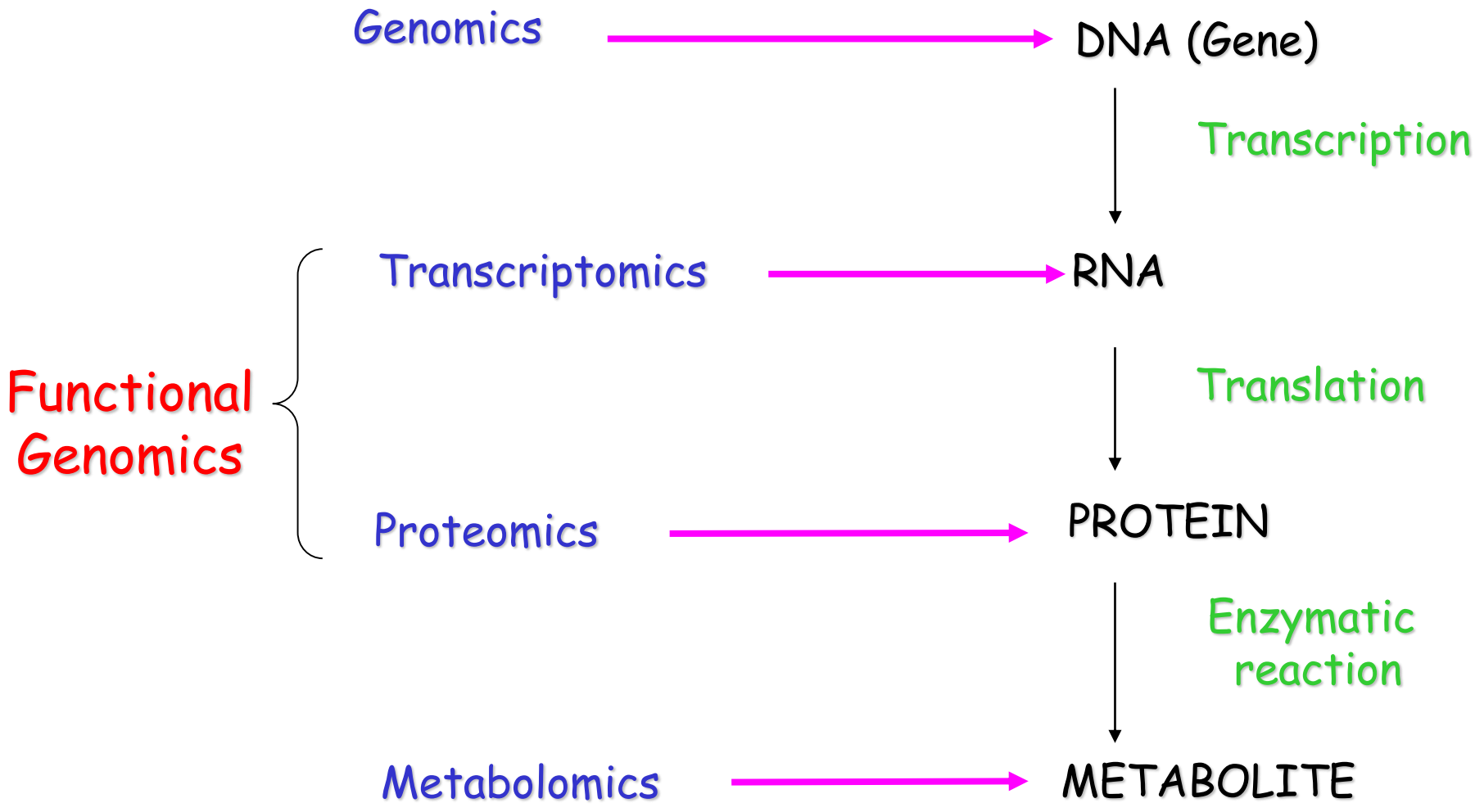
L'utilizzo dell'informazione è differenziale a seconda del tipo di cellula e del particolare contesto biologico considerato

PROTEOMICA

Effettori molecolari

Il complemento proteico è differente a seconda del tipo di cellula e del particolare contesto biologico considerato

The "omics" nomenclature…



- While the **genome** collects the whole genetic material of an organism, the **proteome** represents the set of its proteins

- The nature of genes – their simple chemical composition and their ability to be used as templates to make exact copies of themselves – made them relatively easy to study and analyze with automatic methods

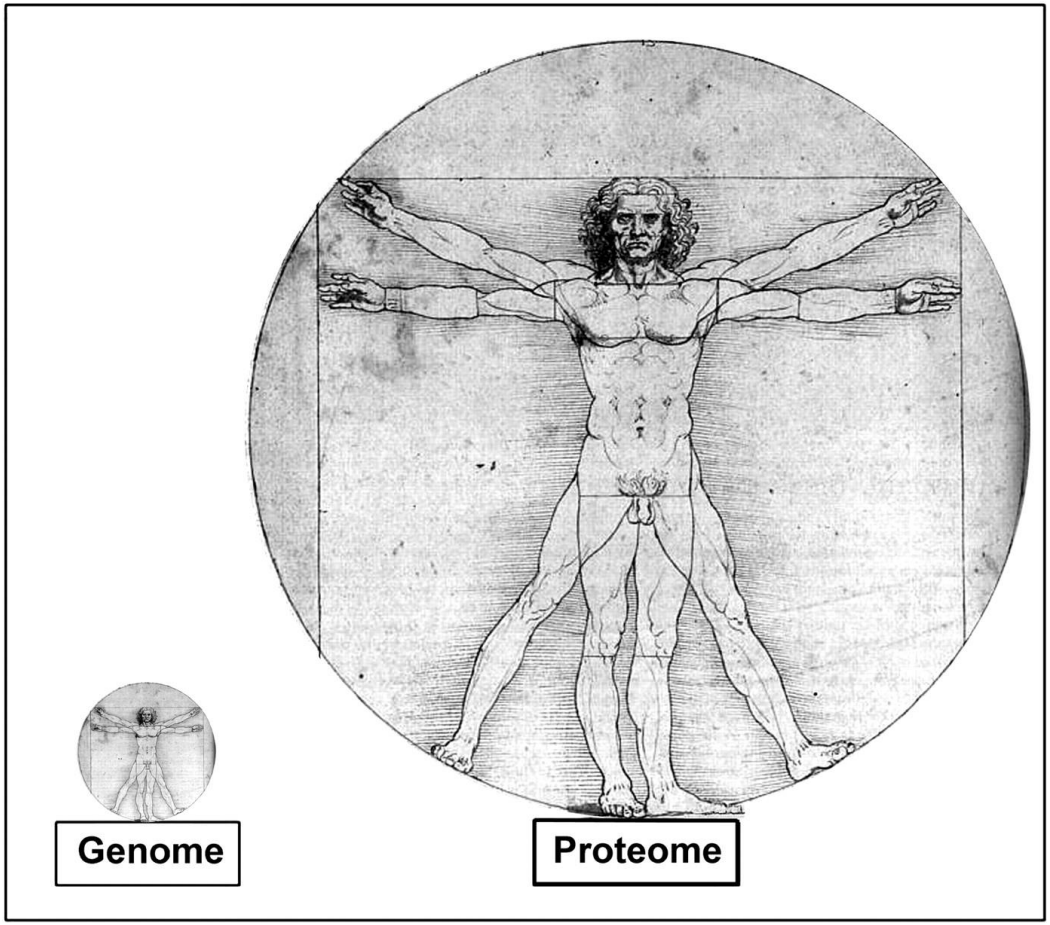
- The nature of proteins – with their twenty elementary components, the complex chemical changes they subdue, together with their replication inability – made them much more difficult to be analyzed

- Proteins are the agents that, within the cell, "do what it is needed to do"
- One of the major findings, of the new post–genomic era, is that the old paradigm according to which a gene codes for a single protein is no longer valid

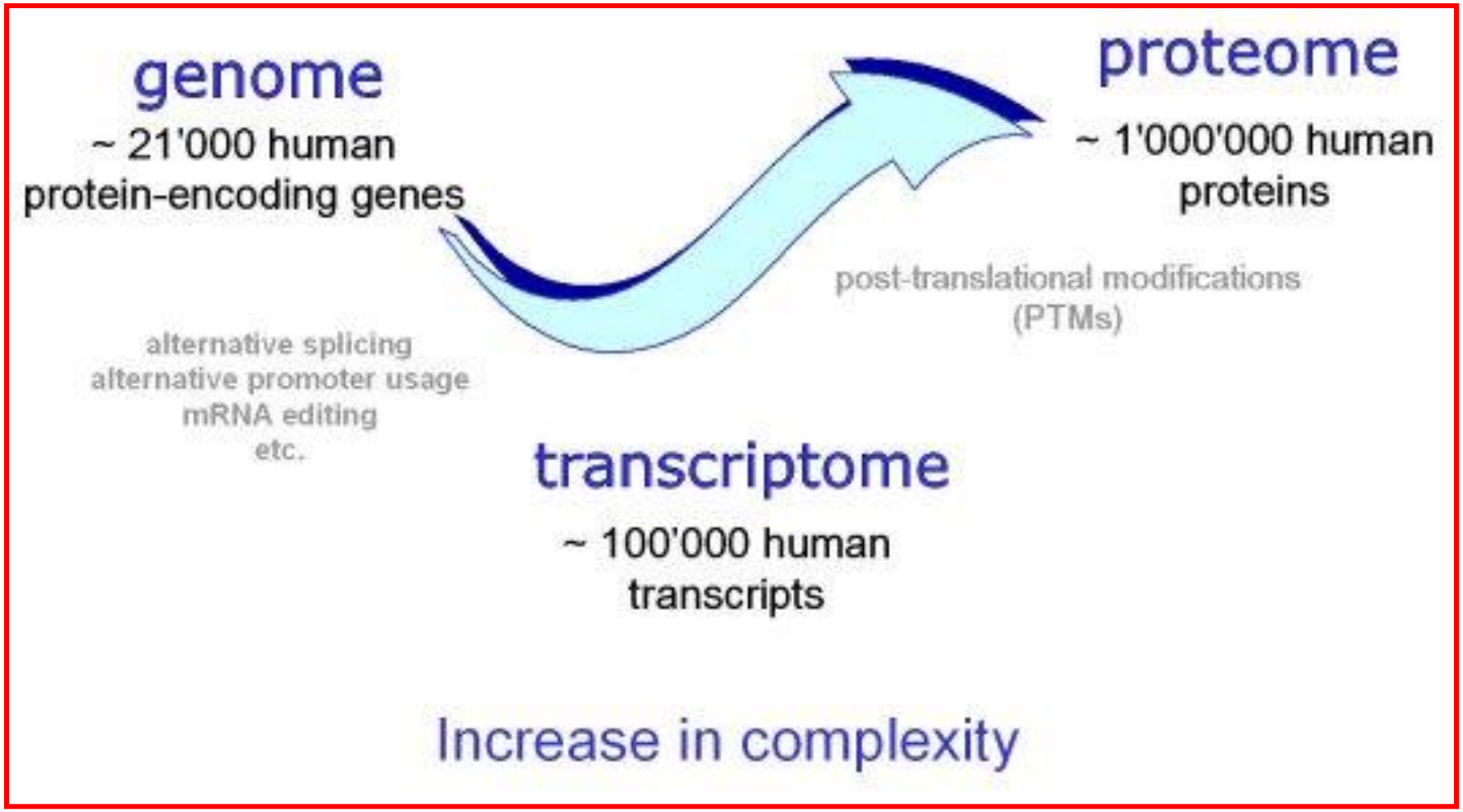
- **Due to alternative splicing and post– translational modifications (proteolytic cleavage, glycosylation, phosphorylation) of proteins, more than one proteome may correspond to a single genome**

- The genome of a living being, even when completely sequenced, doesn't allow to fully understand all the biological functions that characterize an organism, which depend on multiple factors, including regulatory and metabolic pathways of proteins

- Genome ~ 26,000-31,000 protein encoding genes
- Human proteins ≥ 1 million



Zimmermann J and Brown LR. (2001)



The Human Proteome Initiative. (2007) Retrieved March 24, 2009. http://world-2dpage.expasy.org/swiss-2dpage/

- Therefore, **proteomics** appears complementary to genomics, and essential for the understanding of biological mechanisms

- Proteomics allows the study of proteins, both in the forms just translated from genes and in **isoforms** (due to alternative splicing), or after any post–translational change, which may occur in the cell after translation

- The study of isoforms and of post–translational modifications allows to understand interaction mechanisms between proteins: these mechanisms will affect their activity and function

Proteomics

- The proteome is larger than the genome due to alternative splicing and protein modification.

We need to know

- All protein-protein interactions
- Function
  - One protein or peptide may have multiple functions depending on context.
- Regulation of protein function
- Modification
- Location
  - Location will help us to understand the proteins role in the cell, what its function is, and what controls its function.
- Detection and quantitation
  - The concentration of all proteins changes by 10 orders of magnitude within the cell. Currently there are no easy methods for determining the concentrations

Experimental techniques

- from the perspective of proteomics, the very nature of proteins makes the laboratory analyses particularly difficult and much less precise than those available for the genome

  - Two–dimensional electrophoresis
  - Mass spectrometry
  - Protein microarray

Two–dimensional electrophoresis

- The two–dimensional electrophoresis is a technique that allows the separation of proteins according to their molecular weight and charge

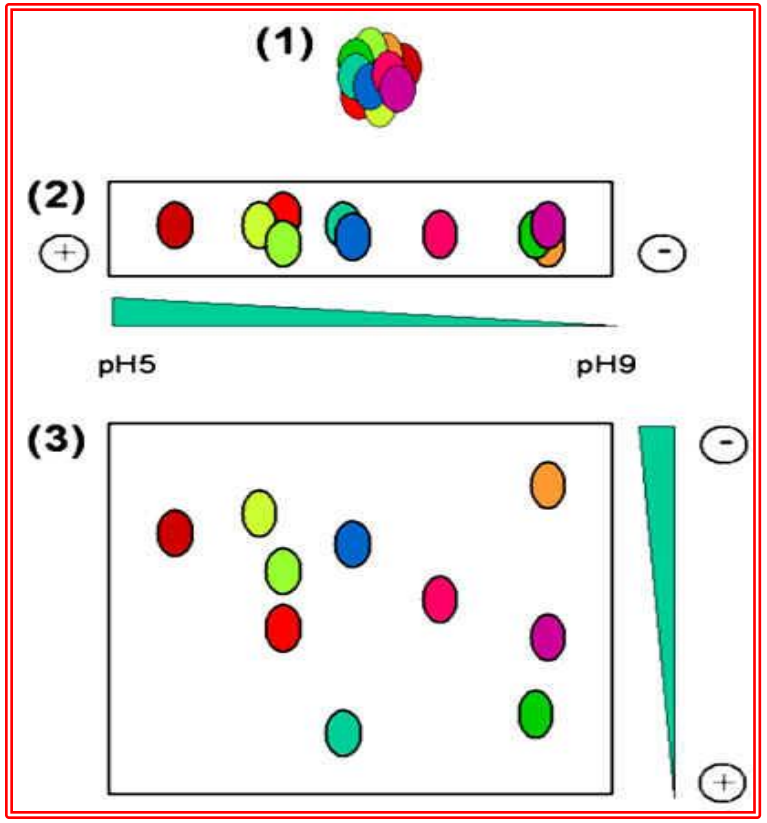
- This process starts from the extraction of proteins from a tissue

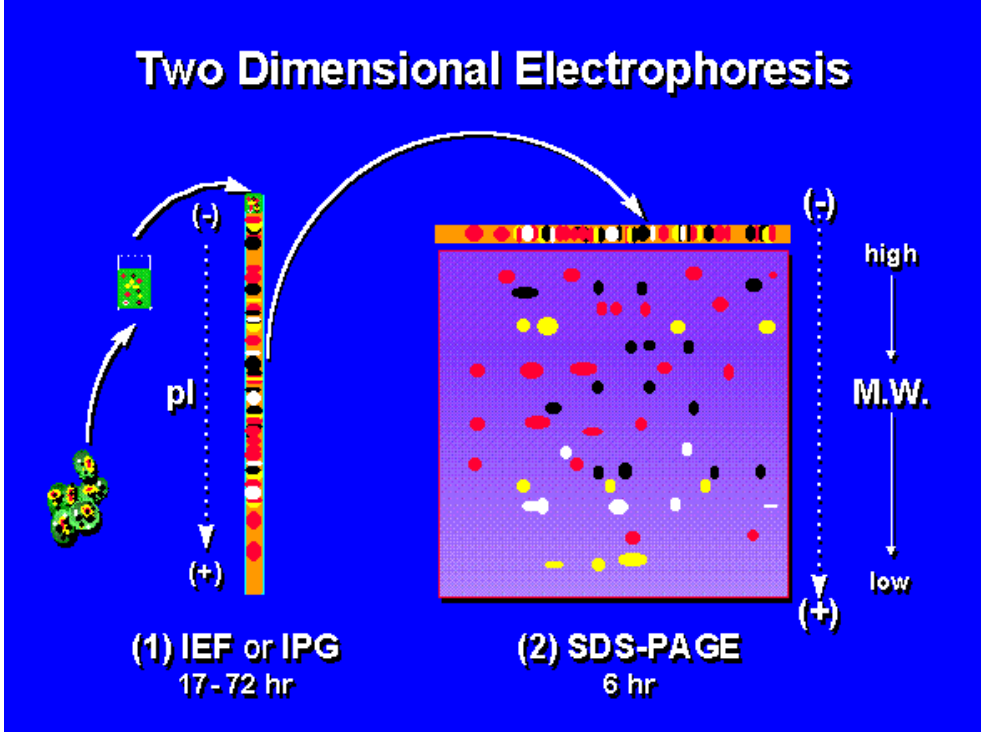
- Proteins, placed on a polymeric support strip, to which an electric current is applied, and in the presence of a gradient of acidity, migrate in a different manner according to their intrinsic electrical charge, reaching their isoelectric point and forming some "bands"

- This "first dimension" is isoelectric focusing

Two–dimensional electrophoresis

- At this point, the support is placed on the margin of an electrophoresis gel that allows the separation of proteins according to their molecular weight, based on the application of an electric field (SDS-PAGE)
- The final result is a gel in which each protein virtually occupies a point in the two– dimensional space; therefore, it is easily detectable by appropriate colors





TWO DIMENSIONAL ELECTROPHORESIS (2DE) is one of the best experimental tools for the reliable separation of thousands of proteins in a single gel. 2DE consists of a tandem pair of electrophoretic separations:

In the first dimension, proteins are resolved in according to their isoelectric points (pIs), isoelectric focusing (IEF). Under standard conditions of temperature and urea concentration, the observed focusing points of the great majority of proteins using IEF approximate the predicted pI calculated from the proteins' amino acid compositions.

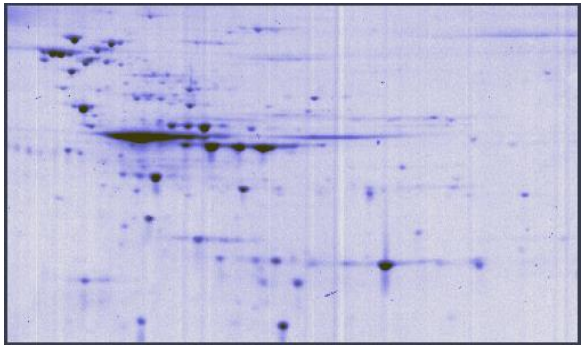
In the second dimension, proteins are separated according to their approximate molecular weight using SDS-PAGE. This technique can provide molecular weight approximations (+/- 10%) for most proteins, with some dramatic exceptions.

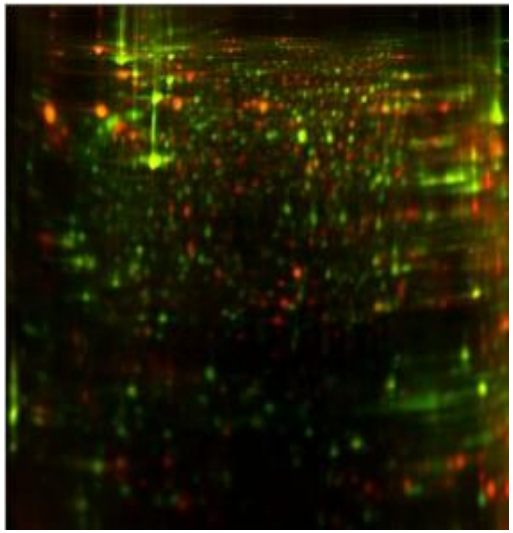
Preparazione del campione e separazione delle proteine

- può consistere solo di pochi passaggi (deve essere quantitativa e veloce)
- per evitare problemi nella focalizzazione isoelettrica, si devono usare solo detergenti non ionici o zwitterionici
- in pratica molte cellule possono essere caricate direttamente - per campioni difficili bisogna elaborare protocolli speciali
- in pratica l'elettroforesi 2D è l' unica tecnica in grado potenzialmente di separare fino a 10000 componenti
- si può giocare molto variando i gradienti di pH o pre-frazionando il campione

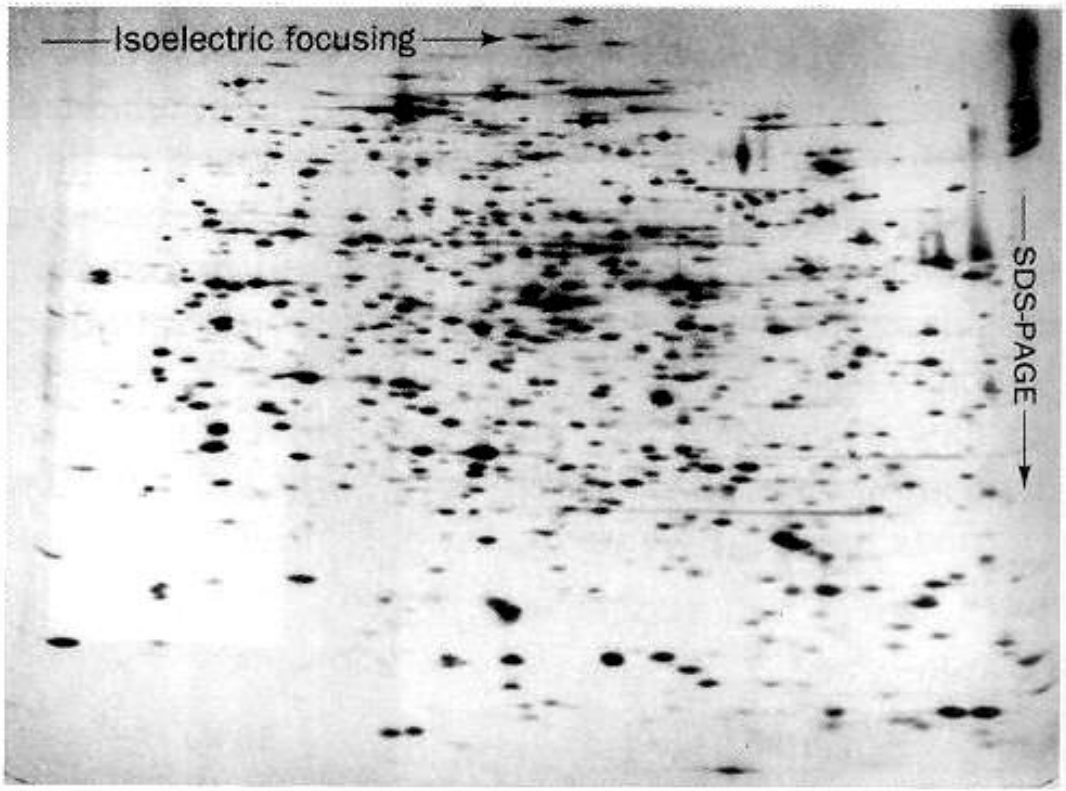
Staining Technology

- **Staining**
  - -Silver
  - -Coomassie blue
  - -Fluorescent dyes
  - -Radioisotopic labeling



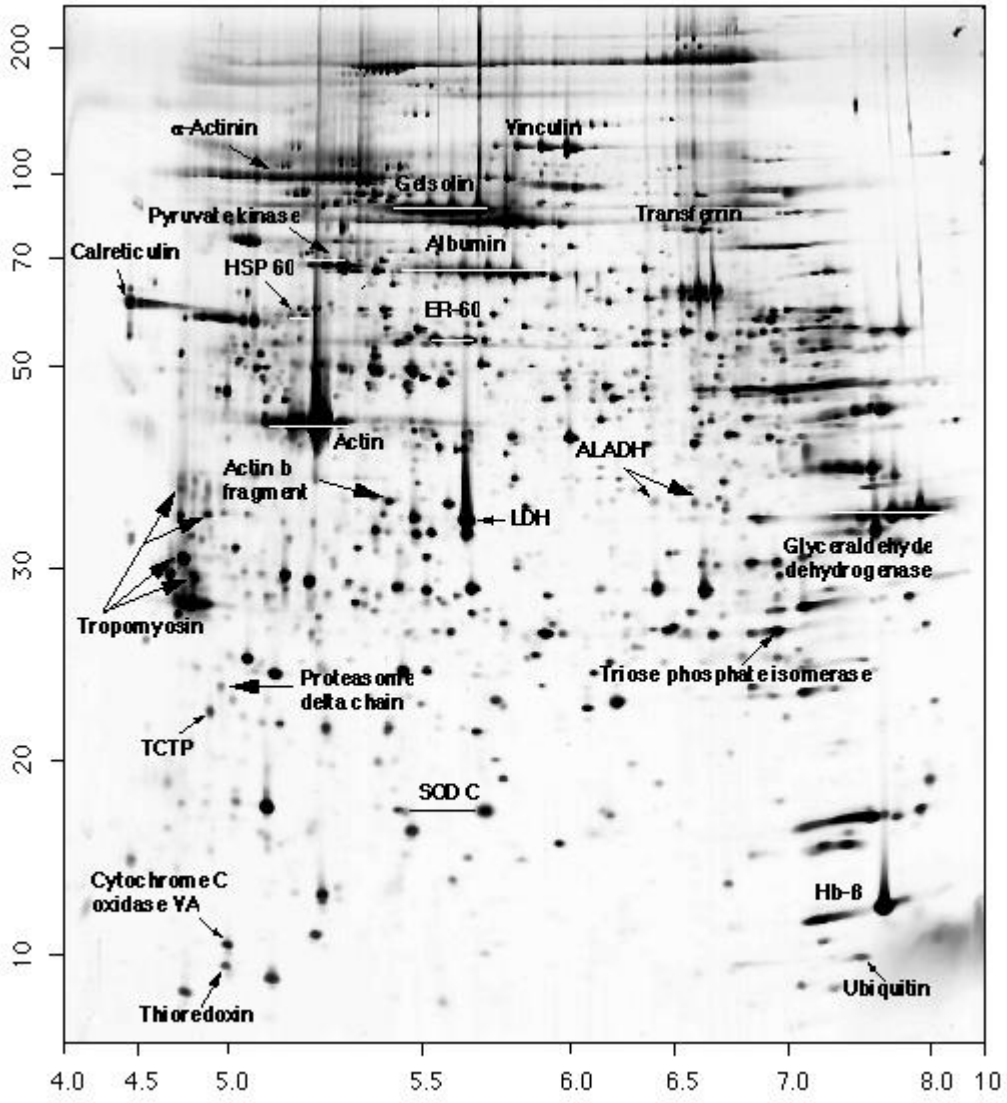


ELETTROFORESI 2D



An autoradiogram showing the separation of *E. coli* proteins using isoelectric focusing in one dimension and SDS–PAGE in the second dimension. A 10-μg sample of proteins from *E. coli* that had been labeled with [^{14}C]amino acids were subjected to isoelectric focusing in a 2.5 × 130-mm tube of urea containing polyacrylamide gel. The gel was then extruded from its tube, placed at one edge of an SDS–polyacrylamide slab gel, and subjected to electrophoresis. Over 1000 spots were counted on the original autoradiogram, which resulted from an 825-h exposure.

Elettroforesi bidimensionale



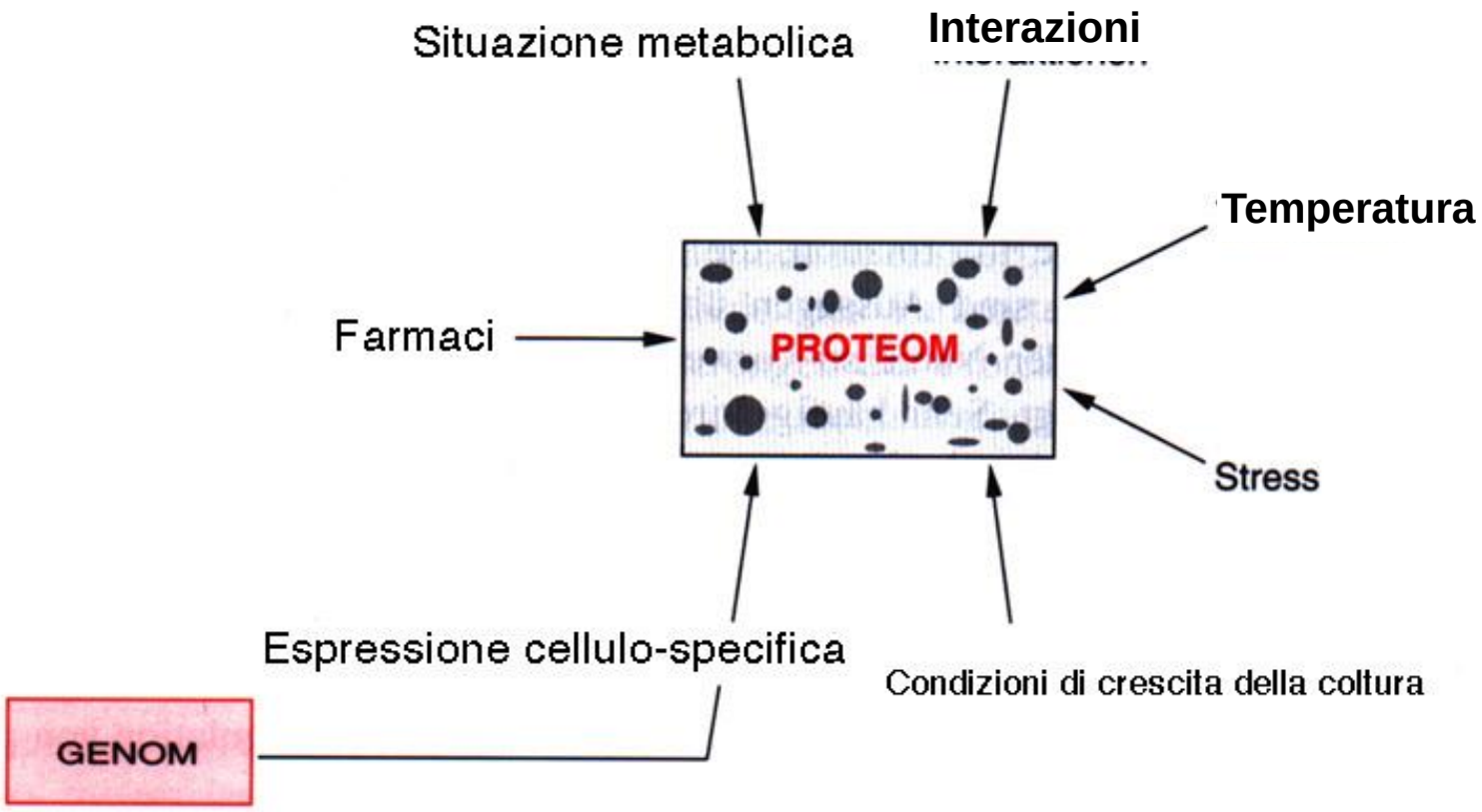
Proteine cellulari (piastrine umane) in una prima dimensione separate per isofocalizzazione (pH sulle ascisse) e successivamente per elettroforesi in condizioni denaturanti.

Two–dimensional electrophoresis

- The last step consists in isolating each protein from the gel, to carry out the analysis that allows its identification
- Such analysis can be achieved either manually, by cutting a small piece of gel containing a single protein, and then proceeding with mass spectrometry, or through automatic techniques (more or less advanced) able to "read" directly from the gel

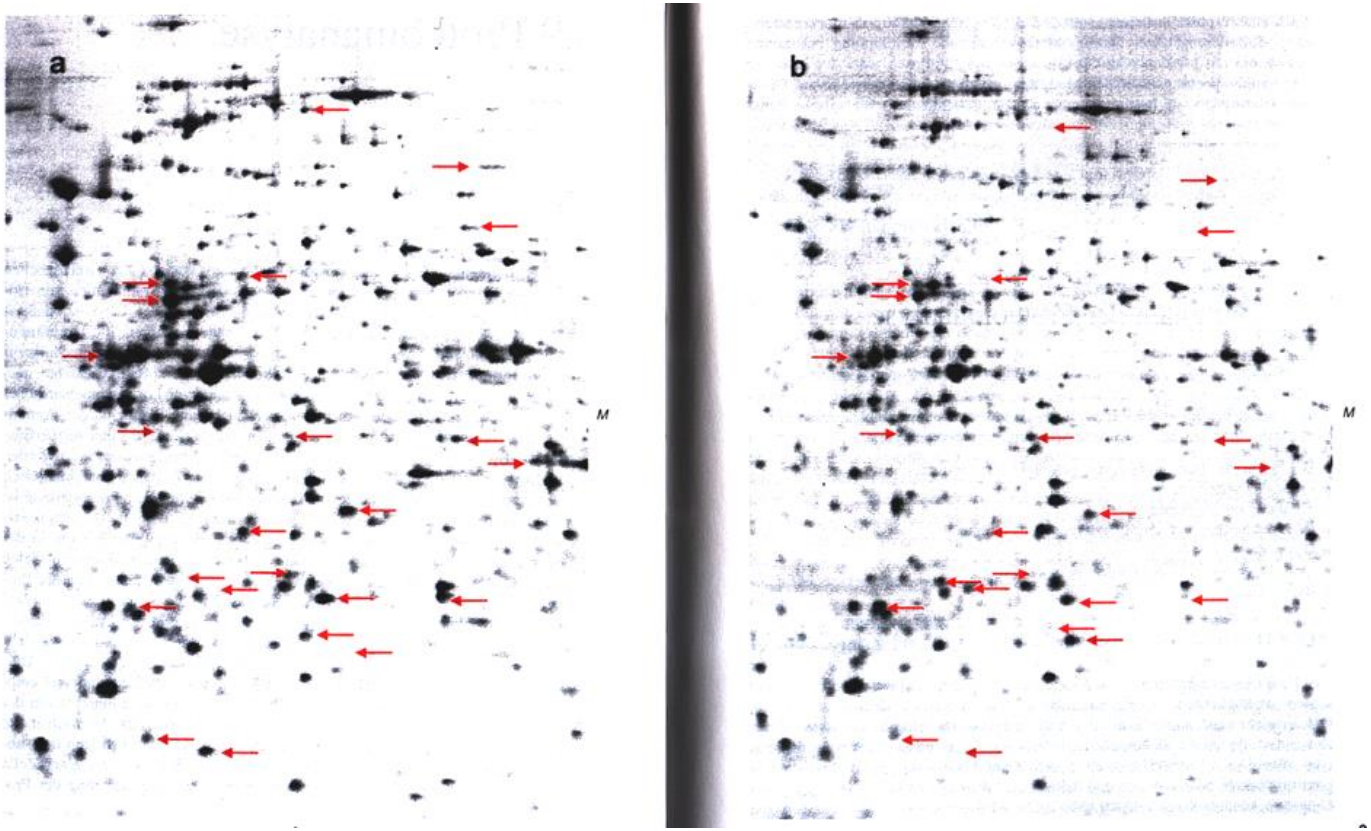
Influenza di vari parametri sull' espressione cellulare

I livelli effettivi di proteina in una cellula sono determinati dai parametri più svariati e sono estremamente sensibili ai cambiamenti di questi parametri.

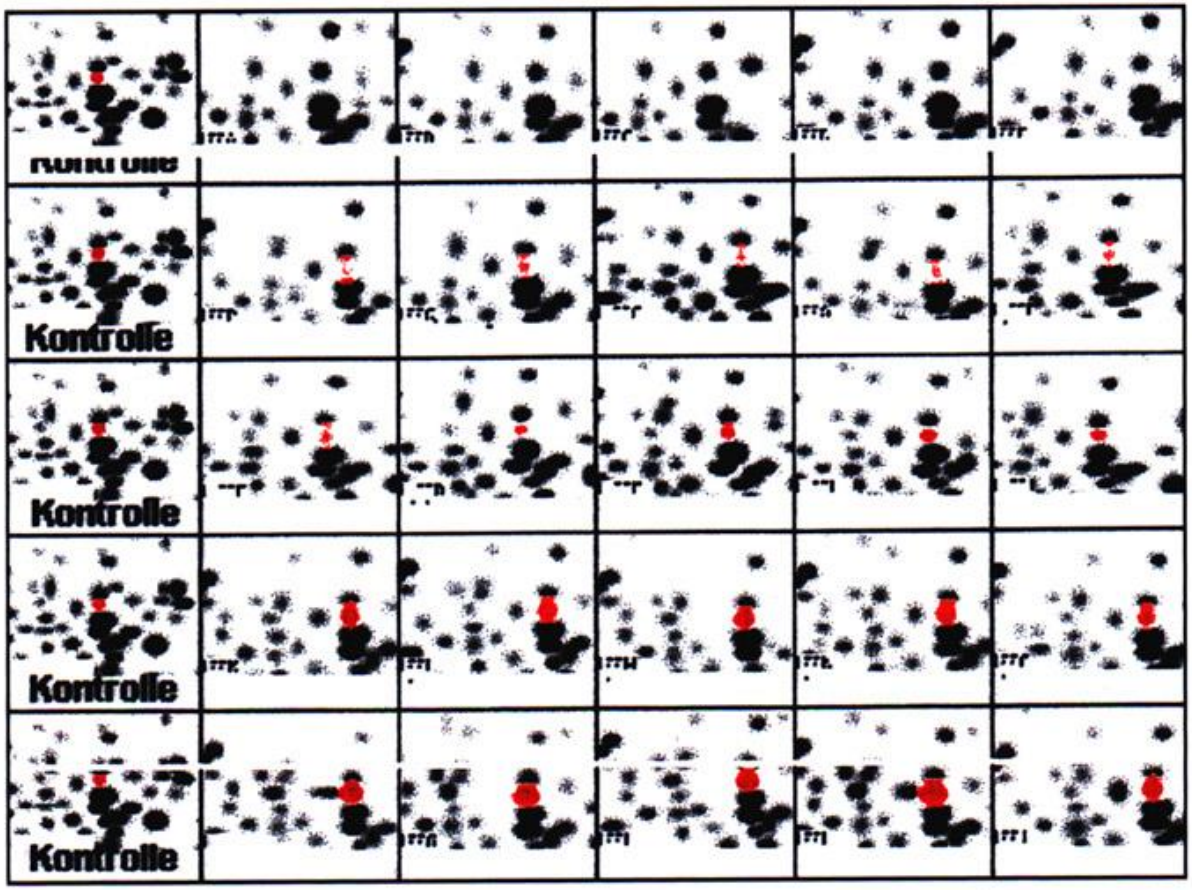


Analisi del proteoma per sottrazione

Una coltura cellulare (*E. coli)* viene portata da uno stato iniziale (a) ad un secondo stato (b) da un cambiamento delle condizioni di crescita.



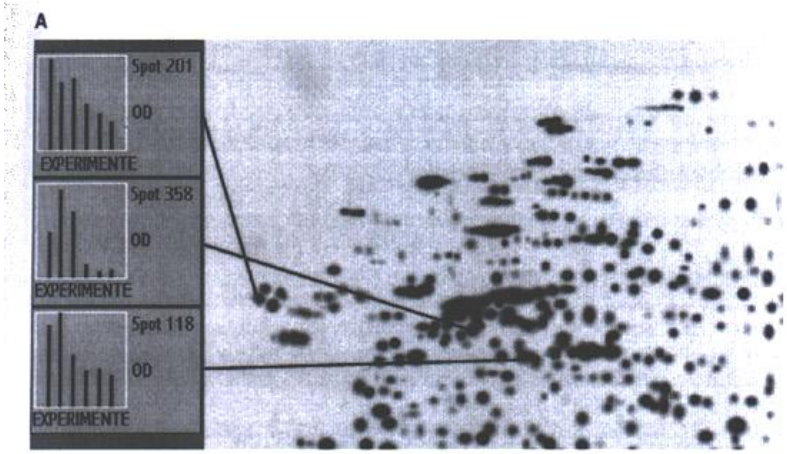
Elaborazione delle immagini e quantificazione delle proteine



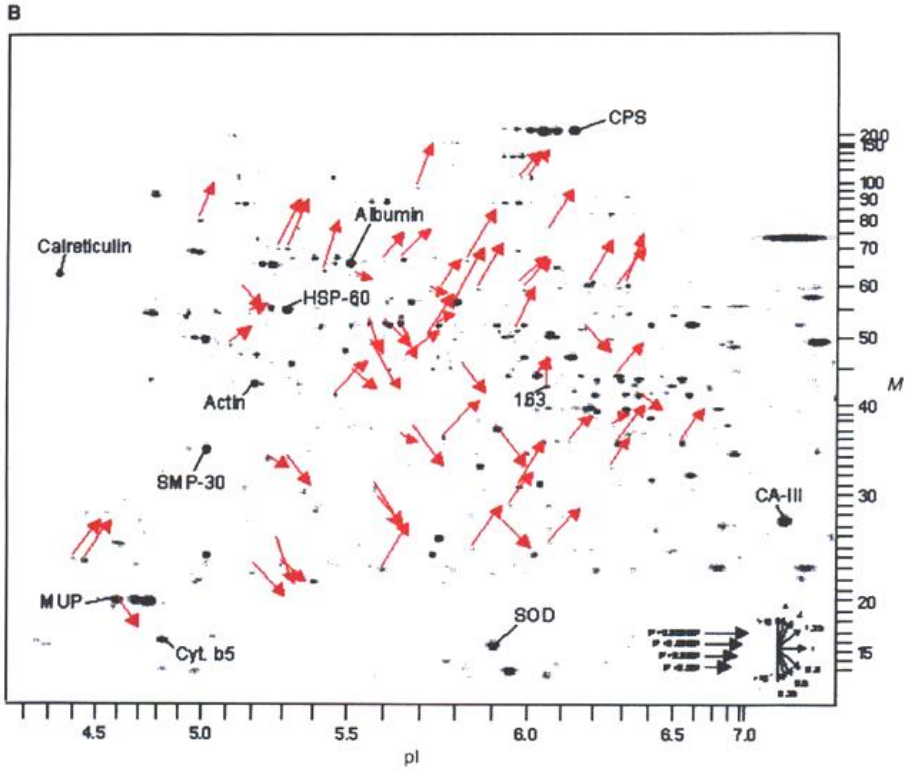
Sezioni da 25 diversi gel confrontati con il controllo con procedura computerizzata.

Si nota la riproducibilità del pattern delle proteine e le significative differenze nell' espressione della proteina contrassegnata.

Rappresentazione dei risultati di una analisi proteomica

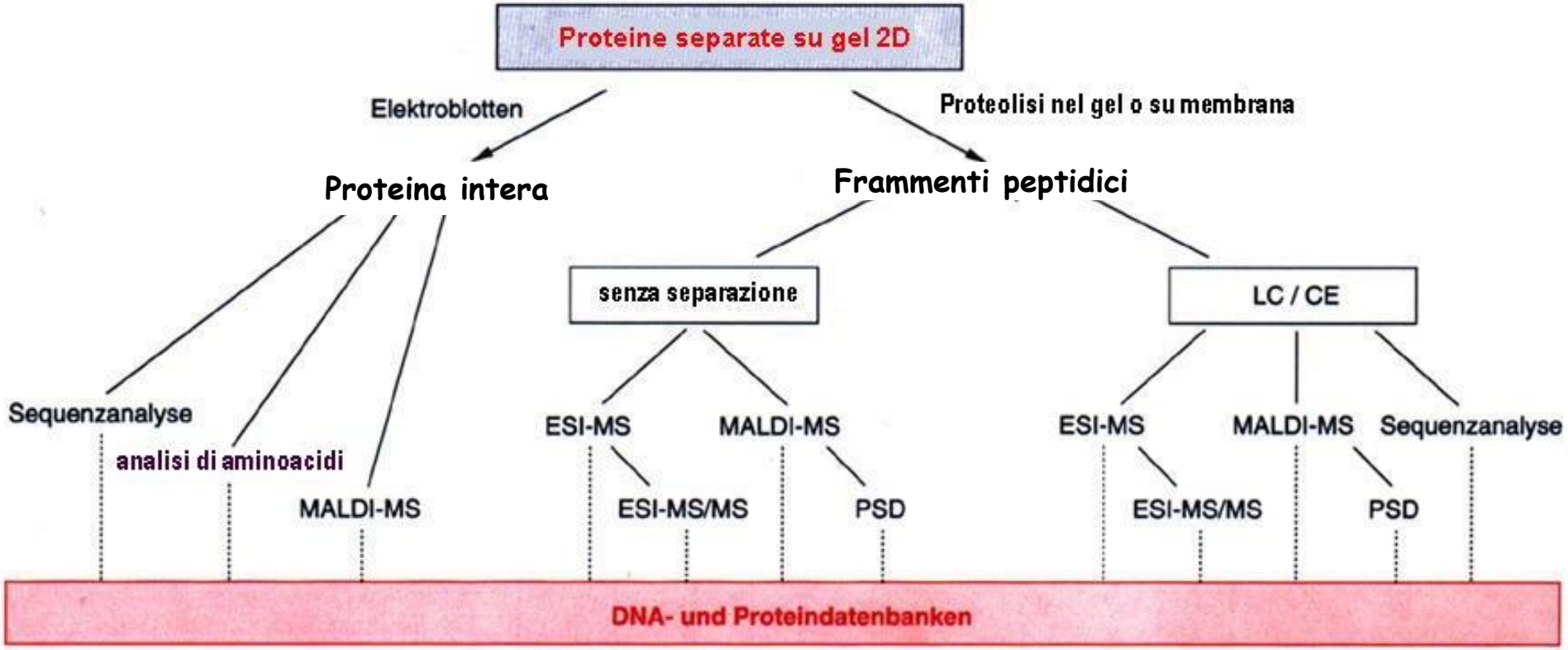


E' mostrata la quantità di singole proteine in funzione dell' età della cellula



Le frecce mostrano i cambiamenti nelle proteine di topo sotto l'influsso del farmaco Nafenopina

Analisi di proteine separate su gel 2D



MS= mass spectrometry; ESI= ElectroSpray Ionization; MALDI= Matrix-Assisted Laser Desorption Ionization;

From genome to proteome

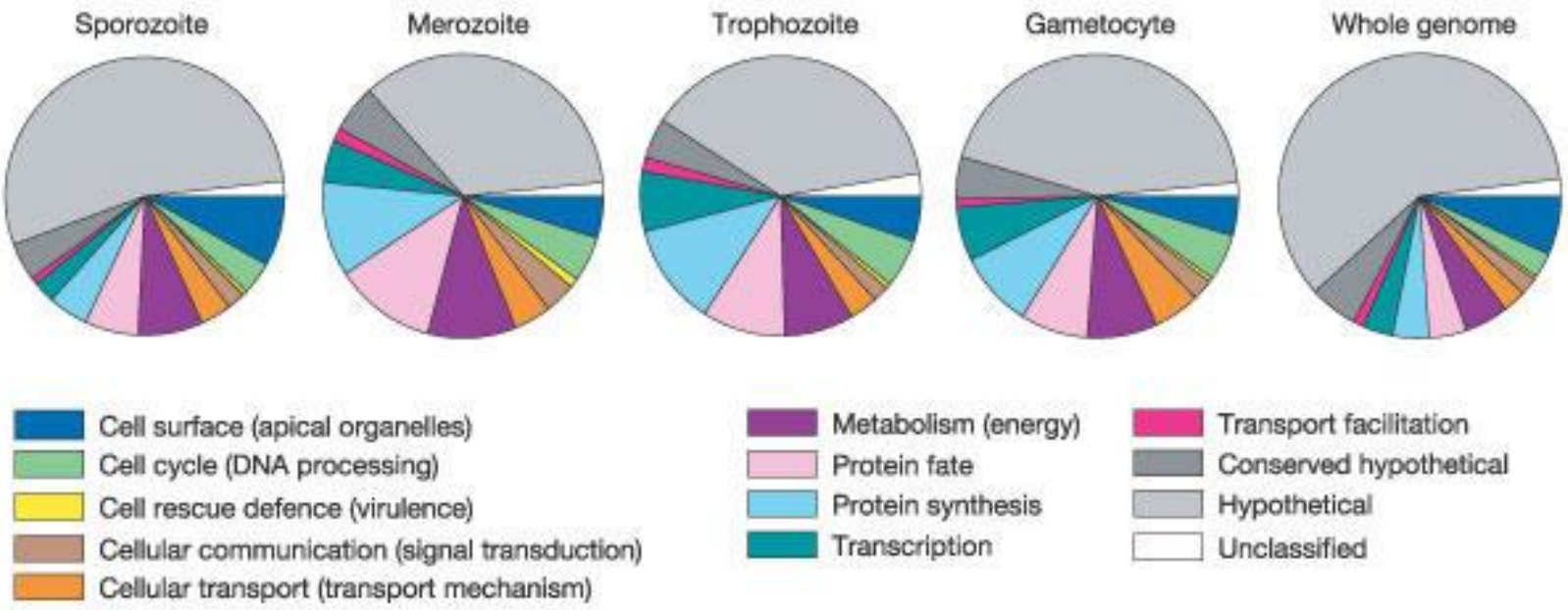
- Proteins require much more accurate manipulations compared to DNA, because their tertiary structure can easily be altered when they come into contact with an inappropriate surface or environment
- Moreover:
  - The ability of nucleic acids to specifically hybridize with other nucleotide sequences make the DNA identification a relatively simple task
  - The identification of proteins is much more difficult and requires complicated analysis of mass spectrometry and advanced software tools, or the generation of specific antibodies

- The current proteomic studies are mainly focused on two principal areas:
  - Functional proteomics
  - Expression proteomics
- **Functional proteomics** aims at defining the biological function of proteins, the role of which is still unknown, and to identify *in vivo* protein–protein interactions, in order to describe cellular mechanisms at the molecular level
- **Expression proteomics** is focused on the qualitative and quantitative study of the expression profiles of different proteins; the expression of proteins can in fact be altered by changes in cellular conditions (different growth conditions, stress, in presence of cellular diseases, etc.)
  - The different protein profiles in a tissue, the absence, the presence or some different quantity levels, are potential biomarkers of a physiological and/or a pathological situation

From genome to proteome

- Genome vs Proteome
  - The caterpillar and the butterfly are genetically identical, but possess very different proteome and phenotype, as well as the tadpole and the frog!

Analysis of *Plasmodium falciparum* (malaria parasite) proteome



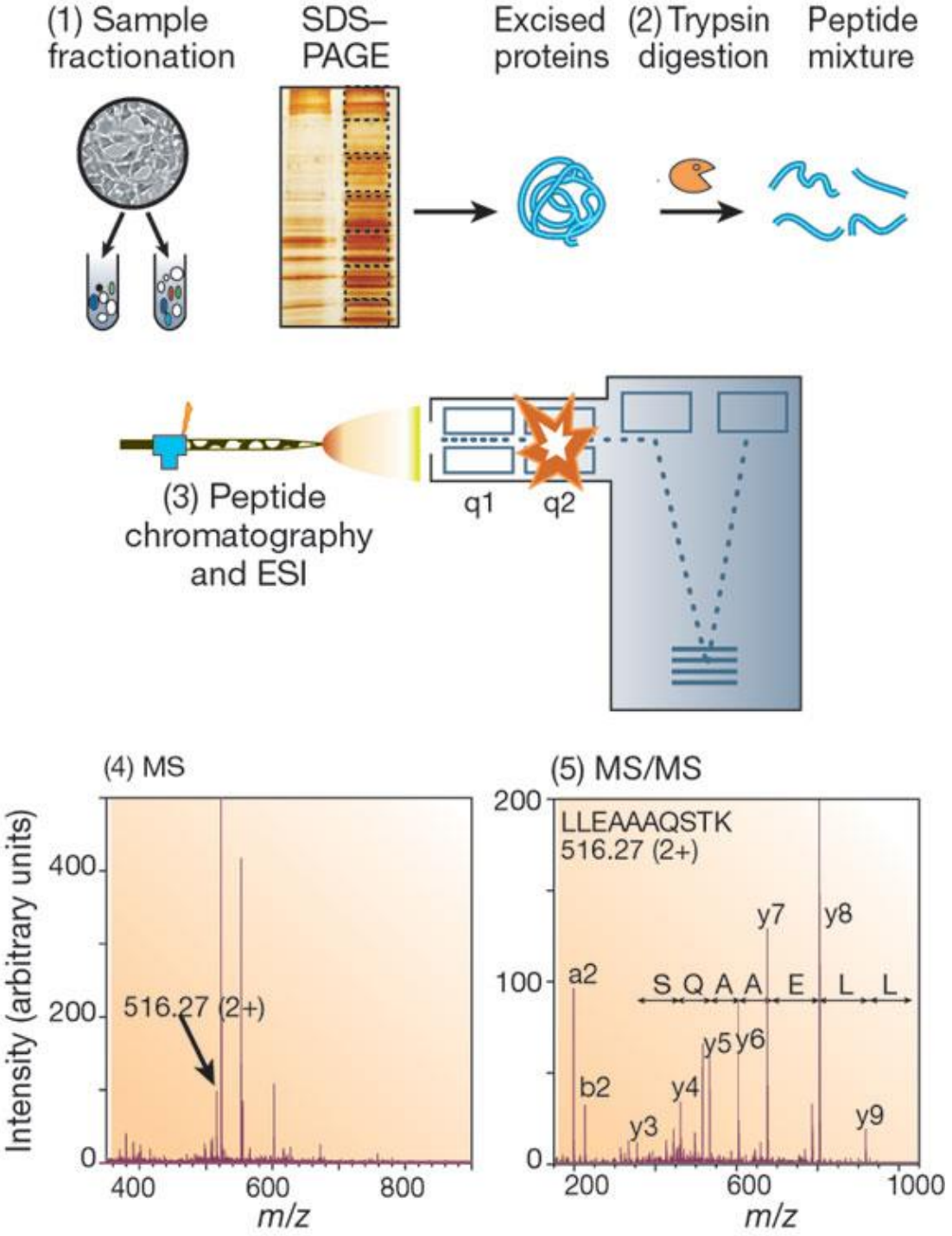
Proteins identified in each stage are plotted as a function of their broad functional classification. To avoid redundancy, only one class was assigned per protein.
Functional profiling of over 2,400 proteins agreed with the physiology of each stage.

Florens, L. et al. (2002) A proteomic view of the *Plasmodium falciparum* life cycle. Nature. 419, 520-526.

Alternative Separation Methods

- Whole proteome is analyzed at once.
- Proteome is digested with protease (trypsin)
- Digested proteome is injected to HPLC with 2 columns in series (mixed bed ion exchange and reverse phase)
- Peptides are eluted from ion exchange onto reverse phase and then separated on reverse phase column.
- Peptides then enter ESI-MS-MS

-Quadrapole MS
- Collider
- TOF MS



Aebersold, R & Mann, M. (2003, March). Mass spectrometry based proteomics. Nature. 422, 198-207

Protein microarray

- Protein microarrays are widely used because of their ability to perform protein analysis on a large scale, in the same way in which genetic chips have revolutionized the transcriptome analysis
- The basic concept of the protein chips is very similar to that of the gene chips: small amounts of individual probes are covalently linked to the surface of the silicon chip in a high–density array
- Proteins extracted from the cells are labeled with fluorophores and flushed on the chip
  - Just as with gene chips, the amount of material (in this case, protein) bound to the probes is determined by the excitation of the fluorophore

Protein microarray

- Apart from microarrays able to detect protein–protein interactions and protein–compound interactions, also arrays of capture probes (for example, antibodies) can be used – that bind to proteins of a sample so as to evaluate the relative expression levels
- Protein microarrays do not have the same impact of gene chips
  - Unlike DNA sequences, with their unique bonds dictated by base coupling, it is reasonable to expect that a single protein can interact with multiple different probes
  - The binding kinetics of each probe may vary, and differences in the intensity of the signal could be due to differences in the intensity of the binding
  - Proteins are known to be sensitive to the chemistry of their environment and to the surface they encounter, and both target proteins and probes may result in an unexpected behavior when subjected to control procedures

Different types of protein microarrays

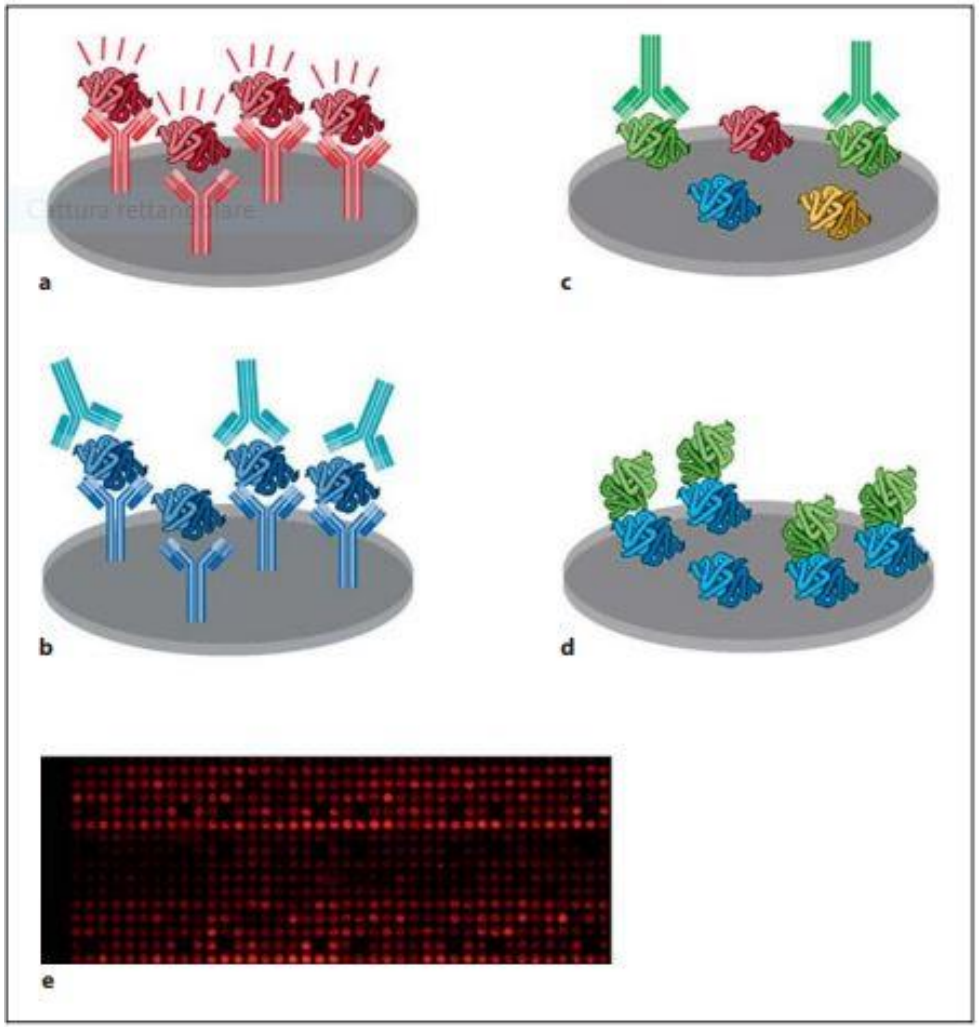


Fig. 1. Protein microarray types. **a** Label-based antibody array – where the analyte is pretreated to allow detection. **b** Sandwich antibody array – where 2 antibodies are used to detect the analyte. **c** Reverse-phase array – where the analyte is immobilized on the surface. **d** Single-protein array – where only 1 protein of interest is used and immobilized on the surface.

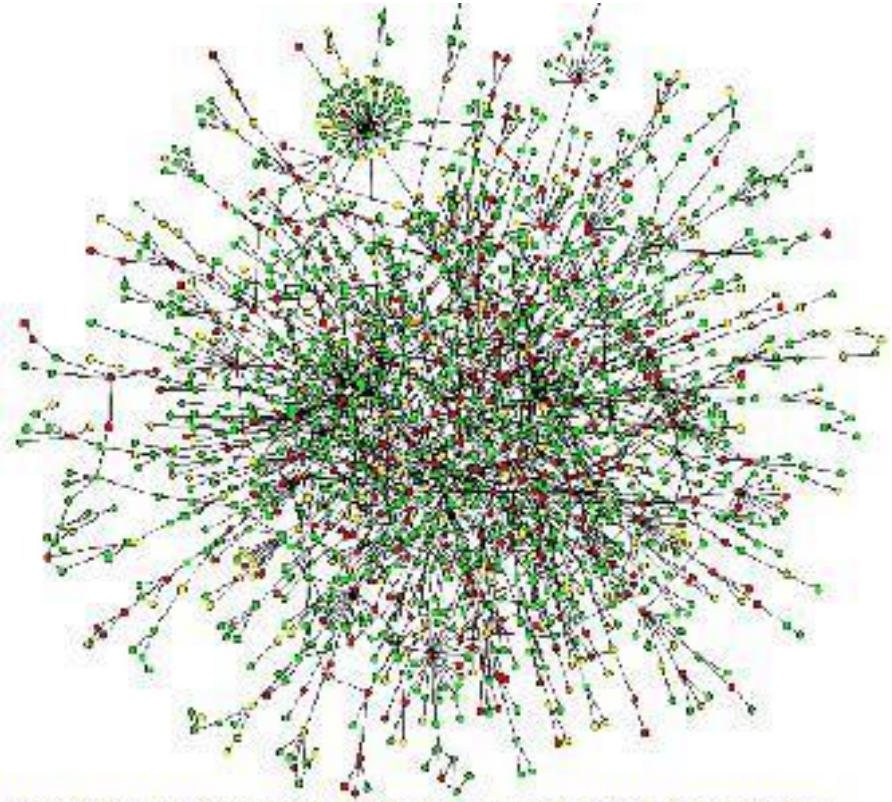
JC–H. Lin, "Protein microarrays for cancer diagnostics and therapy", Medical Principles and Practice, 19(4):247–254 (2010)

Protein Interactions

- When analyzing a new protein, first question to ask is – to what proteins does it bind?
- Method: Use new protein as an affinity agent to isolate its binding partners
  - Will not detect low affinity, transient, or cellular environment specific interactions

Protein Interaction Experiments

- Steps
  1. Bait presentation using endogenous proteins
  2. Affinity purification of complex
  3. Analysis of bound proteins



Model of 1, 58 yeast proteins & their 1,948 interactions.
Work by Hawoong Jeong & colleagues at U. Notre Dame

Proteomics is extremely valuable for understanding biological processes and advancing the field of systems biology.

"The ultimate goal of systems biology is the integration of data from these observations into models that might, eventually, represent and simulate the physiology of the cell."