Lecture 6

How are pure enzymes analyzed?

Enzyme analysis

-**Test for activity**

Unit (U) : µmol/min

Specific activity : units/mg. Number of enzyme units per ml divided by the concentration of protein in mg/ml

- **ELISA** (Enzyme-Linked-Immuno-Sorbent Assay) - utilises suitable antibody

- **Gel electrophoresis** on acrylamide – estimate the molecular weight of the target enzyme

- **Determination of protein concentration**

- Assay by colourimeter - based on the formation of coloured complexes

- Enzyme structural analysis

Protein Assays

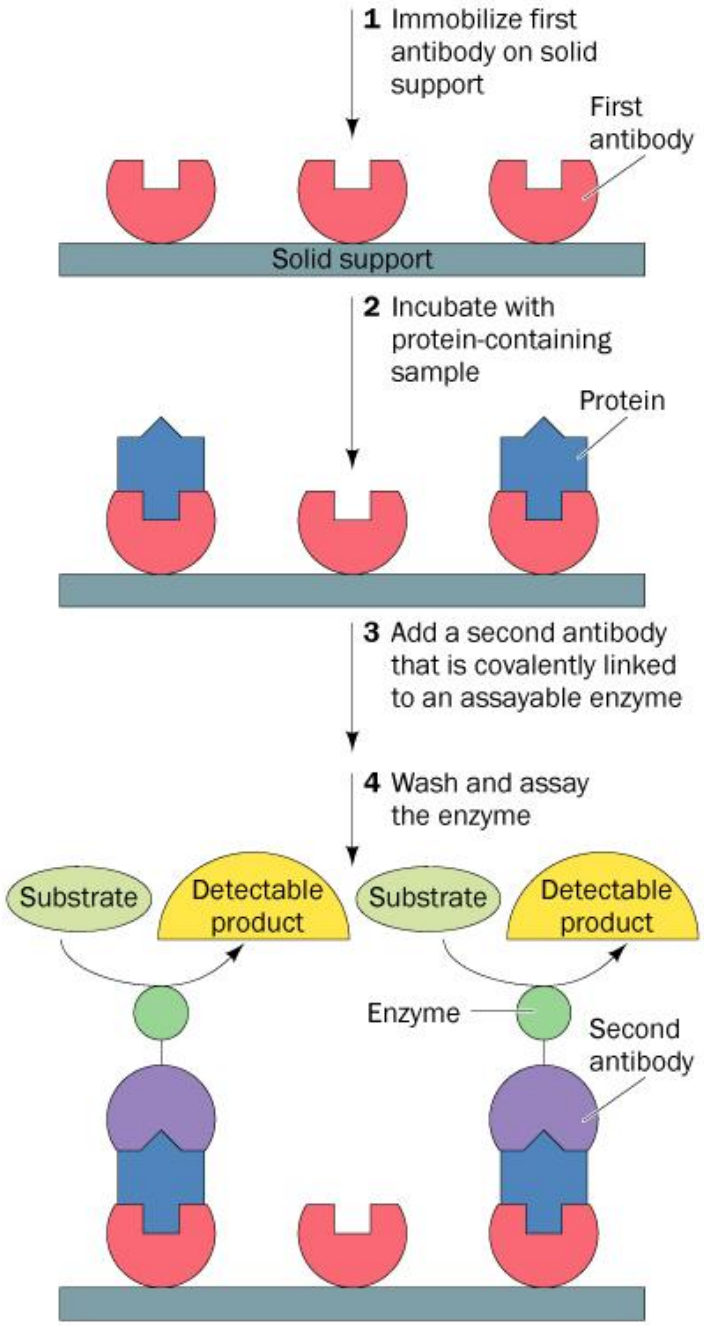
- An assay is a method of detection
- It has to be:
  - **Specific**
  - **Sensitive**
  - **Convenient to use**

ELISA (Enzyme-Linked-Immuno-Sorbent Assay)

- Term Was Coined By Engvall and Pearlmann in 1971

- Different Types
  i. Sandwich
  ii. Indirect
  iii. Competitive

- Similar To RIA, Except No Radiolabel
- Can Be Used To Detect Both Antibody and Antigen
- Very Sensitive, **pg/mL**



Enzyme-linked Immunosorbent Assay

- Usable in a complex mixture
- High sensitivity

Sandwich ELISA protocol

1. Coat primary antibody onto microplate.

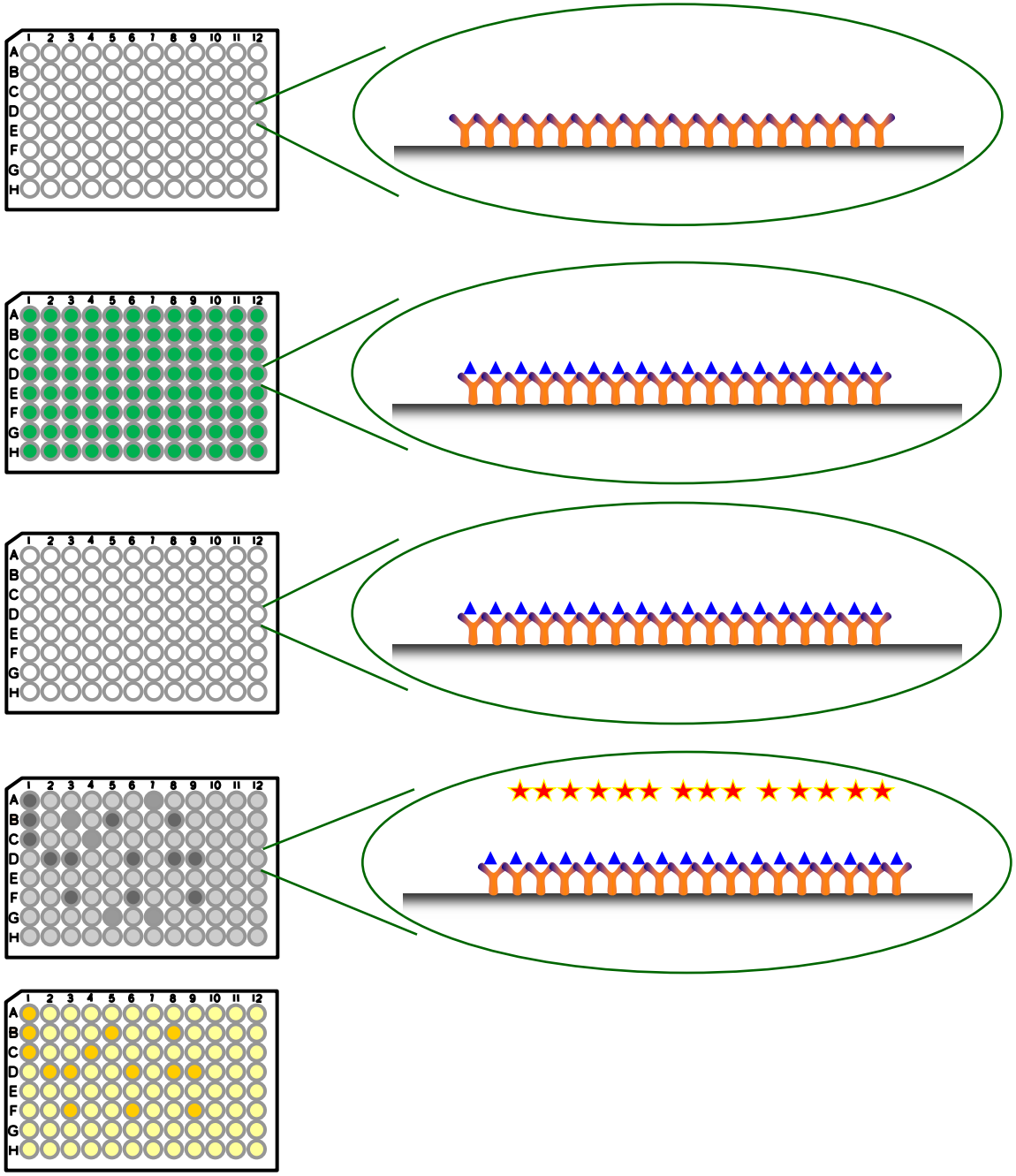
1a. Allow antibody adsorption and block unoccupied sites with neutral protein (BSA).

2. Add antigen sample to be detected into each well. Incubate 30 min at 37^0 C.

3. Add second primary antibody against antigen and HRP-conjugated secondary antibody (antibody mix) into each well. Incubate 30 min at 37^0 C.

4. Develop colorimetric reaction with appropriate substrate. Incubate 15 min at room temperature.

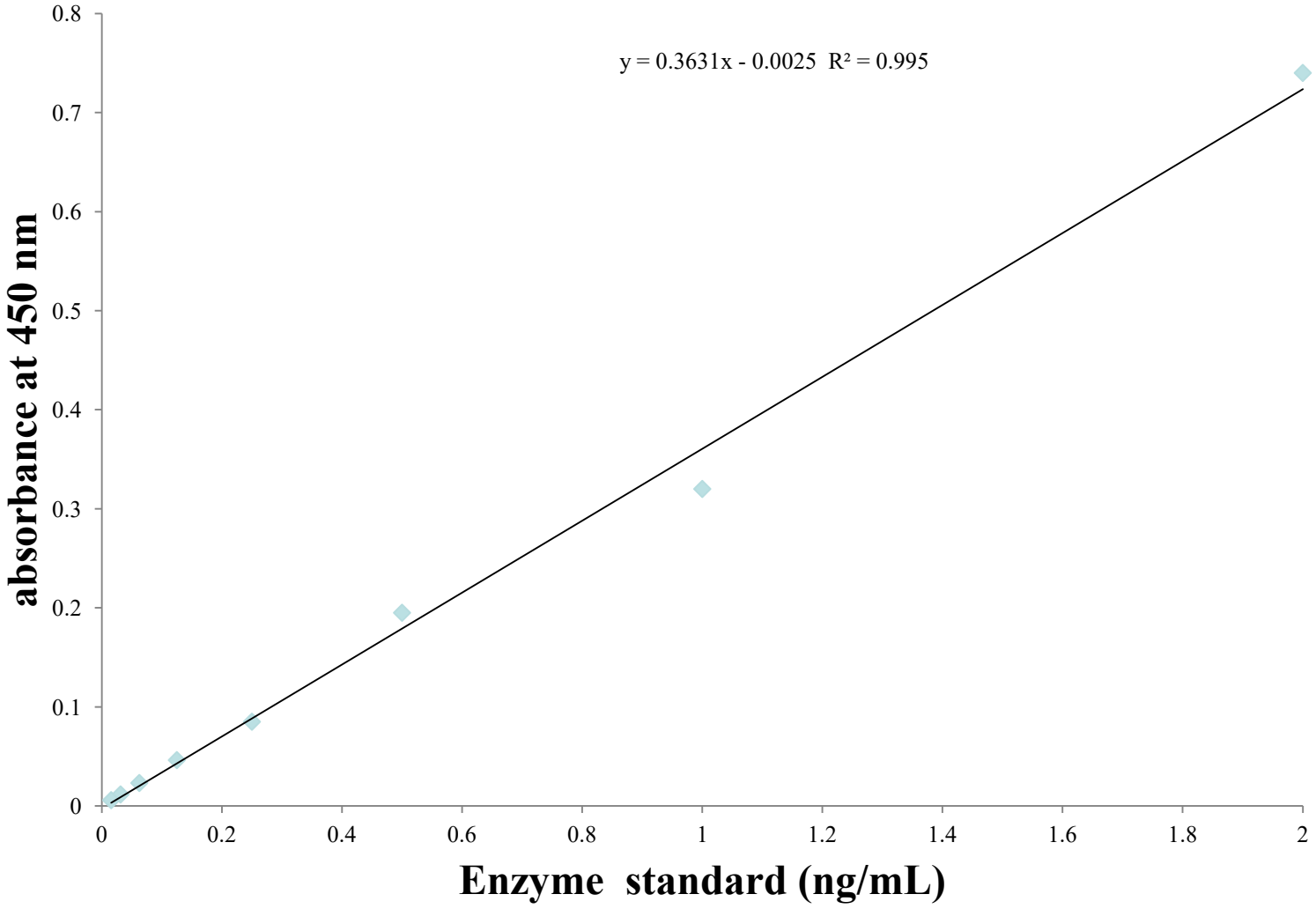
5. Stop reaction with 3M H_2SO_4. Read absorbance in ELISA spectrophotometer and quantitate relative antigen levels.



ELISA steps - Summary

- Add substrate and allow blue color to develop.
- Stop the enzymatic reaction with 3M H_2SO_4.
- Presence of enzyme (Protein of interest) will result in color change

ELISA data analysis



Electrophoresis

- Electrophoresis uses an electric current or field to 'push' analytes through a medium

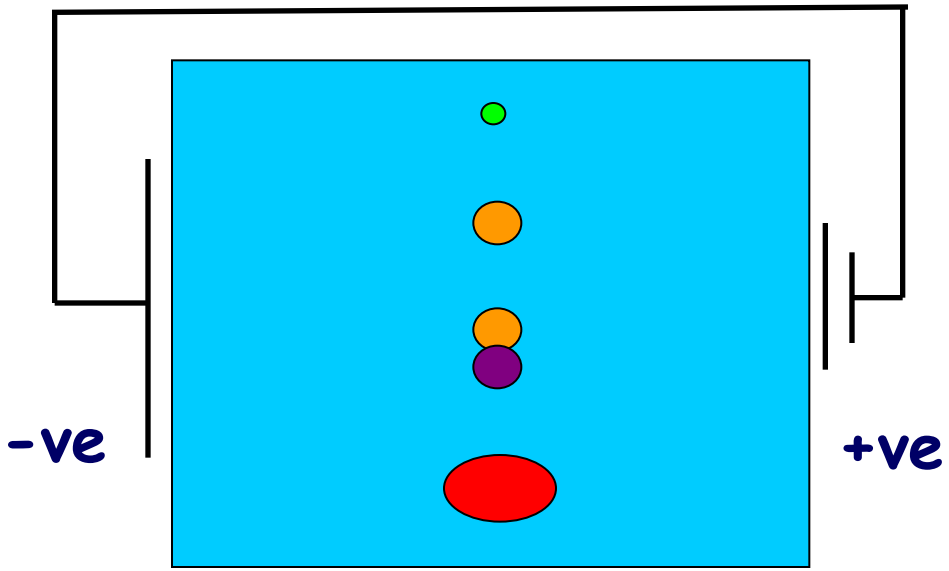
- In the simplest case, the electrophoretic mobility of an analyte μ_e is proportional to the electric field E

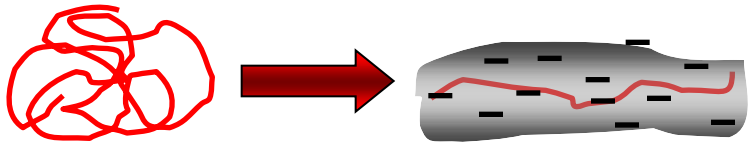
$$\mu_e = \frac{v}{E}$$

Protein Electrophoresis

Proteins even trickier than DNA/RNA:

- They are all effectively supercoiled (3° Structure)
- They can be either positively or negatively charged
- The number of charges depends on the amino acid sequence and is not proportional to size

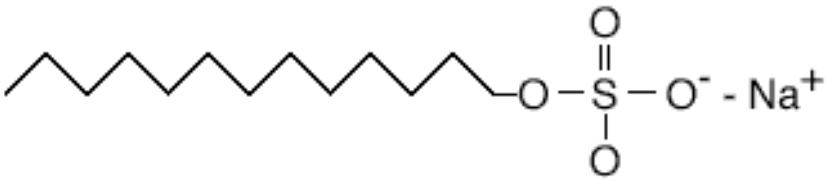




SDS-PAGE

(**S**odium **D**odecyl **S**ulfate-**P**oly**A**crylamide **G**el **E**lectrophoresis)

A variant of electrophoresis in which the buffers contain SDS, a detergent that binds to proteins



Sodium dodecyl sulfate, SDS

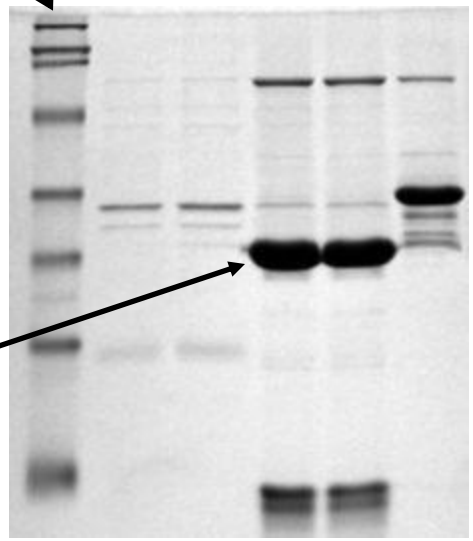
$CH_3(CH_2)_{10}CH_2$-SO_4^-, Na^+

Protein Electrophoresis

- **Proteins will bind an amount of SDS roughly proportional to their size (1.4g/g polypeptide)**

The 'Standard' ladder

The Over-Expressed protein of interest?

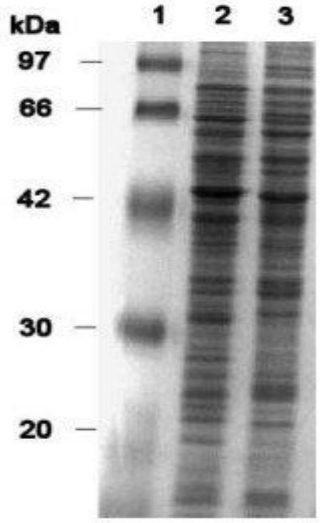


- The 'gel of choice' for SDS-page protein electrophoresis is polyacrylamide. Danger! Acrylamide monomers are potent neurotoxins☠
- Sometimes proteins are pre-boiled in a reducing agent to eliminate disiphide bonds, S-S.

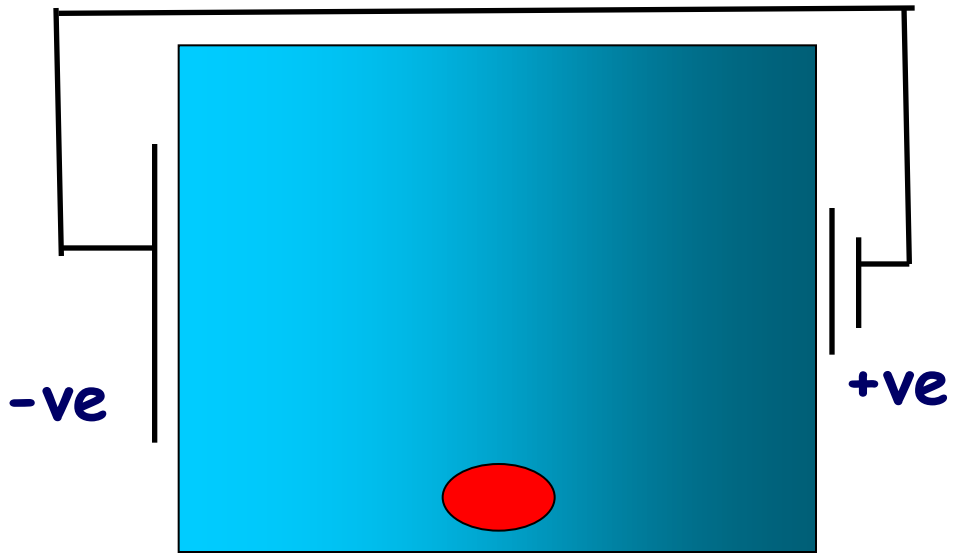
Proteins are visualized using a dye (coommassie blue) or other stain (silver)

2D Electrophoresis

- If you want to analyze the whole protein content of a cell, a 1D separation isn't enough

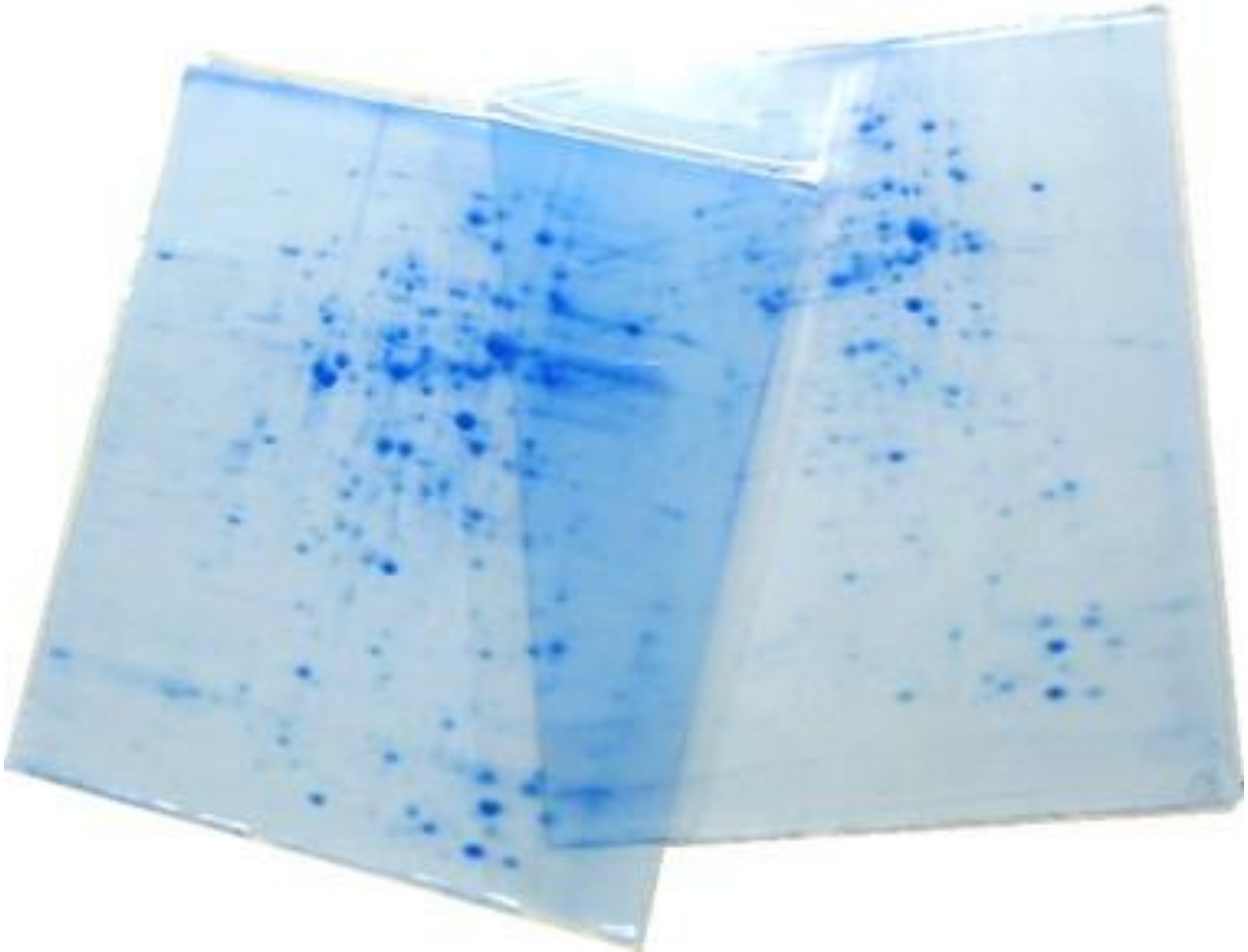


- The solution is to do a 2D separation using a gel that has a pH gradient.
- Proteins will run on this gel (in both directions) until they hit their isoelectric point where they aggregate



2D Gels

2D Electrophoresis is a powerful tool for analyzing the protein complement of simple cells



Determination of protein concentration

Biuret test – Molecules with two or more peptide bonds react with Cu^{2+} ions in alkaline solution and form a **purple complex**. The protein treated with biuret reagent is measured at 540 nm after the purple product is formed

Lowry (Folin) protein assay– similarly to the biuret reaction, but a second reagent (Folin- Ciocalteu reagent) is used in addition to strengthen the colour. The strong **blue colour** is created by two reactions: (1) formation of the coordination bond between peptide bond nitrogens and a copper ion and (2) reduction of the Folin-Ciocalteu reagent by tyrosine (phosphomolybdic and phosphotungstic acid of the reagent react with phenol). The measurement is carried out at 750 nm

Bradford protein assay– method is based on the ability of the Coomassie Brilliant **Blue dye** to bind to proteins in acidic solution (via electrostatic and van der Waals bonds), resulting in a shift of the absorption maximum of the dye from 465 to 595 nm

UV/Vis spectroscopy

Absorption – transfer of energy from a photon (light) to a molecule
Chromophore – a molecule or a group on a molecule that absorbs light

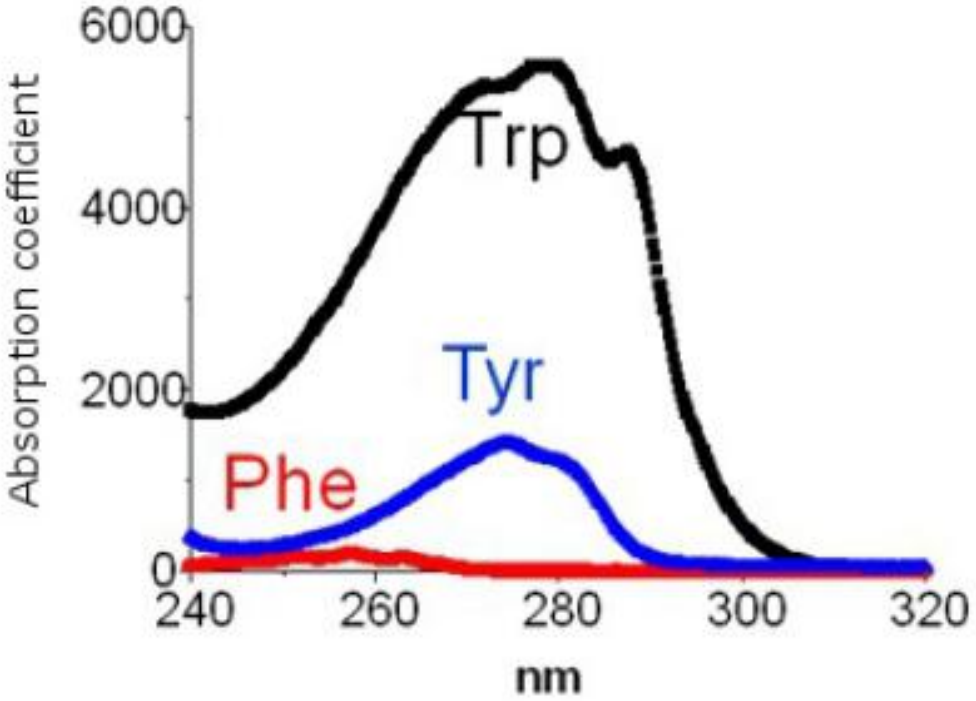
Chromophores in proteins include

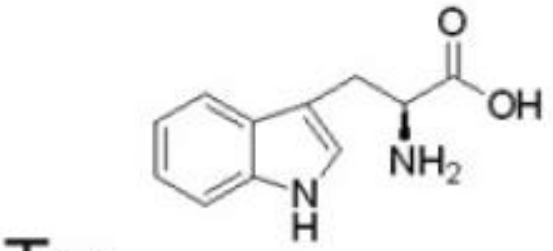
- peptide bond ~220 nm
- aromatic a.a. residues ~280 nm for Trp. Others are Tyr and Phe
- prosthetic groups, e.g., the heme in hemoglobin and myoglobin is red thus it absorbs visible light

USES of absorbance spectroscopy:

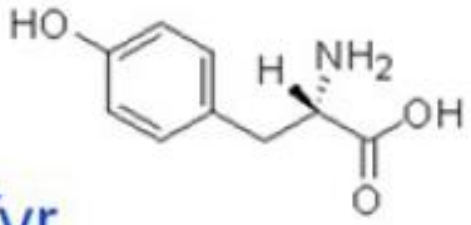
- determine concentration (Beer's Law)
- study conformational changes
- detect and quantitate ligand binding

Absorption spectra of aromatic amino acids

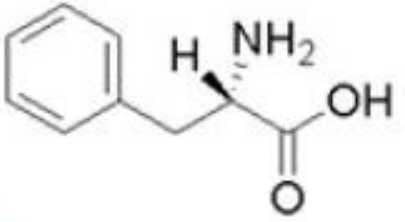




Trp



Tyr

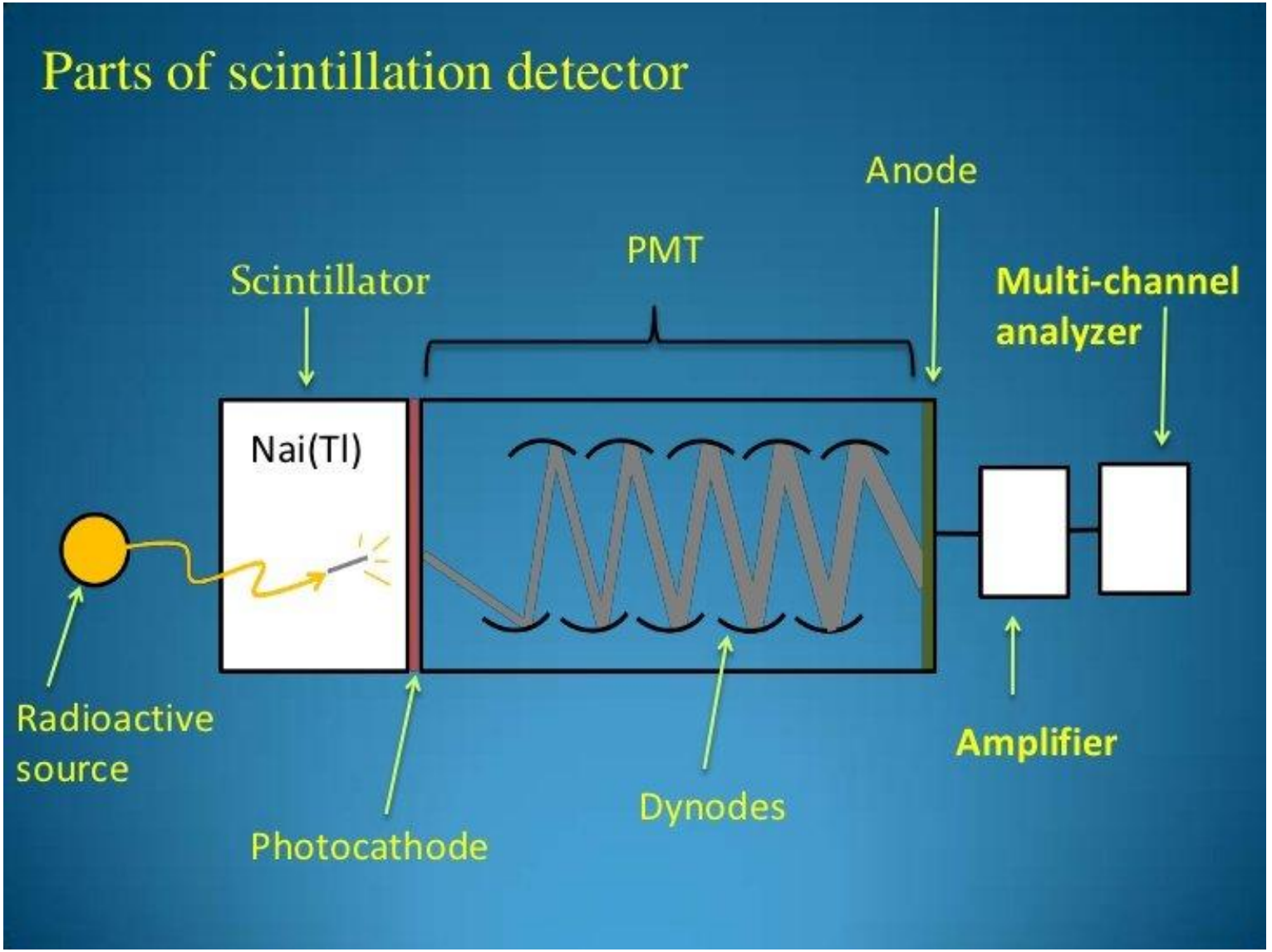


Phe

Absorption is measured at 260 and 280 nm and protein concentration can be calculated with the following equation:

$$c_{prot}(mg/mL) = 1.55 * A_{280nm} - 0,76*A_{260nm}$$

Enzyme Radiometric Methods



Enzyme Radiometric Methods

- Involves the quantification of **radioisotopes** incorporated into the substrate and retained in the product
- Relies on the efficient separation of the radiolabled product from the residual radiolabeled substrate and on the sensitivity and specificity of the radioactivity detection method
- Most commonly used radioisotopes, for example, ^{14}C, ^{32}P, ^{35}S, and ^{3}H
- Detection can be achieved by
  - Scintillation
  - Autoradiography

An example of radiometric assay

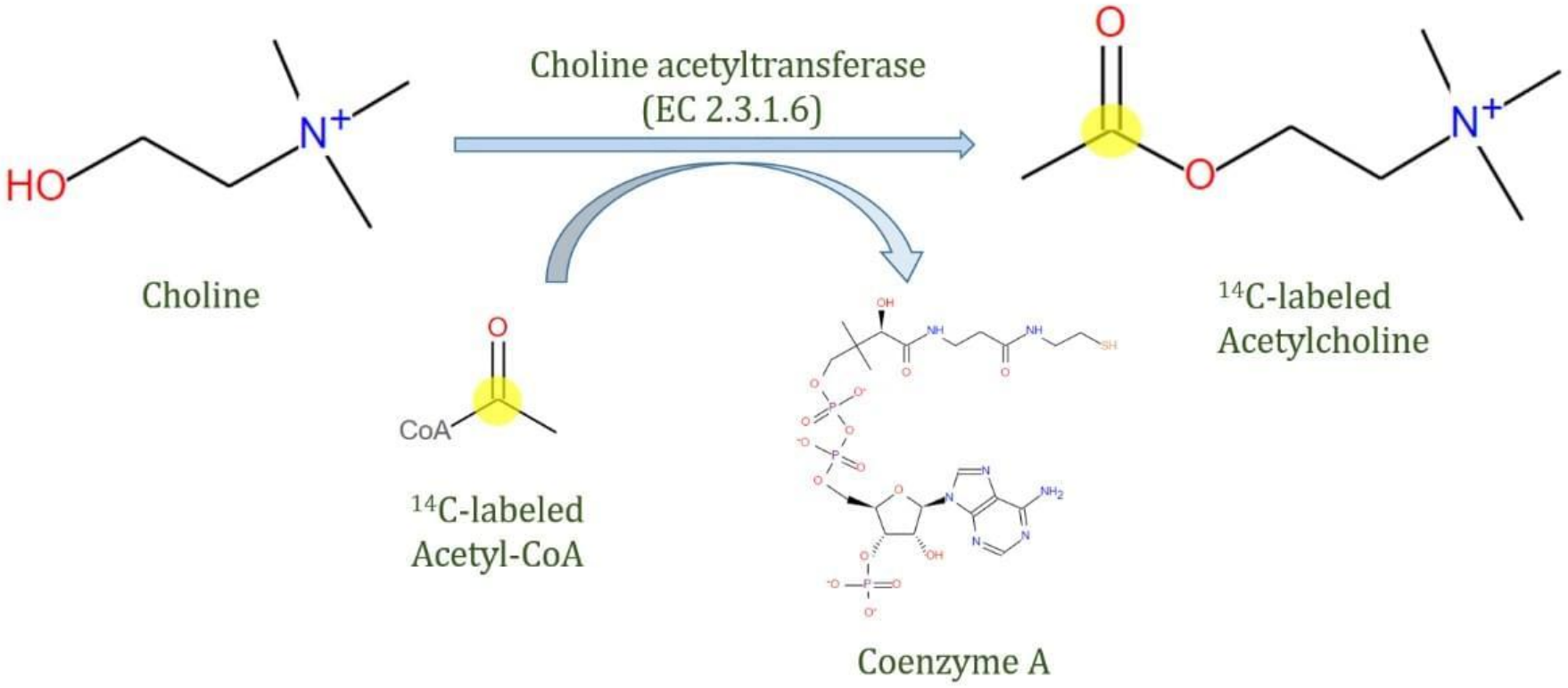
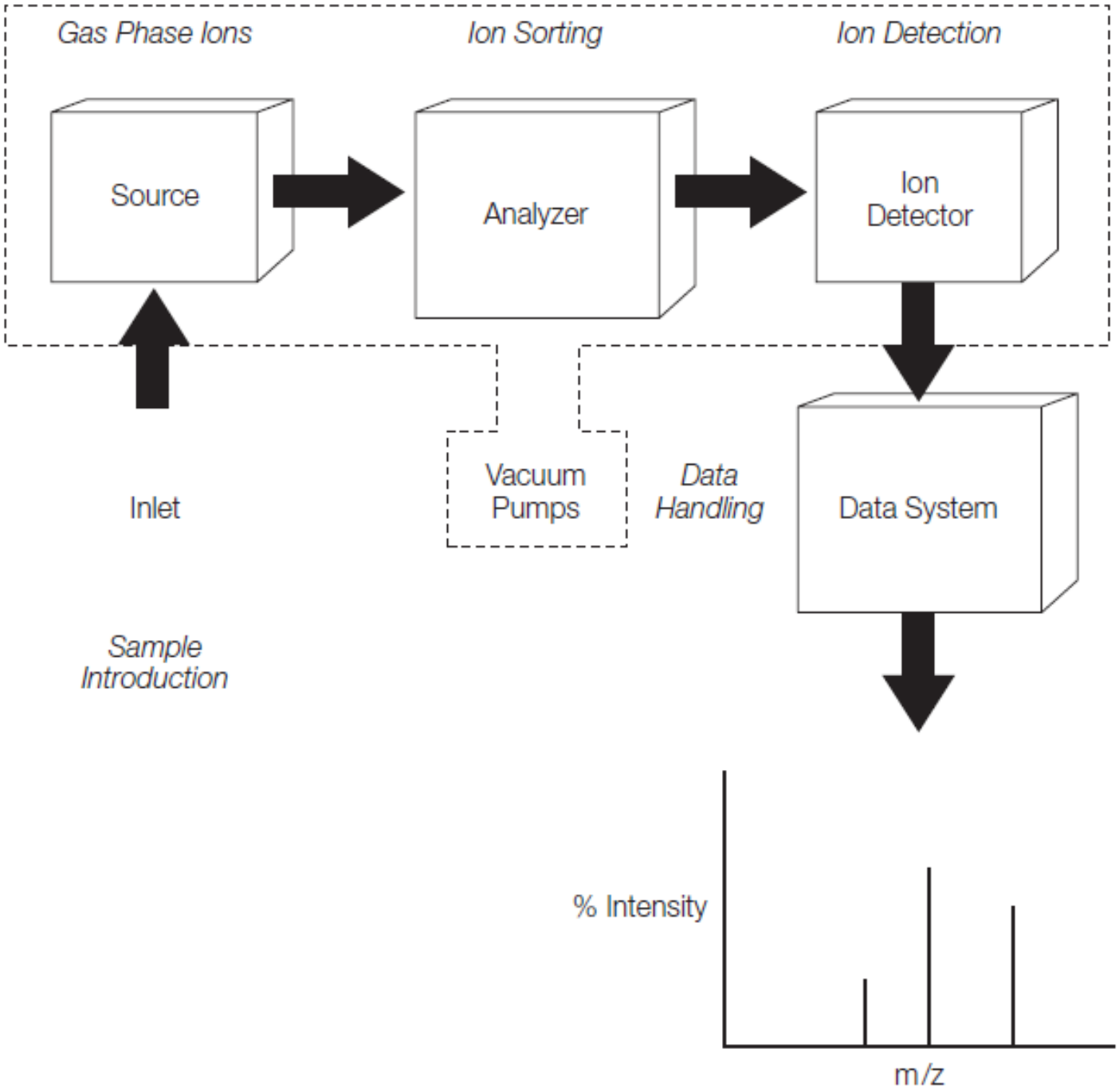


Figure: The reaction catalyzed by choline acetyltransferase using ^{14}C-labeled acetyl-CoA as the substrate.

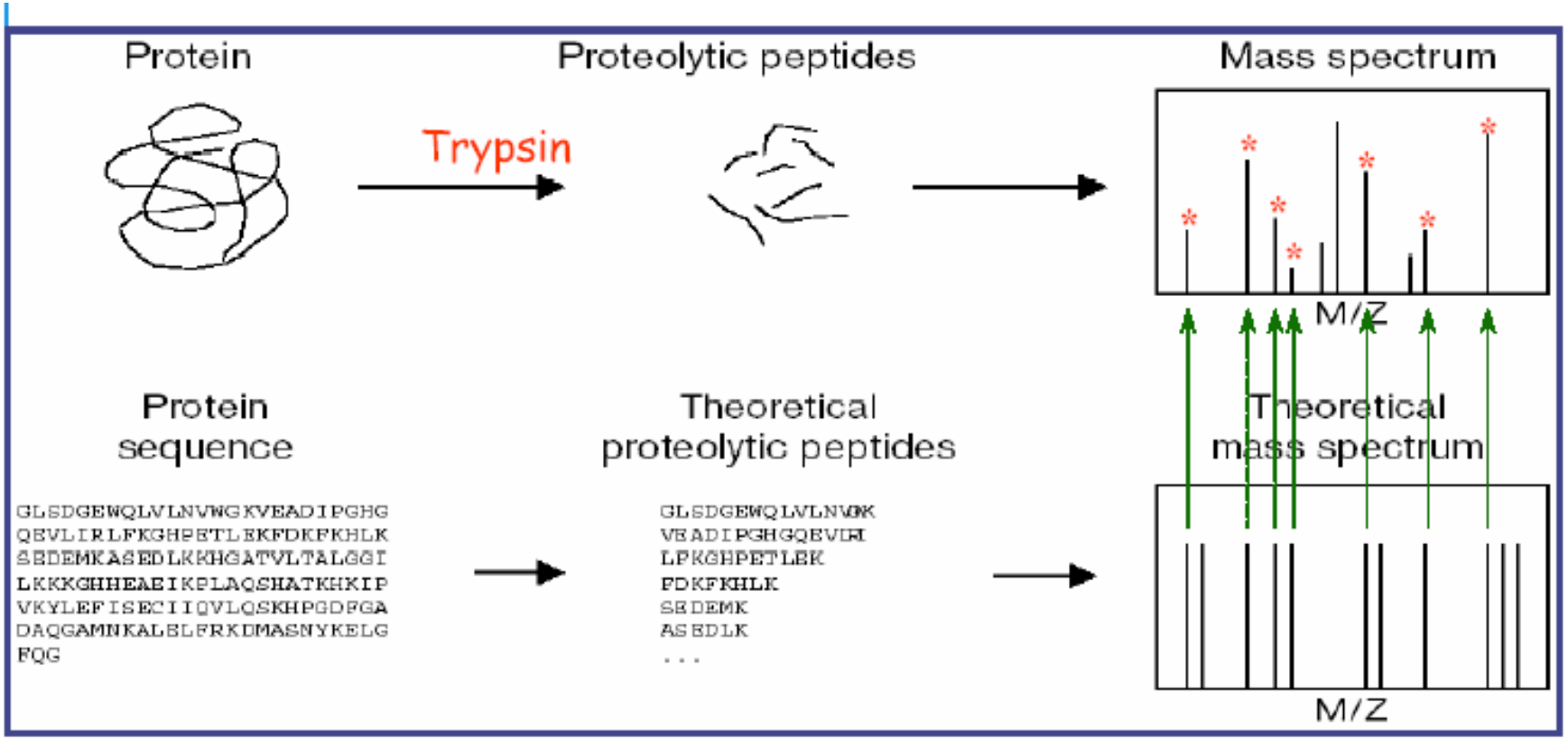
Mass Spectrometry (MS)

- The mass spectrometer consists of three components: an **ionization device, a mass analyzer, and an ion detector** (see scheme next slide)
- Neutral molecules are ionized, and their positively charged ion products are directed through an electric and/or magnetic field, where they are separated (analyzed) on the basis of their **mass-to-charge ratio**
- A detector then records the ions after separation. The "spectrum" generated by MS displays ion intensity as a function of ***m/z*** (mass-to-charge ratio)


Gas Phase Ions
Ion Sorting
Ion Detection
Source
Analyzer
Ion Detector
Inlet
Vacuum Pumps
Data Handling
Data System
Sample Introduction
% Intensity
m/z

Applications of Mass Spec in Biochemistry

1. Identification of peptides and proteins (enzymes)
2. Characterisation of post-translational modification processes
3. Peptide sequencing
4. Protein-protein interactions



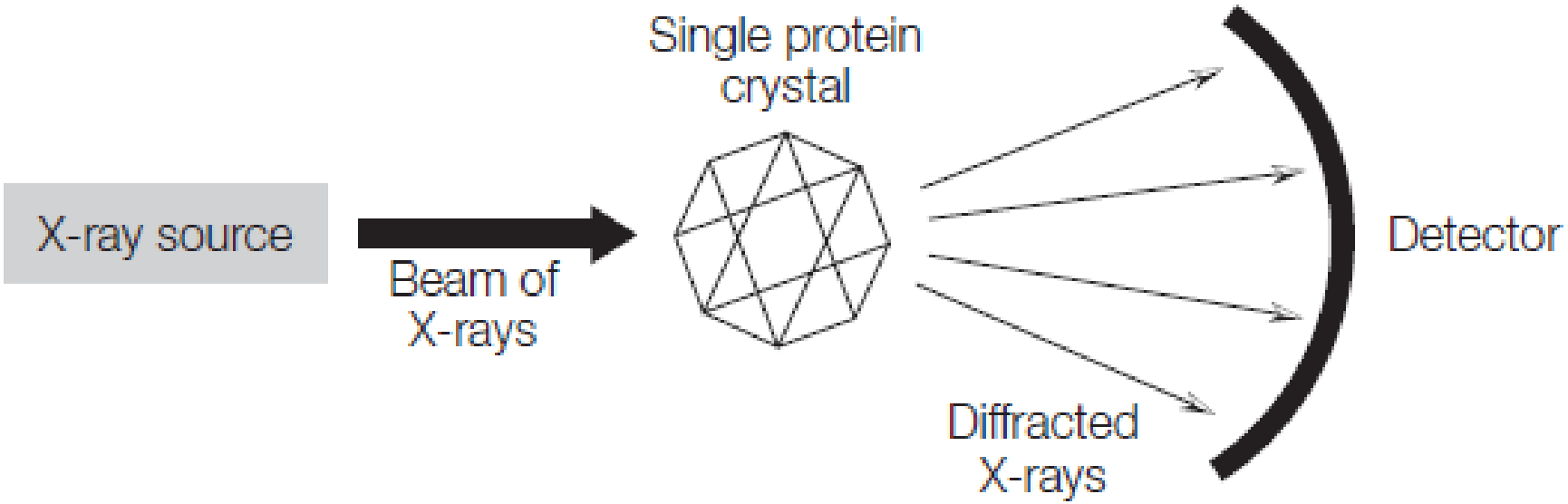
X-ray crystallography

Applied in the study of three-dimensional structures of enzymes

The 3D structures are deposited in the Protein Data Bank (www.rcsb.org/pdb)

An X-ray crystallography analysis requires three components:

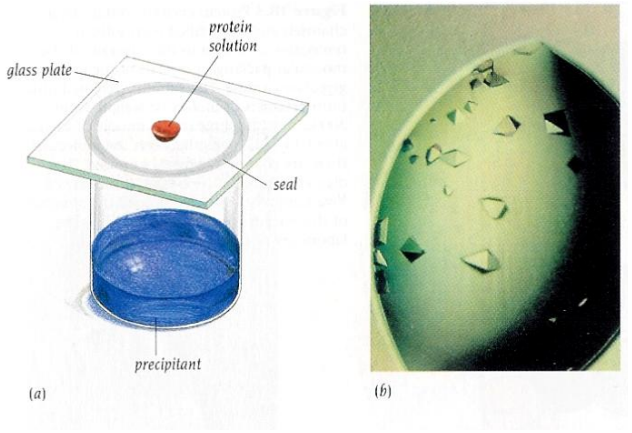
(1) a protein crystal
(2) an X-ray source
(3) a detector (i.e., radiation detector or photographic film)



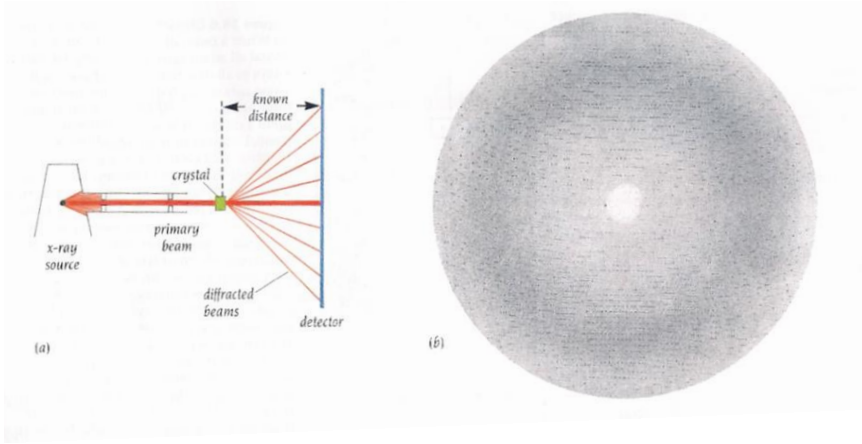
Schematic of X-ray crystallography

The three phases of solving a structure using X-ray diffraction:

1. Obtaining (diffracting) crystals
2. Obtaining high resolution diffraction data
3. Solving the phases and building & refinement of the model

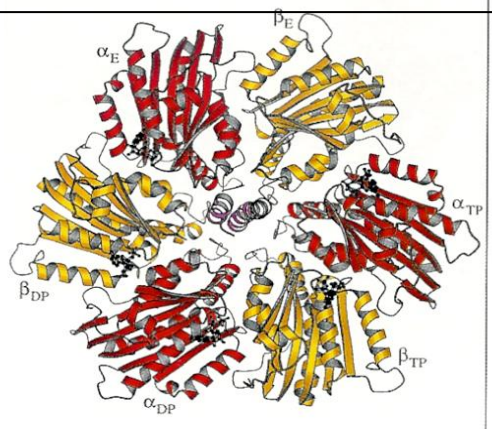


1. Grow crystals



2. Measure diffraction

3. Solve phases and refine structure



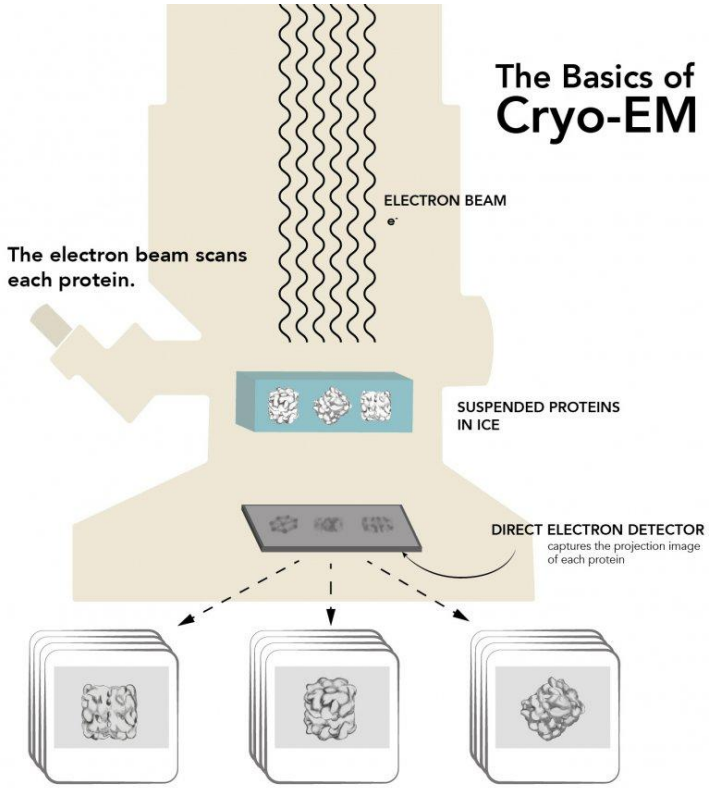
X-ray crystallography - Limitations

- An extremely pure protein sample is needed
- The protein sample must form crystals that are relatively large without flaws. **Generally the biggest problem**
- Many proteins aren't amenable to crystallization at all (i.e., proteins that do their work inside of a cell membrane)

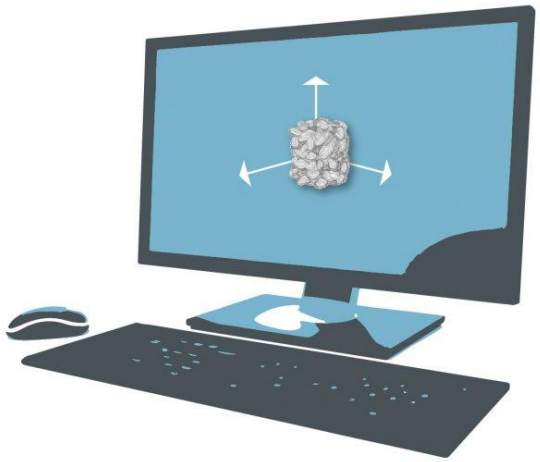
Cryo-Electron Microscopy

- Cryo-EM is a type of transmission electron microscopy that allows for the specimen of interest to be viewed at cryogenic temperatures
- Following years of improvement, the cryo-electron microscope has become a valuable tool for viewing and studying the structures of various biological molecules.

The Basics of Cryo-EM



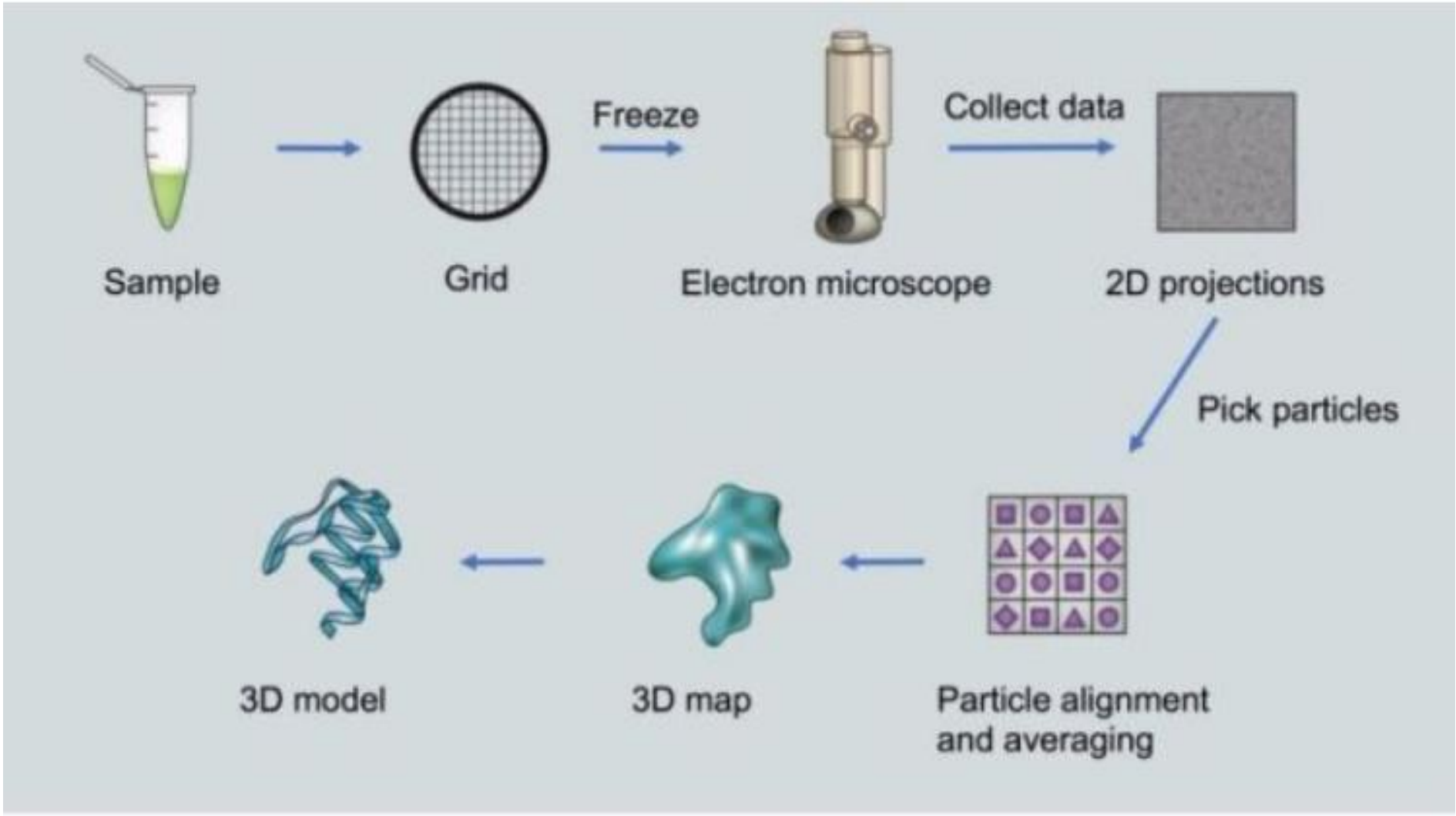
The projection images are categorized into like groups.



Then they are COMBINED to create a hi-res 3D model.

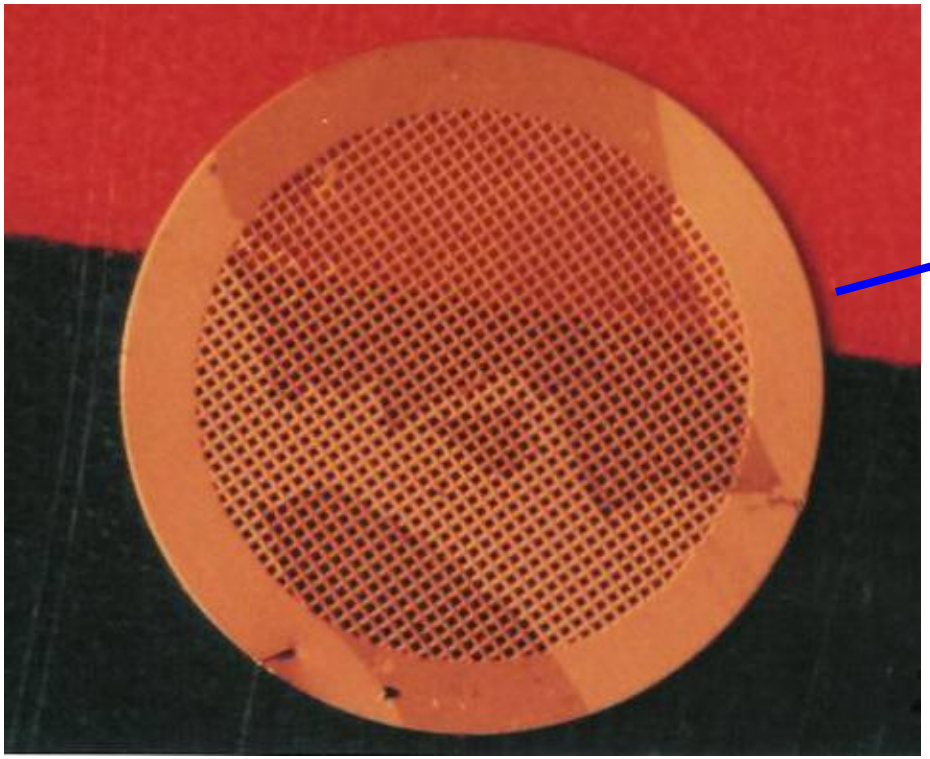
https://cryoem.slac.stanford.edu/what-is-cryo-em

Cryo-Electron Microscopy- steps

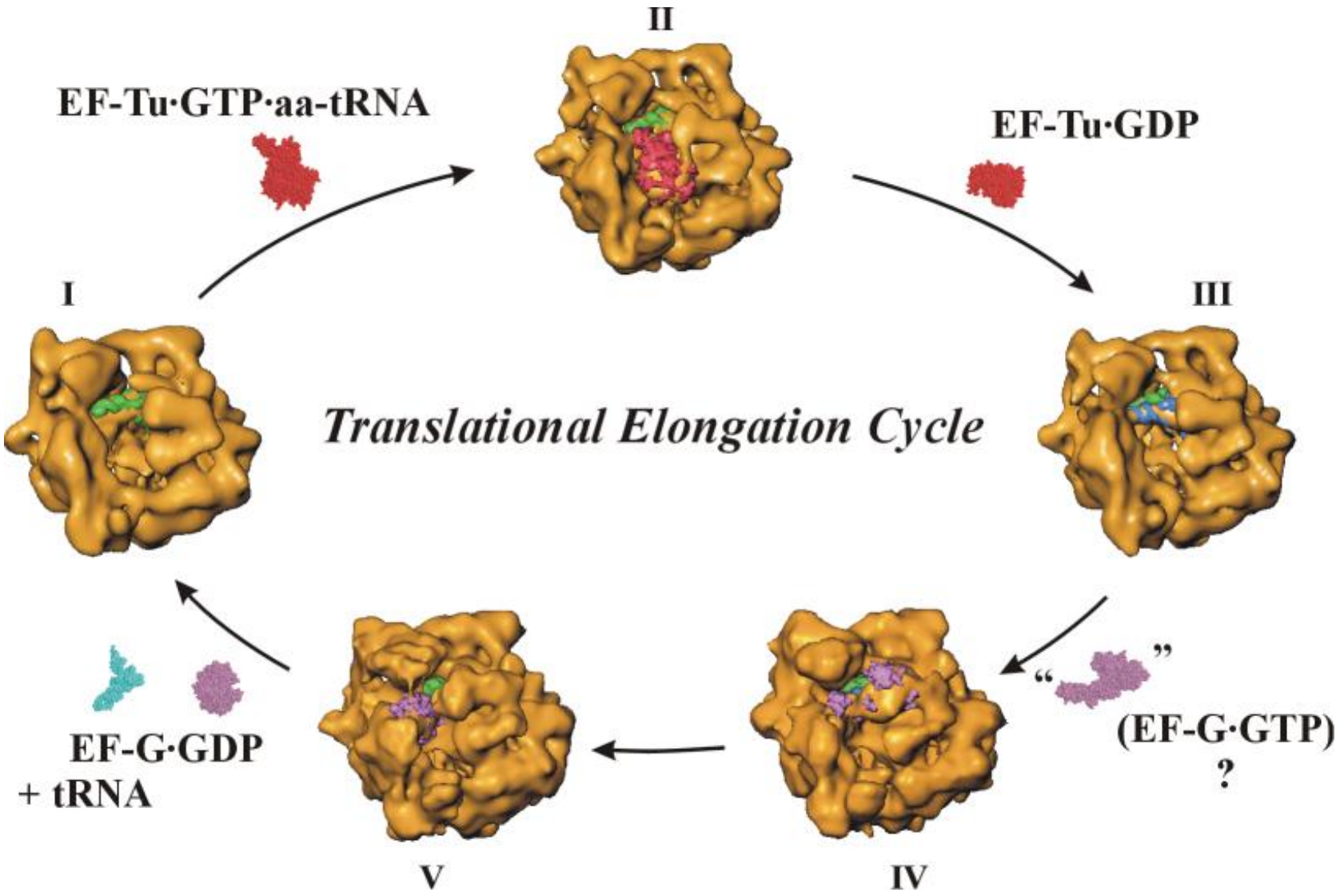


Cryo specimen preparation

- Preserve native environment
- High vacuum→ need frozen specimens!
- Snap freezing for amorphous ice phase, not crystalline ice phase



Examples: Ribosome



Next lecture

Enzyme catalytic Mechanisms