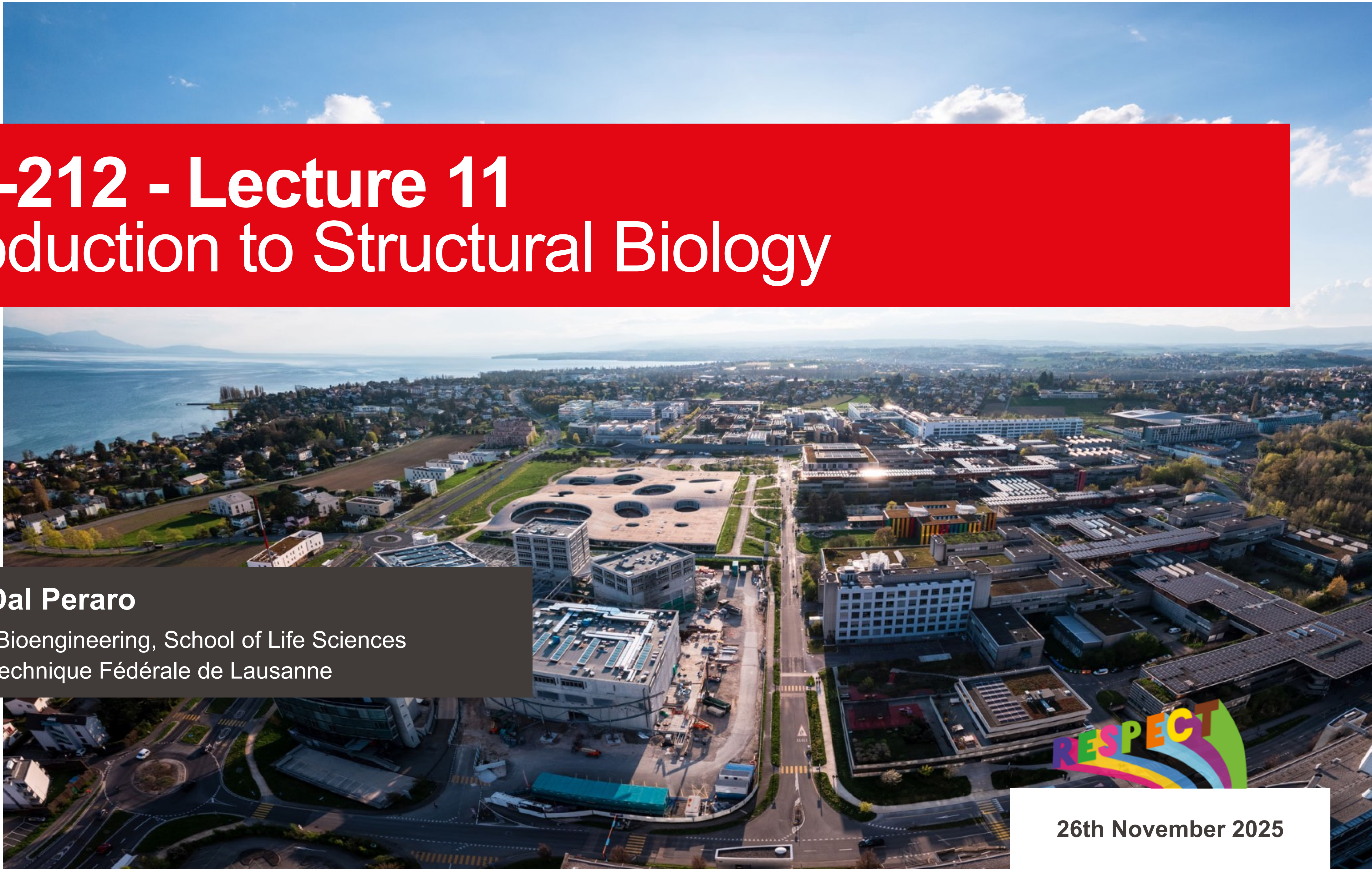


# BIO-212 - Lecture 11
## Introduction to Structural Biology

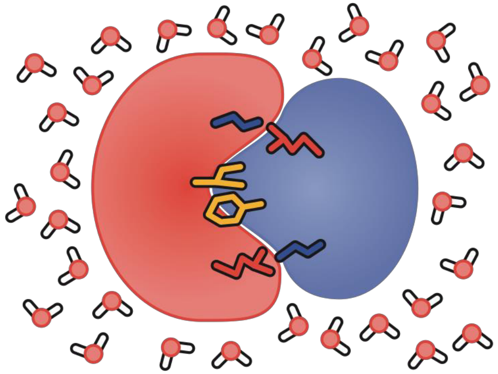
**Matteo Dal Peraro**
Institute of Bioengineering, School of Life Sciences
École Polytechnique Fédérale de Lausanne

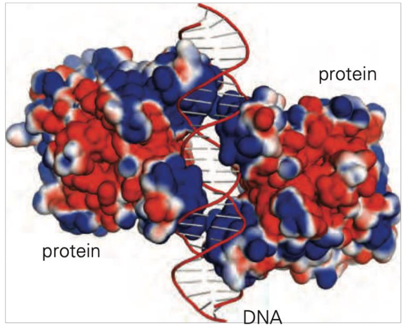
26th November 2025

# Lecture 10 – Quick Summary

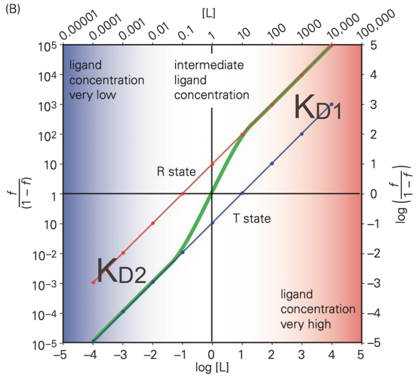
## Binding interfaces

- Their origin and features





- Allostery and cooperativity

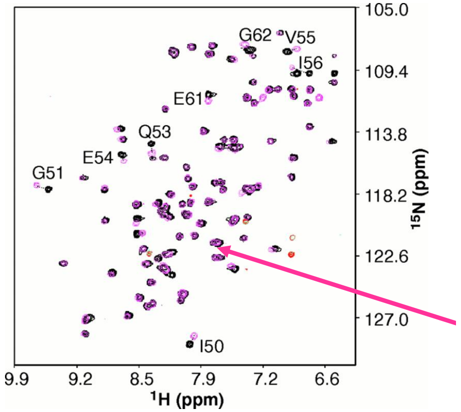


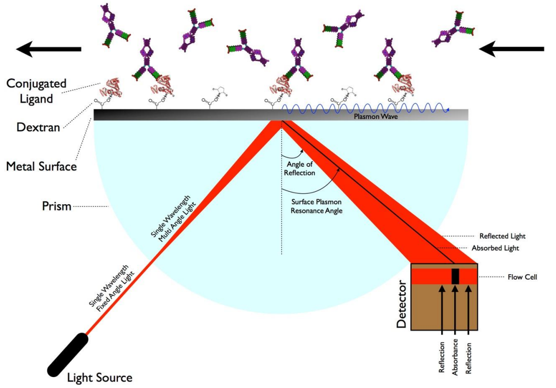
$$n_H = \frac{2}{1+\sqrt{\frac{K_{D2}}{K_{D1}}}}$$

## Experimental methods for studying binding

- Isothermal Titration Calorimetry (ITC)
- Fluorescence polarization
- Nuclear Magnetic Resonance (NMR)
- Surface Plasmon Resonance (SPR)

$$\frac{k_d}{k_a} = \frac{k_{off}}{k_{on}} = \frac{[P][L]}{[P{\bullet}L]} = K_D = \frac{1}{K_A}$$

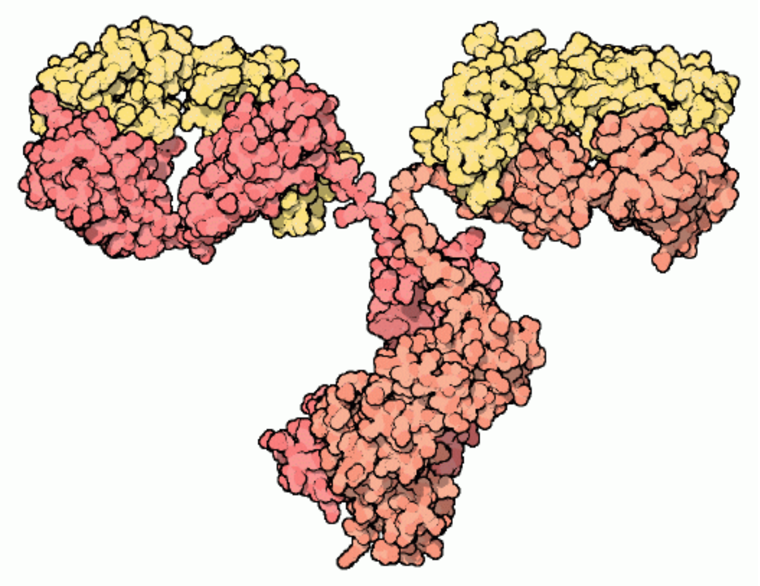




# Why do we care about biomolecule structures?

- Examples of different biomolecules and their functions

Glycerophospholipids
(building membranes)



Antibody (immune defense)

(energy storage)

DNA (carrier of genetic information)

# Structure *is* function

If you want to understand function, study structure

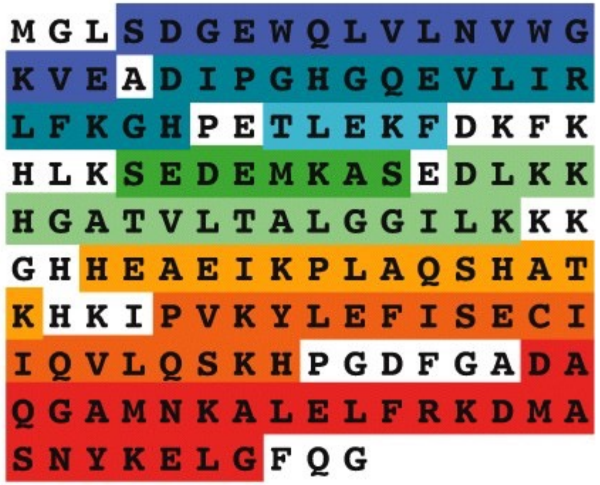
F. Crick

Pauling and Corey (1951)

Franklin, Watson and Crick (1953)

Perutz and Kendrew (1959)

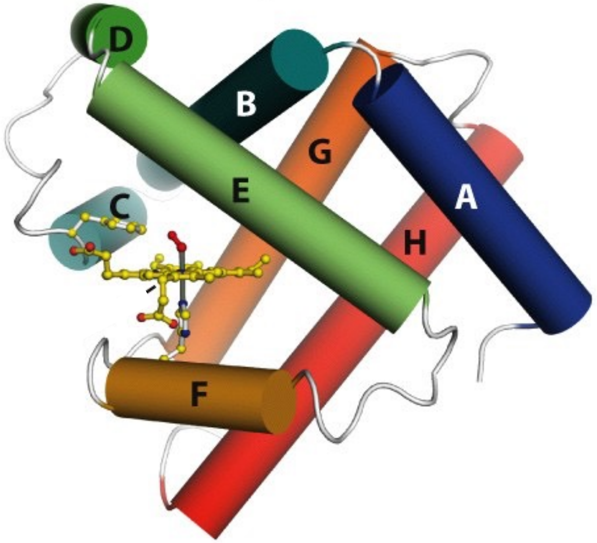
# The structural biology paradigm



M G L S D G E W Q L V L N V W G
K V E A D I P G H G Q E V L I R
L F K G H P E T L E K F D K F K
H L K S E D E M K A S E D L K K
H G A T V L T A L G G I L K K K
G H H E A E I K P L A Q S H A T
K H K I P V K Y L E F I S E C I
I Q V L Q S K H P G D F G A D A
Q G A M N K A L E L F R K D M A
S N Y K E L G F Q G

→

myoglobin



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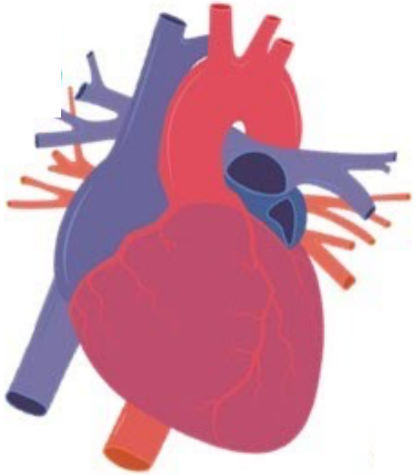


**sequence** **structure** **function**

→

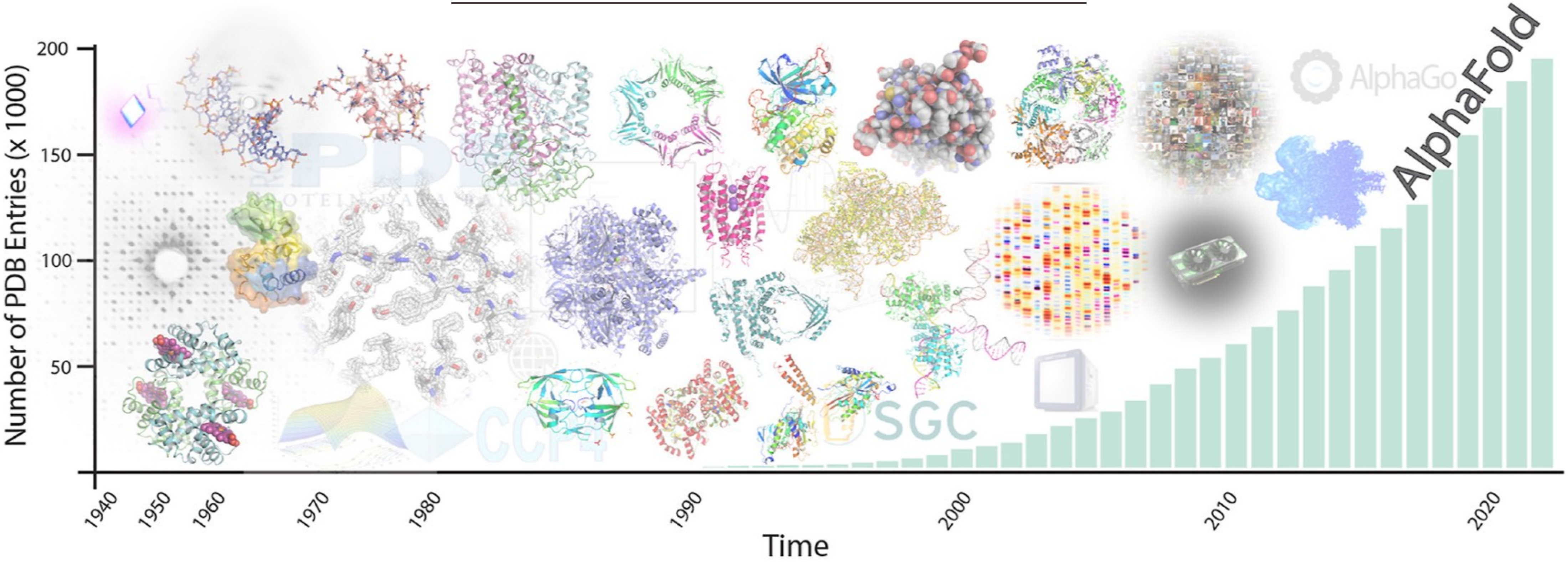
evolution (billion years)

- Anfinsen discovered it in a key experiment already in 1954

# Structural biology - A subfield of Biochemistry

- Structural biology is the study of molecular structure and dynamics of biological macromolecules, particularly proteins and nucleic acids, and how alterations in their structure affect their function.
- Total number of available experimentally determined structures to date is ~225k
- Computational tools (e.g., AlphaFold) can predict macromolecular structures *in silico* allowing to expand the available structure space

Biomolecule structures resolved over time

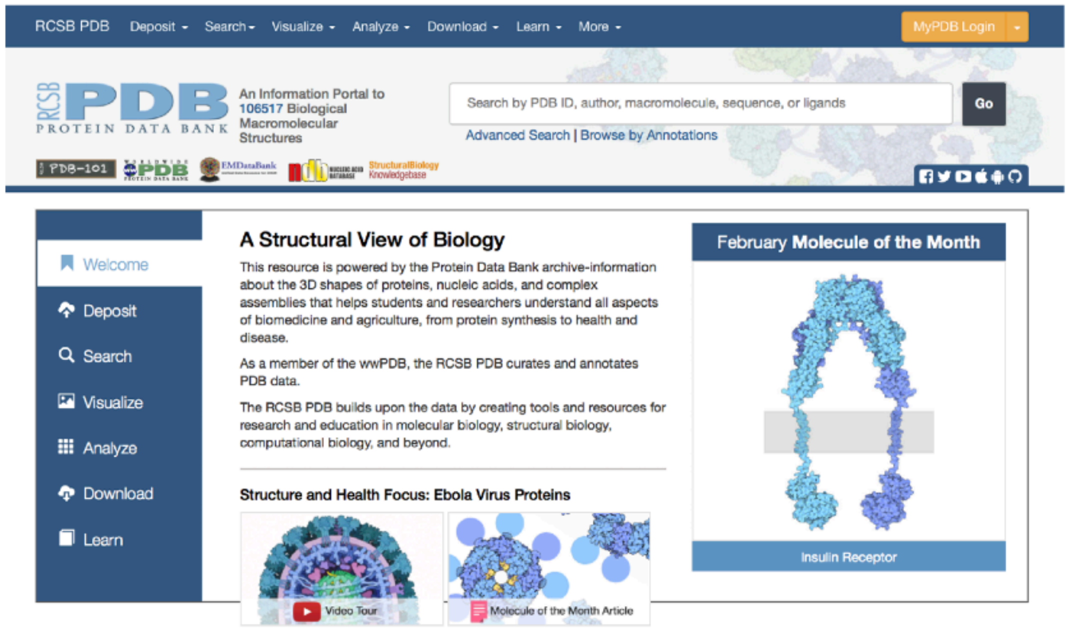


# What is a structure?

- Generally speaking, a structure is a file containing information on (1) atoms comprising the biomolecule, (2) their coordinates in 3D space and (3) connections via covalent bonds
- The files are typically in a *.pdb or *.cif formats and can be opened with programs such as Pymol

## Structures are deposited to Protein Data Bank (PDB)

http://www.rcsb.org



An Information Portal to 106517 Biological Macromolecular Structures

### A Structural View of Biology

This resource is powered by the Protein Data Bank archive-information about the 3D shapes of proteins, nucleic acids, and complex assemblies that helps students and researchers understand all aspects of biomedicine and agriculture, from protein synthesis to health and disease.

As a member of the wwPDB, the RCSB PDB curates and annotates PDB data.

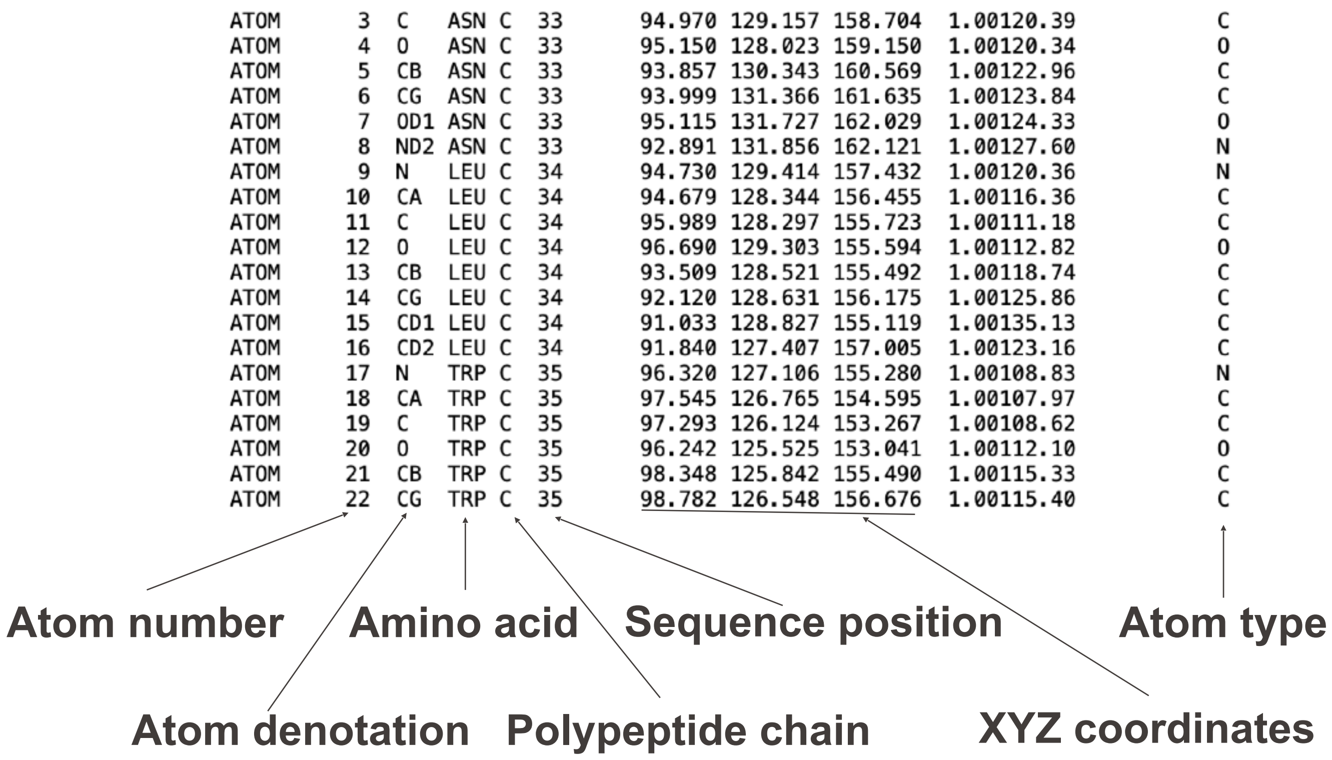
The RCSB PDB builds upon the data by creating tools and resources for research and education in molecular biology, structural biology, computational biology, and beyond.

**Structure and Health Focus: Ebola Virus Proteins**

**February Molecule of the Month**

Insulin Receptor

## Structures are text files with atom information



```
ATOM      3  C   ASN C  33      94.970 129.157 158.704  1.00120.39           C
ATOM      4  O   ASN C  33      95.150 128.023 159.150  1.00120.34           O
ATOM      5  CB  ASN C  33      93.857 130.343 160.569  1.00122.96           C
ATOM      6  CG  ASN C  33      93.999 131.366 161.635  1.00123.84           C
ATOM      7  OD1 ASN C  33      95.115 131.727 162.029  1.00124.33           O
ATOM      8  ND2 ASN C  33      92.891 131.856 162.121  1.00127.60           N
ATOM      9  N   LEU C  34      94.730 129.414 157.432  1.00120.36           N
ATOM     10  CA  LEU C  34      94.679 128.344 156.455  1.00116.36           C
ATOM     11  C   LEU C  34      95.989 128.297 155.723  1.00111.18           C
ATOM     12  O   LEU C  34      96.690 129.303 155.594  1.00112.82           O
ATOM     13  CB  LEU C  34      93.509 128.521 155.492  1.00118.74           C
ATOM     14  CG  LEU C  34      92.120 128.631 156.175  1.00125.86           C
ATOM     15  CD1 LEU C  34      91.033 128.827 155.119  1.00135.13           C
ATOM     16  CD2 LEU C  34      91.840 127.407 157.005  1.00123.16           C
ATOM     17  N   TRP C  35      96.320 127.106 155.280  1.00108.83           N
ATOM     18  CA  TRP C  35      97.545 126.765 154.595  1.00107.97           C
ATOM     19  C   TRP C  35      97.293 126.124 153.267  1.00108.62           C
ATOM     20  O   TRP C  35      96.242 125.525 153.041  1.00112.10           O
ATOM     21  CB  TRP C  35      98.348 125.842 155.490  1.00115.33           C
ATOM     22  CG  TRP C  35      98.782 126.548 156.676  1.00115.40           C
```

**Atom number** **Amino acid** **Sequence position** **Atom type**

**Atom denotation** **Polypeptide chain** **XYZ coordinates**

**visualization programs: VMD, Pymol, Chimera, Rasmol, Jmol, etc.**

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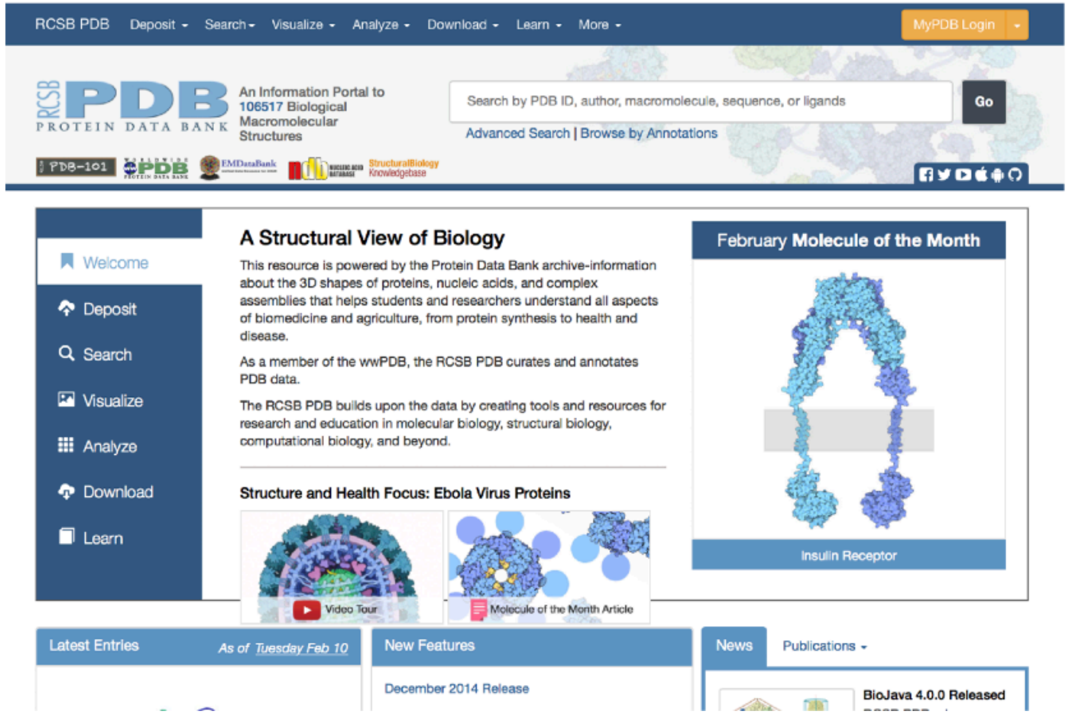

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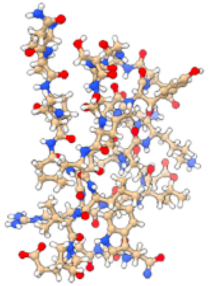
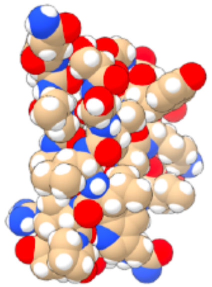
December 2014 Release

BioJava 4.0.0 Released

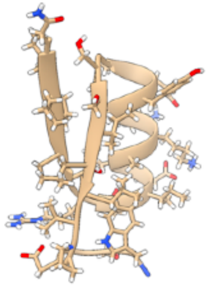
## Different representations of a structure



**All atom (sticks)**

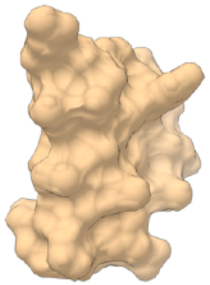


**All atom (balls)**



**Ribbon + Side chains**

**Ribbon**



**Surface**

All atom views can be busy so it is often advantageous to show secondary structure or whole surface view.

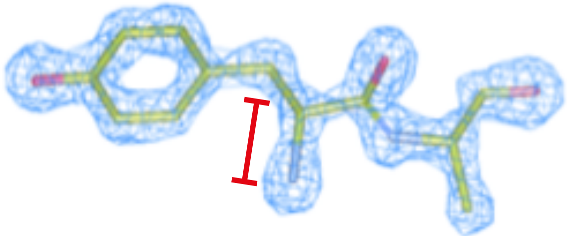
# History of experimental structure determination

- Due to the challenging nature of the experiments and the importance for understanding biological systems, major structural biology efforts have been rewarded with Nobel Prizes

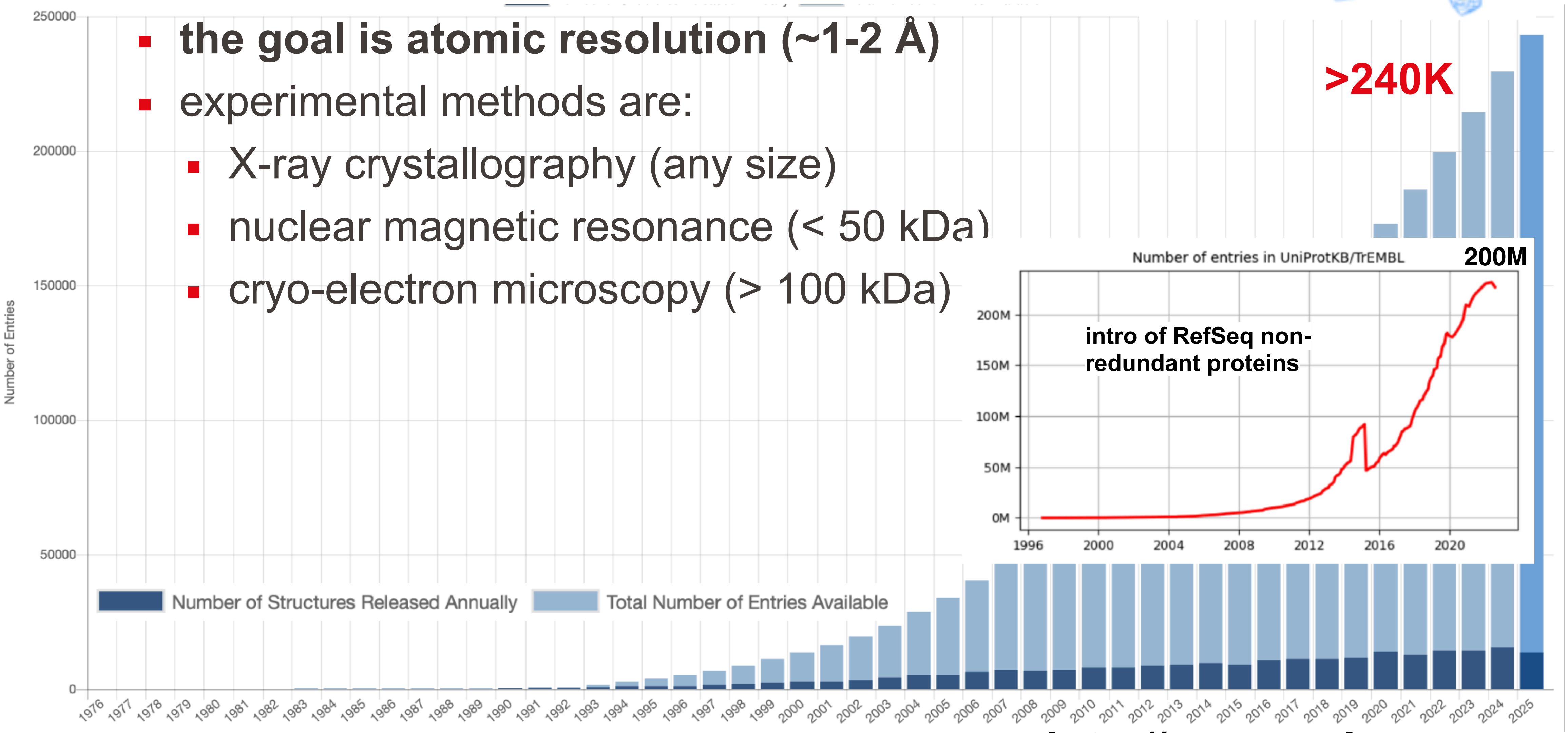
**1962 – Nobel Year of Structural Biology**

| | | |
|---|---|---|
| 2024: Chemistry | Jumper, Hassabis, Baker | Protein prediction and design |
| 2020: Chemistry | Charpentier and Doudna | CRISPR-Cas9x |
| 2017: Chemistry | Dubochet, Henderson, Frank | development of cryoEM |
| 2013: Chemistry | Karplus, Levitt and Warshel | Molecular modeling |
| 2012: Chemistry | Brian Kobilka | G-protein coupled receptors |
| 2009: Chemistry | Venki Ramakrishnan, Thomas Steitz, Ada Yonath | Ribosome |
| 2006: Chemistry | Roger D. Kornberg | RNA polymerase |
| 2003: Chemistry | Roderick MacKinnon | potassium channel |
| 2002: Chemistry | Kurt Wüthrich | development of protein NMR |
| 1997: Chemistry | John E. Walker | FoF1-ATPase |
| 1991: Chemistry | Richard R. Ernst | development of protein NMR |
| 1988: Chemistry | Johann Deisenhofer, Robert Huber, Hartmut Michel | photosynthetic reaction centre |
| 1982: Chemistry | Aaron Klug | development of cryo-EM |
| 1972: Chemistry | Christian B. Anfinsen | ribonuclease |
| 1964: Chemistry | Dorothy Hodgkin | penicillin |
| 1962: Medicine | Francis Crick, James Watson, Maurice Wilkins | DNA double helix |
| 1962: Chemistry | Max Perutz, John Kendrew | myoglobin and haemoglobin |
| 1946: Chemistry | James Sumner | first crystals of an enzyme (urease) |

# Structure determination is key



- **the goal is atomic resolution (~1-2 Å)**
- experimental methods are:
  - X-ray crystallography (any size)
  - nuclear magnetic resonance (< 50 kDa)
  - cryo-electron microscopy (> 100 kDa)



**>240K**

**200M**

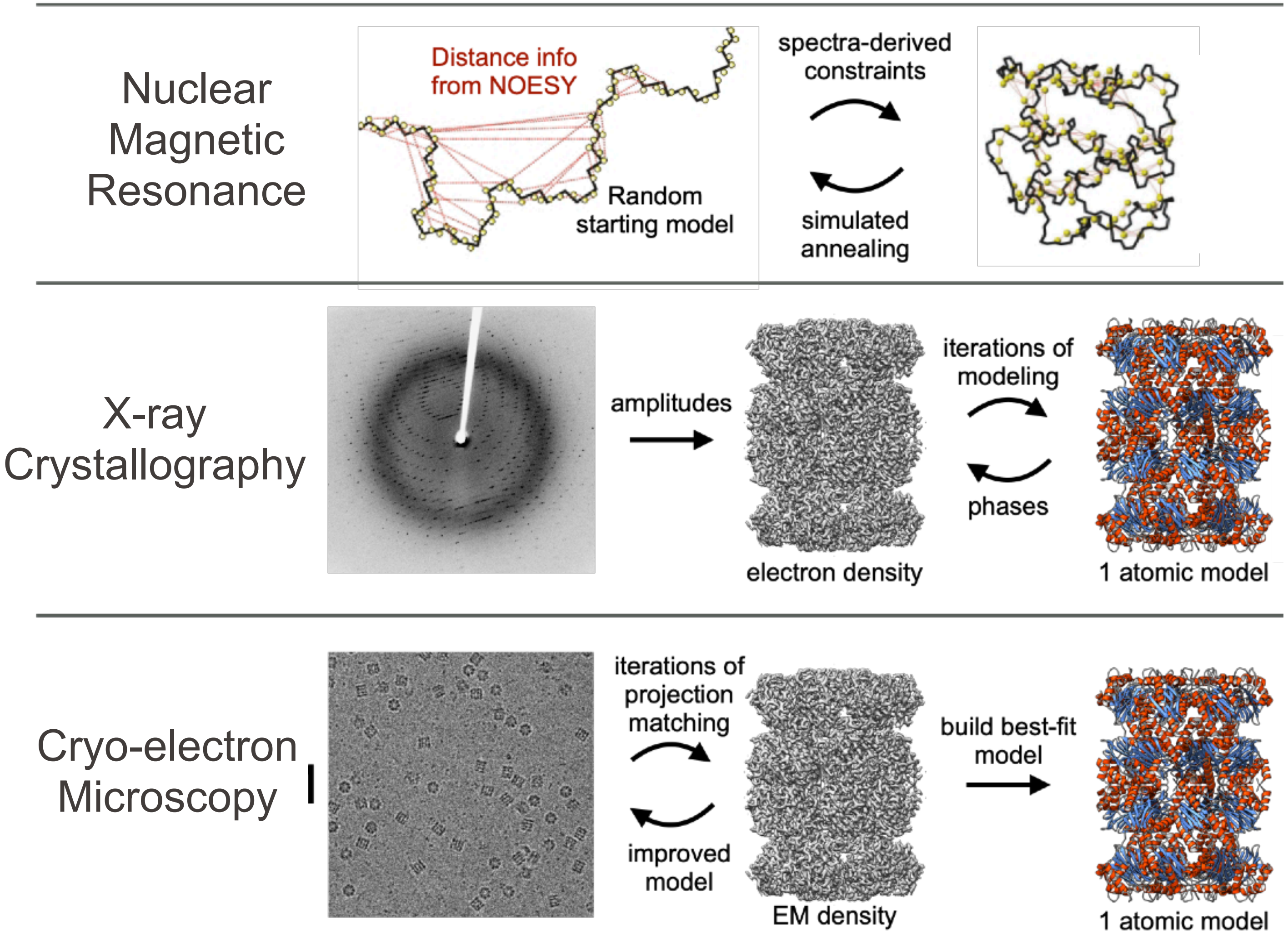
**intro of RefSeq non-redundant proteins**

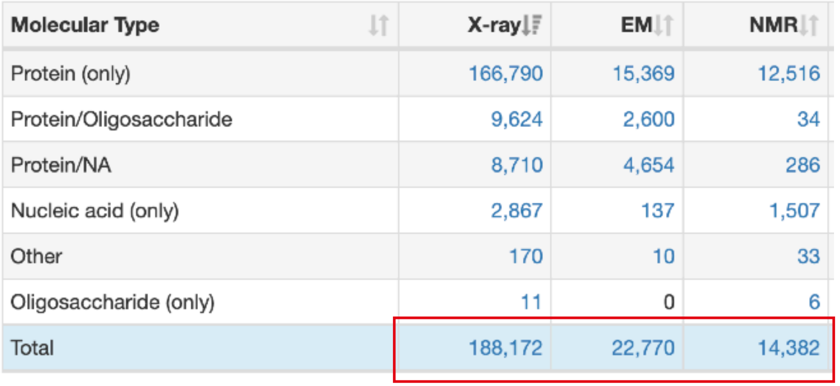
- **The Protein DataBank — PDB**

**http://www.rcsb.org**

# The main structural biology methods

- 3 main methods for experimental structure determination:





| Molecular Type | X-ray | EM | NMR |
|---|---|---|---|
| Protein (only) | 166,790 | 15,369 | 12,516 |
| Protein/Oligosaccharide | 9,624 | 2,600 | 34 |
| Protein/NA | 8,710 | 4,654 | 286 |
| Nucleic acid (only) | 2,867 | 137 | 1,507 |
| Other | 170 | 10 | 33 |
| Oligosaccharide (only) | 11 | 0 | 6 |
| Total | 188,172 | 22,770 | 14,382 |

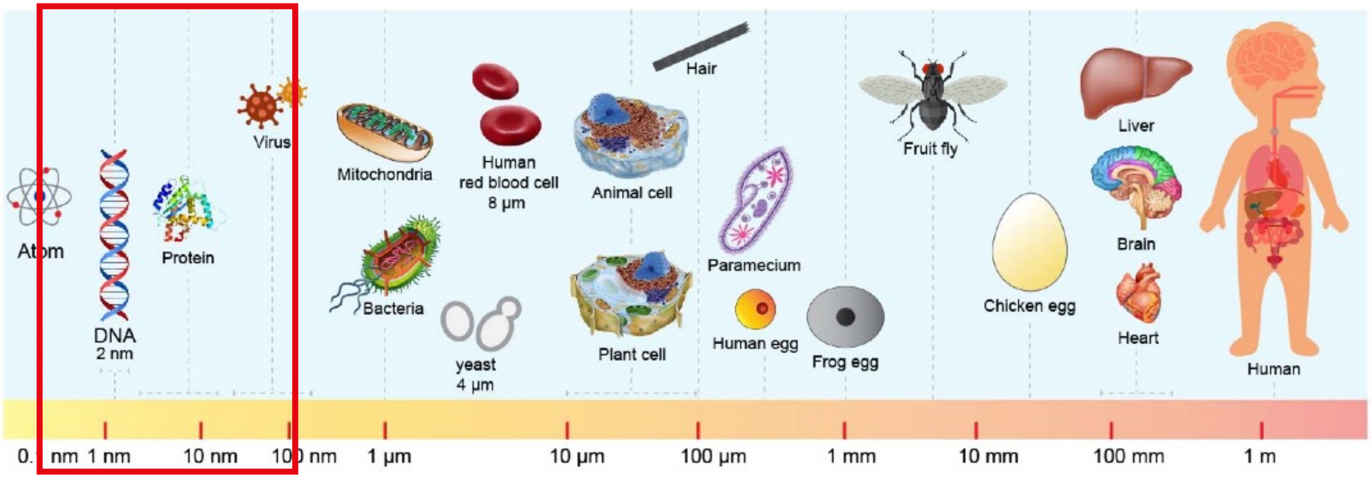
- Most structures came from X-ray crystallography since it is historically the oldest method

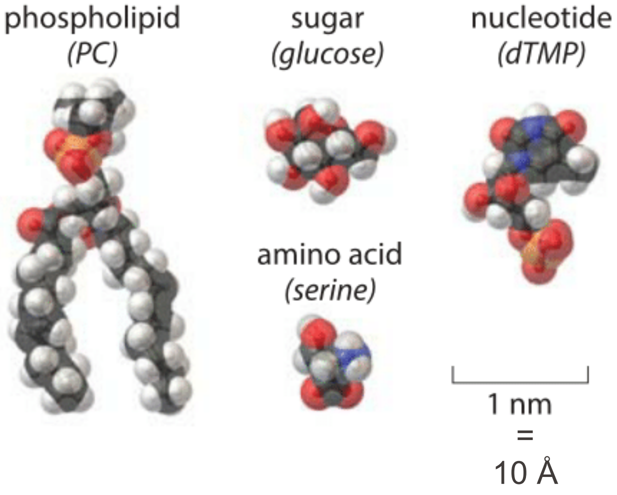
- The outputs of computational (AI) prediction of biomolecular structures are usually not referred to as “structures” but rather as “models”

- This is because they have not been experimentally verified

# Relative sizes of biological macromolecules

- The relevant size range is: ~0.1 nm - 100 nm
- Molecular weight range: 1 Da - 100 MDa





Some reminders:

- Molecular weight of biomolecules is usually expressed in Dalton (1 Da = 1 g/mol)
  - Average amino acid has a MW of ~110 Da
  - Average nucleotide pair has a MW of ~650 Da
- Length and resolutions are often expressed in Angstrom (Å) which is $10^{-10}$ m
  - Average atom diameters and bond lengths in biomolecules are ~1-2 Å
  - Basic building blocks (amino acids, nucleic acids) will be ~5-15 Å

# Abbé’s law on diffraction limited optical systems

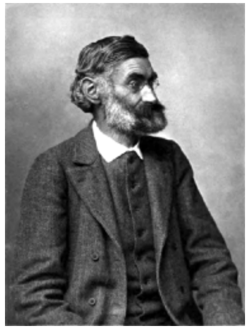
- The wavelength of electromagnetic radiation needs to be at a similar or lower order of magnitude as the features that are visualized (d ~ 250 nm)

**Resolution limit of a microscope**

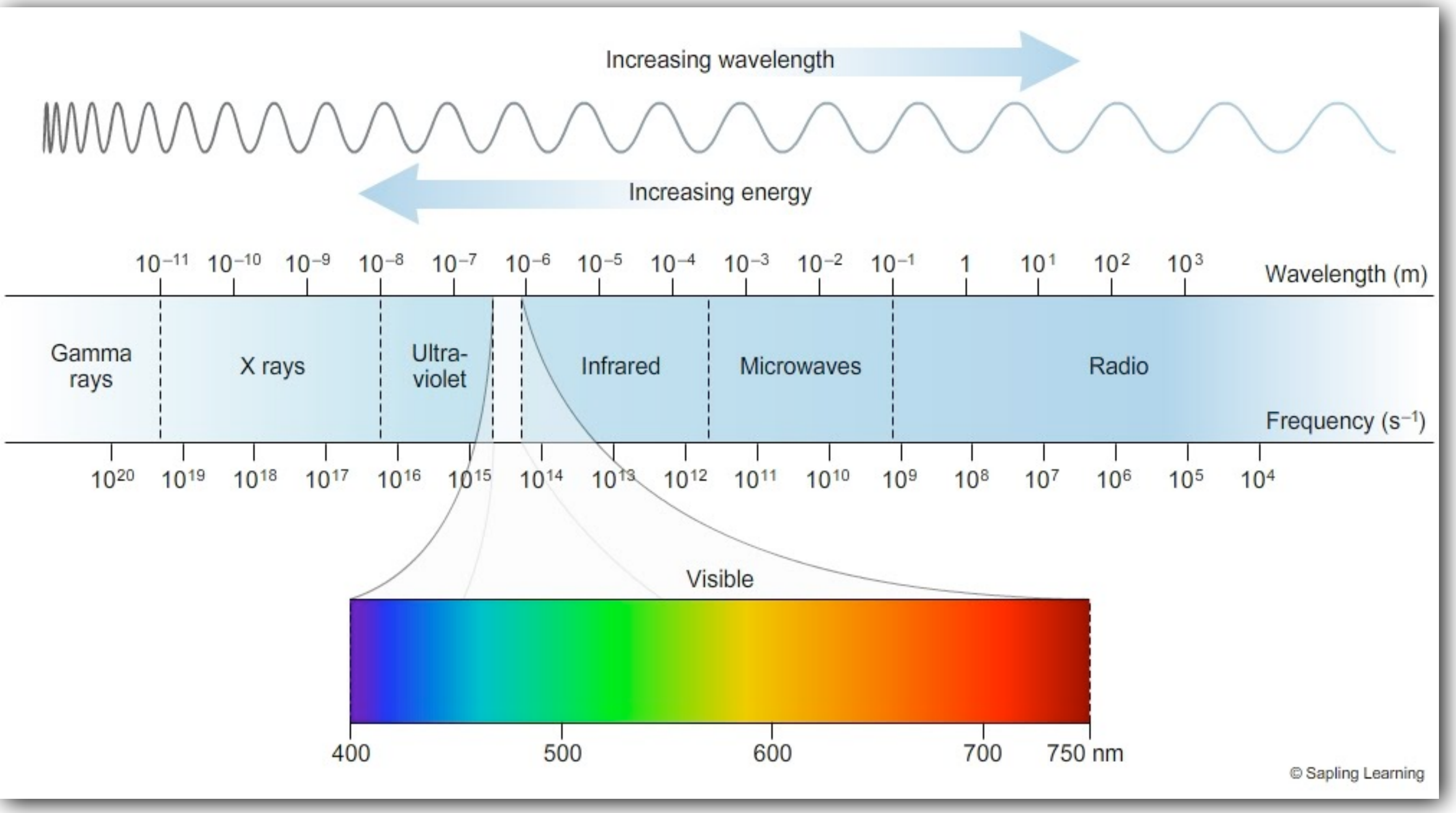
**Light wavelength**

$$d = \frac{\lambda}{2n\sin\theta} = \frac{\lambda}{2\mathrm{NA}}$$

**Numerical Aperture**



**Ernst Abbe**
(1840-1905)



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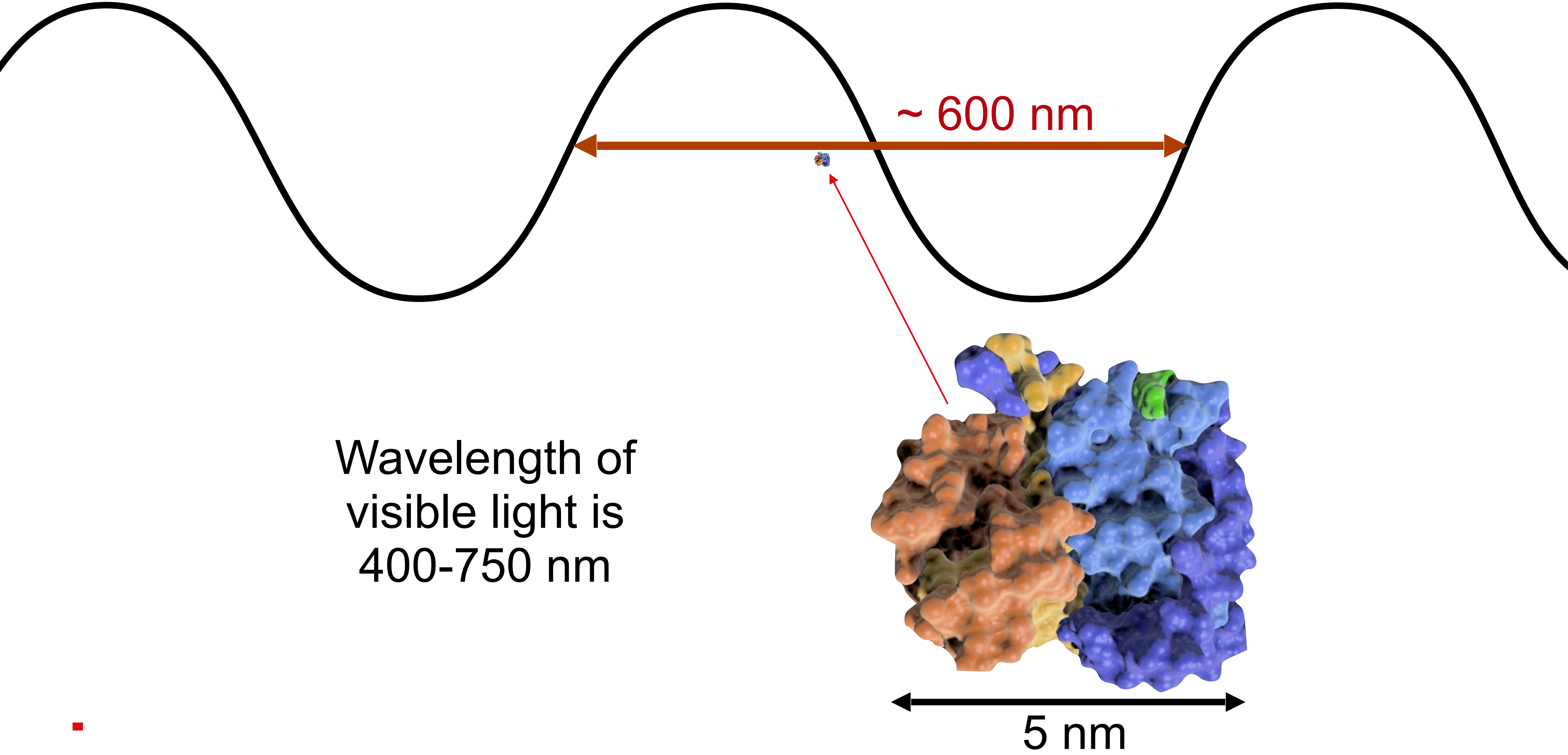
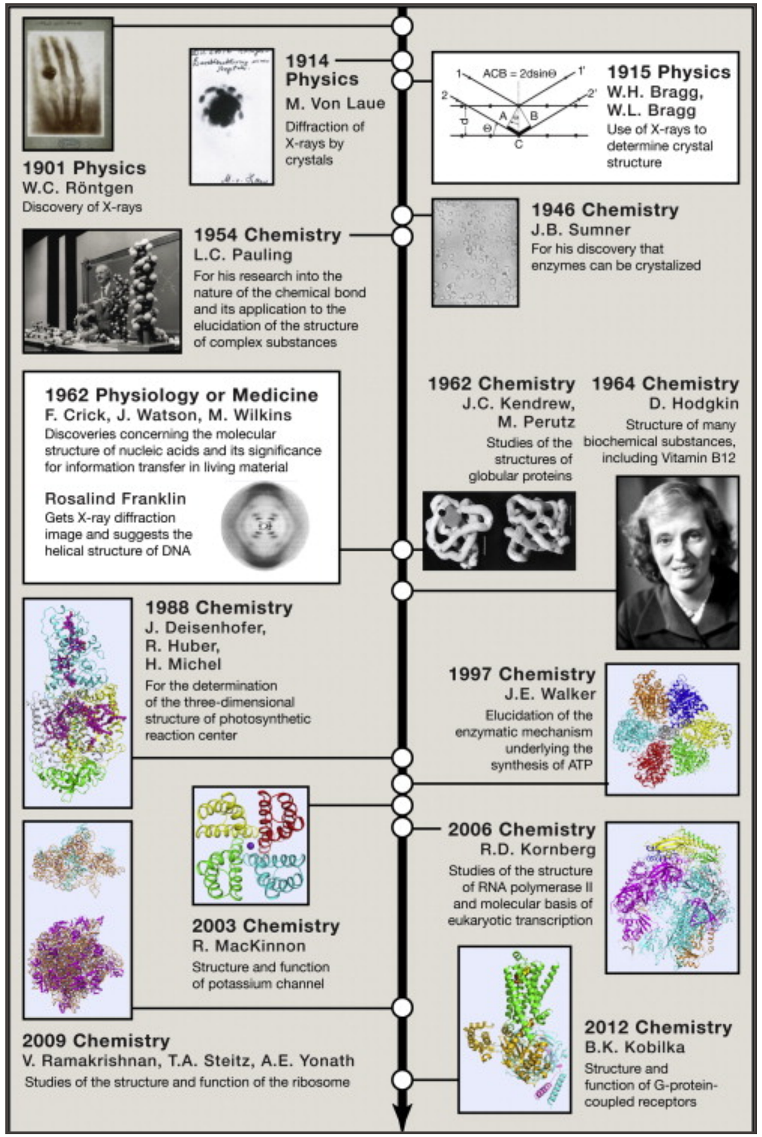
# Visible light is too “coarse” to study biomolecular structures



Wavelength of
visible light is
400-750 nm

# X-ray crystallography



**1901 Physics**
W.C. Röntgen
Discovery of X-rays

**1914 Physics**
M. Von Laue
Diffraction of X-rays by crystals

**1915 Physics**
W.H. Bragg, W.L. Bragg
Use of X-rays to determine crystal structure

**1946 Chemistry**
J.B. Sumner
For his discovery that enzymes can be crystalized

**1954 Chemistry**
L.C. Pauling
For his research into the nature of the chemical bond and its application to the elucidation of the structure of complex substances

**1962 Physiology or Medicine**
F. Crick, J. Watson, M. Wilkins
Discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material

**Rosalind Franklin**
Gets X-ray diffraction image and suggests the helical structure of DNA

**1962 Chemistry**
J.C. Kendrew, M. Perutz
Studies of the structures of globular proteins

**1964 Chemistry**
D. Hodgkin
Structure of many biochemical substances, including Vitamin B12

**1988 Chemistry**
J. Deisenhofer, R. Huber, H. Michel
For the determination of the three-dimensional structure of photosynthetic reaction center

**1997 Chemistry**
J.E. Walker
Elucidation of the enzymatic mechanism underlying the synthesis of ATP

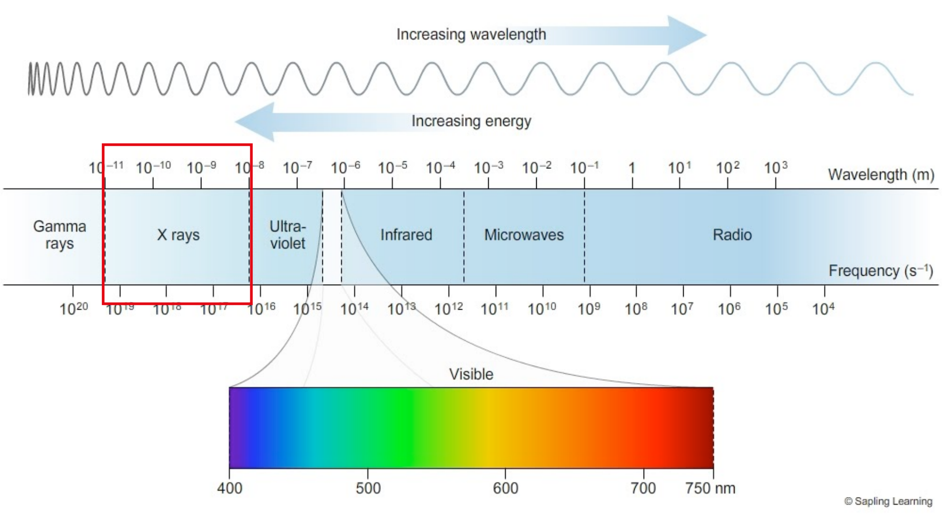
**2003 Chemistry**
R. MacKinnon
Structure and function of potassium channel

**2006 Chemistry**
R.D. Kornberg
Studies of the structure of RNA polymerase II and molecular basis of eukaryotic transcription

**2009 Chemistry**
V. Ramakrishnan, T.A. Steitz, A.E. Yonath
Studies of the structure and function of the ribosome

**2012 Chemistry**
B.K. Kobilka
Structure and function of G-protein-coupled receptors

- X-ray diffraction applied to study the structural properties of crystalline materials in atomic detail



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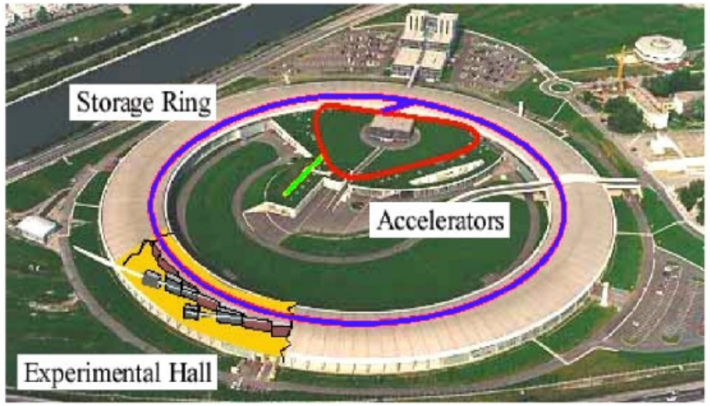
- First applied to determine the structures of inorganic molecules (e.g. salts) or organic molecules
- Relatively easy for small molecules but the proteins are much more challenging. Compare:
  - $C_6H_5OH$ (Phenol)
  - $C_{2717}H_{4155}N_{735}O_{810}S_{19}$ (Protein with ~500 amino acids)

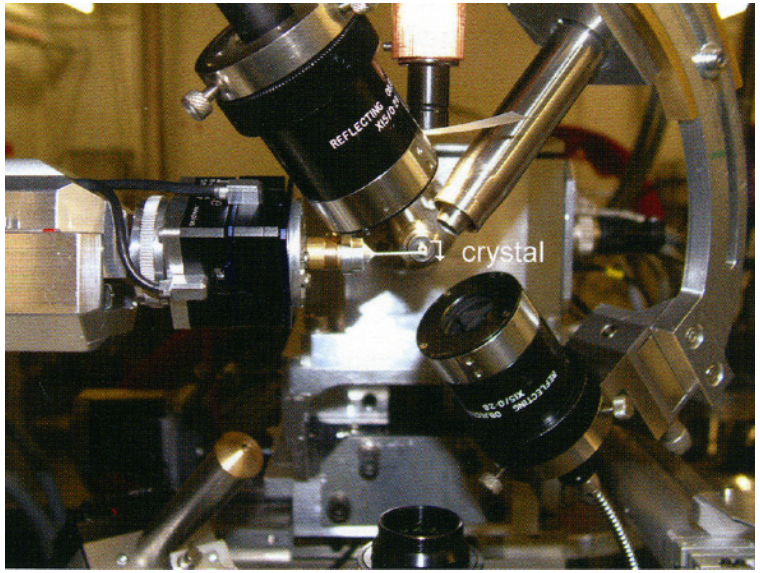
# Sources of X-rays: Synchrotrons

- Synchrotrons are cyclic particle accelerators in which the magnetic field bends the particles (e.g., accelerated electrons) into a closed path which results in production of X-rays.
- The radiation is projected at a tangent to the electron storage ring creating a very intense, coherent beam at a single frequency

**SLS in Villigen**



**ESRF Grenoble**

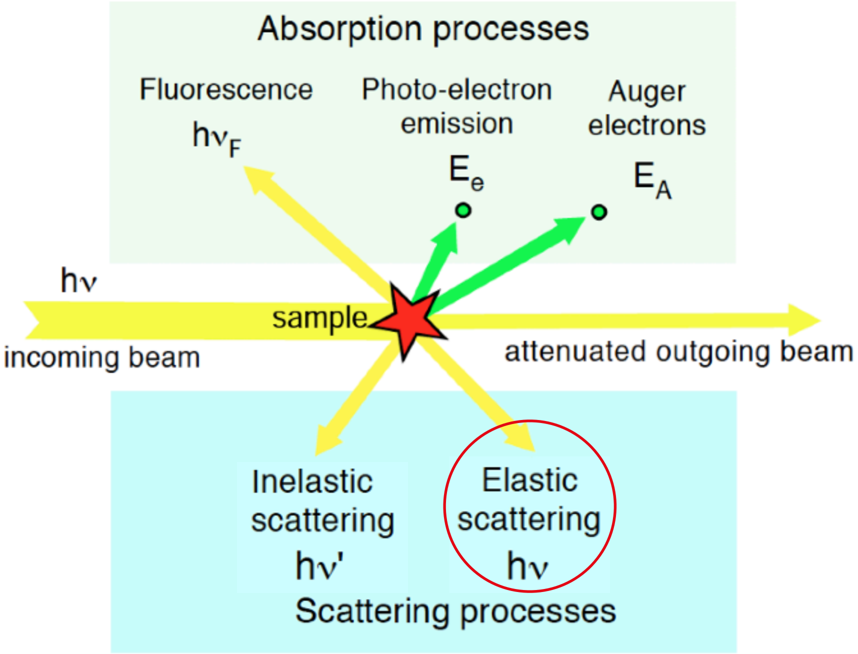


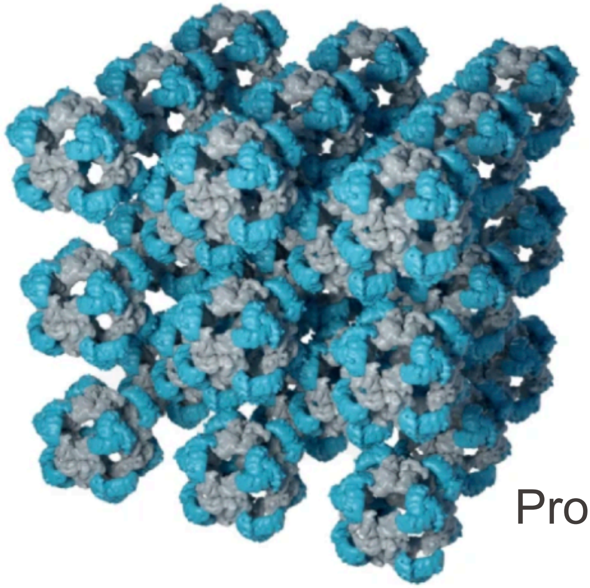
Crystal is mounted onto a goniometer-controlled stage and shot with X-rays while it rotates

Note: There are also smaller X-ray anode-based sources housed in research labs that are very much in use today

# Why do we need crystals?

- X-rays can interact with matter in different ways including **elastic scattering**
- X-rays are **scattered** by the electrons in atoms comprising the sample molecule



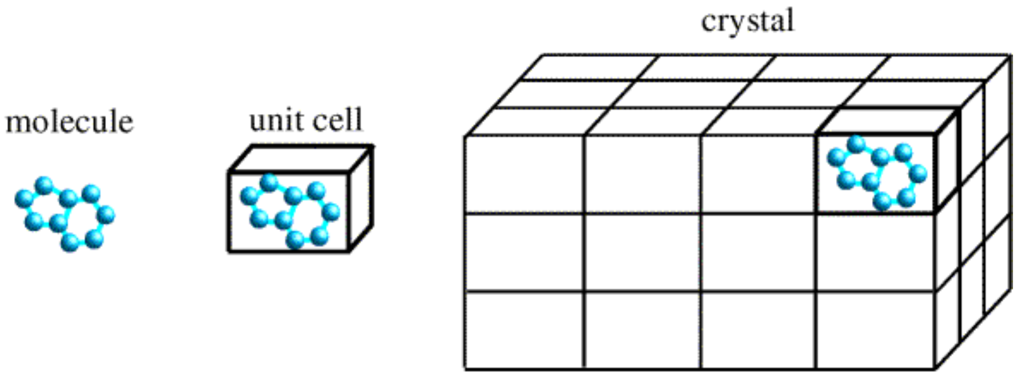


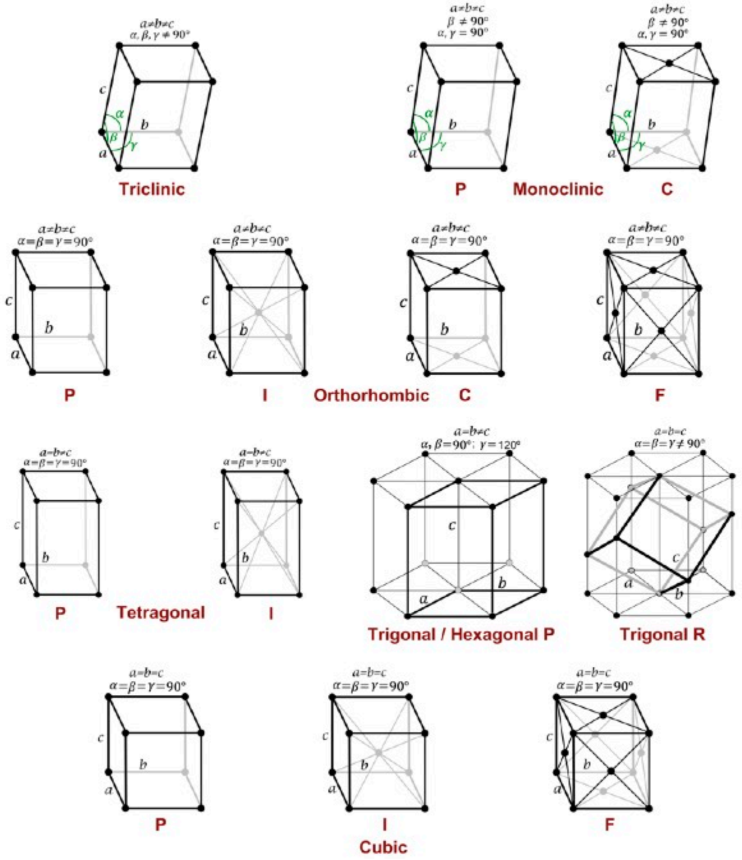
Protein crystal

- X-ray scattering from a single atom/molecule is too low to generate contrast while imaging.
- Protein crystals (0.2 mm cube) contain ~$10^{15}$ molecules. They scatter in phase and **amplify the signal.**

# What are crystals?

- A form of solid state where molecules are packed together in an ordered lattice held together by non-covalent bonds
- **Unit cell** is the smallest repeating structural unit of dimensions (a,b,c) and angles (α, β, γ)
- **Bravais lattice** defines how atoms can assemble into unit cells compatible with highly regular crystal pattern
- **14 different Bravais lattice systems**
- **230 space groups** = lattice + rotational and translational symmetries

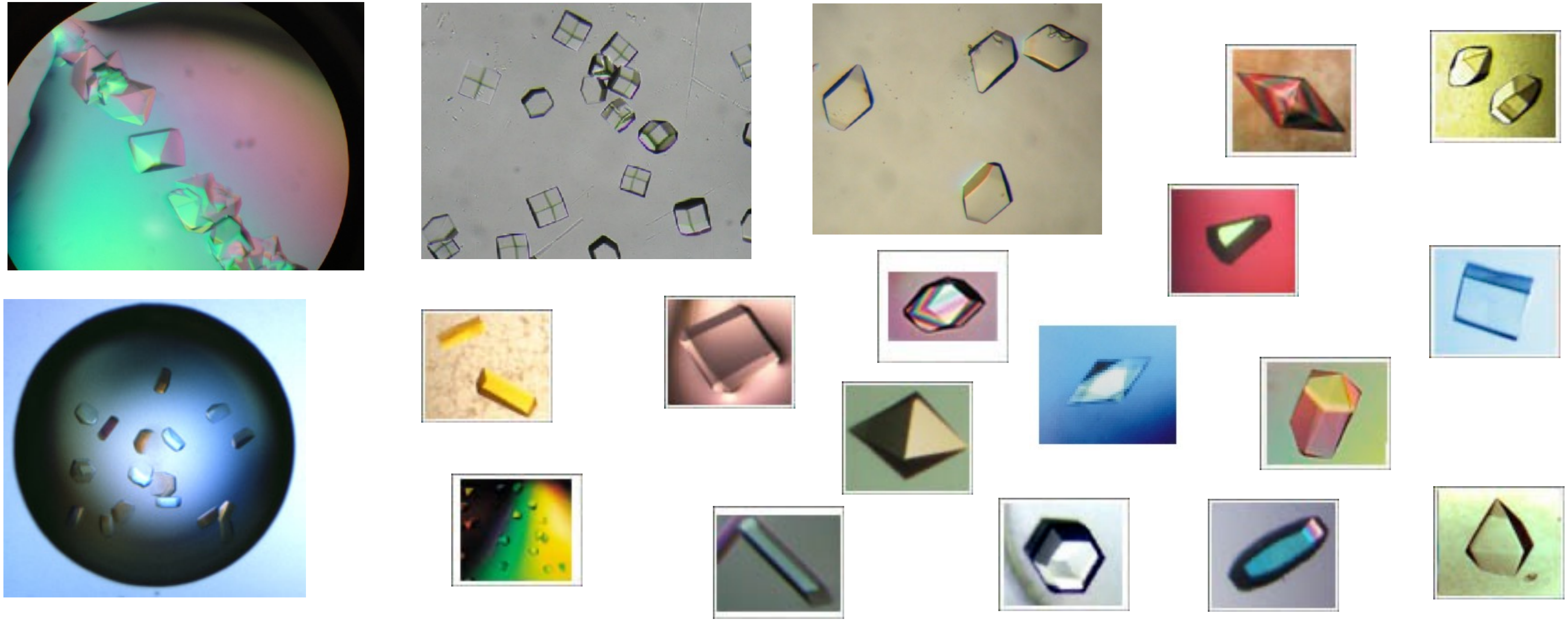




Auguste Bravais
(1811-1863)

# Protein crystals - Difficult to make but very beautiful

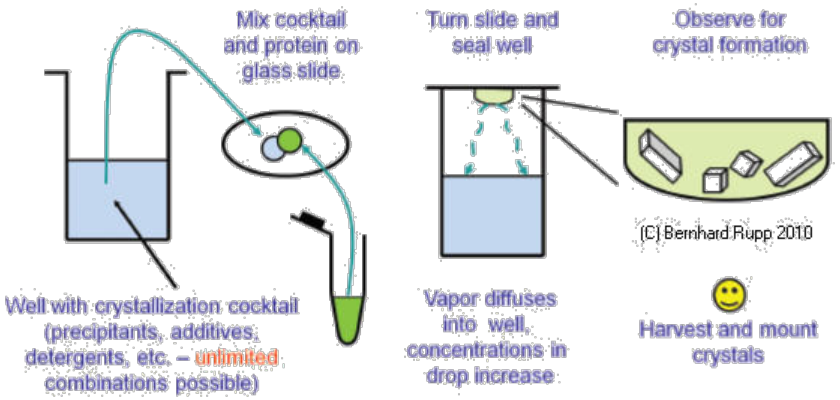
- Proteins do not naturally crystallize, and most of the work in the process is spent on their production



- Proteins are crystallized by **precipitation with high amounts of salts, organic solvents or polymers**
- Typical sizes needed for structural analysis are **0.1 - 0.3 mm**

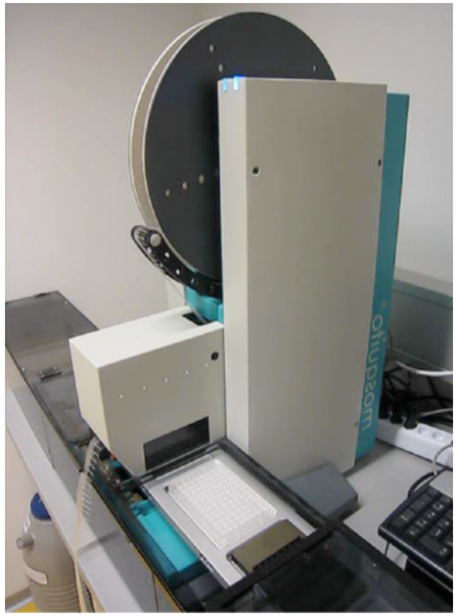
# How to get protein crystals?

- Mix purified concentrated protein with a crystallisation cocktail
- Try a few hundred (or thousand) combinations and vary also temperature (20 °C or 4 °C are most common)
- Wait! (minutes, hours, days, weeks, months, ...)
- Components of protein crystallization solutions:
  - Buffer
  - Salt
  - Precipitant
  - Additive (detergents, organic molecules, ...)
  - Protein concentration (typically high: >2mg/ml, sometimes up to 50mg/ml)



**induce production of crystals using vapor diffusion techniques:**

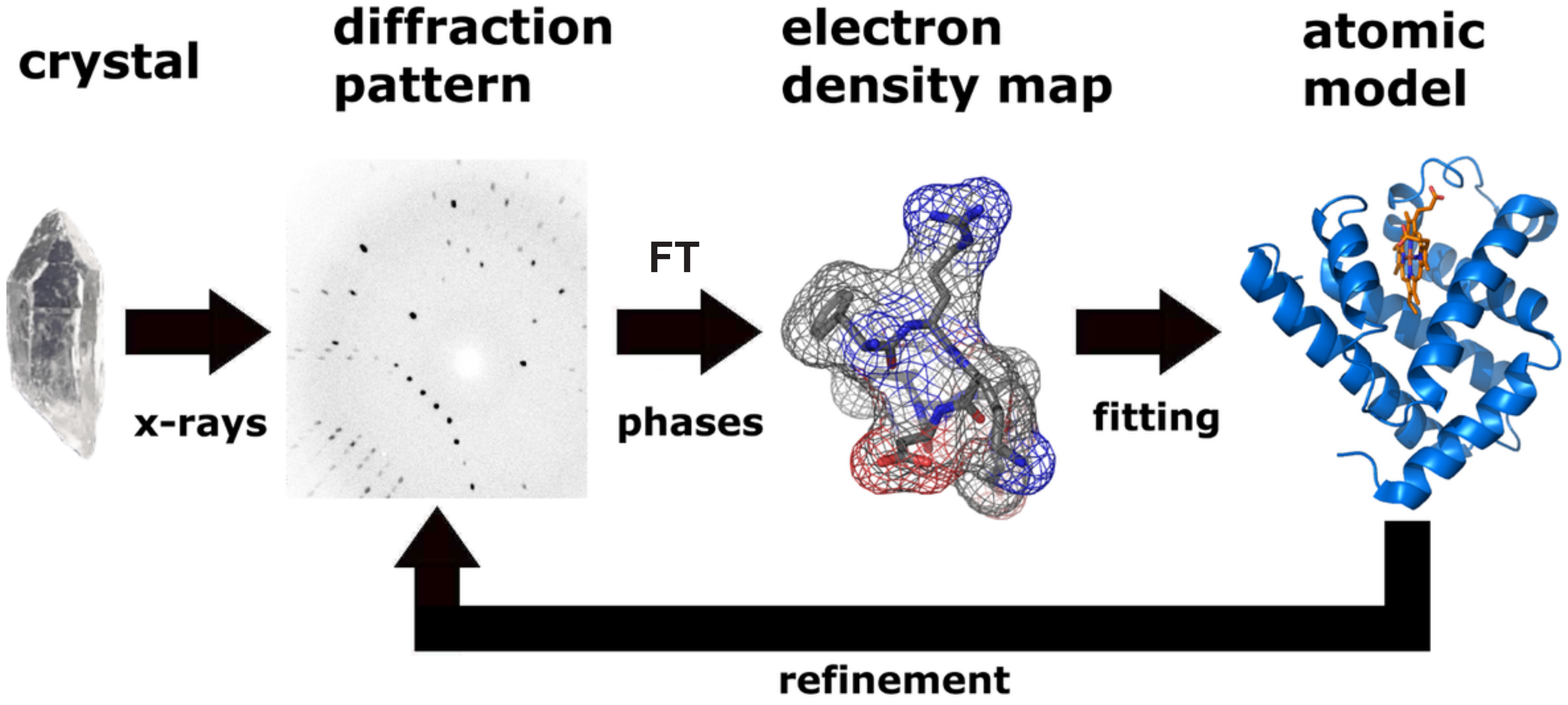
- technique the most used for crystallization
- diffusion of water from drop to well
- allow the screen of thousands of different precipitants in presence of your protein
- goal is to reduce solubility of the sample



**(Mosquito, TTP biotech)**

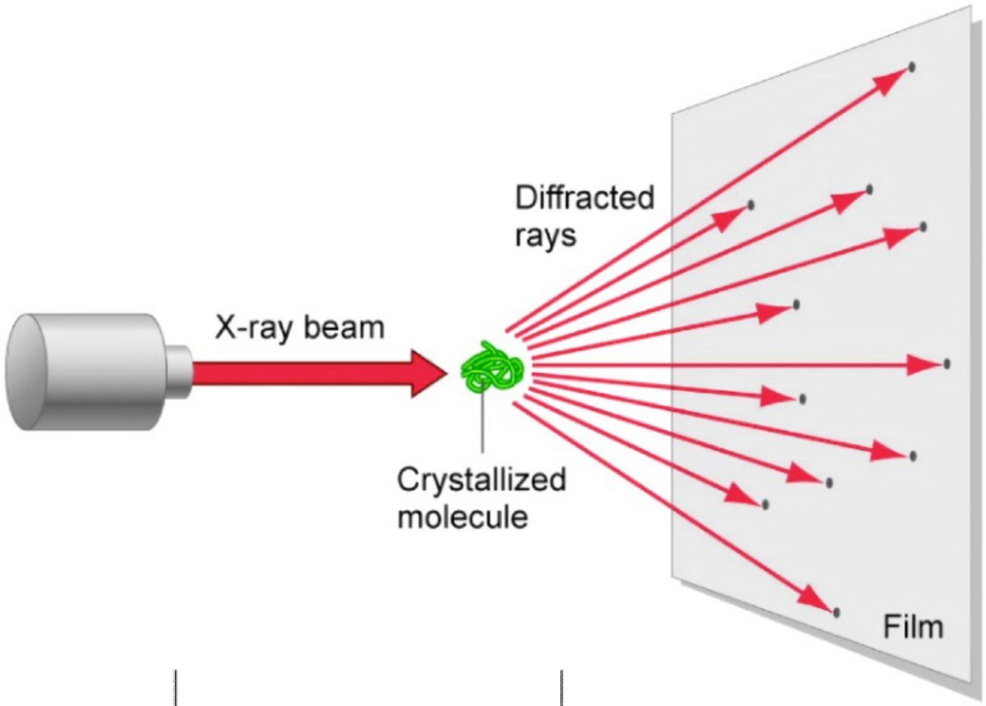
# Structure-solving using X-ray crystallography

- Protein crystals are placed in the X-ray beam which produces a diffraction pattern (diffractogram)
- Fourier transform of the diffractogram produces real-space map that can be used for model building

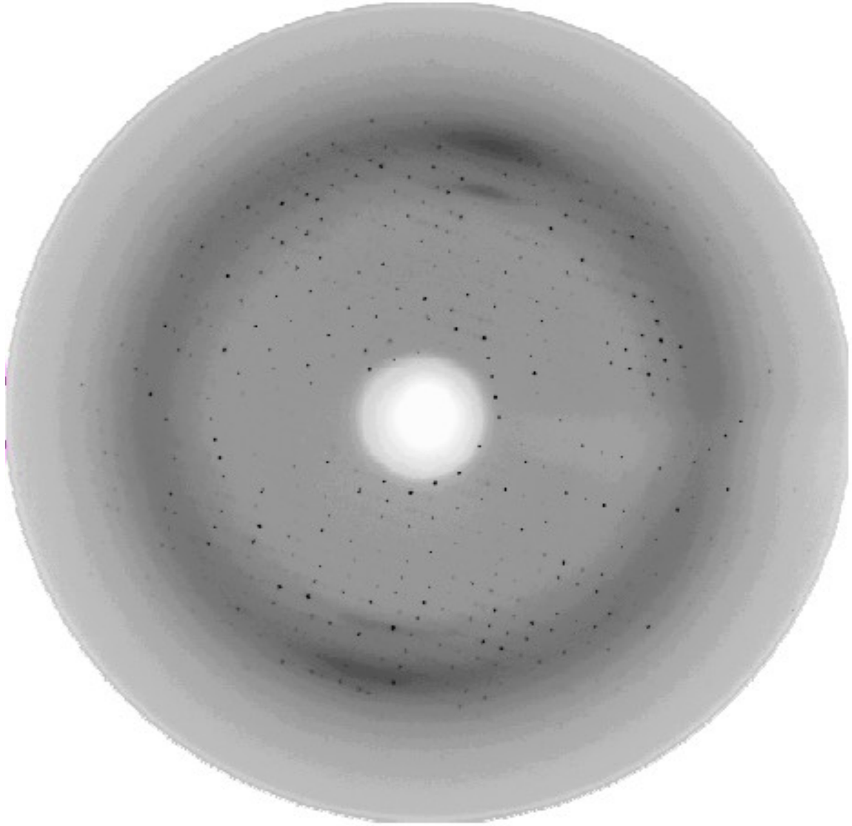


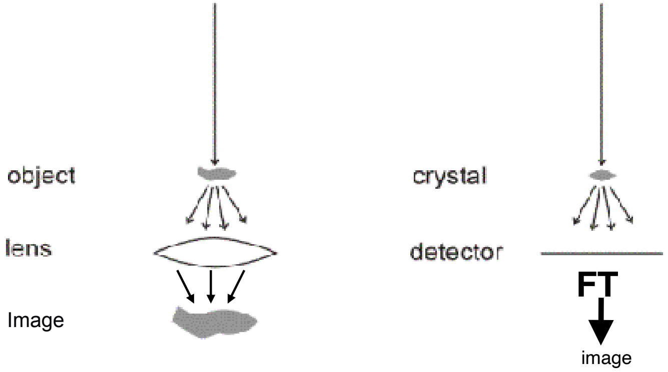
# Diffraction pattern

- Diffraction pattern is the raw output of X-ray scattering experiments from crystalline samples



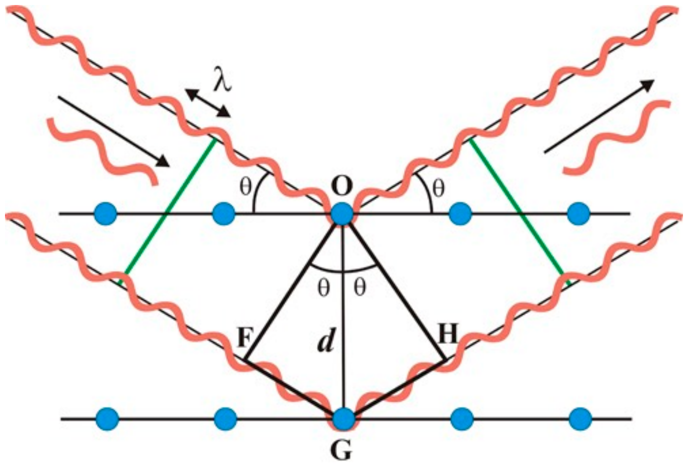
Where are the spots coming from?





# Diffraction pattern origin

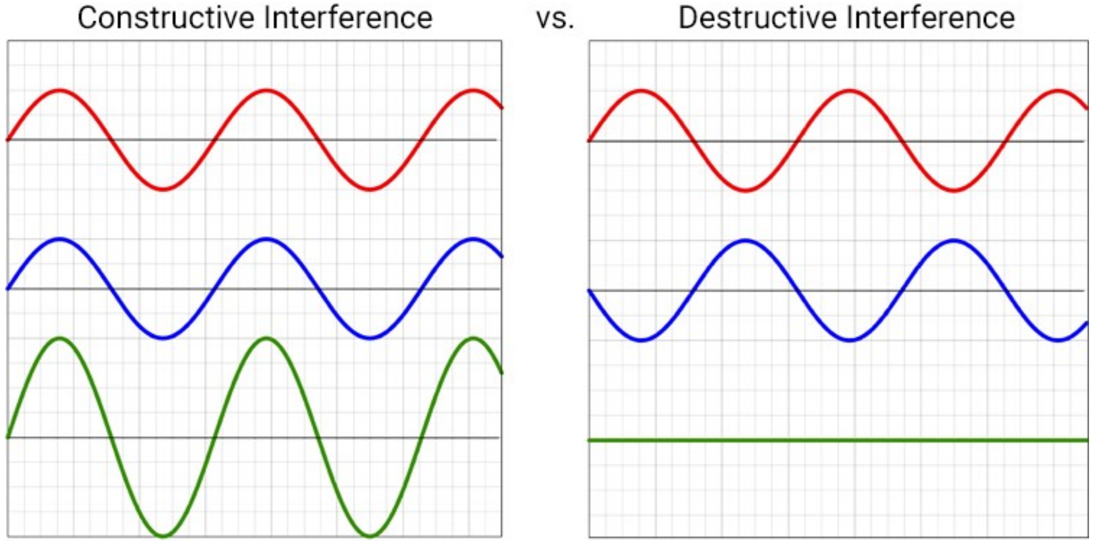
- Diffraction pattern is generated as a result of (constructive) **interference of X-rays elastically scattered from electrons** in the crystallized biomolecule
- Bragg's law connects the scattering angles and intensities of spots to the information on spacing in the crystal, i.e. on the position of atoms in a protein molecule



$$\boxed{n\lambda = 2d \sin\theta}$$

- λ is the wavelength of incident X-rays
- d is the spacing between the planes in the atomic lattice
- θ is the angle between the incident ray and the scattering planes

- Depending on the phase offset between the two X-rays the waves will interfere differently

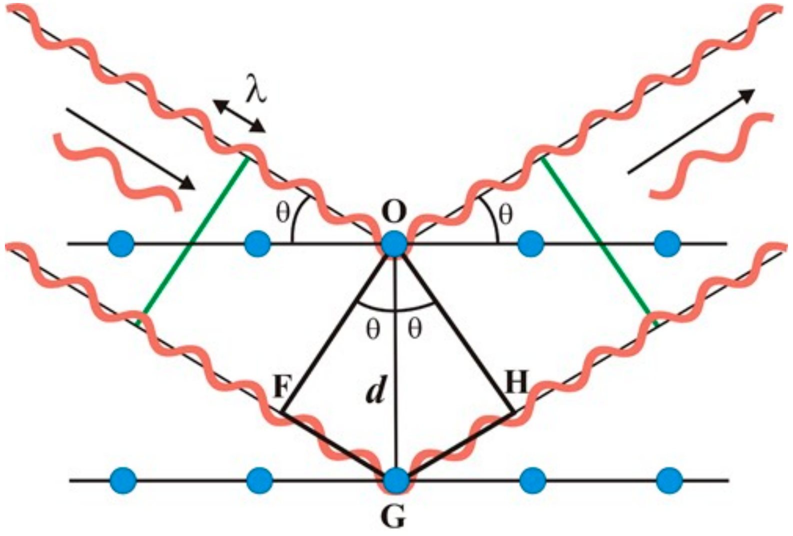


Intensity ~ $(\text{Amplitude})^2$

# Resolution can be estimated from diffraction patterns

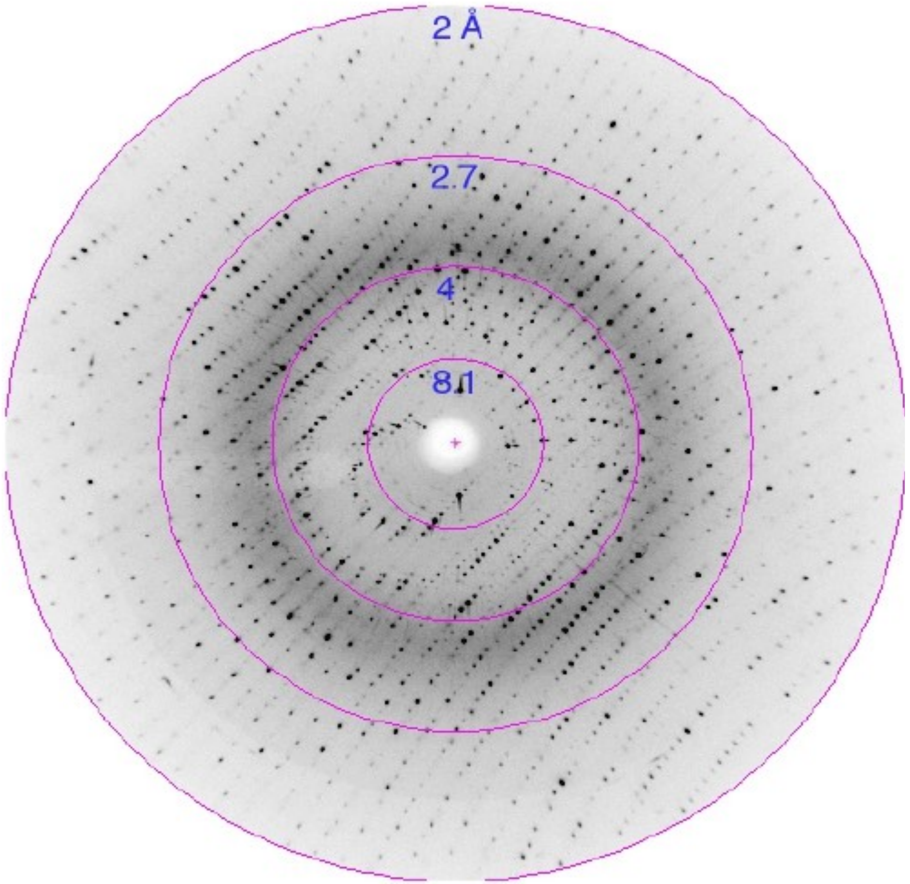
- The further out spots (wider ϴ angles) are visible the finer the distance between diffracting atoms
- This **minimum measurable distance translates into the resolution** of reconstructed 3D map

Bragg's law



**$n\lambda = 2d \sin\theta$**

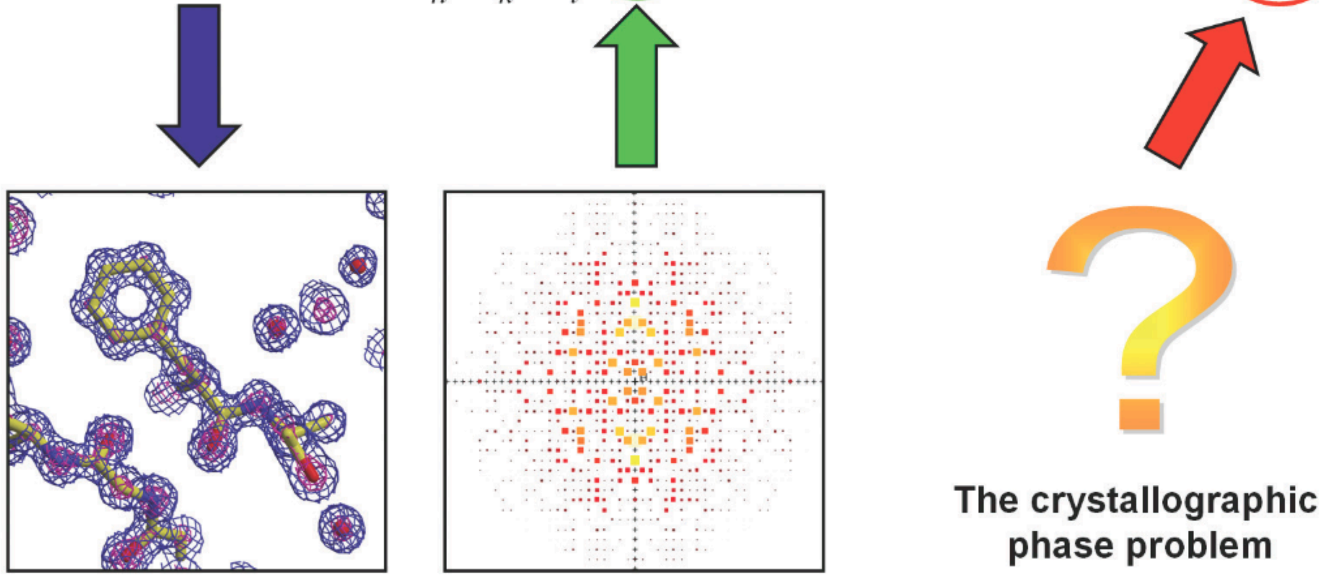
**$d \sim 1/\sin\theta$**



# Reconstructing electron density map

- The diffraction pattern is related to the scattering object by a Fourier transform:

$$\rho(x,y,z)=\frac{1}{V}\sum_{-h}^{h}\sum_{-k}^{k}\sum_{-l}^{l} F_{hkl}\cdot\exp[-2\pi i(hx+ky+lz-\alpha_{hkl})]$$



**The crystallographic phase problem**

**$F_{hkl}$** - Structure Factor at coordinates h,k,l
**h,k,l** - Miller indices (dimensions in reciprocal space)
**$\rho_{xyz}$** - Electron density at position xyz
**x,y,z** - Coordinates in real space
**$\alpha_{hkl}$** - Phase angle of X-rays hitting position h,k,l

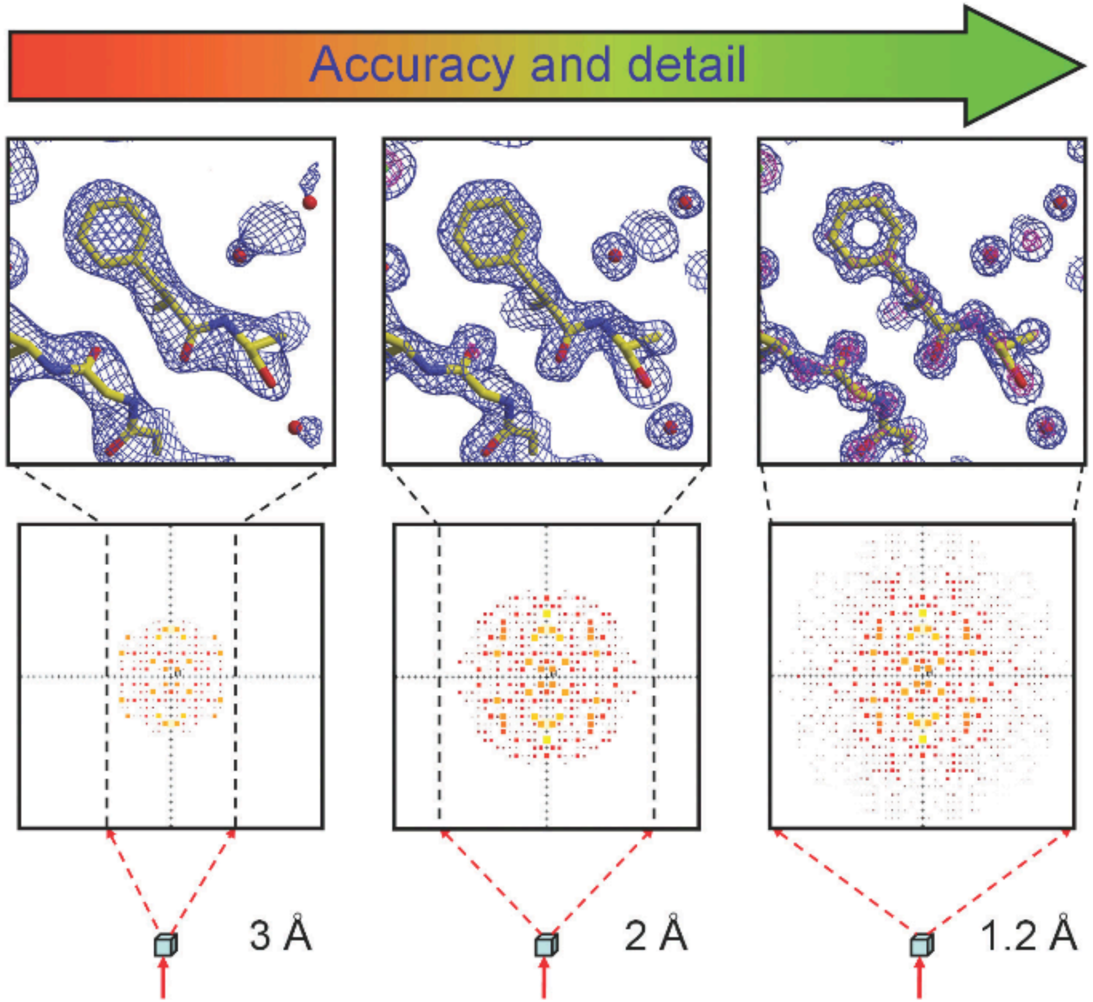
- Detectors measure only intensity (amplitudes) of X-rays but not the phase of the resulting X-rays

**- Phase problem** can be solved using heavy metals during crystallization or a known starting model of the protein during reconstruction.

- Structure factors F(h,k,l) are calculated from the diffraction pattern and “inverted” to reveal the 3D structure of a scattering object in real space (x,y,z)

# Interpreting the map and reconstructing the model

- Resulting electron density maps are used for building atomic models of the biomolecules
- The higher the resolution (lower value in Å) the greater the detail in EM maps



In case of proteins and nucleic acids:

| | |
|---|---|
| **10 - 20 Å** | Molecule shape |
| **5 - 10 Å** | Secondary structure elements |
| **4 - 5 Å** | Main-chain trace (+ bulky side-chains) |
| **1.5 - 4 Å** | Side-chain densities |
| **<1.5 Å** | Atoms |

The interpretation of data is confined by the final map resolution (i.e., you cannot build atoms in a 20 Å map)

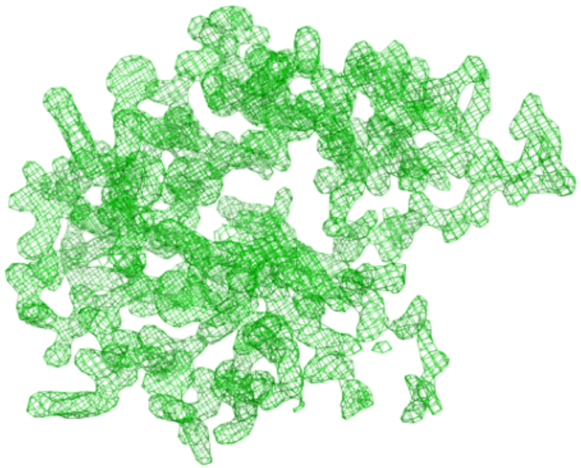
# Reconstructing the atomic model

- The structure is built using:
  - Electron density map restraints
  - Known sequence of the model (e.g., amino-acid sequence)
  - Geometric restraints (e.g., Cis-Trans peptide bonds, allowed Ramachandran angles)

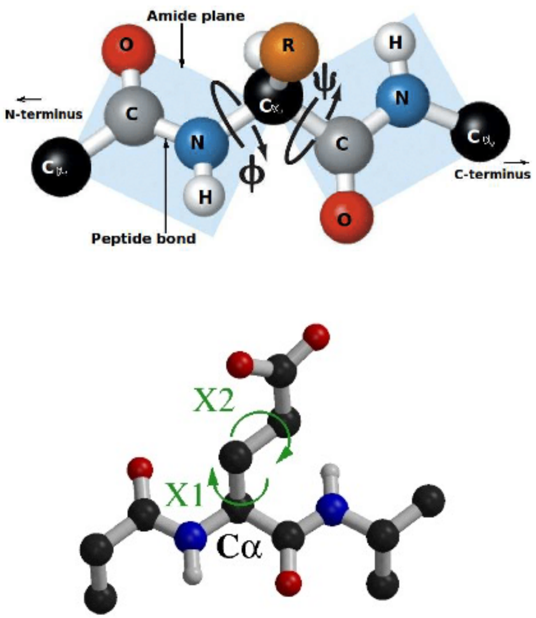
Sequence:

PVNSLEKHSWYHGPVSRNAAEYLLS
SGINGSFLVRESESSPGQRSISLRYE
GRVYHYRINTASDGKLYVSSESRFNT
LAELVHHHSTVADGLITTLHYPAPKRN

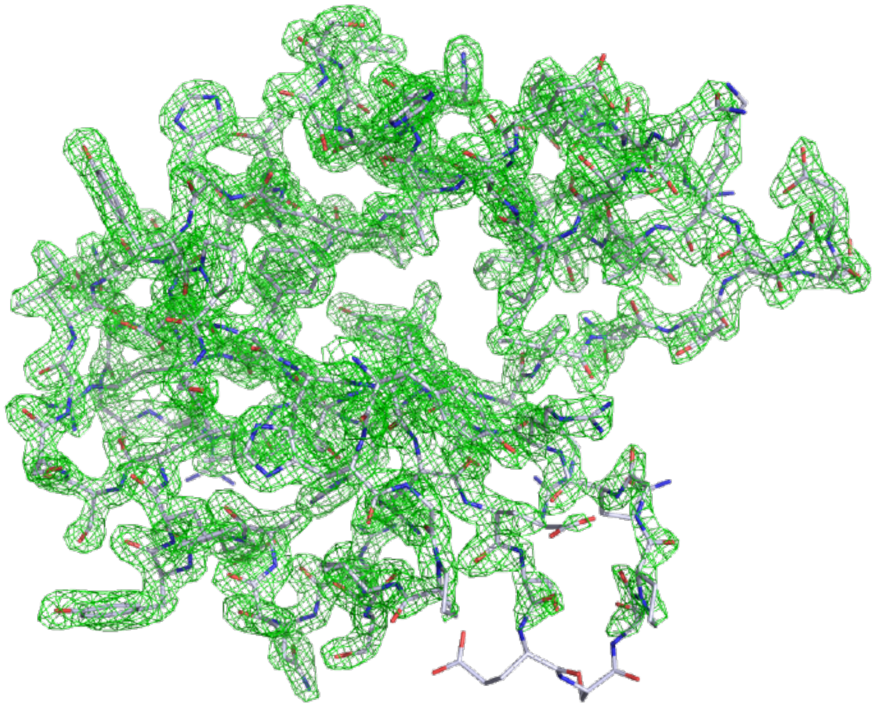
Electron density map

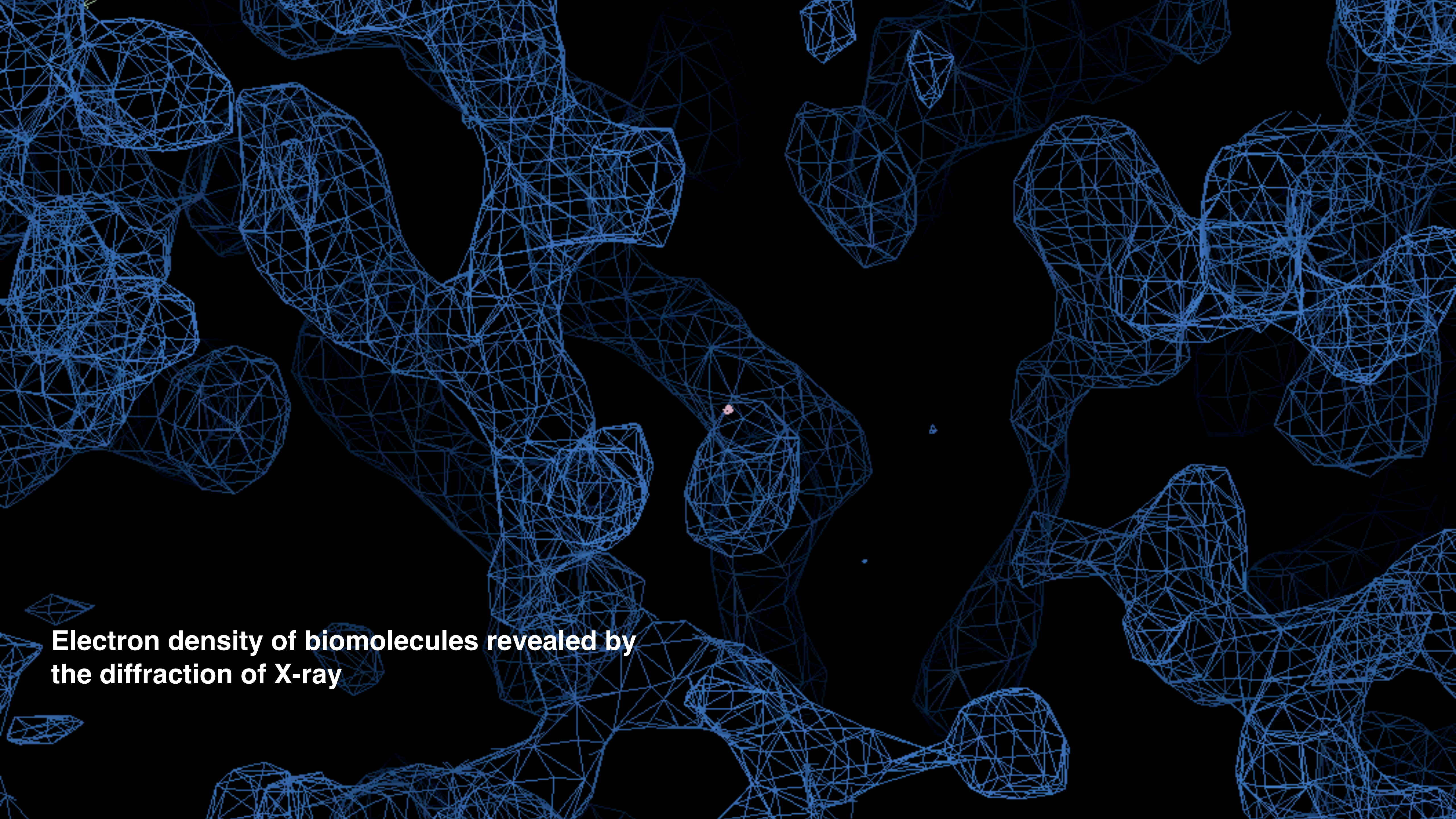


Geometric restraints:

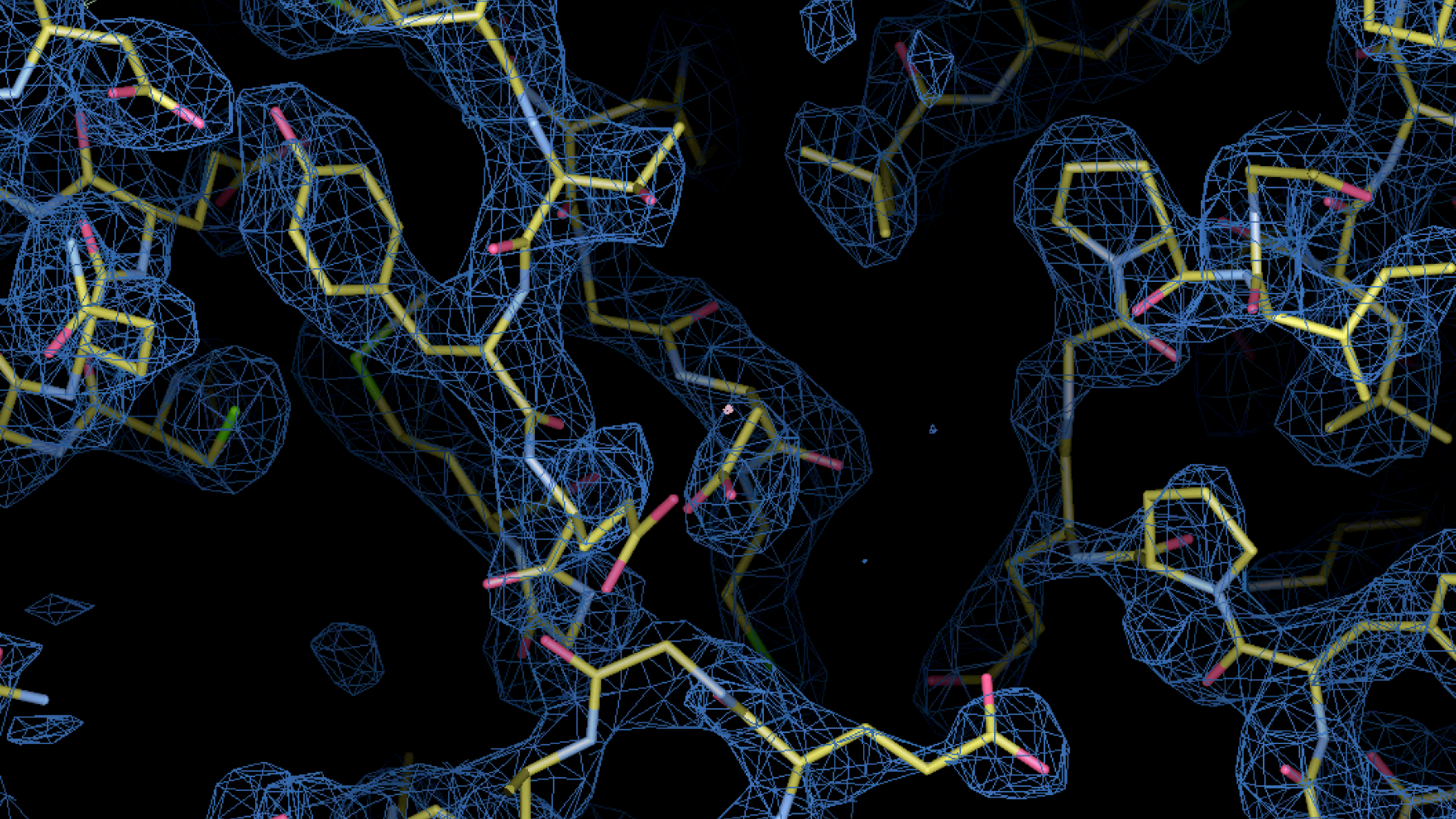


Atomic model



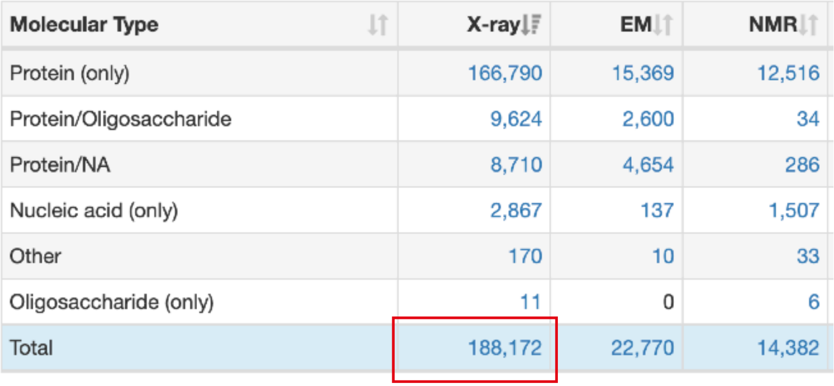


Electron density of biomolecules revealed by the diffraction of X-ray



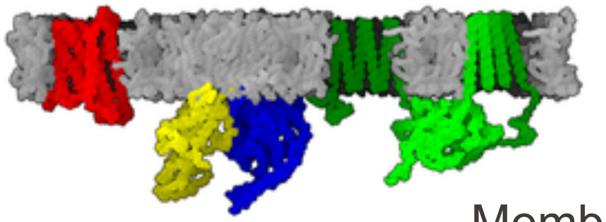
# X-ray crystallography - Pros and Cons

- **Positive sides**
- Versatile and highly applicable
- Historically, it is the most fruitful method for structure determination
- No limitations with respect to molecular weights of biomolecules that are studied
- No limitations in terms of achievable resolutions



| Molecular Type | X-ray | EM | NMR |
|---|---|---|---|
| Protein (only) | 166,790 | 15,369 | 12,516 |
| Protein/Oligosaccharide | 9,624 | 2,600 | 34 |
| Protein/NA | 8,710 | 4,654 | 286 |
| Nucleic acid (only) | 2,867 | 137 | 1,507 |
| Other | 170 | 10 | 33 |
| Oligosaccharide (only) | 11 | 0 | 6 |
| Total | 188,172 | 22,770 | 14,382 |

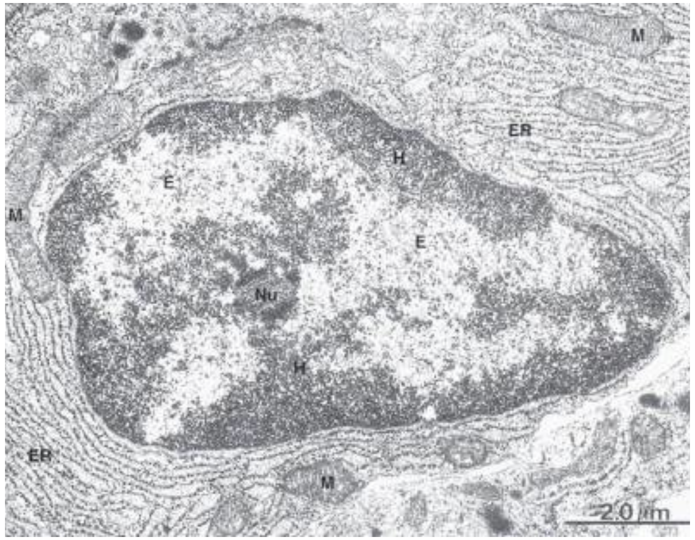
- **Negative sides**
- Needs highly purified and homogenous biomolecule material
- Biomolecule crystallization is a very challenging and unpredictable process
- Cannot be used to study heterogeneous samples (e.g., flexible complexes, pleomorphic viruses)
- High material requirements (many milligrams of purified biomolecule)
- It is a static picture of a molecule and cannot capture dynamic information
- Difficult to solve membrane proteins due to heterogeneous membrane environment



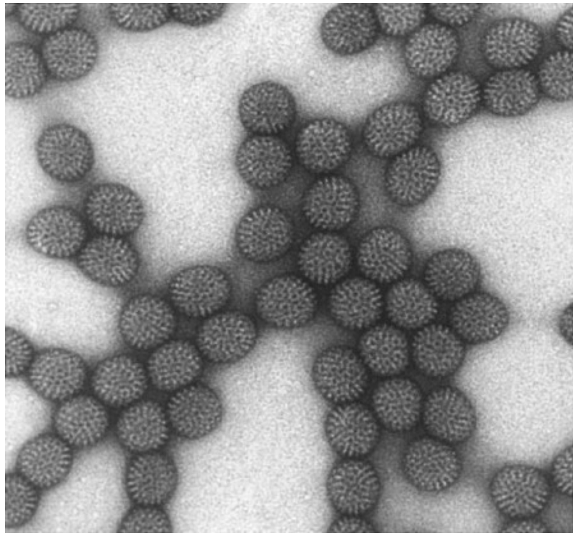
Membrane proteins

# Electron Microscopy

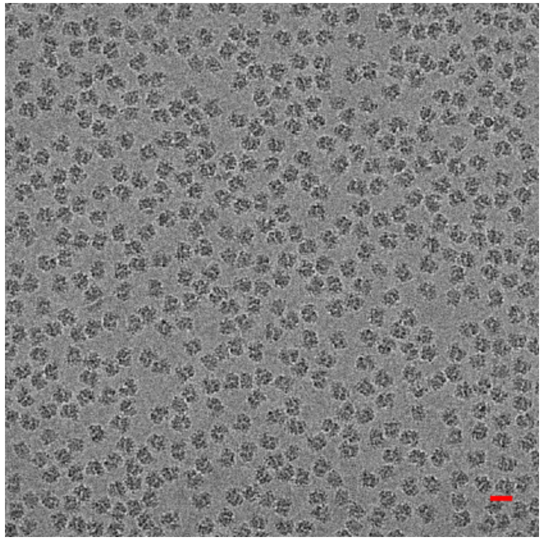
Nucleus of a cell



Rotavirus



Ribosomes

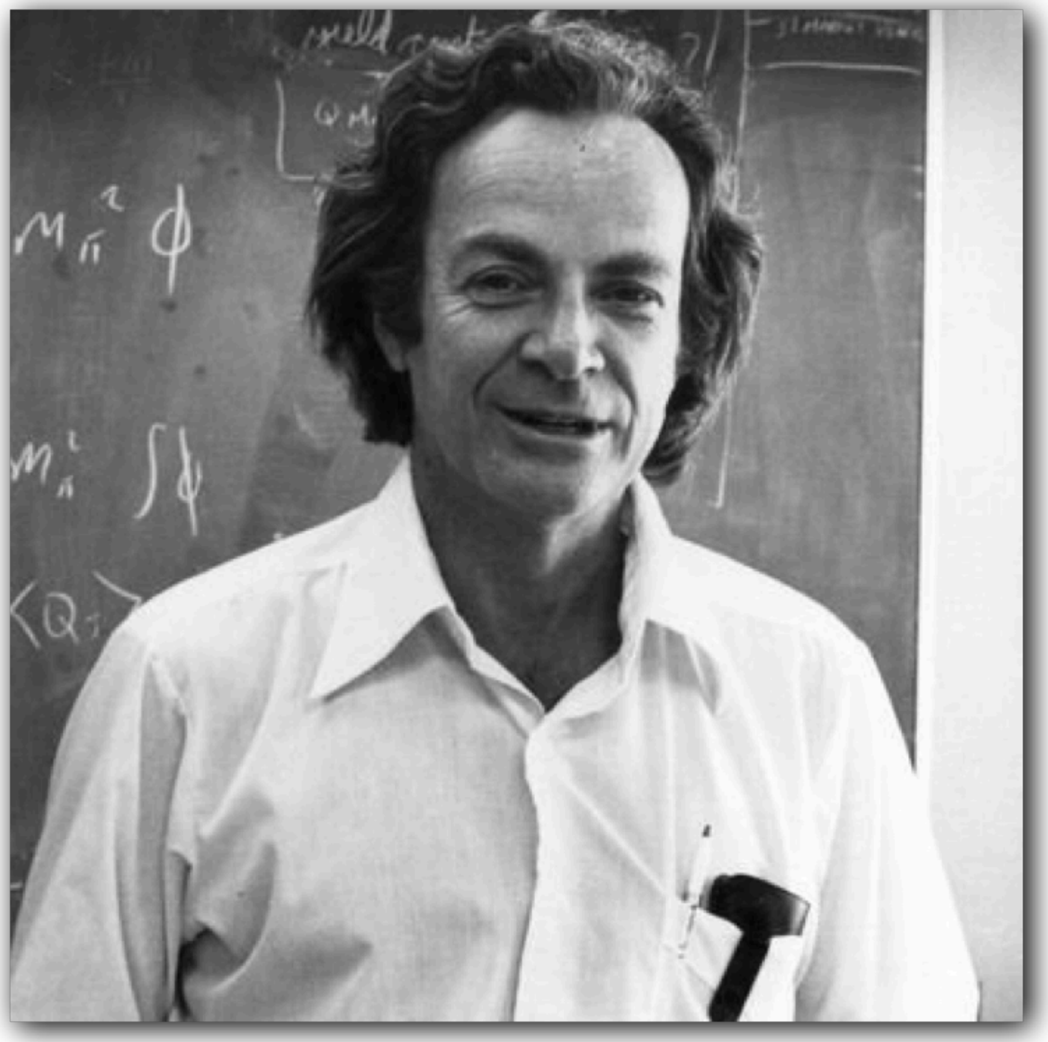


… same technique, but at different magnification, as protein are much smaller than cells!

# How to visualize biomolecules?

"It is very easy to answer many of these
fundamental biological questions;
you just look at the thing!... Make the microscope
one hundred times more
powerful, and many problems of biology would
be made very much easier.
I exaggerate, of course, but the biologists would
surely be very thankful
to you ….."

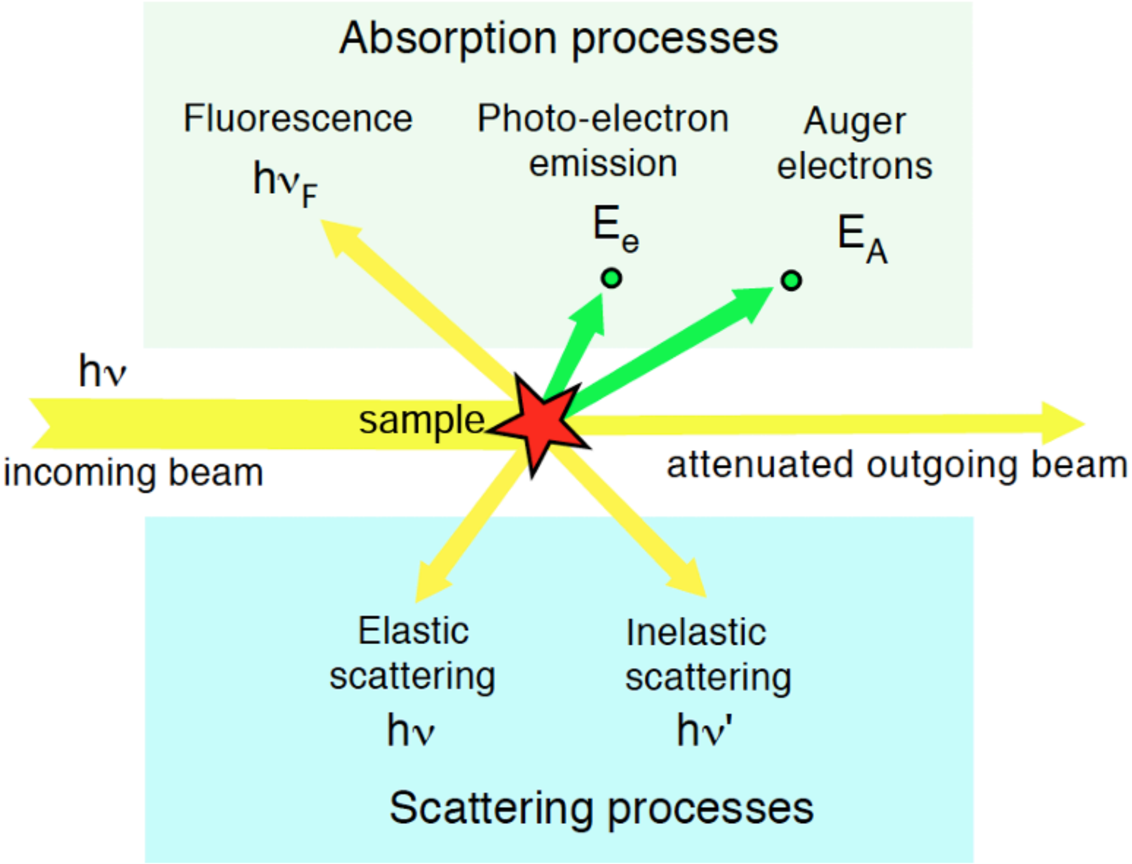
**Richard P. Feynman**



*"There's plenty of room at the bottom"*
lecture given at the APS in 1959

# X-rays for microscopy applications

- X-rays can interact with matter in different ways but are relatively poorly scattered



Absorption processes

Fluorescence

$h\nu_F$

Photo-electron emission

$E_e$

Auger electrons

$E_A$

$h\nu$

sample

incoming beam

attenuated outgoing beam

Elastic scattering

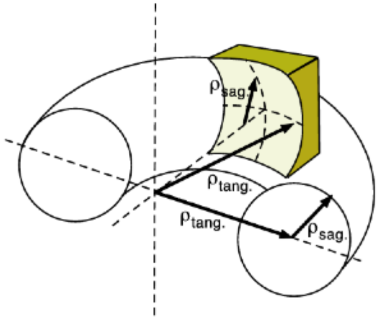
$h\nu$

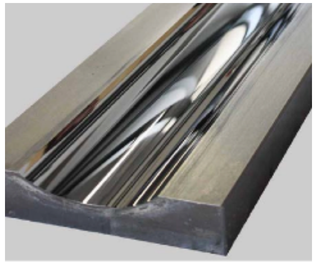
Inelastic scattering

$h\nu'$

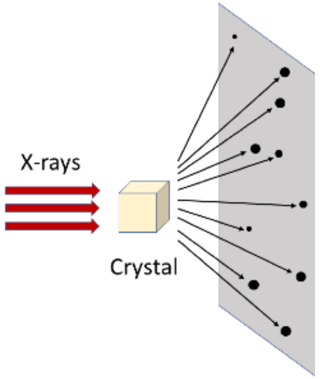
Scattering processes

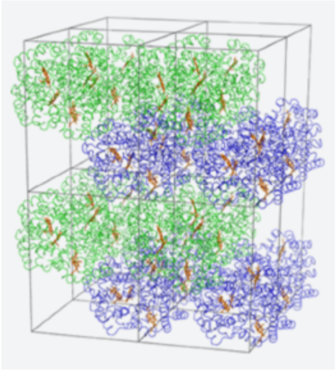
- Difficult to produce good lenses/mirrors





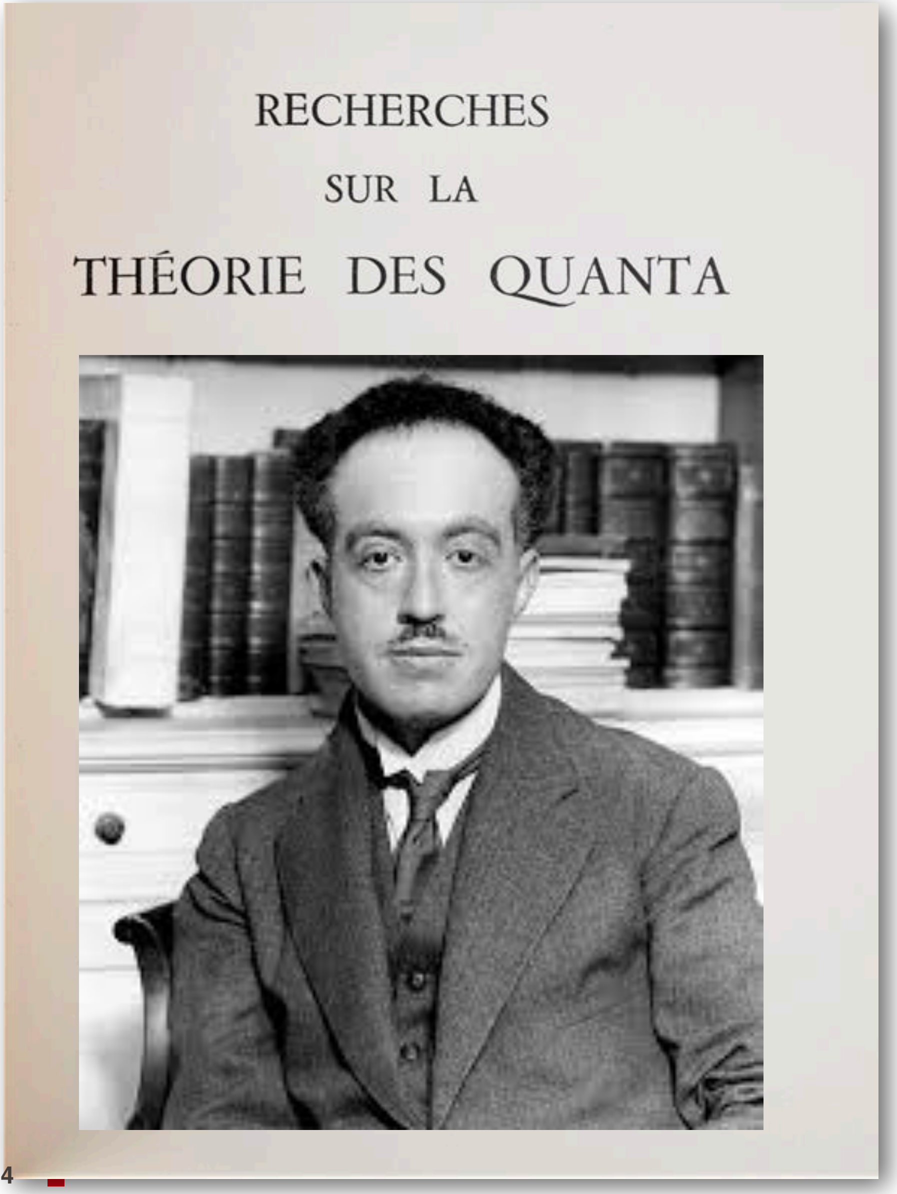
- Poor image contrast in a microscope





- Protein crystals help amplify the scattering signal

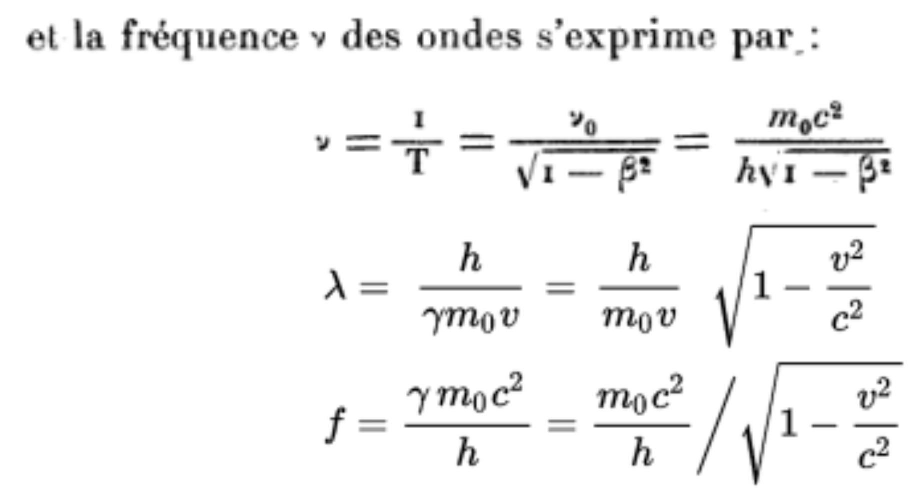
# Accelerated electrons as an alternative for X-rays



RECHERCHES
SUR LA
THÉORIE DES QUANTA

## 1924 thesis of Louis de Broglie

Combined the equations of special relativity & quantum theory to suggest that electrons could be thought of as waves as well as particles.



et la fréquence $\nu$ des ondes s'exprime par :

$$\nu = \frac{1}{T} = \frac{\nu_0}{\sqrt{1 - \beta^2}} = \frac{m_0 c^2}{h\sqrt{1 - \beta^2}}$$

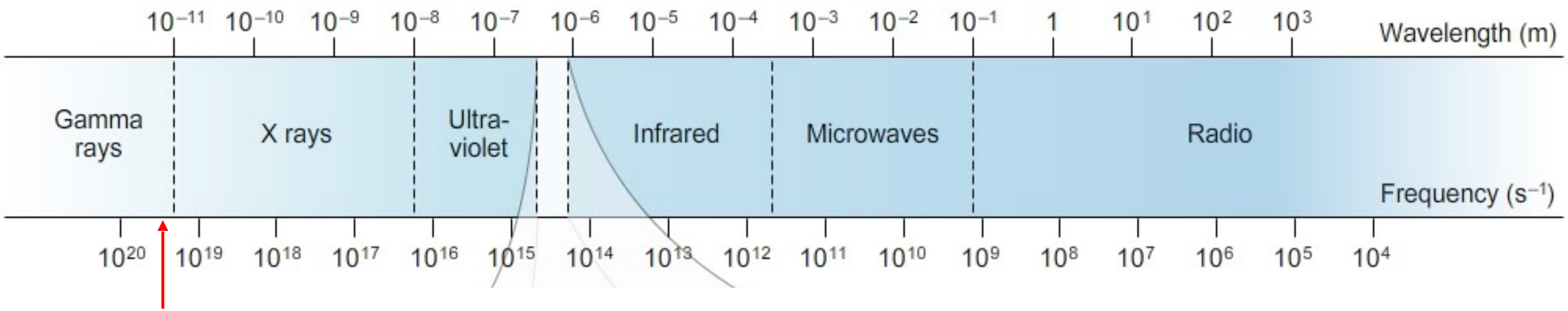
$$\lambda = \frac{h}{\gamma m_0 v} = \frac{h}{m_0 v} \sqrt{1 - \frac{v^2}{c^2}}$$

$$f = \frac{\gamma m_0 c^2}{h} = \frac{m_0 c^2}{h} \Big/ \sqrt{1 - \frac{v^2}{c^2}}$$

$$\lambda = \frac{h}{p}$$

# Accelerated electrons as an alternative for X-rays

- Electrons with energy of **~100 keV** have a wavelength of **3.88 pm**



- Typical voltage in a microscope is 100-300 kV

- 100X shorter λ than typical X-rays in a synchrotron

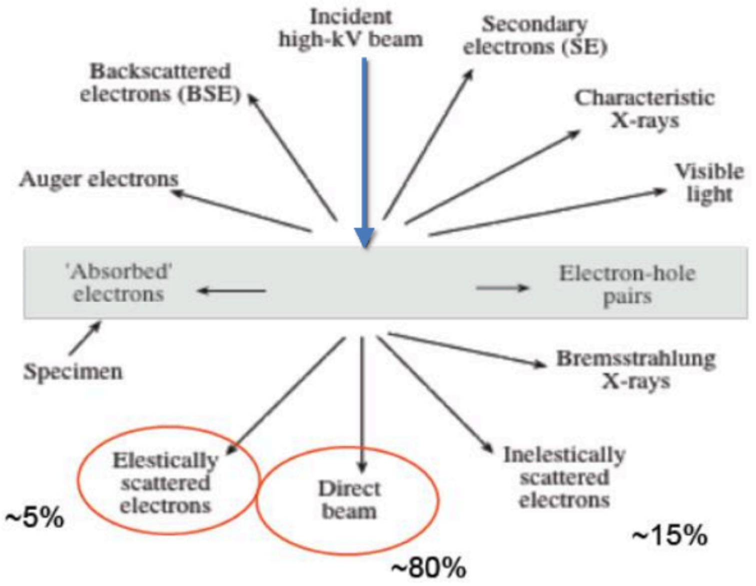
$$\lambda = \frac{6.626\times10^{-34}}{\sqrt{2V\times1.6\times10^{-19}\times9.11\times10^{-31}}}$$

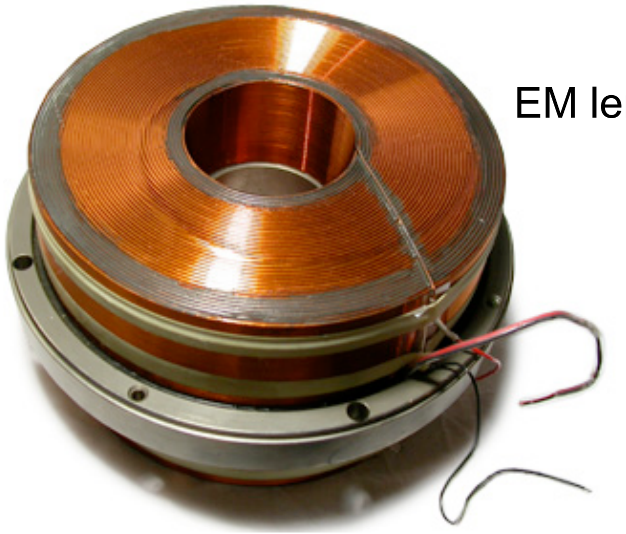
$$\Rightarrow \lambda = \frac{12.27\times10^{-10}}{\sqrt{V}}\text{meter}$$

$$\boxed{(\text{or})\ \lambda = \frac{12.27}{\sqrt{V}}\text{Å}}$$

# What are the advantages of electrons?

- The main benefit comes from the negative charge
- Compared to X-rays, elastic scattering of electrons is ~$10^3$-$10^6$ times stronger
- Electrons are easily accelerated using potential difference
- Electron beam can be readily collimated with electromagnetic lenses
- Readily detected by films and cameras





EM lens

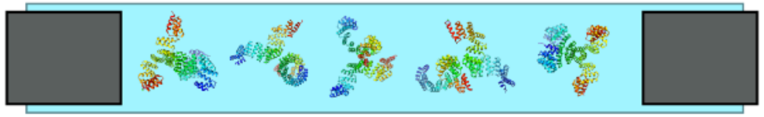
# What are the disadvantages of electrons?

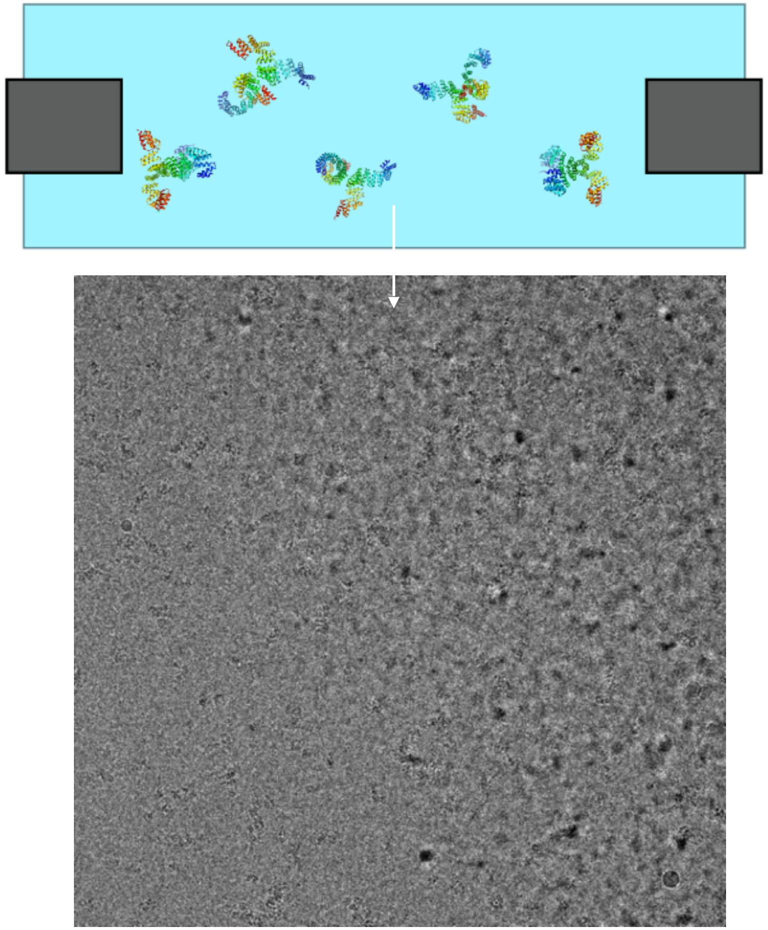
- Need high vacuum within the microscope to reduce interactions with air molecules

- Samples need to be applied as a very thin layer (~10-100 nm) to achieve sufficient contrast against surrounding ice

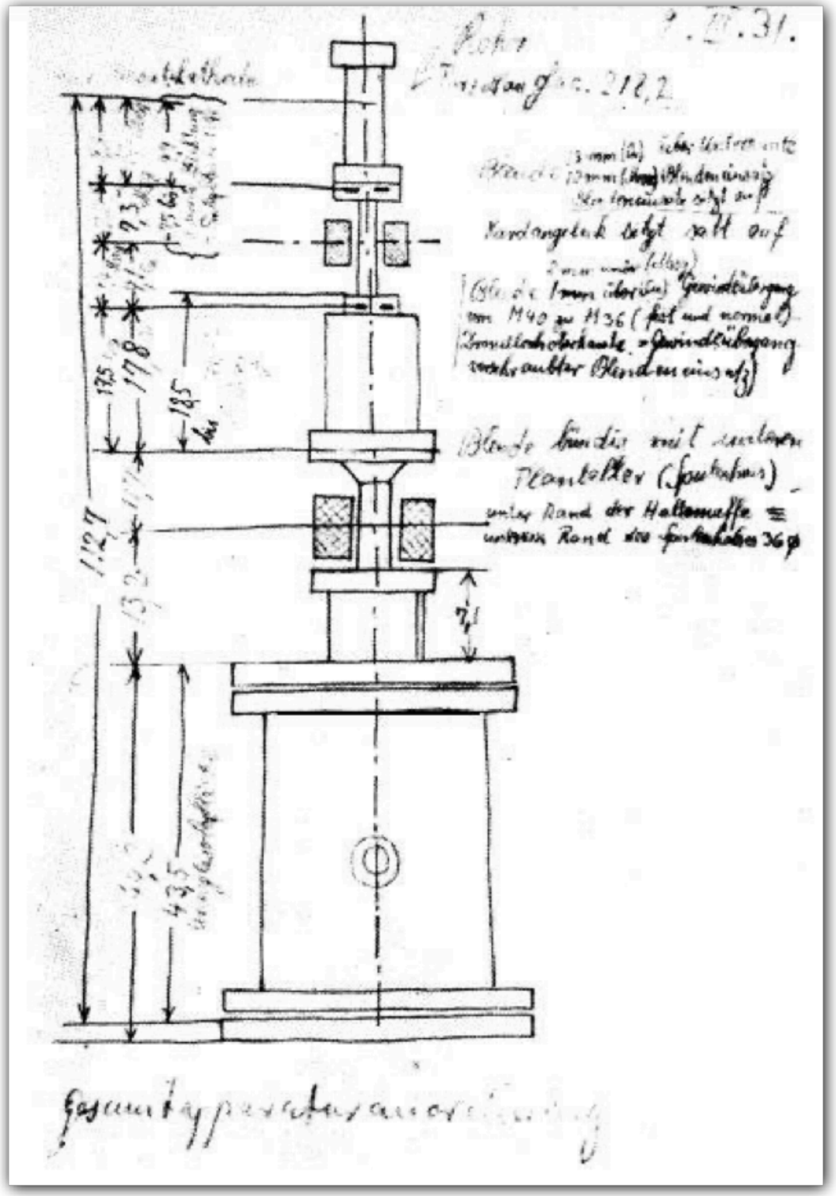
- Radiation damage in the sample caused by inelastic scattering of electrons (i.e., collisions with biological material leading to energy transfer)

- Insufficient contrast when imaging small proteins (lower molecular weight limit for EM is ~50 kDa)

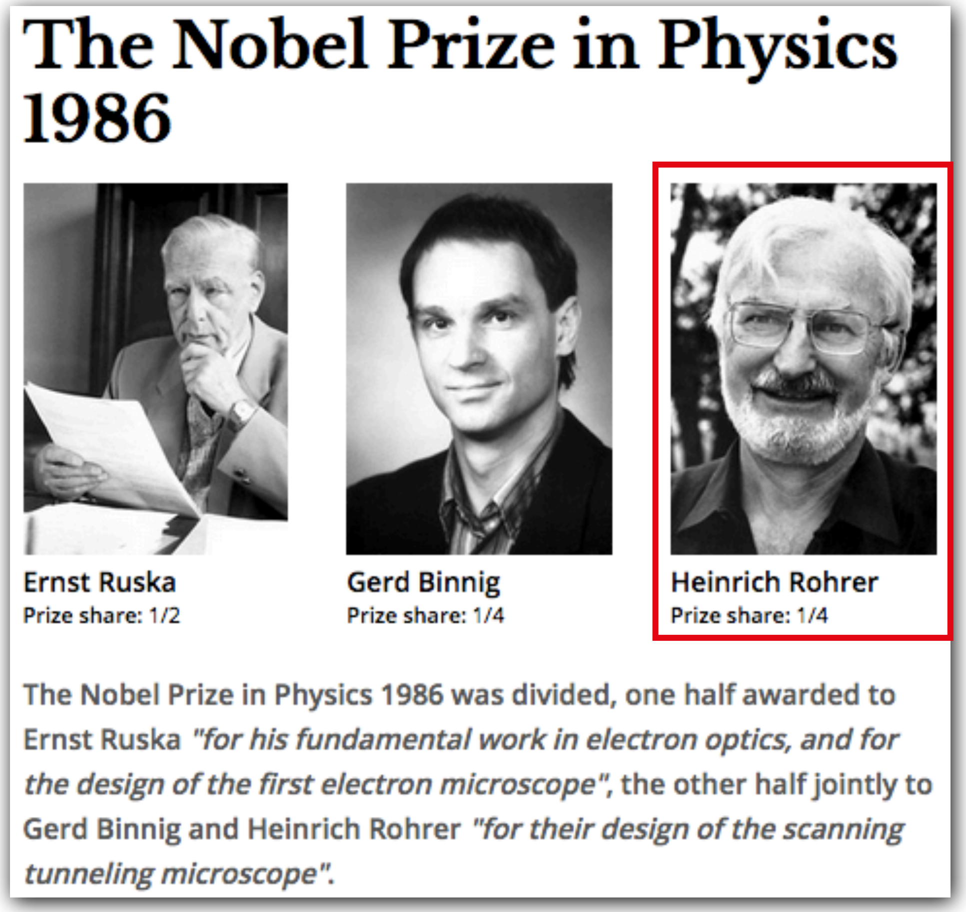




# The first Transmission Electron Microscope



# The first Transmission Electron Microscope



## The Nobel Prize in Physics 1986

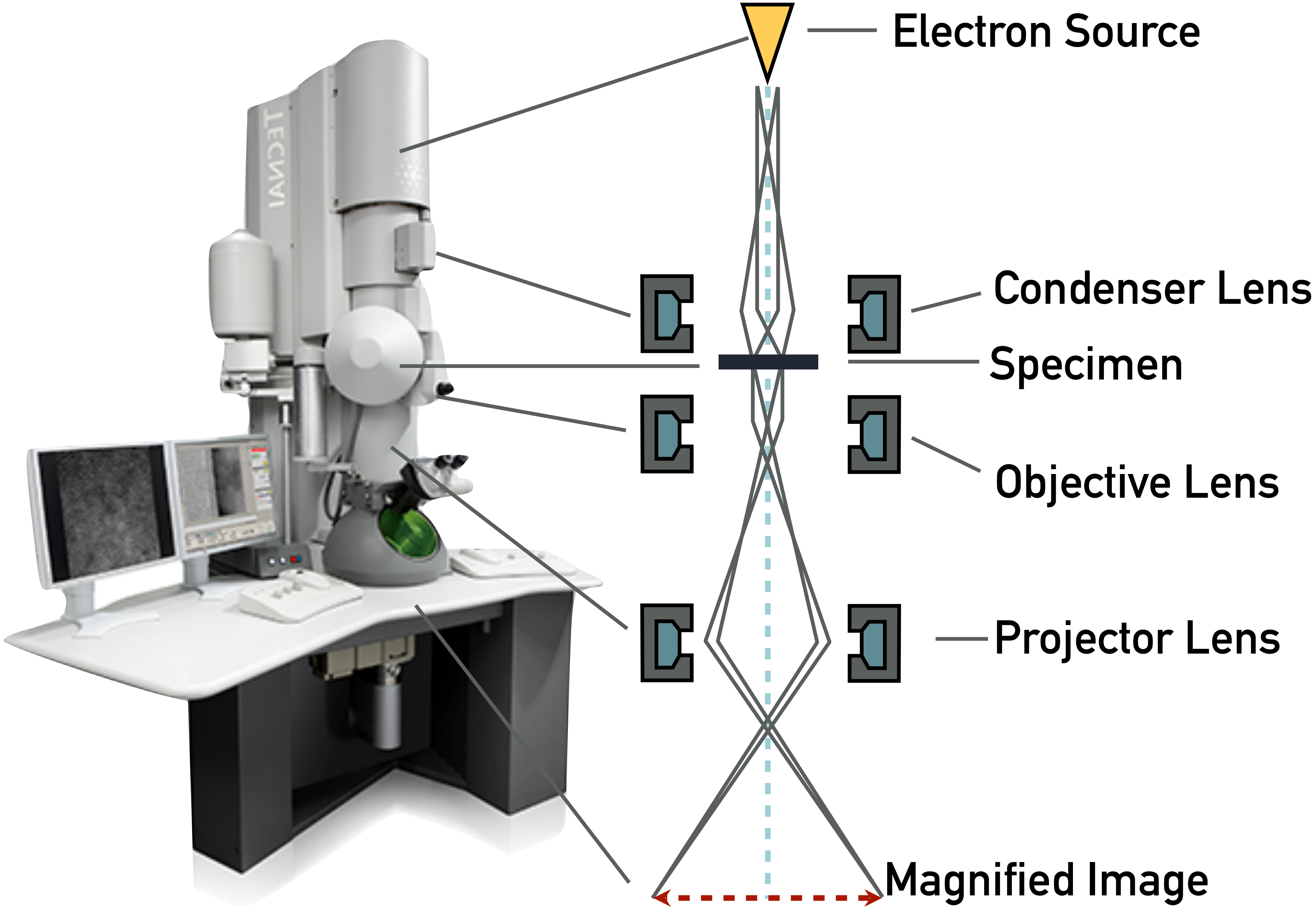
**Ernst Ruska**
Prize share: 1/2

**Gerd Binnig**
Prize share: 1/4

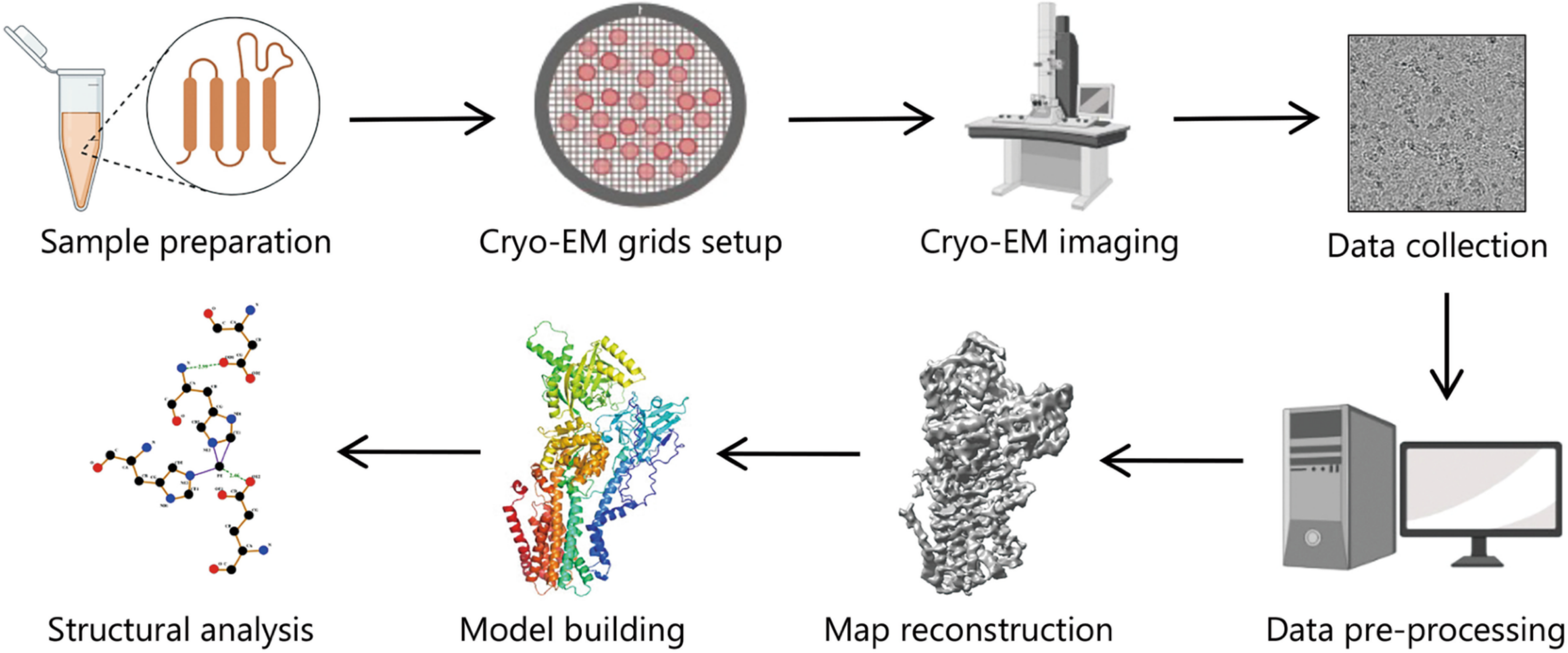
**Heinrich Rohrer**
Prize share: 1/4

The Nobel Prize in Physics 1986 was divided, one half awarded to Ernst Ruska *"for his fundamental work in electron optics, and for the design of the first electron microscope"*, the other half jointly to Gerd Binnig and Heinrich Rohrer *"for their design of the scanning tunneling microscope"*.

# Microscope principle and components



# Typical cryo-EM workflow



- Unlike X-ray crystallography, cryo-EM maps are recovered in real-space

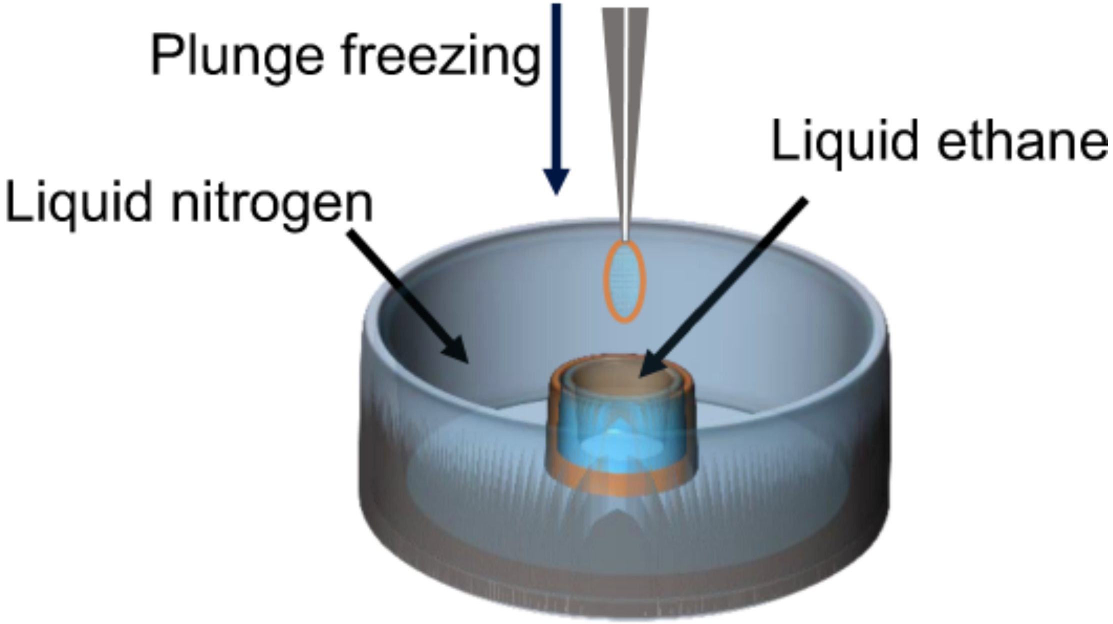
# Preserving Biological Specimens for EM Imaging

- Biological samples are about 80% water
  - Placing biological samples into the vacuum of electron microscope will cause immediate dehydration and destruction of sample
  - Need to preserve ultrastructure to withstand vacuum

- Biological samples are susceptible to radiation damage
  - Conversion of electron beam flux to radiation dose:
    1 Coulomb/m$^2$ @100keV = 40 Mrad (4x10$^7$ rad)
  - To view samples in EM, typically use 30 C/m$^2$/sec (1200Mrad/sec)

Equivalent to standing 30 m from
a 10Mt H-bomb explosion

# Plunge-freezing method (the “cryo” part of cryo-EM)

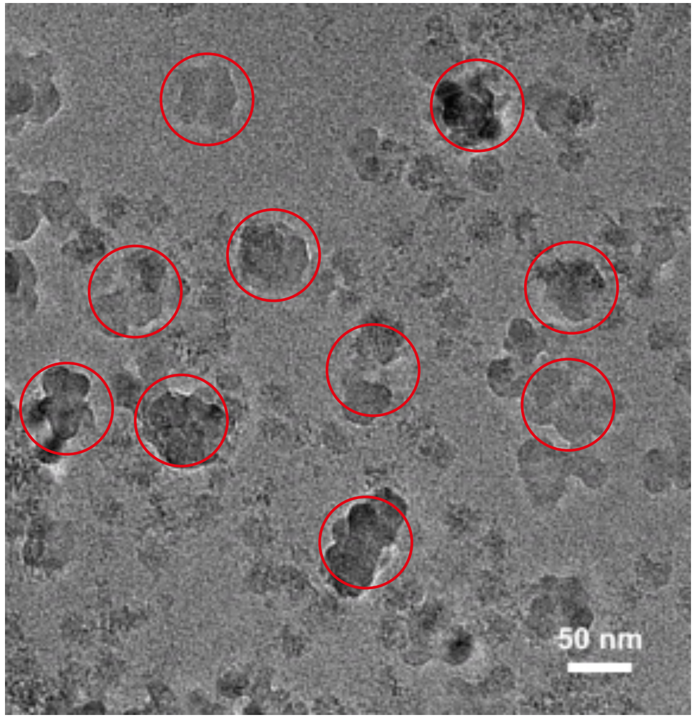


Ethane has a much higher heat capacity than liquid nitrogen.

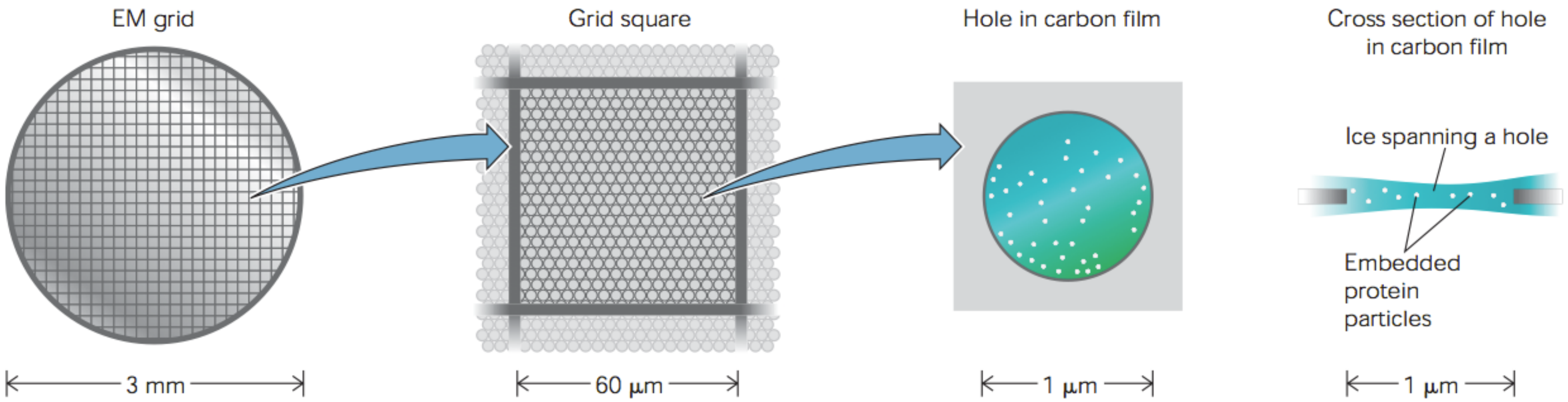
Ethane is liquid at temperatures just slightly above those of liquid nitrogen (its melting point is -188 °C)

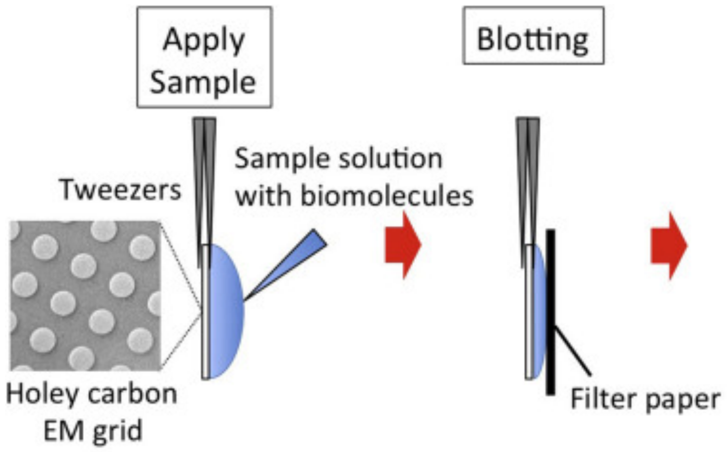
Therefore, liquid ethane is cold enough to vitrify water quickly, while not boiling off in the process.

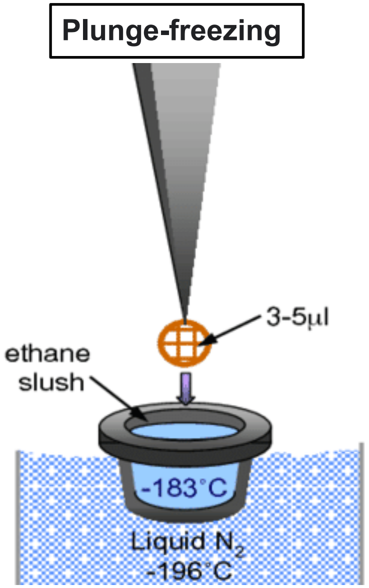
**Rapid vitrification is critical to avoid crystalline ice formation!**



# EM grid preparation





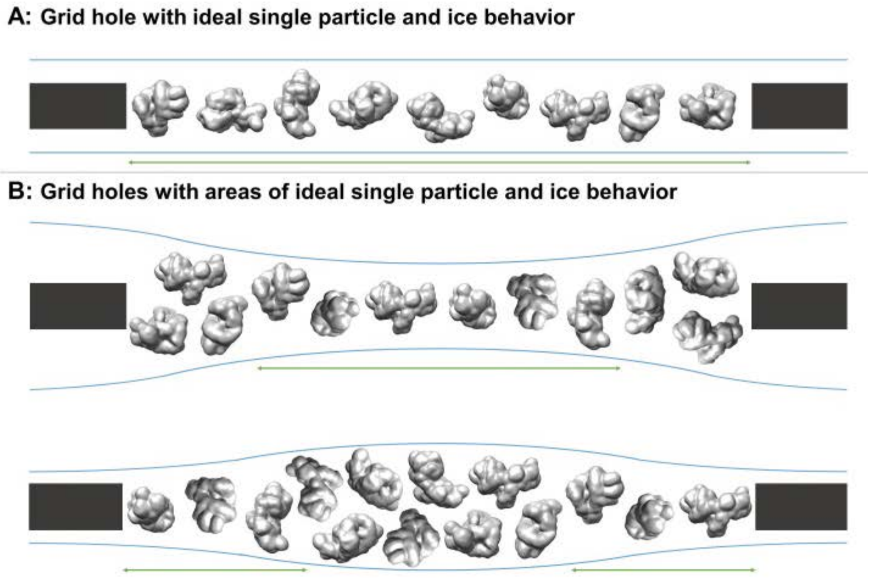


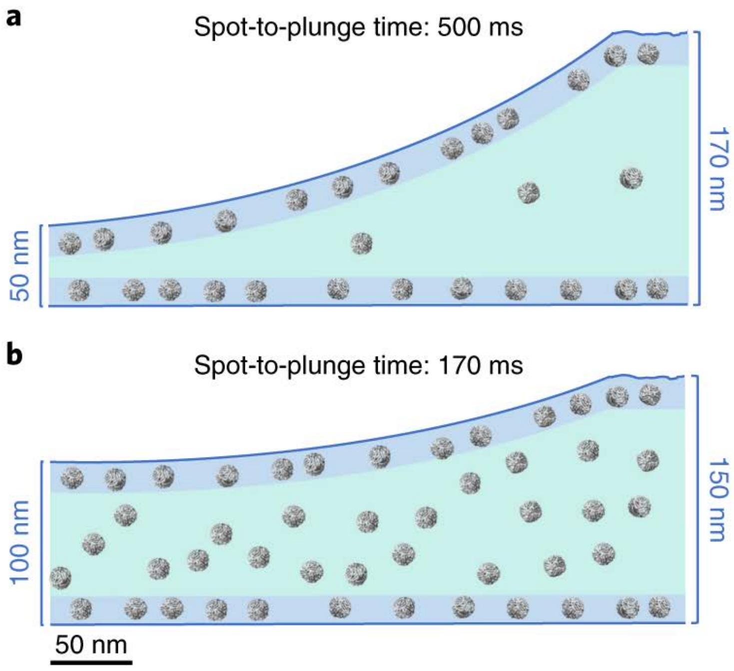
**sample deposition on the grid**

Vitrobot sample freezing machine
(from http://vitrobot.com).

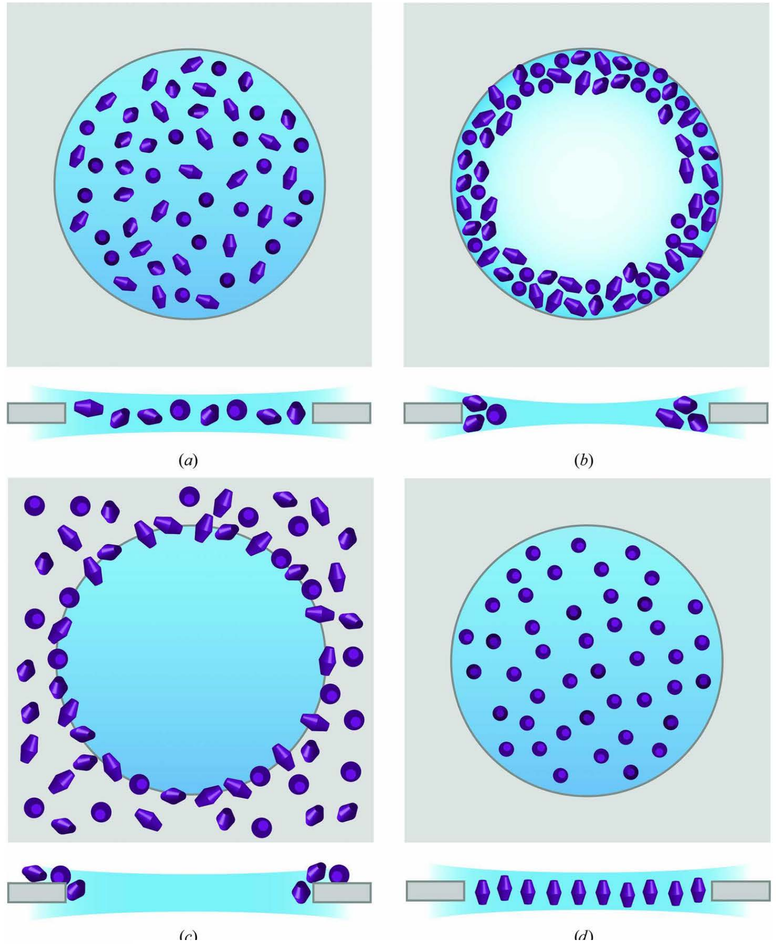
**(usually 3 µL protein solution at a concentration of 0.05 – 5 µM)**

- **Homogeneity**
- **Good distribution on grid**
- **Different orientations**





Noble et al, Nature Methods,15, 793–795 (2018)



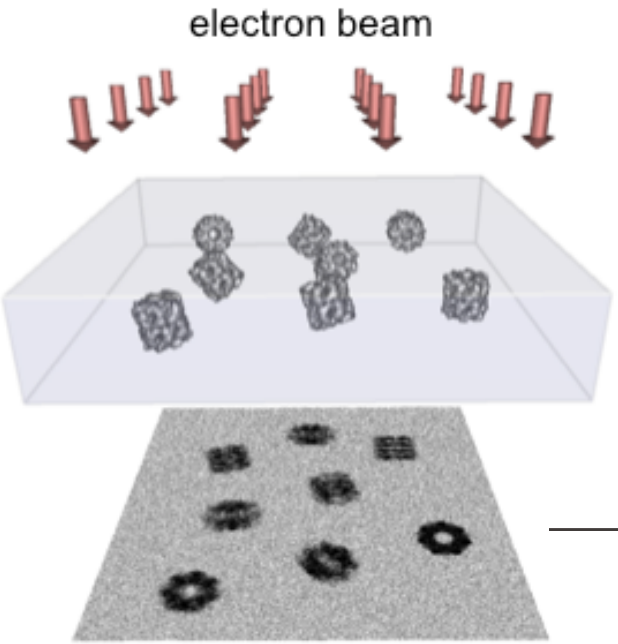
**Drulyte *et al.***

**Volume 74** | **Part 6** | June 2018 | Pages 560–571 | 10.1107/S2059798318006496

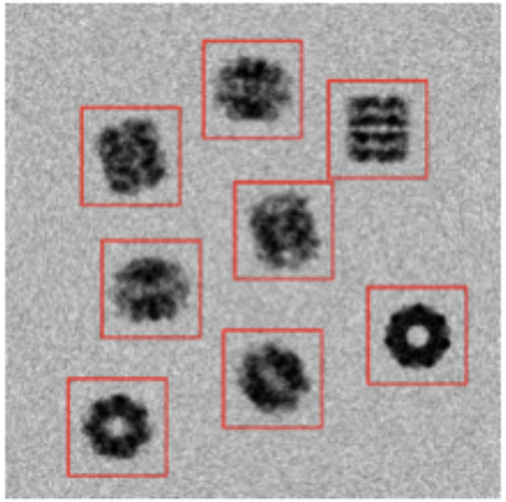
# What does the grid look like?

# Data analysis and reconstruction of 3D models

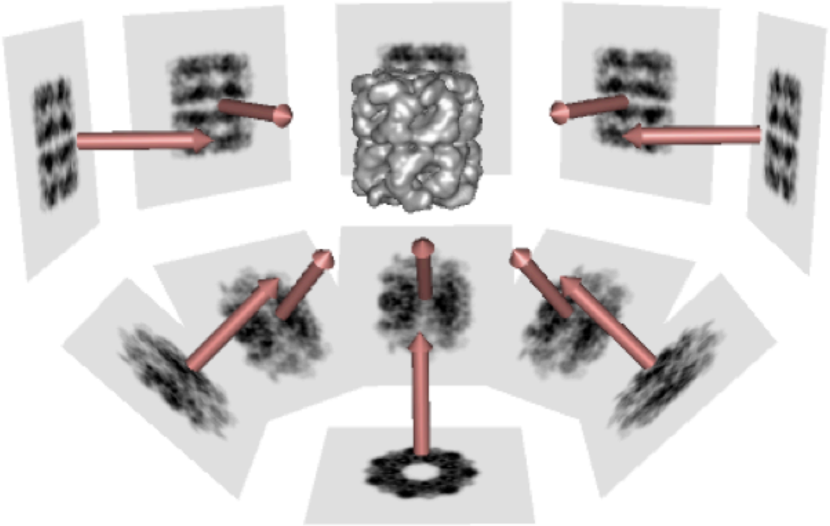
Imaging



2D projection images



3D reconstruction

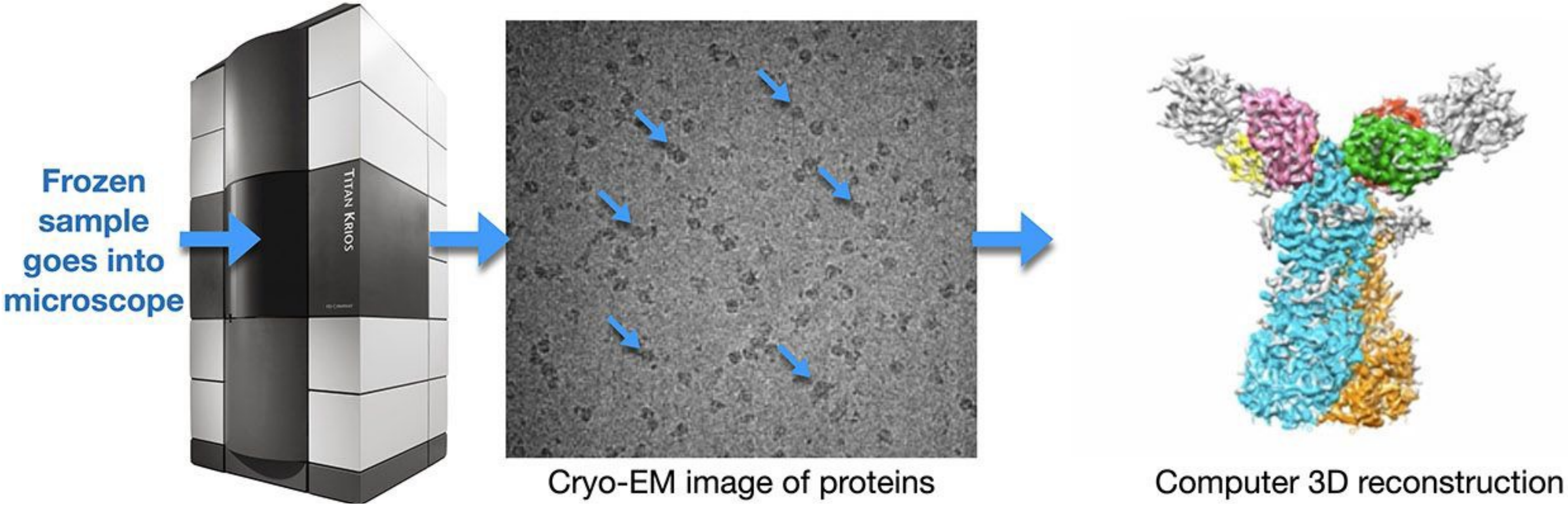


- Samples are vitrified in a thin layer of ice which provides structural support to the layer and reduces radiation damage
- 2D projection images are extracted from each micrograph and used to recover the 3D model of the object**(s)**

Adapted from: https://people.csail.mit.edu/gdp/cryoem.html

# Many particles needed to recover maps from 2D projections

- As many as **$\sim 10^3$ - $10^6$ particles go into final map reconstructions**. Typically, the more -> the better

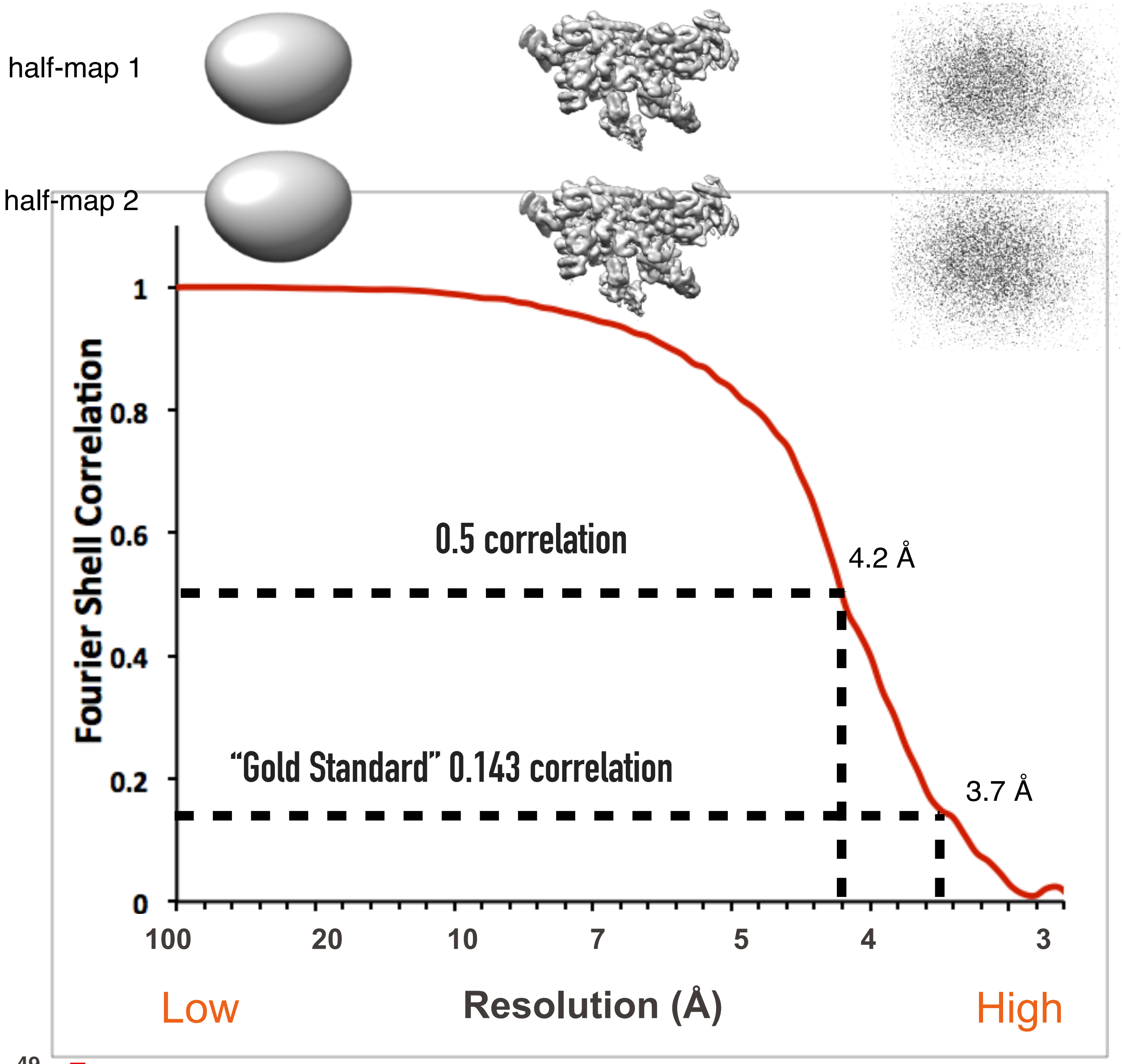


Cryo-EM image of proteins

Computer 3D reconstruction

## But how is map resolution estimated?

# Estimating the resolution of a cryo-EM map



- **Fourier Shell Correlation (FSC) plot**

- Correlation between resolution shells in 2 independently refined half-sets of data (particles)

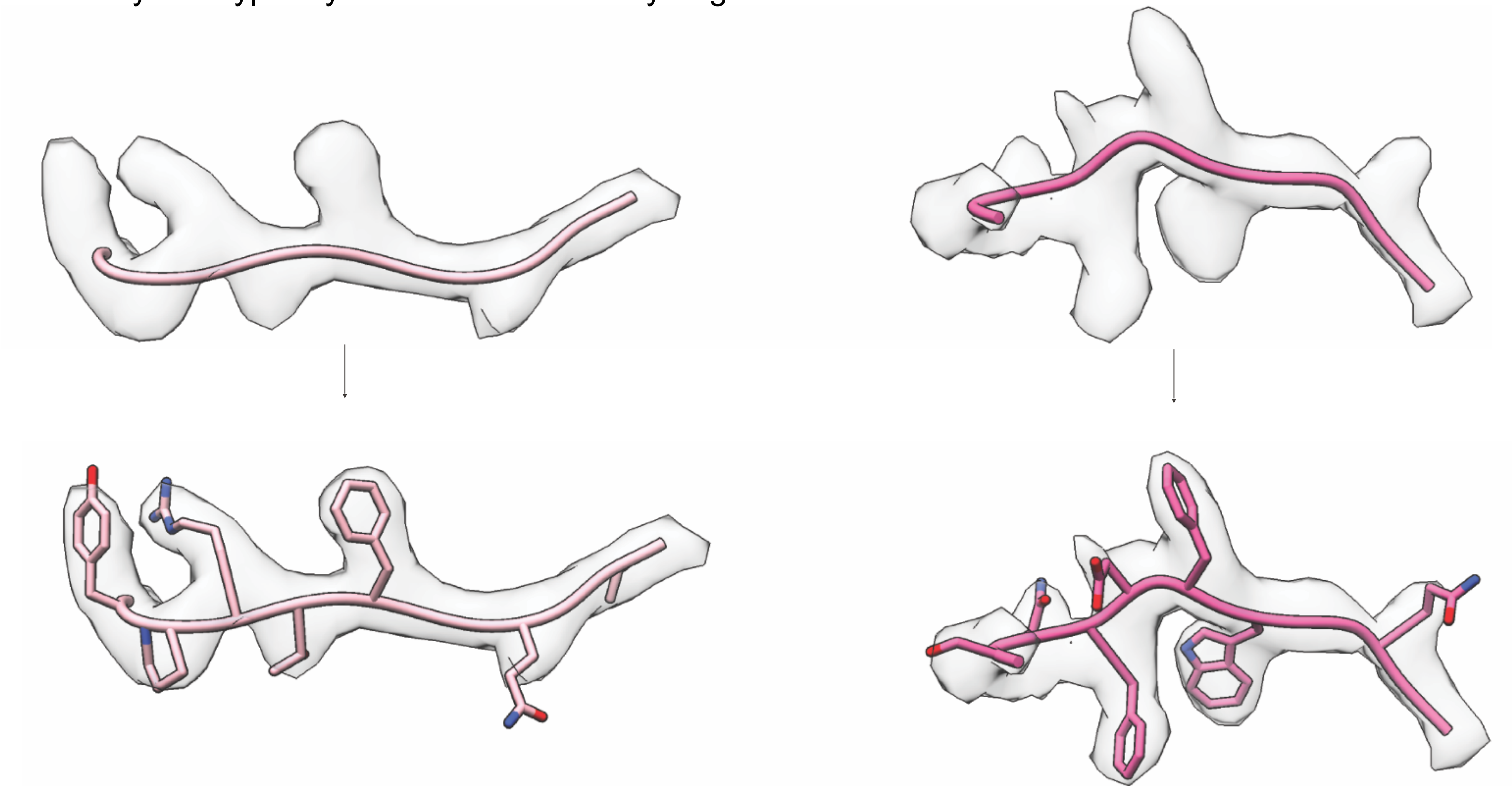
$$FSC(r) = \frac{\sum_{r_i \in r} F_1(r_i) \cdot F_2(r_i)^*}{\sqrt[2]{\sum_{r_i \in r} |F_1(r_i)|^2 \cdot \sum_{r_i \in r} |F_2(r_i)|^2}}$$

**F1** – Structure factors for volume 1
**F2*** – Complex conjugate of the structure factors for volume 2
**$r_i$** – Voxel element at the radius r

- "Gold Standard" is the most commonly used criterion to define global resolution

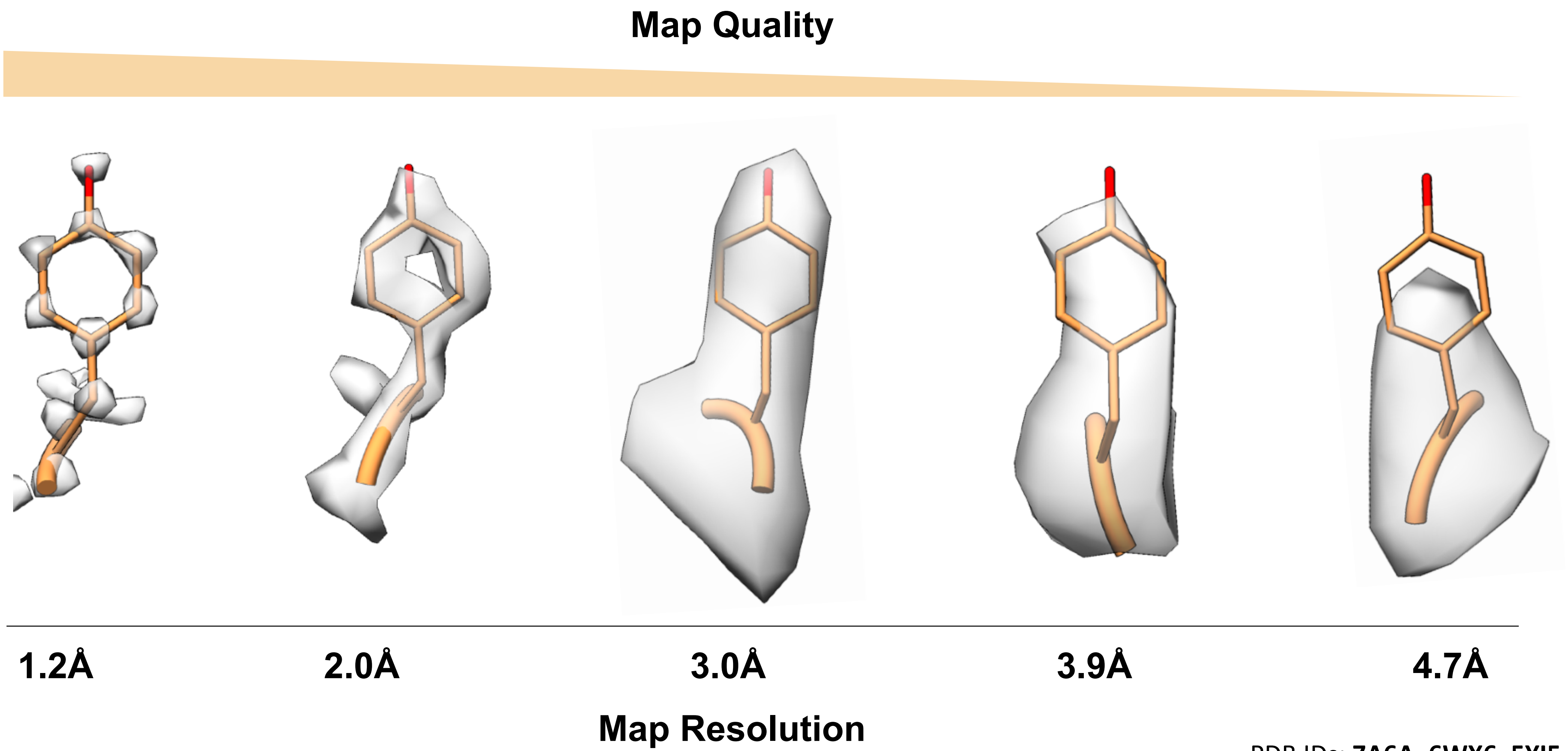
# cryoEM map is used to build atomic models

- cryoEM map is used as a set of 3D restraints to approximate an atomic model that best recapitulates the reconstructed map.
- Amino-acid, nucleotide or monosaccharide sequence (depending on the biomolecule) needs to be known beforehand.
- Resolution in cryoEM typically insufficient to build hydrogen atoms



# cryoEM maps at different resolutions

- Side-chain of residue Y28 of apoferritin at different EM map resolutions
- Structural data alone is insufficient to unambiguously build atomic models in maps > 5 Å



**Map Quality**

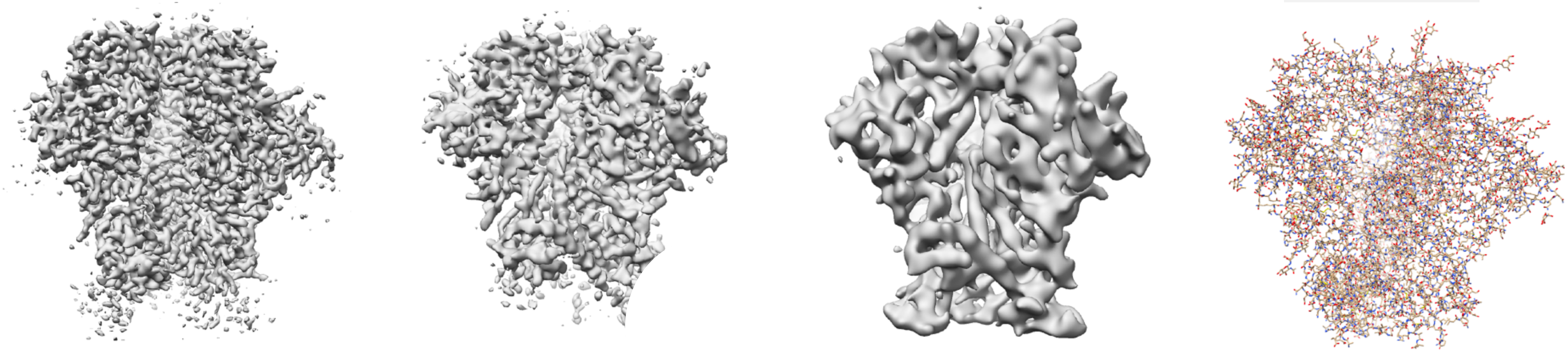
**1.2Å** **2.0Å** **3.0Å** **3.9Å** **4.7Å**

**Map Resolution**

PDB IDs: **7A6A, 6WX6, 5YI5, 6HPI, 4V1W**

# Let’s compare some protein maps

- What map has the highest resolution?
- Can you guess what the values may be?

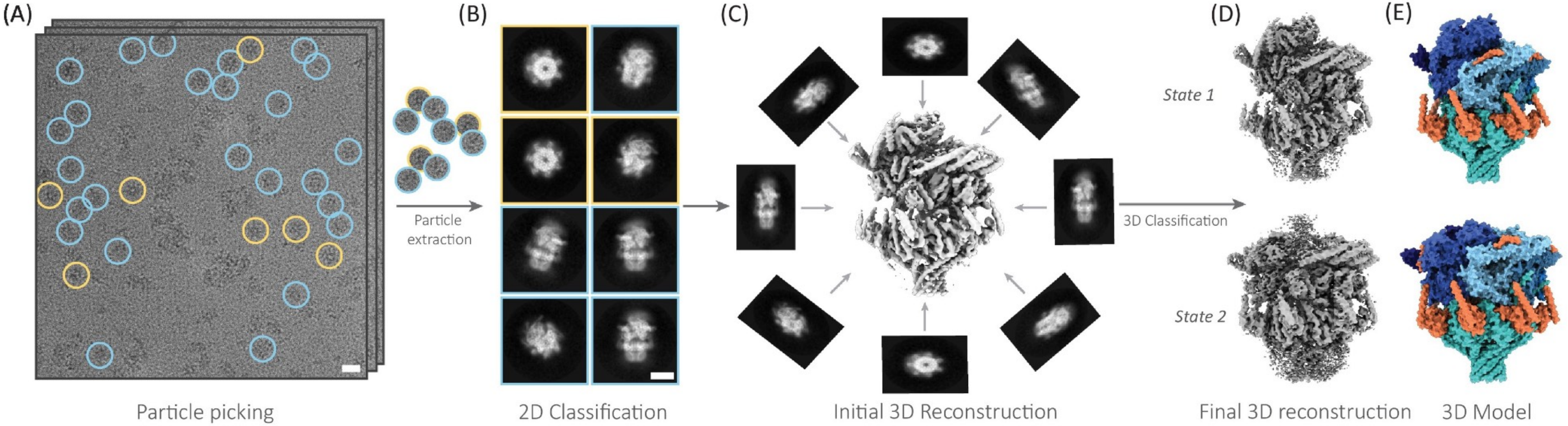


Atomic model

HIV glycoprotein at different resolutions

# cryoEM analysis allows to reconstruct multiple maps

- 2D and 3D image classification algorithms allow to separate biomolecules that are compositionally or conformationally different



- In the above example, the authors identified 2 different states of the same protein from a single dataset

# “Resolution Revolution” and “Democratization” of EM

Improved resolution of EM data

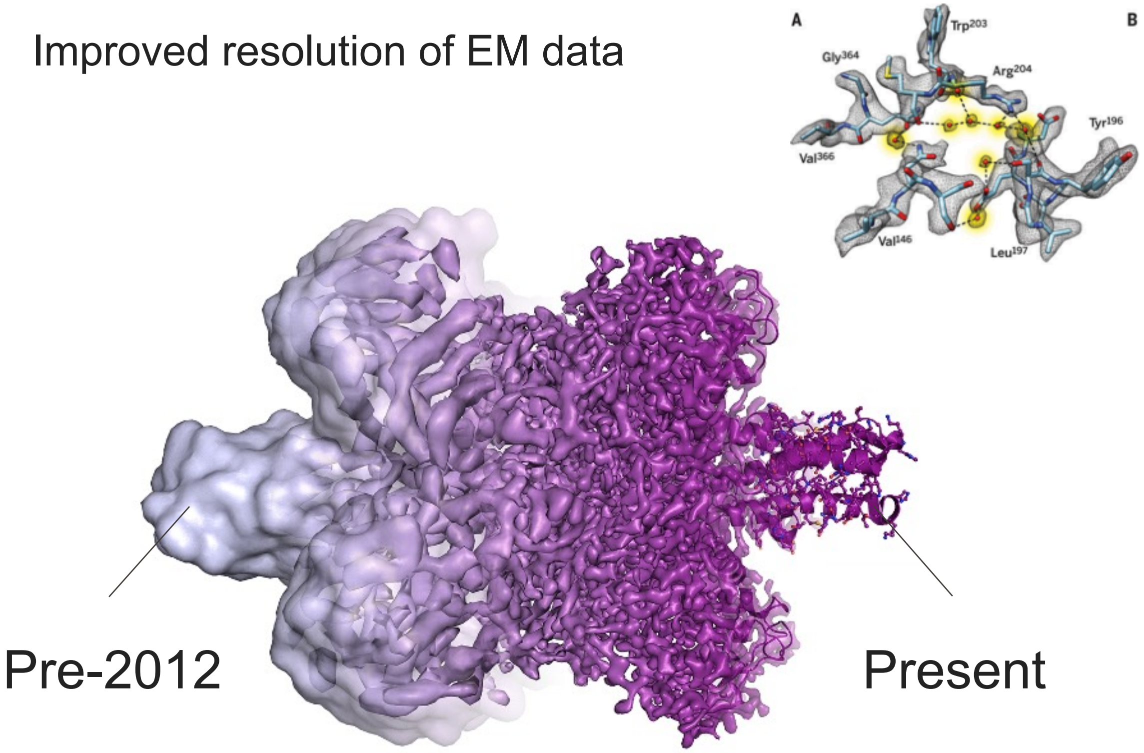
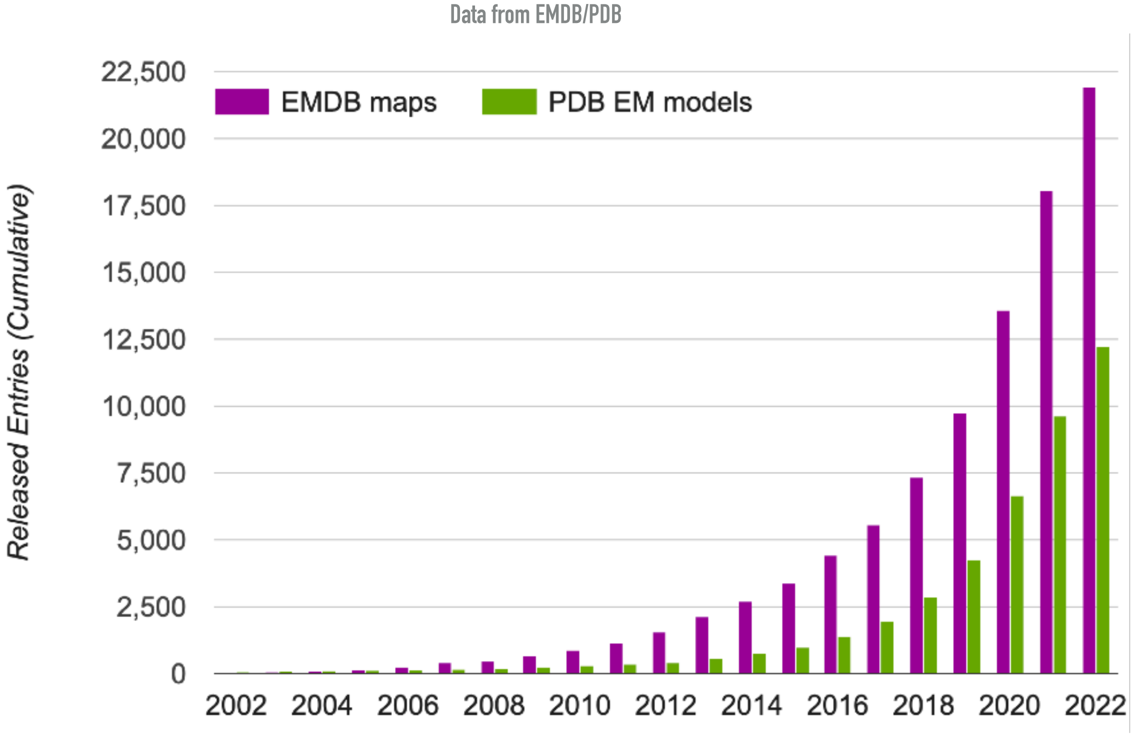


Illustration by Martin Högbom; The Royal Swedish Academy of Science

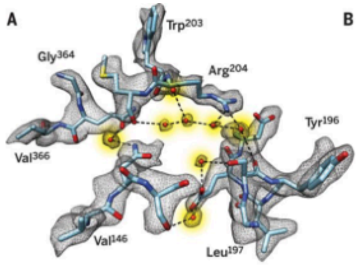
EM map/model depositions



- High-resolution maps became readily attainable starting ~2012 as a result of technological breakthroughs in the field, such as the development of direct electron detectors, new electron sources, 300 kV microscopes and computational tools

# “Resolution Revolution” and “Democratization” of EM

Improved resolution of EM data



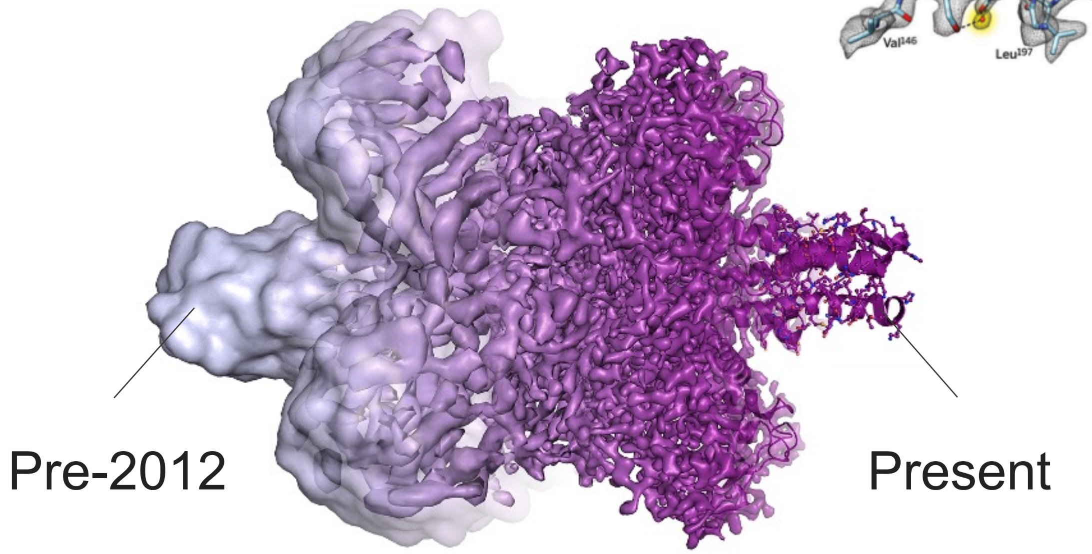
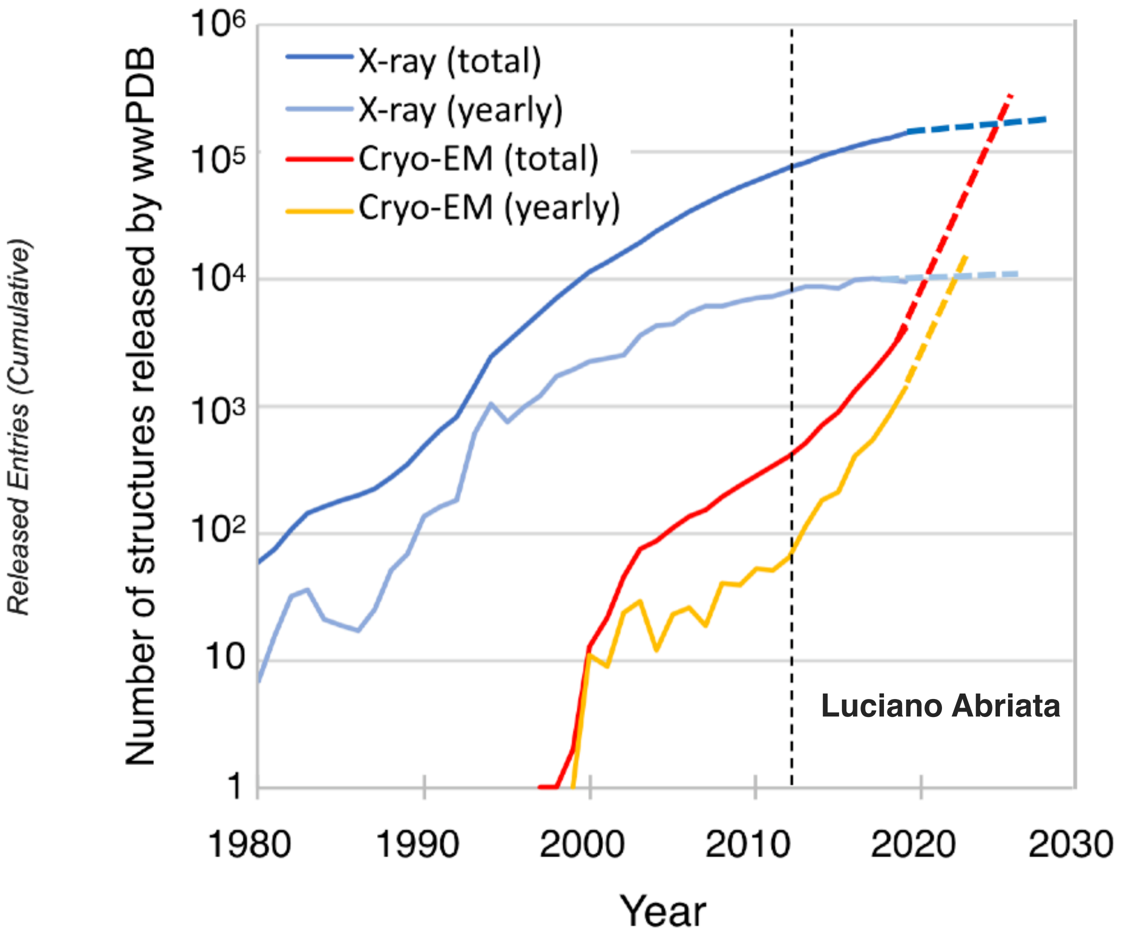


Illustration by Martin Högbom; The Royal Swedish Academy of Science



- High-resolution maps became readily attainable starting ~2012 as a result of technological breakthroughs in [illegible]n as the development of direct electron detectors, new electron sources, 300 kV microscopes and computational tools

Abriata et al. 2020

# “Resolution Revolution” and “Democratization” of EM

Improved resolution of EM data

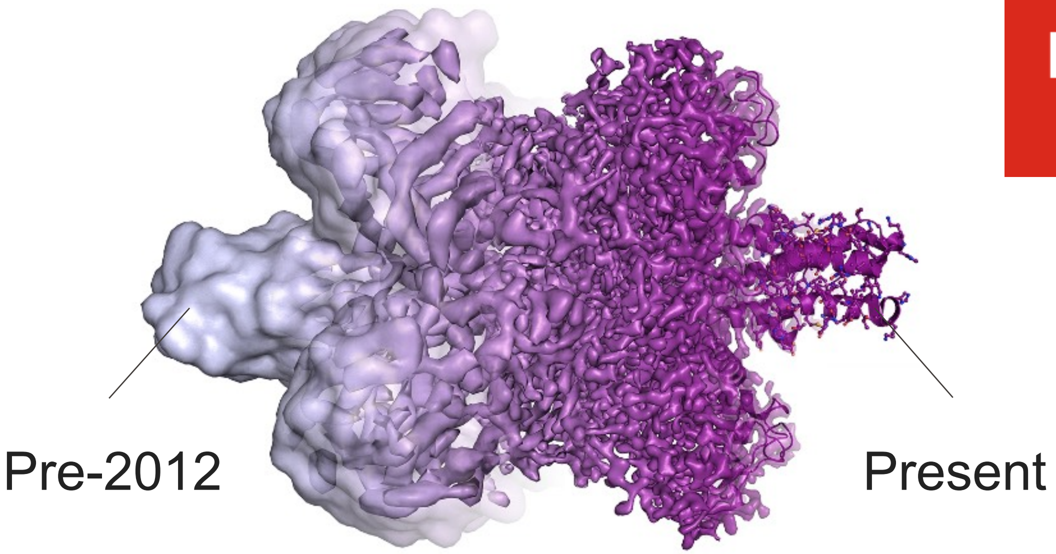
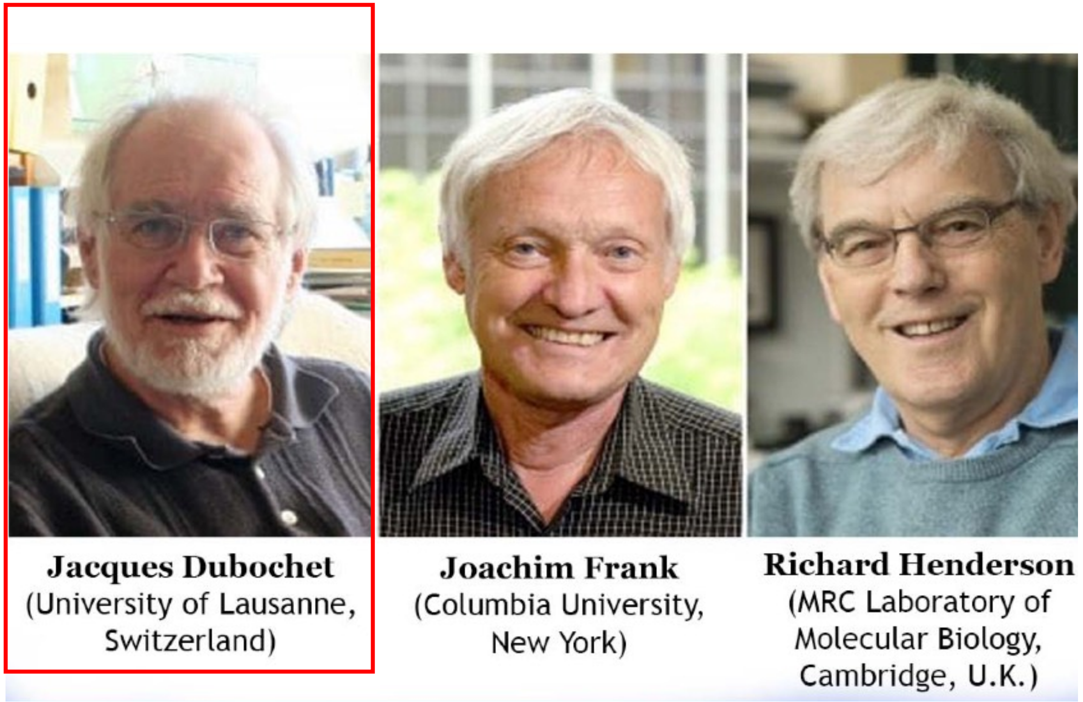


Illustration by Martin Högbom; The Royal Swedish Academy of Science

Nobel Prize in Chemistry, 2017



“for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution”

- High-resolution maps became readily attainable starting ~2012 as a result of technological breakthroughs in the field, such as the development of direct electron detectors, new electron sources, 300kV microscopes and computational tools

Bicycle parking spot at Biophore

ATOMS
MADE
VISIBLE

# ©DCI – DUBOCHET CENTER FOR IMAGING

Instruments at the DCI Lausanne

Titan Krios (300kV), E-CFEG, SelectrisX, Falcon4

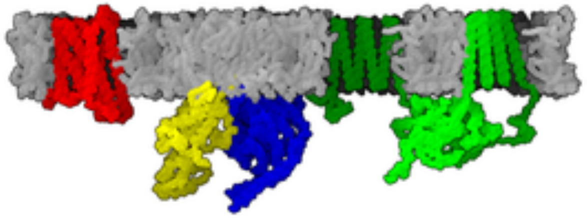
Titan Krios (300kV), E-CFEG, Falcon4

Glacios (200kV), X-FEG, Falcon4

# cryo-electron-microscopy - Pros and Cons

- **Positive sides**
- Very versatile and quick
- No requirement for protein labeling
- No requirement for crystallization
- No requirement for homogenous samples
- Real space imaging – no phase problem
- Can be used to study protein dynamics
- Can be expanded to larger assemblies (e.g., viruses and cells) and very heterogeneous samples (e.g., membrane proteins)

- **Negative sides**
- Grid preparation procedure requires screening
- Limited to samples with MW >40kDa
- Preferred orientation problems (i.e., cannot reconstruct a 3D map if there is limited distribution of orientations in 2D images)
- Computationally heavy (TBs of data + requirement for GPU processing)

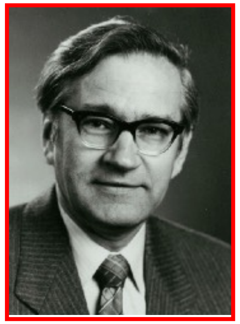


Membrane proteins

# Nuclear Magnetic Resonance (NMR)

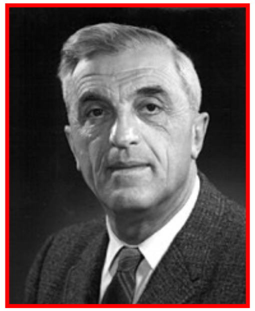
# Nuclear Magnetic Resonance Spectroscopy

- Indirect determination of relative atomic coordinates using nuclear magnetic spins and radio-frequency radiation (~MHz-GHz range)

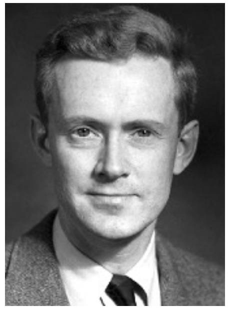


**Richard Ernst**
(1933-2021)

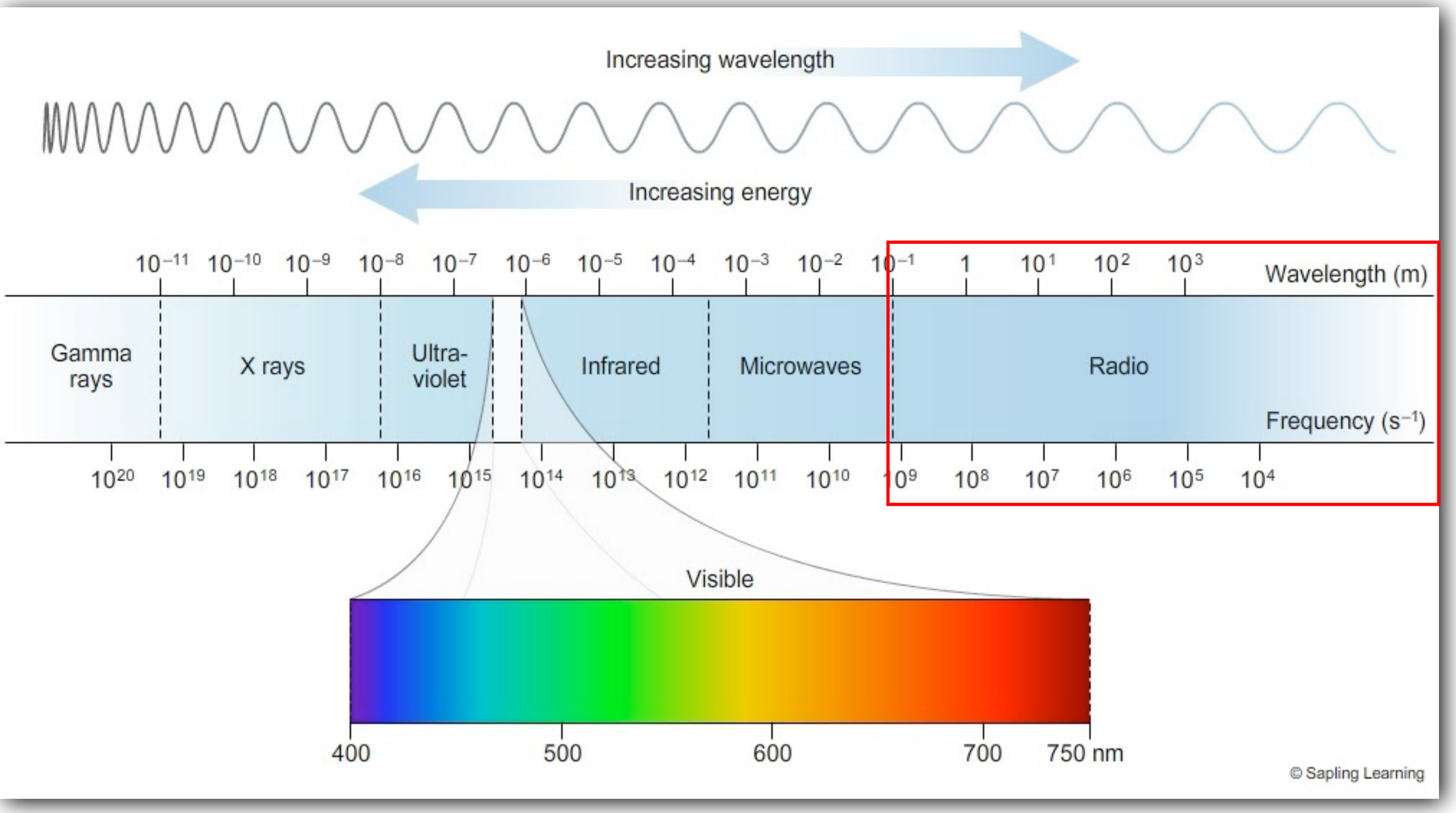
**Isidor Isaac Rabi**
(1898-1988)



**Felix Bloch**
(1905-1983)

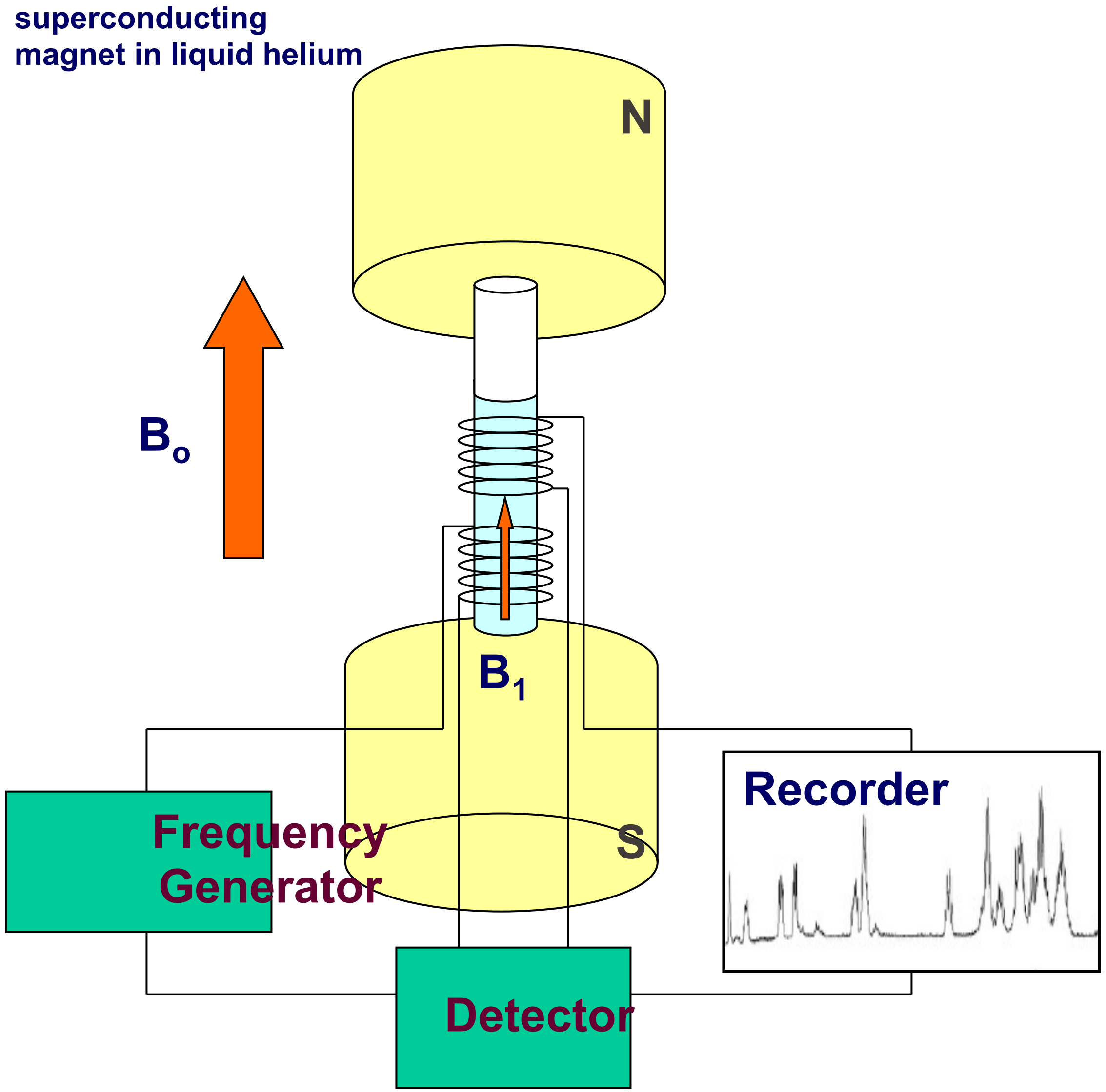


**Edward Purcell**
(1912-1997)



© Sapling Learning

# NMR Spectrometer



900 MHz NMR Spectrometer

**$B_0$ = 21.1 Tesla** magnetic field > $^{1}$H resonates at 900 MHz.
(magnetic field of the Earth is around 50 μT)

NMR sample tube
(400-500 μl protein needed)

# Nuclei in the magnetic field

- NMR is the phenomenon occurring when “NMR active” nuclei at equilibrium in a static magnetic field are exposed to an external oscillating magnetic field.

- NMR active nuclei are those that have a magnetic spin ideally of 1/2 (e.g.,: **$^{1}$H, $^{13}$C, $^{15}$N, $^{31}$P**)

- In a magnetic field, the nuclei orient either parallel or antiparallel to the direction of the field creating energy levels

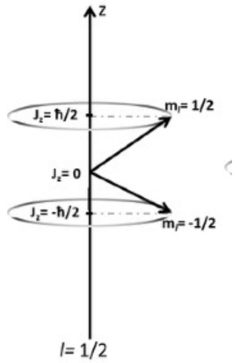
- The difference in energy levels is directly proportional to the strength of the magnetic field

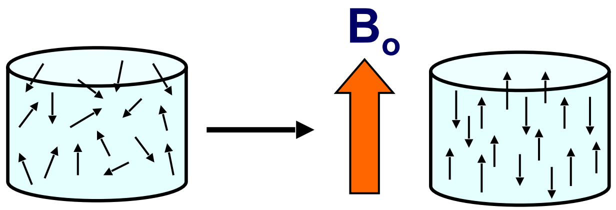
- The difference in energy levels matches the energy of radio-frequent radiation

**S =1/2 implies $m_s$ = ±1/2**

**S = nuclear spin**

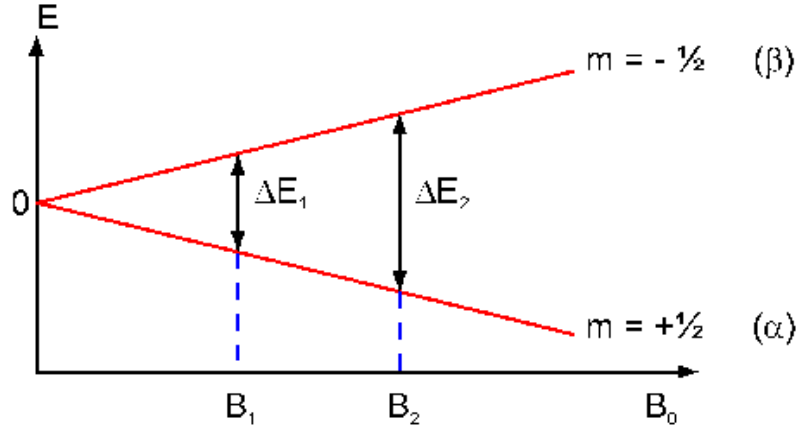
**$m_s$= quantum spin number**





$$\boxed{\Delta E = \gamma h B_o / 2\pi}$$

γ : Gyromagnetic constant



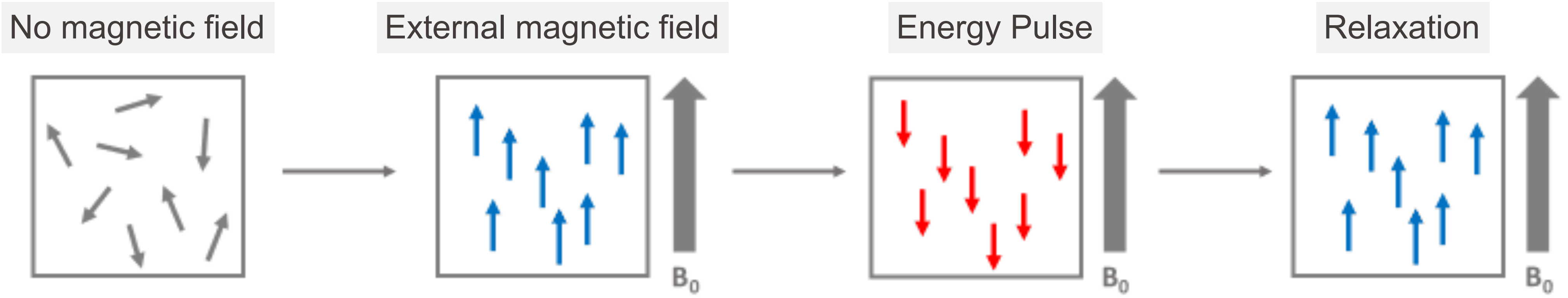
Note: To readily detect C and N atoms in NMR experiments isotope labelling with $^{13}$C and $^{15}$N is necessary

# NMR “visible” atoms

| isotope | abundance [%] | spin | active in NMR? |
|---|---|---|---|
| **$^{1}\mathrm{H}$** | **99.98** | **1/2** | **yes** |
| $^{2}\mathrm{H}$ | 0.02 | 1 | no |
| **$^{12}\mathrm{C}$** | **98.89** | **0** | **no** |
| $^{13}\mathrm{C}$ | 1.11 | 1/2 | yes |
| **$^{14}\mathrm{N}$** | **99.64** | **1** | **no** |
| $^{15}\mathrm{N}$ | 0.37 | 1/2 | yes |
| **$^{16}\mathrm{O}$** | **98.90** | **0** | **no** |
| $^{17}\mathrm{O}$ | 0.036 | 5/2 | no |
| **$^{31}\mathrm{P}$** | **100** | **1/2** | **yes** |

# Nuclear Magnetic Resonance Spectroscopy

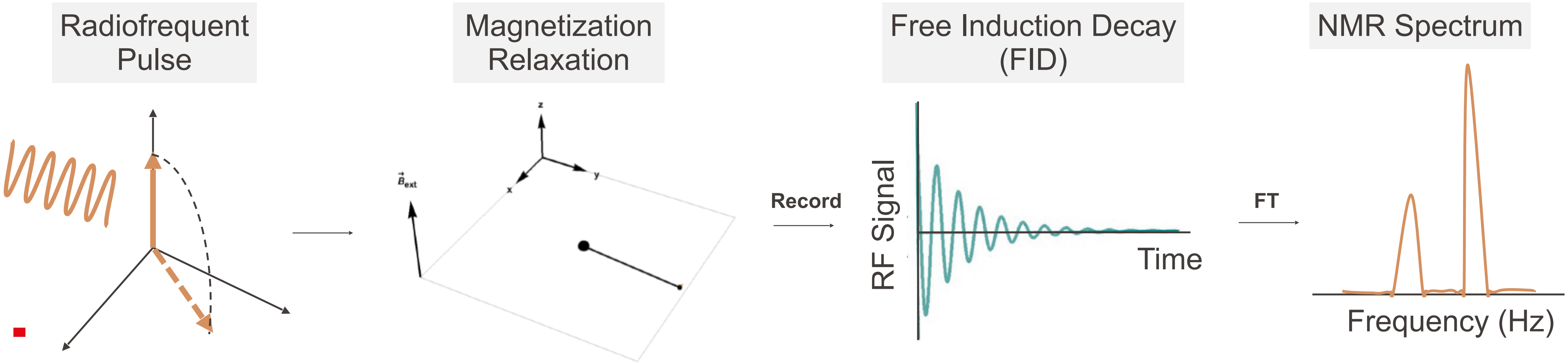
- Radio-frequent pulse is used to deflect the nuclei out of their equilibrium distribution



Larmor Frequency

$\nu = \gamma B_o / 2\pi$

- To return to equilibrium perturbed nuclei emit RF radiation which is recorded as FID and then converted to frequencies by Fourier transform (FT)

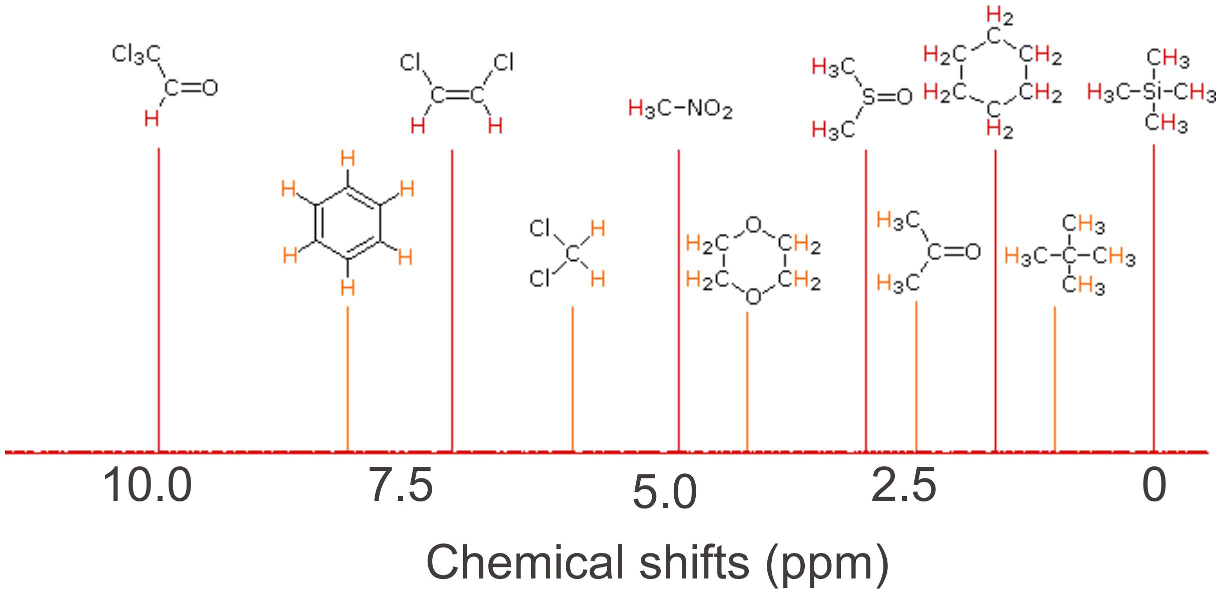


# Chemical shifts (δ)

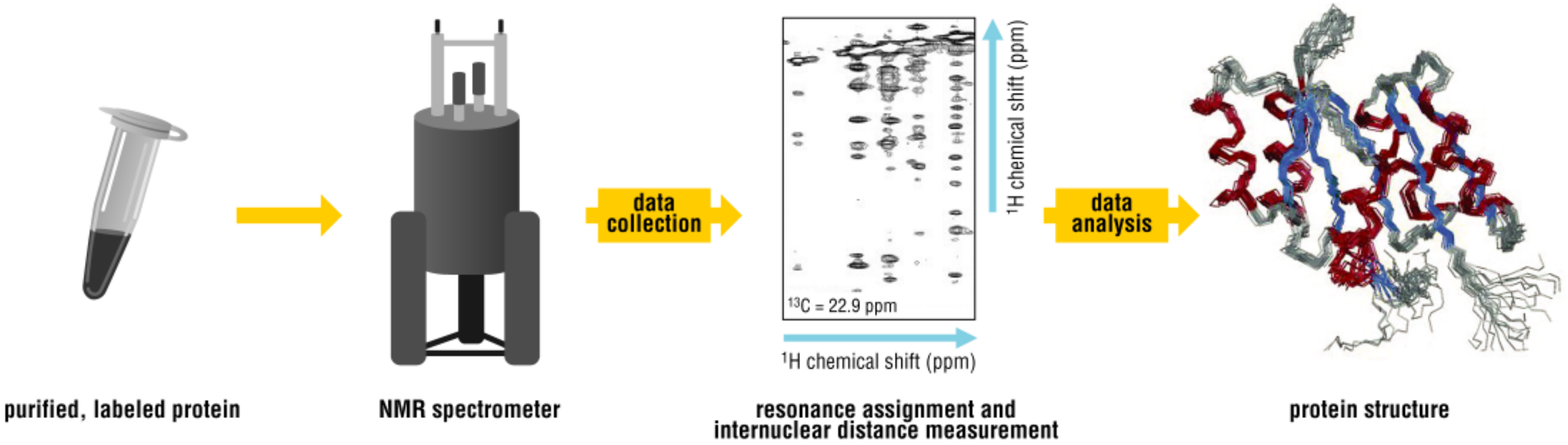
- All “NMR active” nuclei resonate at a characteristic frequency depending on their chemical environment (i.e., the degree of electron shielding)

- Chemical shifts are proportional to frequency δ reported in ppm with respect to a standard resonance (ppm=“parts per million”)
  - $^1$H range ~ 10 ppm or 5000 Hz of 500,000,000 Hz or 9000 Hz of 900,000,000 Hz
  - $^{13}$C range ~ 200 ppm or 25000 Hz of 125,000,000 Hz
  - $^{15}$N range ~ 70 ppm or 3500 Hz of 50,000,000 Hz

* The detected frequencies (in Hz) are referenced against TMS (tetramethylsilane, $Me_4Si$), which is assigned the chemical shift of zero.



# From a protein in solution to a structure using NMR

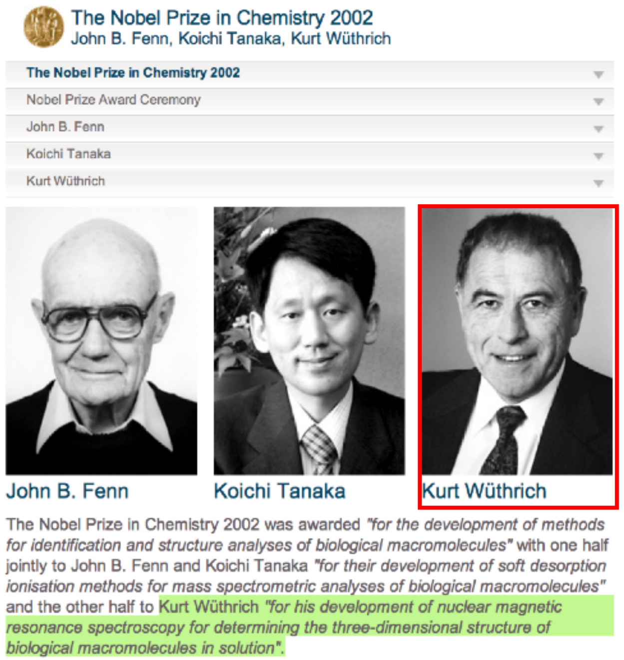


- Completely different to crystallography and cryoEM, as proteins are studied **in solution**
- Several different NMR experiments give you information on spatial restraints of particular nuclei (e.g. proton of NH group of Ala-25 is close to C alpha of Tyr-88)
- The collection of several hundreds of such restraints is used to calculate **several models of the protein** that adheres to the experimental restraints

# $^1$H-NMR spectrum of a protein is very complex

- 60 kDa protein has >4000 H-atoms which makes the $^1$H spectrum “busy”
- One can distinguish folded from unfolded (denatured) proteins, but otherwise not too useful to get the structure of a protein since we cannot connect resonance to the atomic nuclei.
- **How can we identify the corresponding atomic nuclei in a protein?**

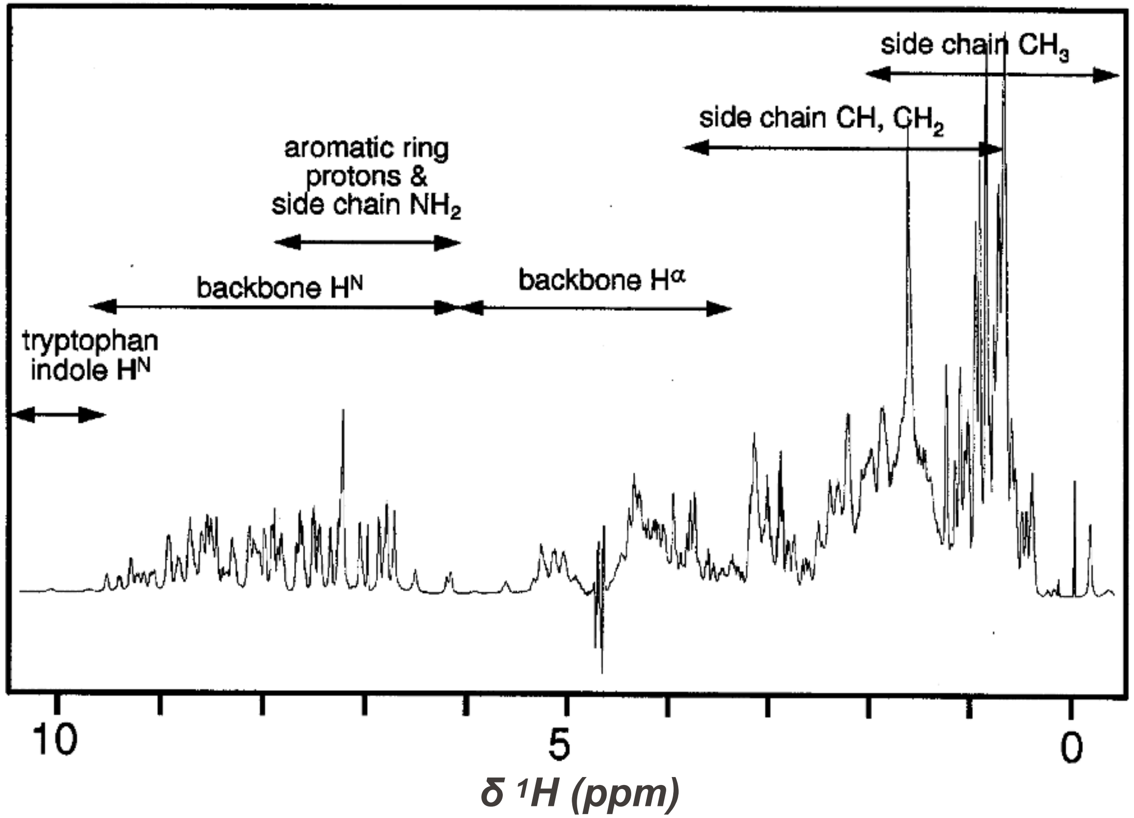
Enter these guys:



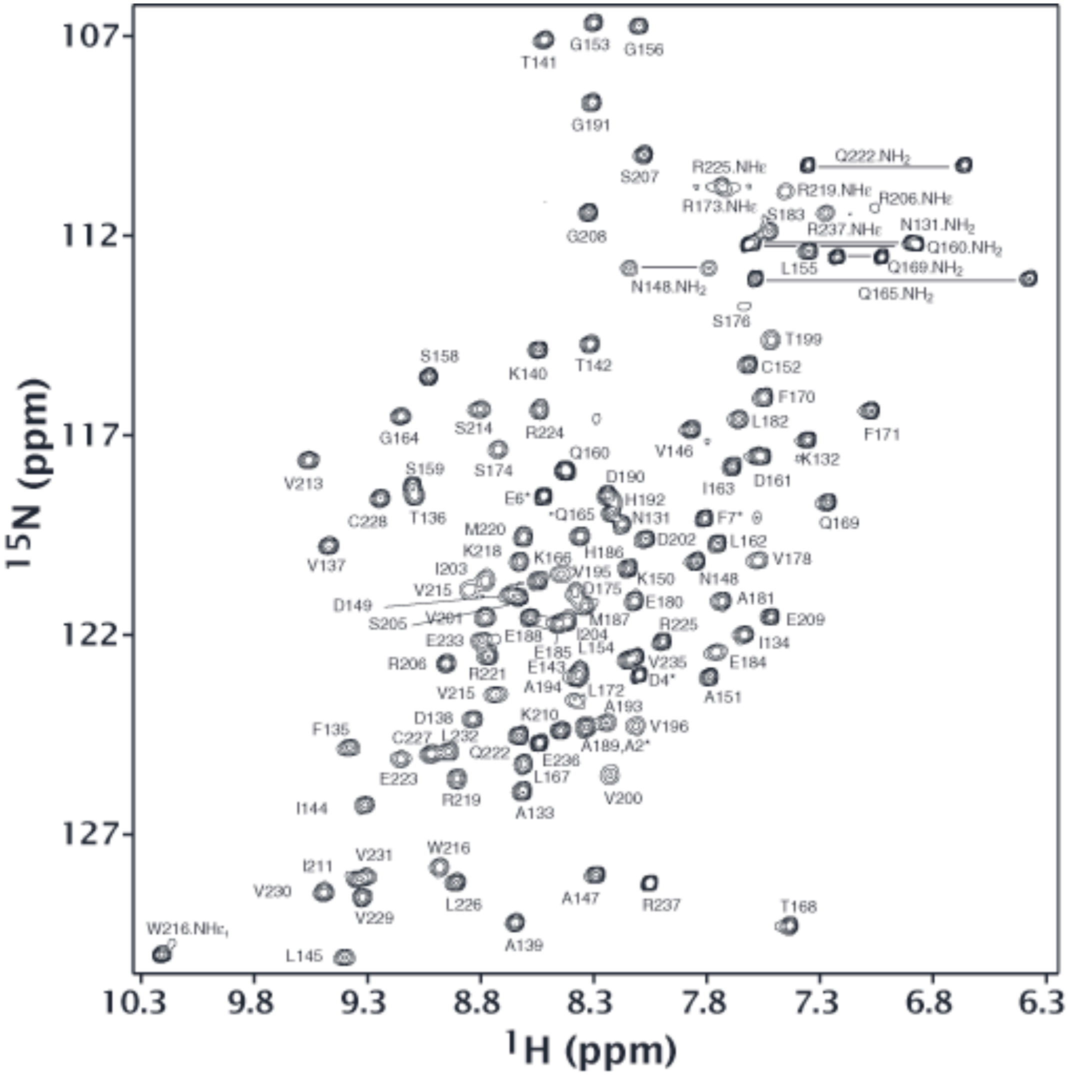
The Nobel Prize in Chemistry 2002
John B. Fenn, Koichi Tanaka, Kurt Wüthrich

John B. Fenn Koichi Tanaka Kurt Wüthrich

The Nobel Prize in Chemistry 2002 was awarded *"for the development of methods for identification and structure analyses of biological macromolecules"* with one half jointly to John B. Fenn and Koichi Tanaka *"for their development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules"* and the other half to Kurt Wüthrich *"for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution"*.



# 2D NMR experiments: $^{15}N/^{1}H$-HSQC



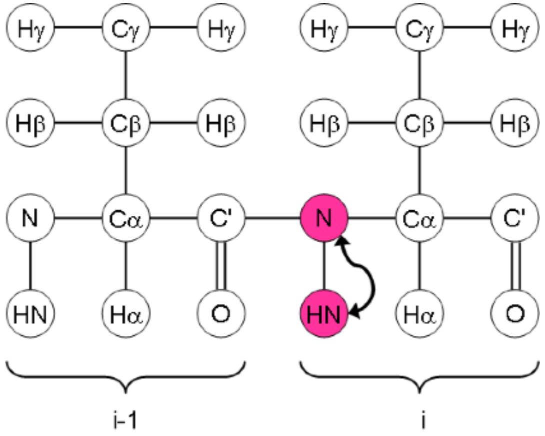
- **Correlates amide nitrogen with its proton**
- One peak for every amino acid (except Pro) i.e. **‘fingerprint’ of protein**
- Additional peaks for aa with NH group in sidechain, i.e. N, Q, K

Commonly used in protein NMR:

- Rapidly acquired (ca. 10 min)
- Cheap uniform $^{15}N$ labeling

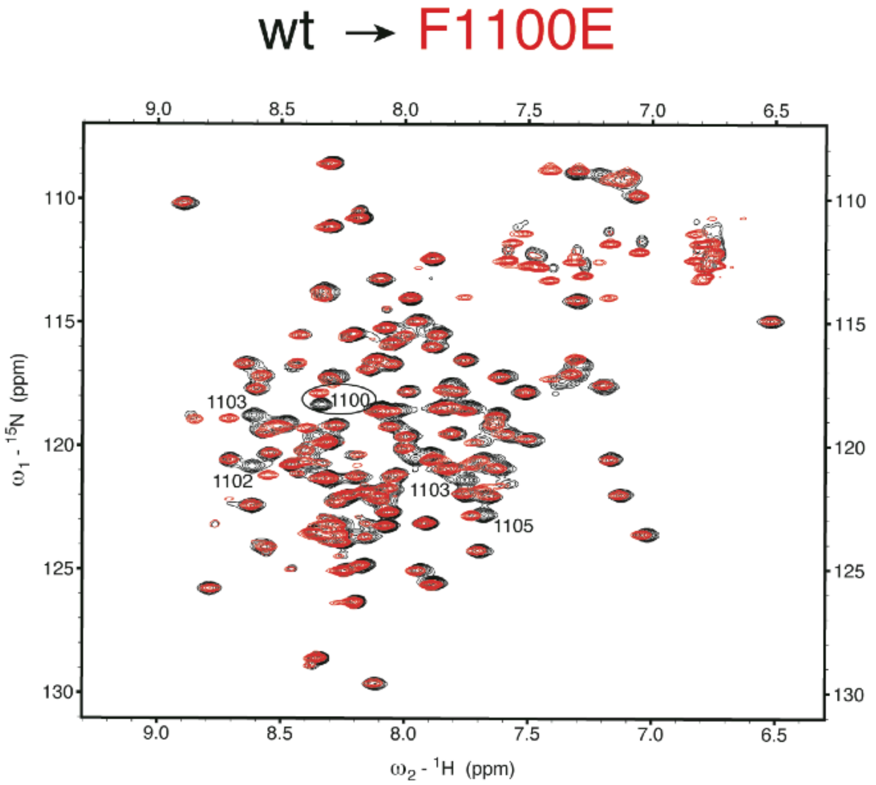
What it can assess:

- Interactions (ligand, protein)
- Conformational changes
- Folding

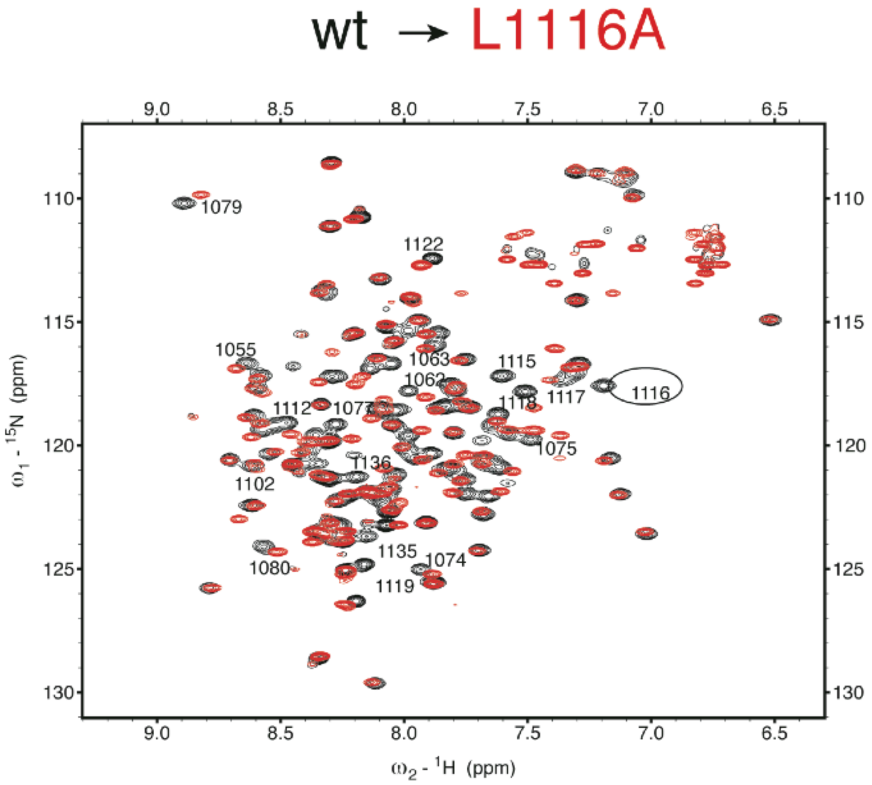


*** Heteronuclear Single Quantum Correlation**

# $^{15}$N/$^{1}$H-HSQC: Example 1
# Structural changes of mutant proteins



F1100E mutant has no global effect on folding*



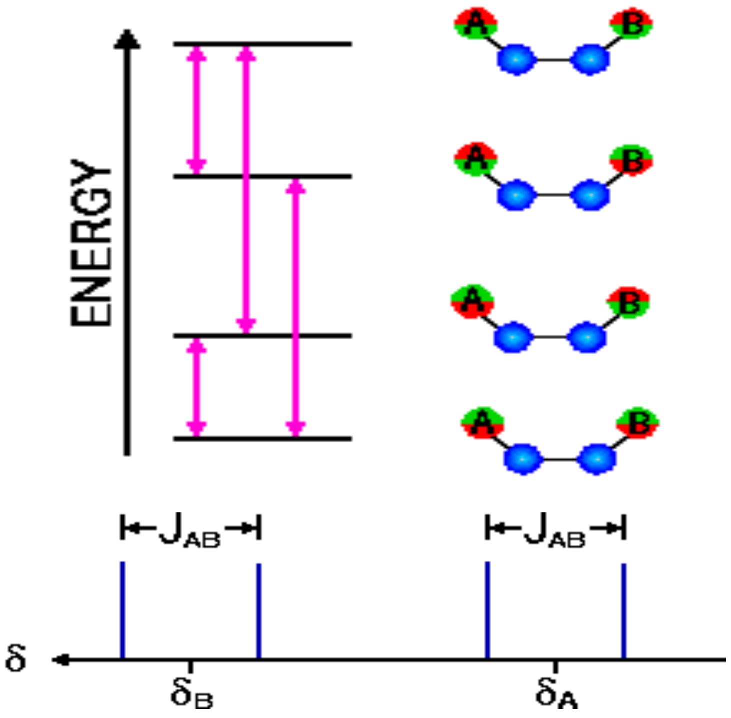
L1116A mutant causes global disturbance of tertiary structure

* this is the F-actin binding domain of Abl.

# Magnetic coupling in NMR

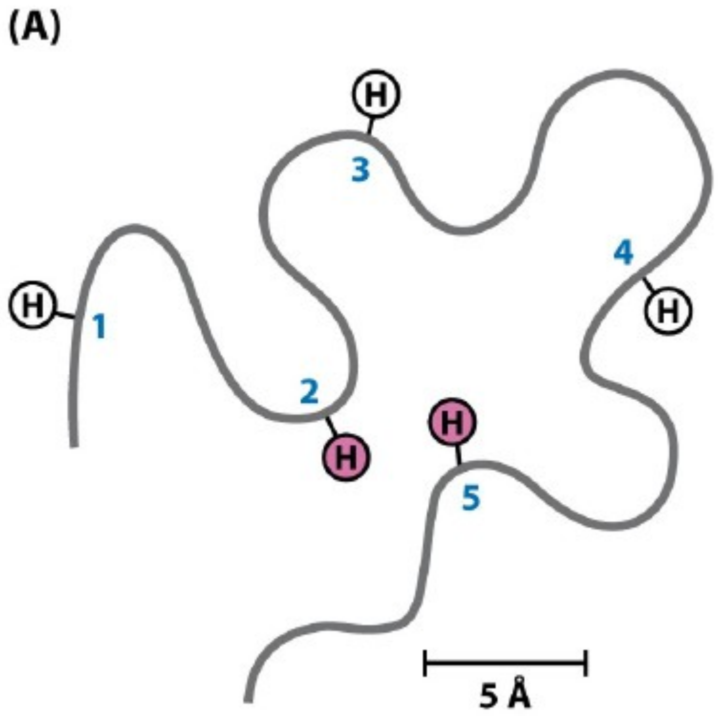
- NMR-active nuclei can “sense” each other if close in 3D space

- **Scalar (J) coupling**



Coupling of nuclei connected by 1-3 chemical bonds

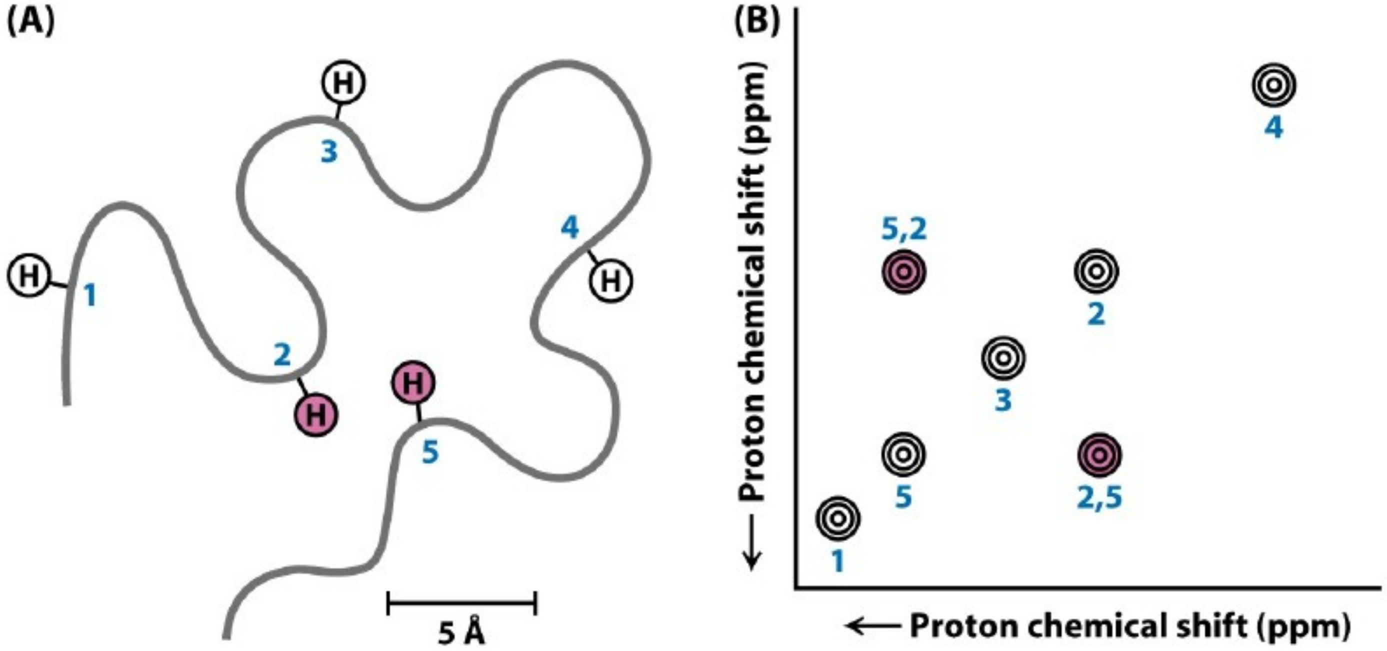
- **Dipolar (NOE) coupling**

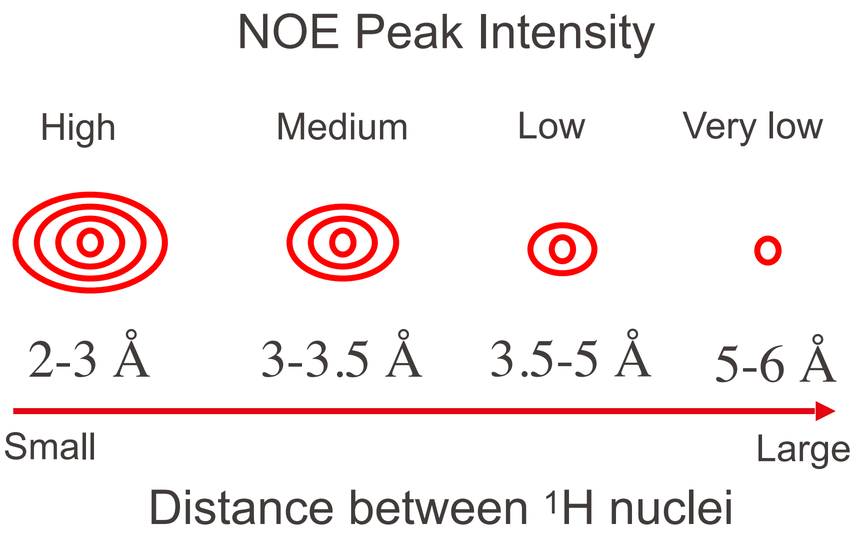


Coupling of nuclei proximal in 3D space

- The coupling can be detected by the methods developed by Wutrich and others, allowing to determine which atoms are in close proximity via bonds or through space

# Nuclear Overhauser Effect Spectroscopy (NOESY)




NOE Peak Intensity

| High | Medium | Low | Very low |
|---|---|---|---|
| 2-3 Å | 3-3.5 Å | 3.5-5 Å | 5-6 Å |

Small → Large

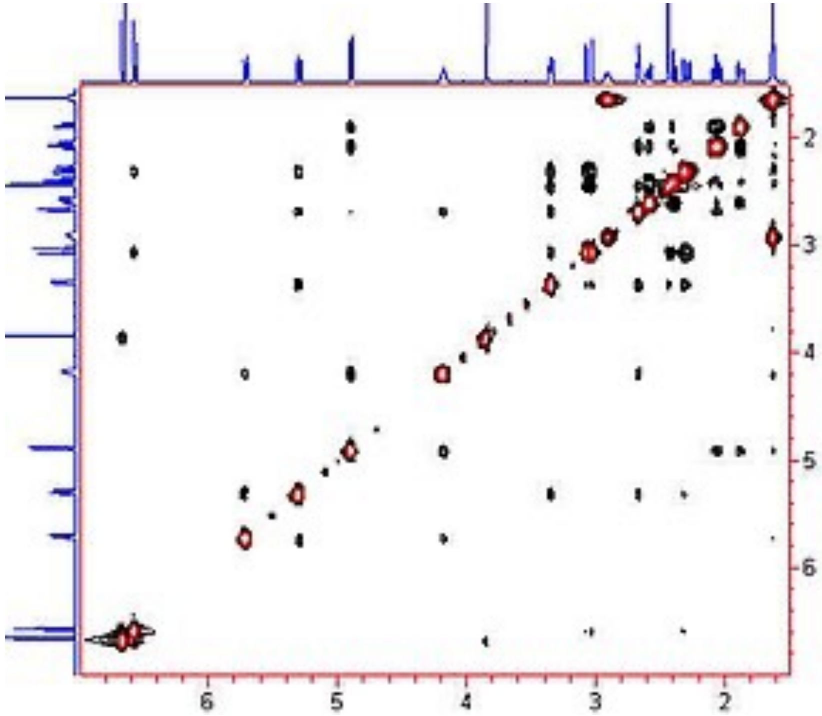
Distance between $^1$H nuclei

- Also protons that are not coupled directly via a few bonds, but are close in space influence each others' chemical shift (Nuclear Overhauser Effect)
- **The NOE is strongly distance dependent (~$1/r^6$), therefore detectable only for distances < 5-6 Å**
- Provides information on **spatial proximity of protons** in a protein


Kurt Wüthrich

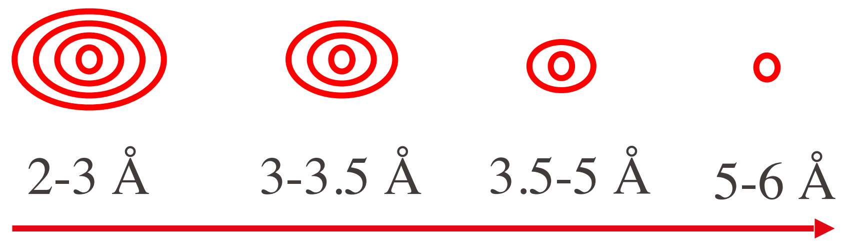
# Nuclear Overhauser Effect Spectroscopy (NOESY)

Real example



All photons off-diagonal originate from $^1H$-$^1H$ pairs close in 3D space

NOE Peak Intensity



| High | Medium | Low | Very low |
|---|---|---|---|
| 2-3 Å | 3-3.5 Å | 3.5-5 Å | 5-6 Å |

Small → Large

Distance between $^1H$ nuclei

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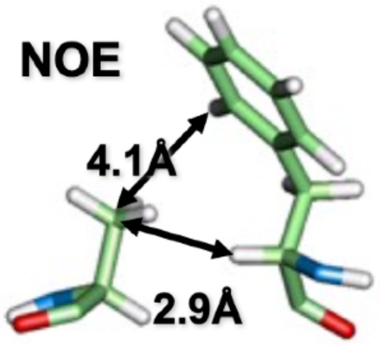
- **The NOE is strongly distance dependent (~$1/r^6$), therefore detectable only for distances < 5-6 Å**
- Provides information on **spatial proximity of protons** in a protein

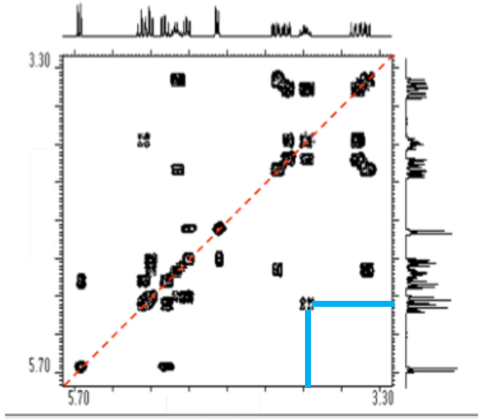
Kurt Wüthrich

# How to get a structure from NMR experiments?

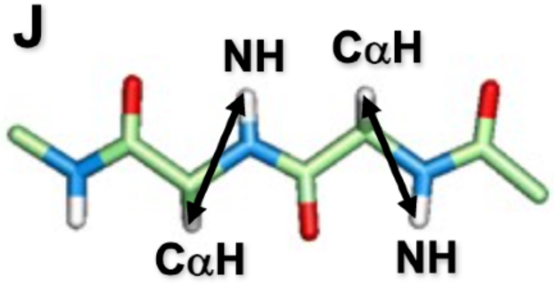
- The essence of NMR structure solving is to acquire as many connections between atoms (either through covalent bonds or through space) which then serve as restraints for model building



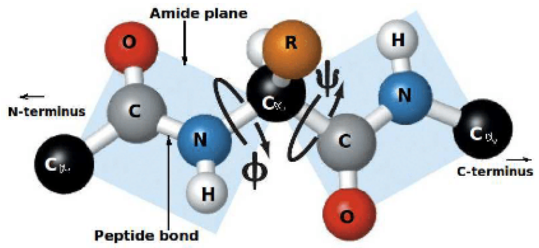
Atom proximity in 3D space



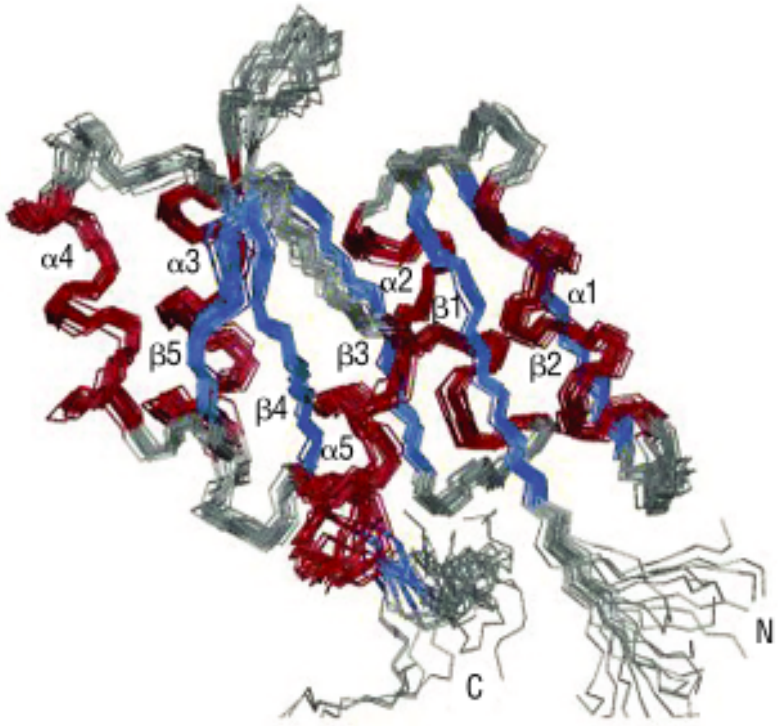
Chemical shifts (ppm)



Atoms connected by bonds



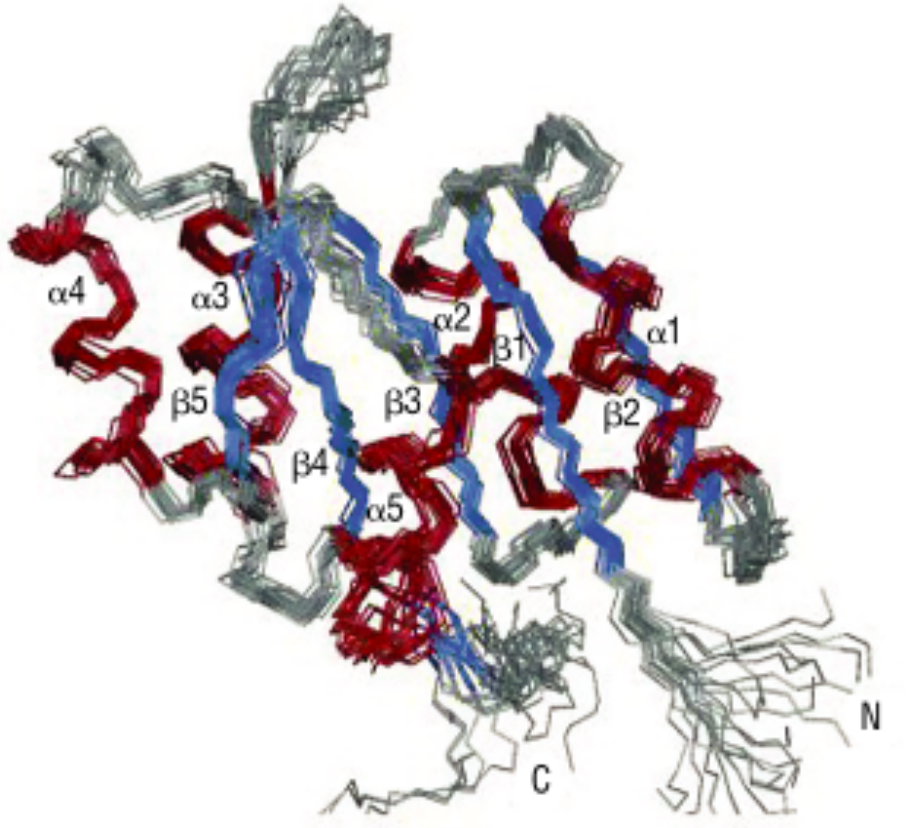
Geometric restraints:



Ensemble of structures that satisfy all the imposed restraints.

# NMR Structure is an ensemble

- An NMR structure is in reality a **model** based on the available restraints (e.g., recovered by restrained simulated annealing)
- In contrast to crystal structure, ensemble of 10-20 lowest energy structures
- Gives appreciation of rigidity and dynamics of structure
- Does not have a given resolution
- It is usually modelled with reported H atoms (in comparison to X-ray crystallography and cryoEM that usually do not have enough resolution to solve H atoms)



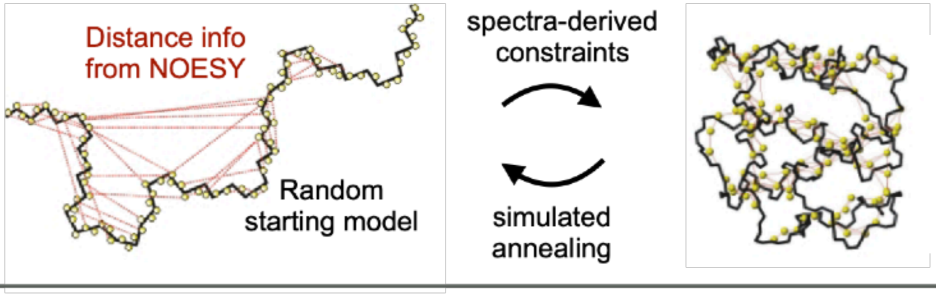
# Nuclear Magnetic Resonance - Pros and Cons

- **Positive sides**
- Proteins studied in solution
- Applicable to many more questions than just the structure determination
- Computationally light
- Can be used to measure/approximate protein dynamics
- Hydrogens are “visible” by this method

- **Negative sides**
- Limited to studies of biomolecules with MW < 50kDa
- Complex procedure to assign resonances to atoms
- Requires isotopic labeling ($^{13}C$, $^{15}N$) which is expensive and not very practical

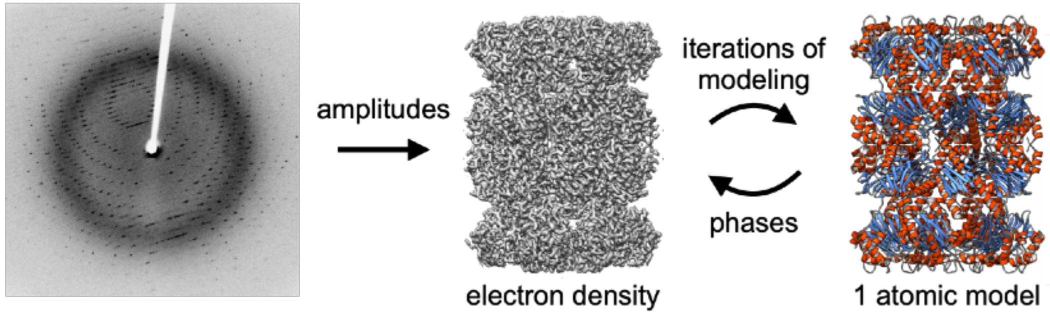
# Methods for determining biomolecular structures

**NMR**



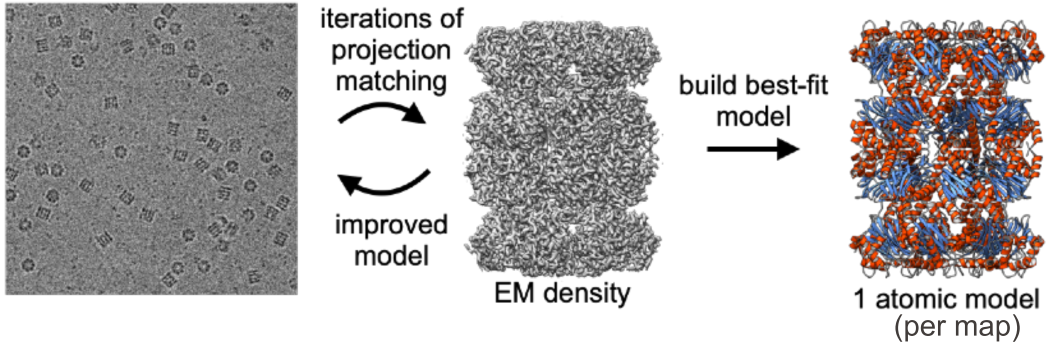
- **Versatile tool for studying protein structure and dynamics**
- Computationally light
- Full structural analysis limited to smaller proteins (<50kDa)
- Requires isotopic labeling
- Results in model ensemble

**X-ray**



- Gold-standard method for solving protein structures
- **Not limited in size or achievable resolution**
- Computationally light
- Requires highly homogenous, crystallizable sample
- Requires screening of crystallization conditions
- Phase problem
- Results in a single model

**CryoEM**



- Versatile tool for studying protein assembly, structure, dynamics
- Limited to proteins >40kDa
- No requirement for protein labeling
- **Does not require homogenous samples**
- Grid preparation procedure requires screening
- Real space imaging – no phase problem
- Can be used to study protein dynamics
- Can be expanded to larger assemblies (e.g., viruses and cells)
- Results in 1 or more models per dataset
- Computationally heavy (TBs of data + requirement for GPU processing)

# Additional learning resources

Extra material:

- Grant Jensen course on EM
https://cryo-em-course.caltech.edu/

- Lectures and courses on X-ray crystallography:
https://www.youtube.com/watch?v=mDbmfyOGLIM

- Lectures and Courses on NMR:
https://www.youtube.com/watch?v=_rjYI8XxEOs
https://www.khanacademy.org/science/