Methods to solve protein 3D structure

- X-ray crystallography
  - Solubilization of the over-expressed protein
  - Obtaining crystals that diffract
  - Structure determination by diffraction of protein crystals
  - Size of a molecule: no theoretical limit

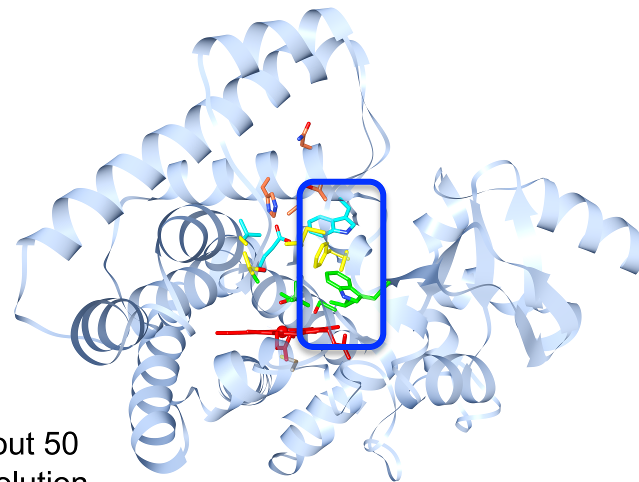
- Nuclear Magnetic Resonance spectroscopy
  - Solubilization of the over-expressed protein
  - Structure determination of a molecule as it exists in solution
  - Size-limit is a major factor (< 40 kDa)

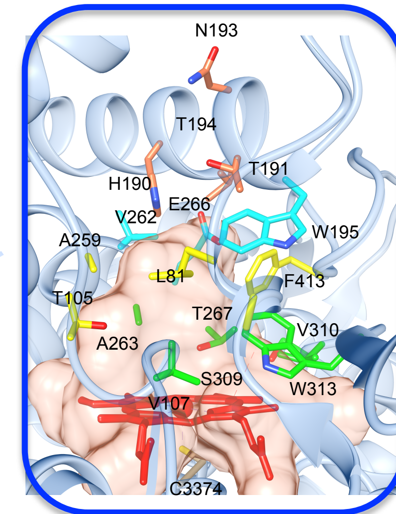
- Cryo Electron Microscopy (cryoEM)
  - Solubilization of the over-expressed protein
  - Structure determination of a molecule as it exists in solution
  - Size-limit is a major factor (proteins smaller than about 50 kDa are currently too small to be imaged at high resolution by cryo-electron microscopy)

Methods to predict protein 3D structure

- Homology modelling
  - The 3D structure of a homolog protein

- Threading or fold recognition
  - The 1D (protein sequence) must be compatible with a 3D fold present in the Protein Fold Databases (CATH and SCOP)

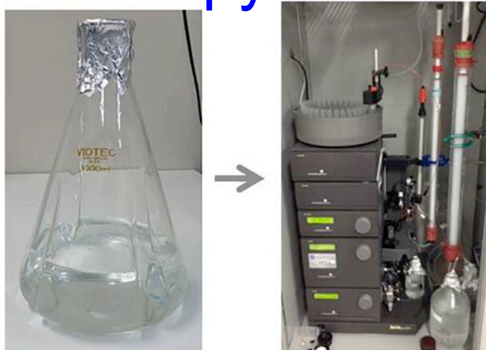




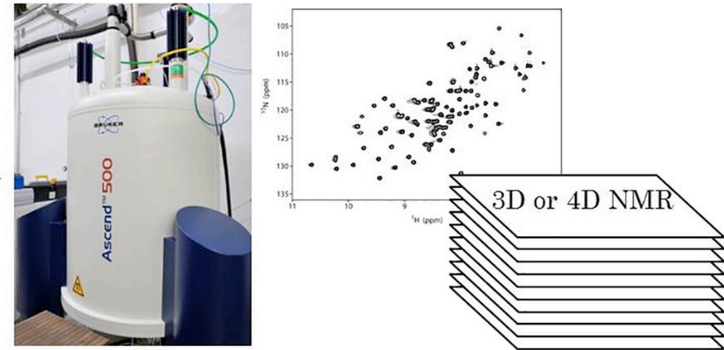
Methods to solve protein 3D structure

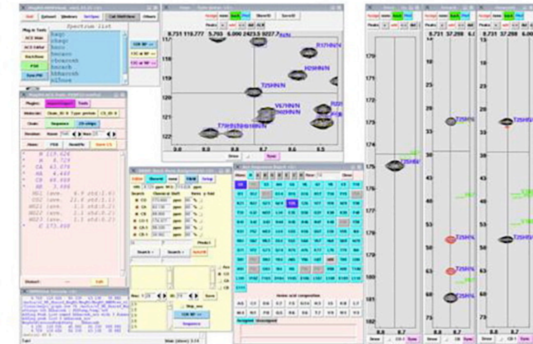
- Nuclear Magnetic Resonance spectroscopy

(A) Isotope labeled protein preparation (section 2)

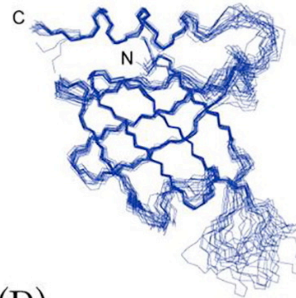


(B) NMR data collection (section 3)

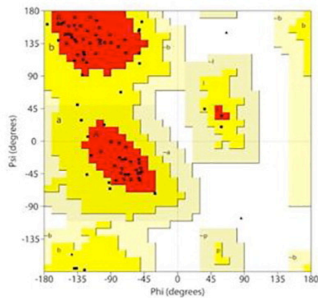




(C) Signal assignments (section 3)



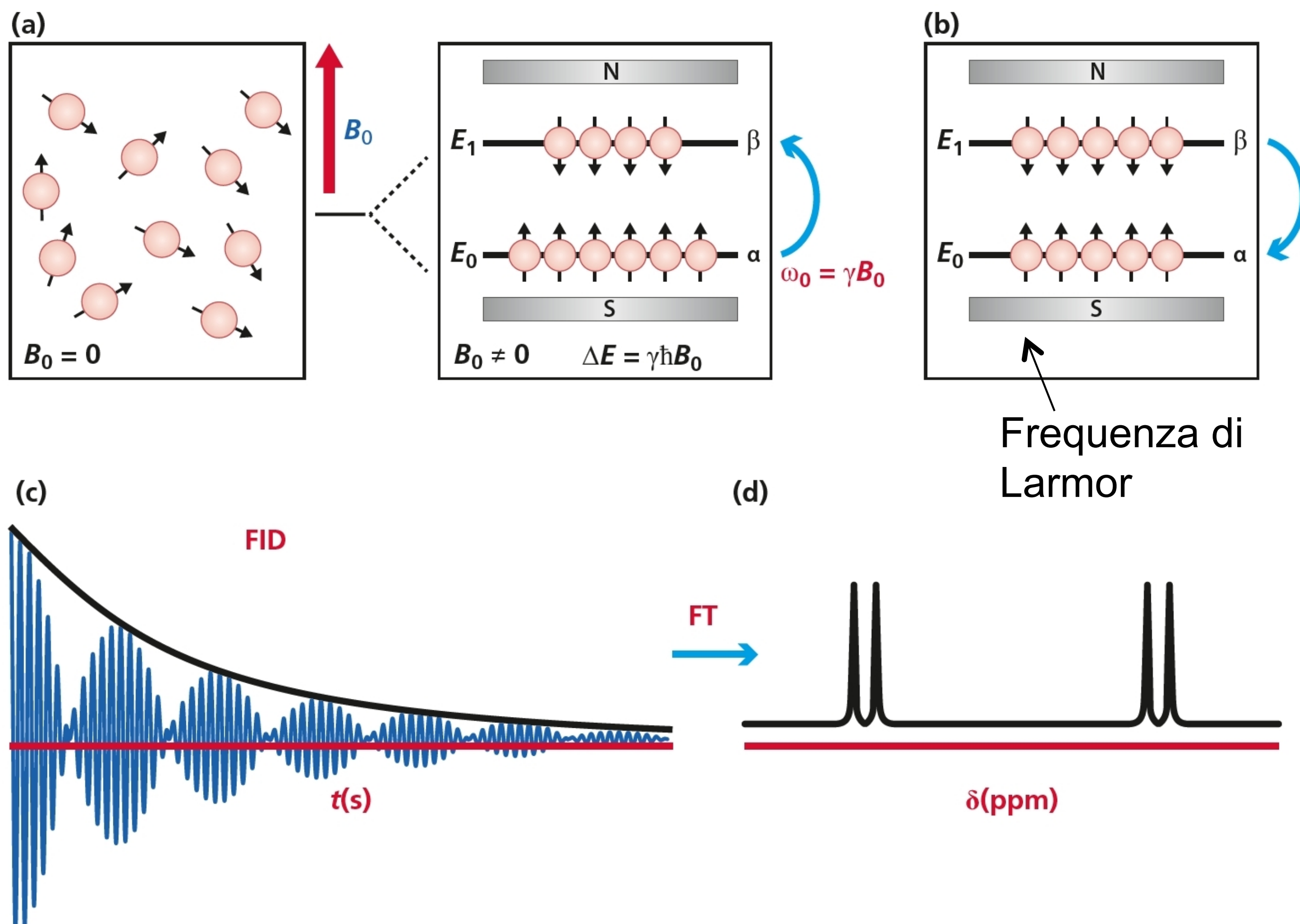
(D) Simulated annealing with NMR-based restraints
e.g. ^1H-^1H distance
Dihedral angle
(section 4, 5)



(E) Validation of precision and accuracy of the structure models (section 6)

Sugiki T, Kobayashi N, Fujiwara T. Modern Technologies of Solution Nuclear Magnetic Resonance Spectroscopy for Three-dimensional Structure Determination of Proteins Open Avenues for Life Scientists. Comput Struct Biotechnol J. 2017, 15:328-339. doi: 10.1016/j.csbj.2017.04.001.

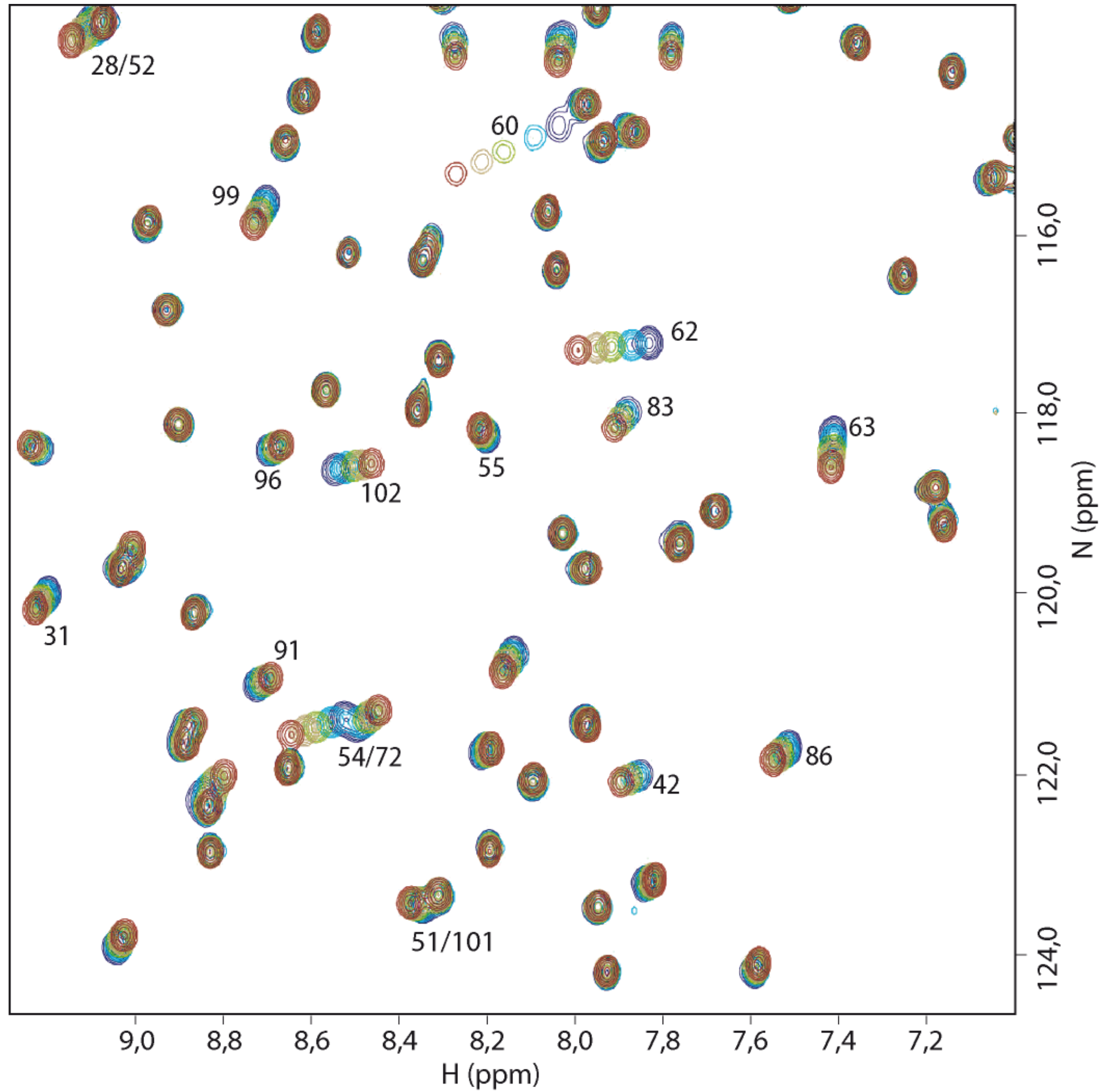
Nuclear magnetic resonance (NMR)



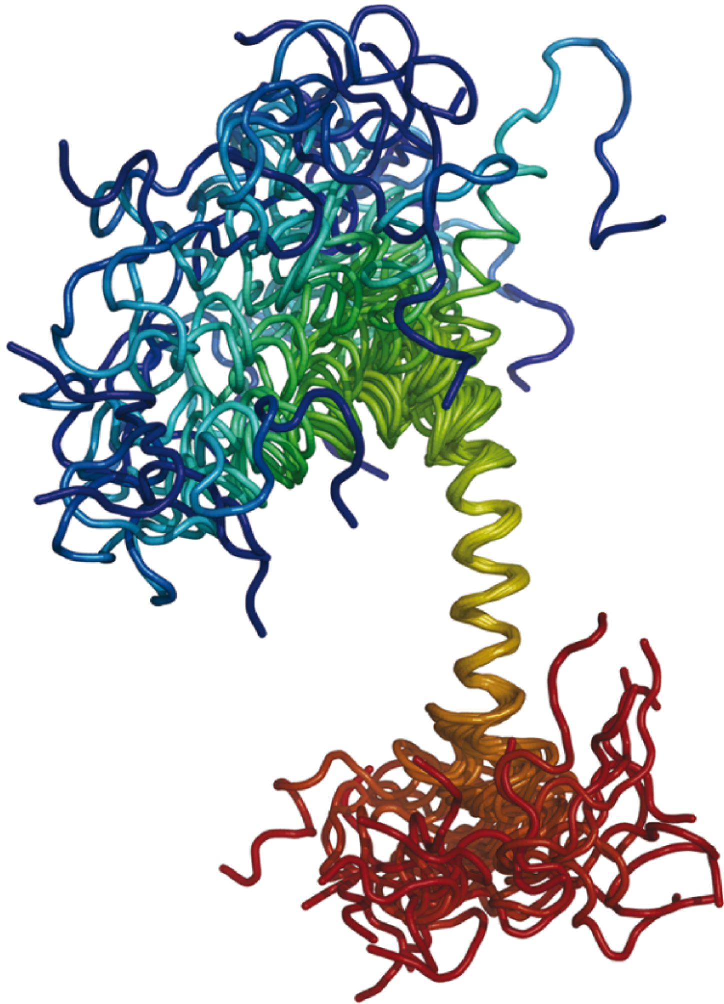
FID= free induction decay

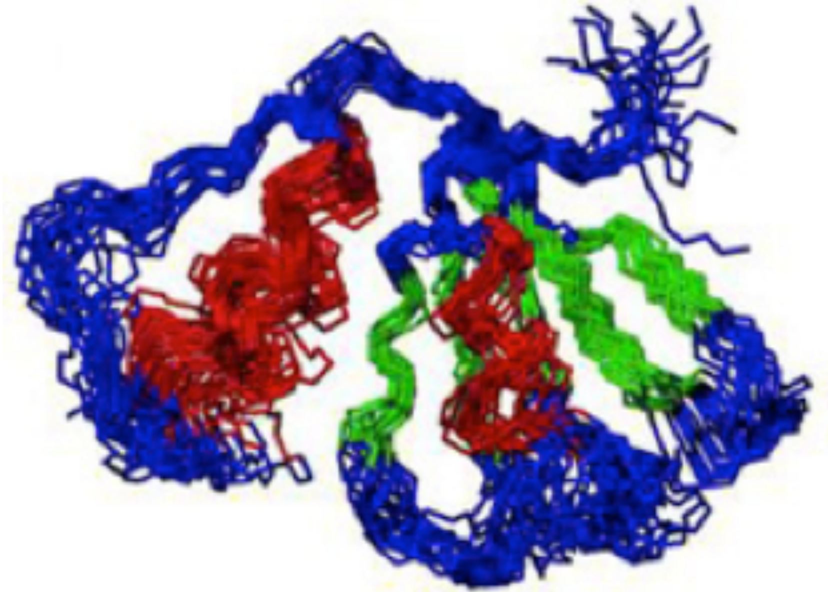
ZANICHELLI

Ligand binding experiment: titration



NMR structures - protein dynamics and flexibility -





The Protein Data Bank

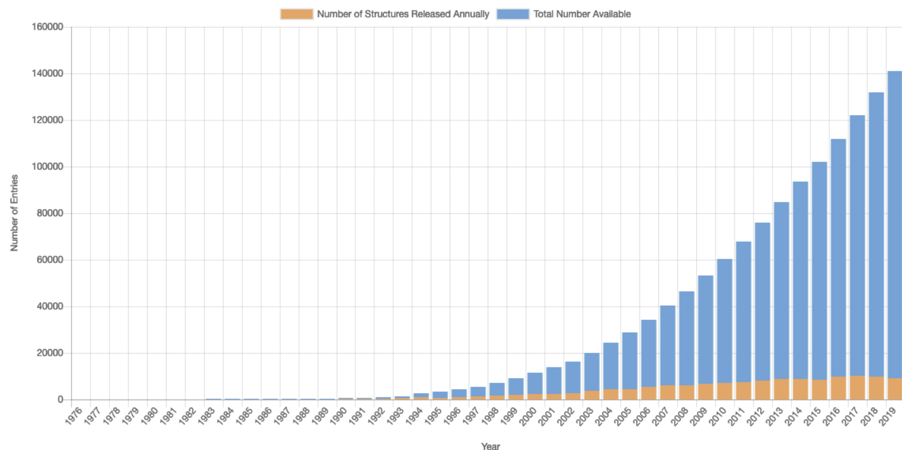
Other Statistics

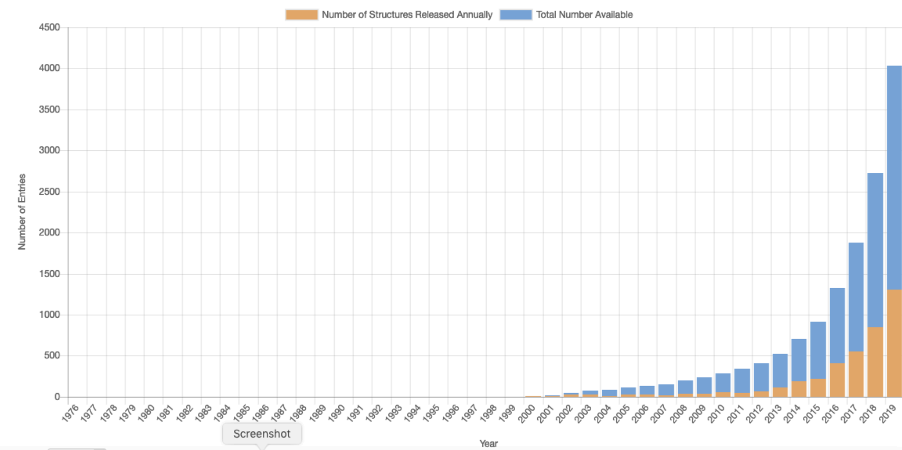
PDB Data Distribution by Experimental Method and Molecular Type

Copy | CSV

Experimental Method	Proteins	Nucleic Acids	Protein/NA Complex	Other	Total
X-Ray	132004	2073	6787	8	140872
NMR	11248	1306	262	8	12824
Electron Microscopy	2974	33	1021	0	4028
Other	281	4	6	13	304
Multi Method	144	5	2	1	152
Total	146651	3421	8078	30	158180

- X-ray crystallography
- Cryo Electron Microscopy (cry





Methods to solve protein 3D structure

- Cryogenic electron micoscopy (cryo-EM)

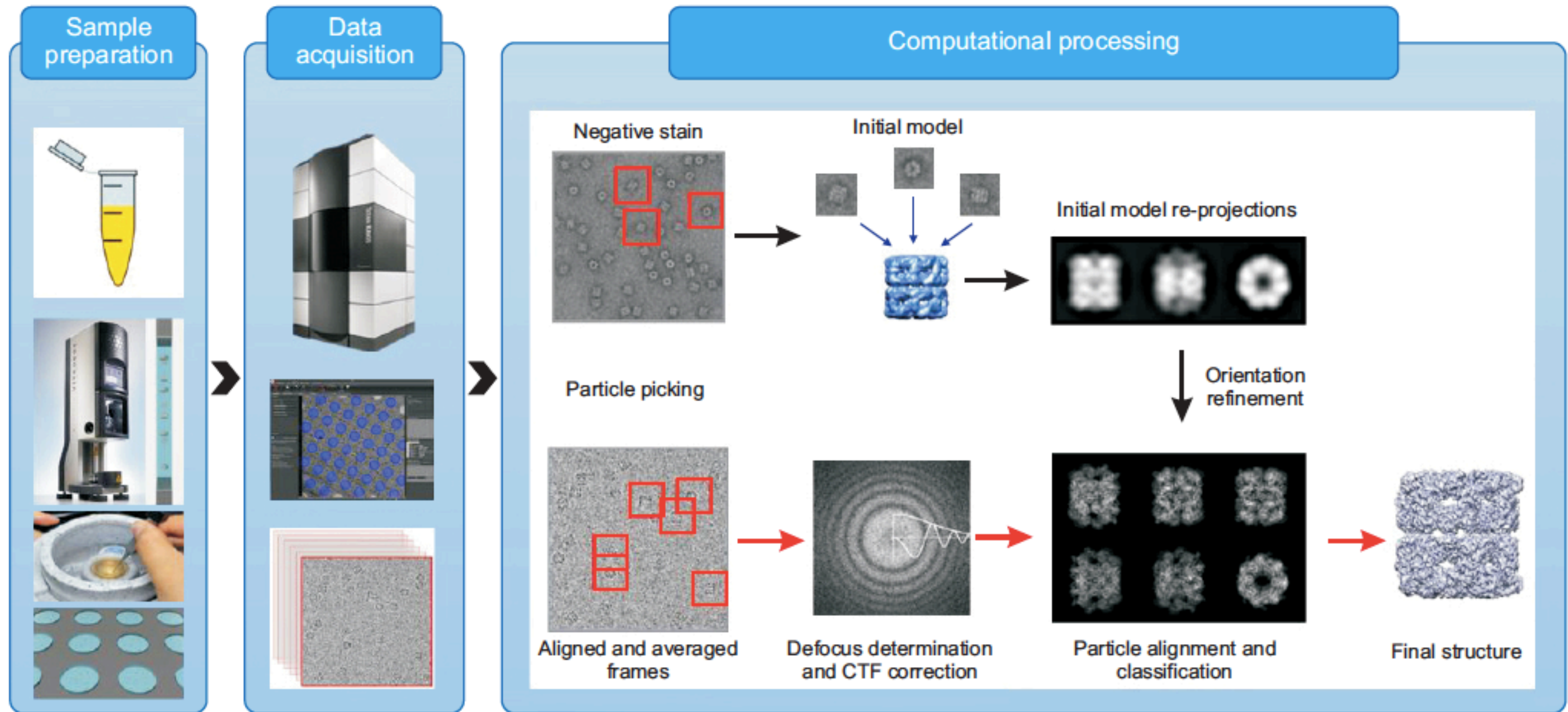


Fig. 2. Workflow of single-particle analysis for protein reconstruction. First of all, purified samples should be screened through negative staining to confirm adequate concentration and homogeneity. Classification and averaging can create better contrasting images. Used to construct initial models for orientation refinement, which is done by class averaging. In sequence, vitrified specimens of proteins are prepared by plunge freezing, after which three-dimensional images are created by computational processes such as particle two-dimensional classification, averaging from aligned and drift-corrected subframe images, defocus determination and contrast transfer function (CTF) correction, and orientation refinement with initial models to determine resolution. Adapted and modified from Carroni and Saibil (2016) (*Methods* 95, 78-85) in accordance with the Creative Commons Attribution 4.0 International (CC BY 4.0) license.

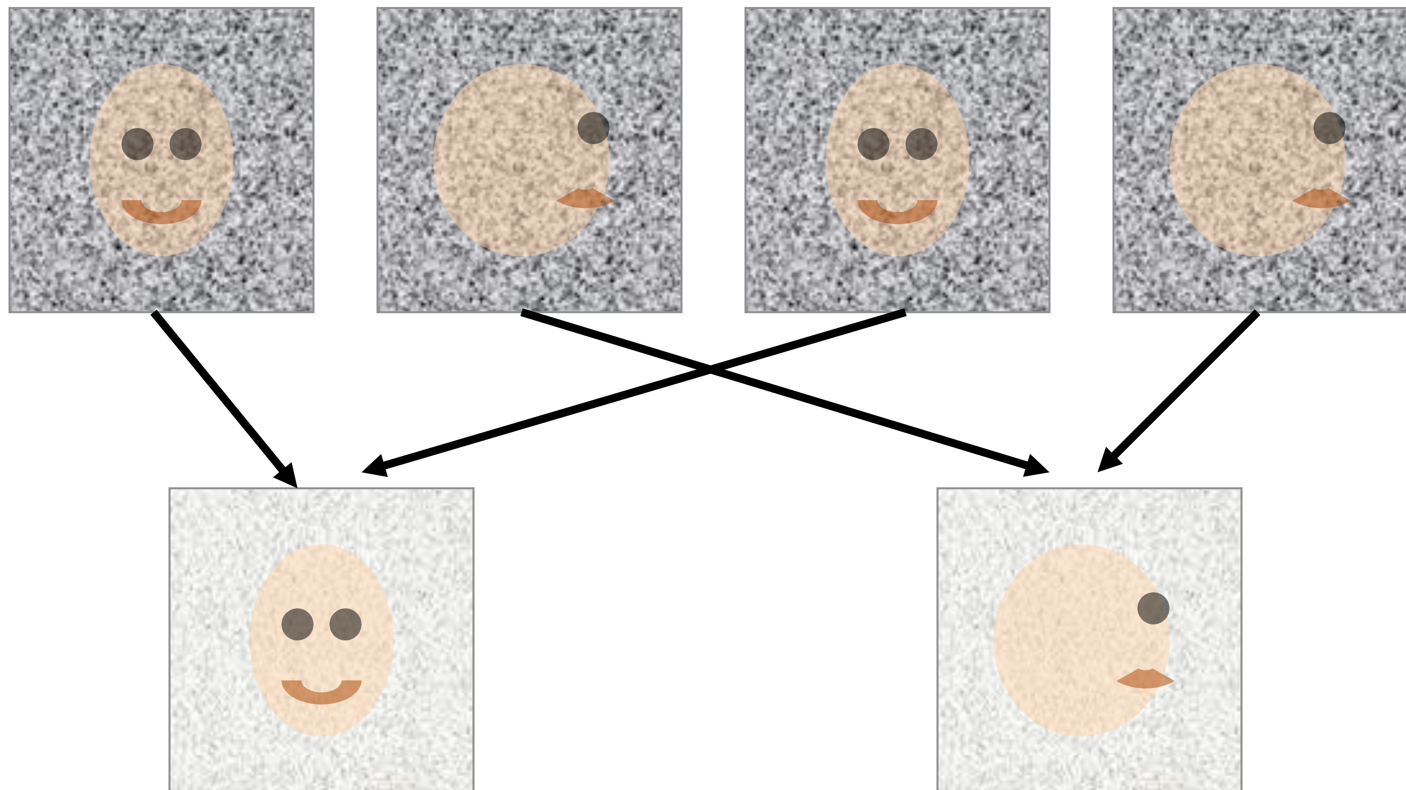
Choi et al., Workflow of Cryo-Electron Microscopy and Status of Domestic Infrastructure Applied Microscopy https://doi.org/10.9729/AM.2018.48.1.6

Cryogenic electron micoscopy (cryo-EM)

- Transmission electron microscopy (TEM): the sample is irradiated by a focused electron beam, with very short wavelengths (0.02-0.025 Å)
- The electronic "bombardment" is generated by intense potentials (200-300 kV) possible only in high vacuum.
- The sample must be frozen to avoid radiation damage (T = <-175 ° C).
- 2D images of "single particles" are obtained.
- The images of hundreds of thousands of single particles are collected in different orientations.
- The images of the iso-oriented ones are added together to obtain homogeneous 2D classes, increase the contrast and obtain the 3D structure through the Fourier transform.

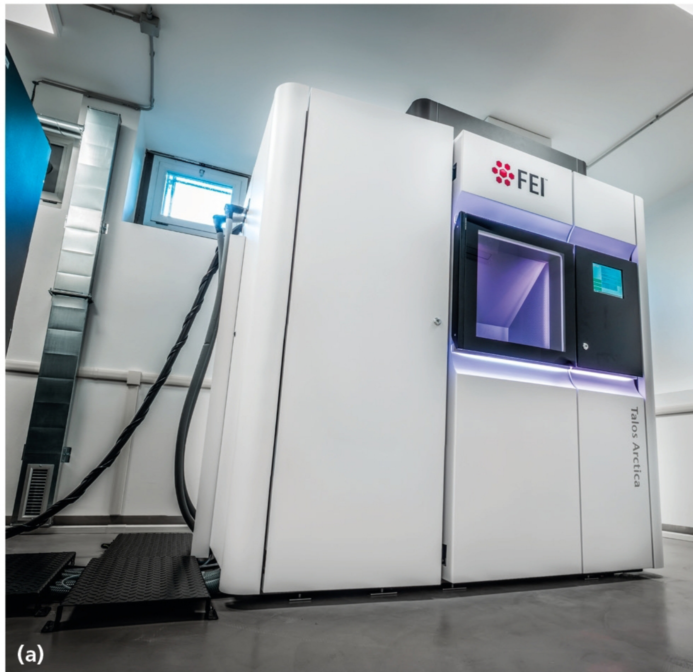
Cryogenic electron micoscopy (cryo-EM)

- The images of the iso-oriented ones are added together to obtain homogeneous 2D classes, increase the contrast and obtain the 3D structure through the Fourier transform.



Methods to solve protein 3D structure

- Cryogenic electron micoscopy (cryo-EM)



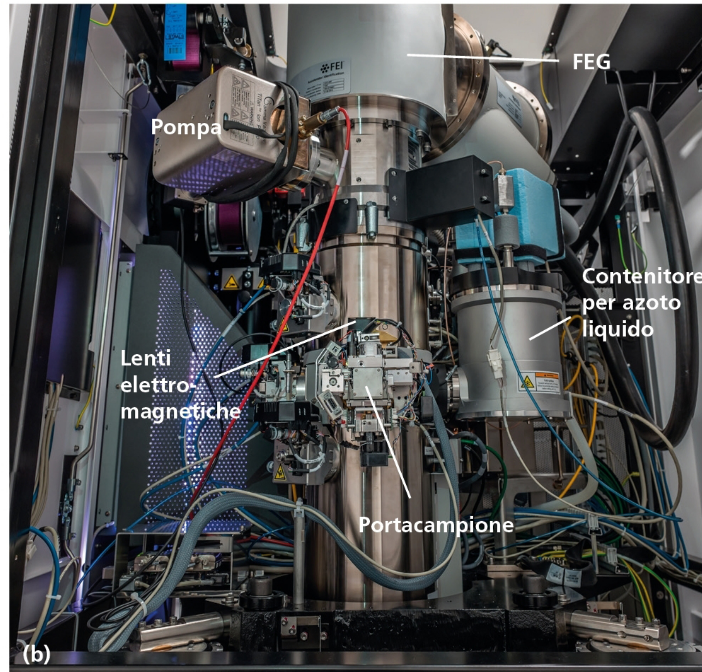


Figura 23.8
(**a**) Immagine del ThermoFisher Scientific™ Talos™ Arctica S/TEM (200keV FEG), un moderno crio-microscopio elettronico in dotazione all'Università degli Studi di Milano e parte del Centro per la Ricerca Pediatrica "Romeo ed Enrica Invernizzi". (**b**) Visione interna del microscopio elettronico. La fonte di elettroni, in questo caso una FEG, è posta in alto. Seguono ai lati della colonna, composta da diverse lenti elettromagnetiche, un sistema di pompe e contenitori per azoto liquido per il mantenimento di alti livelli di vuoto ($< 10^{-6}$ Pa) e basse temperature (< -175 °C). Il portacampione è posto centralmente alla colonna e ortogonalmente all'asse ottico del microscopio.

Milne JL, Borgnia MJ, Bartesaghi A, Tran EE, Earl LA, Schauder DM, Lengyel J, Pierson J, Patwardhan A, Subramaniam S. Cryo-electron microscopy--a primer for the non-microscopist. FEBS J. 2013 Jan;280(1):28-45. doi: 10.1111/febs.12078.

ZANICHELLI

Main steps in protein crystallography.

1. Protein purification

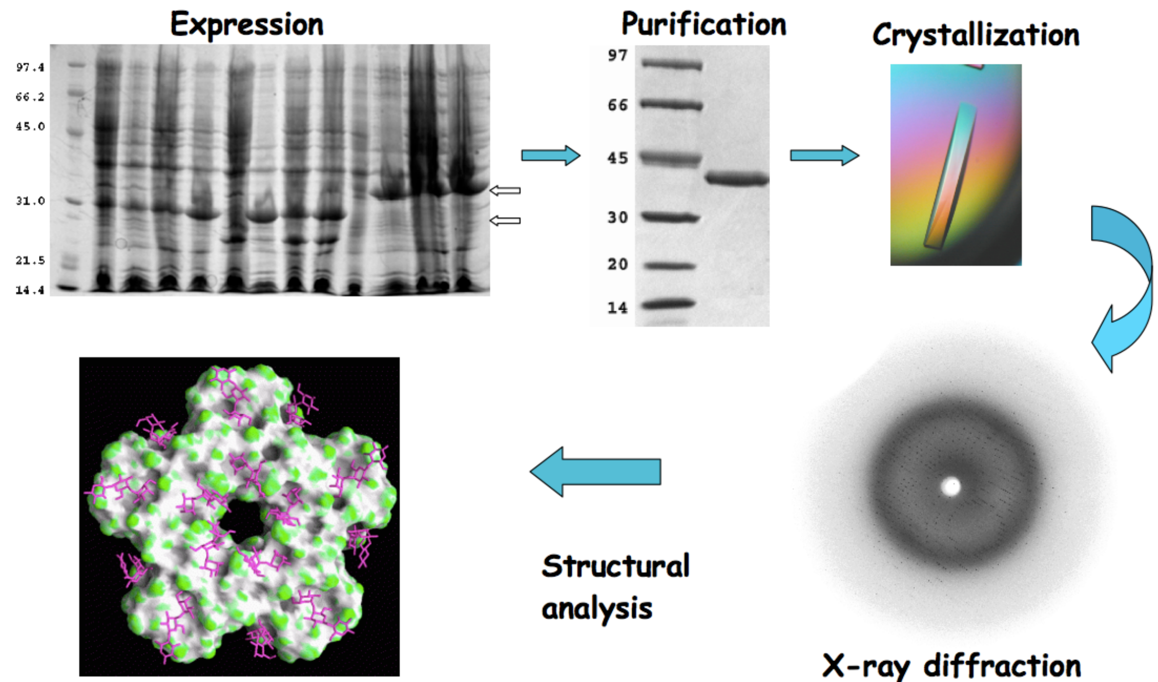
2. Protein crystallization

3. Testing crystals

4. Data collection

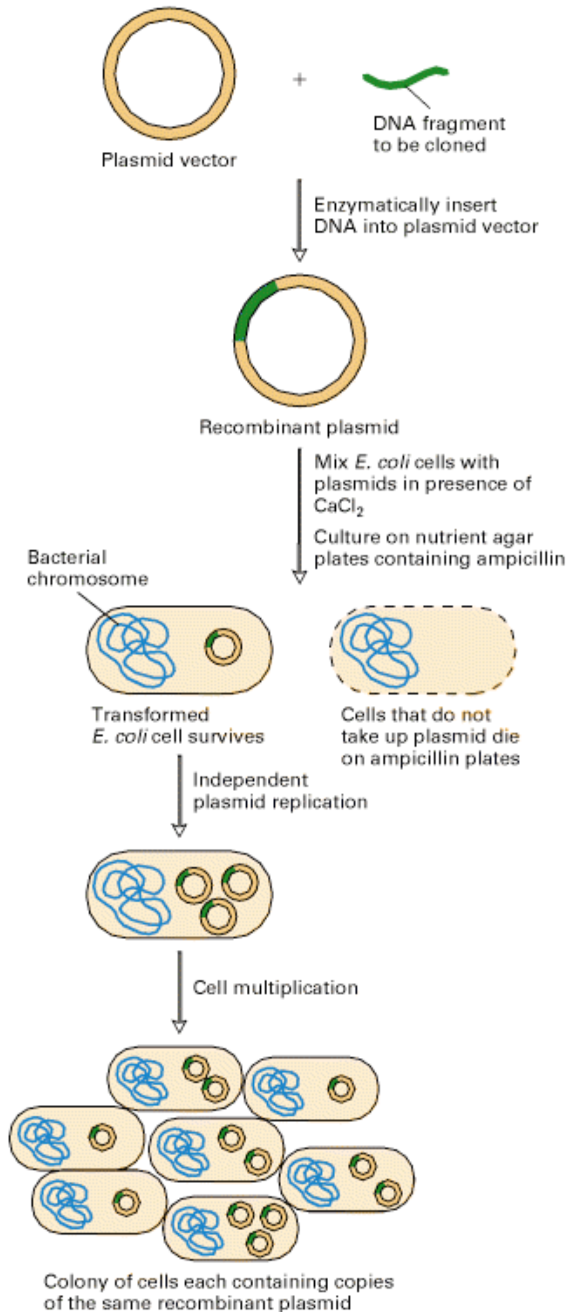
5. Data processing

6. Structure solution



- Obtaining single crystals that diffract to high resolution remains the primary bottleneck of protein crystallography!!!

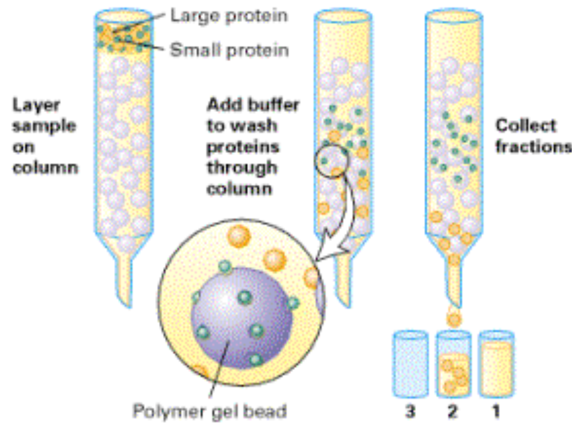
1. Protein preparation

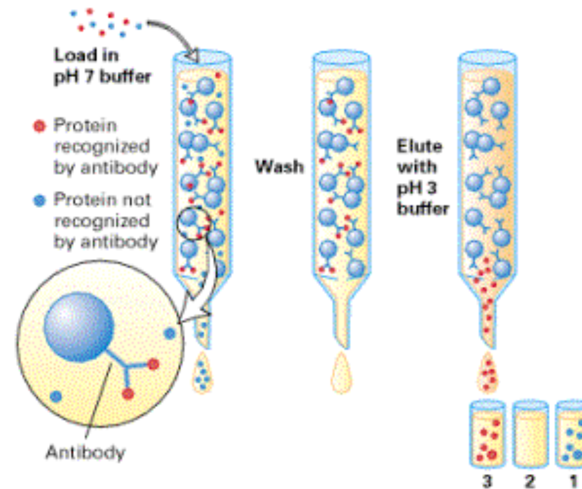


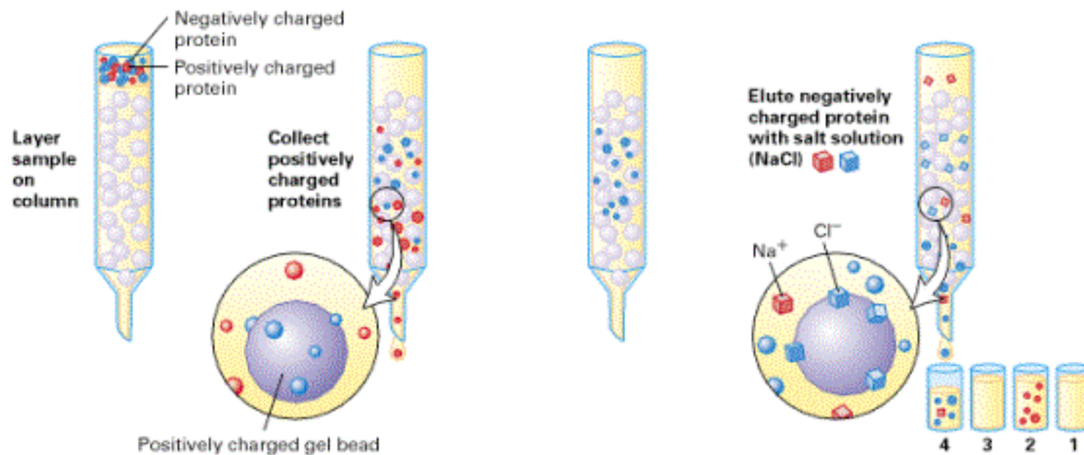
- **Preparing protein for crystal trails usually requires a large amount of highly purified protein.**
- Using recombinant DNA technique, it is now possible to instruct a variety of cells and organisms to make a large amount of almost any protein chosen by investigator.
- Not only can specific proteins be expressed in large quantities also recombinanat proteins can be modified in ways that make the task of the crystallographer simpler and in some cases, dramatically improve the quality of the resulting crystals.

Expression systems.
1.E. coli
2.Yeast
3.Insect Cell
4.Animal Cell

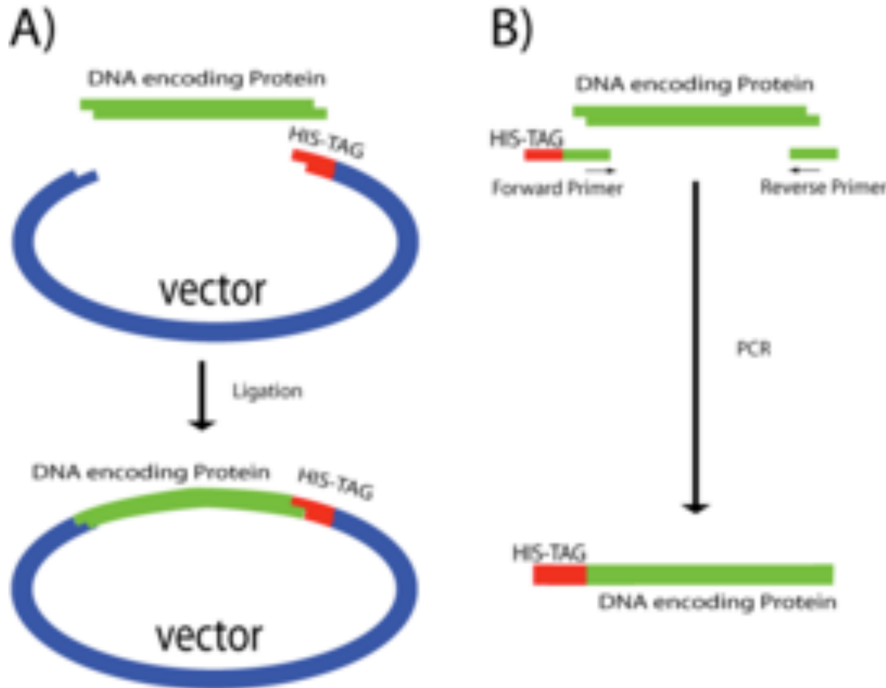
The goal of a protein separation is to obtain a protein in a pure, active form with minimum steps and within shortest time. Affinity choromatography can allow efficient purification of fusion protein or proteins with well defined ligand-binding domains.

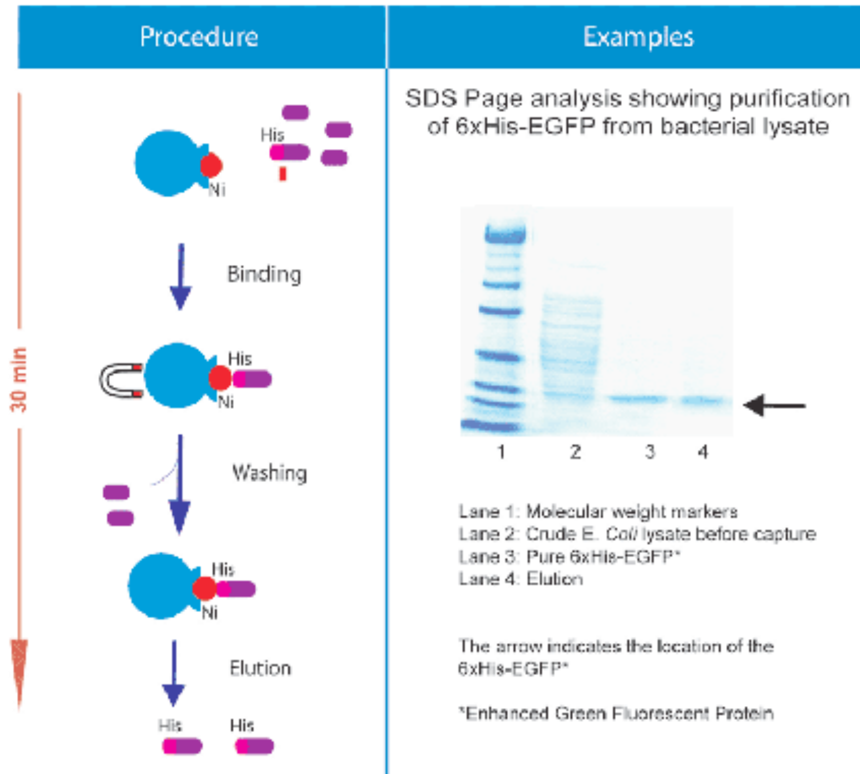


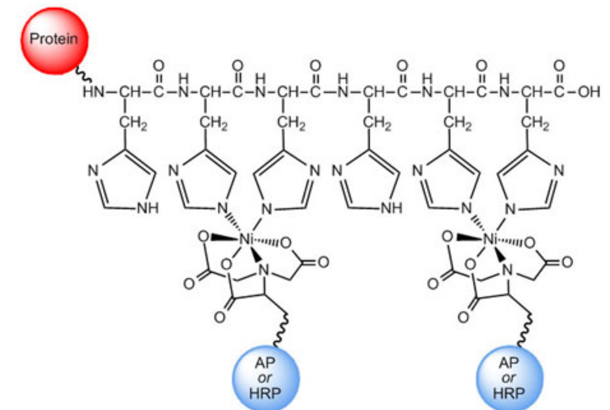




Histidine-tagged proteins







USEFUL TECHNIQUES FOR CRYSTALLOGRAPHERS

The most important thing you will ever put in your crystallization trials is your protein. Some basic things to check before you start are:

•*Is it pure?*

SDS-PAGE with Silver staining

•*Is it homogenous?*

Absence of post-translation modifications with mass spectroscopy

•*Is it folded?*

Circular dichroism

•*Is it fresh?*

•*Is it monodispersed?*

Gel filtration and light scattering

•*Does your protein need to be kept reduced?*

Gel filtration in presence and absence of reducing agent

•*Does your protein need the addition of something (eg salt) to stay in solution?*

•*Is your protein stable at room temperature?*

•*Does your protein break down rapidly*?

•*Has anything similar been crystallized before?*

Check the PDB and look in the header records for crystallization details

If you are getting nowhere with your protein, try crystallizing something else.

• *Ligand-protein complex.* Does your protein bind a ligand? If it does, it can help because the binding of a ligand

- is likely to order the region of the protein that binds ligand
- may bring two subdomains together and reduce flexibility
- will change the surface properties of the protein
- may cause a conformational change in your protein

• *Different constructs.* Heterogeneous tertiary/quaternary structure can hinder crystallization

• Does your protein break down to a stable proteolytic fragment (either "spontaneously" or with the help of an added protease)?

• Does the homology of your protein to others in the same family drop off at the N- and C-termini?

• Does your protein have a domain structure?

- Check the Pfam database
- Check the ProDom database

•Does your protein have low complexity regions?

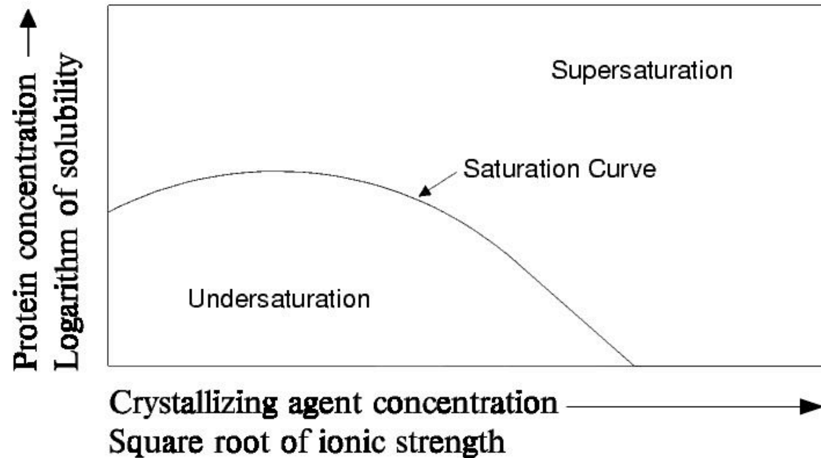
• *Different species.* Sometimes, a point mutation is all that is required to stop/start a protein crystallizing. Working on a different species is the easiest way to get a collection of point mutations that do not affect function.

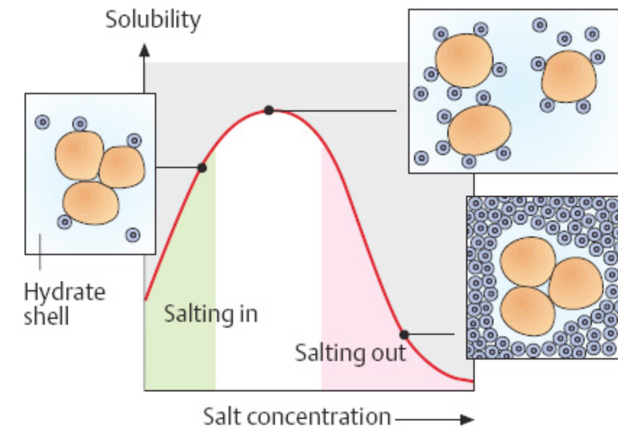
• *Deglycosylation.* If your protein is glycosylated the floppy and heterogeneous carbohydrates may be interfering with the crystallisation. Try enzymatic deglycoylation.

Crystallizability is inversely proportional to biological interest. *Murphy's crystallization law*

2. Protein crystallization

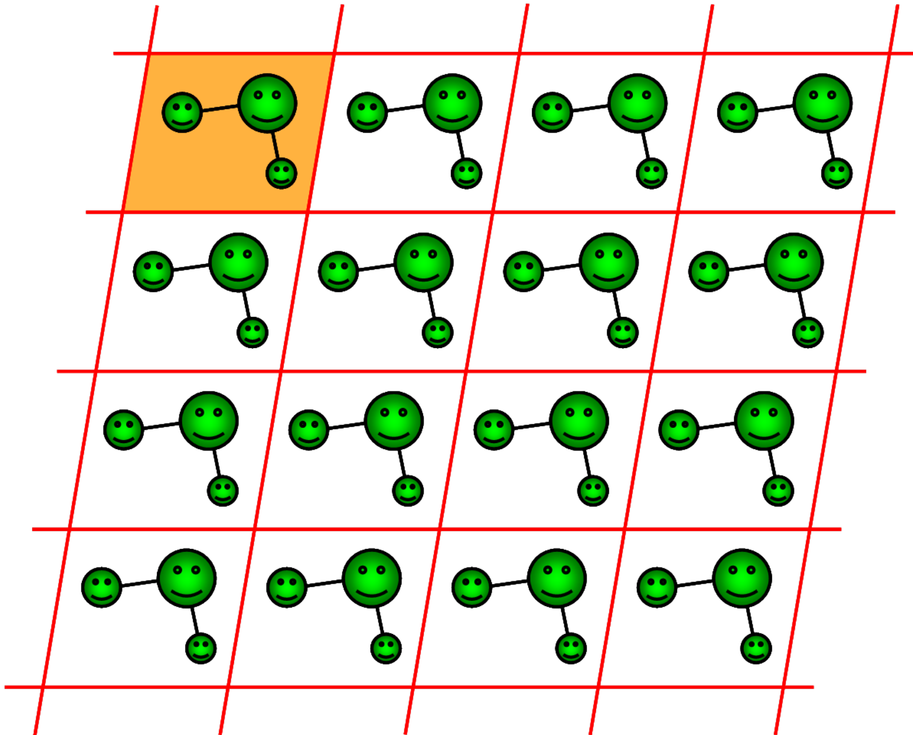
The solubility of proteins can be represented in **phase diagrams**.
The phase diagram plots the solubility curve of a protein.
•the horizonal axis shows the parameter being varied (usually precipitant concentration)
•the vertical axis shows the protein concentration.





•Saturation occurs when the rate of loss and gain of both the solid and solution phases of the protein are equal, and the system is in equilibrium.
•*Salting-out* is seen on the right hand side of the diagram where there is a reduction in protein solubility as the concentration of salt increases
•*Salting-in* is seen on the left hand side of the diagram where there is an increase in protein solubility as the concentration of salt increases.

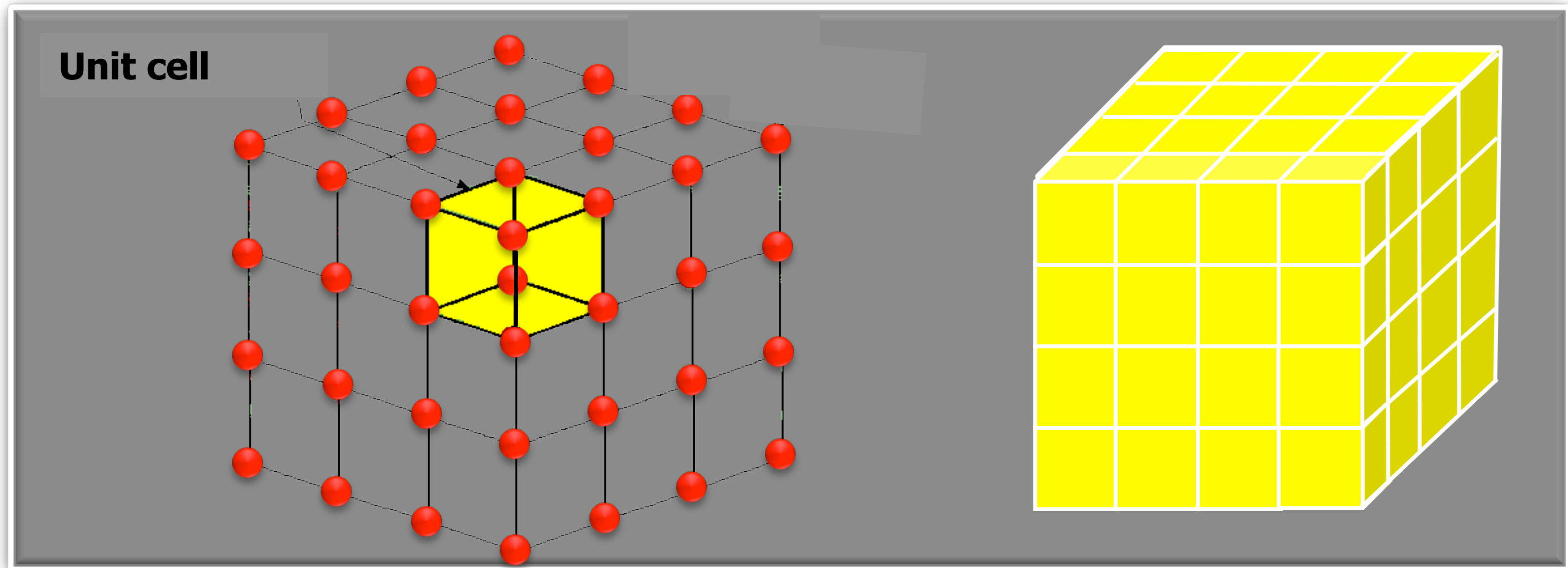
Crystals

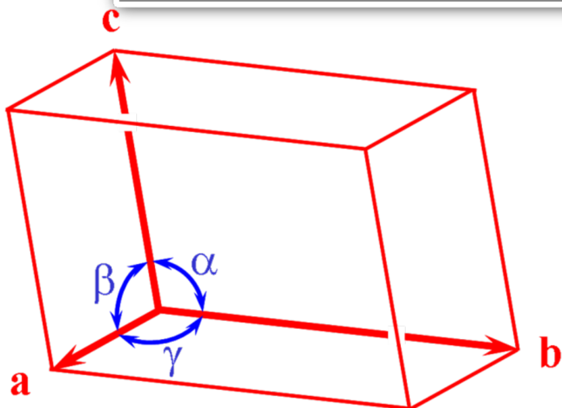


In the crystal lattice, all the unit cells have the sameshape, size and content.

Crystals

In three dimensions, the unit cell is the smallest portion of volume of the lattice which, translated parallel to itself, reconstructs the entire crystal.



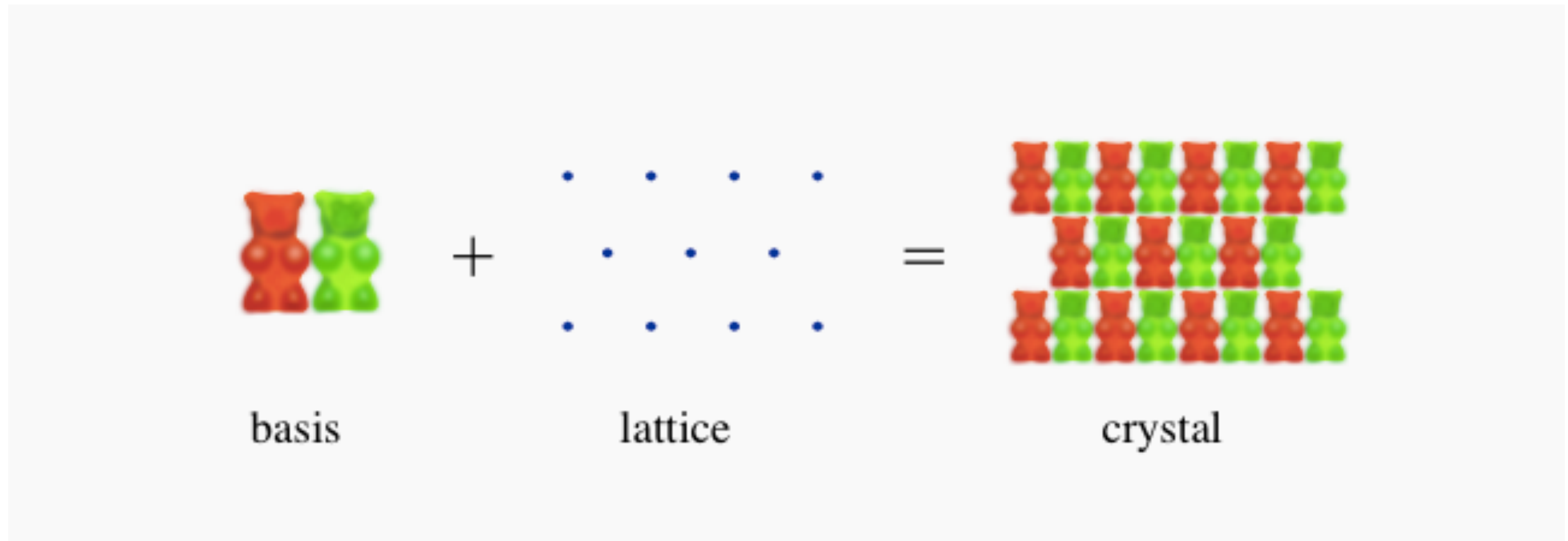


To fully describe the elementary cell, a total of six scalar amounts must be specified, thatare called lattice parameters and are indicated with the symbols:

a, b, c	length of the sides
α, β, γ	angles between sides

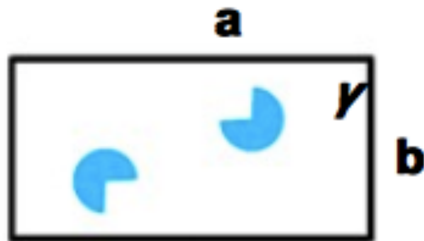
Crystals

Now one could go ahead and replace the lattice points by more complex objects (called basis), e.g. a group of atoms, a molecule. This generates a structure that is referred to as a crystal:



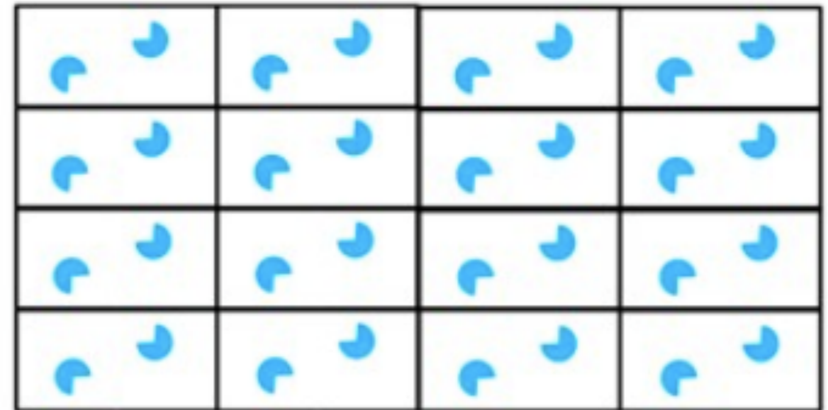
A crystal is defined as a lattice with a basis added to each lattice site. Usually the basis consists of an atom, a group of atoms or a molecule.

The asymmetric unit and the unit cell

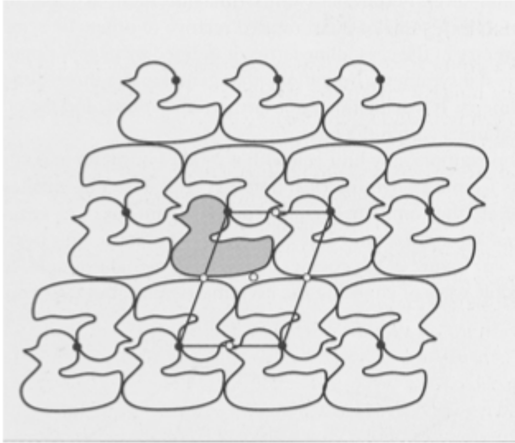


Unit cell:
characterized by edge length and angles
can contain several molecules
is repeated multiple times along the axis of the crystal

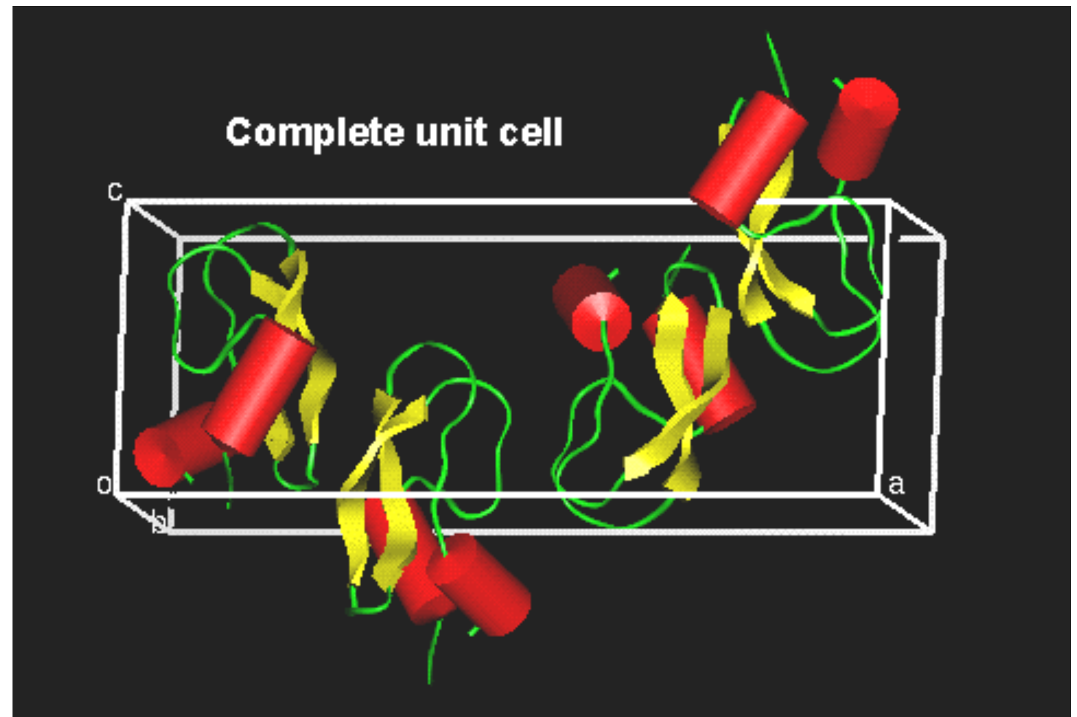
Assymetric Unit: minimal part of the unit cell which is related to other parts by defined symmetry operations



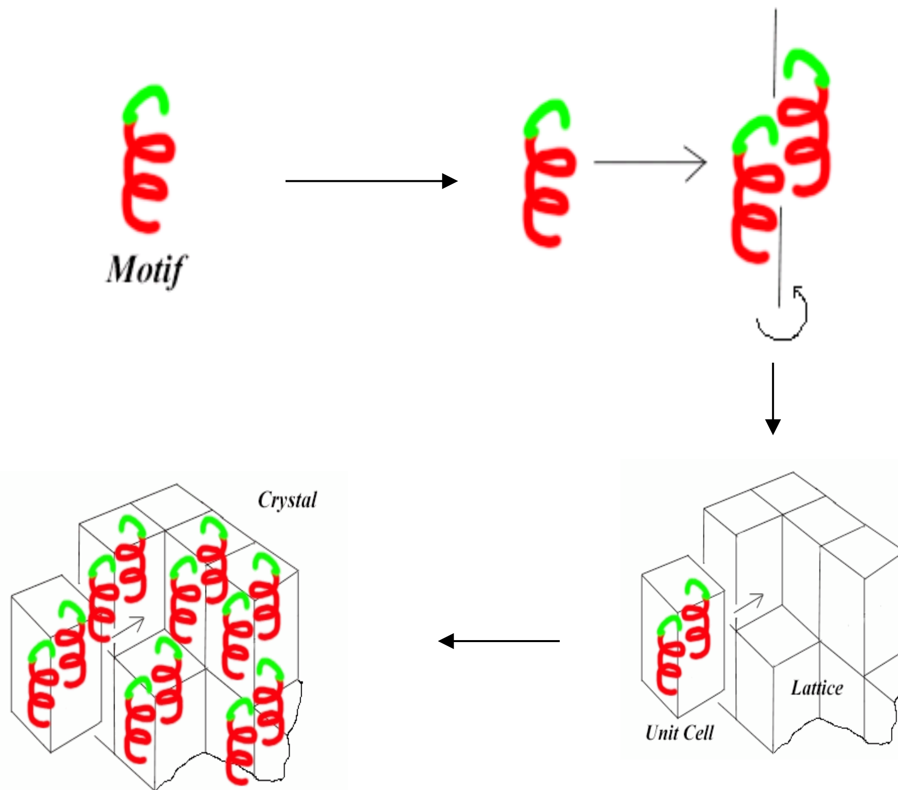
The asymmetric unit

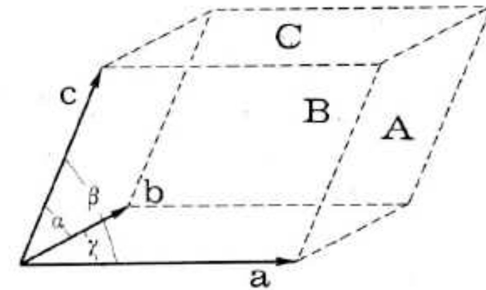


The asymmetric unit (shaded duck) is the smallest unit of structure that can generate the whole crystal after application of the crystal symmetry.



Protein crystals symmetry.





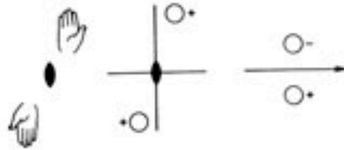
The unit cell is outlined by three cell axes, **a**, **b**, and **c** along the x, y and z directions respectively. The use of bold font indicates that the cell axes are vectors, with length and direction.The unit cell is often described by the length of the axes and the angles between them: *a* = |**a**|, *b* = |**b**|, *c* = |**c**|

α= angle between **b** and **c**; β= angle between **a** and **c**; γ= angle between **a** and **b**

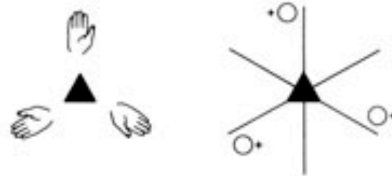
Symmetry operators in crystals

Rotation axes

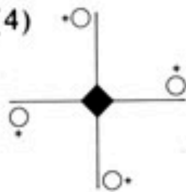
$C_2(2)$



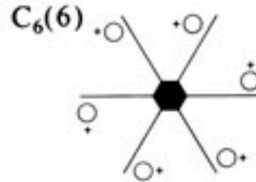
$C_3(3)$



$C_4(4)$



$C_6(6)$

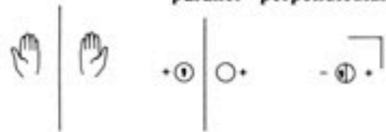


Mirror plane

$\sigma(m)$

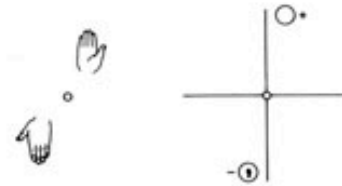
Looking

parallel perpendicular



Inversion through a center

$i(\bar{1})$

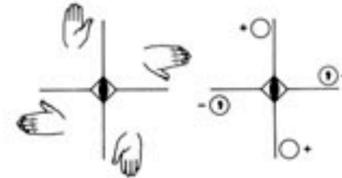


Rotatory inversion axes

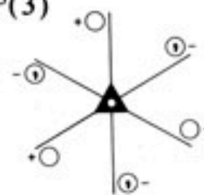
$i(\bar{1})$ = inversion

$\sigma(\bar{2})$ = mirror plane

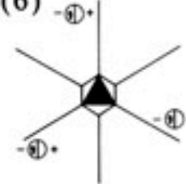
$S_4^3(\bar{4})$



$S_6^5(\bar{3})$



$S_3^5(\bar{6})$



A **point group** is a group of geometric symmetries (isometries) leaving a point fixed (origin).

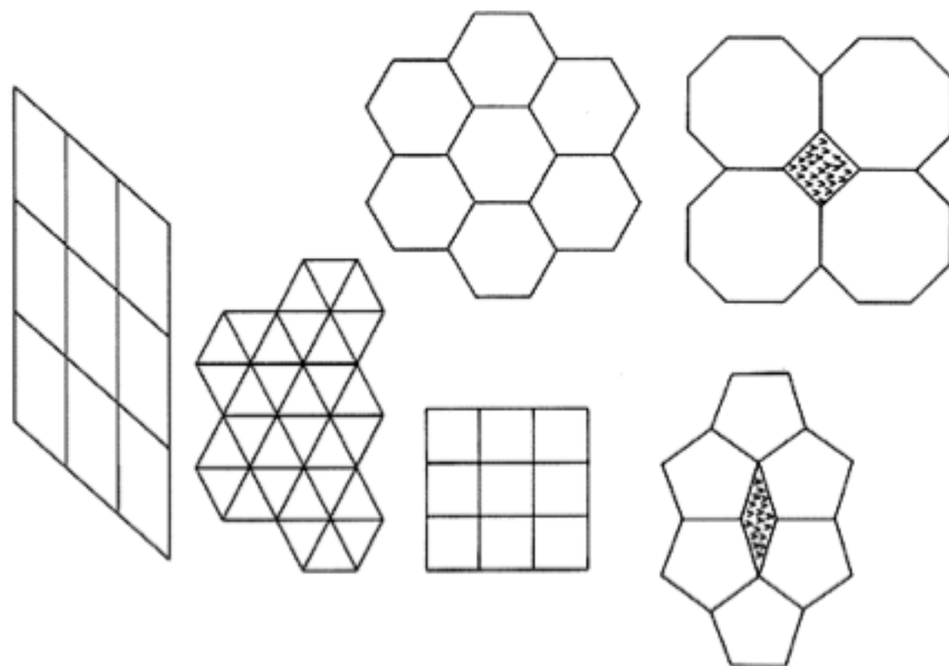
Asymmetric unit: the minimum portion of the unit cell that can generate all the unit cell by applying the simmetry operations.

The **space groups** derive from point groups with the addition of translation operations.

A space group contains all the simmetry operations of the atoms in a crystal.

Not all rotational symmetries are allowed in a crystal

The only rotational symmetries possible in a crystal lattice are 2, 3, 4 and 6, because it is not possible to fill space with other symmetries.



(I am excluding "quasi-crystals" which can show 5-fold symmetry, discovered by Dan Schechtman in 1982, Nobel prize in Chemistry 2011)

Note this restriction does not apply to molecular symmetry, for example C-reactive protein has 5-fold symmetry, GroEL has 7-fold etc

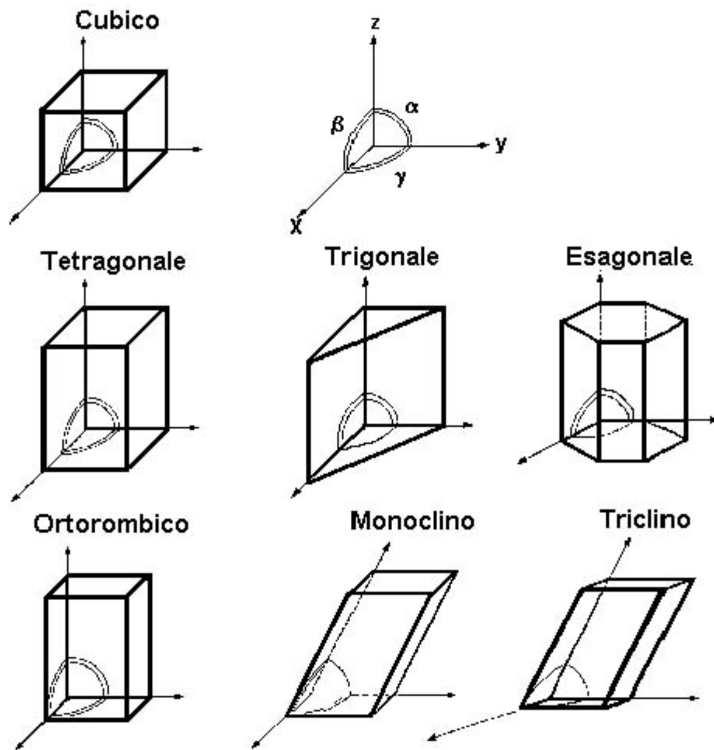
Crystal systems

There are restrictions in simmetry that have to be applied to the axes and angles of the unit cell. This constrains the simmetry of the lattice and gives rise to the 7 crystal systems.

Crystals can be described by shape of unit cell (called lattice type) and symmetry type of asymmetric unit

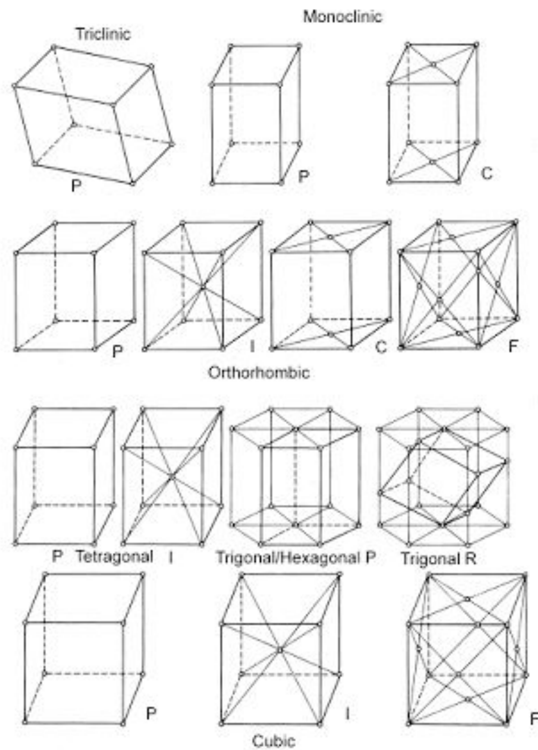
65 different space groups possible for natural biomolecules

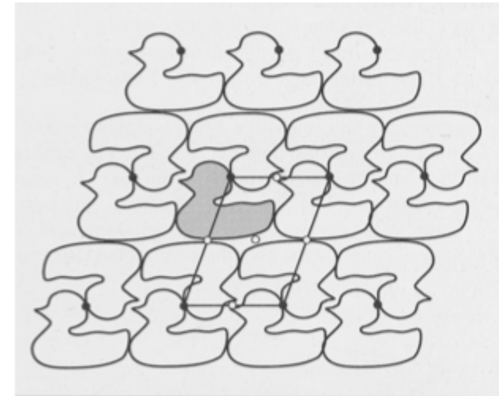
Example $P2_12_12_1$

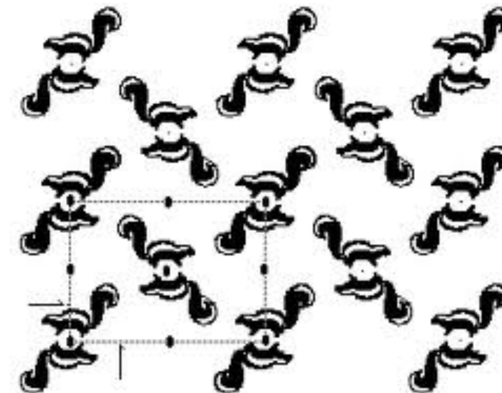


Triclinic	$a \neq b \neq c$ $\alpha \neq \beta \neq \gamma$
Monoclinic	$a \neq b \neq c$ $\alpha = \beta = 90° \neq \gamma$ (1st setting) $\alpha = \gamma = 90° \neq \beta$ (2nd setting)
Orthorhombic	$a \neq b \neq c$ $\alpha = \beta = \gamma = 90°$
Tetragonal	$a = b \neq c$ $\alpha = \beta = \gamma = 90°$
Cubic	$a = b = c$ $\alpha = \beta = \gamma = 90°$
Hexagonal	$a = b \neq c$ $\alpha = \beta = 90°; \gamma = 120°$
Trigonal (Rhombohedral)	same as hexagonal ($a = b = c; \alpha = \beta = \gamma$)

Crystal systems and Bravais lattices



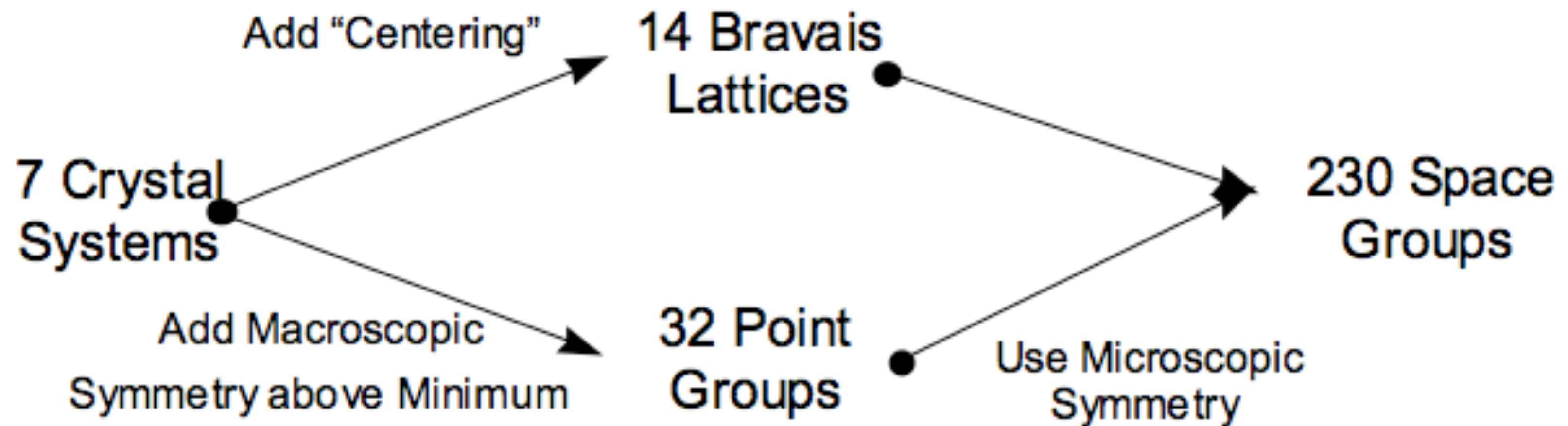




The French crystallographer Auguste Bravais established that in three-dimensional space only fourteen different lattices may be constructed.

The Bravais lattices are of three different types. A **primitive lattice** has only a lattice point at each corner of the three-dimensional unit cell. A **body-centered lattice** contains not only lattice points at each corner of the unit cell but also contains a lattice point at the center of the three-dimensional unit cell. A **face-centered lattice** possesses not only lattice points at the corners of the unit cell but also at either the centers of just one pair of faces or else at the centers of all three pairs of faces.

A space group is a representation of the ways that the macroscopic and microscopic symmetry elements (operations) can be self-consistently arranged in space. There are 230 unique manners in which this can be done and, thus, 230 space groups.

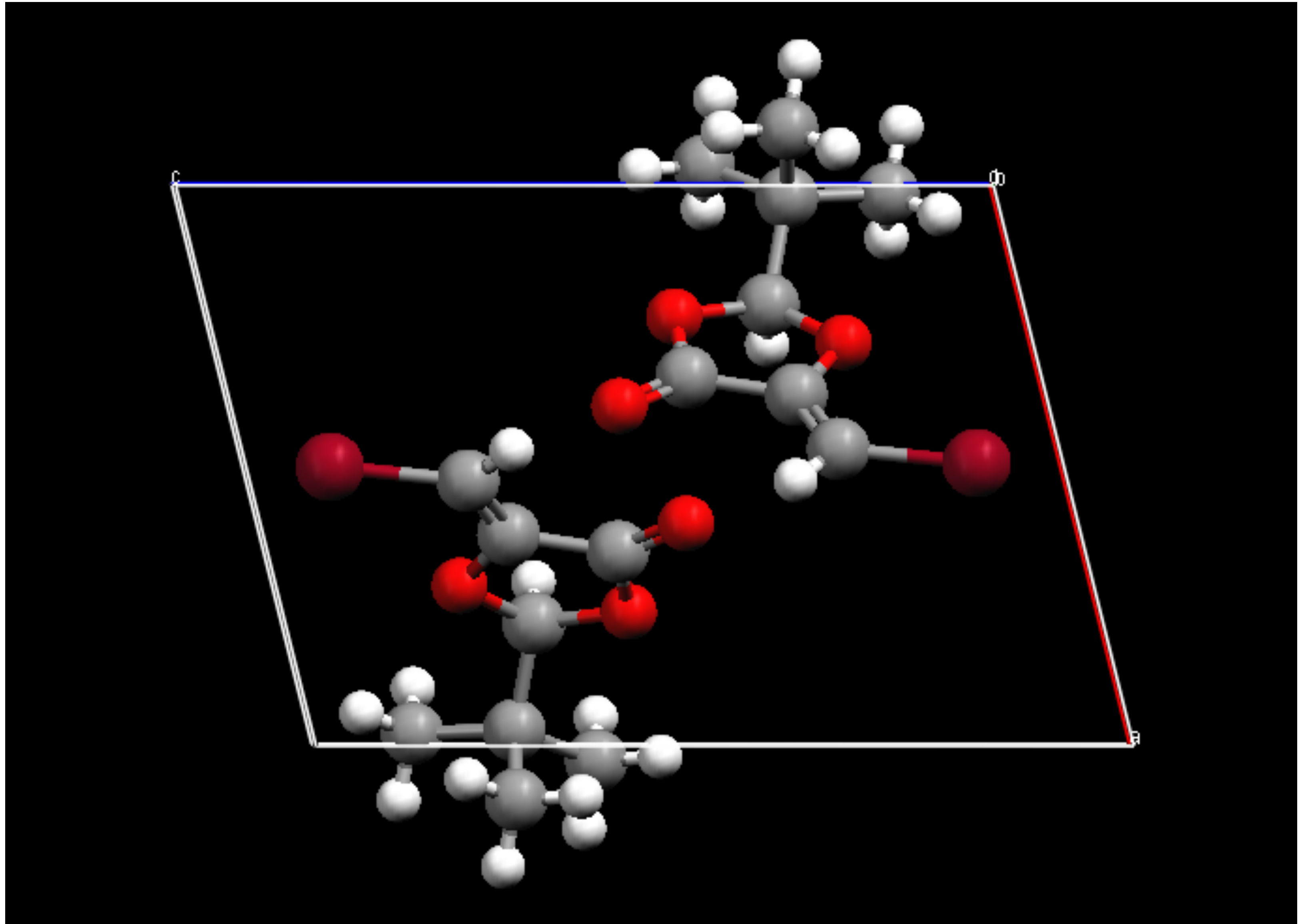


-Not all 230 space groups are allowed for protein crystals.

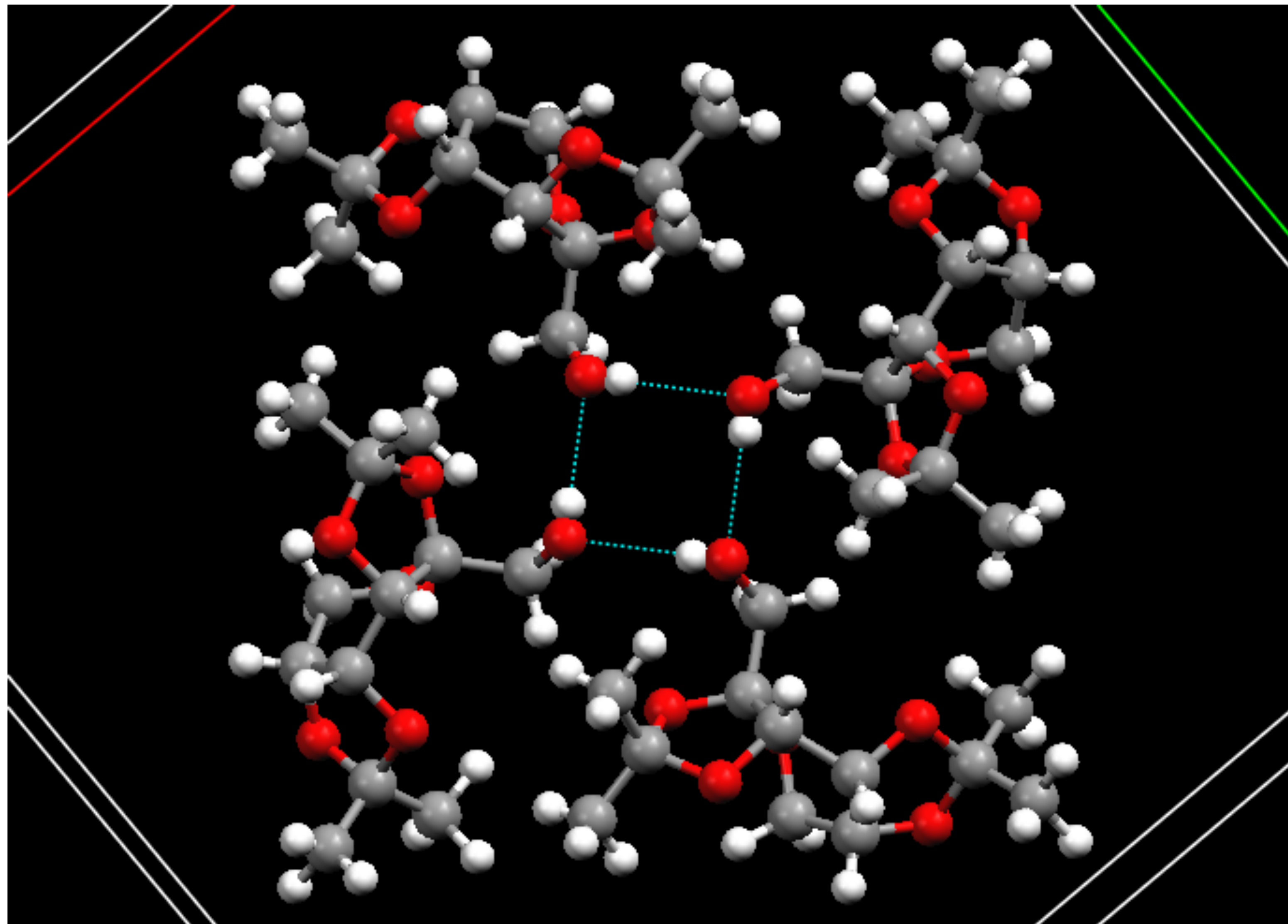
- Mirror planes and inversion centers would change the symmetry of the aminoacids from L- to D-aminoacids, never found in proteins.

- Rotation and screw axis are allowed.

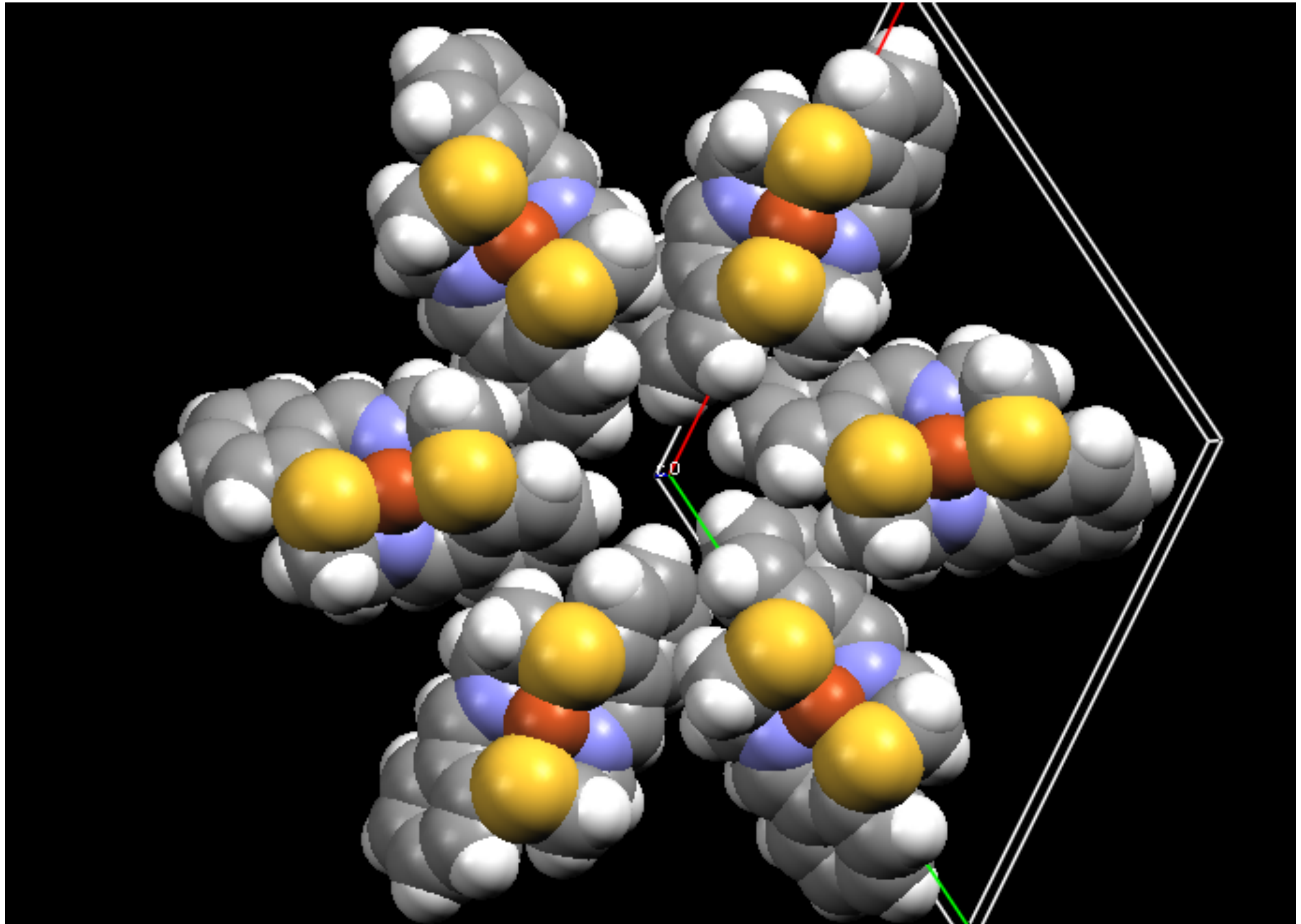
2 Rotation Axis (ZINJAH)

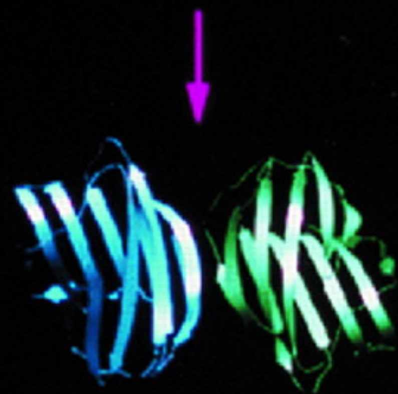


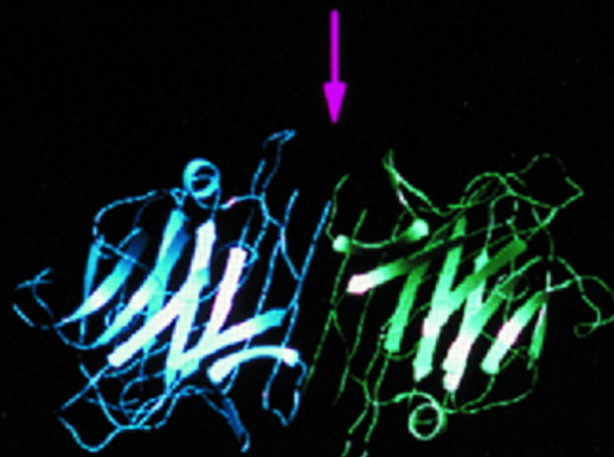
4 Rotation Axis (FOYTAO)



6 Rotation Axis (GIKDOT)

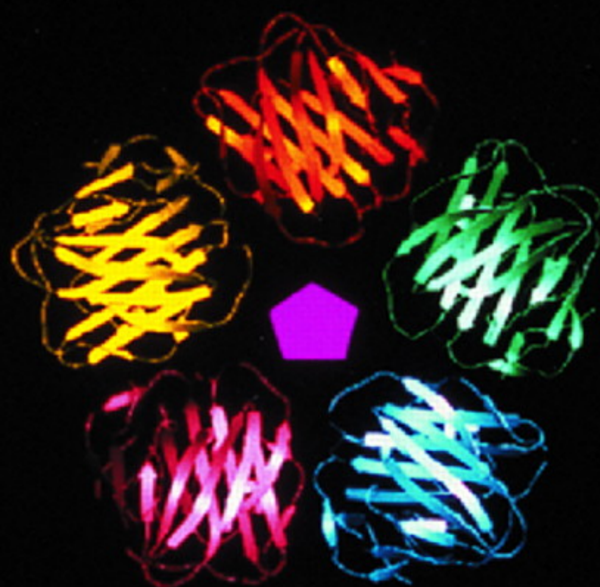



Bovine galectin


Pea lectin

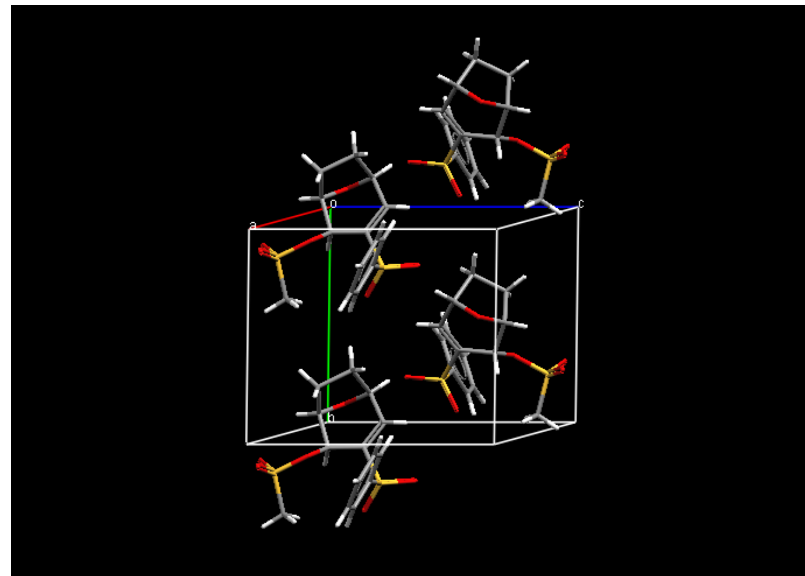
Concanavalin A

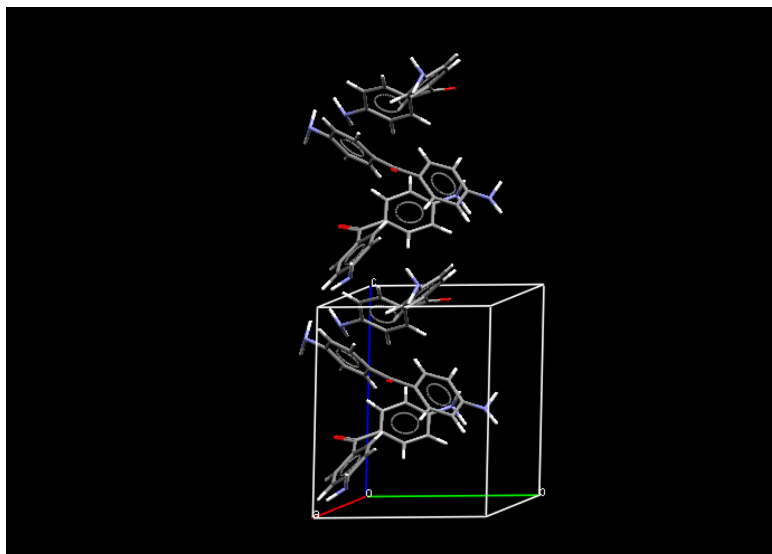
Peanut lectin


Serum amyloid P-component

Limulus CRP

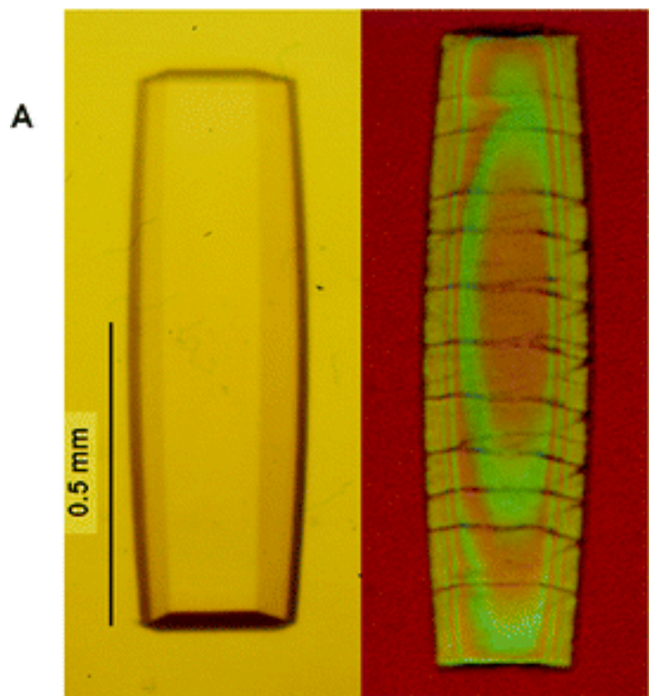
2_1 Screw Axis (ABEBIS)



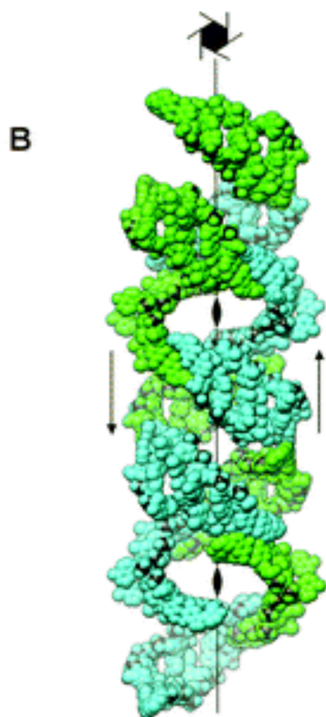


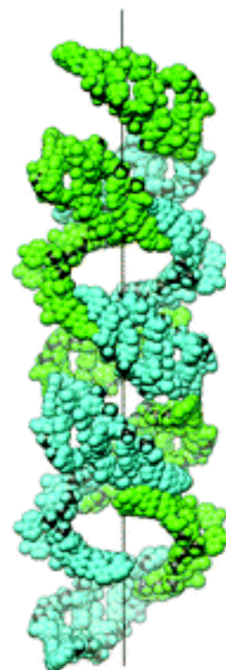
3_1 Screw Axis (AMBZPH)

A

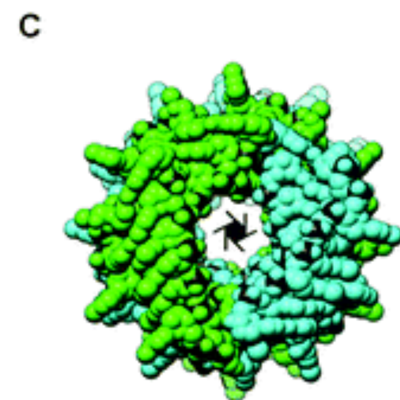


B

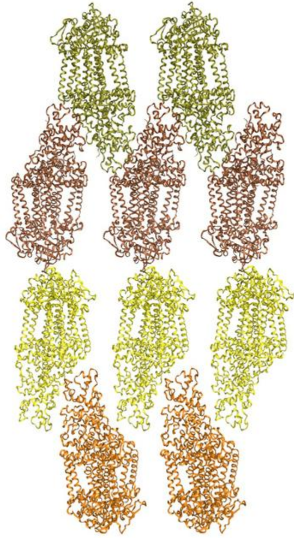


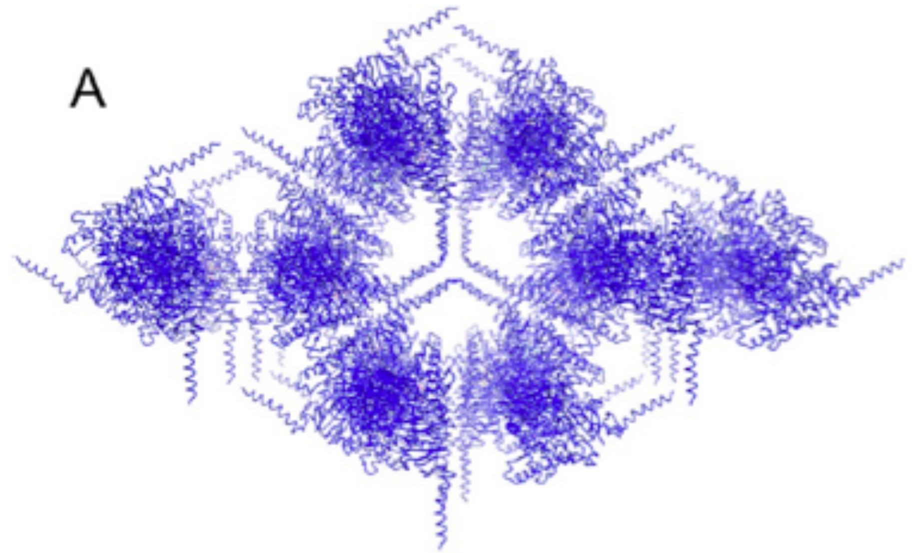


C

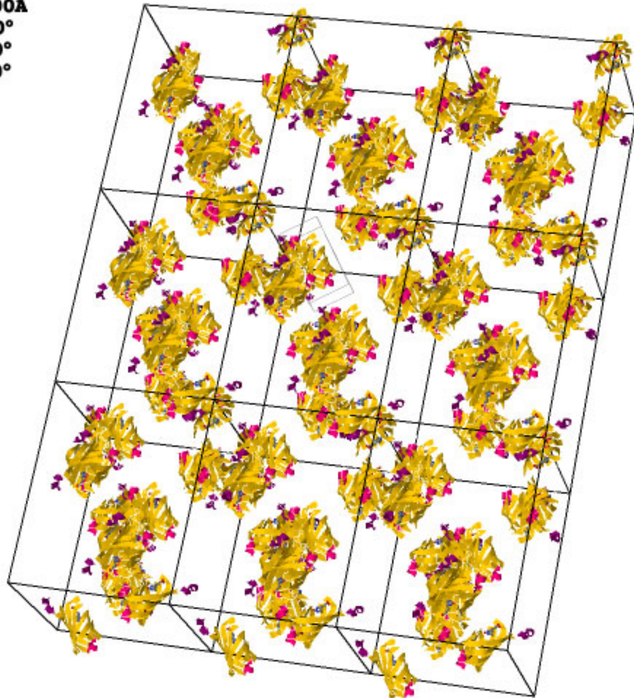


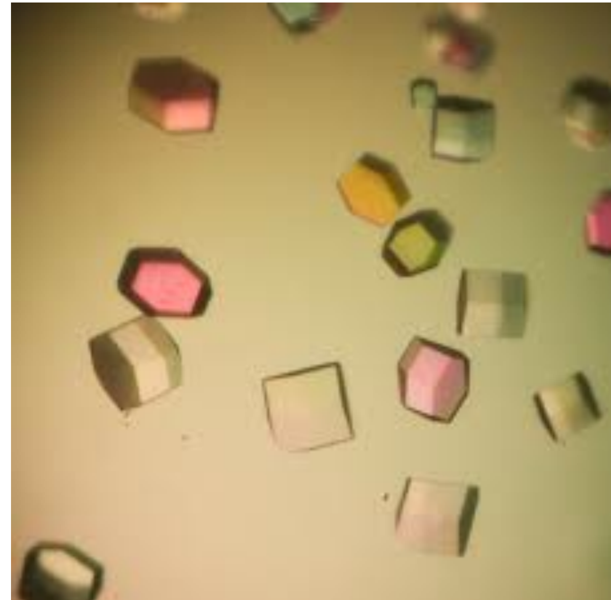
Protein crystals





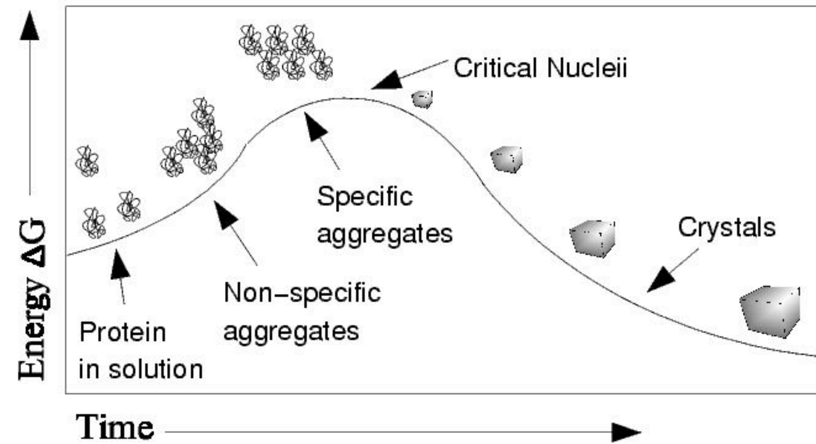
I 4bw 2bw [I 41 2 2] #98
a=99.400Å
b=99.400Å
c=125.800Å
α=90.000°
β=90.000°
γ=90.000°

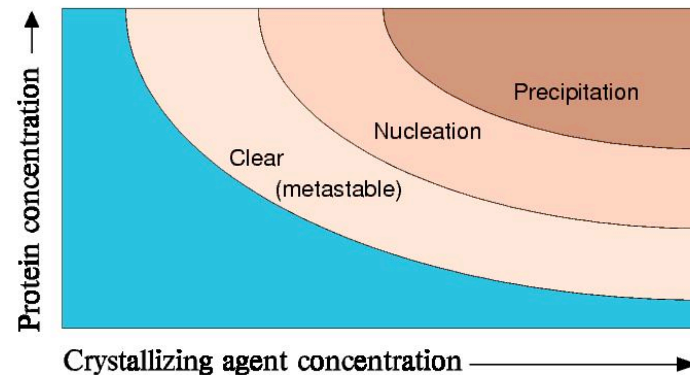


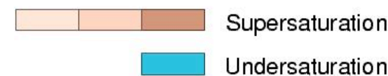


Protein crystallization

There is an energy barrier to crystallization.
In order for a protein to crystallize it must overcome an energy barrier analogous to that for conventional chemical reactions.



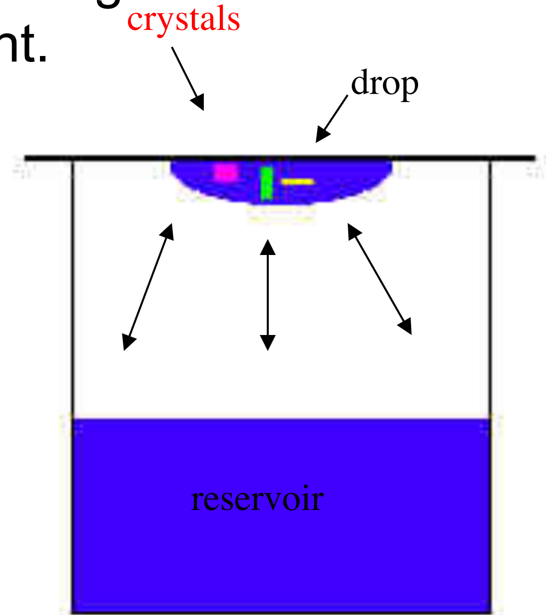




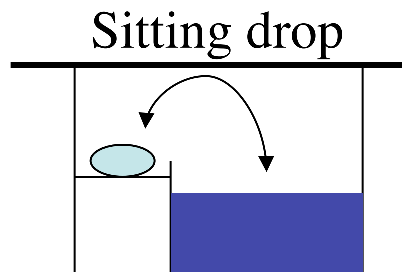
Methods of Protein Crystallization

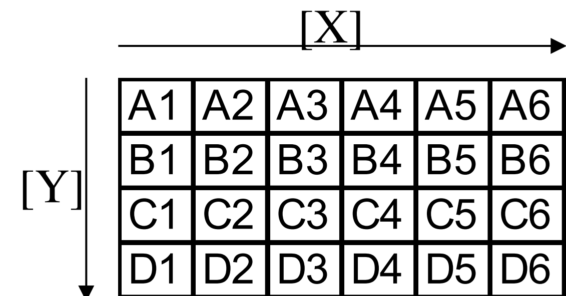
Vapour Diffusion

In a vapour diffusion experiment, small volumes of precipitant and protein mixed together and the drop equilibrated against a larger reservoir of solution containing precipitant or another dehydrating agent.



- Drop equalizes with reservoir
- Volume of drop slowly decreases
- Protein concentration slowly increases

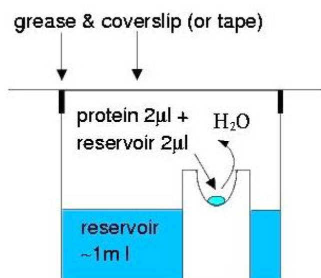


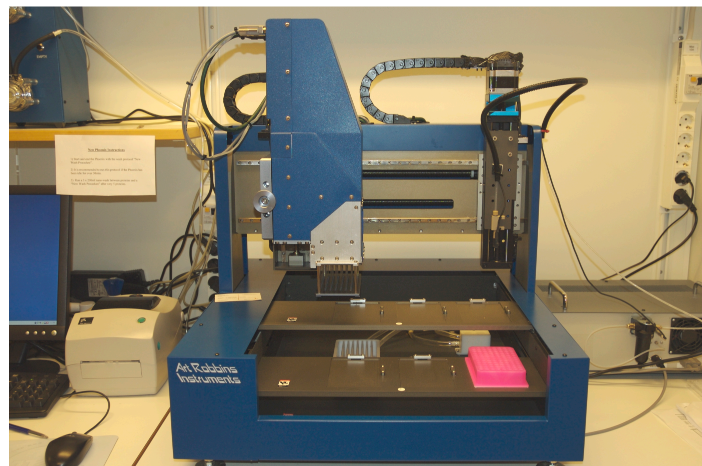


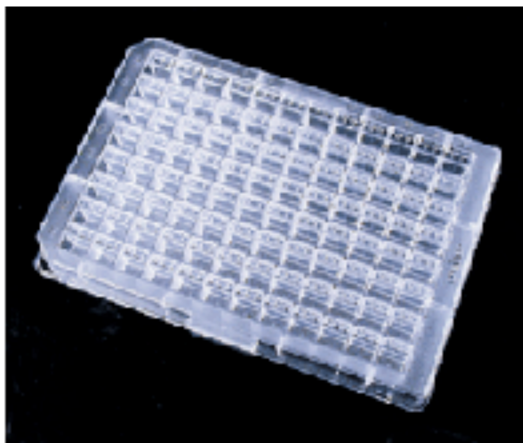
Crystallization tools

24- and 96-well plates.

Robot







Low molecular weight PEG based

Data Sheet

JBScreen Classic 1

Cat.-No: CS-101L

Number	Precipitant 1	Precipitant 2	Buffer	pH	Additive
A 1	15 % w/v PEG 400	None	100 mM Sodium Acetate	4.6	100 mM Calcium Chloride
A 2	15 % w/v PEG 400	None	100 mM MES Sodium Salt	6.5	None
A 3	15 % w/v PEG 400	None	100 mM HEPES Sodium Salt	7.5	200 mM Magnesium Chloride
A 4	15 % w/v PEG 400	None	100 mM Tris-HCl	8.5	200 mM Sodium Citrate
A 5	25 % w/v PEG 400	None	100 mM Sodium Acetate	4.6	100 mM Magnesium Chloride
A 6	25 % w/v PEG 400	None	100 mM Tris-HCl	8.5	200 mM Lithium Sulfate
B 1	28 % w/v PEG 400	None	100 mM HEPES Sodium Salt	7.5	200 mM Calcium Chloride
B 2	30 % w/v PEG 400	None	100 mM Sodium Acetate	4.6	100 mM Calcium Chloride
B 3	30 % w/v PEG 400	None	100 mM MES Sodium Salt	6.5	100 mM Sodium Acetate
B 4	30 % w/v PEG 400	None	100 mM MES Sodium Salt	6.5	100 mM Magnesium Chloride
B 5	30 % w/v PEG 400	None	100 mM HEPES Sodium Salt	7.5	200 mM Magnesium Chloride
B 6	30 % w/v PEG 400	None	100 mM Tris-HCl	8.5	200 mM Calcium Citrate
C 1	30 % w/v PEG 550 MME	None	100 mM Bicine	9.0	100 mM Sodium Chloride
C 2	28 % w/v PEG 550 MME	None	100 mM MES Sodium Salt	6.5	10 mM Zinc Sulfate
C 3	25 % w/v PEG 1000	None	100 mM HEPES Sodium Salt	7.5	None
C 4	30 % w/v PEG 1000	None	100 mM Tris-HCl	8.5	None
C 5	15 % w/v PEG 1500	None	None		None
C 6	20 % w/v PEG 1500	None	100 mM HEPES Sodium Salt	7.5	None
D 1	30 % w/v PEG 1500	None	None		None
D 2	20 % w/v PEG 2000 MME	None	100 mM Tris-HCl	8.5	10 mM Nickel (II) Chloride
D 3	25 % w/v PEG 2000 MME	None	None		None
D 4	30 % w/v PEG 2000 MME	None	100 mM MES Sodium Salt	6.5	100 mM Sodium Acetate
D 5	20 % w/v PEG 3000	None	100 mM HEPES Sodium Salt	7.5	200 mM Sodium Acetate
D 6	30 % w/v PEG 3000	None	100 mM Tris-HCl	8.5	200 mM Lithium Sulfate

Jena Bioscience GmbH | Löbstedter Str. 80 | 07749 Jena, Germany | Tel.:+49-3641-6285 000 | Fax:+49-3641-6285 100
http://www.jenabioscience.com
Last update: May 08, 2009

High molecular weight PEG based

Data Sheet

JBScreen Classic 5
Cat.-No: CS-105L

Number	Precipitant 1	Precipitant 2	Buffer	pH	Additive 1	Additive 2
A 1	12 % w/v PEG 8000	5 % w/v Glycerol	None		100 mM Potassium Chloride	None
A 2	12 % w/v PEG 8000	10 % w/v Glycerol	None		500 mM Potassium Chloride	None
A 3	15 % w/v PEG 8000	None	None		200 mM Ammonium Sulfate	None
A 4	15 % w/v PEG 8000	500 mM Lithium Sulfate	None		None	None
A 5	15 % w/v PEG 8000	None	100 mM MES Sodium Salt	6.5	200 mM Sodium Acetate	None
A 6	15 % w/v PEG 8000	None	None		50 mM Ammonium Sulfate	100 mM Sodium Citrate
B 1	18 % w/v PEG 8000	None	100 mM HEPES Sodium Salt	7.5	200 mM Calcium Acetate	None
B 2	18 % w/v PEG 8000	2 % w/v 2-Propanol	100 mM HEPES Sodium Salt	7.5	100 mM Sodium Acetate	None
B 3	18 % w/v PEG 8000	None	100 mM Tris-HCl	8.5	200 mM Lithium Sulfate	None
B 4	20 % w/v PEG 8000	None	100 mM HEPES Sodium Salt	7.5	None	None
B 5	20 % w/v PEG 8000	None	100 mM MES Sodium Salt	6.5	200 mM Magnesium Acetate	None
B 6	20 % w/v PEG 8000	None	100 mM CHES	9.5	None	None
C 1	22 % w/v PEG 8000	None	100 mM MES Sodium Salt	6.5	200 mM Ammonium Sulfate	None
C 2	25 % w/v PEG 8000	None	None		200 mM Lithium Chloride	None
C 3	30 % w/v PEG 8000	None	None		200 mM Ammonium Sulfate	None
C 4	8 % w/v PEG 10000	None	100 mM Sodium Acetate	4.6	None	None
C 5	14 % w/v PEG 10000	None	100 mM Imidazole-HCl	8.0	None	None
C 6	16 % w/v PEG 10000	None	100 mM Tris-HCl	8.5	None	None
D 1	18 % w/v PEG 10000	20 % w/v Glycerol	100 mM Tris-HCl	8.5	100 mM Sodium Chloride	None
D 2	20 % w/v PEG 10000	None	100 mM HEPES Sodium Salt	7.5	None	None
D 3	30 % w/v PEG 10000	None	100 mM Tris-HCl	8.5	None	None
D 4	10 % w/v PEG 20000	None	100 mM MES Sodium Salt	6.5	None	None
D 5	17 % w/v PEG 20000	None	100 mM Tris-HCl	8.5	100 mM Magnesium Chloride	None
D 6	20 % w/v PEG 20000	None	None		None	None

Ammonium sulfate based

Data Sheet

JBScreen Classic 6

Cat.-No: CS-106L

Number	Precipitant 1	Precipitant 2	Buffer	pH	Additive
A 1	500 mM Ammonium Sulfate	1.0 M Lithium Sulfate	None		100 mM Sodium Citrate
A 2	1.0 M Ammonium Sulfate	None	None		None
A 3	1.0 M Ammonium Sulfate	None	100 mM Sodium Acetate	4.6	None
A 4	1.0 M Ammonium Sulfate	2 % w/v PEG 400	100 mM HEPES Sodium Salt	7.5	None
A 5	1.0 M Ammonium Sulfate	None	100 mM Tris-HCl	8.5	None
A 6	1.2 M Ammonium Sulfate	3 % w/v 2-Propanol	None		50 mM Sodium Citrate
B 1	1.5 M Ammonium Sulfate	15 % w/v Glycerol	100 mM Tris-HCl	8.5	None
B 2	1.6 M Ammonium Sulfate	500 mM Lithium Chloride	None		None
B 3	1.6 M Ammonium Sulfate	1.0 M Lithium Sulfate	None		None
B 4	1.6 M Ammonium Sulfate	None	100 mM HEPES Sodium Salt	7.5	200 mM Sodium Chloride
B 5	1.6 M Ammonium Sulfate	2 % w/v PEG 1000	100 mM HEPES Sodium Salt	7.5	None
B 6	1.8 M Ammonium Sulfate	None	100 mM MES Sodium Salt	6.5	None
C 1	2.0 M Ammonium Sulfate	2.0 M Sodium Chloride	None		None
C 2	2.0 M Ammonium Sulfate	None	100 mM Sodium Acetate	4.6	None
C 3	2.0 M Ammonium Sulfate	5 % w/v PEG 400	100 mM MES Sodium Salt	6.5	None
C 4	2.0 M Ammonium Sulfate	None	100 mM Tris-HCl	8.5	None
C 5	2.2 M Ammonium Sulfate	None	None		None
C 6	2.2 M Ammonium Sulfate	20 % w/v Glycerol	None		None
D 1	2.4 M Ammonium Sulfate	None	None		100 mM Sodium Citrate
D 2	3.0 M Ammonium Sulfate	1 % w/v MPD	None		None
D 3	3.0 M Ammonium Sulfate	10 % w/v Glycerol	None		None
D 4	3.5 M Ammonium Sulfate	None	100 mM HEPES Sodium Salt	7.5	None
D 5	3.5 M Ammonium Sulfate	1 % w/v MPD	100 mM MES Sodium Salt	6.5	None
D 6	3.5 M Ammonium Sulfate	None	None		None

Variables to consider:

-Temperature (usually 4 and 20 °C)

- Protein concentration (can vary from 2 mg/ml to 60 mg/ml)

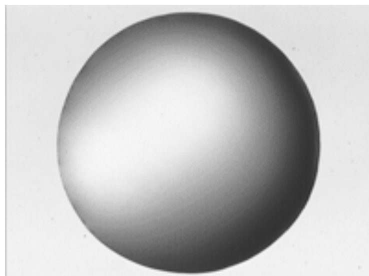
- Protein buffer (pH, ionic strength, detergent, reucing agents, etc…)

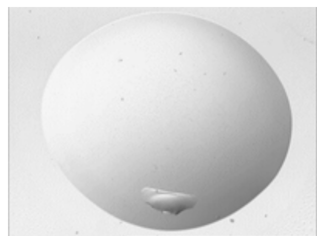
- Method of crystallization

- Volume ratio protein:reservoir

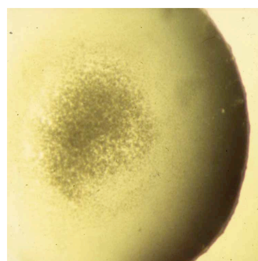
Observation

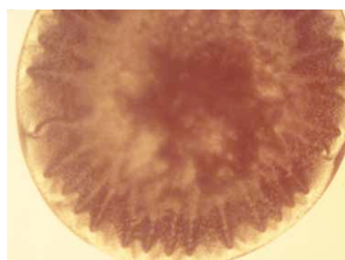
Clear Drop



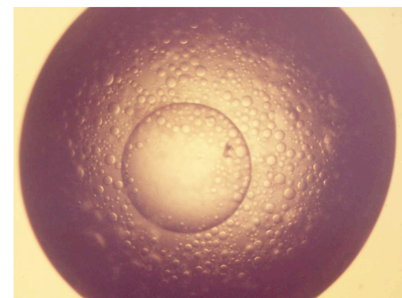


Precipitate

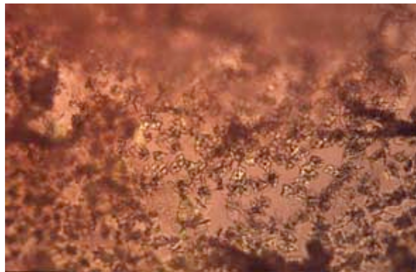


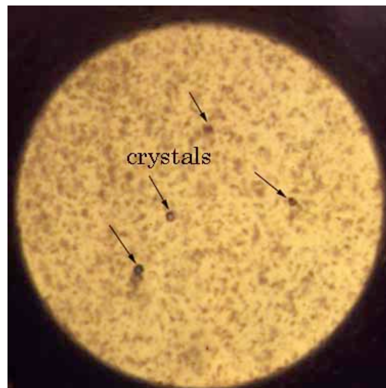


Phase Separation

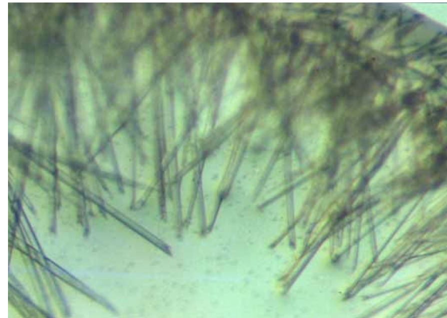


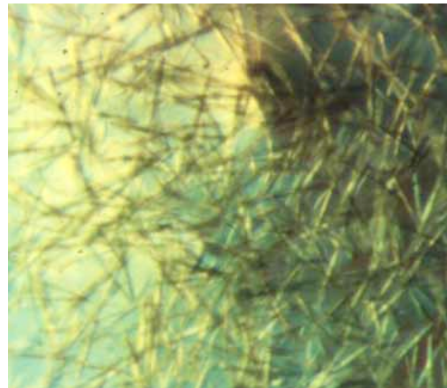
Microcrystalline Precipitate





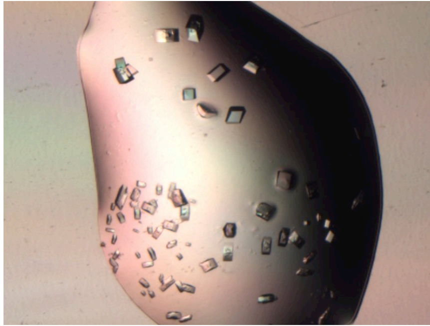
Needles

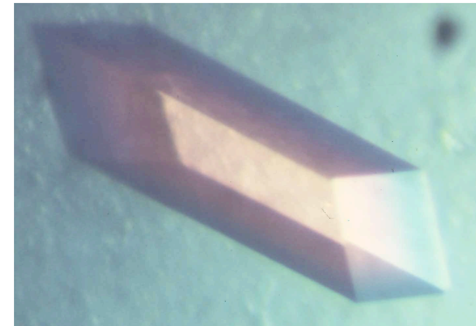


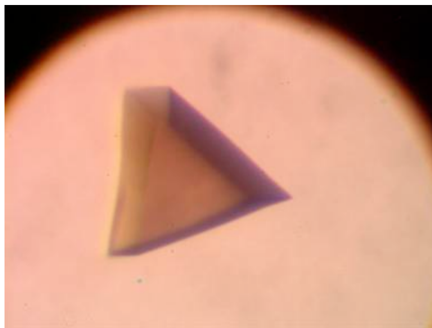


Plates

Crystals







Optimization

When you get a crystal in a screening, an optimization is usually required to increase size and quality of the crystals.

Screening around the conditions

Screening around the conditions that give microcrystals is done by varying

- *Precipitant concentration*
- *pH*
- *Protein concentration*
- *Temperature*
- *Method*

Additives and detergent screening

Popular additives are: glycerol, which may stop nucleation and may give you fewer, larger crystals, and has the advantage of doubling as a cryo-protectant.

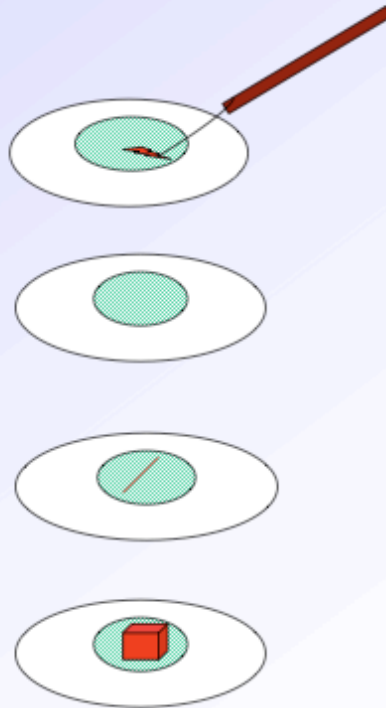
Oil on the reservoir

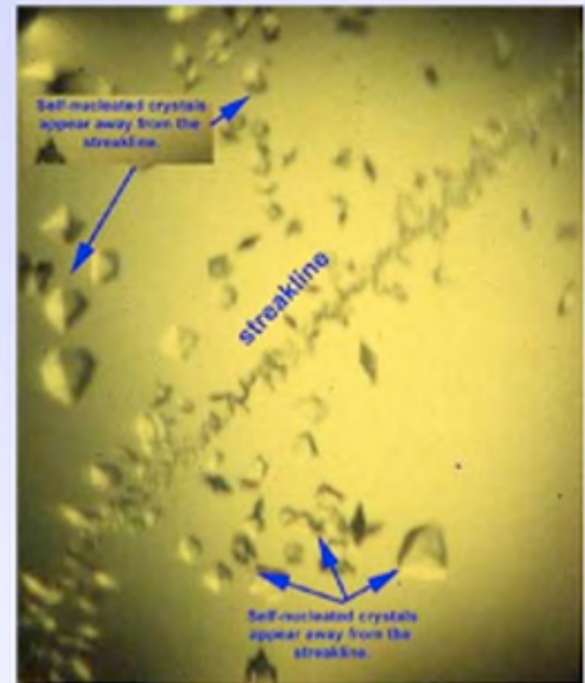
Diffusion can be slowed down by adding a layer of oil on the top of the hanging drop well

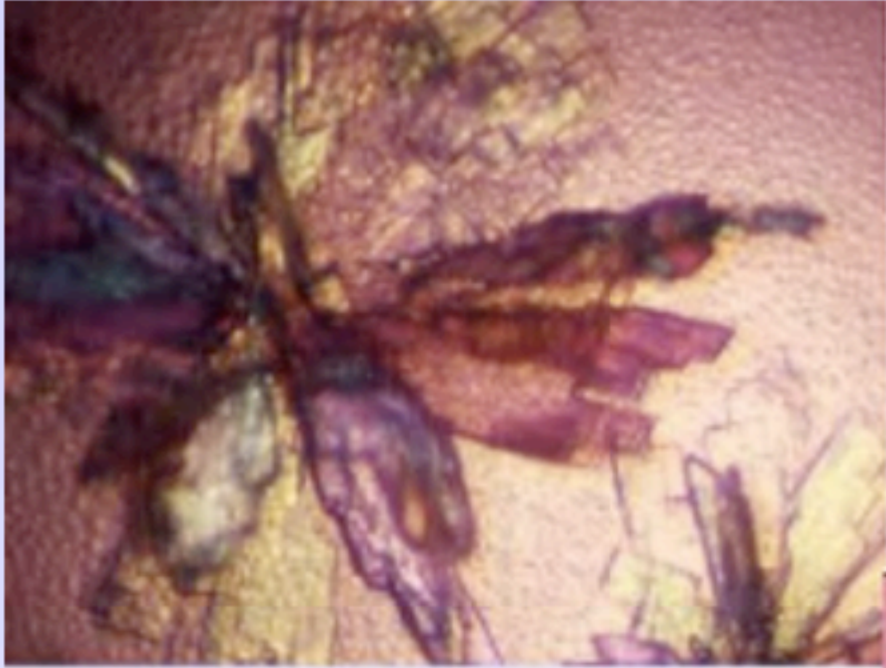
The proportion of light silicone oil to heavy paraffin oil must be mixed in different proportions to get an appropriate rate of diffusion

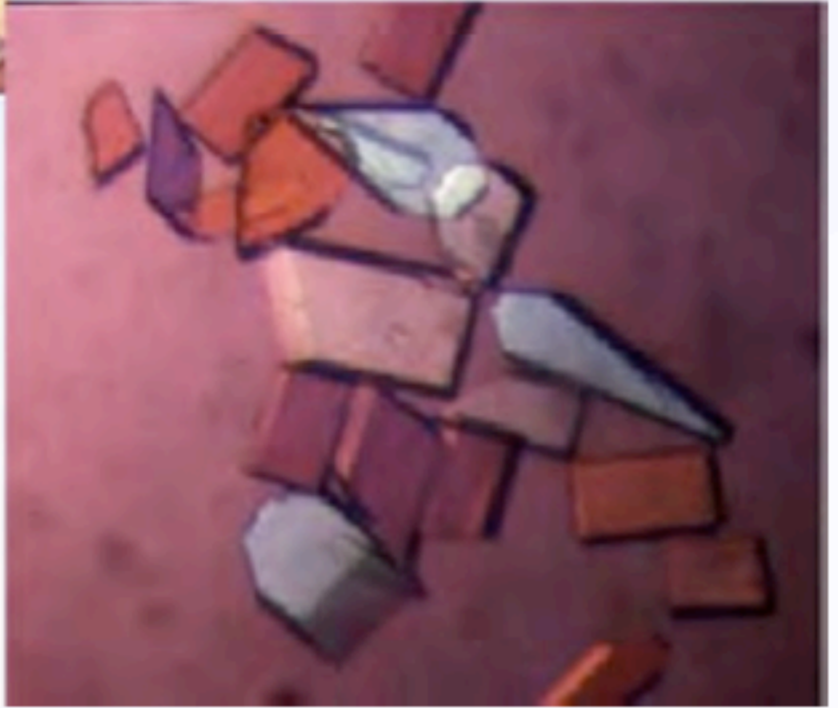
Streak seeding

- Make up a new drop with lower protein or precipitant
- Touch a whisker to the seed crystal
- Draw a line in the equilibrated drop
- Wait









Crystallization of protein-ligand complexes

Strategies for obtaining crystals of protein-ligand complexes:

1. Co-crystallization:

(i) co-expression of the protein with the ligands of interest,

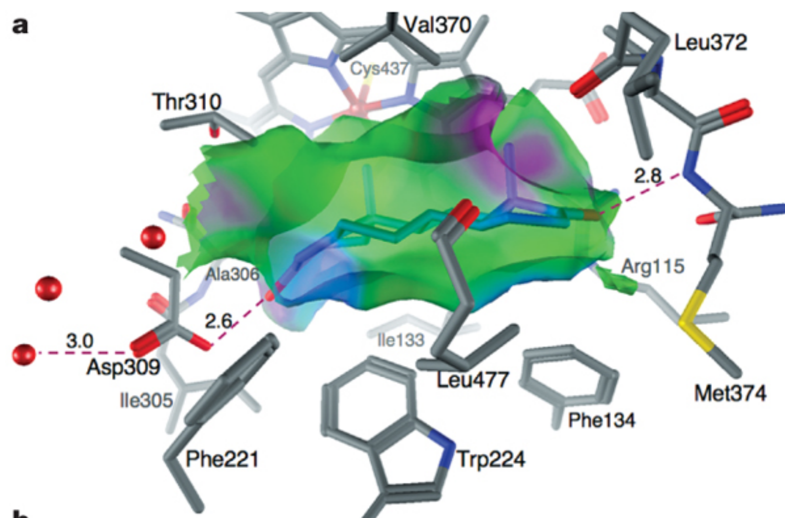
(ii) use of the ligands during protein purification,

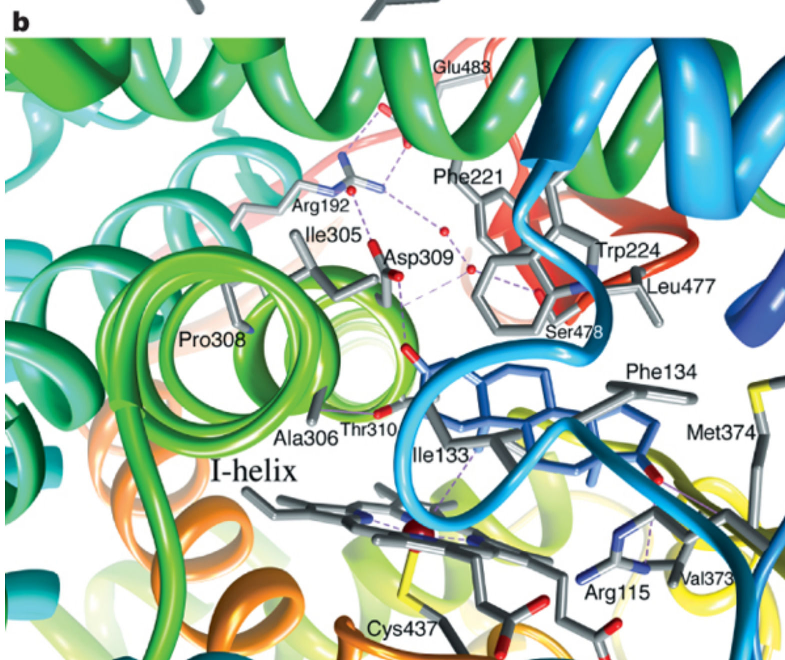
(iii) the ligand is added before crystallization trials

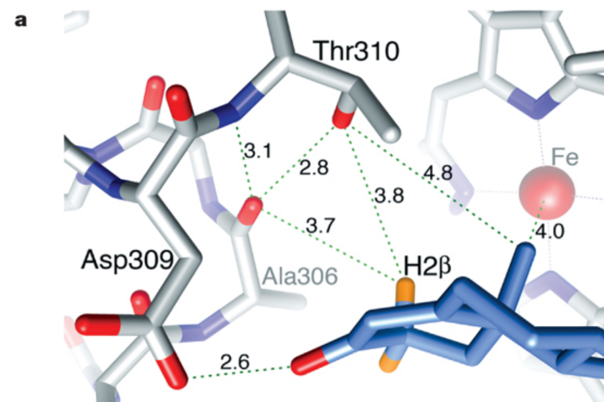
2. Soaking:

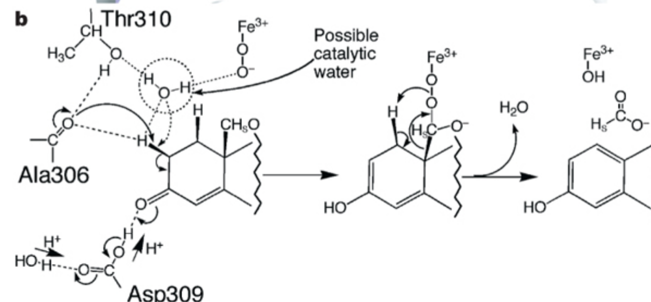
Crystals are often put into stabilization buffers before they are immersed in the ligand solution. These buffers may contain increased concentrations of the precipitant(s) and a stepwise/gradual increase in reagent concentration or the introduction of a cryoprotectant may be required so that the crystals are not damaged.

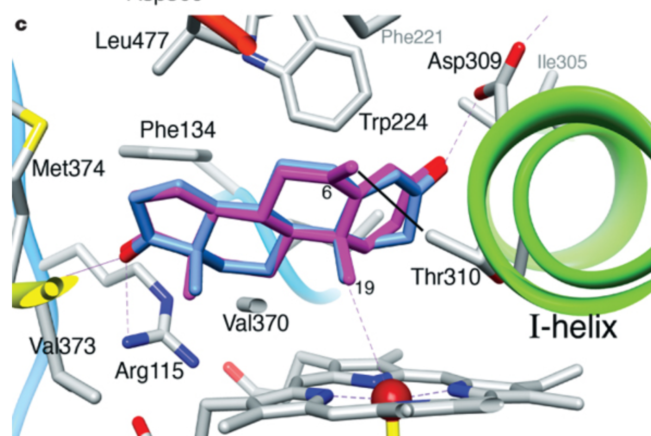
Crystallization of protein-ligand complexes











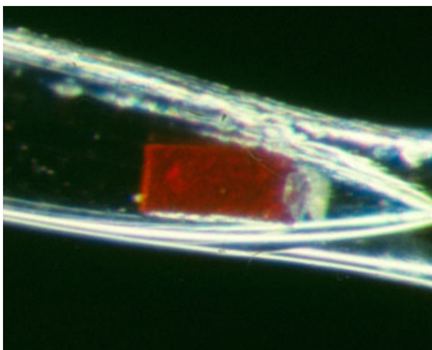
Ghosh et al., Nature 2009

Crystal mounting

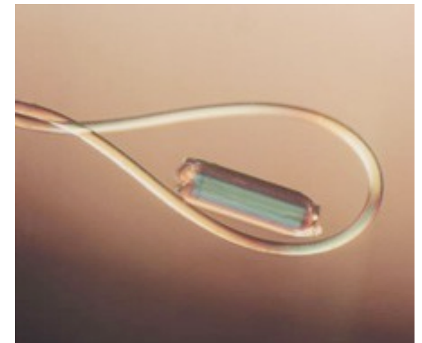
- Crystals should be kept in the saturated vapour of their mother liquor or at a sufficiently low temperature to prevent evaporation of the solvent (40-60% of the crystal volume is occupied by solvent).

- Flash freezing or shock cooling (use of cryoprotectant such as glycerol, MPD, ethyleneglycol and low molecular weight PEG) in liquid nitrogen or in a stream of cold nitrogen gas at a temperature range of 100-120°K.

Capillary



Loops



Cryo-mounting

Cryoprotection is effectively accomplished during harvesting, when the crystals are scooped up from the drop in cryo-loops and briefly swept though a cryoprotectant before being dipped into liquid nitrogen. Common cryoprotectants are ethylene glycol (the anti-freeze in automobile radiators), glycerol, higher alcohols, polyethylene glycols (PEGs), or high concentration solutions of sucrose or salts. Once the protein crystals are flash-cooled and stored in pucks in a liquid nitrogen dewar (dry shipper), they can be safely sent to a synchrotron for data collection.

Radiation damage.

Proteins are sensitive to X-ray radiation damage. The energy range of X-rays used for diffraction is in the 6 to 15 keV range, which is in fact severely ionizing radiation. The ionizing absorption events create radicals, which rapidly destroy any protein crystal, particularly at dose rates experienced at synchrotrons. An efficient way to suppress radiation damage by slowing down the kinetics of the radical reactions is cryogenic cooling. Rapidly quenching or flash-cooling crystals to liquid nitrogen temperatures, either in cold nitrogen gas streams or directly into liquid nitrogen, will strongly reduce radiation damage. To prevent the formation of crystalline ice during flash-cooling of the crystals, cryoprotectants, present in the mother liqor or added to the mother liquor, are necessary.

Benefits of Cryo-Cooling

Many factors contribute to improvements in data quality during cryo-protection:

- reduced thermal vibrations,
- enhanced signal-to-noise ratio,
- reduced conformational disorder,
- a higher limiting resolution.