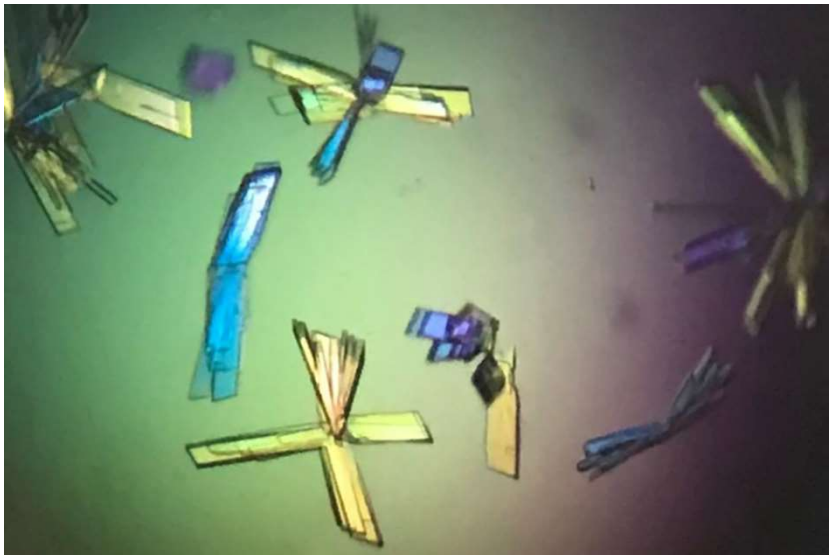
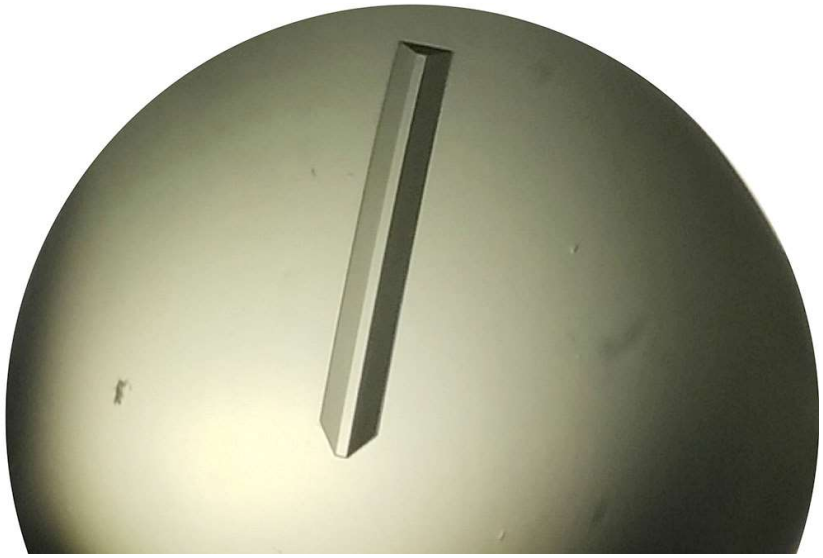


Protein crystallization



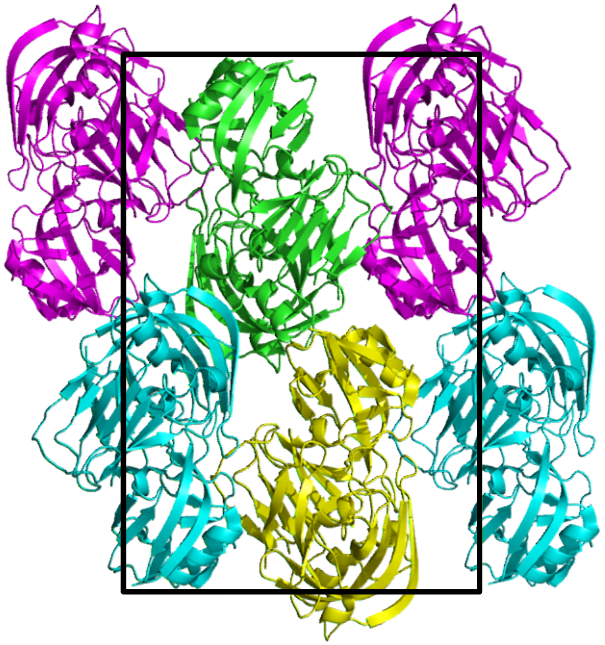
Corso di Biocristallografia e Microscopia Elettronica rdezorzi@units.it

Protein crystallization for structural studies

For biocrystallography, crystallization is the first condition – often not easy to obtain.

Protein crystal: array of ordered protein molecules, held together by weak, non-covalent interactions.

Self-assembly of protein molecules.



Are protein crystals representative of the native conformation of proteins?

1) Protein crystals contain a **high amount of solvent** (water): 30-80% of crystal volume occupied by water. Solvent in the crystal maintains the protein molecules in a *close-to-native* state.

2) **Enzymatic activity** has been measured in protein crystals, indicating active conformation.

3) Evaluation of local conformations influenced by crystal contacts and, when different crystal forms are available, evaulation of **crystallization artifacts**.

However, structure should always be validated!

Protein crystallization

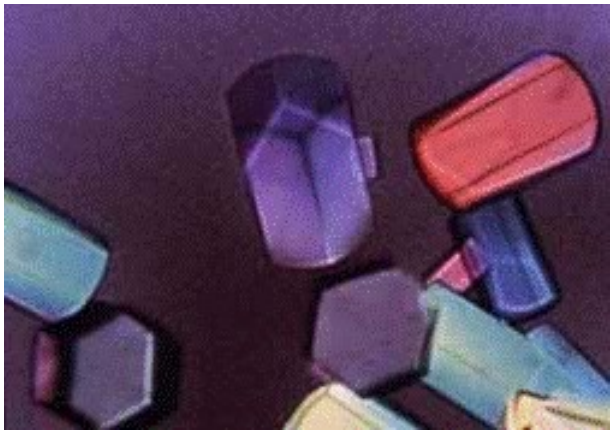
Crystals: ordered aggregates of molecules, anisotropic. Crystal appearance: sharp edges and plane faces.

Crystallization: phase transition between solution and solid state. The crystallization process competes with the formation of isotropic, amorphous solids.

Factors affecting crystallization:

- **Purity of the sample:** impurities (1) hamper the crystallization process, (2) create disorder within the crystals, reducing their diffraction power, (3) reduce the dimension of the crystals
- **Sequence and conformational homogeneity:** crystallization is statistically more probably when the sample is homogeneous, including post-translational modifications
- **Protein folding:** before starting crystallization experiments, check if the protein has its native folding
- **Quantity:** crystallization experiments require a large amount of protein (> 5 mg)

Crystal requirements



Not all crystals are suitable for biocrystallography:

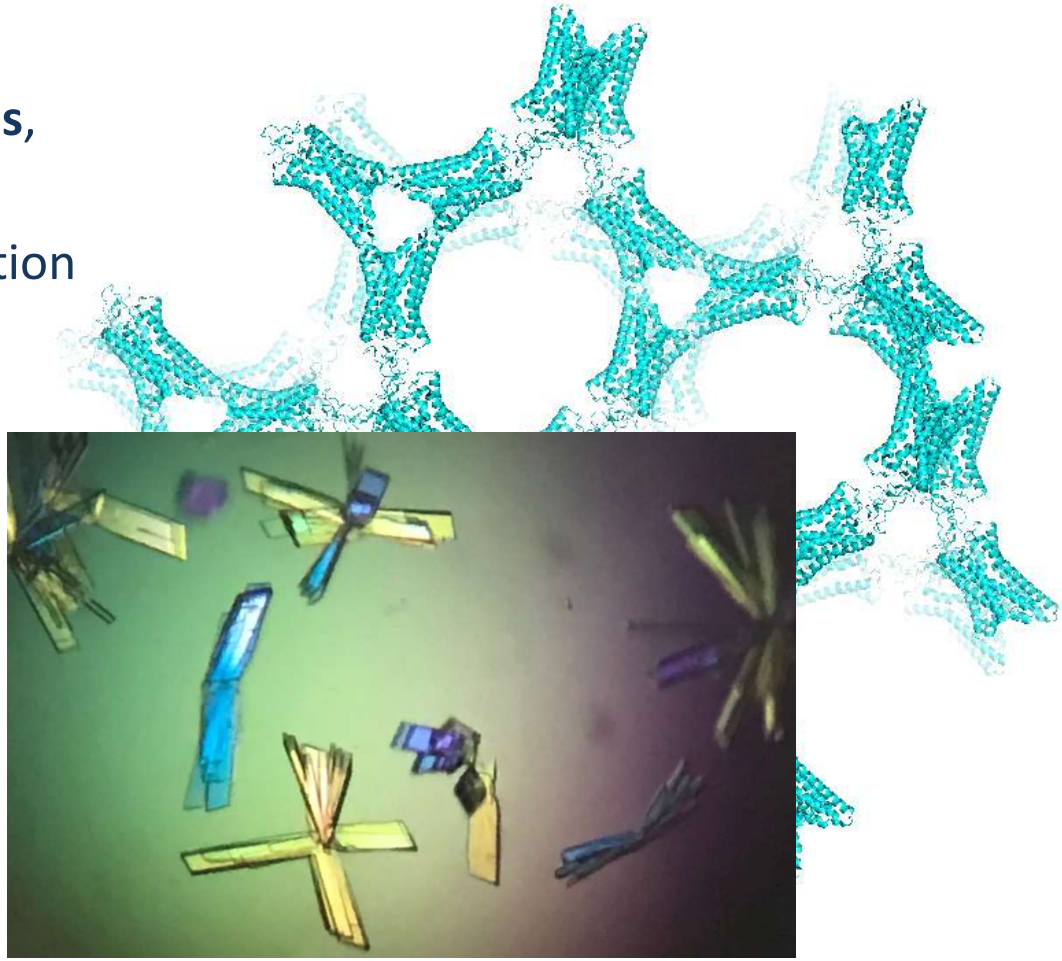
- Dimension: for typical diffraction analysis, lateral dimension of >10 μm in all directions (for microfocus beamlines)
- Quality: well-ordered crystals yield good patterns and signal to high resolution
- Single: no crystal aggregates! If crystals grow as aggregates, they need to be separated.

Don't trust the looks! Good looking crystals may not give good diffraction data. Always test crystals!

Poorly diffracting crystals require optimization of crystallization conditions.

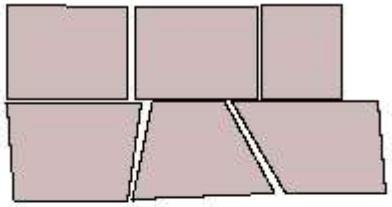
Due to **high solvent content** and **weak protein-protein interactions**, protein crystals are:

- Fragile and need extreme caution when handled
- Small
- With low diffraction power
- Often aggregate
- Sometimes twinned

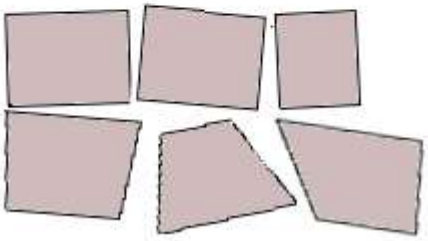


Mosaicity

0.4°



1.4°



Crystal arrays are not completely regular, but are collections of slightly rotated domains (**mosaic crystals**). The average rotation (in °) is known as **mosaicity**.

For good diffraction: 0.1° < mosaicity < 2°

Crystallization experiment

Protein solution: - High concentration of protein, close to saturation (optimal value depends on protein: from 1 mg/mL to 100 mg/mL!
- Use fresh protein!
- Purity > 95%! Check chemical and conformational homogeneity.
- Centrifuge solution before use to remove aggregates.

During the crystallization experiment the protein concentration has to reach a value above its **solubility**, to drive the equilibrium

$$\text{protein}_{(\text{solution})} \overset{K_{eq}}{\Longleftrightarrow} \text{protein}_{(\text{crystal})}$$

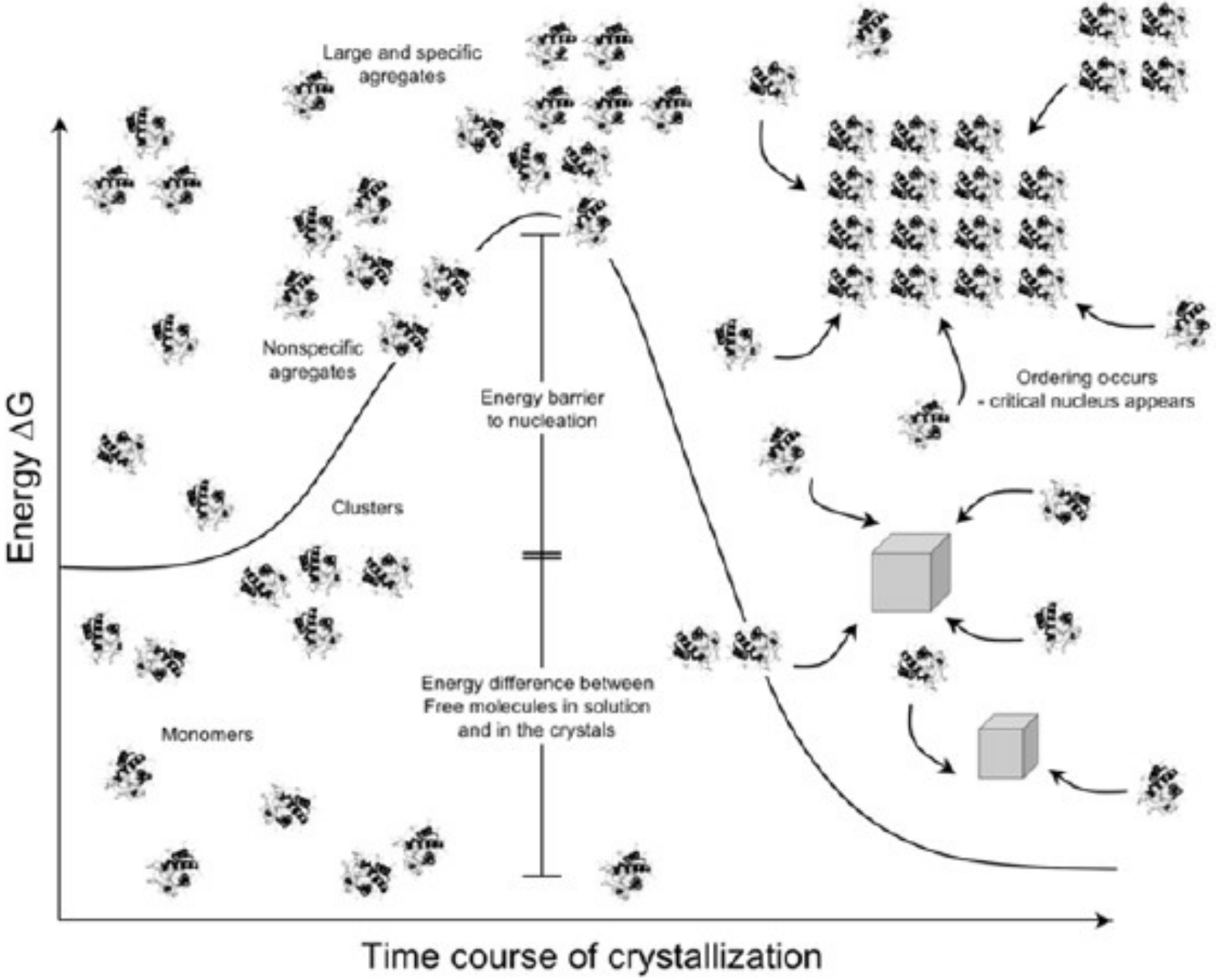
towards the formation of the protein crystal.

Precipitant: a chemical compound that reduces the solubility of the protein, it may induce crystallization through different effects.

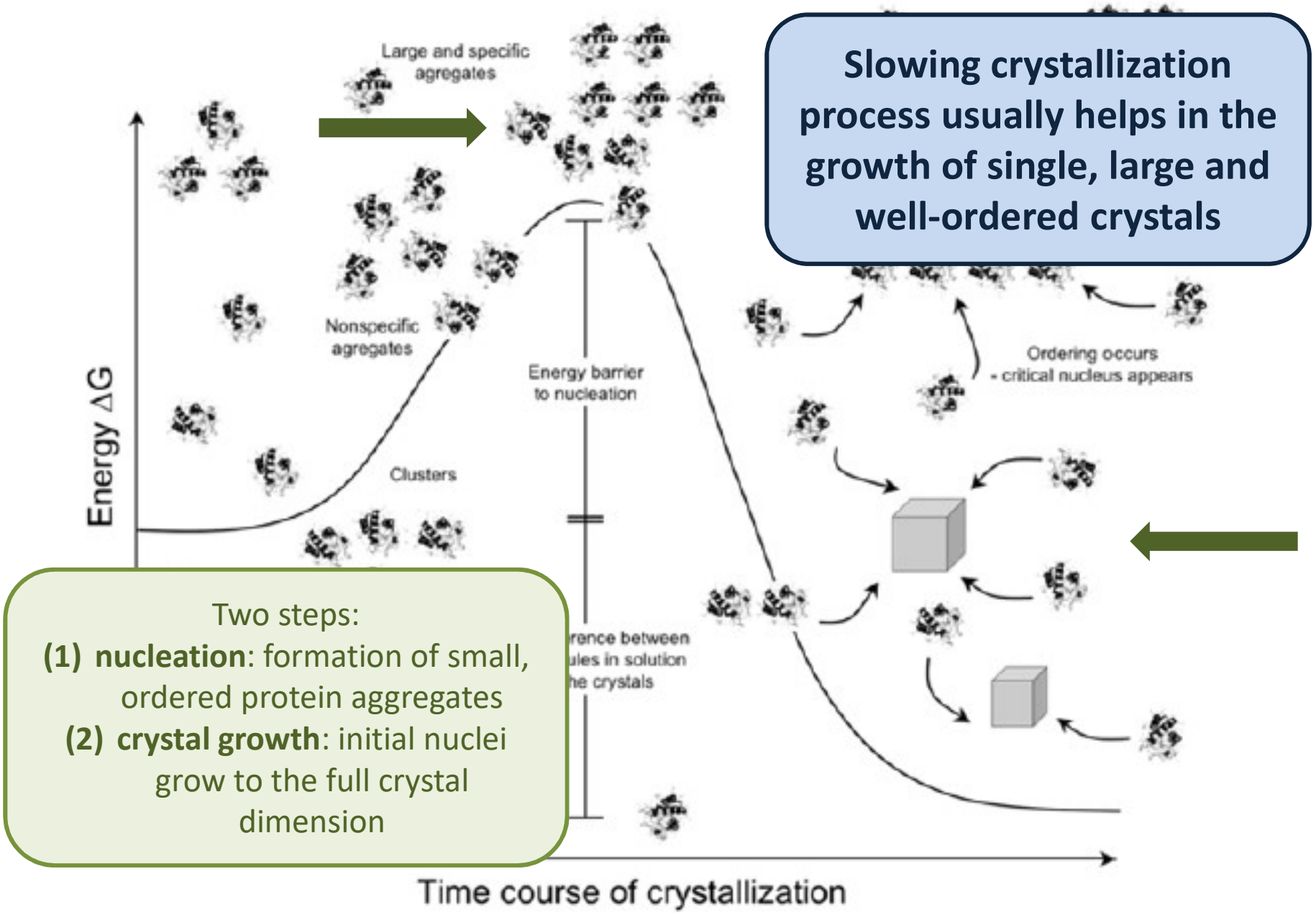
Interactions that lead to protein formation are weak, specific and difficult to predict. No known method to identify the ideal crystallization conditions.

Crystallization is mostly a trial-and-error process, but analysis of physical and chemical aspects gives important tips for crystallization/crystal optimization.

Kinetics of crystallization



Kinetics of crystallization



Slowing crystallization process usually helps in the growth of single, large and well-ordered crystals

Two steps:

(1) **nucleation**: formation of small, ordered protein aggregates

(2) **crystal growth**: initial nuclei grow to the full crystal dimension

Thermodynamics of crystallization

Enthalpic and entropic contributions in the crystallization process:

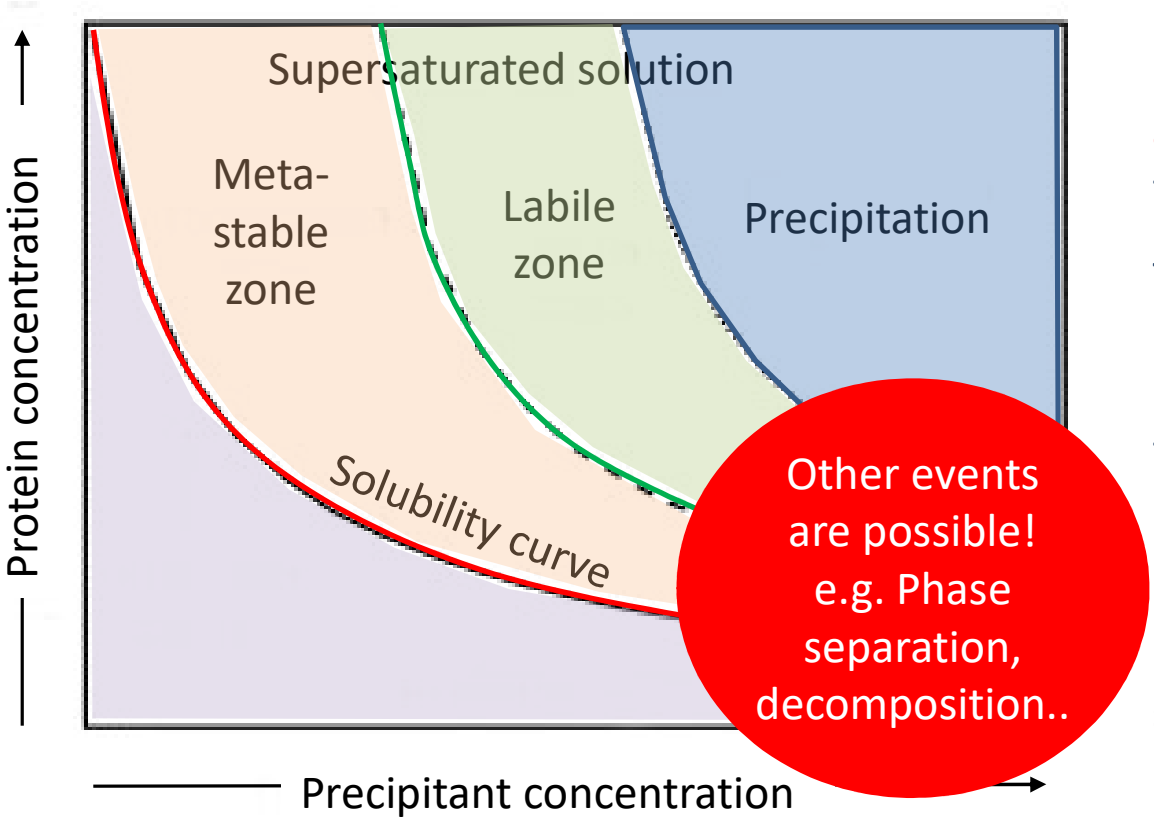
$$\Delta G = \Delta H - T\Delta S$$

- Weak protein-protein interactions: small negative ΔH contribution
- Loss of protein entropy (ordering in arrays!): negative $\Delta S_{protein}$ contribution
- Large gain in solvent entropy! Due to release of ordered water molecules interacting with the protein: positive $\Delta S_{solvent}$ contribution

$$\Delta G_c = \Delta H_c - T\,(\,\Delta S_{protein} + \Delta S_{solvent}\,)$$

[Mutation of high-entropy residues on the surface, such as Arg or Lys, with low-entropy residues, such as Ala, **may** improve crystallization chances…]

Thermodynamics + Kinetics



Phase diagram

Solubility curve: thermodynamic curve

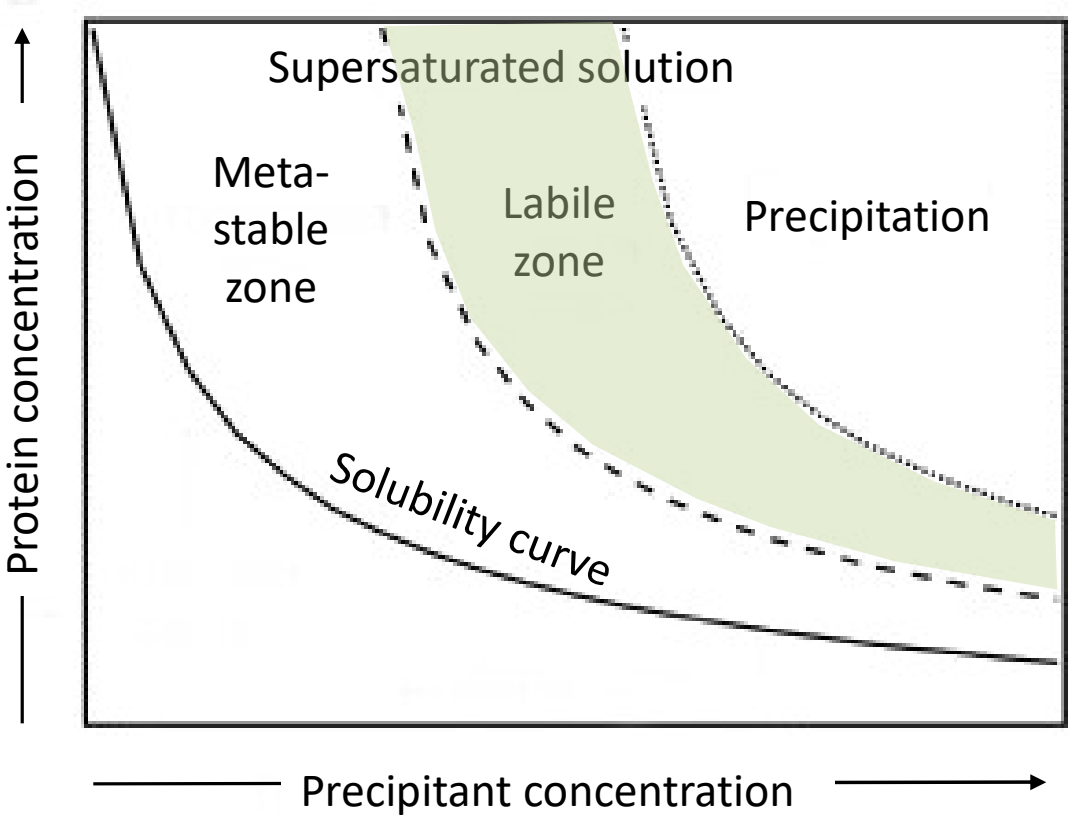
The **supersolubility curve**, dividing labile and metastable zones, is NOT a thermodynamic curve, but is determined by nucleation kinetics.

In conditions **below the saturation curve**, a clear and stable solution is present.

A solution in the **metastable zone** DOES NOT crystallize, unless crystallization nuclei are present. No nucleation in this region, however pre-formed crystals can grow.

In the **labile zone**, both nucleation and crystal growth are possible. Ideal conditions

In the **precipitation zone**: massive protein precipitation in microcrystalline or amorphous form.



Ideally, a crystallization experiment should start with a solution in the labile zone (suitable concentrations of protein and precipitant).

In time, few small, ordered aggregates (crystallization nuclei) start to form in the solution.

If conditions are close to the precipitation zone, more nuclei form and they are less likely to grow as single crystals.

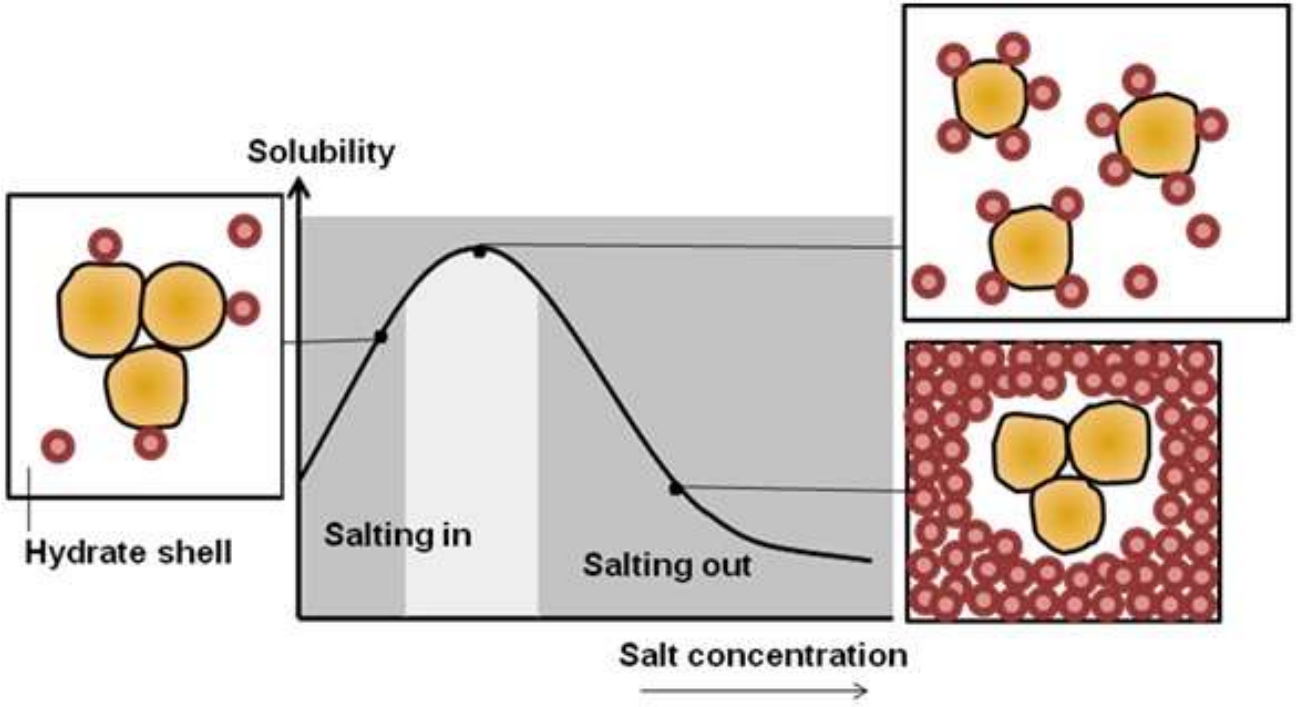
Different setups allow to reach the labile zone and to grow large single crystals.
Among the different crystallization techniques used for protein crystallography:

- Vapor diffusion techniques: hanging and sitting drops
- In batch crystallization under oil
- Dialysis

Crystallization conditions

- **Protein concentration** — If too low, experiment does not reach supersaturation; if too high, massive nucleation/precipitation leads to microcrystals
- **Precipitant** — Polymers such as PEGs (very good also for cryo!): compete with the protein for water binding

  Organic precipitants such as MPD, other alcohols or other compounds (DMSO, glycerol…): reduce dielectric constant of the solution
- **Salts** — Divalent salts bridging protein-protein contacts, avoid low solubility salts



Salts may act as precipitants through **salting-in** and **salting-out** effects:

small amounts of salt increases protein solubility,

but larger amounts compete with protein for water interactions

Crystallization conditions

- **Buffer**

  Influence protein crystallization; isoelectric point is not always ideal for crystallization. Statistically, optimal pH for crystallization: ≈7

  Low buffer concentration in protein solution allows pH change by addition of precipitant, avoid low solubility buffers (phosphate)

- **Additives**

  Their effect on crystallization is usually hard to predict, but crystallization experiments usually include various additives

  May promote crystal contacts (metal ions)

  May promote protein stability and conformational homogeneity (including binding to inhibitors, ligands)

  Detergents, particularly for membrane proteins, but also for soluble proteins (below CMC)

  Reducing agents (β-mercaptoethanol, DTT, TCEP, glutathione): improve stability and avoid oxidation effects during crystallization

  Other additives with antibacterial properties may help preserving crystallization trials for long incubation periods (NaN_3, EDTA, EGTA)

- **Temperature**

  Low temperature slows crystal growth, improving quality, but it has also an effect on thermodynamics! Hard to predict!

Crystallization conditions: additional variables

- **Amount of protein solution** for each experiment
- **Protein/precipitant ratio** at the beginning of the experiment
- **Crystallization method**
- **…**

In addition, **poor reproducibility** in crystallization experiments, due to small variations difficult to control (e.g. crystallization drop shape, convective currents in the crystallization experiment…). Even different protein preps may lead to different results…

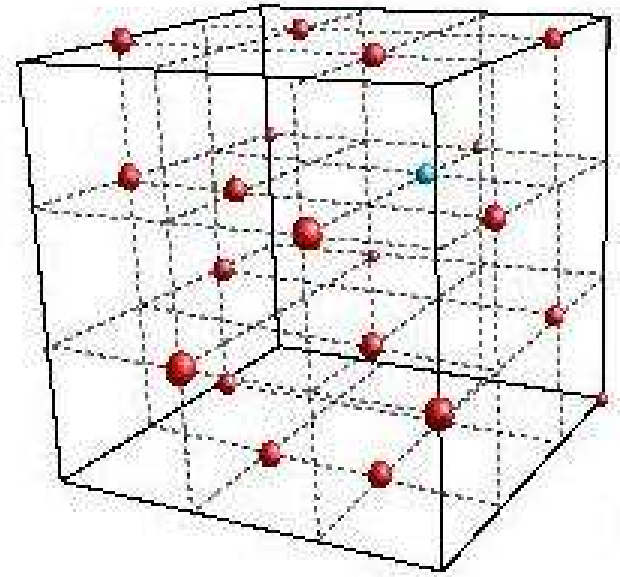
Possible solutions: - Repeat crystallization tests and use large grids in optimization.
- Test as many conditions as possible.
- Test crystallization at different purification stages (small molecules co-purified may help in stabilization…).

If results are still unsatisfying, go back to construct design for optimization: add/remove tags, choose orthologs, truncate extremities, shorten loops…

Crystallization screens

If information on crystallization of related proteins is available, conditions may be chosen according to literature.

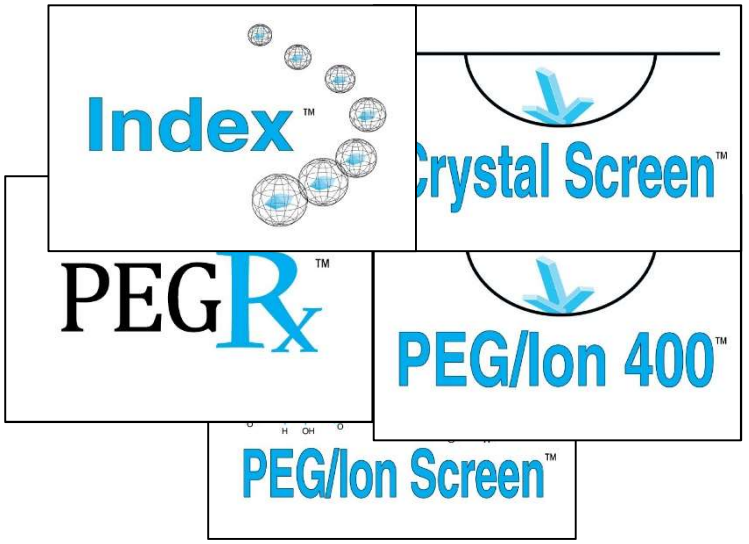
But if no information is available…



Many parameters can be varied to obtain crystals!

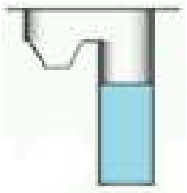
Too many different combinations of conditions in a multidimensional space that needs to be sampled.

Commercial crystallization screens are available, based on **sparse matrices of conditions** and on statistical analysis of successful crystallization experiments

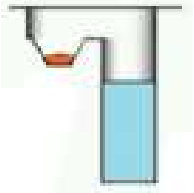


Vapor diffusion techniques

Supersaturation conditions are reached by concentrating the protein solution against a precipitant solution, through vapor diffusion.

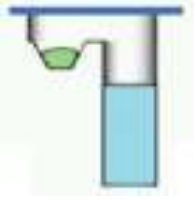


1. Dispense precipitant in suitable well *(reservoir)*

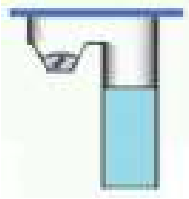


2. Dispense a drop of protein solution

3. Add a small amount of precipitant to protein



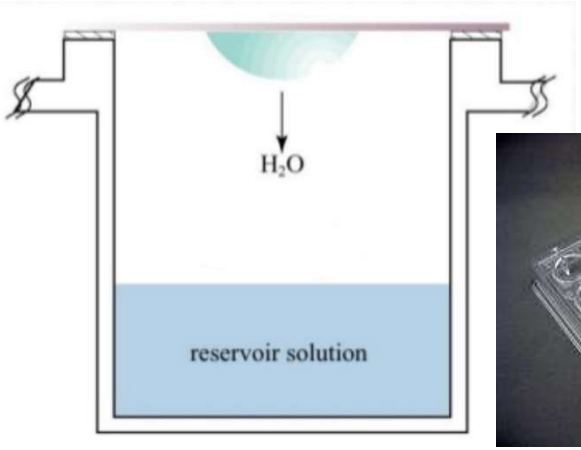
4. Seal the well and allow to equilibrate

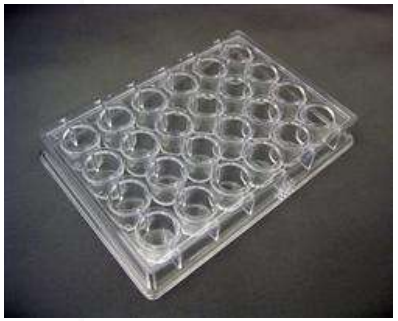


5. If successful, crystals will appear in the protein drop

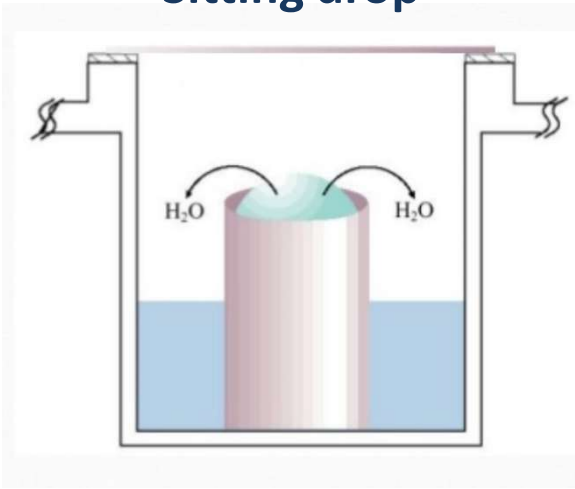
Two possible configurations:

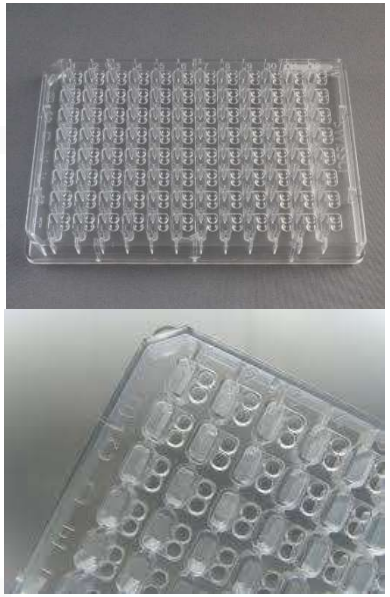
Hanging drop

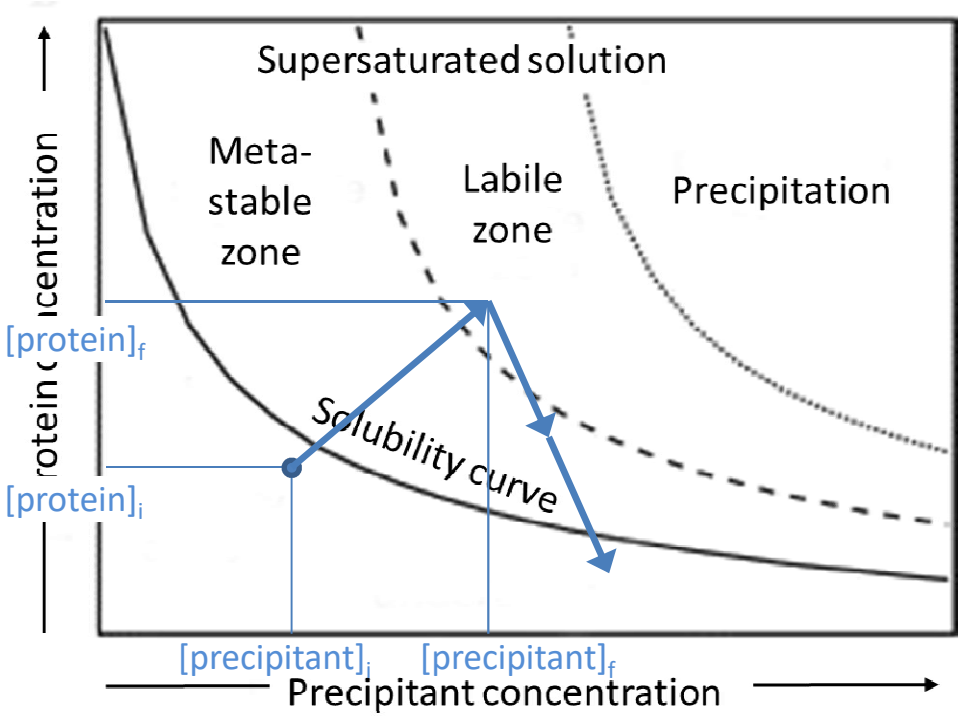




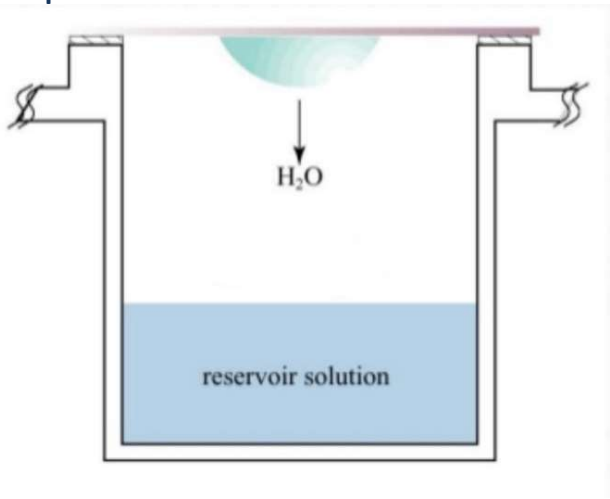
Sitting drop



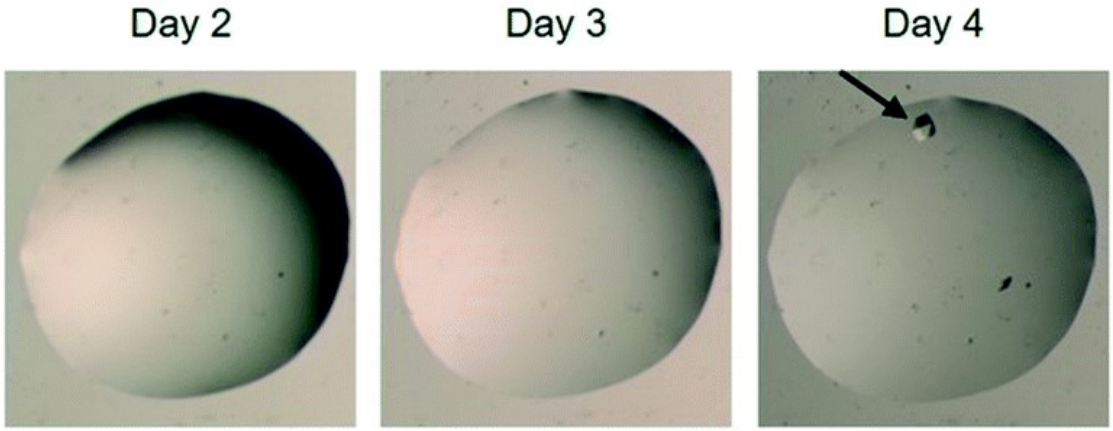




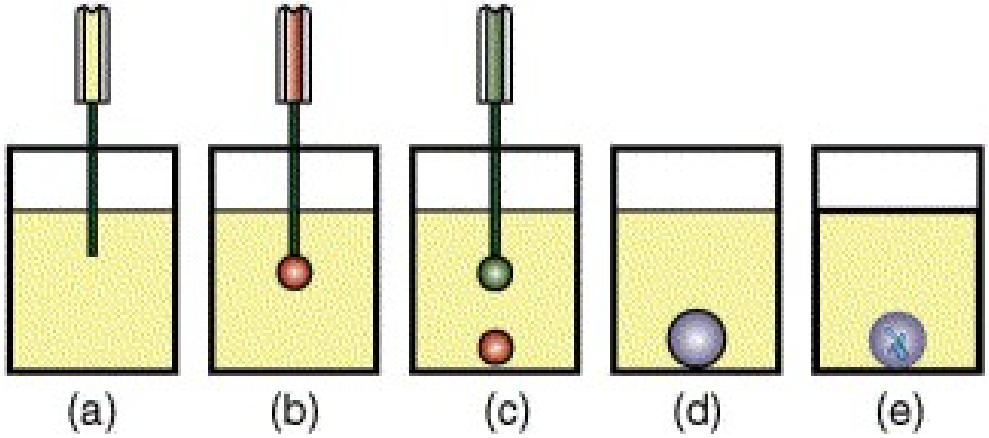
In a typical vapor diffusion experiment, equal volumes of protein solution and precipitant solution are mixed.



1) Water diffuses *from the drop to the reservoir*, increasing protein and precipitant concentrations.
2) The system reaches the labile zone, nuclei start to form and the protein in solution decreases, reaching the metastable zone.
3) Crystals grow until the protein concentration decreases below the solubility level.



Batch technique



(a) Small amount of oil dispensed in the well

(b) Protein drop dispensed under oil

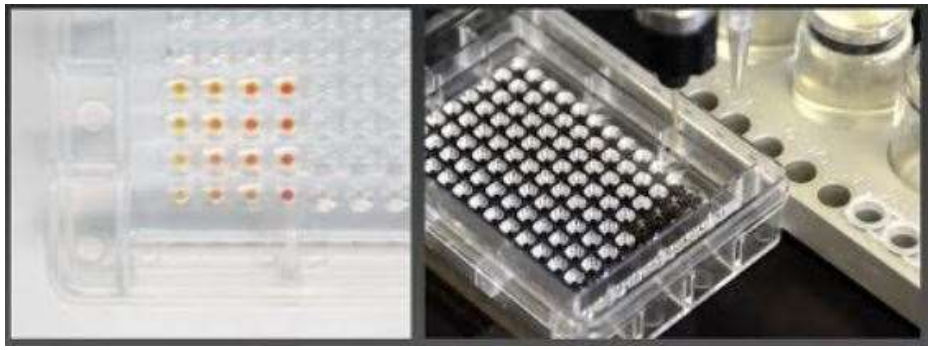
(c) Precipitant drop added

(d) Mixing of the two solutions and incubation

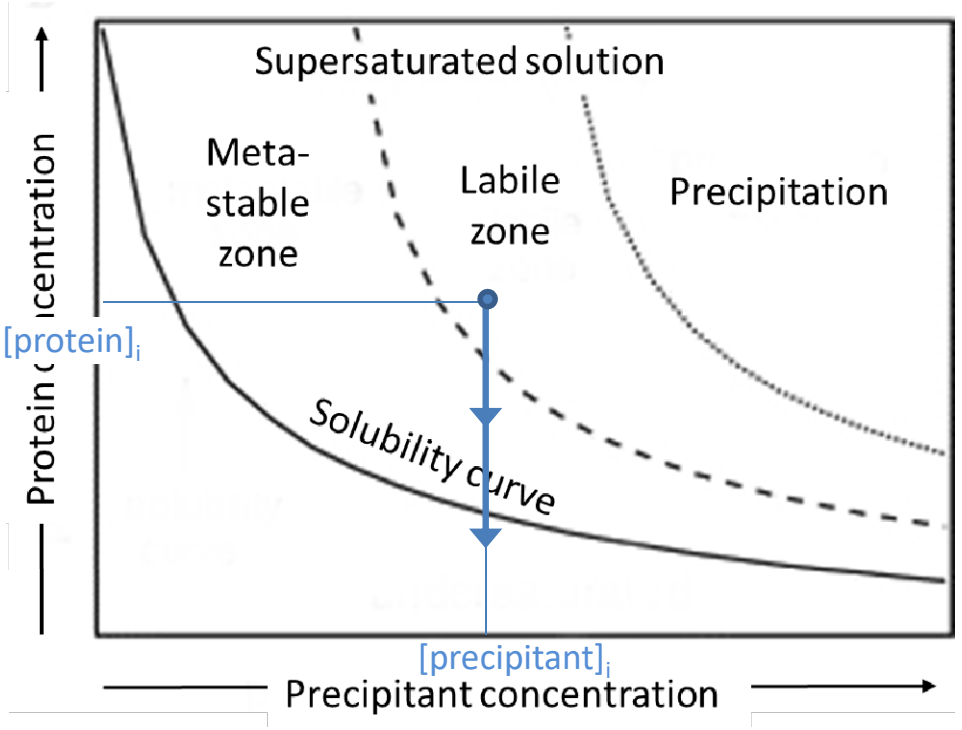
(e) Formation of crystals in the drop

Initial protein concentration above solubility curve!

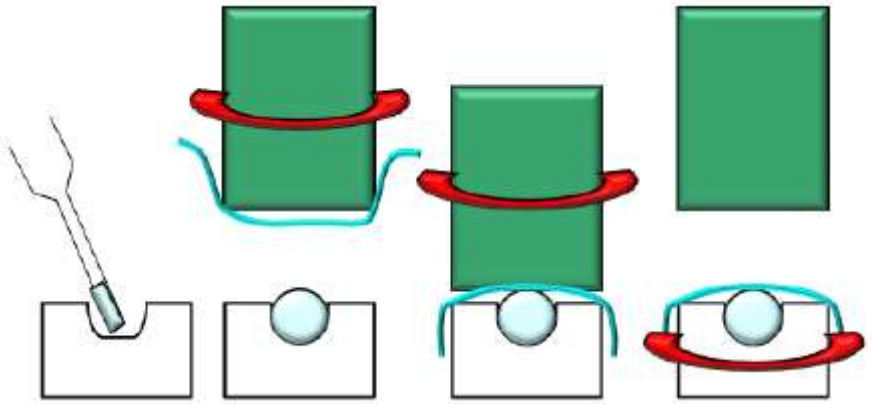
Oil layer avoids water evaporation from the drop.



Small volume and automation!!

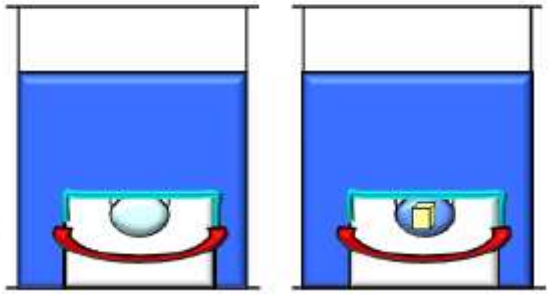


Microdialysis technique

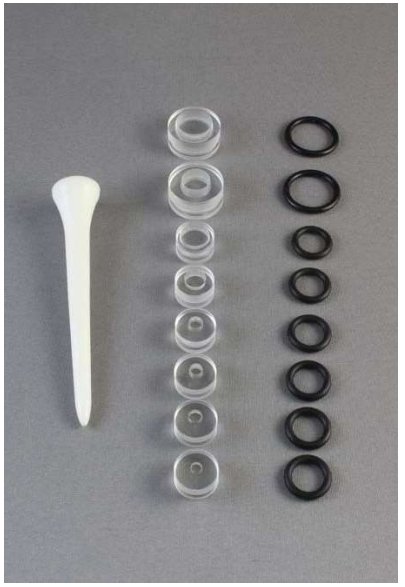


(a) Small volume of protein solution in a dialysis device

(b) Dialysis button closed with membrane (cutoff < protein size, > precipitant size)

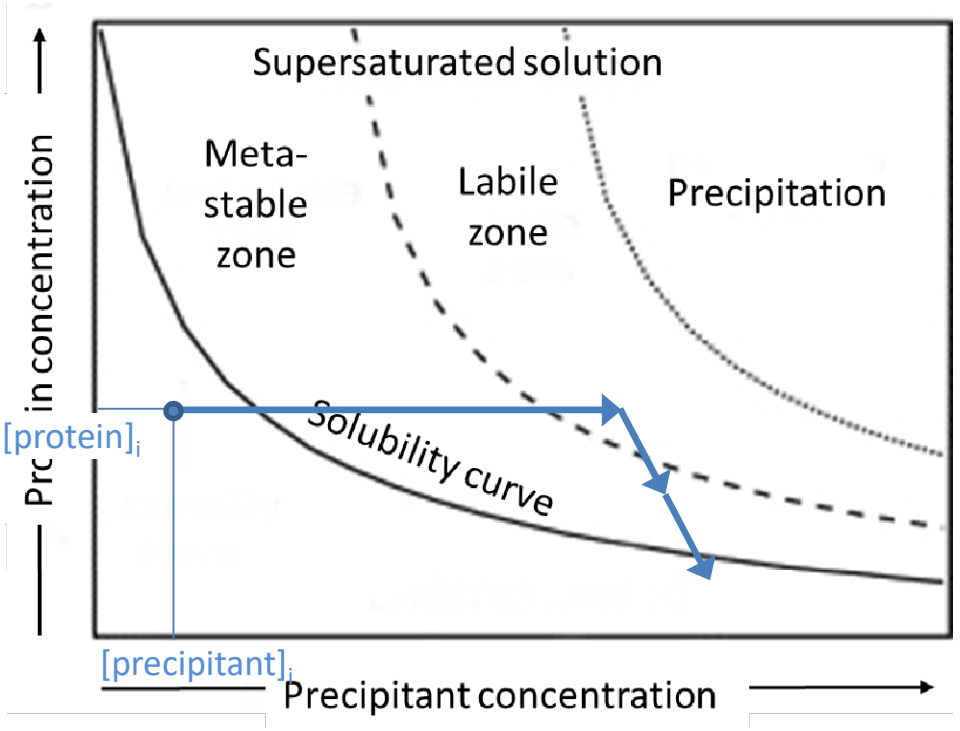


(c) Dialysis button in suitable solution



Protein concentration is constant during crystallization experiment

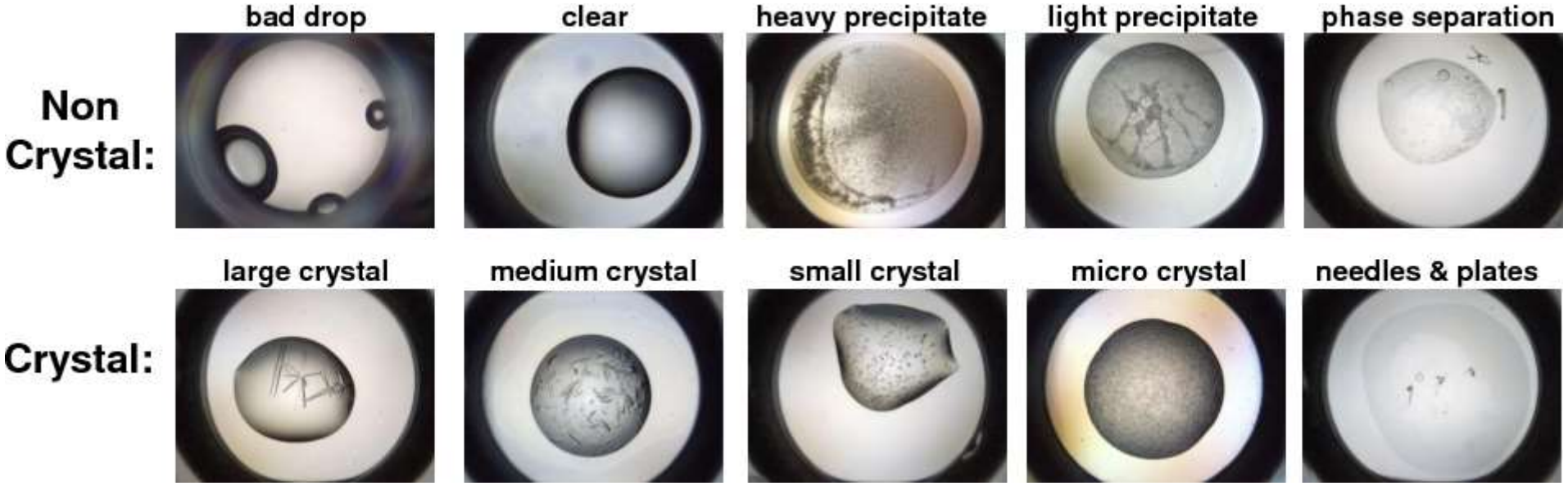
Kinetics can be controlled by stepwise change of dialysis solution



Comparison between crystallization techniques

	Protein amount	*Automation*	*Seeding*	*Harvesting*	**Results change with method!!**
Vapor diffusion: hanging drop	Small to large	Possible	Easy	Very easy	• *Particularly used in optimization tests* • *not suitable for low surface tension reagents*
Vapor diffusion: sitting drop	Small!	Easy	Possible	Easy	• *Ideal for initial screening of crystallization conditions* • *can be used with alcohols*
In-batch method	Small!	Possible	Not possible	Difficult	• *Oil may give unwanted interactions with protein*
Dialysis	Larger	No	Not possible	Easy	• *Yields large crystals!*

Analysis of experiments



Careful evaluation of results help optimization of crystallization conditions

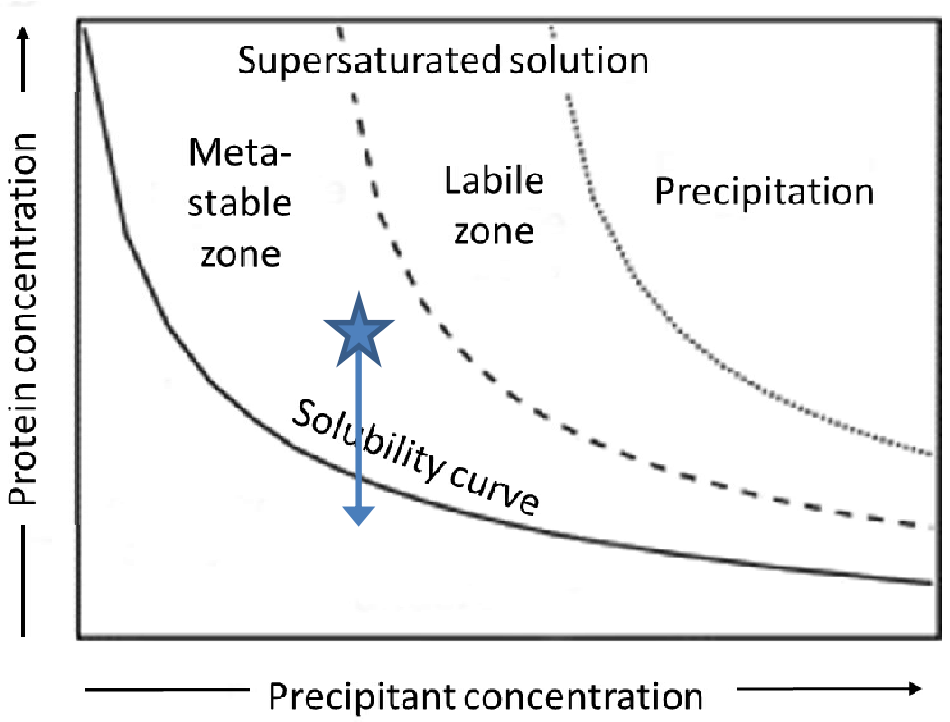
- If most of crystal drops are clear, protein concentration may be too low. If large amounts of precipitate are present in many crystallization drops, reduce protein concentration
- Many positive results with a precipitant suggest to use it in optimization screens

Experiments should be checked often to observe changes in the drops, particularly at the beginning of experiment incubation.

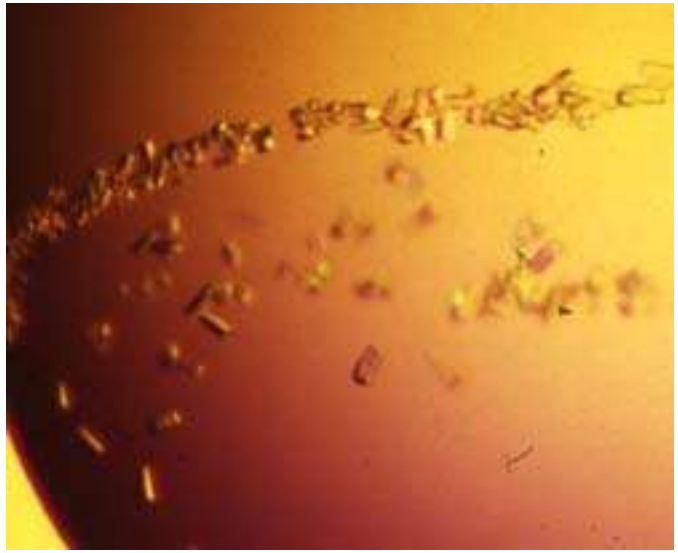
Observation under polarized light: birefringence effects allow to distinguish salt crystals from protein crystals and to highlight twinned crystals.

Seeding

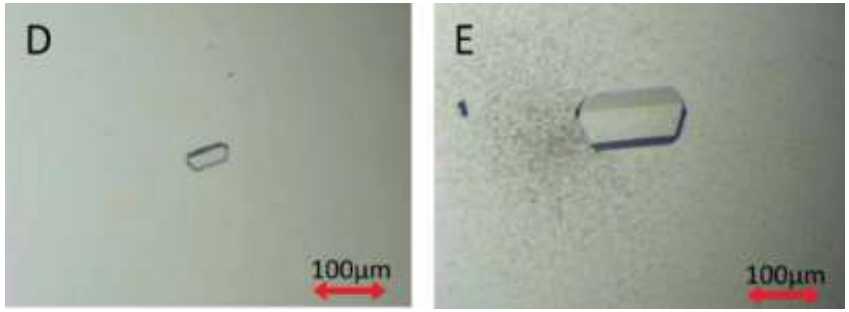
Introduction of crystallization nuclei in a clear solution (above saturation curve, but below the labile region)



1) Microseeding: streak the solution with a whisker to introduce small crystalline fragments – or dirt, or any other small particle that can induce crystallization



2) Macroseeding: small crystals are introduced in a clear drop; if aggregates, crystals are crushed before seeding



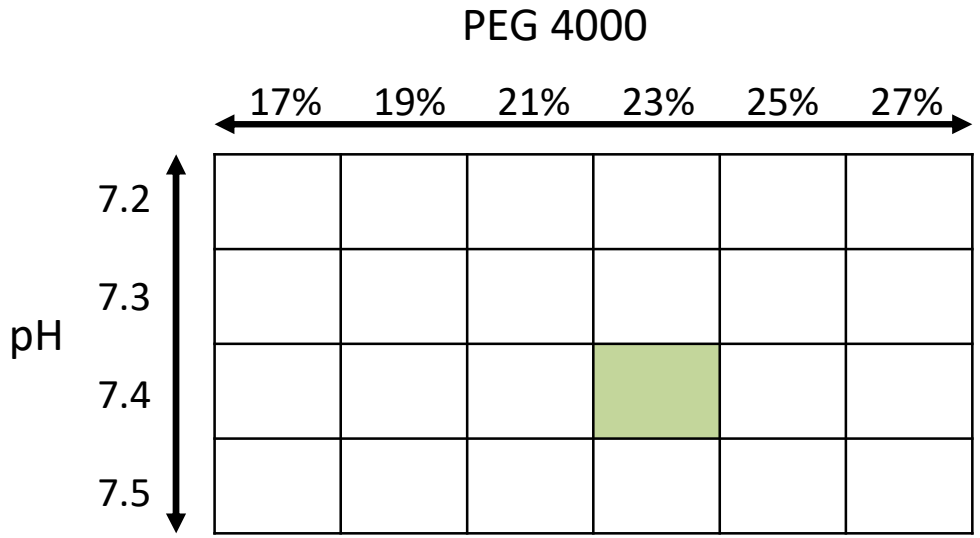
Optimization philosophy

If crystallization conditions of a related protein are known, or if a sparse-matrix screen has yielded initial hits: OPTIMIZATION!

1) Grid screen from the successful conditions:

E.g.:

Initial conditions:
buffer Tris pH 7.4,
precipitant PEG 4000 23%,
NaCl 150 mM

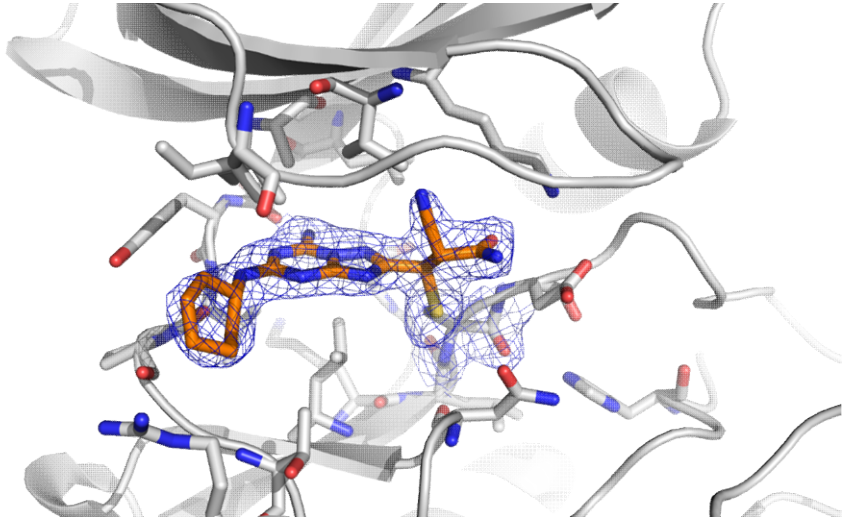


2) Use additives (salts, organic volatile/non-volatile solvents, amino acids, polyamines, chaotropic agents…) to improve crystal quality

Commercial additive screens are available.

Protein-ligand crystals

Structures of protein bound to ligands are essential for drug design studies. Two methods are available to obtain protein-ligand crystals:



Soaking

After crystallization, transfer protein crystals in a solution of the ligand. Incubate (hours to days) to allow diffusion of ligand in crystal channels (up to 100 Å large!).

Useful also for: cryoprotectants, heavy metals for phasing, substrates, …

Not suitable for large ligands.

Soaking may damage crystal order.

Co-crystallization

After checking binding affinity, mix protein solution and ligand and set up crystallization trials.

Usually, > stoichiometric amount of ligand.

Crystallization conditions may be different from conditions of the *apo* protein.

If ligand induces conformational homogeneity, it may improve crystal quality/dimension.

Automation

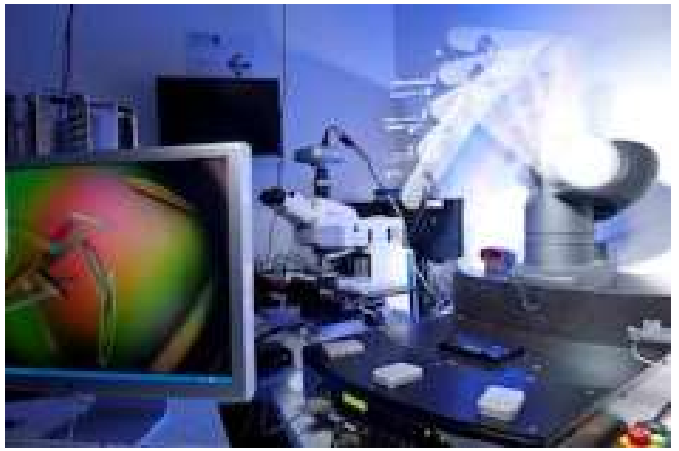
Automated systems exist for both crystallization setup and analysis of experimental results. Instruments available @Elettra, BioLab:

To dispense reservoir solutions in suitable plates: TECAN FredomEvo

Reservoir solution for sparse matrix commercial screens are sold in pre-mixed 96-deep-well blocks:

To prepare protein/precipitant drop with both hanging and sitting drop configurations: **Mosquito**

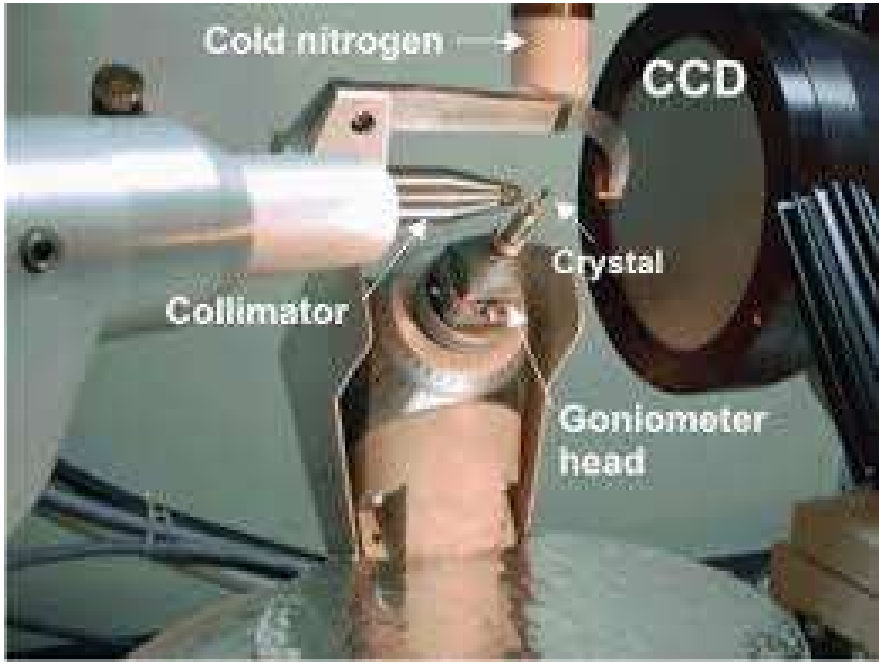
Automated system to monitor crystallization experiments and take pictures: Explora Nova Xtal Focus



Software are available (but not widely used) to assess crystallization result (e.g. giving to each experiment a rating)

Crucial instrument for biocrystallography!! Not only for reducing manual work and allowing a large number of trials, but also for reproducibility in handling **very** small volumes (down to 50 nL)

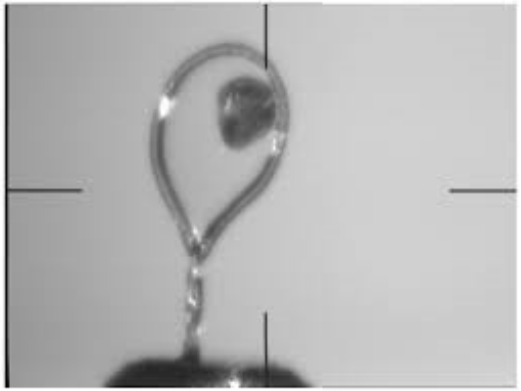
Biocrystallography experiment



Crystals are **harvested** from the crystallization experiment, **mounted** on the diffractometer goniometer and analyzed using X-ray.

Crystals are: small, sensitive to loss of solvent (dehydration), sensitive to radiation damage

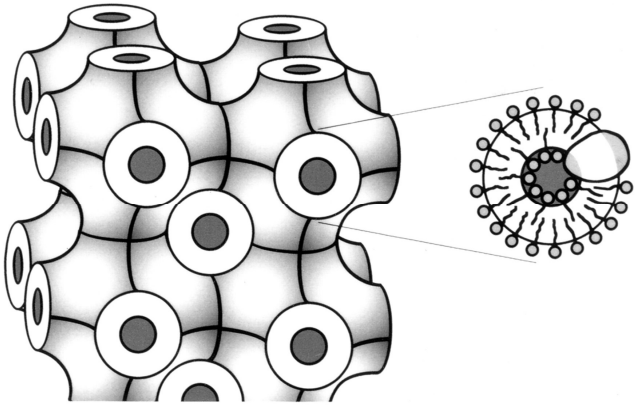
Loops are often used to harvest crystals keeping them in their mother liquor



To reduce radiation damage in long data collections, **freezing** of loops: during data collection, stream of cold nitrogen to keep sample frozen

Freezing may induce **solvent crystallization** (additional powder diffraction from solvent microcrystals, disruption of the crystalline order): **cryoprotection**

Membrane protein crystallization: Lipid Cubic Phase



Also known as ***in meso* method**

Complex 3D matrix obtained by thoroughly mixing a detergent solubilized protein with monoacylglycerol monolein; thick, sticky and viscous phase

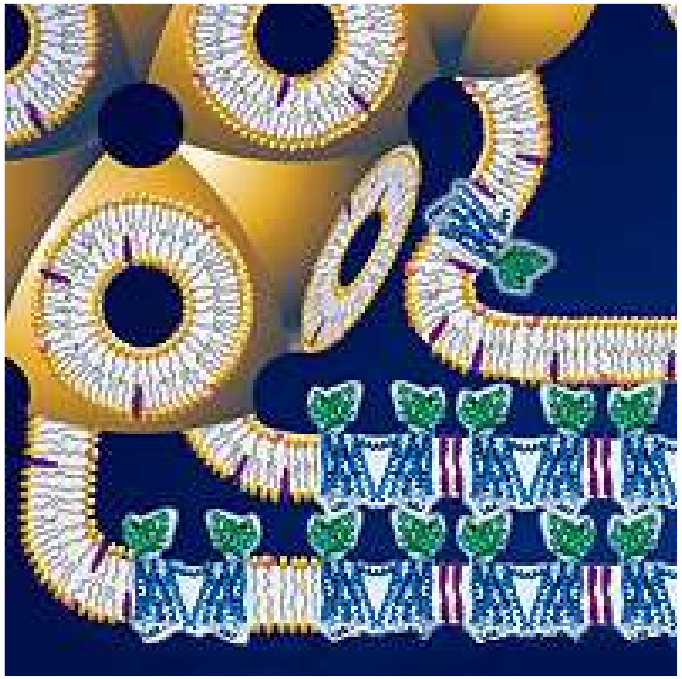
The matrix is bicontinuous: both lipid component and aqueous one are continuous in space

Crystallogenesis:

1) Reconstitution of protein in the curved bilayer
2) Phase separation induced by precipitant
3) Protein diffuses in the lamellar-like phase
4) Crystal growth

Advantages: small amounts of protein, native-like environment with possibility to add lipids

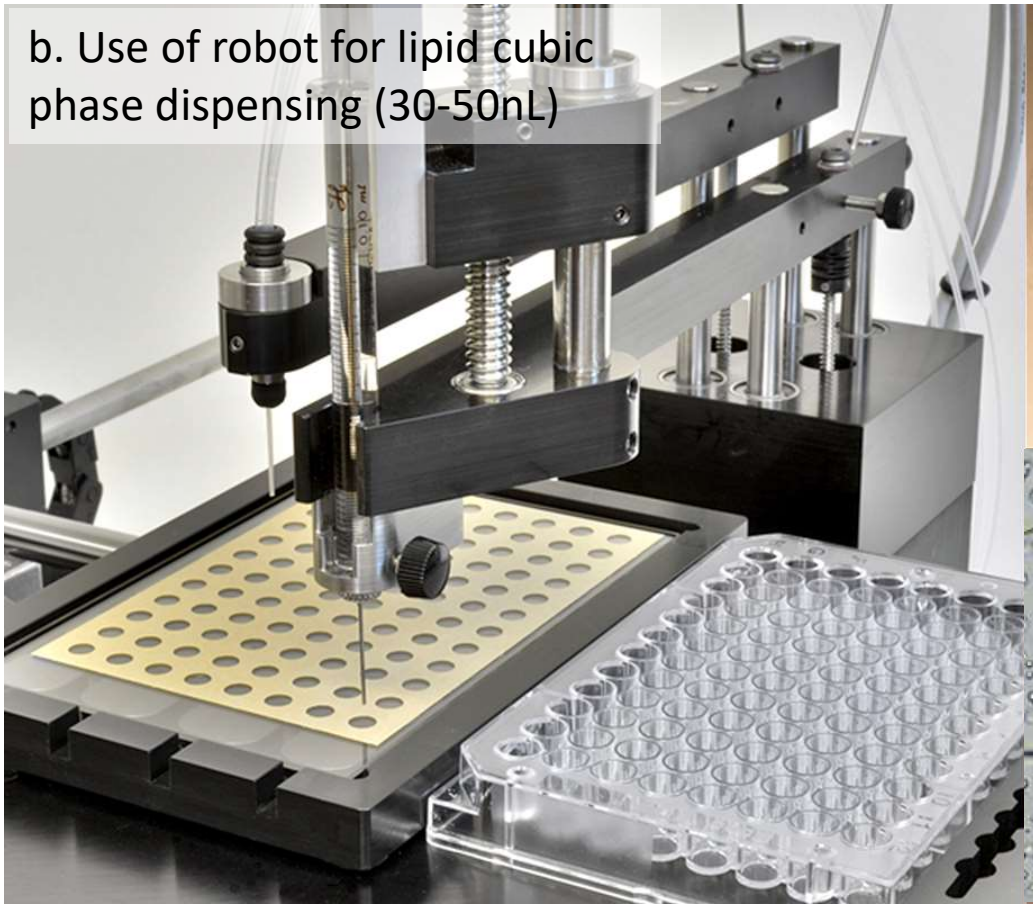
Drawbacks: small crystals and often hard to see

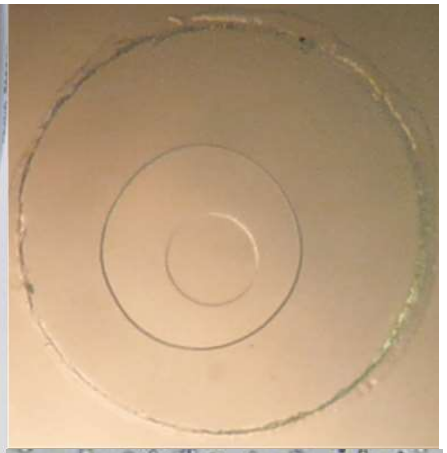


Lipid Cubic Phase

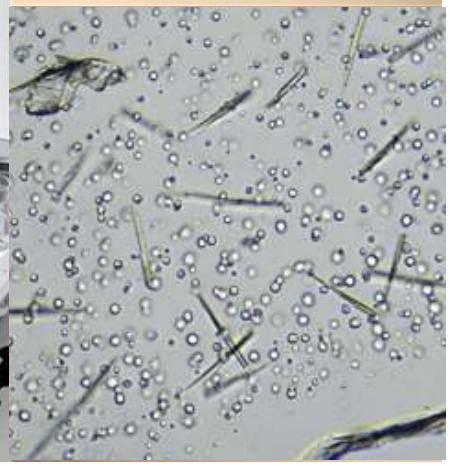
a. Mixing of protein and monolein

b. Use of robot for lipid cubic phase dispensing (30-50nL)





c. Addition of precipitant solution and sealing of the plate



d. Crystal formation!