Crystallization Methods and Screening

Juan Manuel García-Ruiz

Laboratorio de Estudios Cristalográficos
CSIC-Universidad de Granada, Spain
jmgruiz@ugr.es
Crystallization methods

Solvent is water (plus detergent in membrane protein crystallisation)

Water removal, either by:
- Evaporation.
- Dialysis.

Solubility change driven by:
- Temperature
- pH
- Dielectric constant
- Ionic strength
- Polymers

Because of lack of material protein crystallization techniques are microscale or nanoscale design

Low reproducibility
Lack of understanding

**Crystallizability:**

the propensity of a compound to crystallize, i.e. to enter the attractive regime to make ordered arrays of their molecules, both with orientational and translational symmetry.

**Crystallizability** depends on the properties of the macromolecule itself and on the _chemical_ cocktail used to bring them into the attractive regime, including ligands that are assumed to help crystallizability.

However, the way to mix the macromolecules and the molecules of the precipitating cocktail, the rate at which they mix and how fast supersaturation is achieved, depends on the crystallization technique.

Thus, the output from any crystallization experiment depends, in some extent, on the crystallization technique used. This is particularly true for screening.
Vapour diffusion technique

Advantages

• Easy to understand by the users
• Simple to implement
• Inexpensive
• Robotic available for H-T structural genomics
• Crystals can be handled for cryoXtallography

Drawbacks

• The solution moves from equilibrium to far from equilibrium
• Ambiguous interpretation of drop’s crystallization phenomena
• Problems of reproducibility and scale-up
• Each drop experiment screens a narrow crystallization space (measured by visited values of supersaturation and supersaturation rate)
Batch method

Direct mixing of protein and precipitant

- Protein
- Precipitant
- Slow evaporation
- Oil thin layer

Trial is blind
- Super(saturation) is immediately achieved
- Screening performed by repeating a number N of experiments using different initial conditions
- Higher the number N of experiments higher the possibility of success
- Number of visited crystallization conditions per trial: One supersaturation value, One (infinite) rate of supersaturation
Evaporation of water increases the concentration of precipitating agent and protein at the same rate.
As a result protein precipitates hopefully as crystals …
and the concentration of protein decreases

Note that the solution moves from equilibrium to out of equilibrium: It means initial conditions and/or rate of evaporation need to be tuned
Experimental set-up

A Mach-Zehnder interferometer study

The drop was sandwiched between two glass plates with a gap of 0.4 mm.

Juan Ma. García-Ruiz

XIII International Conference on the Crystalization of Biological Macromolecules

Cancun, 6-9 of May, 2008
Low concentration = Low density solution

Dynamics of a crystallising drop
A demonstration of the role of gravity

Crystals nucleate at the surface of the drop
Crystals sedimentate

Convective flow motion

Mach-Zehnder interferometry

Juan Ma. García-Ruiz
XII International Conference on the Crystallization of Biological Macromolecules
Cancun, 6-9 of May, 2008
Nucleation frequency $J$ is defined as the number of stable nuclei forming per unit of time and unit of volume. It is given by:

$$J = \kappa \exp \left( - \frac{16\pi v^2 \gamma^3}{3(kT)^2 \ln S} \right)$$
### The most common precipitants used in macromolecular crystallization

<table>
<thead>
<tr>
<th>Salt</th>
<th>Volatile organic compounds</th>
<th>Polymers</th>
<th>Non-volatile organic solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium phosphate and sulfate</td>
<td>1. Propanol and isopropanol</td>
<td>1. Poly(ethylene glycol) 1000, 3350, 6000, 8000, 20000</td>
<td>1. 2-methyl-2,4-pentanediol</td>
</tr>
<tr>
<td>Lithium sulfate</td>
<td>2. 1,3-propanediol</td>
<td>2. Ethylene glycol 400</td>
<td></td>
</tr>
<tr>
<td>Sodium or potassium or ammonium chloride, phosphate, acetate, tartrate</td>
<td>3. Dioxan</td>
<td>3. Jeffamine</td>
<td></td>
</tr>
<tr>
<td>Magnesium or calcium sulfate, chloride</td>
<td>4. Acetone</td>
<td>4. Polyamine</td>
<td></td>
</tr>
<tr>
<td>Sodium or magnesium formate</td>
<td>5. Methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>6. Ethanol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The chemical cocktail is in most cases:

Protein + water + buffer + precipitant

**Problem:** Low reproducibility; same chemistry yields different results within a single drop with chemically identical drops: Lack of understanding

**The first step of a Protein Crystallization experiment is always mixing.**

The way to mix the molecules and the molecules of the precipitating cocktail, the rate at which they mix and how fast supersaturation is achieved, depends on the crystallization technique.
Problems of Reproducibility

¿Precipitant over protein or protein over precipitant?

Protein heavier

Protein lighter

Relative viscosities also matter

Precipitant

Protein
Case 1: An interferometric video corresponding to the experiment of pouring a lighter NaCl solution (1%) over a denser Lysozyme solution (30 mg/ml). The diffusivity of the protein molecules towards the upper salt solution is slower than the diffusivity of the salt molecules towards the lower protein solution. This triggers a convective mechanism that enhances mixing. Time scale, 2 frame per second.
Case 2: An interferometric video corresponding to the experiment consisting of pouring a NaCl solution (2%) over a lighter lysozyme solution (30 mg/ml). Notice that the system becomes turbulent as a result of the development of Rayleigh-Taylor instability and the cocktail becomes homogeneous in less than one minute. Time scale, 2 frame per second.
Case 3: An interferometric video recording an experiment consisting of pouring a lighter lysozyme protein solution (30 mg/ml) over a NaCl solution (3%). It is clearly observed the phenomenon of double-diffusive salt fingering instability. The salt molecules diffuse faster into the pockets of proteins than the protein molecules into the pockets of salts. That simple mechanism creates density differences between the pockets and the surrounding solution that provokes the formation of upwards salt-rich fingers and downwards protein-rich fingering. Time scale: 2 frame per second.
Case 4: An interferometric video corresponding to an experiment consisting of pouring a Lysozyme protein solution (60 mg/ml) over a NaCl solution (9%). The interferometric mode switch to normal optical illumination between 27 and 34 seconds. Then, it can be clearly noticed that due to high concentration of the solutions, the fluid is mixed by drag of the precipitated protein. Time scale, 2 frame per second.
Time sequence of interferograms. A solution of Lysozyme 30 mg/ml poured on top of a solution of NaCl 2% m/v.

- Protein lighter, more viscous
- NaCl heavier, less viscous
\[ \Delta \text{density} < 0 \]

Salt

\[ \Delta \text{density} > 0 \]

Protein
To Mix or not to Mix. Is that the question?

As a practical corollary of the results, it is recommended to perform mixing when seeking for reproducibility while avoid mixing when seeking for a more complex and extensive screening of the crystallisation space.

From: On the mixing of protein crystallisation cocktails
Eduardo I. Howard, José Miguel Fernandez and Juan Manuel García-Ruiz
Submitted for publication
Batch Method

Batch Method no mixing = liquid-liquid diffusion

Capillary counter diffusion
The counter-diffusion technique

Based on diffusion-reaction patterns forming when two reactants are allowed to diffuse one against the other under initial conditions very far from equilibrium:

Requirements:

- Convection must be removed
- One reactant must flow faster than the other one
- It must be room to display the spatial pattern
- Initial conditions far from equilibrium

A physical buffer (optional)

A precipitant chamber

A long protein chamber

The diffusion step:

\[
\frac{\partial c}{\partial t} = \frac{1}{2} \frac{\partial}{\partial x} \left( \frac{D_\text{ppt} \partial c}{D_\text{ppt} + D_\text{p}} \right) + \frac{1}{2} \frac{\partial}{\partial x} \left( \frac{\partial c}{\partial x} \right)
\]

The precipitation step is governed by supersaturation with respect to the solubility curve of the protein

\[
\frac{\partial c}{\partial t} = \frac{1}{2} \frac{\partial}{\partial x} \left( \frac{D_\text{ppt} \partial c}{D_\text{ppt} + D_\text{p}} \right) - \frac{1}{2} \frac{\partial}{\partial x} \left( \frac{\partial c}{\partial x} \right) - \frac{\partial c}{\partial x} \frac{D_\text{ppt}}{D_\text{p}}
\]

with \(D_\text{ppt} >> D_\text{p}\)

How do counter-diffusion works?


Juan M. Garcia-Ruiz

XII International Conference on the Crystallization of Biological Macromolecules

Cancun, 6-9 of May, 2008
Counter-diffusion technique: How does it work?

Typical counterdiffusion crystallization patterns
Numerical simulation of the counter-diffusion technique

A supersaturation wave for protein crystallisation


The counter-diffusion technique

Based on diffusion-reaction patterns forming when two reactants are allowed to diffuse one against the other under initial conditions very far from equilibrium:

Requirements:
- Convection must be removed
- One reactant must flow faster than the other one
- It must be room to display the spatial pattern
- Initial conditions far from equilibrium

A precipitant chamber | A long protein chamber

The diffusion step:
\[ c_{x,t} = \frac{1}{2} c_{x0} \text{erfc} \left( \frac{x + d}{2 \sqrt{D_t t}} \right) \quad \text{and} \quad c_x = \frac{1}{2} c_{x0} \text{erfc} \left( \frac{x}{2 \sqrt{D_0 t}} \right) \]

with \( D_{\text{ext}} \gg D_s \)

The precipitation step is governed by supersaturation with respect to the solubility curve of the protein.
A demonstration of the dynamics of pattern formation in counterdiffusion technique

Advantage of CCD: Diffusive mixing assures reproducibility

Juan Ma. Garcia-Ruiz
XIII International Conference on the Crystallization of Biological Macromolecules
Cancun, 6-9 of May, 2008
In batch + evaporation and vapor diffusion techniques the system moves towards far from equilibrium.

In counter-diffusion technique the system moves from far from equilibrium to equilibrium:

The technique self-search the best crystallization conditions.
How to perform counter-diffusion experiments?

Looking for diffusion mass transport scenarios

At high $Gr_N$ convection dominate

$$Gr_N = \frac{\text{buoyancy forces}}{\text{viscous drag forces}} = L^3 \cdot \alpha \cdot \Delta c \cdot g \cdot \nu^{-2}$$

At low $Gr_N$ convection dominate

- Gels ($10^{-7}-10^{-6}$ cm) and capillaries ($2 \times 10^{-2}$ cm)
- Viscosity $0.015$ g cm$^{-1}$ s$^{-1}$
- Microgravity $981 - 9.8 \times 10^4$ cm$^{-1}$ s$^{-1}$
- Density gradient $\approx 10^{-3}$ gr cm$^{-3}$ mol$^{-1}$

Juan Ma. García-Ruiz

XII International Conference on the Crystalization of Biological Macromolecules

Cancun, 6-9 of May, 2008
For capillaries of 0.1 mm internal diameter the required volume of protein solution is 314 nanoliters for 4 cm long capillaries and 235 nanoliters for 3 cm long capillaries. For a capillary diameter of 0.75 mm (the limit for a good visualization) the volume of protein solution is just 166 and 133 nanoliters respectively.
Or by counterdiffusion techniques, since you have already seen a good collection of crystal, here there are some more from other laboratory.
A partir de un análisis de secuencia se han determinado las mutaciones más conservadas evolutivamente. Para entender el por qué de esta conservación se llevan a cabo estudios termodinámicos y estructurales que nos permitan en un futuro diseñar proteínas funcionales de mayor robustez.
Crystal structure of dihydropyrimidinase de *S. meliloti*...

...and mutants C76A y C181A of the hidantoin racemase of the

Dpto. Química, Área de Bioquímica y Biología Molecular. UAL. Dr. Felipe Rodríguez-Vico y Dr. Sergio Martínez-Rodríguez
In situ data collection and structure determination at room temperature

NI14A Abl-SH3 domain mutant complexed with a high affinity peptide ligand

A demonstration of the use of the method to solve structures at room temperature directly from 0.1 mm capillary screening.

Table 1: X-ray data collection statistics

<table>
<thead>
<tr>
<th>Space group</th>
<th>P2_1 2_1 2_1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit cell dimensions (Å)</td>
<td>48.170 50.093 56.431 90.00 90.00 90.00</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50-1.75</td>
</tr>
<tr>
<td>Number of observations</td>
<td>84702</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>13266 (1638)</td>
</tr>
<tr>
<td>Data completeness (%)</td>
<td>92.2 (75.3)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>0.07 (0.24)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>16.4 (4.4)</td>
</tr>
</tbody>
</table>

*The values in parentheses are for the highest resolution bin

Cryocrystallography with crystals grown by counterdiffusion technique

Keeping crystals in the capillary (Method 1)

**Precipitant + glycerol (cryo solution)**

Protein and low concentration agarose

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>IP</th>
<th>Precipitant</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>14.3 KDa</td>
<td>9.3</td>
<td>NaCl</td>
<td>4.5</td>
</tr>
<tr>
<td>Thaumatin</td>
<td>22 KDa</td>
<td>8.5</td>
<td>Na/K Tart.</td>
<td>7.0</td>
</tr>
<tr>
<td>HLFBPase</td>
<td>147 KDa</td>
<td>6.6</td>
<td>PEG 4000</td>
<td>9.0</td>
</tr>
<tr>
<td>Ferritin</td>
<td>456 KDa</td>
<td>5.4</td>
<td>CdSO₄</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Each protein was concurrently grown, derivatized with a halide, treated with a cryogenic solution in a single capillary tube and used directly for data collection using synchrotron radiation source without ever handling the crystals.

The structures were determined ab initio by Iterative Single Anomalous Scattering (ISAS) yielding to de novo crystallographic models. This procedure is proposed as a direct application towards high thorough-put screening and structure determination for proteins in general.


In situ data collection and structure determination
Example of capillary mounted crystal for cryo-cooling
Example of a SH3 Alpha-Spectrin crystal collected at room temperature and then cryo-cooled at 100K.

<table>
<thead>
<tr>
<th>SH3 Alpha-Spectrin pH 9.0</th>
<th>mixPEGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>0.977</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁,2₁,2₁</td>
</tr>
<tr>
<td>Cell parameters (Å)</td>
<td>34.46, 42.47, 50.04</td>
</tr>
<tr>
<td>Resolution limit (Å)</td>
<td>1.55</td>
</tr>
<tr>
<td>T data collection (K)</td>
<td>RT</td>
</tr>
<tr>
<td>Precipitant</td>
<td>mixPEGs</td>
</tr>
<tr>
<td>Crystallization Technique</td>
<td>CD Capillary</td>
</tr>
<tr>
<td>Crystallization pH</td>
<td>9.0</td>
</tr>
<tr>
<td>Mosaicity</td>
<td>0.8-1.0</td>
</tr>
<tr>
<td>B factor</td>
<td>19.7</td>
</tr>
</tbody>
</table>
Dip one capillary into the protein solution. The protein solution will rise by capillarity and the capillaries will be filled. Seal the upper end with the putty.

Dip the filled capillary into the GCB-Domino. Just punch the unsealed end of the capillary across the gel located on top of the precipitant.
Low-cost plastic constructs fabricated from cyclic olefin polymers shown non-birefringent and compatible materials for microfluidic crystallization.

In situ data collection and structure determination

Sauter et al., From macrofluidics to microfluidics for the crystallization of biological macromolecules CGD 7 2007.


Our aim is to reduce the screening for crystallization conditions as much as possible based on understanding.

Could a mixture of PEGs do as well as each PEG separately?
Is it the pH of the crystallization experiment important when used salts as well as PEG as precipitating agent?

Study base on 20 proteins along one year:

- PEG 400K (30%); six pH values
- PEG 4000 (PEG 30%); six pH values
- PEG 8000 (30 %); six pH values
- PEG 400K (20%); PEG 4000 (PEG 15%); PEG 8000 (10 %); six pH values

Ammonium sulphate 3M at six different pH values.
The precipitant cocktail contains:

a) A buffer that keeps the solution within one unit of pH
b) A low molecular weight polyethylene glycol
c) A high molecular weight polyethylene glycol
d) An additive that may help to precipitate the protein.

According to Einstein-Stokes relation the diffusivity depends on the radius of the molecule. Therefore:

Buffer molecules diffuse faster and change the pH of the protein solution
Low MW PEG molecules go later on
Finally high MW PEG molecules scan the capillary

Several effects in one single experiment!
All concentrations tested in one experiment!
Crystallizability is very sensitive to pH—as expected. The bad news is that there is not a clear correlation with pI. When crystals form at any pH, precipitation occurs in any other pH.
Crystallizability is sensitive to pH. There is not a clear correlation with pI.

The 3PEG mixture does as well as each of them. However, it is required to increase the concentration of PEG 8K.
Some additional results observed:

1. It is confirmed that, for the same screening matrix, counterdiffusion yields at least as many hits as vapor diffusion, within the first three weeks after set-up of the experiments.

2. It is confirmed that for similar crystallization conditions, nucleation is retarded in the case of counterdiffusion with respect to drop techniques.

3. It has been also proven that counterdiffusion yields crystals under screening conditions that drop techniques never produced. Noticeably the number of new crystallization conditions increases with time, sometime with waiting periods of month(S).
The way in which supersaturation is achieved has an effect on the crystal form. 

<table>
<thead>
<tr>
<th>Protein</th>
<th>Crystallisation technique</th>
<th>Crystal form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cablys3*lysozyme</td>
<td>hanging drop PEG P2,2,2,1</td>
<td>67 69 113 90 90 90</td>
</tr>
<tr>
<td></td>
<td>hanging drop formate C2</td>
<td>133 73 39 90 105 90</td>
</tr>
<tr>
<td></td>
<td>APCF C2</td>
<td>129 73 38 90 107 90</td>
</tr>
<tr>
<td></td>
<td>Counterdiffusion formate P2,2,2,1</td>
<td>68 69 113 90 90 90</td>
</tr>
<tr>
<td>TIM</td>
<td>hanging drop AS P322,1</td>
<td>125 104</td>
</tr>
<tr>
<td></td>
<td>Counterdiffusion AS P3x12</td>
<td>215 106</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>sitting drop P2,2,2,1</td>
<td>51 50 35</td>
</tr>
<tr>
<td></td>
<td>Counterdiffusion P2,2,2,2</td>
<td>59 59 26</td>
</tr>
<tr>
<td></td>
<td>Counterdiffusion P1</td>
<td>26 32 53 85 83, 79</td>
</tr>
</tbody>
</table>

From I. Zeggers, J.M. Garcia-Ruiz and coworkers, unpublished data.
<table>
<thead>
<tr>
<th>PDB ID</th>
<th>Variant</th>
<th>S G</th>
<th>Condition</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1KEB</td>
<td>P40S</td>
<td>P 1</td>
<td>pH: 3.8</td>
<td>Diffusion-Vd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH: 25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CuAc2: 10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaNAc: 100 mM</td>
<td></td>
</tr>
<tr>
<td>1SRX</td>
<td>Forma oxidada</td>
<td>C 2</td>
<td>pH: 3.3</td>
<td>Diffusion-Vd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH: 25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CuAc2: 10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaNAc: 100 mM</td>
<td></td>
</tr>
<tr>
<td>2TRX</td>
<td>Silvestre</td>
<td>C 2</td>
<td>pH: 4.7</td>
<td>Micro-dialysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH: 25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CuAc2: 10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaNAc: 100 mM</td>
<td></td>
</tr>
<tr>
<td>2FCH</td>
<td>G74S</td>
<td>P 1</td>
<td>pH: 5.4</td>
<td>Counter-diffusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH: 25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CuAc2: 10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaNAc: 100 mM</td>
<td></td>
</tr>
<tr>
<td>1ZZY</td>
<td>L7V</td>
<td>Pi</td>
<td>pH: 3.3</td>
<td>Counter-diffusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH: 25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CuAc2: 10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaNAc: 100 mM</td>
<td></td>
</tr>
<tr>
<td>2FD3</td>
<td>P34H</td>
<td>P 1</td>
<td>pH: 3.4</td>
<td>Counter-diffusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH: 25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CuAc2: 10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaNAc: 100 mM</td>
<td></td>
</tr>
<tr>
<td>2H6X</td>
<td>Wild</td>
<td>P61</td>
<td>pH: 6.9</td>
<td>Counter-diffusion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EtOH: 25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CuAc2: 10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NaNAc: 100 mM</td>
<td></td>
</tr>
</tbody>
</table>

Counterdiffusion produces new polymorphs

Vrije Universiteit Brussel, Université de Liège, Université Catholique de Louvaine
Screening with counter-diffusion technique

Simple and fast preparation of the experiments
  No tools required. Just fill the capillary and punch it into the precipitation cocktail

Extensive search of the crystallization space for pH and precipitant concentrations
  Actual grid screen can be made possible and are recommended

Requires minimal amount of protein
  Even less than 250 nanoliters per capillary.

No pictures and image analysis required.
  The whole growth history is stored in the capillaries.

Unambiguous interpretation of the results
  The technique does not yield single results but a trend

The number of conditions for effective screening can be reduced
  Mix of PEGs can be used to replace singular MW PEGs.
Coauthors in the work presented in this talk:

Luis Antonio Gonzalez: Crystallization experiment implementation
José Luis Gavira: Handling of crystals and X-ray diffraction analysis
Eduardo Howards: Work on mixing experiments
Joe Ng: First screening and in situ X-ray diffraction analysis
Fermin Otalora: Computer simulations

Financial support from and help from:

European Community VI Framework Program
Spanish Government (MEC)
Triana Science and Technology
This is a product of La Factoría de Cristalización
Thank you

This is a product of La Factoría de Cristalización