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Understanding the thermal environment in your lab and managing temperature to improve protein crystals

RAMC 2011

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Agenda

- Why is temperature important in biological crystallization
- Sources of temperature fluctuations in your lab
- Our solution:TG40 System
- Results

Three aspects of the use of temperature

(1) a crystallisation parameter, to be included in the parameter space search alongside the type and concentration of precipitating agent, pH and buffer, protein concentration etc.

(2) a means to induce crystallisation by increasing the supersaturation (commonplace in small molecule crystallisation, too rare for macromolecules)

(3) a means to optimise crystal growth, by separating nucleation from the growth stage

The solubility of many proteins depends on temperature

- 86% of proteins tested by Christopher *et al.* (1998) *J. Cryst. Growth* **191**,820, out of which 54% have direct temperature dependence and the rest have retrograde dependence. 9 of 13 retrograde solubility cases where in high salt, 4 of 13 in low salt, none from PEG.

- Results corroborated by Zhu et al. (2006) J. Struct. Biol. 154,297: 80% of proteins tested displayed temperature dependance.

- Temperature dependence is often shallow and can become virtually insignificant at high ionic strength, but becomes much steeper at low ionic strength, with PEG, MPD and organic solvents. Also retrograde solubility is more frequent at high ionic strength, in the cases where there still is temperature dependance (see Lloyd Haire in Bergfors (*ed*) *Protein Crystallization*, IUL 1999.

- Thus the temperature - solubility function is not a property of the protein itself but of the protein-salt system.

Temperature can influence the kinetics of crystallisation by:

(1) changing the speed of mass and heat diffusion in the crystallisation solution. Low temperature can therefore sometimes increase the rate of nucleation, whilst reducing the rate of growth (see eg. Lorber & Giegé (1992) *J. Cryst. Growth* **122**,168).

(2) changing the rate of equilibration in vapour diffusion, dialysis, free interface diffusion…

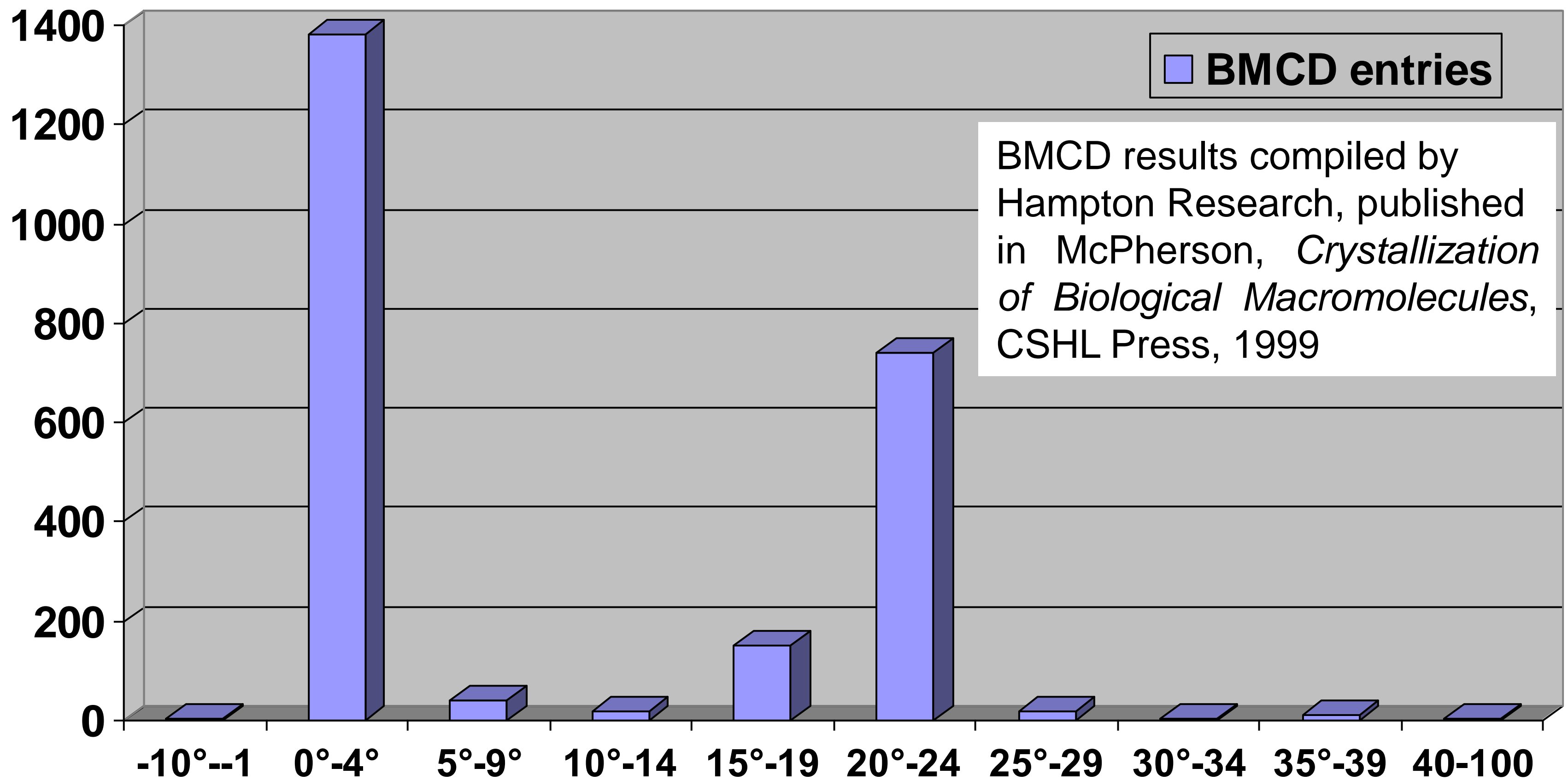
Temperature as a means for inducing crystallisation

- Small-molecule crystallisers often use a controlled, slow drop in temperature to induce crystallisation
- It is a minimally invasive, reversible method to modify the level of supersaturation
- It is however much less used in the macromolecular crystallisation, due in part to the belief that the protein-dependence is too shallow (which is generally true only in high salt) and in part to the lack of dedicated apparatus
- For reports of the use of temperature shift in macromolecular crystallisation, see L. Lloyd Haire, *in* T.M. Bergfors (*ed*) *Protein Crystallization*, I.U.L. 1999, pp. 65-68 and refs therein.

Temperature as a means to separate the nucleation and growth stages of crystallisation

- The temperature-dependence of solubility can be used for modifying the supersaturation level with respect to protein of the solution during the course of the experiment – thus uncoupling the nucleation from the crystal growth stage

Temperature as one more parameter to test



"there is clearly room for more creative use of temperature" (McPherson,1999)

Current Methods

No Temperature control

Air conditioned rooms

Fridges/Incubators

Sources of temperature fluctuations

- Type of air conditioning system
- Position air conditioning system
- Windows and doors
- Heating
- Sunlight
- Seasons
- Other lab equipment , inc: microscopes and PCs

TG40 System

Screening 5 different temperatures
4°C to 60°C at ambient temperatue of 20°C

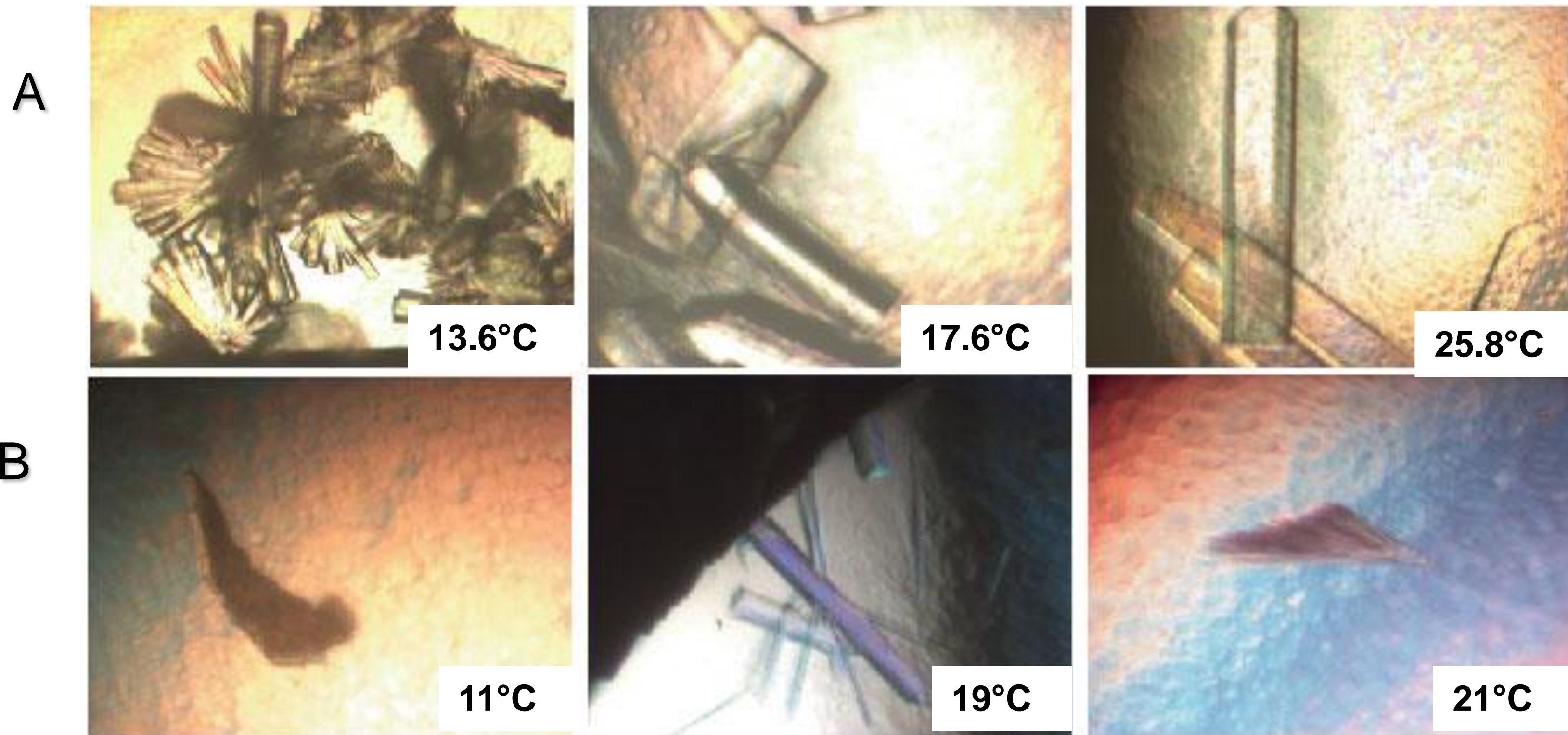
Portable

Programmable

Temperature accuracy 1°C

Compatible with other lab instruments

Reproducibility

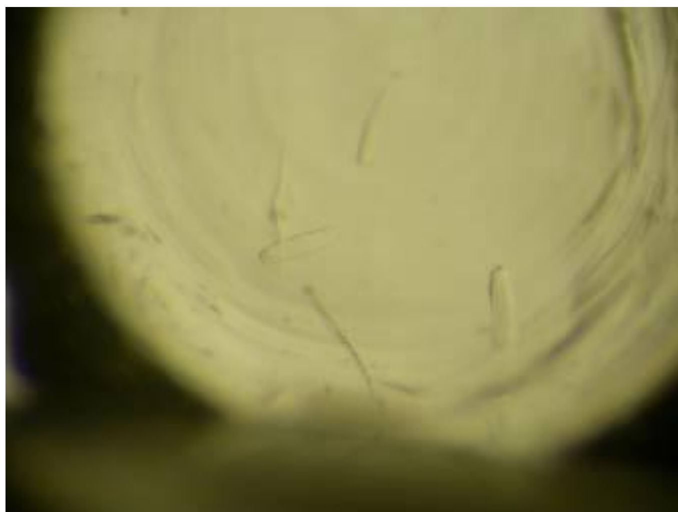


100μm

A) Lysozyme (0.4M NaNO3)

B) C-terminal Fragment of tetanus toxin TetC (0.2M Ammonium Sulphate & 20% PEG 4K)

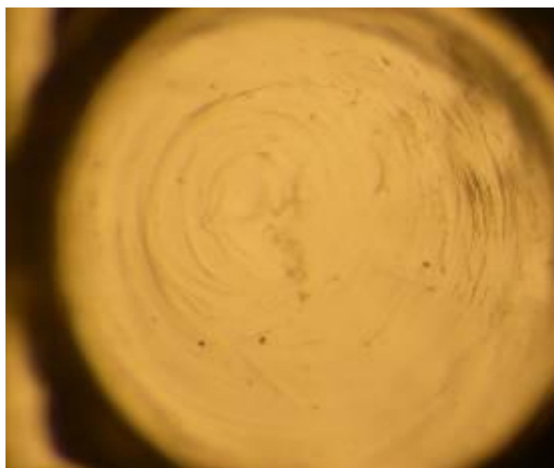
T-G40 System – Temperature Optimization I



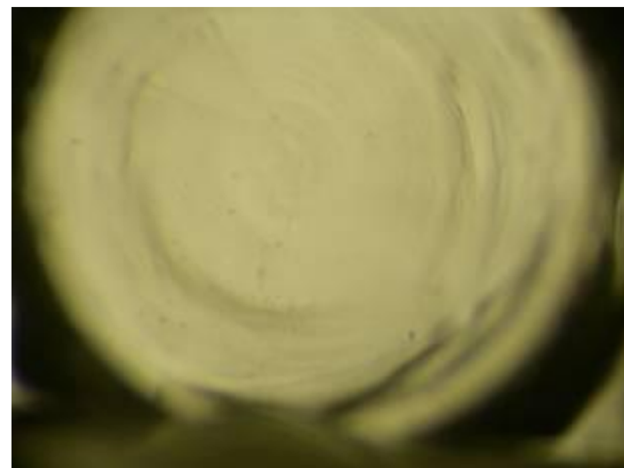
6°C – Twinned Crystals

10°C – Twinned Crystals (better)

14°C - Single Xtals



18°C - Precip



22°C - Precip

Courtesy of Andrew Bent, Protein Crystallization Scientist

Using the TG40 System to Grow Large Crystals for Neutron Diffraction at the ILL - France

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Dr. Susana Teixeira is a lecturer at Keele University in the UK. She has a joint appointment with the Institut Laue Langevin (ILL) in Grenoble, France, where she is an Instrument Scientist at 2 crystallography beam lines: the low resolution neutron diffractometer (D16) and a high resolution Laue diffractometer (LADI-III).

She is also editor of the Grenoble Partnership for Structural Biology (PSB) Newsletter[1] . Dr. Teixeira's research focuses on structural studies of DNA/anticancer drug complexes as well as food-related systems such as thaumatin or caseins. Key to her research is the use of deuterium labeling and neutron diffraction techniques, for which the Deuteration Laboratory[2] and the neutron instruments at the ILL are uniquely suited.

Neutron protein crystallography is a powerful complementary technique to X-ray crystallography. It allows locating key hydrogen atoms in biological structures that in most cases cannot be observed through X-ray analysis alone. This can provide important insights into an enzyme catalytic mechanism[3].

A major difficulty in neutron protein crystallography is that relatively large crystals (typically larger than 0.5mm³) are required to compensate for the relatively weak flux of available neutron beams. Varying the temperature at critical points of the crystallization process can be used to drive a system under study towards optimal crystal volumes.

Why is the TG40 particularly useful for neutron crystallography experiments?

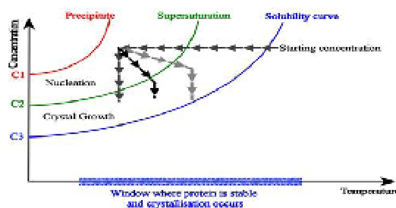
"Since neutrons are non-destructive, the corresponding crystallography experiments benefit from the privilege of collecting data at room temperature (although lower temperatures can be used). There is therefore a strong preference for crystals that are stable at room temperature (~20°C) and this has significant weight in choosing a crystallisation strategy.

To grow larger crystals there are a number of approaches possible, but most involve using relatively high concentrations of protein and/or relatively large volumes of solution. Most crystallographers find it difficult to optimise the delicate balance between the kinetics of vapour diffusion and the best protein/precipitant concentration profile."

Dr. Teixeira used the TG40 System to grow larger crystals for neutron crystallography studies. The usual problem observed when up-scaling crystallisation conditions to significantly larger volumes/ concentrations of sample is that almost inevitably one changes other parameters, namely the rate at which concentration occurs or the size/shape of the surface through which vapour diffusion takes place when such methods are used. To avoid having to re-optimise conditions using large volumes of sample (which is often not an option due to limited availability), choosing a batch method and fine tuning the crystallisation conditions using temperature screenings has been found to produce good results.

A successful strategy was used by Dr. Teixeira to get a handle on nucleation, i.e. to produce as few crystal "seeds" as possible, as summarized briefly below for a protocol involving batch crystallisation using sealed wells of the TG40 System at a range of temperatures.

"1- Pre-screen using either hanging drop or sitting drop vapour diffusion crystallisation techniques with smaller volumes. If possible, choose the combination of sample and precipitating agent concentrations, at starting temperature T_0, for which crystals could be observed under the microscope in a short period of time (ideally 1-2 days; a few hours is usually too short). This tends to be a concentration above C2 in the plot below.

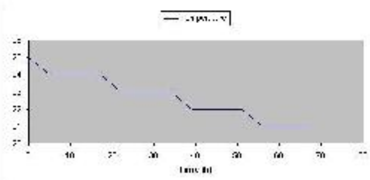


2- Set a few batch conditions on the TG40 by filling in the reservoir with a mixture of your sample and crystallisation buffer that matches your chosen condition in 1 (temperature T_0). Set a range of 3 or 4 similar conditions of concentration of protein (e.g. 5, 6, 7, 8 mg/ml). At this point I did not use more than 10 microlitres of total volume per well.

[1] See http://www.psb-grenoble.eu/
[2] See http:www.ill.eu/deuteration
[3] See for example the recent press release highlight: http://physicsworld.com/cws/article/news/45675

3- Observe the wells for up to 5 days. If crystal showers appear, the concentration of protein and/or precipitant is too high (if possible within the range of temperature your protein is stable on, you can check if it is possible to re-dissolve the crystals by increasing the temperature, but my experience is that this gives poor results). Lower the concentrations and redo another test at T_0.

4- If you set up your batch conditions and no crystals grow after 5 days, it is probably safe to assume you are close to but not at the nucleation condition. Cool down your sample slowly to bring it into the nucleation condition (I used ramps of 1 degree in 5 hours, with intermediate 12 hours equilibration time before another ramp started - see figure below).

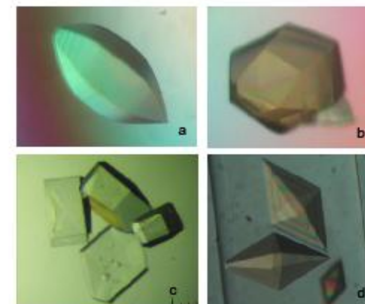


The starting temperature (in the example plot above 25°C) should be different for different rows in the TG40 System. I typically chose a set of 5 rows with temperatures T_0-2°C, T_0-1°C, T_0, T_0+1°C, T_0+2°C.

5- Observe the reservoirs as regularly as possible. As soon as you observe crystals, stop the cooling down process and bring the temperature back to T_0. I successfully used rates of 1 degree per hour.

6- Leave the system at T_0 for as long as you observe crystal growth. The best conditions found can directly be up-scaled to larger volumes following steps 4-6. I used up to 60 microlitres of sample + crystallization buffer in the wells of the TG40 System."

The following images show some examples of crystals grown at the ILL Deuteration Laboratory on the TG40 System using the described method.



The micrographs show a) and b) A-DNA crystals (10bps long), c) Z-DNA crystals (6bps long) and d) Thaumatin crystals. DNA crystals circa 1.5mm in length in the longest direction.

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TG40 System

Temperature controlled microplate for optimization of biological crystals.

For more information and sales contact us at: info@centeo.com Tel. 0044 (0)1389 750 939

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Thanks

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