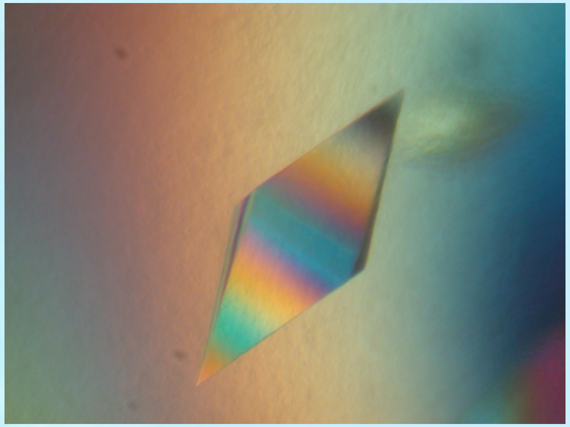
Crystal quality: myths and realities for a better protein X-ray crystallography



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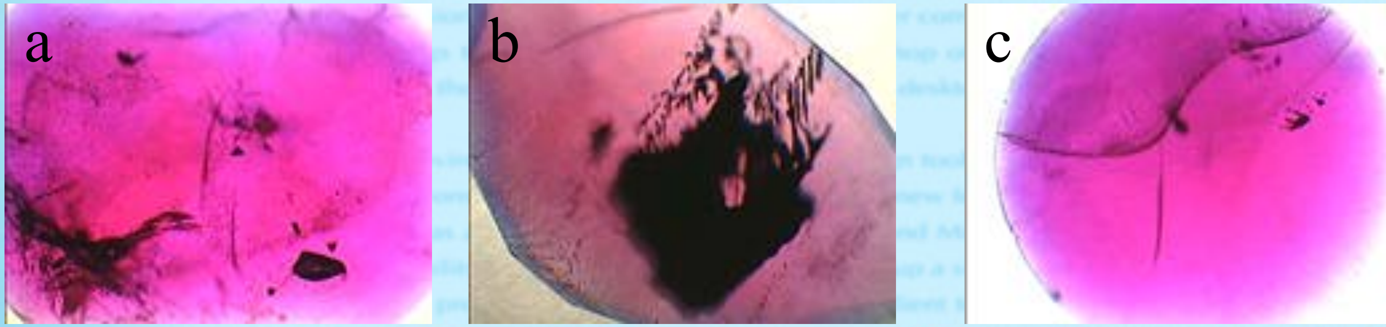
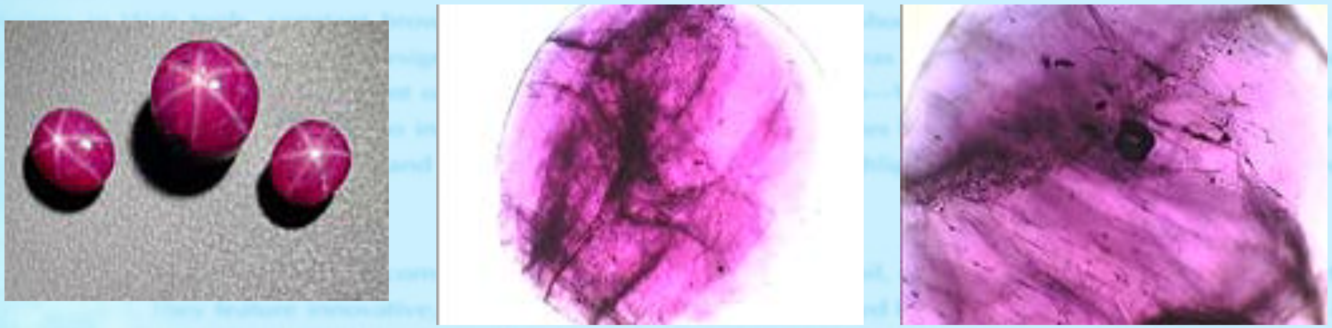
Outline of the talk

1. What does crystal quality mean?
2. Is really a beauty crystal a good crystal? From inorganic to biological macromolecular view point.
3. Crystal mosaicity and crystal defects
4. Methods to estimate the crystal quality
5. Post crystal treatments that can improve crystal diffraction
6. Myths and realities
7. Conclusions

In jewellery the crystal quality is relative for a pure natural crystals…the perfection is not fine!



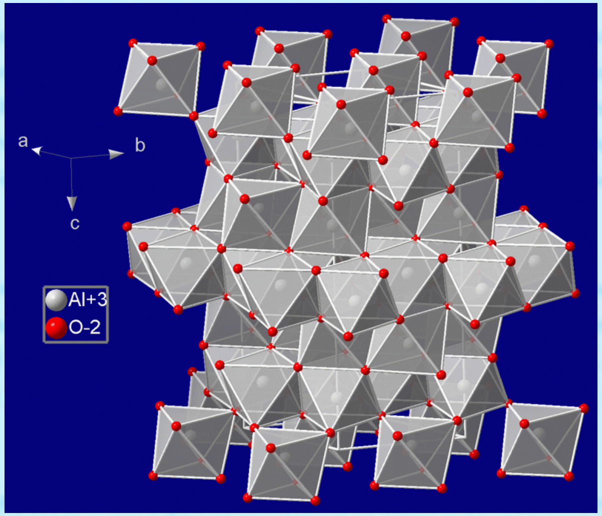
Synthetic Rubies: a) Chatham, b) Kashan, c) Ramaura



Natural Ruby, Thailand: see inclusions and defects!

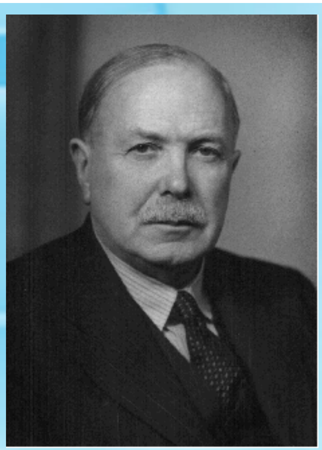
General	
Category	Mineral variety
Chemical formula	aluminium oxide with chromium, Al_2O_3:Cr
Identification	
Color	Red, may be brownish, purplish or pinkish
Crystal habit	Varies with locality. Terminated tabular hexagonal prisms.
Crystal system	Trigonal (Hexagonal Scalenohedral) Symbol (□3 2/m) Space Group: R□3c
Cleavage	No true cleavage
Fracture	Uneven or conchoidal
Mohs Scale hardness	9.0
Luster	Vitreous
Refractive index	nω=1.768 - 1.772 nε=1.760 - 1.763, Birefringence 0.008
Pleochroism	Orangey red, purplish red
Ultraviolet fluorescence	red under longwave
Streak	white
Specific gravity	4.0
Melting point	2044 °C
Solubility	none
Diaphaneity	transparent

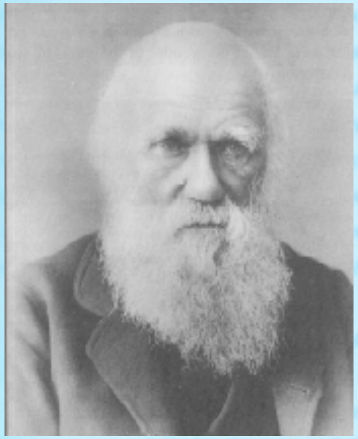




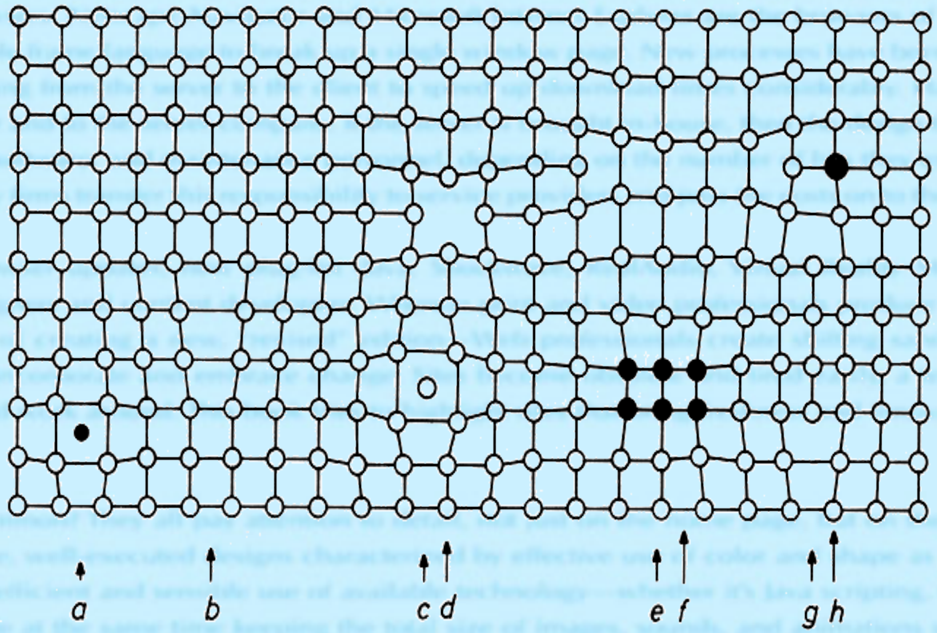
Crystal defects and more

In the 1922 C.G. Darwin proposed for the first time an explanation for the mosaicity of a crystal. In his proposal crystals are described as being composed of several blocks, mosaic blocks, with slightly different orientations and variations in unit-cell and lattice (Phil. Mag. 43 (1922) 800-829). In the late 1980s this concept was extended to the crystallographic studies of proteins.





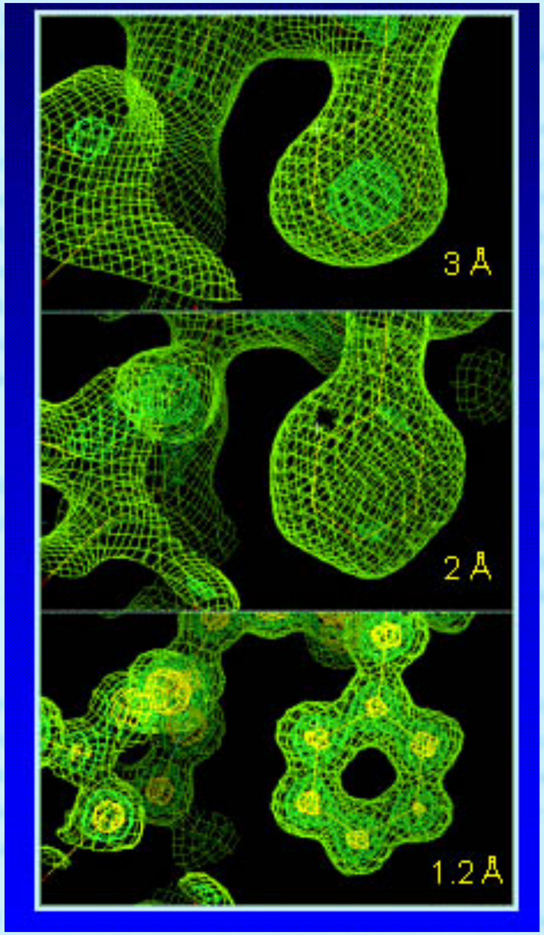
Charles Galton Darwin (1887-1962)
His two 1914 papers on diffraction of X-rays from perfect crystals became often cited classics.



a) Interstitial impurity atom, b) Edge dislocation, c) Self interstitial atom, d) Vacancy, e) Precipitate of impurity atoms, f) Vacancy type dislocation loop, g) Interstitial type dislocation loop, h) Substitutional impurity atom

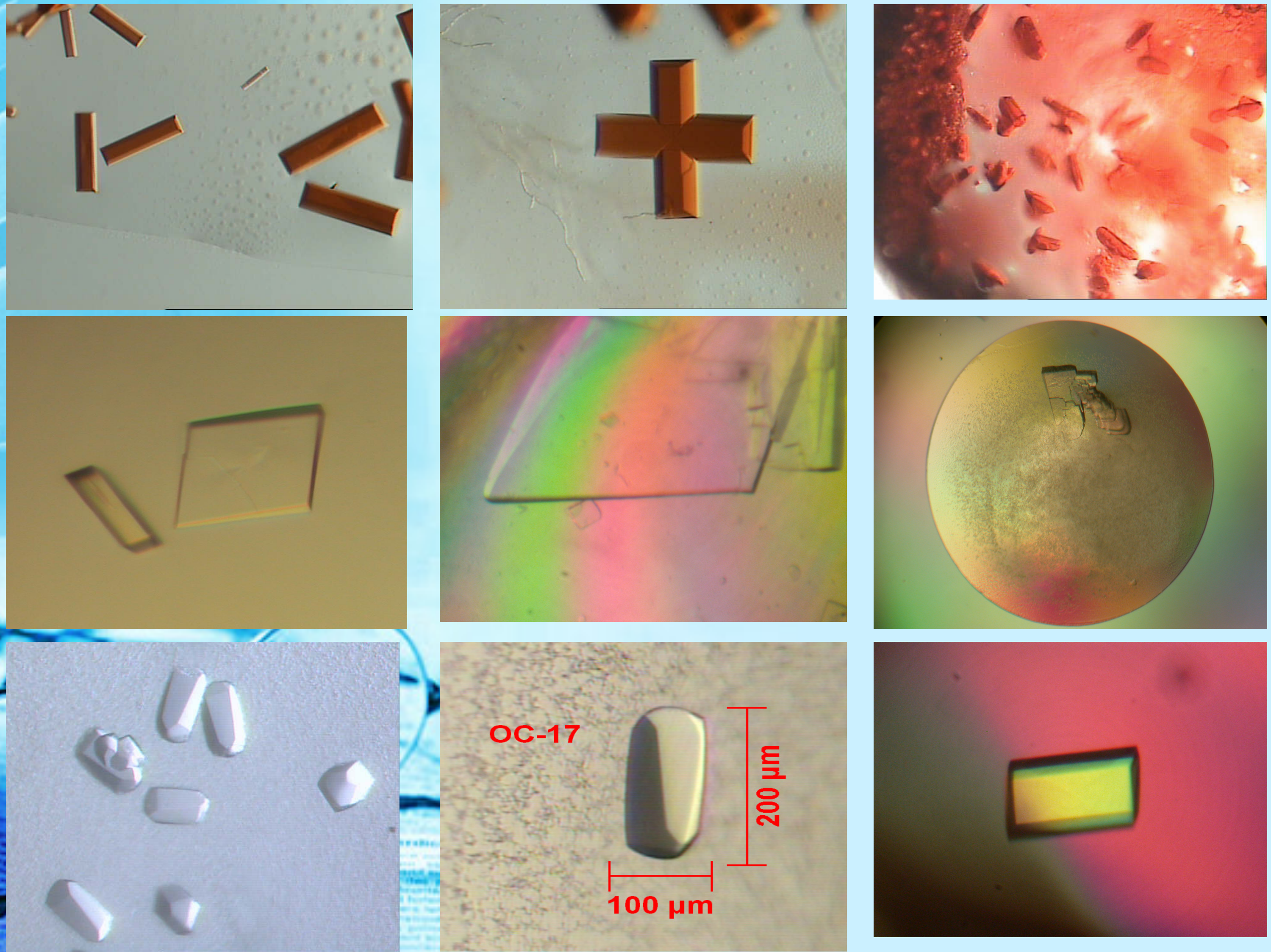
BUT FOR PROTEINS, WHAT DOES CRYSTAL QUALITY MEAN?

High quality single crystals

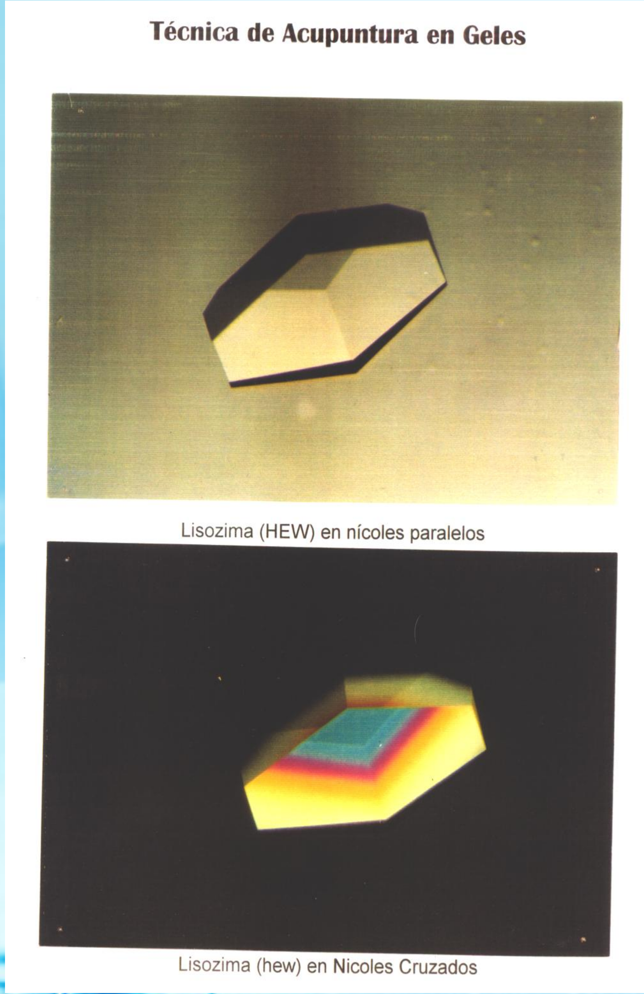


Ultrapure protein samples

PROTEIN CRYSTALS:
the good, the bad and the ugly in crystal growth



A quick birth's eye: using the optical properties



- The **refractive index** is the ratio of the velocity of light in a vacuum to that in the material under investigation.
- When crystals show differently coloured when viewed in different directions, and are described as **pleochroics**. They show interesting effects in plane-polarized light.
- Birrefringence is the anisotropy of the refractive index of a protein crystals.
- Birrefringence implies that there is double refraction as lights passes through a crystal, and light is split into two component (the ordinary and extraordinary ray) that travel with different velocities and have different properties (those of the ordinary ray being normal).

Examination of a crystal under crossed Nicols prisms can also provide information on crystal quality. For example, sometimes the components of a twinned crystal extinguishing plane-polarized light independently.

Optical properties:

- If birefringence is measured as the difference between the refractive indices for the ordinary and extraordinary rays:
- a) a **crystal is positively birefringent** if $\eta_E > \eta_O$ (it can be assumed to contain rod-like bodies lying parallel to the single vibration).
- b) a **crystal is negatively birefringent** if $\eta_O > \eta_E$ (it can be assumed to contain plate-like bodies lying perpendicular to the single vibration direction of least refractive index (η_E).

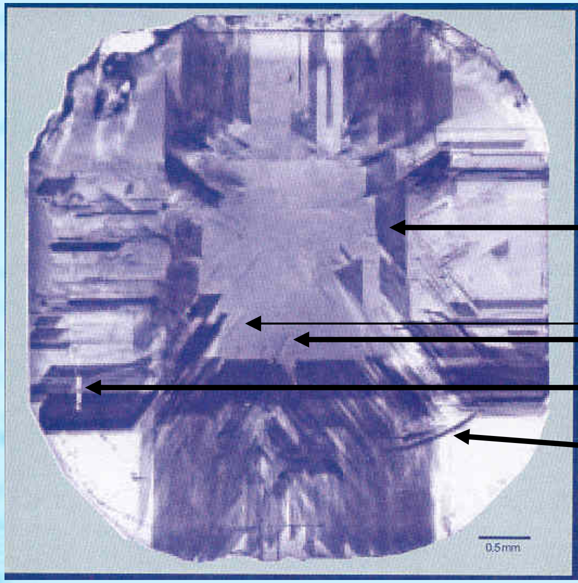
- Some crystals are found to have one, and only one, direction (the optic axis) along which there is no double refraction (crystals with this property are called **uniaxial**). They have two principal refractive axes and are: TETRAGONAL, HEXAGONAL or RHOMBOHEDRAL. Other crystals are found to have two directions along which there is no double refraction (two optic axes), and these are called **biaxial**. Such crystals are either: ORTHORHOMBIC MONOCLINIC or TRICLINIC and have three principal refractive indices.

Crystal quality criteria

1. X-ray Topography experiments
2. Crystal growth mechanisms
3. I /σ (I) (this is related to the internal crystal packing)
4. Structure validation
5. B Factor analysis and mosaicity

1. X-ray topography applied to diamond characterization

X-ray topography is a non-destructive characterization technique, by means of X-ray diffraction, the micrometer-sized to centimetre-sized defect microstructure of crystals.



SF: Stacking faults

D: individual dislocations

I: Inclusions

SD: Surface damage

1. X-RAY TOPOGRAPHY ANALYSIS OF A PROTEIN CRYSTAL

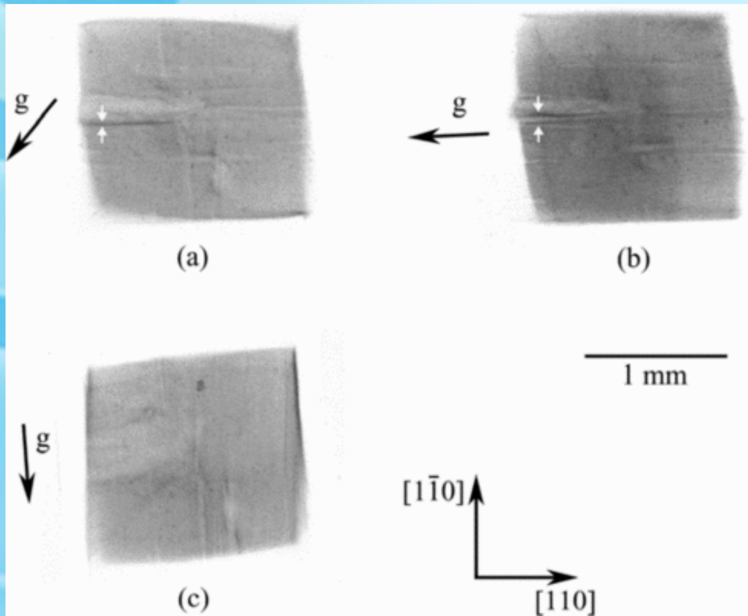


Figure 1. Laue topographs of (a) 080, (b) 004, 040 and c) 120, 102 0 reflections.

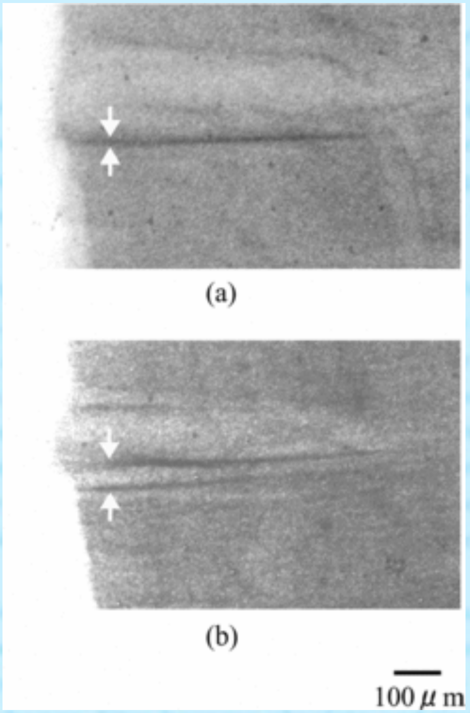


Figure 2. Enlarged topographs showing (a) single contrast and (b) double contrast of the dislocation image, respectively, observed in (a) 080 and (b) 004, 004 0 from Fig 1

Koizumi et al, Phil Mag. 85 (2005) 3709-3717.

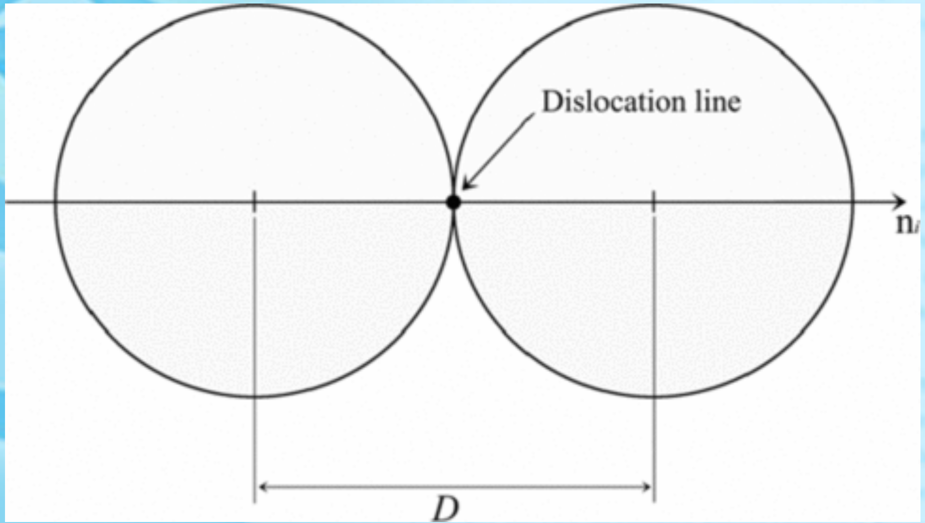


Figure 3. Schematic contour of equal absolute value of effective misorientation |δ| for a screw dislocation running normal to the reflecting plane (paper) in elastically isotropic crystal, where n_i is the vector normal to the incident plane on the reflecting plane. The contour consists of two equal curves touching at the dislocation.

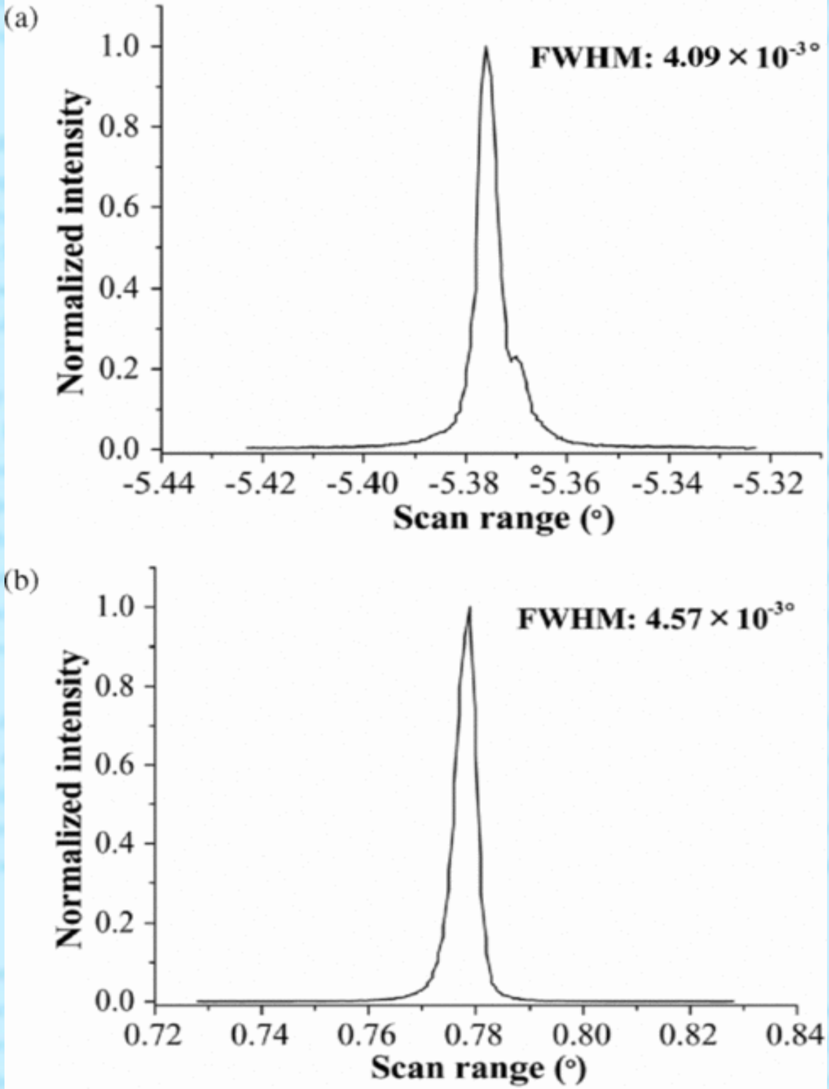
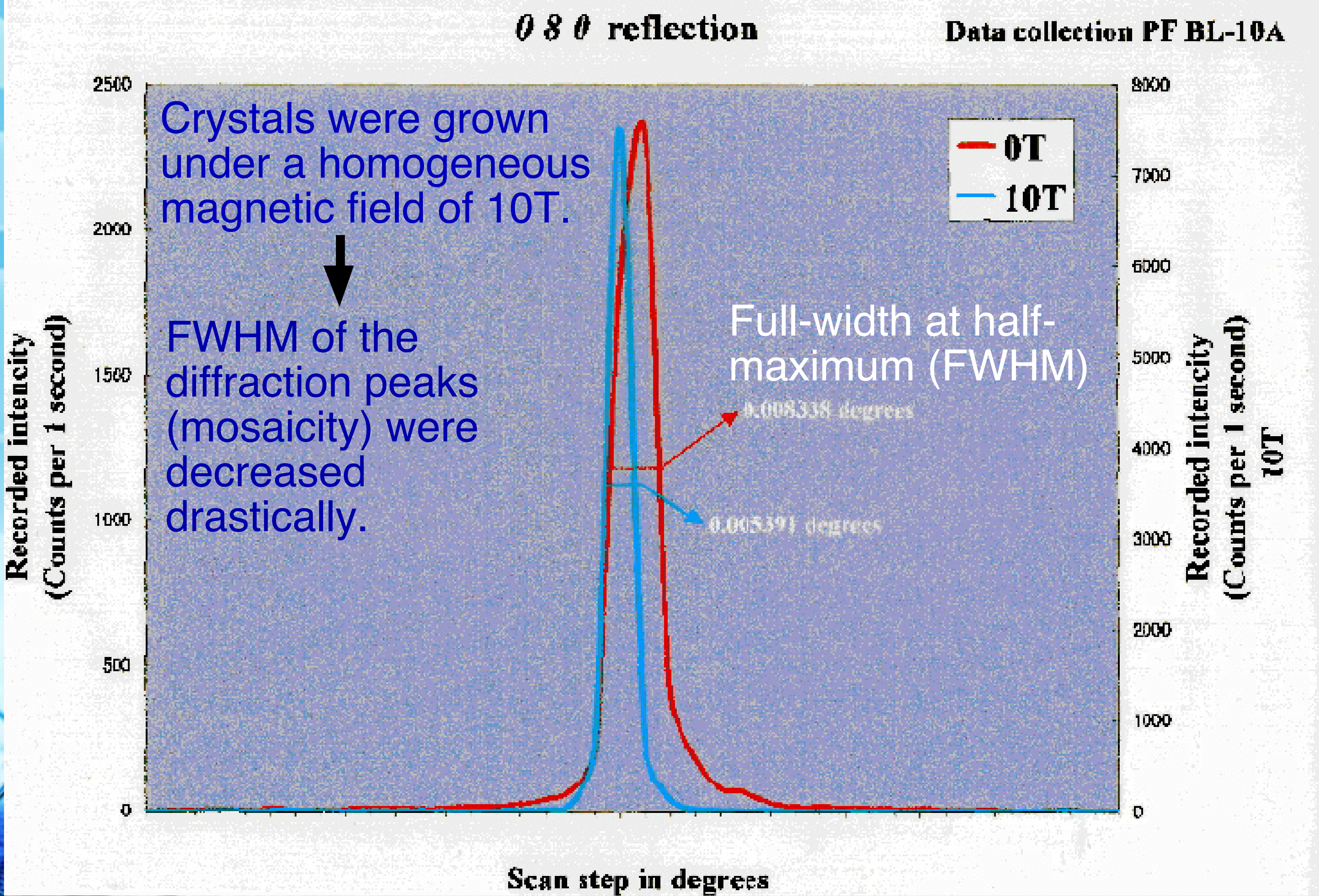


Figure 4. Rocking curve profiles of (a) 0 8 0 and (b) 4 4 0 reflections in tetragonal HEW lysozyme crystal.

Koizumi et al, Phil Mag. 85 (2005) 3709-3717.

Enhancement in the perfection of orthorhombic lysozyme crystals by a magnetic field



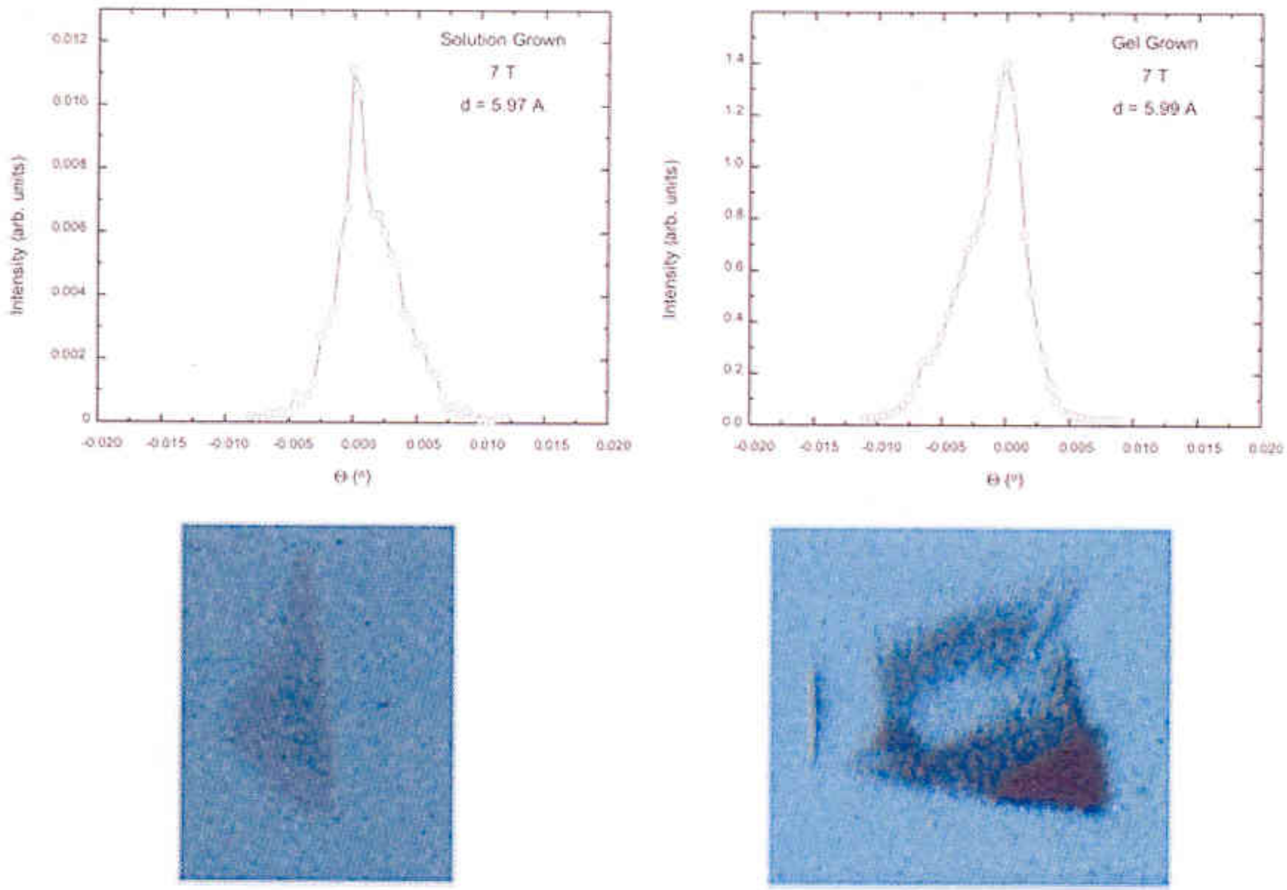
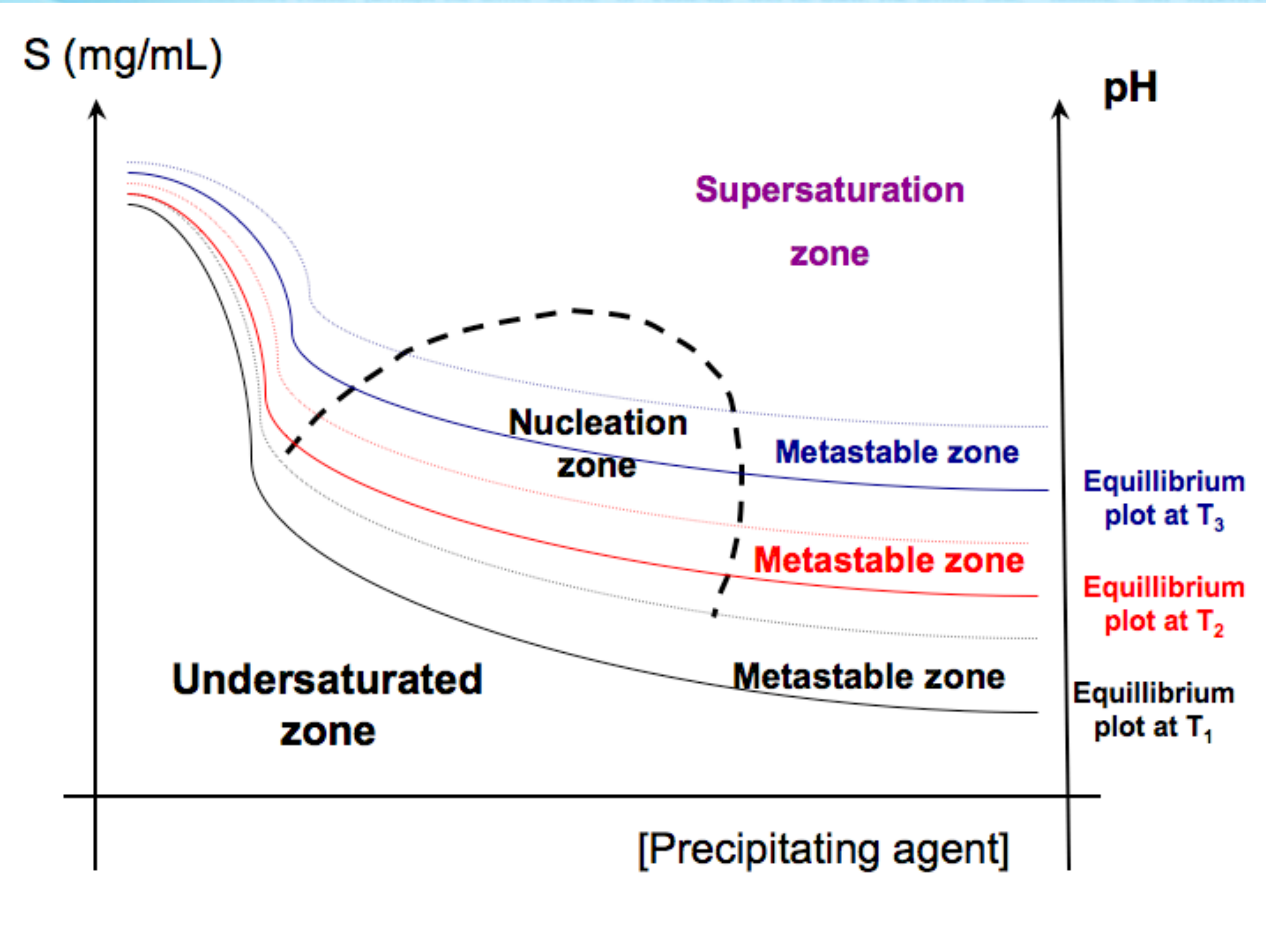


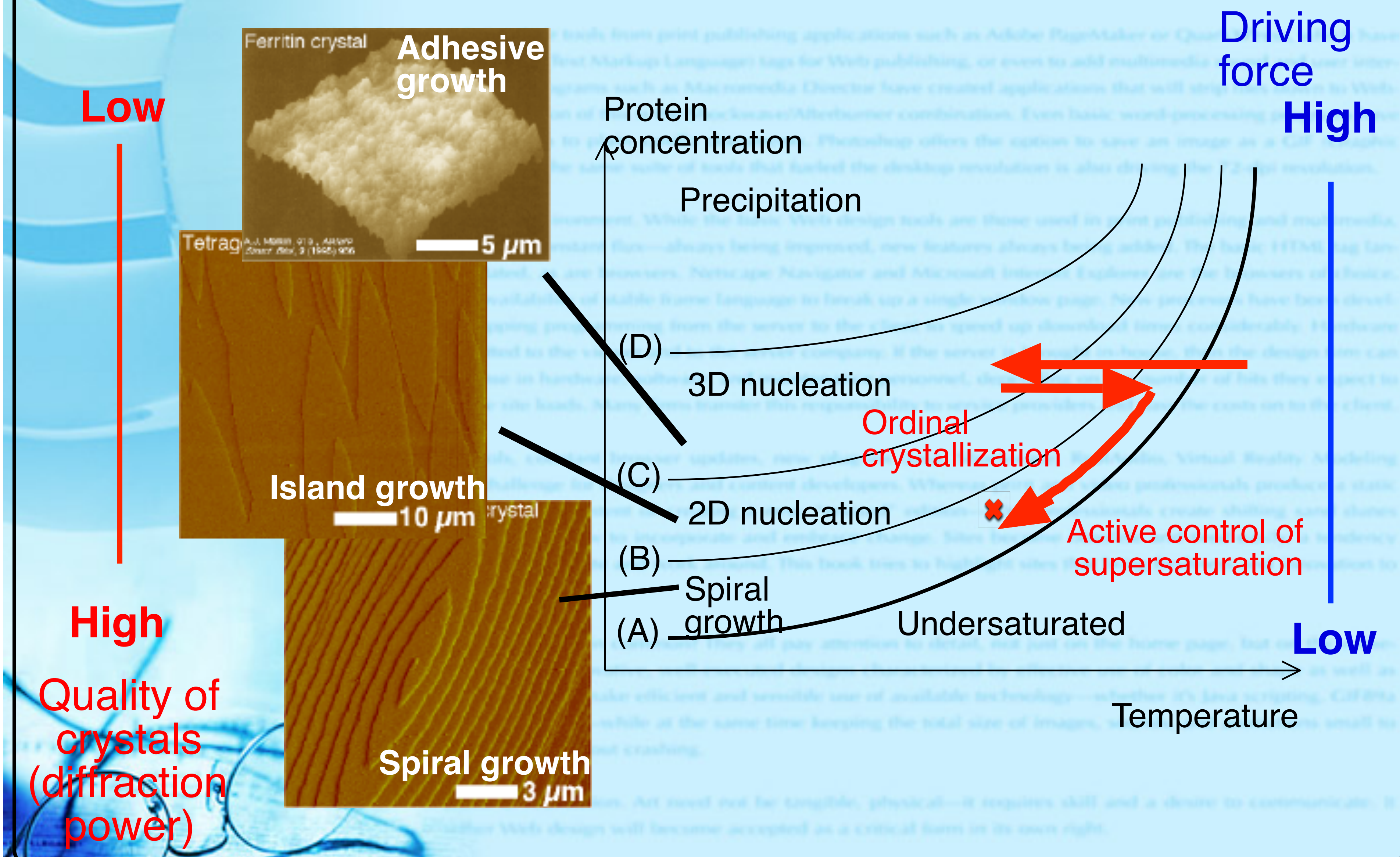
Figure 5. High-resolution imaging results, rocking curve, and corresponding topographic image. Left, solution; right, gel-grown crystal. In the topographic image for the solution-grown crystal two regions can be distinguished. This finding is further supported by the rocking curve measurement, for which two Gaussian curves could be fitted, indicating two regions diffracting at slightly different Bragg angles. (In color in *Annals* online.)

Moreno et al., Annals of the New York Acad. Sci. 1161 (2009) 429-436

2. Crystal Growth Mechanisms


S (mg/mL)
pH
Supersaturation
zone
Nucleation
zone
Metastable zone
Equillibrium
plot at T3
Metastable zone
Equillibrium
plot at T2
Undersaturated
zone
Metastable zone
Equillibrium
plot at T1
[Precipitating agent]

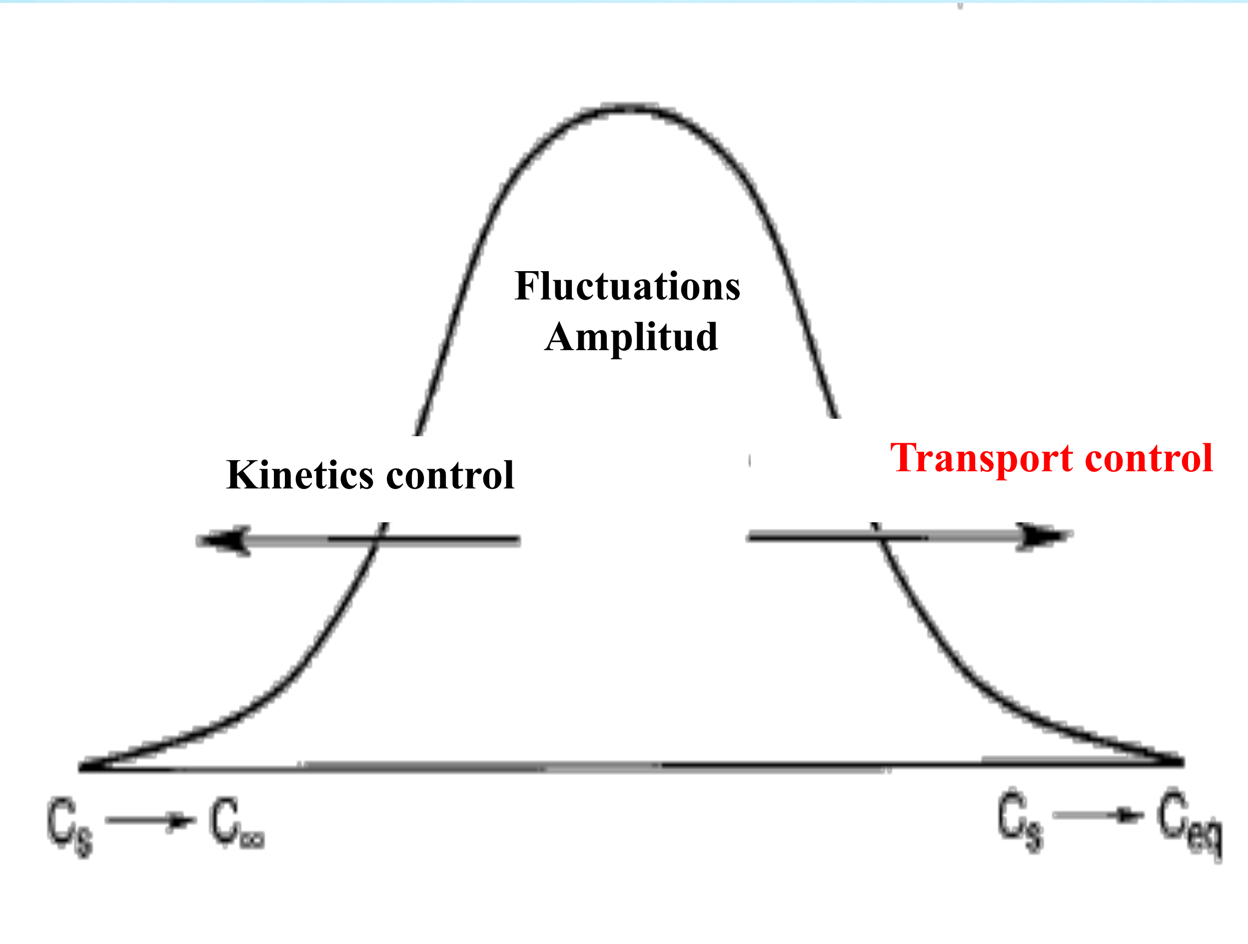
Active control of supersaturation



Obtaining high quality single crystals

This is possible only when the convective transport is eliminated or at least minimized:

1. Growth in gels
2. Growth in capillary tubes
3. Growth in microgravity conditions.



Allègre C.J., Provost, A. And Jaupart C. (1981) *Nature* 294, 223

Myth: The majority of crystals grown in capillary tubes will diffract much better in capillary tubes of diameter less than 0.5mm.

Reality: The problem is to detach the crystals grown in the capillary tubes, if you do not have the expertise, it could be worst, what can you do?

Application of a Femtosecond Laser Ablation for Detaching Grown Protein Crystals from Glass Capillary Tubes

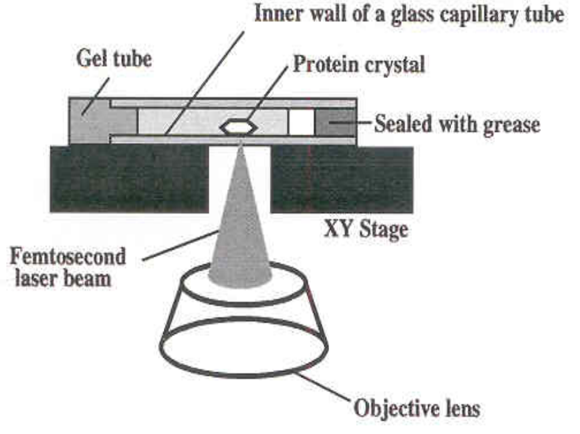


FIG. 1. Schematic illustration of experimental setup for detaching protein crystals from glass capillary tube.

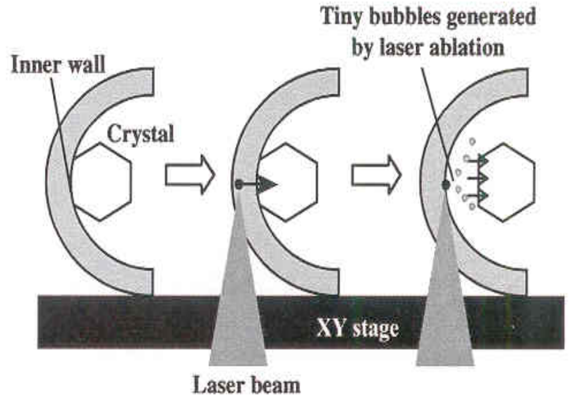


FIG. 2. Schematic illustration of detaching technique.

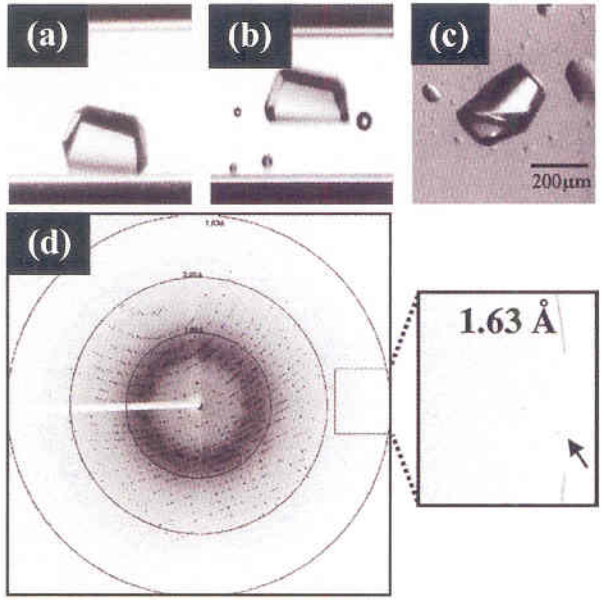
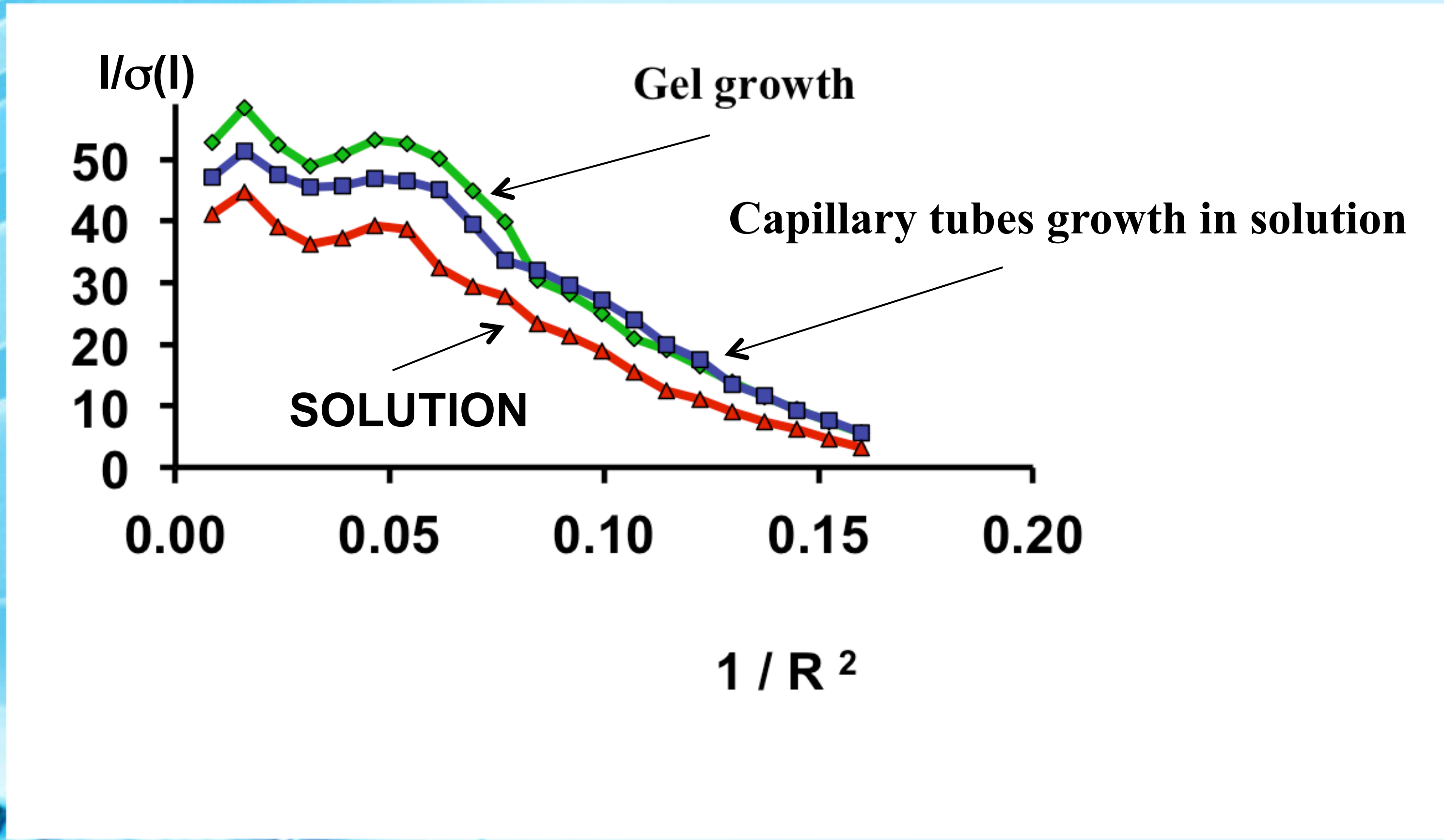


FIG. 3. Photographs of processed HEWL crystal. (a) Before laser irradiation. (b) After laser irradiation. (c) After being packed in another glass capillary tube. (d) XRD pattern of harvested crystal.

Kashii et al. Journal of Bioscience and Bioengineering 102(2006) 372-374.

3. Plot I / I (σ) versus resolution



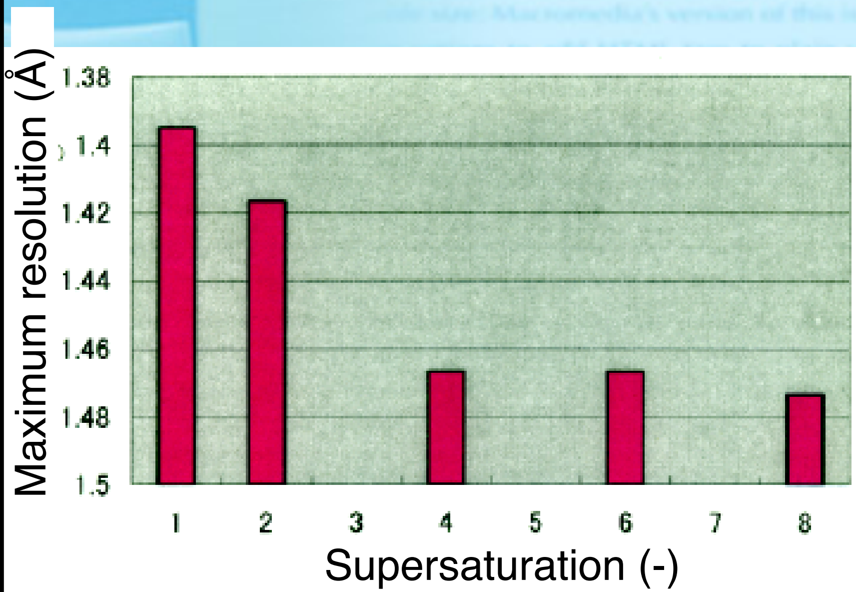
The higher the values of the ratio I/σ(I) the better the crystal quality is. A. Moreno et al. Acta Cryst. D61 (2005) 789-792

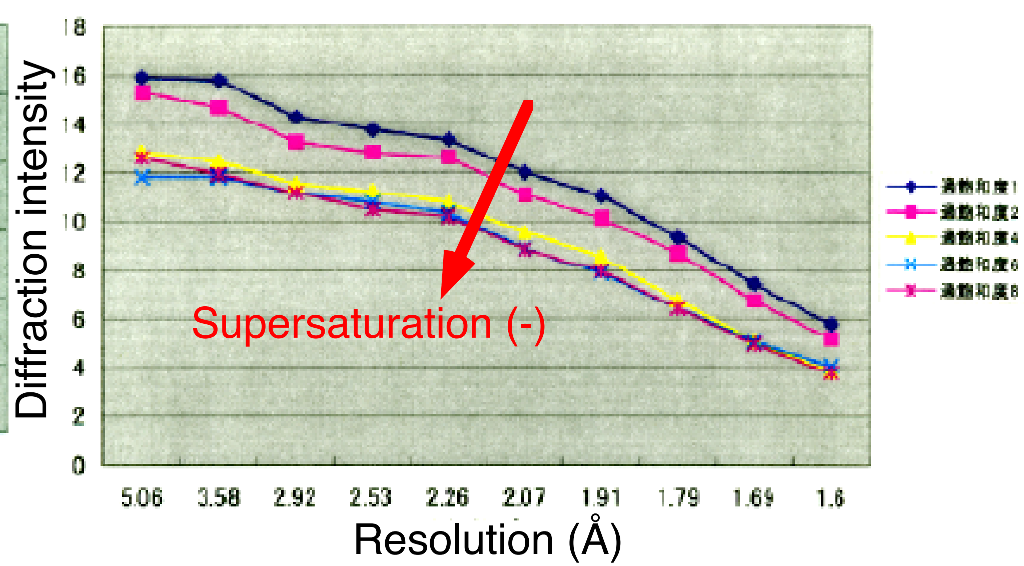
What is it necessary to know in terms of knowledge?

Control mechanism	Concepts
Chemical	Solubility Supersaturation Nucleation Growth

Effect of driving force on the quality of protein crystals

I. Yoshizaki et al., Acta Cryst. D57 (2001) 1621-1629.





Supersaturation ↘ Maximum resolution ↗ Supersaturation ↘ Diffraction intensity ↗

As a supersaturation decreases, the quality of protein crystals (maximum resolution & diffraction intensity) increases.

4. Structure validation

1. Merging R values
2. Completeness
3. Redundancy
4. Signal strength
5. Resolution
6. Unit-cell parameters
7. Symmetry
8. Model Quality, coordinates
9. Geometry and stereochemistry
10. Torsion angles
11. C^{α} -only models
12. Contacts and envoronments
13. Noncrystallographic symmetry
14. Solvent molecules
15. Miscellaneus
16. Model quality, temperature factors
17. Model versus experimental data
18. Real space fits
19. Coordinate error estimates
20. Difference density quality
21. Accountancy

B factor and mosaicity as indicators of crystal quality of proteins.

$$B_{\text{factor}} = ADP_i = 8\pi^2 < u_i^2 >$$

ADP: Atomic Displacement Parameter.
$<u_i^2>$ is the mean square atomic displacement of the atomic vibration of atom i.

Mosaicity is the width of the distribution of misorientation angles of all the units cells in a crystal, usually described in terms of macroscopic domains of the crystal. The measured mosaicity is a convolution of crystal and experimental properties. Typically, unless special conditions are set up, the crystal mosaicity is masked by geometric and spectral parameters of the beam.

Reference: Macromolecular Crystallization and Crystal Perfection. Naomi E. Chayen, John R. Helliwell, Edward H. Snell. Oxford University Press 2012.

$$B_{\text{factor}} = ADP_i = 8\pi^2 < u_i^2 > \tag{1.3}$$

where $< u_i^2 >$ is the mean square atomic displacement of the atomic vibration for atom i. The overall temperature factor for the crystal, averaged over the whole structure, can be calculated from a Wilson plot (1942) where E_{obs} the observed intensity of the reflection, is plotted against $(\sin\theta/\lambda)^2$. The $<B_{\text{factor}}>$ is extracted from the slope of this plot as:

$$E_{\text{obs}}(h,k,l) = E_{(h,k,l)}\mathrm{e}^{(-2<B_{\text{factor}}>\sin^2\theta/\lambda^2)} \tag{1.4}$$

The Wilson plot also provides a scale factor, where it crosses the vertical axis, allowing intensities to be put on an absolute scale.

The atomic-displacement parameter is a measure of how much dynamic disorder of an atom is within the crystal lattice on the molecular level. X-rays interact with the electron cloud, which depends on the atomic position. Each atom vibrates around an equilibrium position so the nuclear position and electron charge cloud both move.

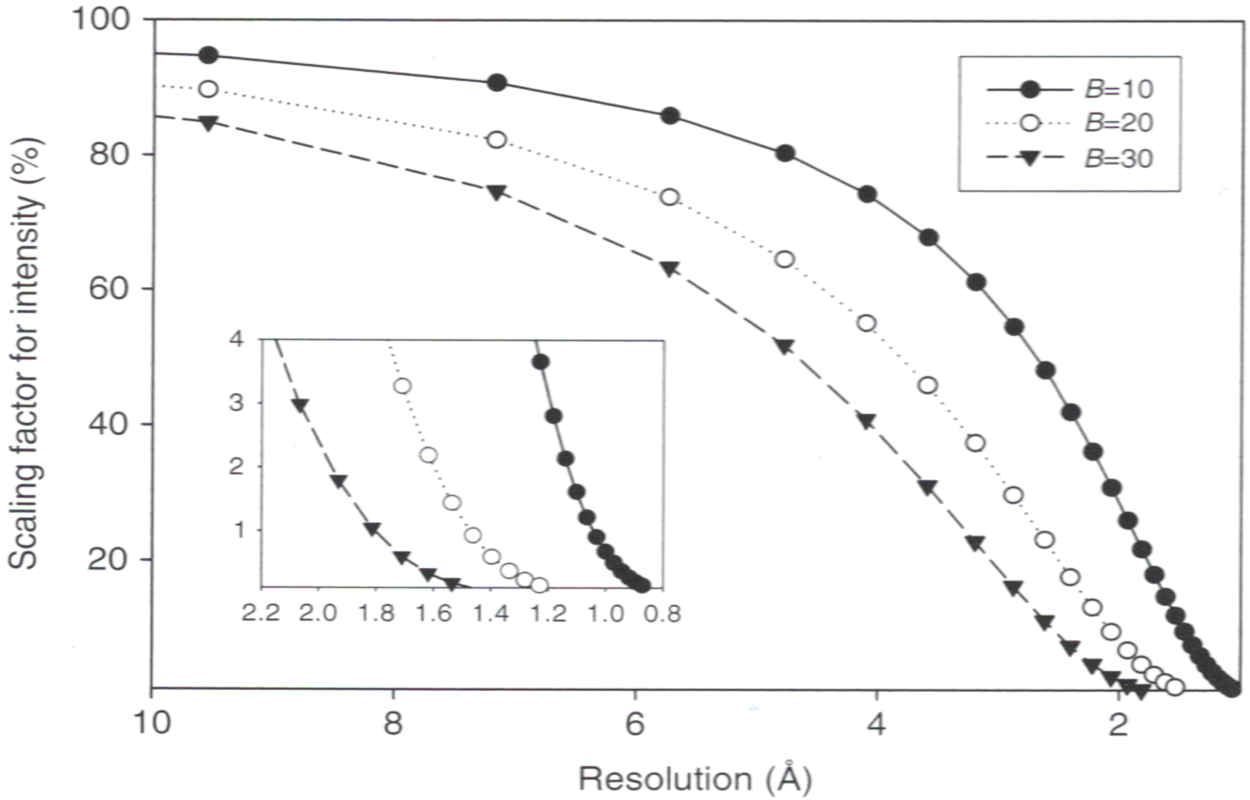


Fig. 1.3. Graph of scaling factor for intensity with resolution (eqn (1.5)) showing that a decrease in the <B_{factor}> causes a significant increase in the resolution. This is a simplified example neglecting crystal path length absorption differences and Bragg reflection signal-to-noise smearing at higher resolutions.

Note: the lower the value of B the higher the crystal quality is.

R factor and crystal quality

R factor is the reliability factor, which is a measure of the agreement between the amplitudes of the structure-factor amplitudes calculated from a crystallographic model and those from the original X-ray diffraction data. The R factor is calculated after each cycle of least-squares structures refinement to asses process. The final R factor is one measure of final model structure quality. A related, very important, parameter is R_{free} reliability factor calculated from a subset of reflections, and give an independent measure of progress towards divergence to a final model.

Note: reflections means spots in data collection.

MOSAIC SPREAD and MOSAICITY

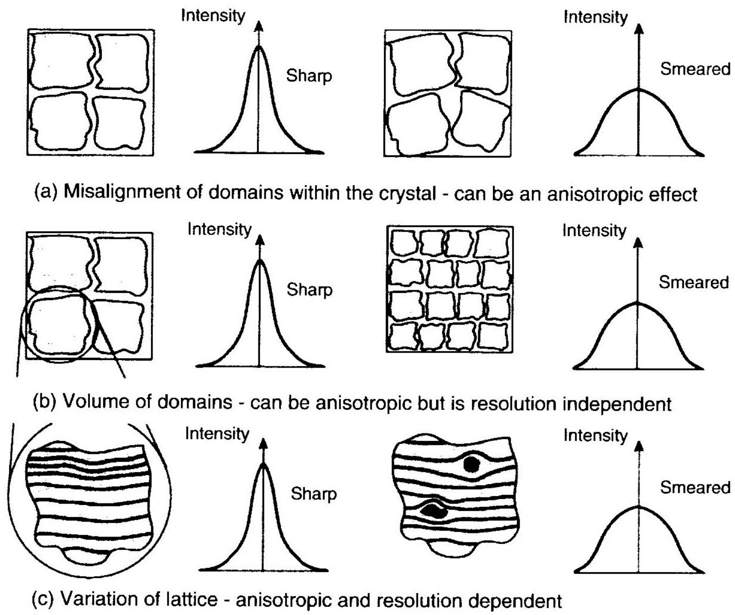


Fig. 1.4. Long-range disorder and the resulting effect on the diffraction profile.

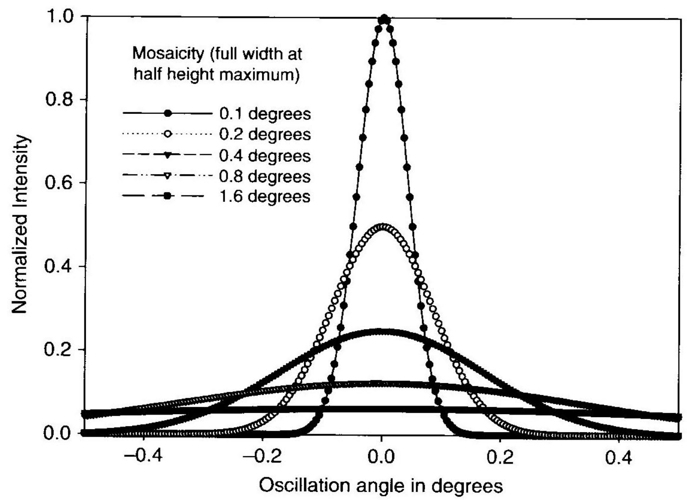
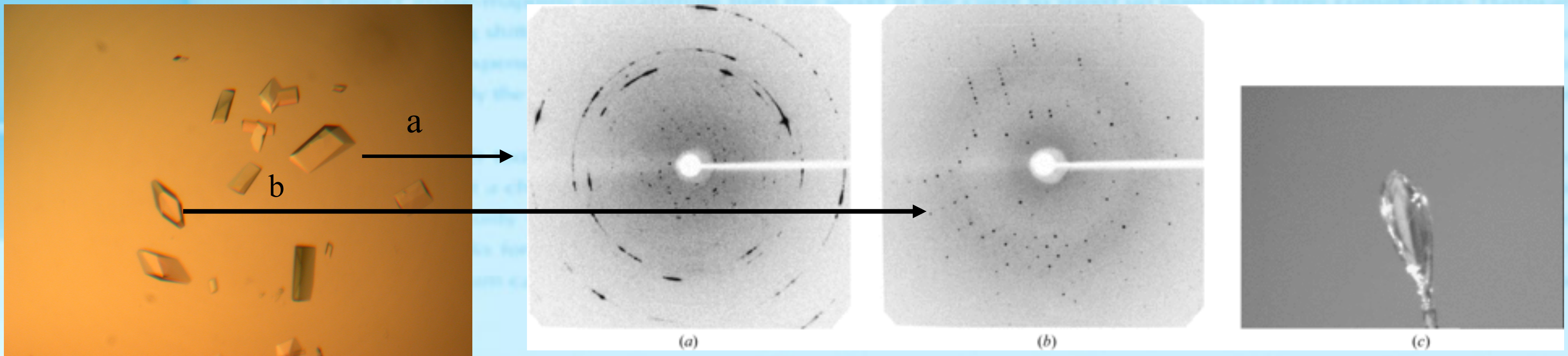


Fig. 1.5. Graph showing a Gaussian reflection profile for identical integrated intensities but differing full width at half-height maxima to illustrate how an increase in mosaicity causes a corresponding decrease in the reflection peak intensity signal.

POST-CRYSTAL TREATMENTS THAT CAN IMPROVE CRYSTAL DIFFRACTION

Despite technical and methodological advances, availability of new methods, obtaining high-quality single crystals is sometimes a major problem.



c) Crystalshowing the presence of ice formation in the loop.

Methods to Improve Crystal Diffraction

1. **Different crystallisation conditions: new crystal forms.**
2. **New construct: crystallise a different form of the protein**
3. **Grow larger crystals**
4. **Post-crystallisation soaking**
5. **Crystal annealing**
6. **Crystal Dehydration**

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3. To grow larger crystals...

◊ Increase the size of the crystal growth cell:

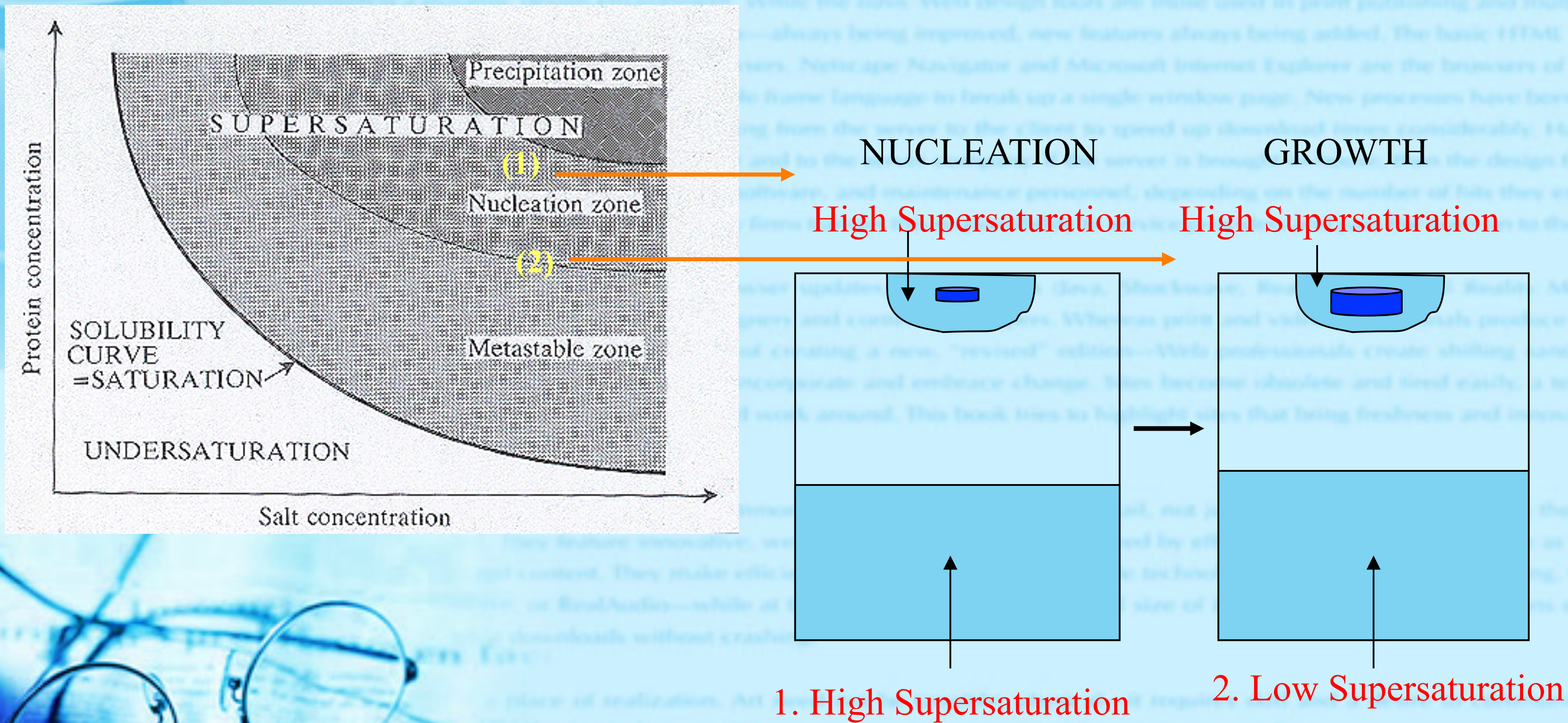
1. Use of gels
2. Use of capillary tubes
3. Larger droplets (reduce the supersaturation value to a value around 3 (supersaturation= C/C_e)

◊ Seeding (take the induction time).

1. From your experiments, please measure the induction time when your crystals appear for the first time.
2. Pre-equilibrate droplet and seed the tiny crystals (for this the induction time for nucleation is very important to take into account).

◊ Decoupling nucleation and growth

1. Incubate coverslip holding the hanging drop at conditions normally giving many small crystals
2. Transfer coverslip over reservoirs at concentrations that normally yield clear droplets.



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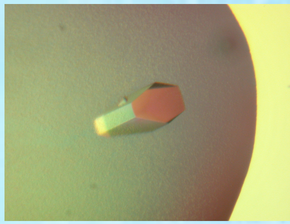
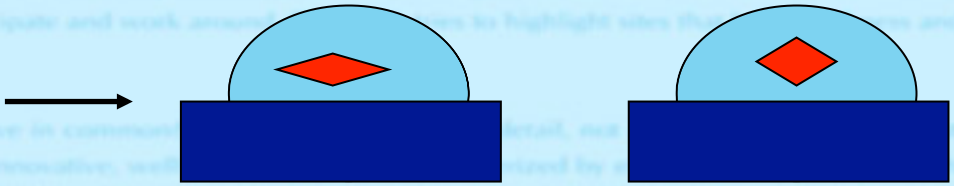
Post-crystallisation soaking…

◊ Higher ionic strength solution

1. Increase the precipitating agent concentration stepwise. The crystals should be incubated for a few weeks to allow the crystals not to shrink after soaking.
2. Shorter incubation time with salts and cryo-protectors (ethylene glycol).





50 µl mother liquor + precipitating agent (high supersaturation)

Cryo-solution at increasing concentrations 5,10,15,20,25,and 30%

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5. Crystal annealing

FLASH COOLING

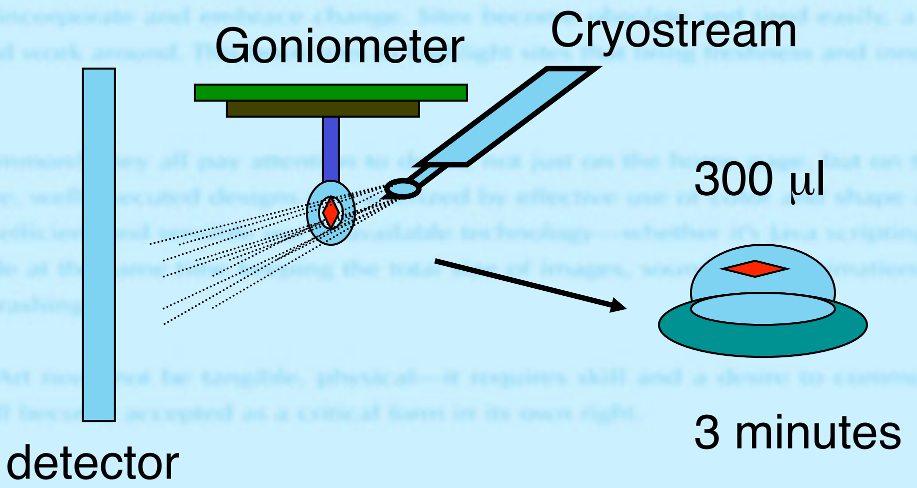
Reduces radiation damage (mosaic broadening).
Increases crystal lifetime (resolution degradation).

CRYSTAL ANNEALING

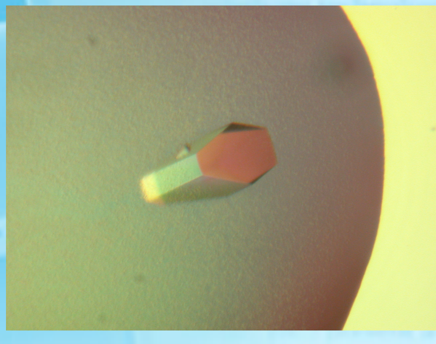
Diffraction properties of flash-cooled crystals can be improved by warning and then cooling a second time.

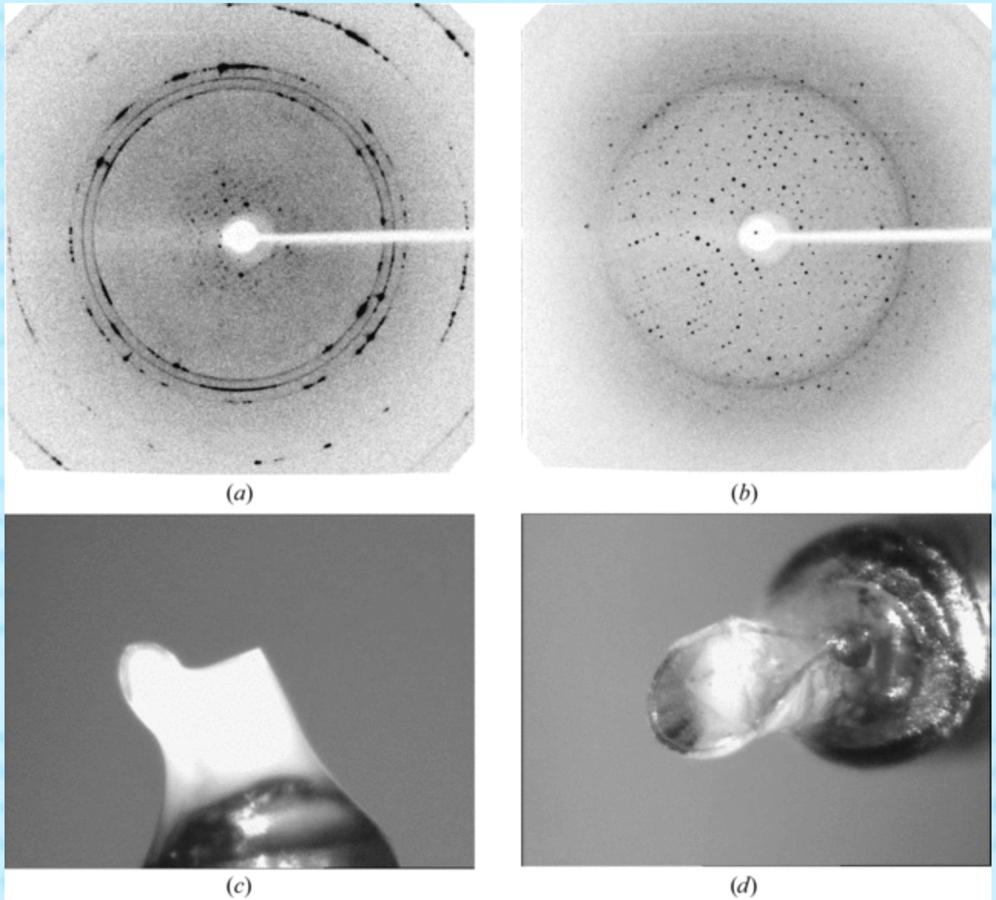
PROTOCOLS.

1. Remove crystal from cold gas stream, place it in cryoprotectant (300 μl: 3 minutes)
2. Re-cool the crystal.

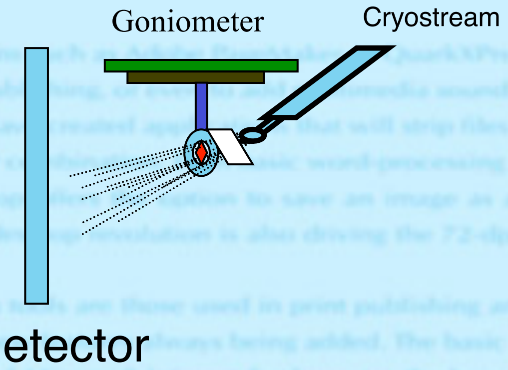


MACROMOLECULAR CRYSTAL ANNEALING





PROTOCOLS



3. Flash annealing:

Block the cold stream (2 seconds)

Recool the crystal (3X)

4. Annealing on the loop:

Block the cold stream (until crystal becomes clear)

Re-cool the crystal (1X)

MYTHS AND REALITIES:

a. Macromolecular crystal annealing: Successful in the majority of times

b. Flash-annealing: Inadequate for most crystals

c. Annealing on the loop: Low solvent content and small.

Annealing examples

Protein	Precipitant	Space Group	Resolution (Å) Before-after	Mosaicity Before-after	Annealing Method	Ref.
Nucleosome Core Part.	$MnCl_2$	$P2_12_12_1$	N.R - 3.1	0.82 - 0.34	MCA	Harp Et al., 1998
Histone	$(NH_3)_2SO_4$	$P3_221$	N.R. - 3.0	0.34 - 0.22	MCA	Harp Et al., 1998
PPase	NaCl	R32	1.8 - 1.2	0.70 - 0.30	MCA (Salt↑)	Samigyna et al, 2000
GlcNAc6P	NaH_2PO_3	$P2_12_12_1$	Med -2.0		MCA	Ferreira et al., 2000
DmpFG	PEG 8k	$P2_12_12_1$	> 6 -2.1		MCA	Manjasety et al., 2001
Glycerol Kinase	PEG 4k/ PEG 200	C2	3.7 - 2.8	> 2 ---1	FA	Yeh & Hol 1998
AR	PEG 4k	$P2_12_12_1$	6 - 2.3		FA	Guan et al., 2001
NiR	PEG-MME550	$P6_3$	2.5 - 1.0	1.5 - 0.30	FA	Elis et al., 2002

Ppase: Pyrophosphatase; **GlcNAc6P**: N-Acetylglucosamine 6 Phosphate; **DmpFG**: 4-hydroxy-2-ketovalerate aldose-aldehyde dehydrogenase; **AR**: Arsenate reductase; **NiR**: Nitrate reductase.

Table adapted from Dr. Begoña Heras, The University of Queensland, Australia

Methods to Improve Crystal Diffraction

1. Different crystallisation conditions: new crystal forms.
2. New construct: crystallise a different form of the protein
3. Grow larger crystals
4. Post-crystallisation soaking
5. Crystal annealing
6. Crystal Dehydration

Crystal Dehydration

In general large solvent content and loose packing of molecules result in low-resolution diffraction

Crystal Dehydration

Reduction of the solvent content by putting the crystal in dehydrating solutions such as:

(1) Increased concentration of precipitant

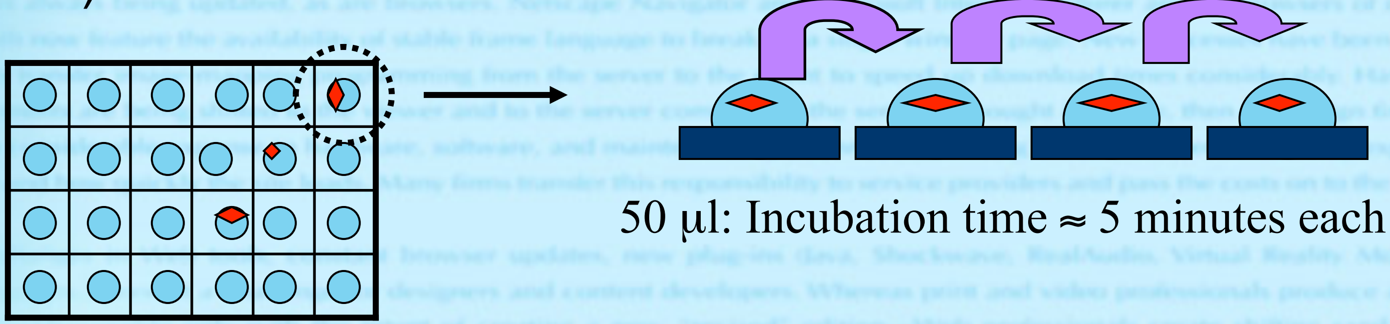
(2) Mother liquor + cryo-protective agents (PEG400, PEG6000, Glycerol, Ethylene glycol, MPD).

REALITY:

Crystal dehydration alone or combined with other treatments has produced dramatic improvements in resolution.

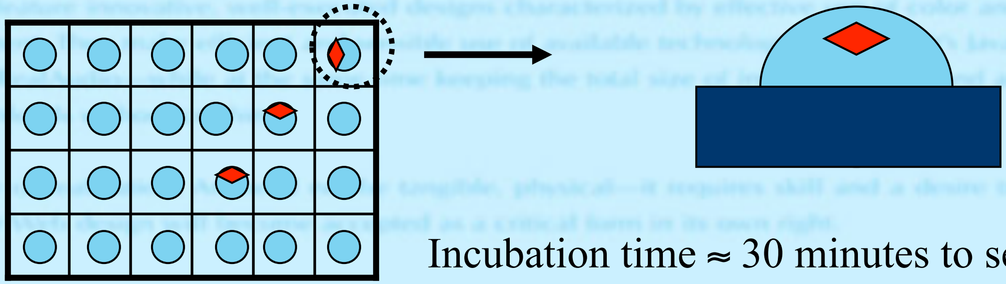
Crystal Dehydration: Protocols

Protocol 1: Serial transfer to increasing concentration of precipitant, incubation time of 5 minutes (Schink & Jurnak, 1994).



50 μl: Incubation time ≈ 5 minutes each

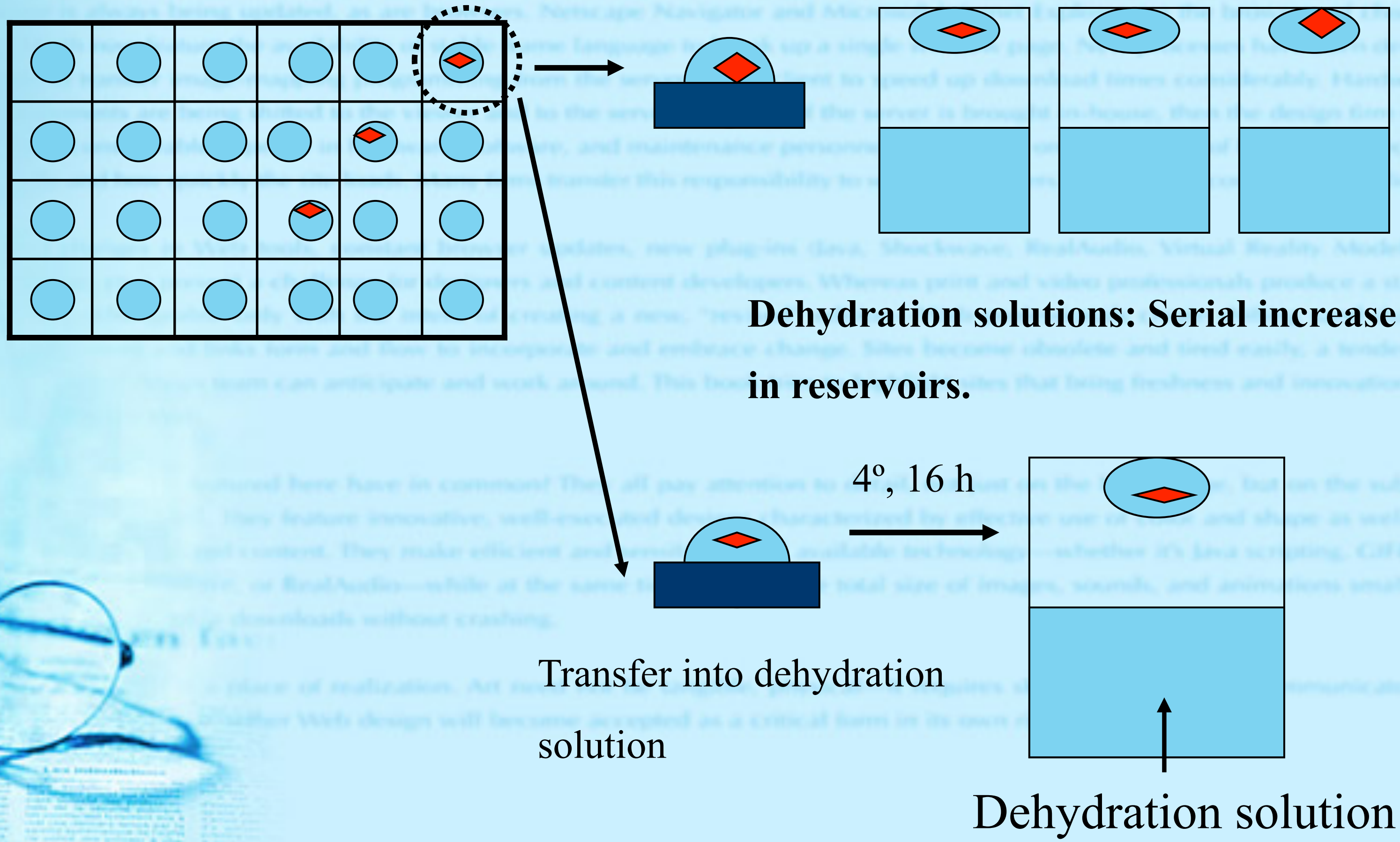
Protocol 2. Transfer to dehydrating solution and air dehydration (Haebel et al., 2001)



Incubation time ≈ 30 minutes to several hours

Crystal Dehydration: Protocols

Protocol 3. Transfer to dehydrating solution containing wells (Pang et al., 2002).



Dehydration examples:

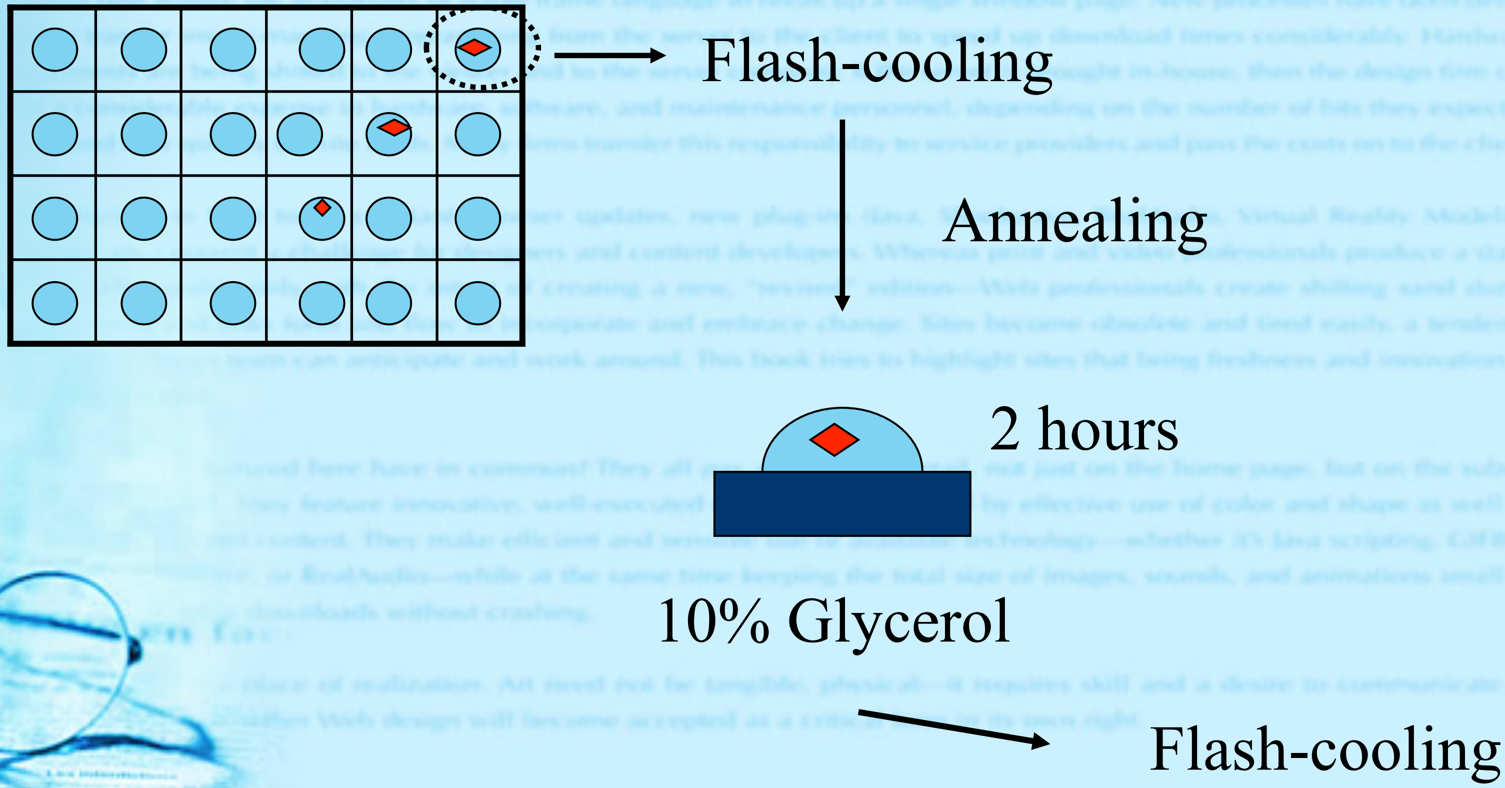
Protein	Precipitant	Dehydrating Agent	Resolution before-after (in Angstroms)	Solvent before-after (%)	Dehydration method
DsbG	20% PEG 4k	30% PEG 4k	>10 - 2.0	≈ 90-53	Protocol 3
EF-Tu-Ts	20% PEG 4k	28%-40% var PEG	4.0 - 2.7	61 - 55	Protocol 1
NF-kBP52:DNA	4-6% PEG 4k	Ppt + 30% PEG 400	3.5 - 2.0	52 - 49	Protocol 1
HIV-RT:inhib	6% PEG 3.4k	46% PEG 3.4k	3.7-2.2	56 - 48	Protocol 2
HCMV protease	16% PEG 4k	30% PEG 4k + $(NH_3)_2SO_4$	3.0 - 2.5	58 -56	Protocol 2
FAD-indep ALS	6-8% PEG 8k, 6-8% EG8k	Ppt + 30% PEG 6k	2.9 - 2.6	Na -52	Protocol 2
DsbC-DsbDα	25% MPEG 5k, 5% glyc	40% MPEG 5k, 10% gly	7.0 - 3.8 (2.3 synchrotron)	55 -41	Protocol 1

EF-Tu-Ts: Guaninenucleatide-exchange elongation factor complex; **NF-kBP52: DNA**: Transcription factor-DNA Complex; **HIV-RT: HIV-Reverse** transcriptase-inhibitor complex; **HCMV protease**: Human cytomegalovirus Protease; **FAD-indep ALS**: Flavin adenine dinucleotide independent acetolactase.

Table adapted from Dr. Begoña Heras, The University of Queensland, Australia

Combining methods

Protocol 4. Spectacular improvement in X-ray diffraction combining annealing and dehydration (Abergel. 2004)



Glycerol and Polyols

- Addition before crystallisation: stabilise protein structure and suppress conformational flexibility.
- Soaking protein crystals in polyols containing solutions: enhance order in disordered regions and increase diffraction resolution.

Malonate

- Evaluation effect of 50-100% saturated Na Malonate on 5 protein crystals.
- PROPERTIES:

  1. Versatile cryoprotectant for salt-grown protein crystals (100% success)

  2. Resolution improvement: Xylose Isomerase from 1.84Å to 0.84 Å.
- Allows derivatiosation with many heavy-atom compounds; soak with ligands.

Myths and realities…

- ◊ The production of poor diffracting crystals may not require returning to time consuming steps.
- ◊ Quick and simple methods can convert an otherwise useless crystals into diffraction quality crystals.
- ◊ Mounting crystals in situ, as it has been done in the past, is the most promising way to increase the crystal quality.
- ◊ Do not use the robots facilities, they are quicker, but do not permit to manipulate your crystals to apply these methods I have described in here!

Conclusions:

- There are three possibilities of controlling the crystal quality via transport phenomena (gels, capillary tubes and microgravity). Gels are easier to use, recommended, but you might be sure to remove all remaining amounts of gel around the crystals, otherwise it is worst.

- Controlling the crystal growth kinetics by physical parameters: electric/magnetic fields, laser pulses, etc., has demonstrated to be an excellent tool to control the crystal quality.

- All these methods are plausible to be applied to your crystals to improve the crystal quality, only if the protein is highly pure and you manipulate your crystals in situ properly!

Thank you so much…

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