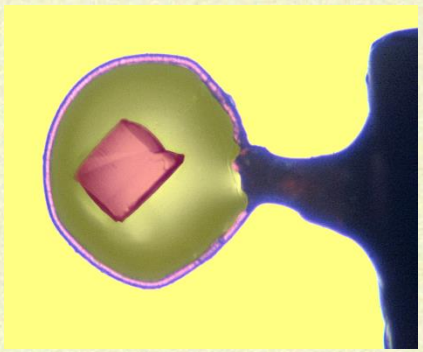
SAMPLE PREPARATION
CRYO-COOLING: why and how?

CCP4/DLS Workshop
November 6th 2023



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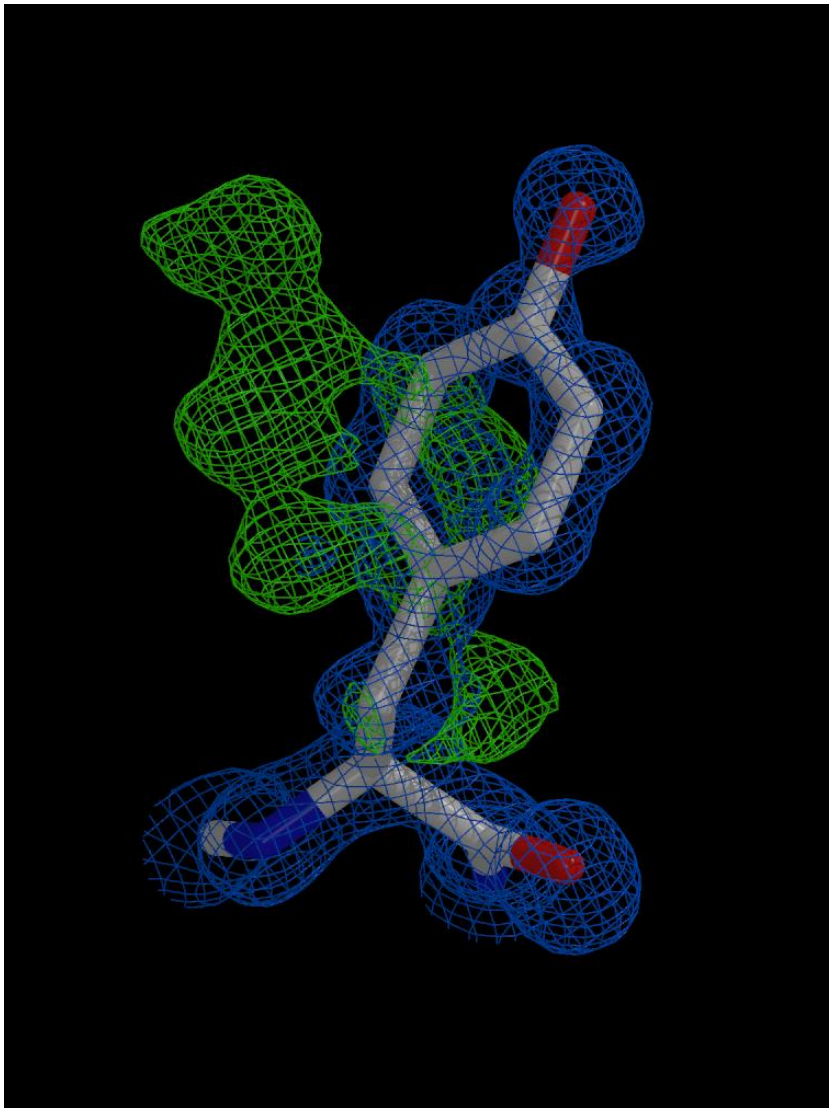
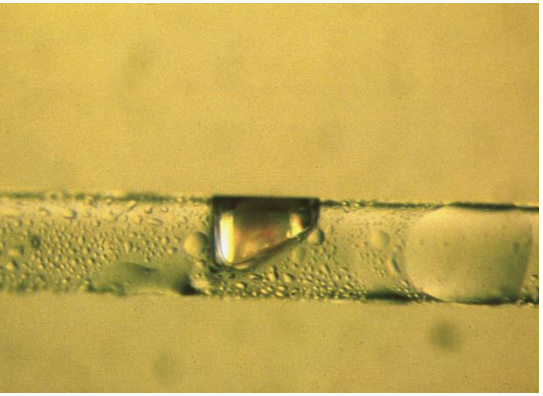
Data quality pivotally affects the amount of biological information we obtain.

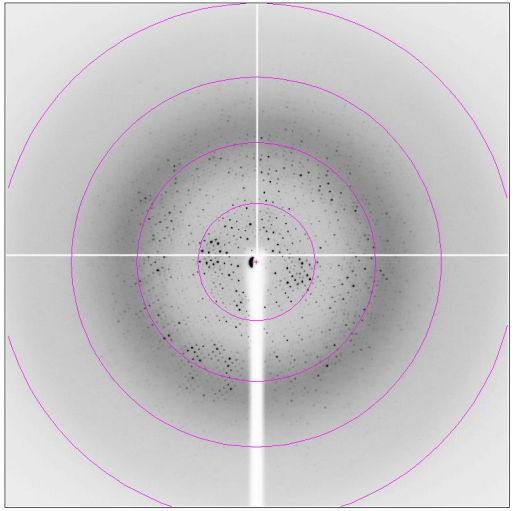
Copyrighted
Gary Larson
Cartoon of cave man finding Structure of dirt.



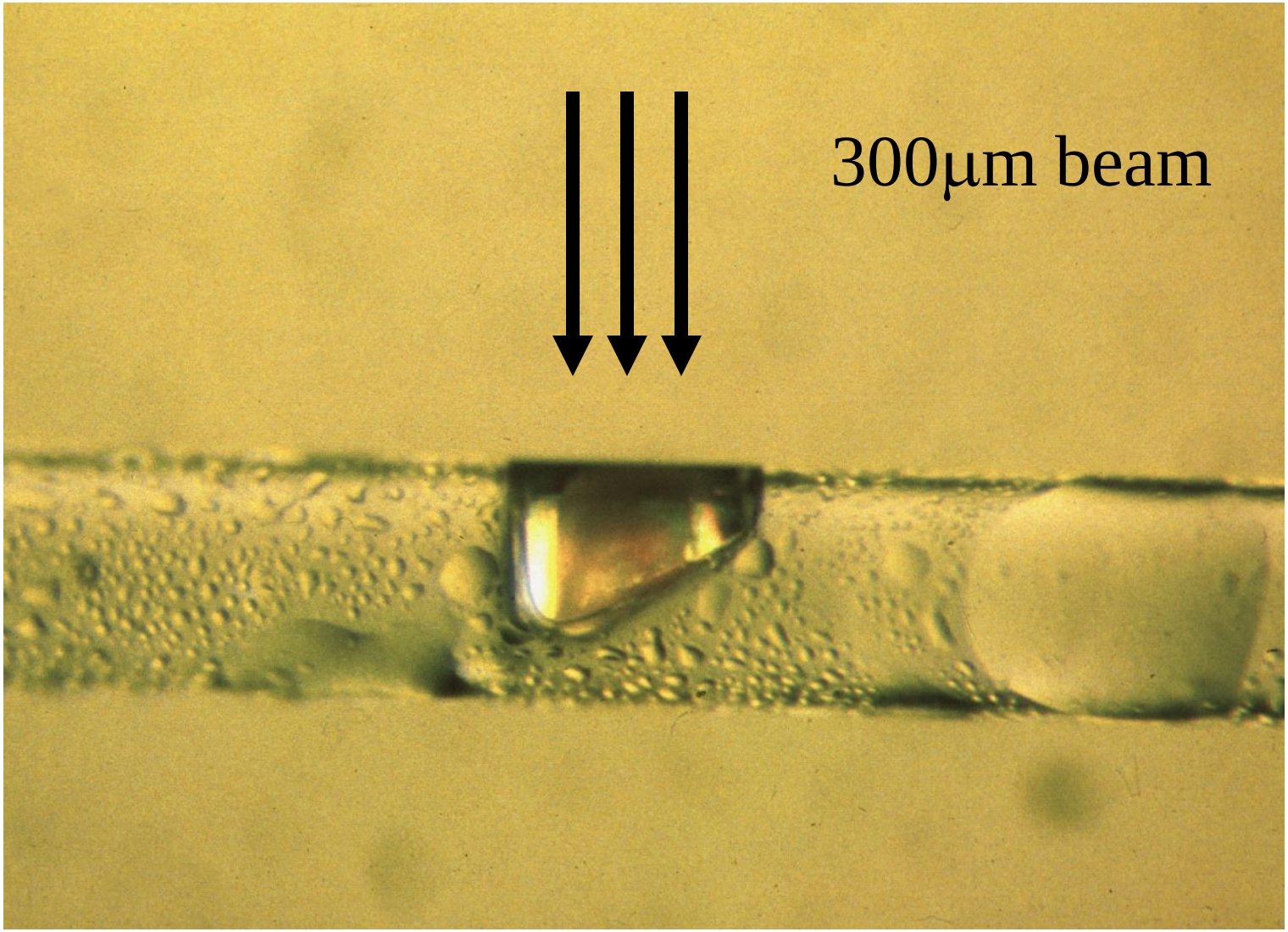
Detailed biological information



The Plan:

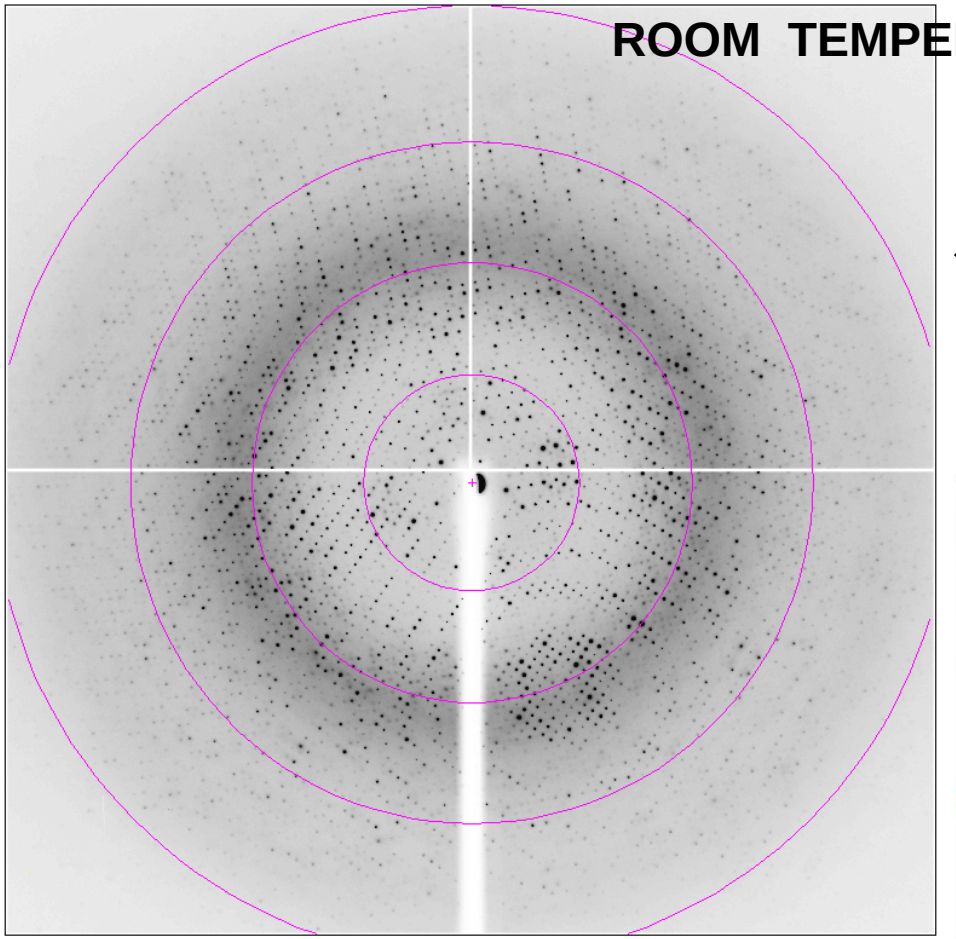


- Cryo techniques
  - **Why cool? Radiation damage (more detail on 21st November).**
  - Optimising cryoprotection.
  - Testing at room temperature.
  - Storage and retrieval.
  - If nothing works…



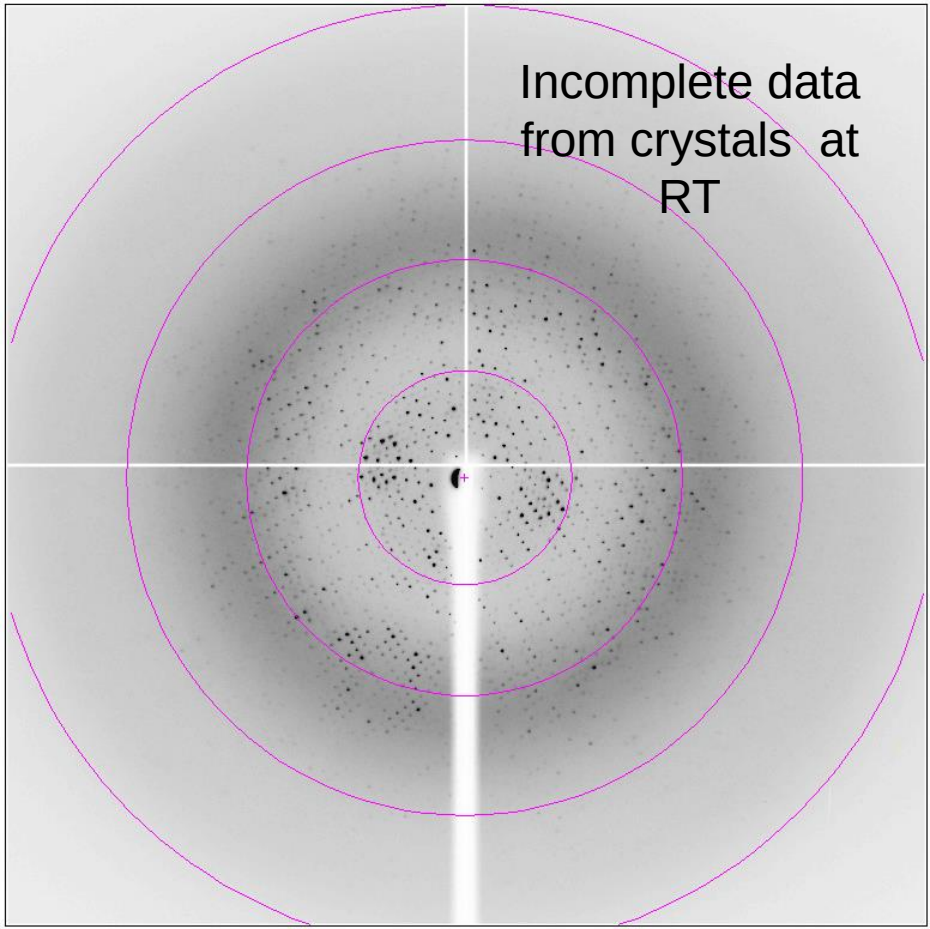
Room temperature: HEWL crystal after 3 hours in a 2nd generation synchrotron beam.

ROOM TEMPERATURE

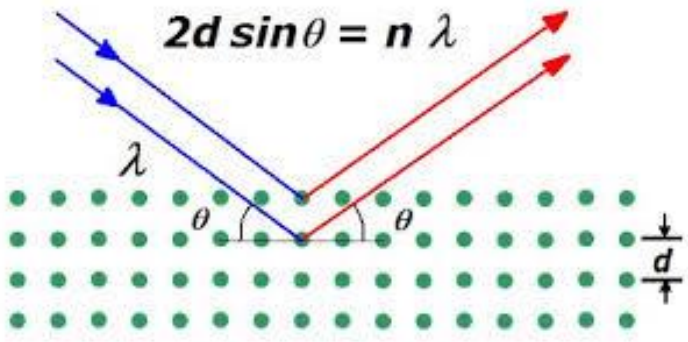


Intensity decrease with dose

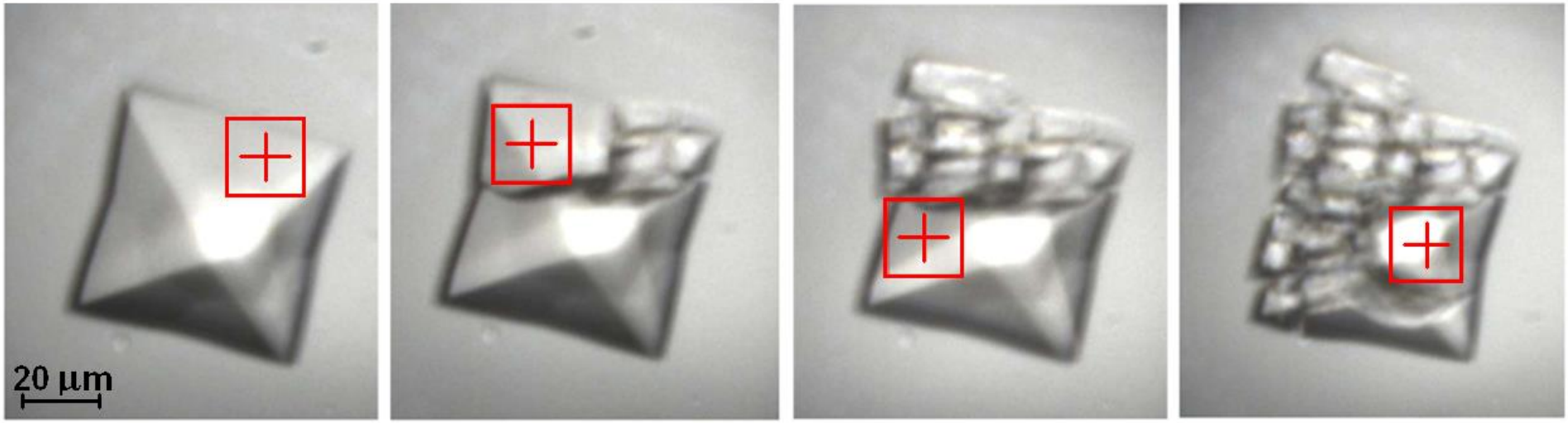
Loss of diffraction

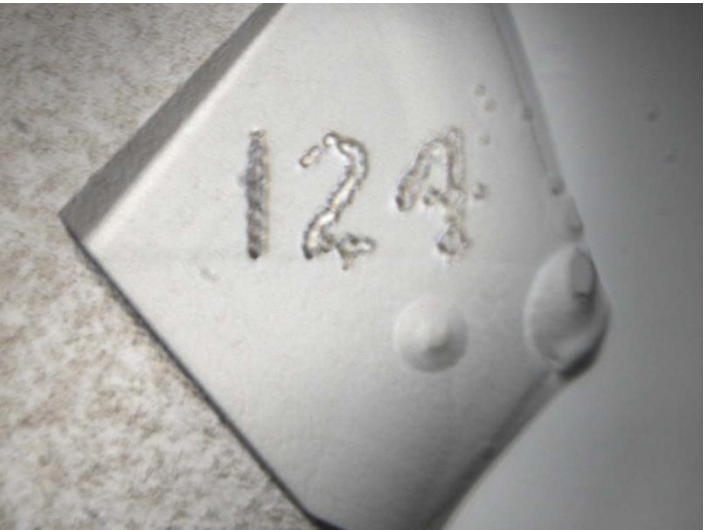


Incomplete data from crystals at RT



I24, Diamond, *in situ* data collection from a Bovine Enterovirus 2 crystal, room temperature, 0.5 s 20 μm x 20 μm beam





Axford *et al.*,
Acta Cryst D (2012) 592

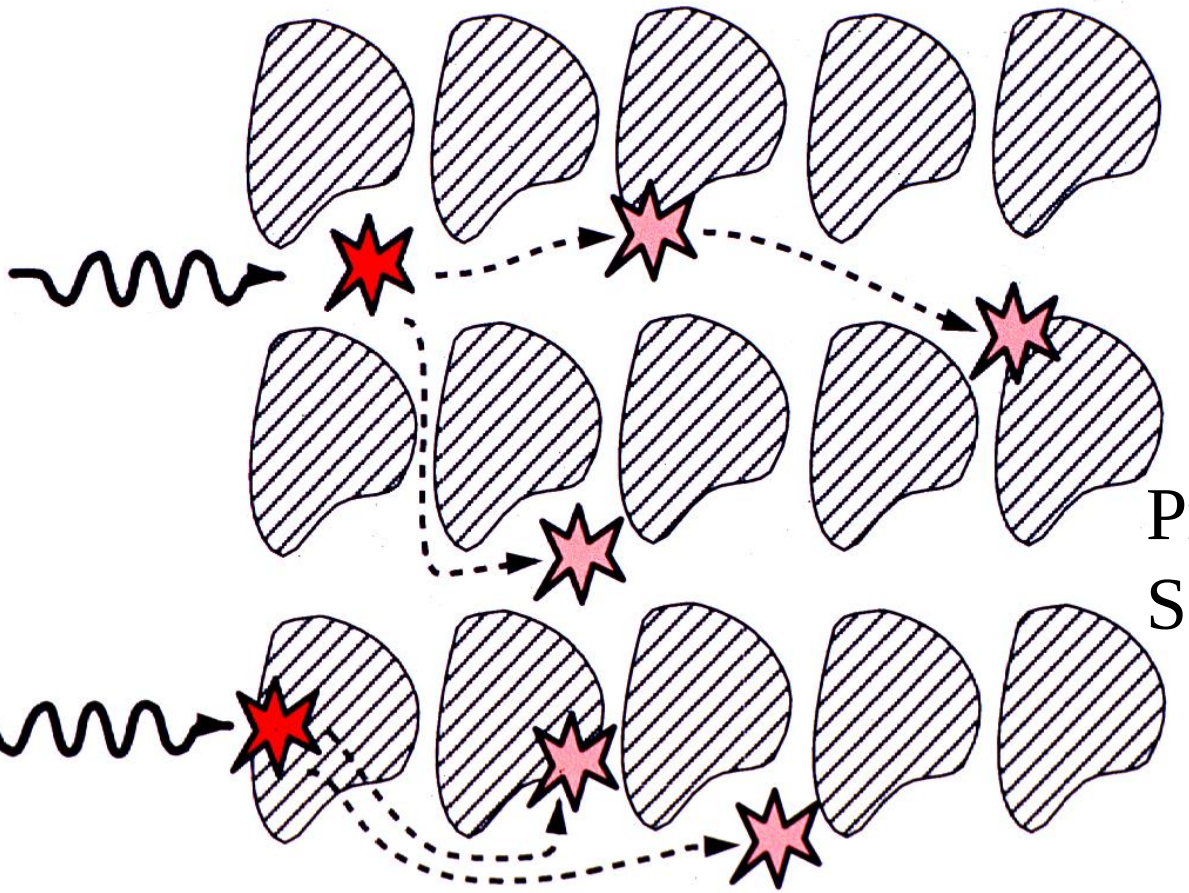
Beamline logo I24
(Gwyndaf Evans *et al.*)

Crystal costs the same as a diamond!!

Radiation Damage

Primary

Secondary



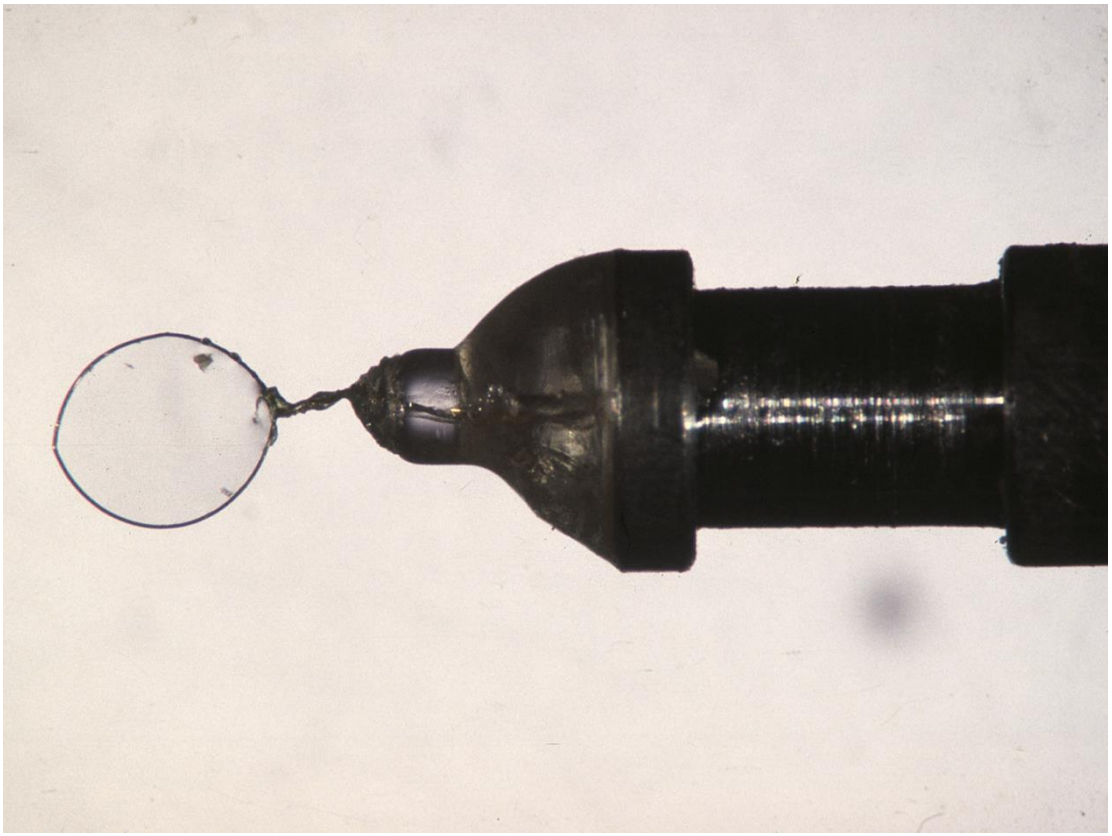
Protein: direct

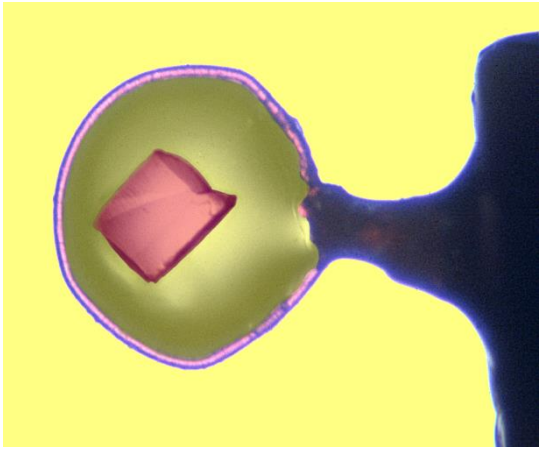
Solvent: indirect

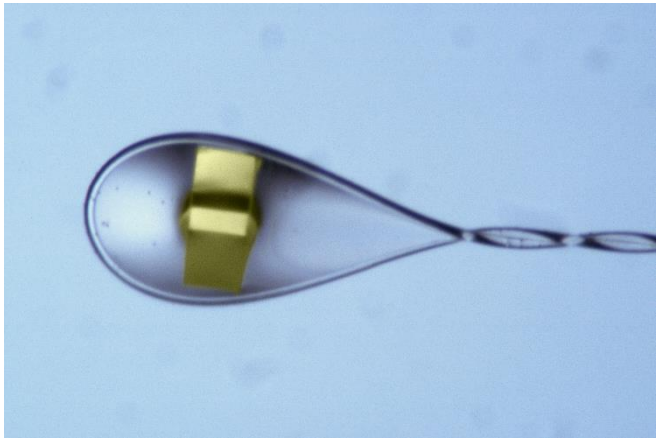
PRIMARY; inevitable, a fact of physics! Neutralise it?

SECONDARY, can we control it?

Loop mounting: T-Y.Teng (1990) J.Appl.Cryst, 23, 387-391.
Used wire loops



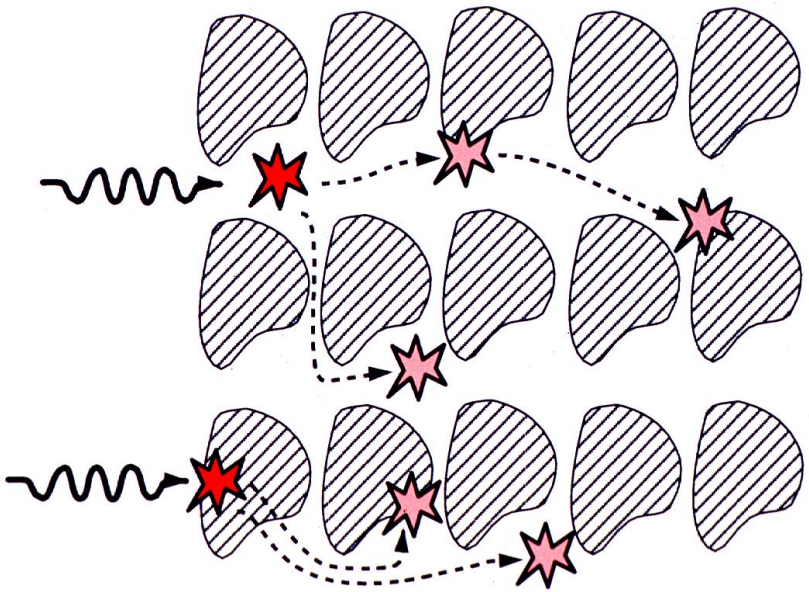


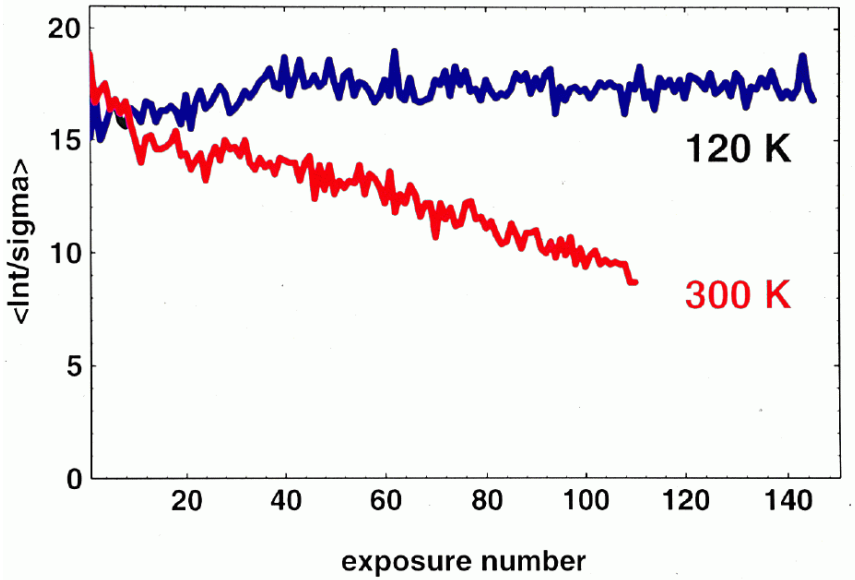


Also, a commercially available and easy to use cryostat (Cosier and Glazer 1986) made the technique accessible to many labs.

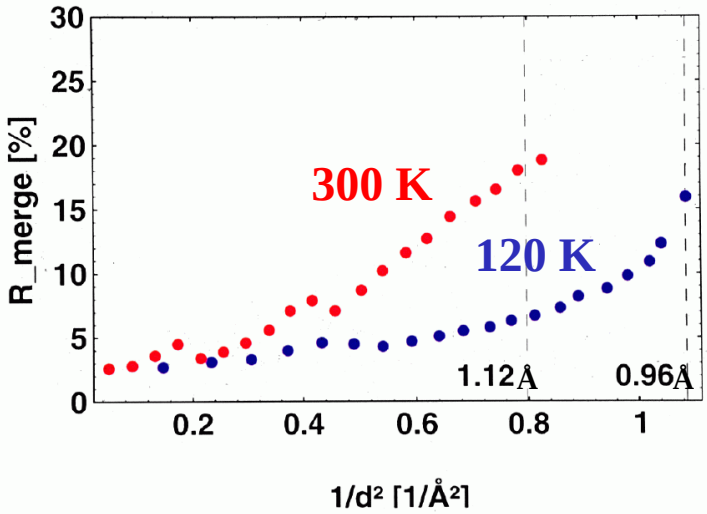
[Garman and Schneider, J.Appl.Cryst, (1997) **23**]

Radiation Damage





SP445: Data Quality [T.Schneider]



Significantly reduced at 100 K: time factor of ~70

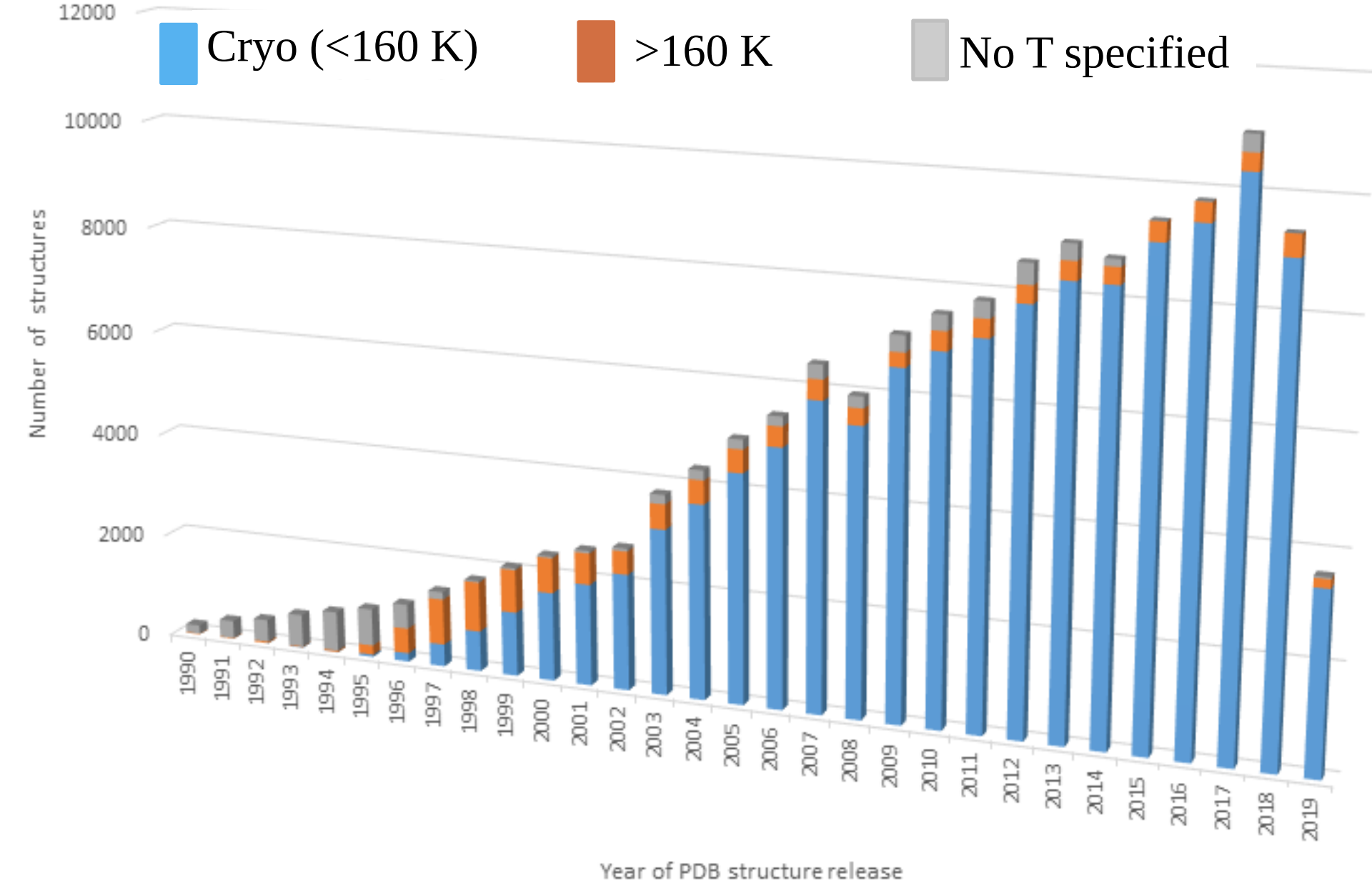
[Nave and Garman *JSR* (2005), **12**, 257-260].

PRIMARY; inevitable, a fact of physics! Proportions?

SECONDARY, can we control it?

Garman and Schneider J Appl Cryst 1997

Temperatures of X-ray structures released by the PDB



[Garman and Gerstel in Haas IUCr 2020]

Also see degradation at 100 K

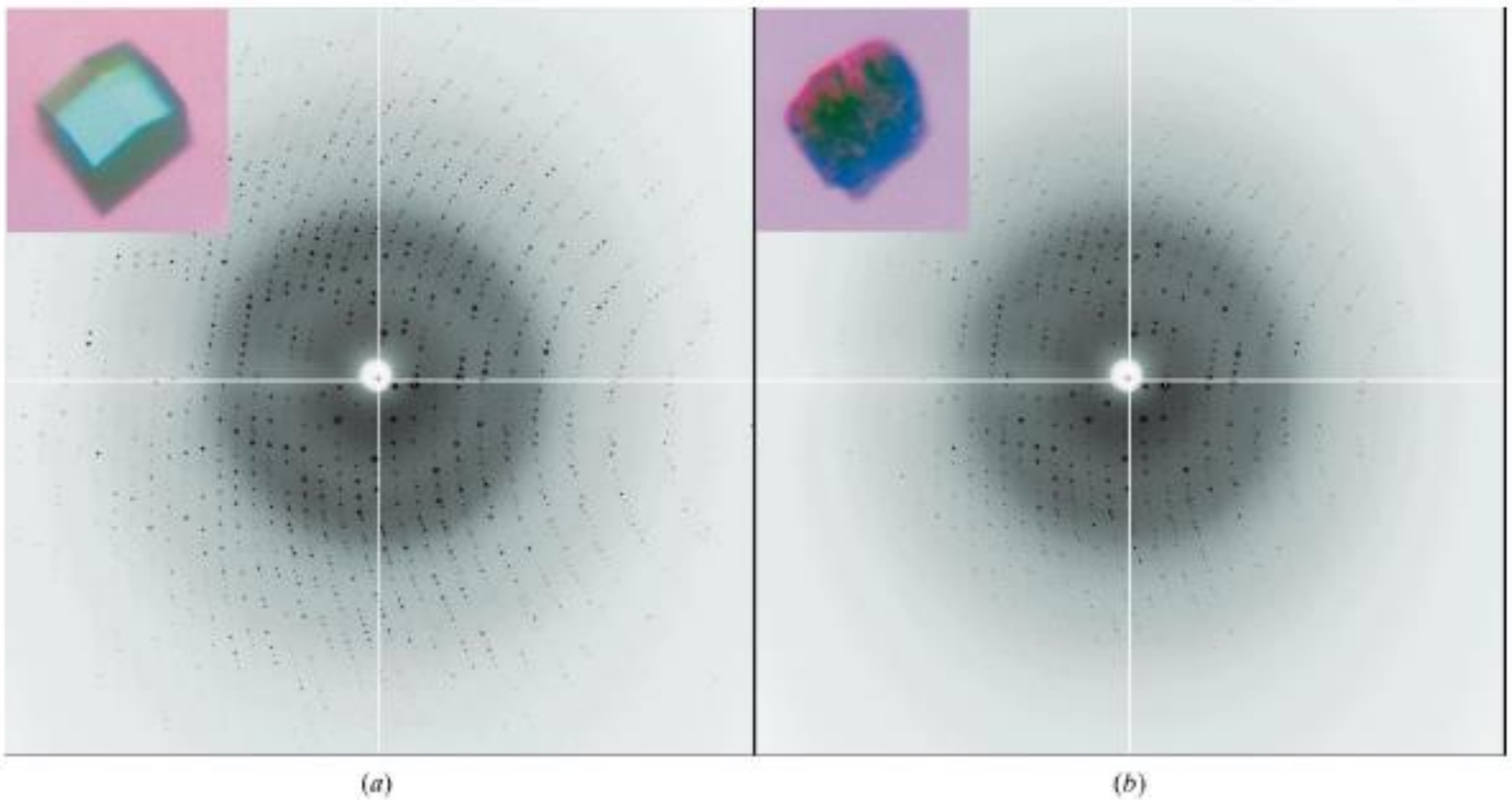
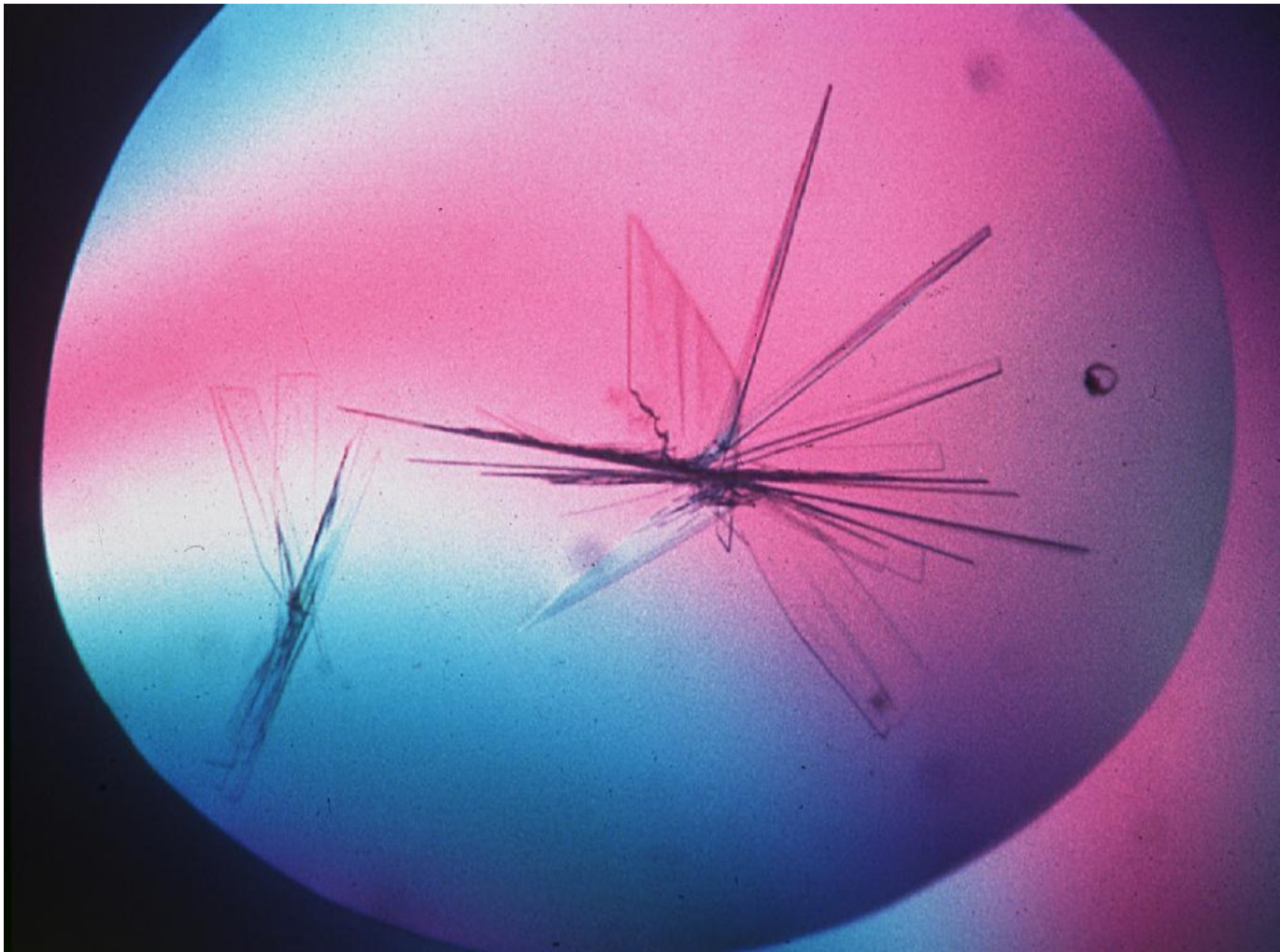


Figure 1
Diffraction images of a lysozyme crystal during a 1.36×10^3 s X-ray exposure at the 14-BM-C beamline. The resolution of diffraction is 1.6 Å at the edge of the image. Two images were taken with identical X-ray dosage. (*a*) The first image; during its exposure 1.2×10^4 Gy were absorbed. (*b*) The last image; after accumulating 1.6×10^7 Gy of absorbed energy. The inserts are photomicrographs of the crystal before and after X-ray exposures. The size of this crystal is ~$110 \times 110 \times 60$ µm. The crystal was maintained at 100 ± 1 K during the experiment.

Teng & Moffatt JSR (2000)

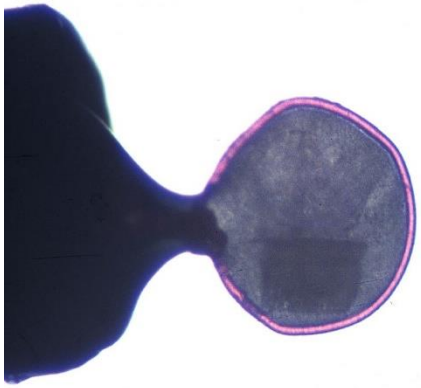
Loop mounting is a MUCH
gentler technique than capillary mounting.
e.g. cyclin A, 5μm x 100μm x 300μm



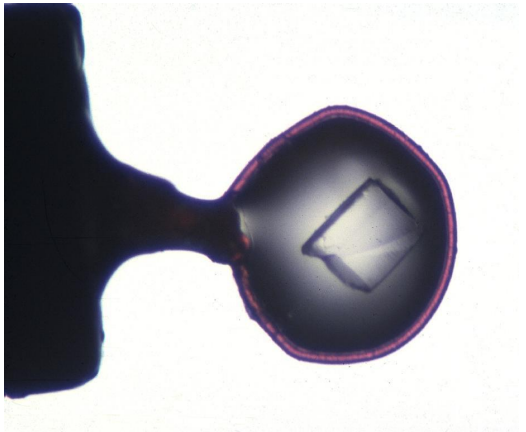
Other advantages:

- Usually get a whole data set from a single crystal $\Rightarrow$ higher QUALITY data.

  For MAD, the systematic errors are minimised by using only one crystal.
- Can harvest and store crystals while they are in peak condition.
- Small crystals and flat plates can be mounted easily.
- Reduced disorder and thermal movement
- Much lower solvent background.
- No secondary radiation damage during storage.
- New experiments are possible.



The Plan:



- Cryo techniques
  - Why cool? Radiation damage.
  - **Optimising cryoprotection.**
  - Testing at room temperature.
  - Storage and retrieval.
  - If nothing works…

Cryocooling: HOW?

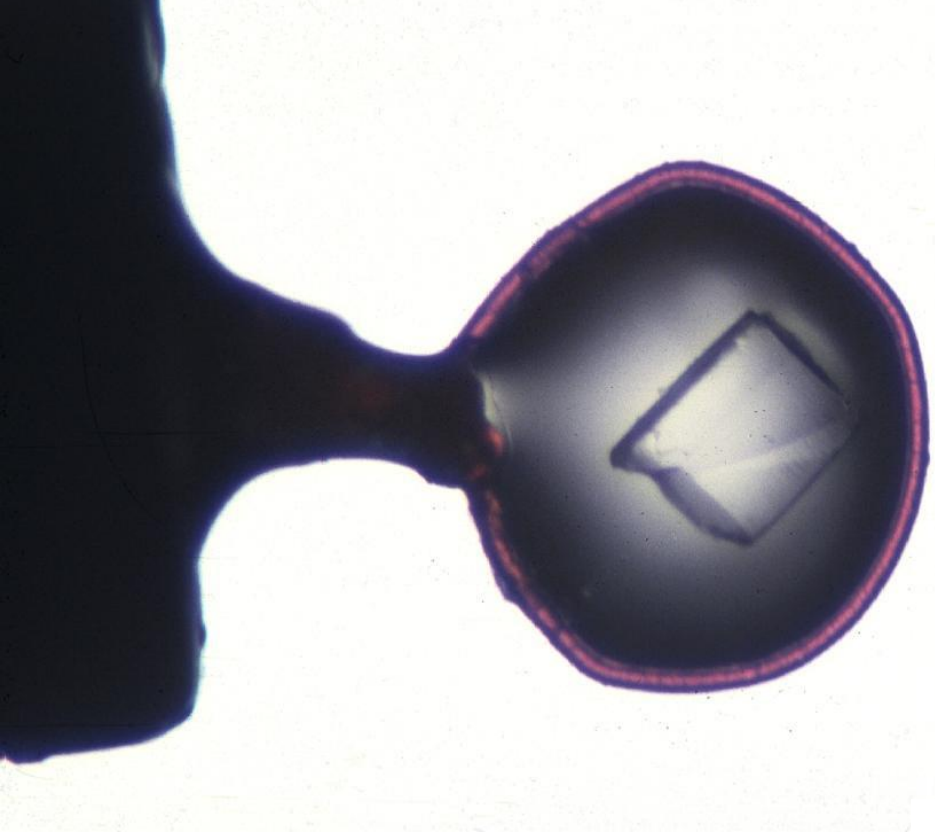
- Cool the crystal so fast that the water in the solvent channels is vitrified and does not form crystalline ice. [Pure water: ~10^{-5} s for typical protein crystal sized drop]

- Add `antifreeze' to increase time for cooling process. [1-2 s]

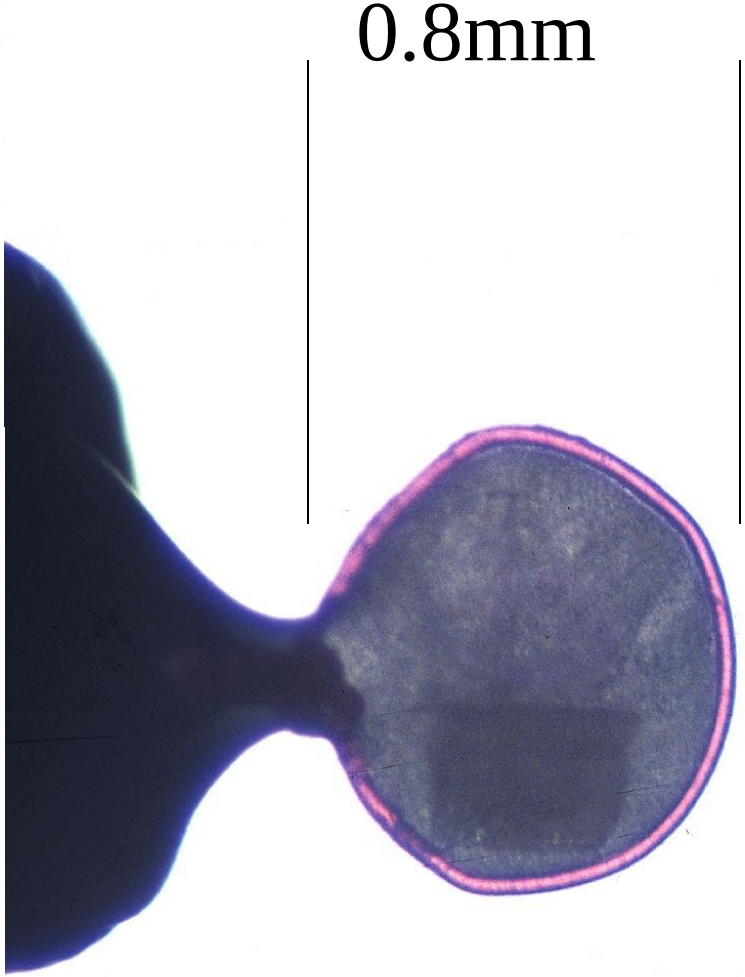
i.e. `cryoprotect'

N.B. WE DO **NOT** WANT TO 'FREEZE' the crystal!

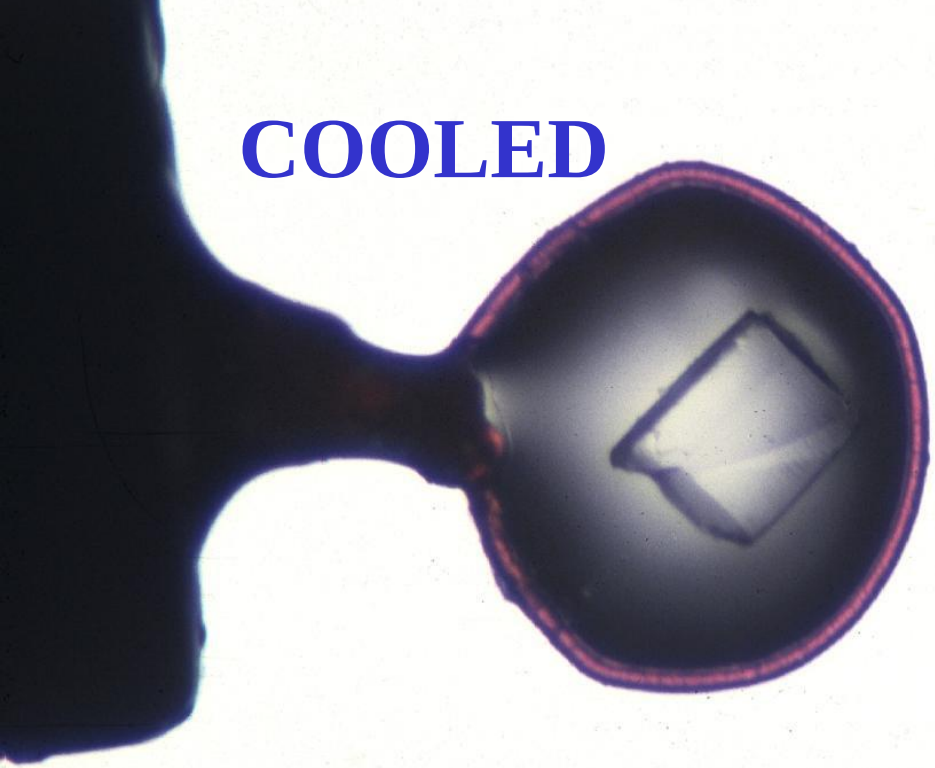
- Collect data at around 100K [below 130K and absolutely below 155K [Weik, 2001]]



Crystalline ice has 7%
more volume than water.
Thus all the solvent channels
will expand if no cryoprotectant
is added, and crystal order is
compromised

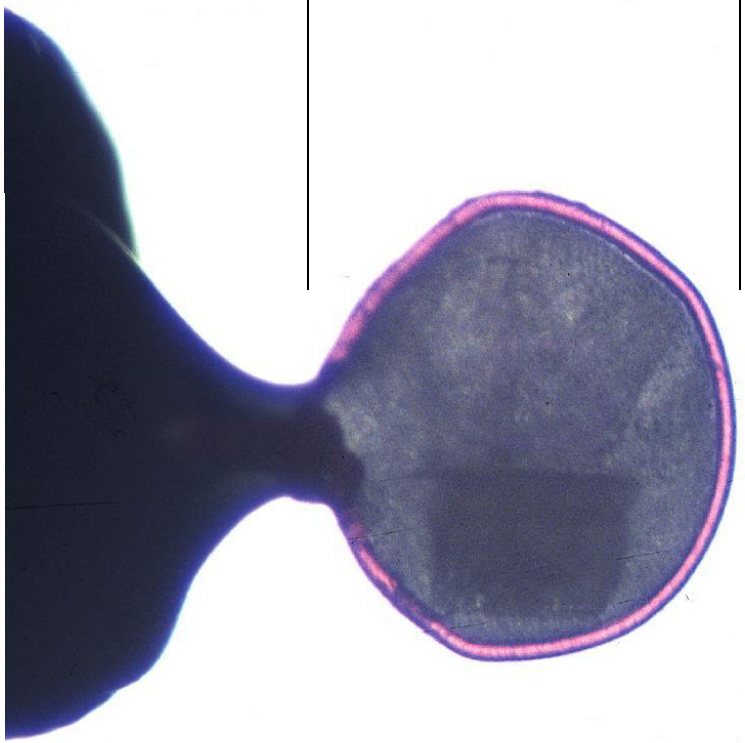


COOLED



FROZEN

0.8mm



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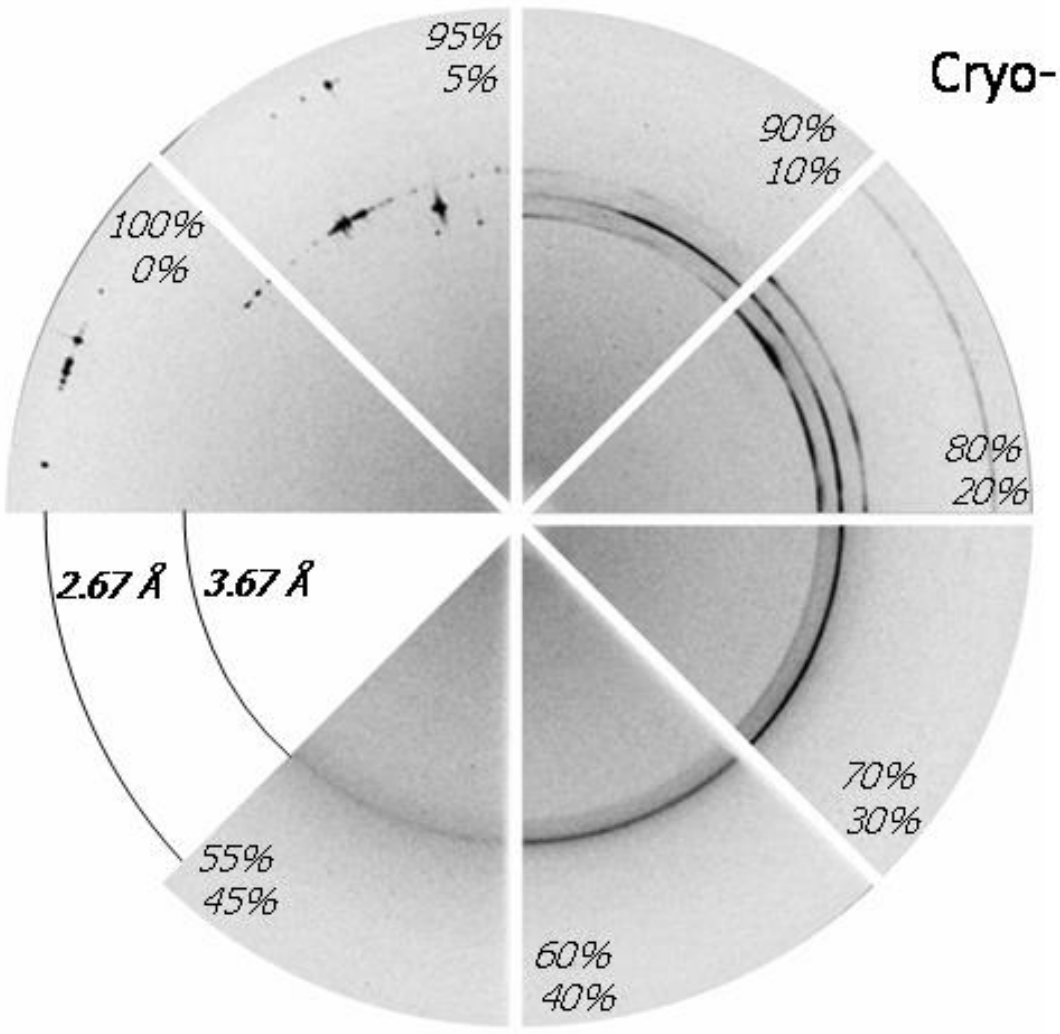


Cryo-buffer optimisation

hkl	d(Å)
100	3.9
002	3.67
101	3.44
102	2.67
110	2.25
103	2.07
200	1.95
112	1.92
201	1.88
202	1.72

- **replace the water in mother liquor with cryo-agent, instead of diluting the mother liquor.**
- test cryo-buffer alone in loop.

Cryo-buffers.

- PEG < 4K→ increase PEG, add small PEGs
- PEG ≥ 4K→ add small PEGs
- 2/3rds of cases → add 15 - 25% glycerol
- MPD → harvesting buffer, increase MPD concentration.
- Salt → add MPD and/or ethylene glycol or glycerol

  → increase conc/add salt. Lithium salts good. [Rubinson et al, Acta D (2000), D56, 996-1001.]

  → Exchange salt. e.g. 100% 8M Na Formate.

N.B. Low salt needs > concentration of cryoprotectant than high salt.

Sugars, paratone N, combinations, +++

Illegible table of minimum concentrations of glycerol required to cryoprotect Hampton Screen I.

For emergency use only (done by dilution)

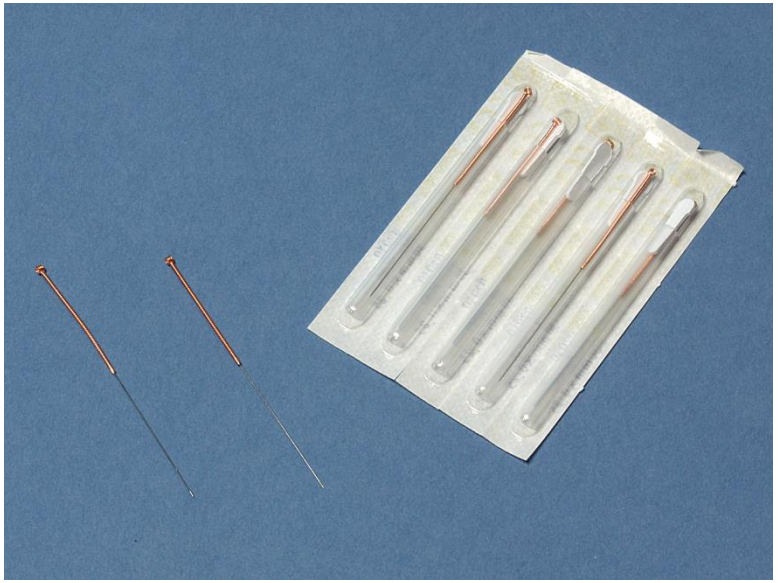
[Mitchell and Garman, J.Appl.Cryst. (1996) 29, 584
McFerrin and Snell J.Appl.Cryst (2002) 35, 538]

N.B. CDK2: 8M Na Formate excellent cryoprotectant: affects salt bridge ionisation?

TABLE 1. Minimum concentration of glycerol to be added to solutions 1 to 50 of the Hampton Research Crystal Screen$^{(TM)}$ I Reagent Components to provide cryoprotection when frozen to 100K. It must be noted that glycerol was added to the Crystal Screen solutions resulting in a DILUTION of the original components. From Garman and Mitchell J. Appl. Cryst.(1996) 29, 584-587

Solution number, SALT	BUFFER	PRECIPITANT	GLYCEROL CONC. v/v
1. 0.02 M Ca Chloride	0.1 M Na Acetate pH 4.6	30% v/v 2-methyl-2,4-pentanediol	0
2. None	None	0.4 M K, Na Tartrate	35
3. None	None	0.4 M NH4 Phosphate	35
4. None	0.1 M Tris HCl pH 8.5	2.0 M NH4 Sulphate	25
5. 0.2 M Na Citrate	0.1 M Na Hepes pH 7.5	30% v/v 2-methyl-2,4-pentanediol	0
6. 0.2 M Mg Chloride	0.1 M Tris HCl pH 8.5	30% w/v PEG 4000	20
7. None	0.1 M Na Cacodylate pH 6.5	1.4 M Na Acetate	30
8. 0.2 M Na Citrate	0.1 M Na Cacodylate pH 6.5	30% v/v 2-propanol	30
9. 0.2 M NH4 Acetate	0.1 M Na Citrate pH 5.6	30% w/v PEG 4000	15
10. 0.2 M NH4 Acetate	0.1 M Na Acetate pH 4.6	30% w/v PEG 4000	15
11. None	0.1 M Na Citrate pH 5.6	1.0 M NH4 Phosphate	30
12. 0.2 M Mg Chloride	0.1 M Na Hepes pH 7.5	30% v/v 2-propanol	10
13. 0.2 M Na Citrate	0.1 M Tris HCl pH 8.5	30% v/v PEG 400	0
14. 0.2 M Ca Chloride	0.1 M Na Hepes pH 7.5	28% v/v PEG 400	5
15. 0.2 M NH4 Sulphate	0.1 M Na Cacodylate pH 6.5	30% w/v PEG 8000	15
16. None	0.1 M Na Hepes pH 7.5	1.5 M Li Sulphate	25
17. 0.2 M Li Sulphate	0.1 M Tris HCl pH 8.5	30% PEG 4000	15
18. 0.2 M Mg Acetate	0.1 M Na Cacodylate pH 6.5	20% PEG 8000	20
19. 0.2 M NH4 Acetate	0.1 M Tris HCl pH 8.5	30% v/v 2-propanol	20
20. 0.2 M NH4 Sulphate	0.1 M Na Acetate pH 4.6	25% w/v PEG 4000	20
21. 0.2 M Mg Acetate	0.1 M Na Cacodylate pH 6.5	30% v/v 2-methyl-2,4-pentanediol	0
22. 0.2 M Na Acetate	0.1 M Tris HCl pH 8.5	30% w/v PEG 4000	15
23. 0.2 M Mg Chloride	0.1 M Na Hepes pH 7.5	30% v/v PEG 400	0
24. 0.2 Ca Chloride	0.1 M Na Acetate pH 4.6	20% v/v 2-propanol	30
25. None	0.1 M Imidazole pH 6.5	1.0 M Na Acetate	30
26. 0.2 M NH4 Acetate	0.1 M Na Citrate pH 5.6	30% v/v 2-methyl-2,4-pentanediol	0
27. 0.2 M Na Citrate	0.1 M Na Hepes pH 7.5	20 % v/v 2-propanol	30
28. 0.2 M Na Acetate	0.1 M Na Cacodylate pH 6.5	30% w/v PEG 8000	15
29. None	0.1 M Na Hepes pH 7.5	0.8 M K,Na Tartrate	35
30. 0.2 M NH4 Sulphate	None	30% w/v PEG 8000	15
31. 0.2 M NH4 Sulphate	None	30% w/v PEG 4000	15
32. None	None	2.0 M NH4 Sulphate	25
33. None	None	4.0 M Na Formate	10
34. None	0.1 M Na Acetate pH 4.6	2.0 M Na Formate	30
35. None	0.1 M Na Hepes pH 7.5	1.6 M Na, K Phosphate	25
36. None	0.1 M Tris HCl pH 8.5	8% w/v PEG 8000	35
37. None	0.1 M Na Acetate pH 4.6	8% w/v PEG 4000	30
38. None	0.1 M Na Hepes pH 7.5	1.4 M Na Citrate	10
39. None	0.1 M Na Hepes pH 7.5	2% v/v PEG 400 and 2.0 M NH4 Sulphate	15
40. None	0.1 M Na Citrate pH 5.6	20% v/v 2-propanol and 20% w/v PEG 4000	5
41. None	0.1 M Na Hepes pH 7.5	10% v/v 2-propanol and 20% w/v PEG 4000	15
42. 0.05 M K Phosphate	None	20% w/v PEG 8000	20
43. None	None	30% v/v PEG 1500	20
44. None	None	0.2 M Mg Formate	50
45. 0.2 M Zn Acetate	0.1 M Na Cacodylate pH 6.5	18% w/v PEG 8000	20
46. 0.2 M Ca Acetate	0.1 M Na Cacodylate pH 6.5	18% w/v PEG 8000	20
47. None	0.1 M Na Acetate pH 4.6	2.0 M NH4 Sulphate	20
48. None	0.1 M Tris HCl pH 8.5	2.0 M NH4 Phosphate	20
49. 1.0 M Li Sulphate	None	2% w/v PEG 8000	20
50. 0.5 M Li Sulphate	None	15% w/v PEG 8000	20

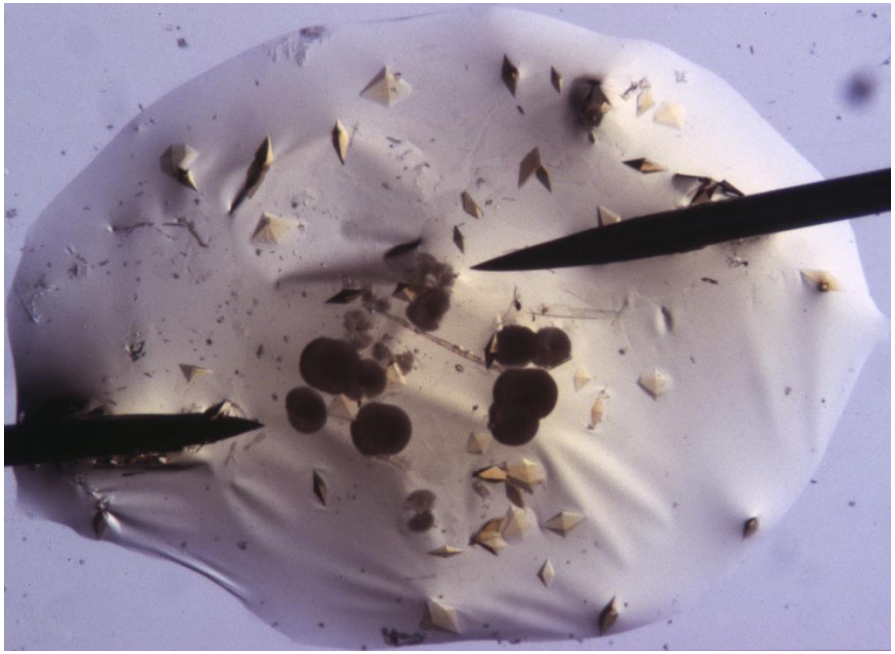
CRYSTAL MANIPULATION:



Acupuncture needles:
[free samples available from me]

- No loss of liquid
- Slightly flexible
- Fine
- Different sizes available.

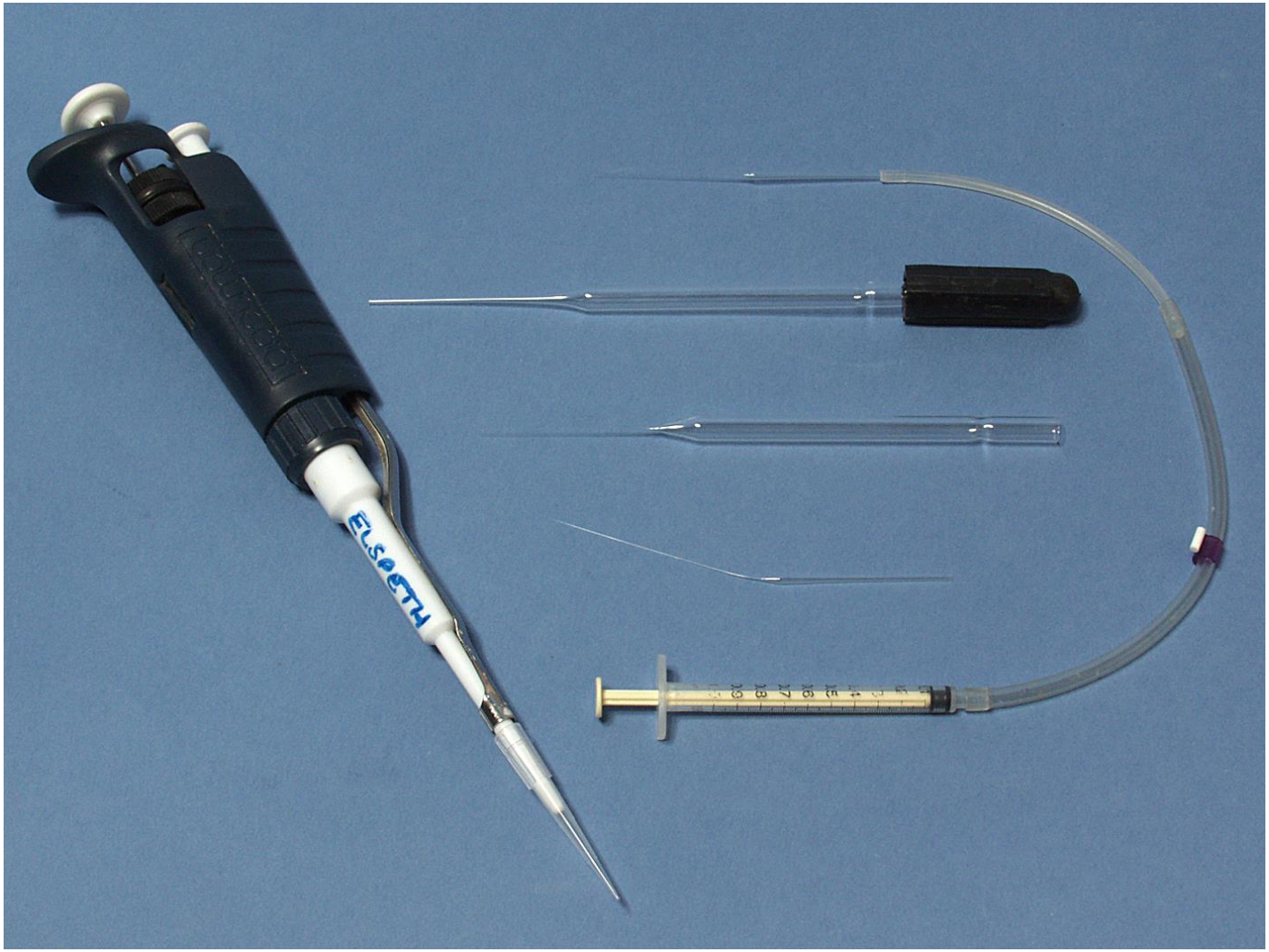
e.g. `Skin' on protein drop:



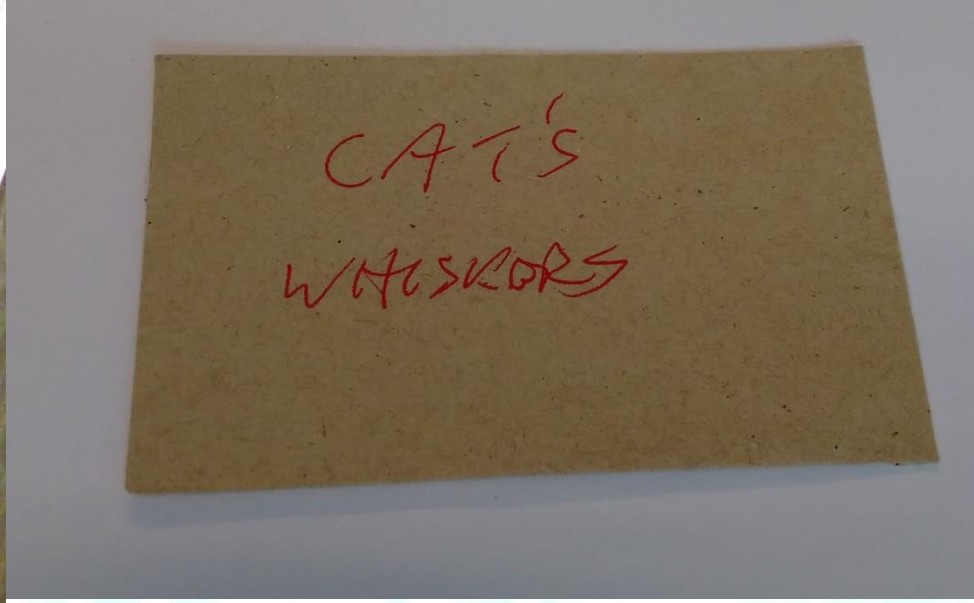
Gentle surgery
Skin doesn't diffract: just increases background

Cats' whiskers, horse eye lashes, horse tail hairs, etc etc

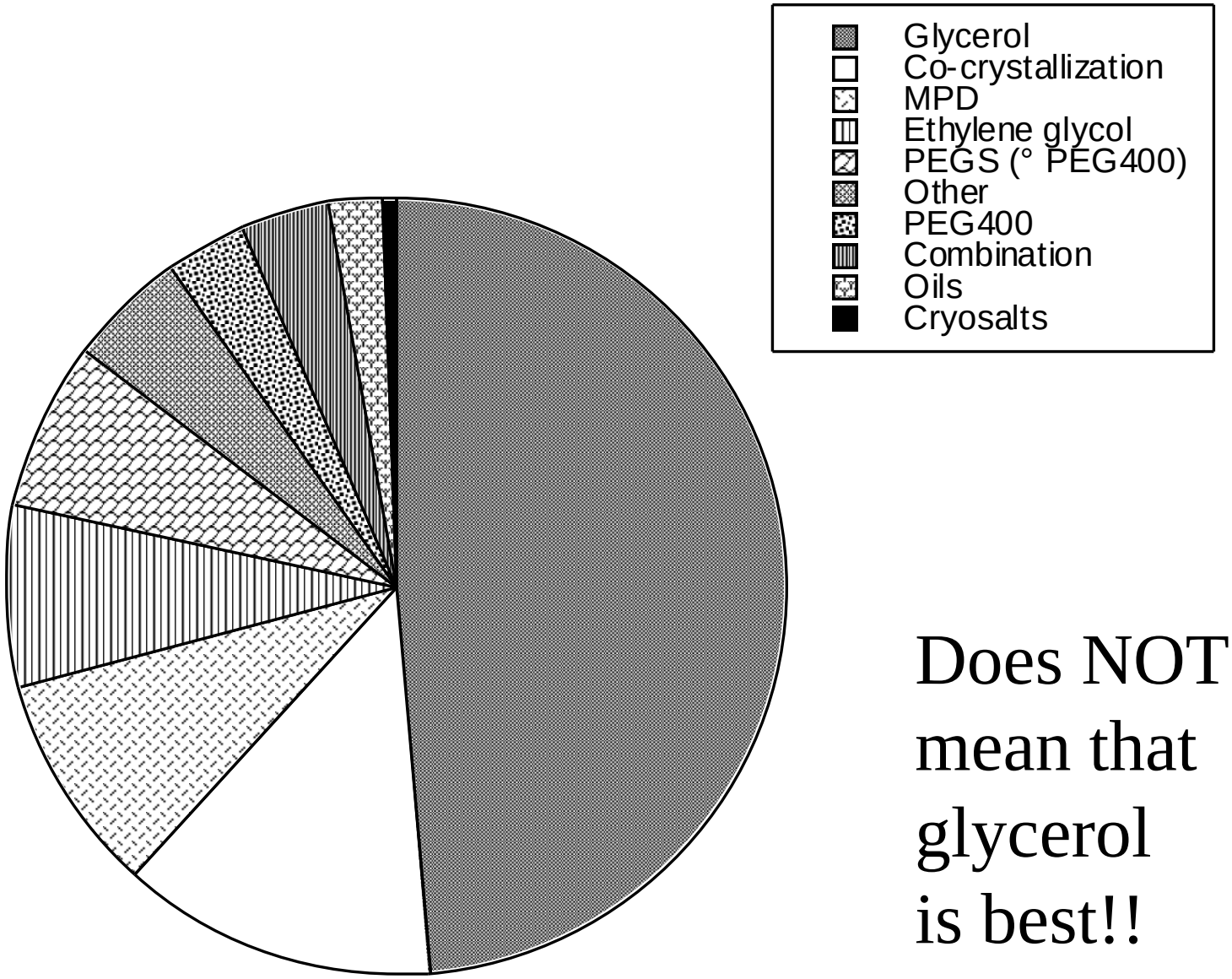
Manipulation of crystal from growth drop. A cryo-loop, or a syringe with flexible hose give much better control than a Gilson.



Saridakis and Chayen, Trends in Biotechnology (2008) 27, 99.
Beta –lactamase grown on bio-glass



Megan, 1988-2017



Does NOT mean that glycerol is best!!

Acta D structures 2001. [Garman and Doublié, Methods in Enzymology 2003, 368, 188.]

Transfer of crystal into cryobuffer:

1) Dialysis of cryoprotectant.

[Fernandez *et al*, JAPC (2000) 33, 168-171]

2) Co-crystallisation with cryoprotectant agent:
   glycerol is already known to help in some cases

[Sousa, Acta Cryst D51 (1995) 271-277.]

3) Rapid transfer
   - Straight into final concentration for up to 5 mins
   - can just `sweep through'- 0.5 sec
   - Sequential soaks in increasing concentrations.

WANT TO MINIMISE HANDLING, as handling can increase the mosaic spread. i.e. 2) is BEST:….

SOLUBILITY versus OSMOTIC shock

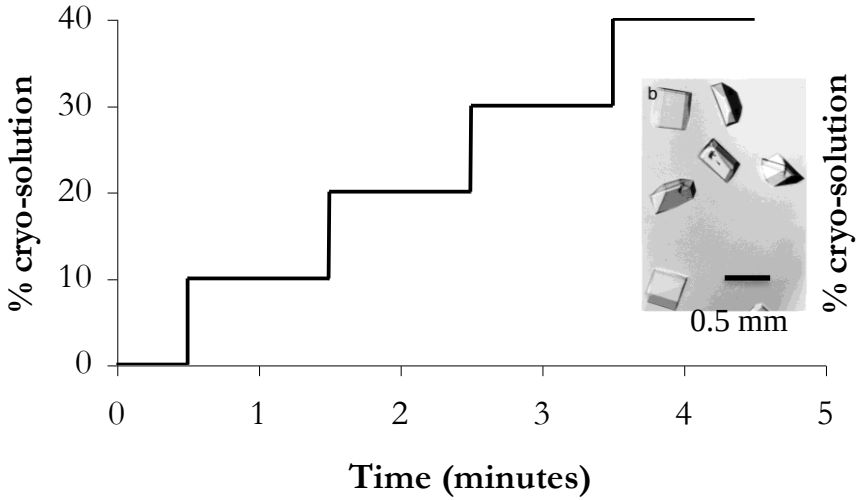
Crystal transfer optimisation:

- balance osmotic shock and attack of crystal surface
- serial transfers: minimise handling and dehydration

Quick ‘dunk’: 3.2 Å N6 influenza NA

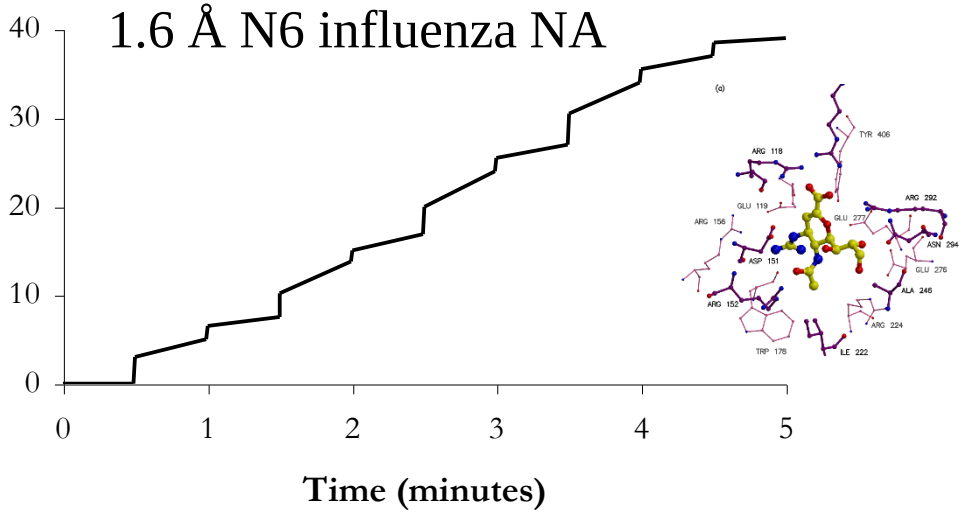
a) Move crystal between solutions

2.4 Å N6 influenza NA

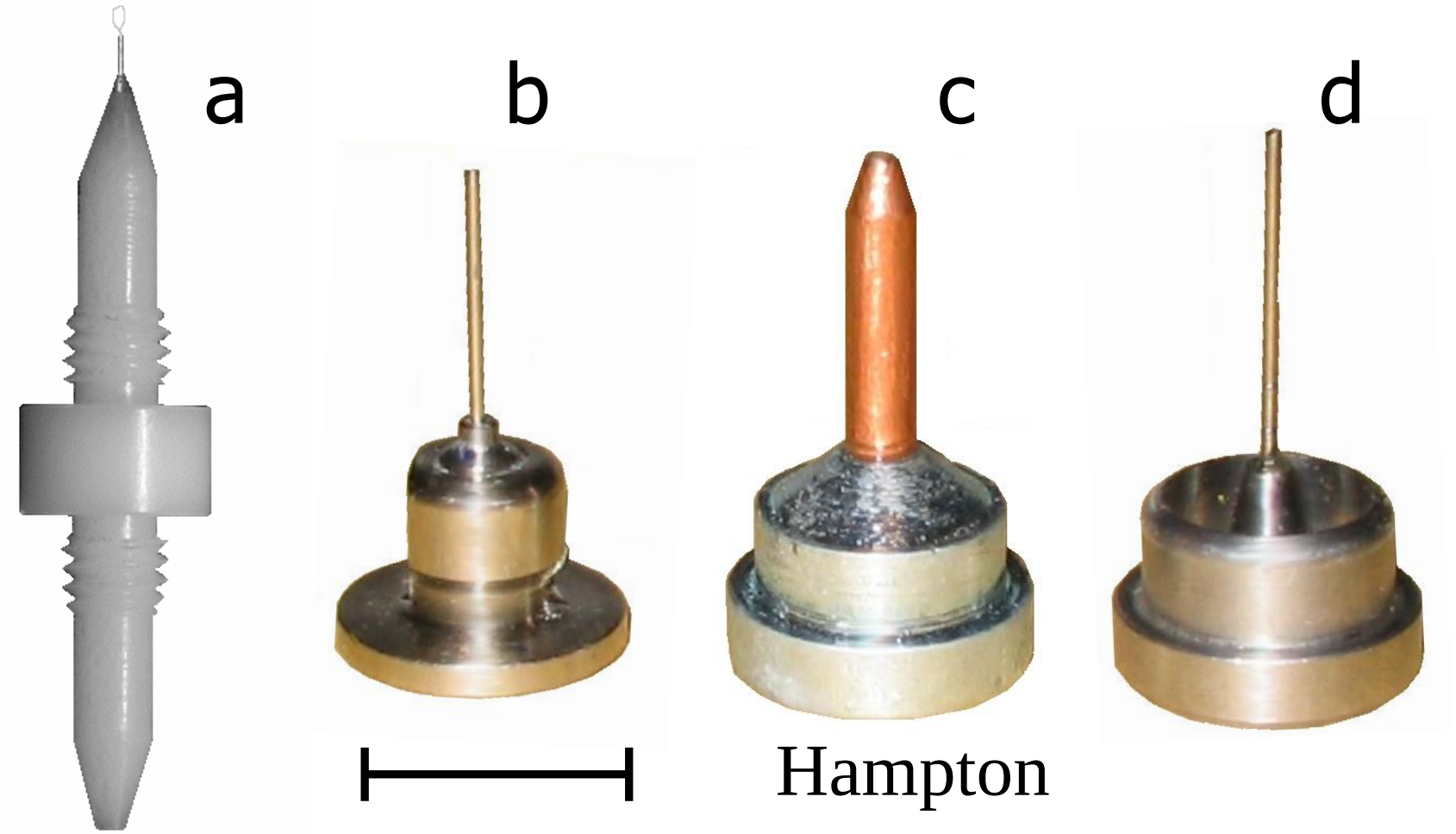


b) Solution pipetted onto crystal

1.6 Å N6 influenza NA



Hardware development: standardisation…

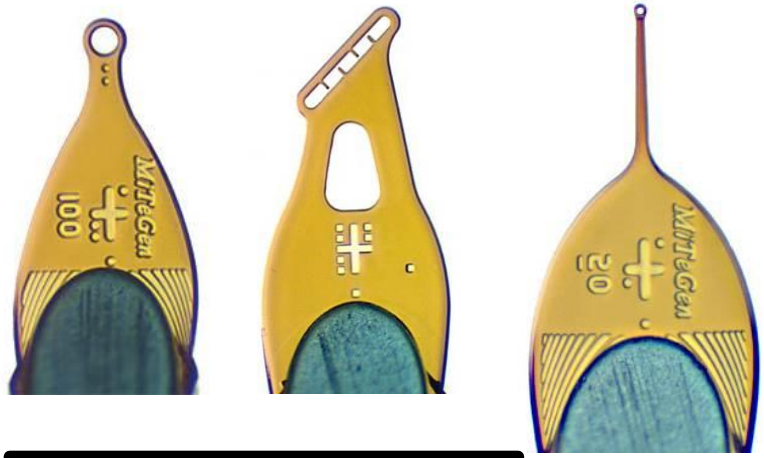


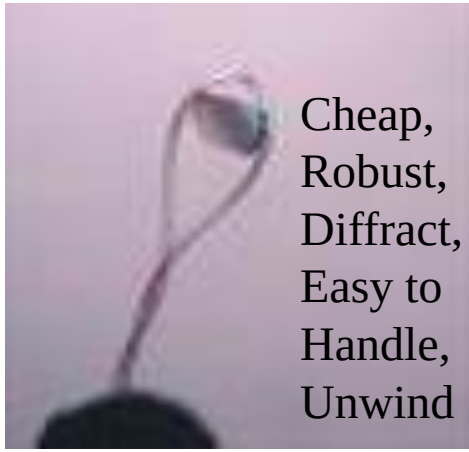
SPring8 pin (Japan)

Old OxfordCryosystems/ LMB Oxford nickel pin

Hampton pin

Spine pin



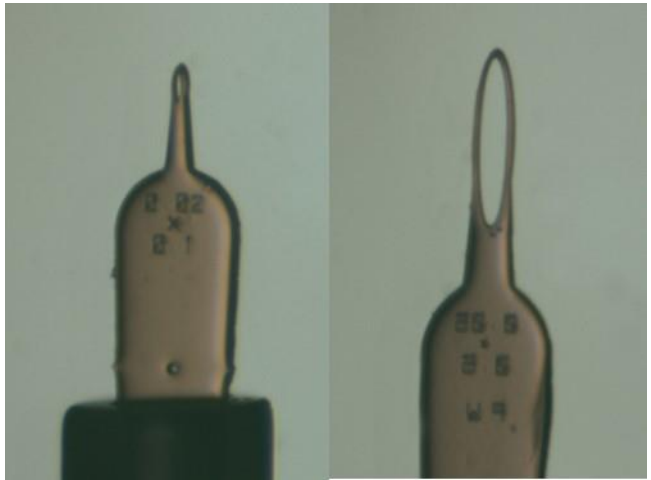


Cheap,
Robust,
Diffract,
Easy to
Handle,
Unwind

Litho-loops,
etched mylar,
ActiLoop polymer
Molecular Dimensions

Check loop vs crystal size BEFORE opening drop

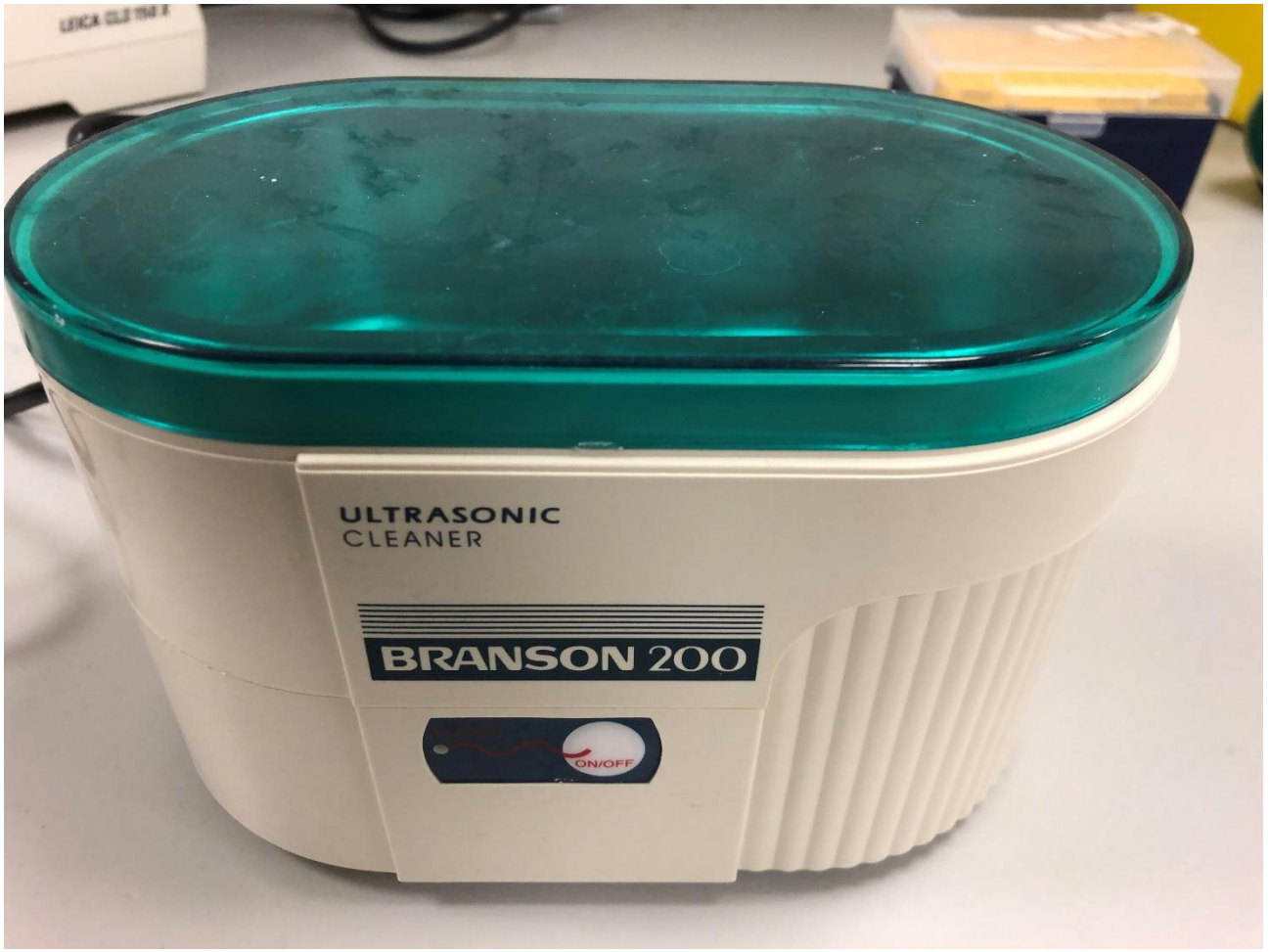
Nylon loops
Hampton Research
NB: CAN VIBRATE IN N_2 STREAM!



Expensive and fragile but stable N_2 in stream.

Micro-mounts, microfabricated polyimide film. **MiTeGen**
[Thorne *et al* (2003), JAPC 36, 1455.]

Loop Cleaning



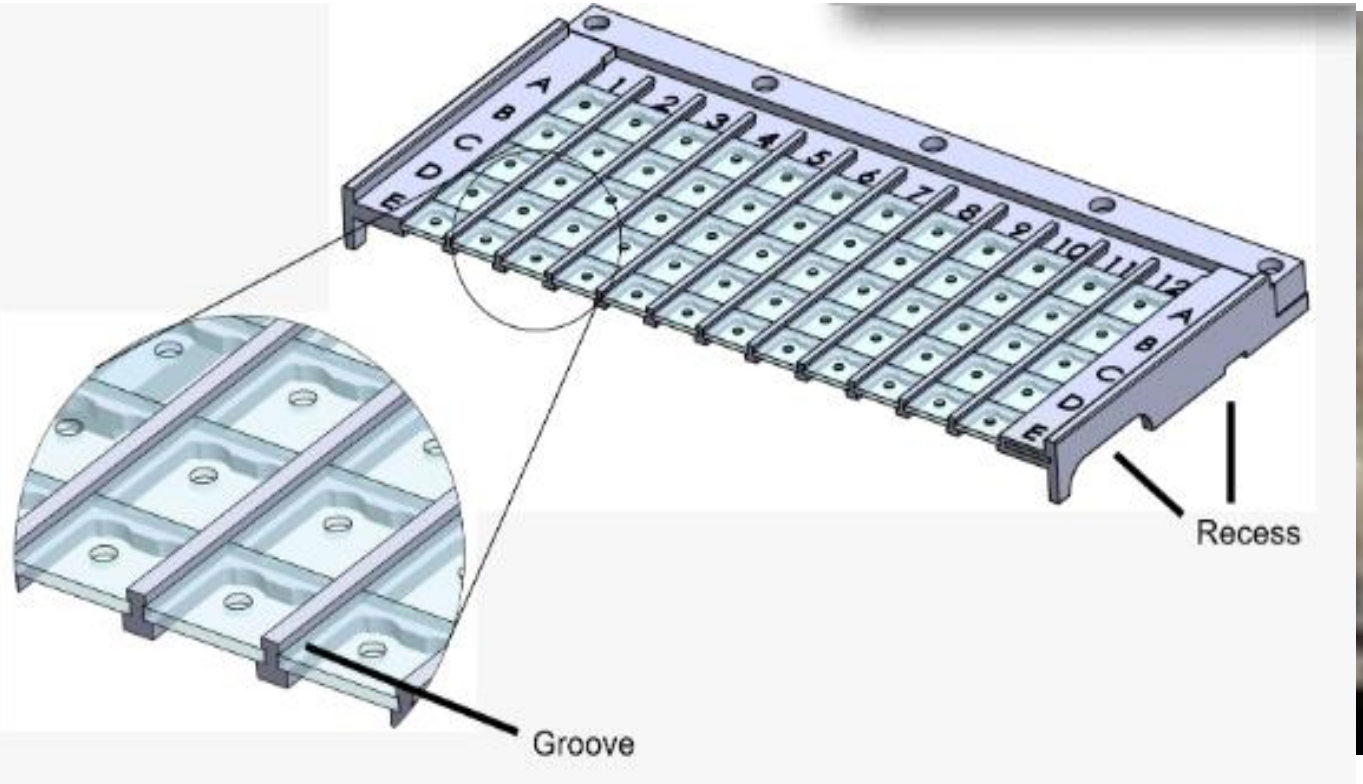
2 mins/cycle, 2 cycles.
Detergent (1 cycle) then water (1 cycle)

Crystallisation trays:

- 96 well plates –small volumes!
- Round bottom wells MUCH easier than square cross sections
- Dehydrate quickly when you take off the sticky tape lid: easy access frame, https://cgf-biotech.de/easy-access-frame/

Crystallisation trays:

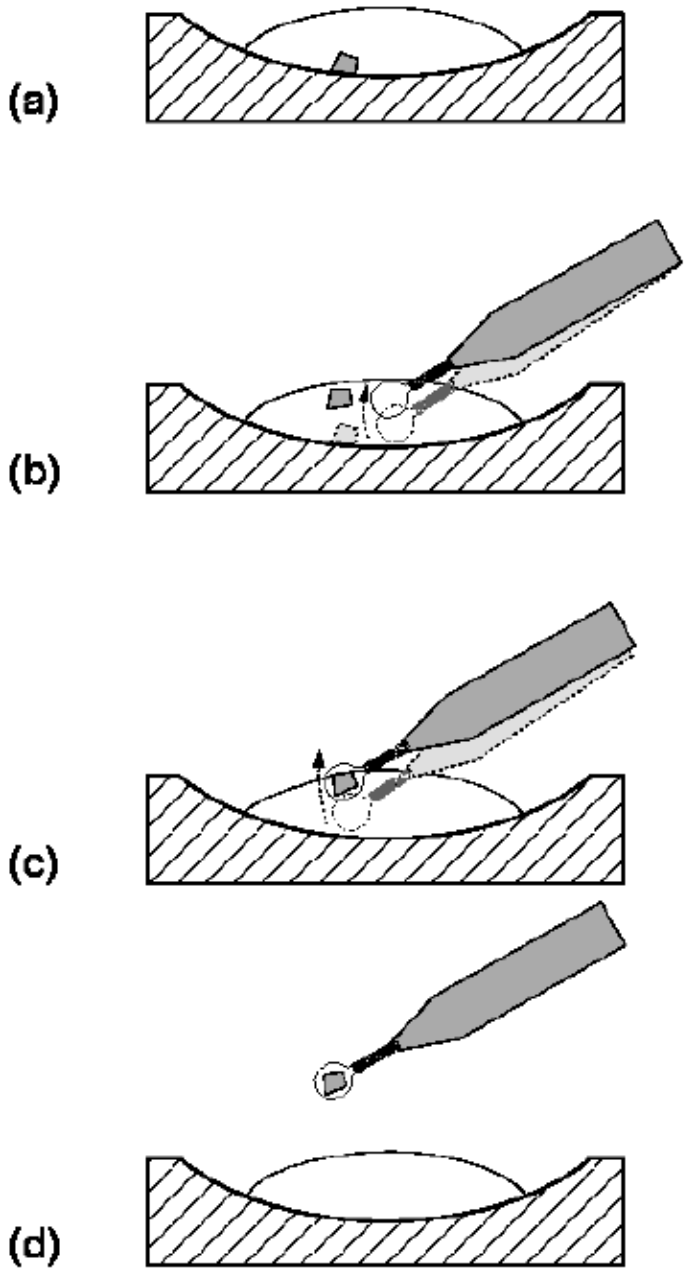
- 96 well plates –small volumes!
- Round bottom wells MUCH easier than square cross sections
- Dehydrate quickly when you take off the sticky tape lid: easy access frame, https://cgf-biotech.de/easy-access-frame/
- Fits MRC plates



Fishing:

- minimise handling
- minimise liquid round crystal
- fish near cryogen
- loop perpendicular to liquid

N.B. Acupuncture needles. Salt crystals in loop.



Garman & Schneider J.App.Cryst. 1997

Flash cool into cryogen:

- Straight into liquid nitrogen:

  have a standard pin length AND

  blow/flap away surface cold nitrogen from FULL Dewar.

- Stream cool into nitrogen gas stream held at around 100K: pre-align pin.

  have a standard pin length!

PRACTICE!!!

(but not with your most precious crystals)

Fishing: what can go wrong?

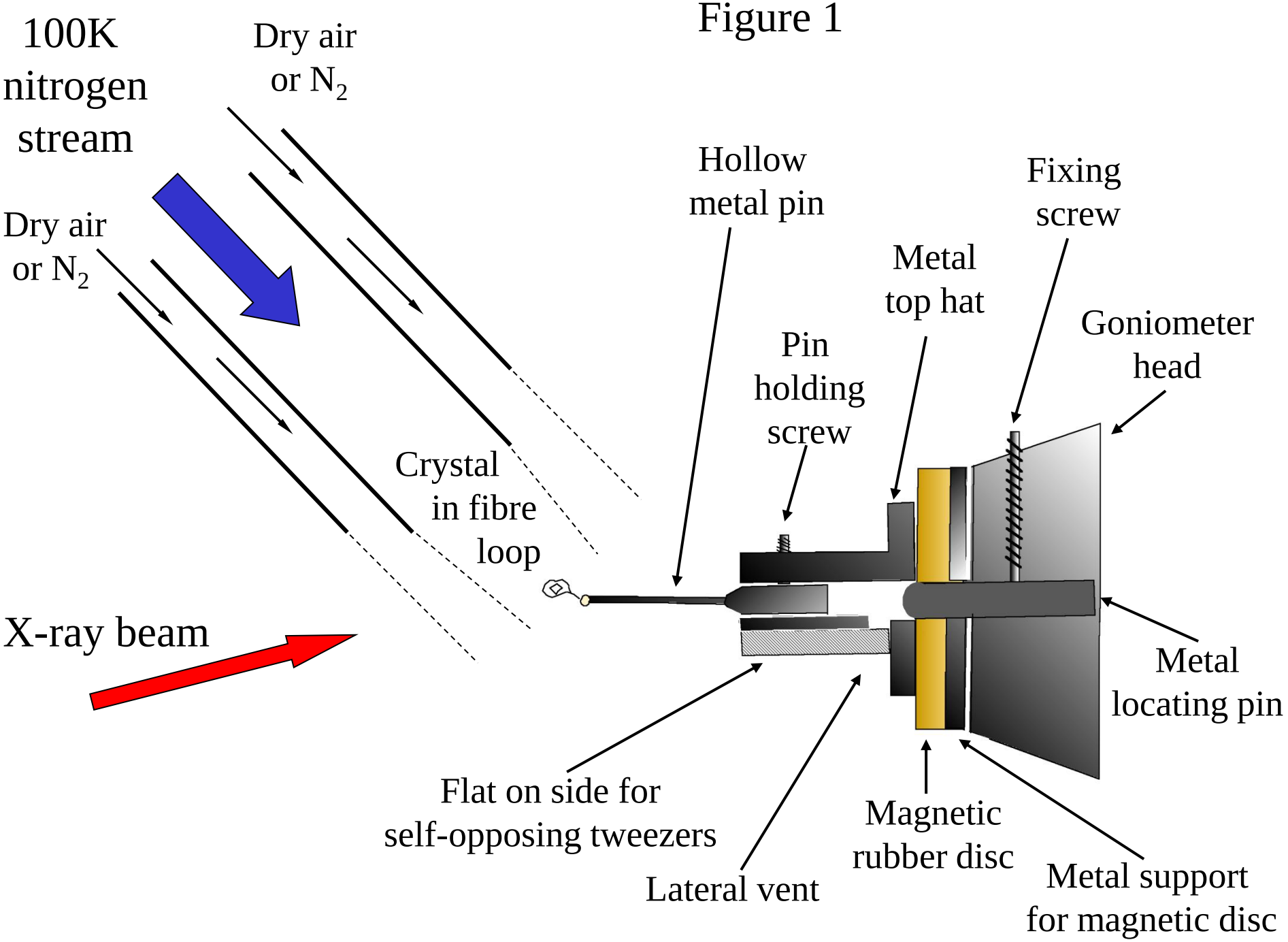
- Skin on drop
- Crystal stuck to plastic
- Loop size is wrong
- Takes too long: drop dries out
- Damage crystals trying to catch them
- Too much solvent on loop

Humidity device to avoid dehydration during mounting

Especially effective for those mother liquors which phase separate when crystallisation drop is opened.

[Farley et al., Juers Acta Cryst D (2014) 70:2111-24]

Figure 1


100K nitrogen stream
Dry air or N_2
Dry air or N_2
Hollow metal pin
Fixing screw
Metal top hat
Goniometer head
Pin holding screw
Crystal in fibre loop
X-ray beam
Metal locating pin
Flat on side for self-opposing tweezers
Lateral vent
Magnetic rubber disc
Metal support for magnetic disc

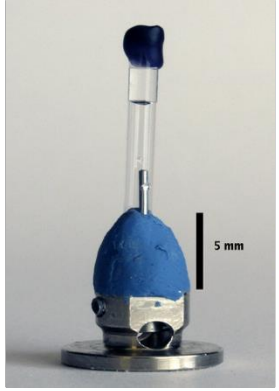
DIFFRACTION?

- NO …

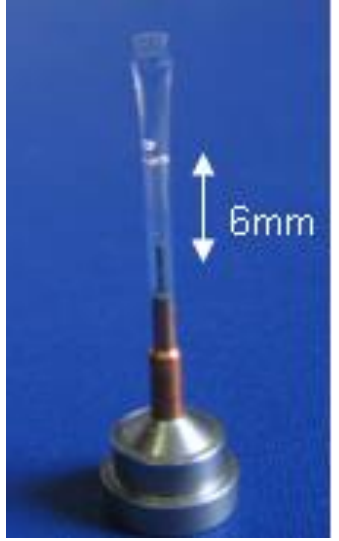
  Does the crystal diffract at room temperature??

NO	YES
↓	↓
Back to crystallisation trials	Change cryo-protocol

- YES… then take data and/or store it in a Dewar.



The Plan:

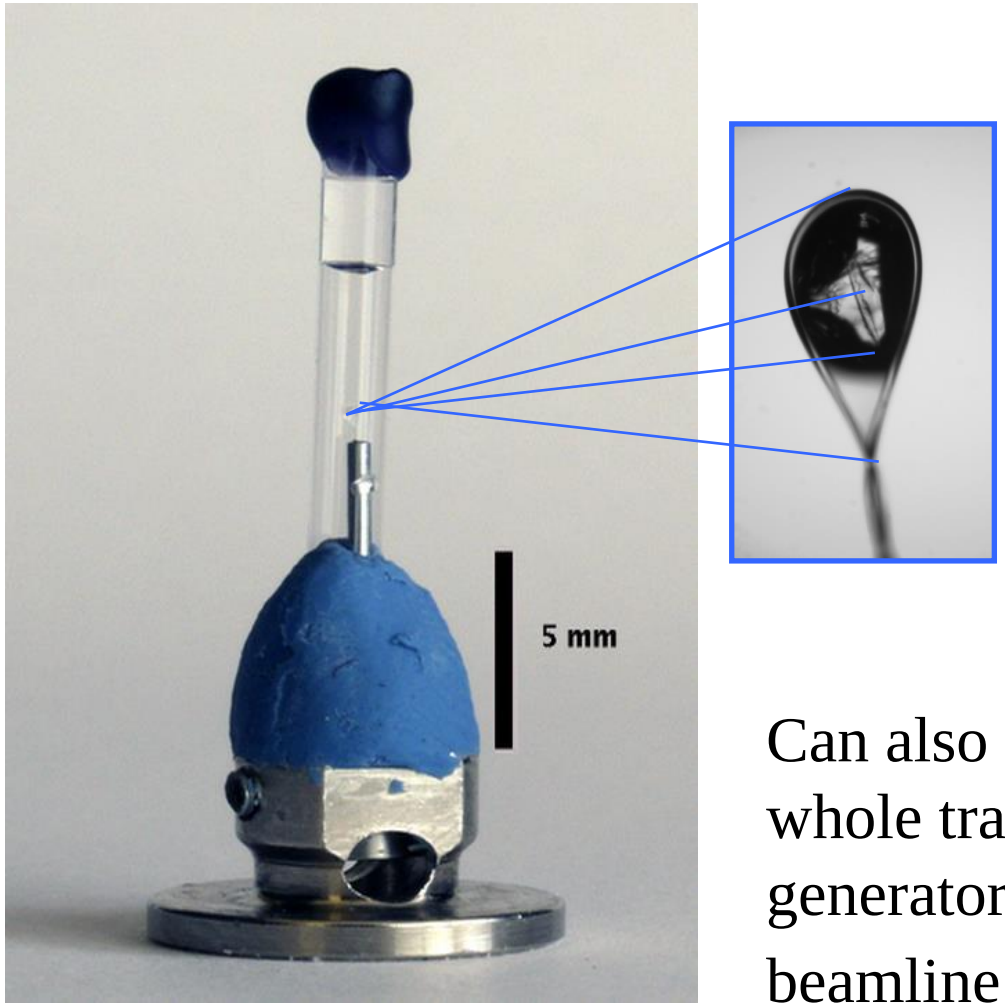


- Cryo techniques
  - Why cool? Radiation damage.
  - Optimising cryoprotection.
  - **Testing at room temperature.**
  - Storage and retrieval.
  - If nothing works…

Easy RT mounting method

Allows protein crystals to be mounted at room temperature in loop.

Skrzypczak-Jankun *et al* Acta. Cryst. (1996) **D52**, 959

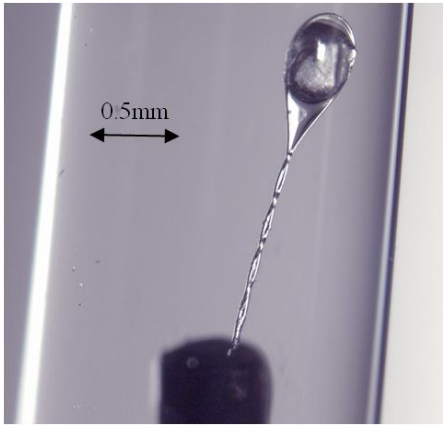


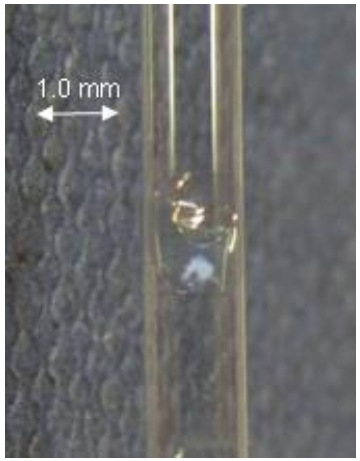
Capillary prevents drop drying out at room temperature.

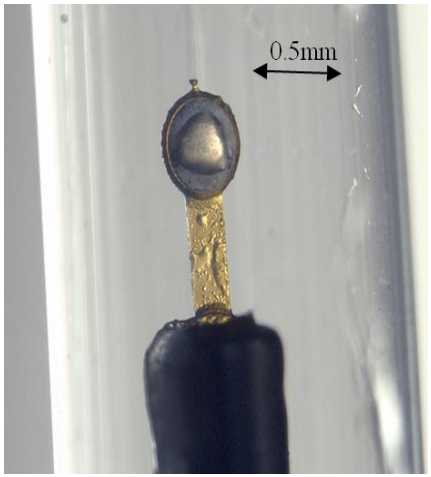
Also eliminates crystal manipulation between room temperature and cryo-cooled datasets.

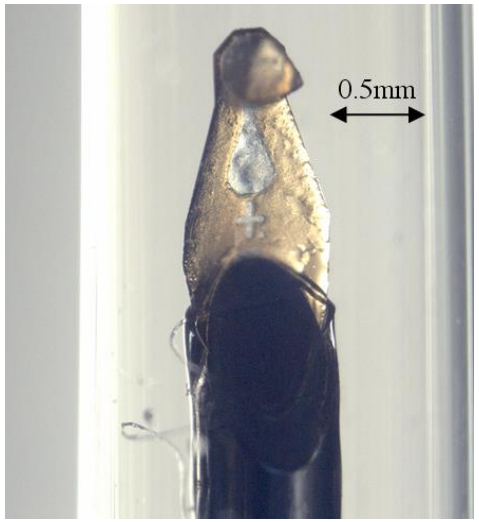
Can also mount whole tray on X-ray generator or on beamline.

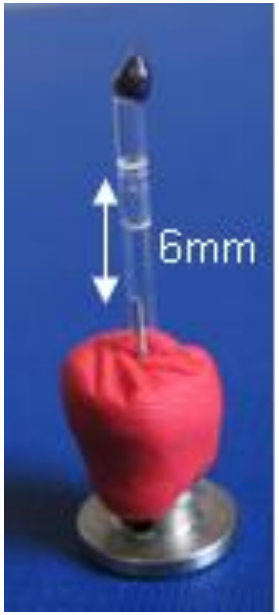
Room temperature (RT) crystal mounting methods

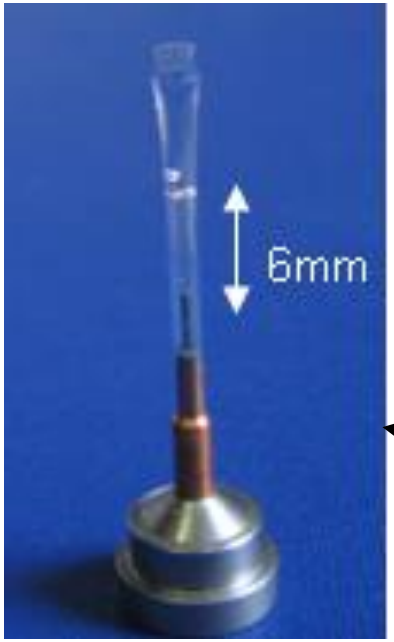




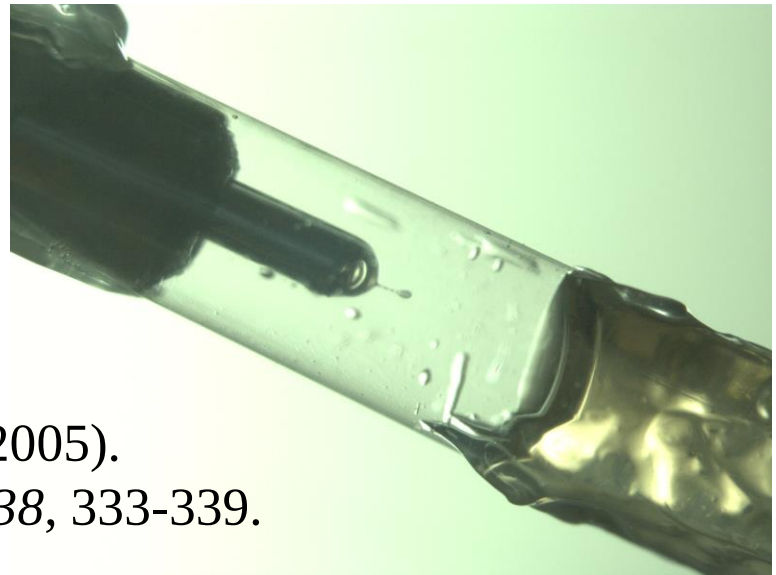






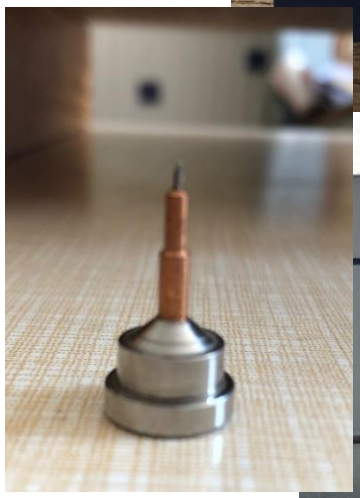


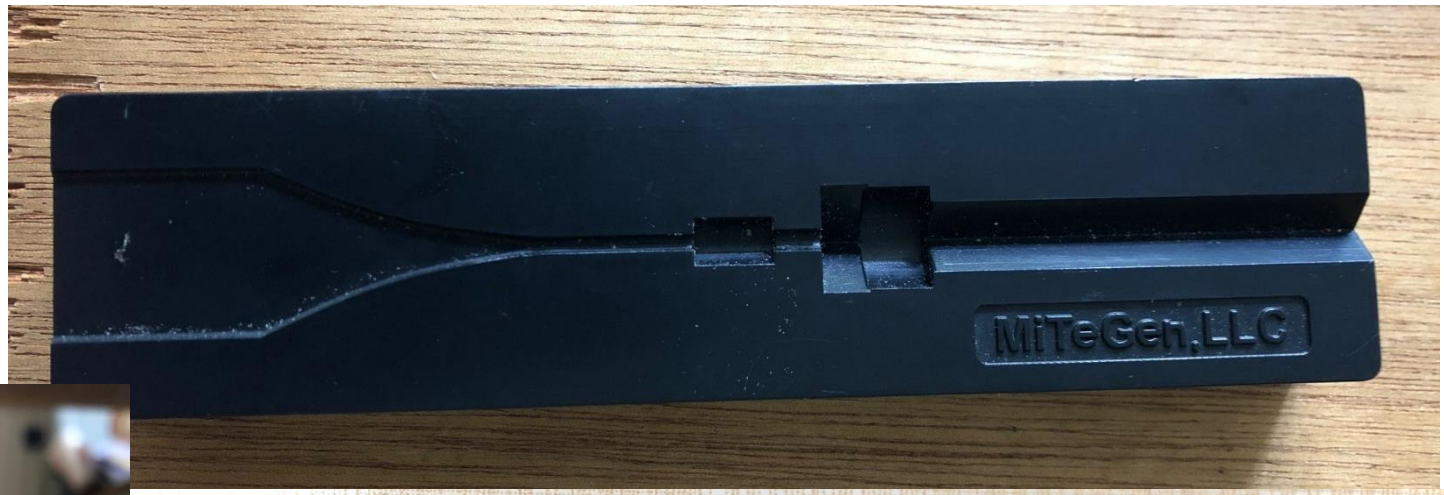
MiTeGen



Kalinin *et al*. (2005).
J. App. Cryst. *38*, 333-339.

RT mounting





The black MiTeGen plastic former helps you thread the thin plastic sleeve over the copper pin holding the crystal.
Then seal the bottom of the tube against the pin with grease.

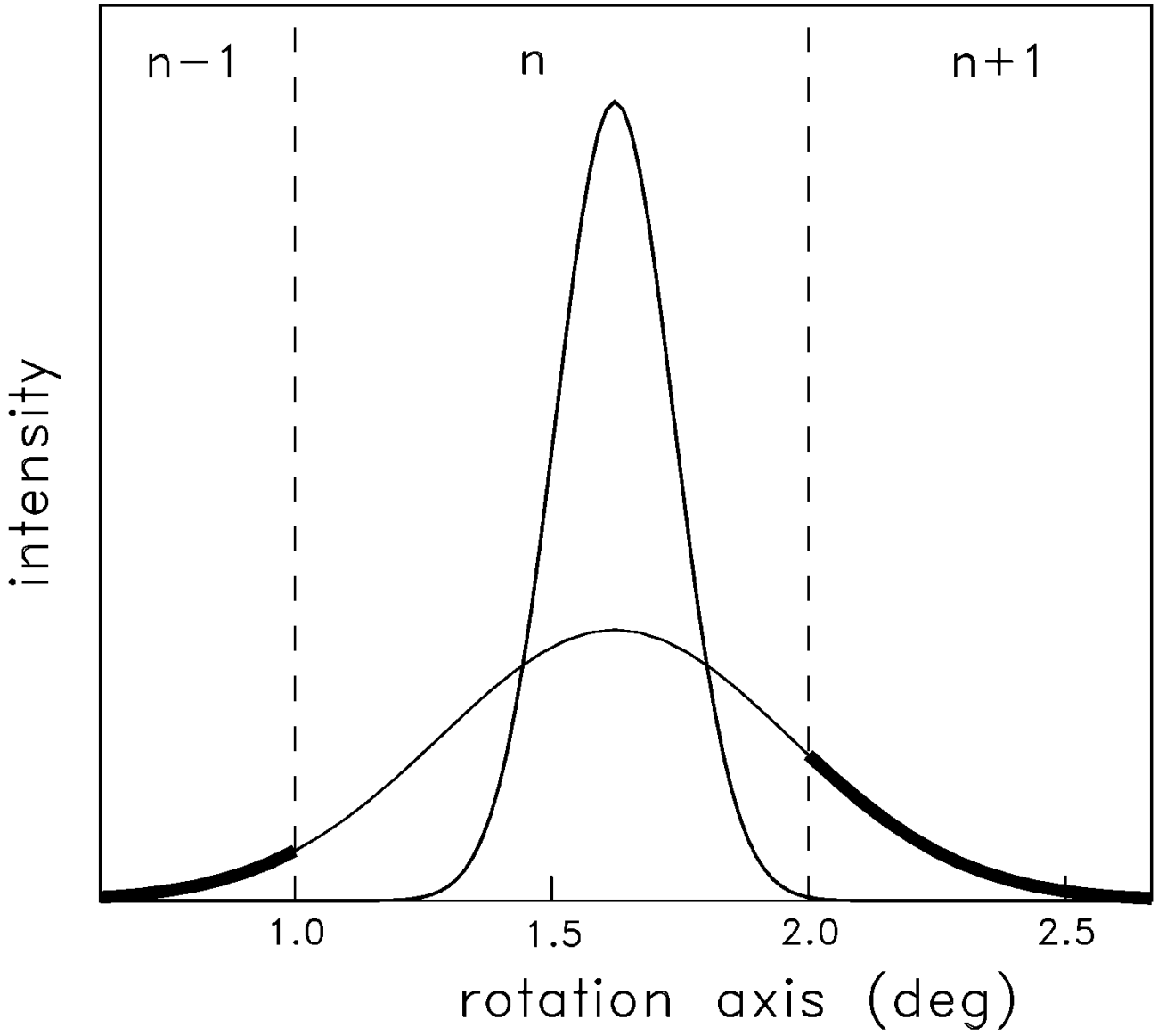
MOSAICITY:

- Want to minimise mosaicity but it often increases from RT values.
- Are cryoconditions optimised?
  - solutions
  - transfer
- Speed of transfer to cryogen
- Speed of cooling
- Size of crystal:

Should be able to reproduce RT mosaicity [need to know it!].

Minimise mosaic spread to optimise data quality.

Should be able to reproduce room temp mosaicity [need to know it!].



Cryo-protocol Optimisation:
maximising resolution, [minimise mosaicity]

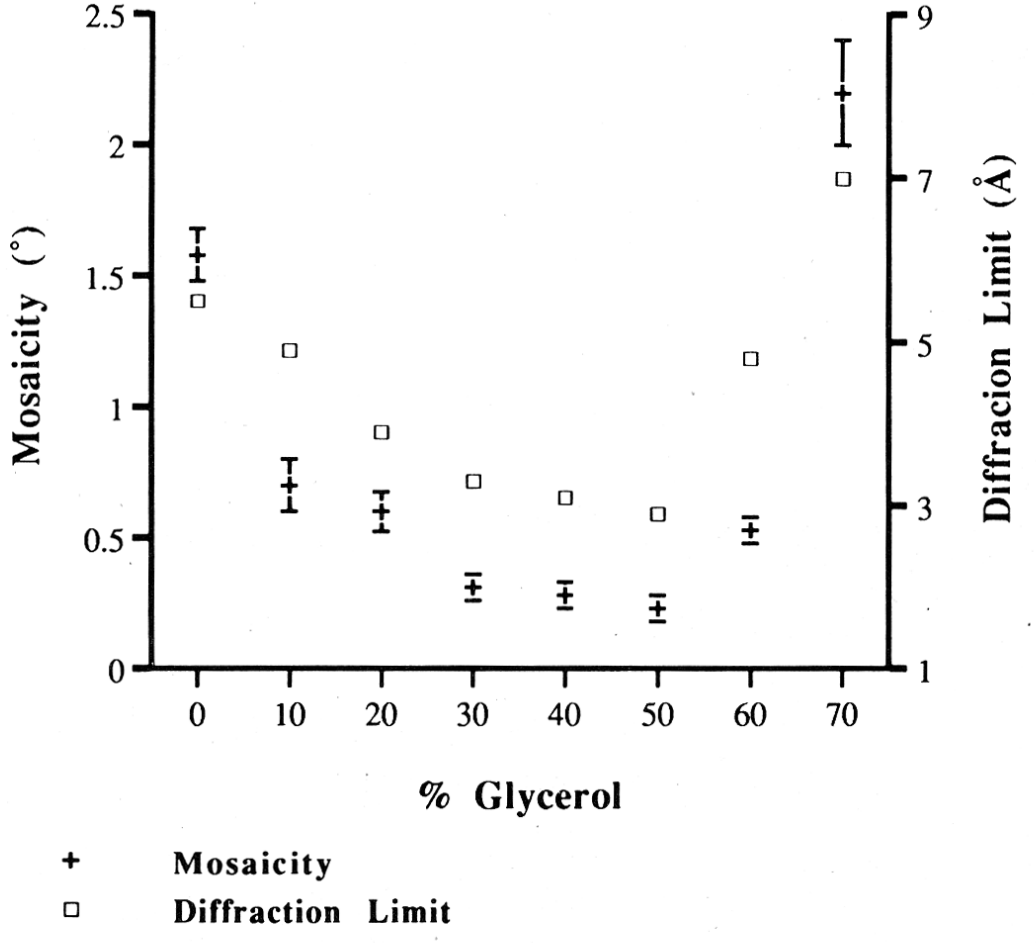


Fig. 2. Variation of mosaicity and diffraction limit of GPb crystals with percentage of glycerol in the buffer. The error bars represent statistical counting errors only.

Mitchell and Garman, J.Appl.Cryst. (1994) 27, 1070-1074

The Plan:

- Cryo techniques
  - Why cool? Radiation damage.
  - Optimising cryoprotection.
  - Testing at room temperature.
  - **Storage and retrieval**
  - If nothing works…

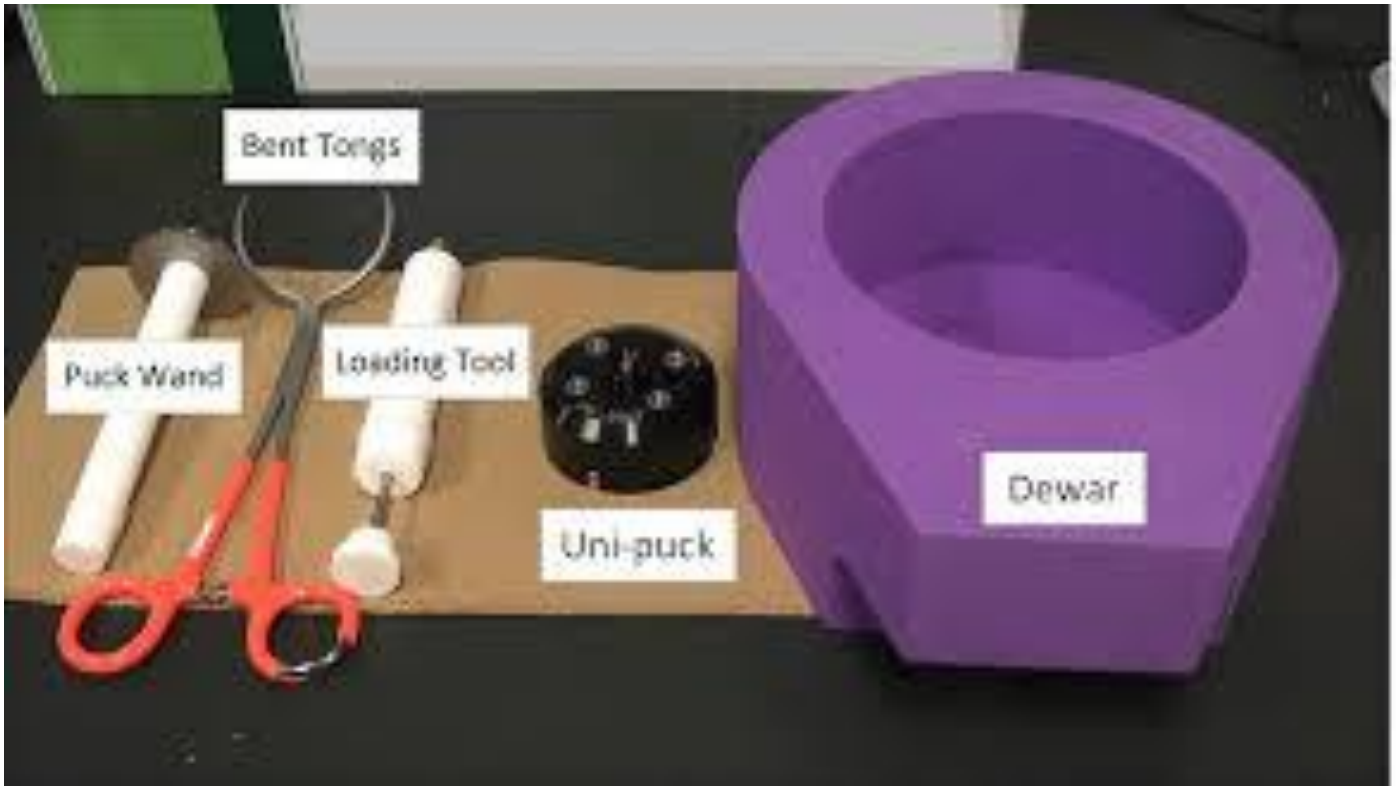
Crystal
storage
and
retrieval

Copyrighted
Gary Larson
Cartoon showing
Man pulling freezer
power out of wall:
don’t forget to fill
up your storage
Dewars regularly!

Hyperquenching: want a FAST cool

Keep Dewar full or remove gas layer from above liquid.

ALSO **keep hand moving** in liquid nitrogen to escape insulating bubble layer round sample.



[Warkentin et al., Thorne, J. Appl. Cryst. (2006) 39, 805]

STORAGE:

- Label the vials beforehand.
- Label the canes.
- Allow transport Dewars to dry out after each trip.

Dewar drying rack, Dept Biochemistry, Oxford, UK

Excellent Dewar testing protocol

http://smb.slac.stanford.edu/facilities/hardware/cryotools/shipping-dewar-testing.html

GOOGLE search (first hit):

ssrl dewar testing

[See also Owen, Pritchard & Garman. *J. Appl.Cryst.* (2004) **37**, 1000-1003]

Change storage LN_2 every 3 months.

Crystal Handling under LN2

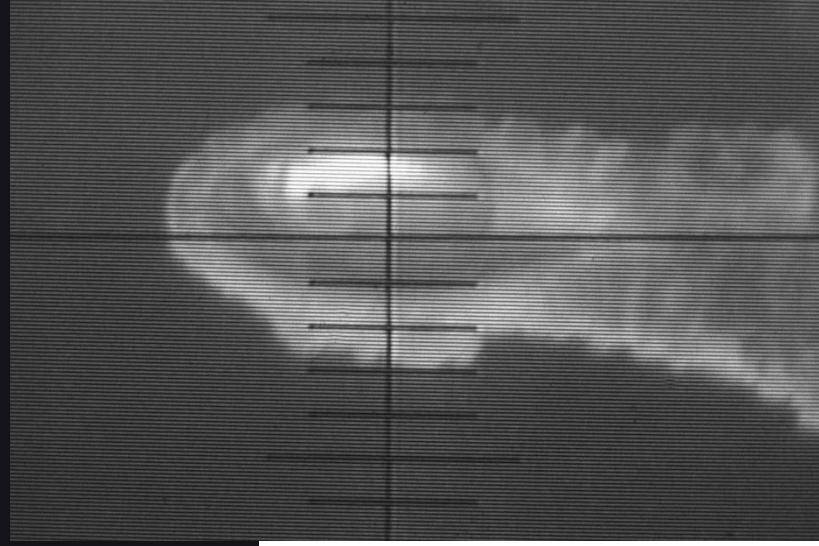
- Use appropriate tools
- Have a small working Dewar: change the LN2 in it often.
- Wait for LN2 to stop bubbling before trying any manipulations.
- Only move one hand at a time
- Steady the stationary hand on edge of Dewar

Step by step instructions:

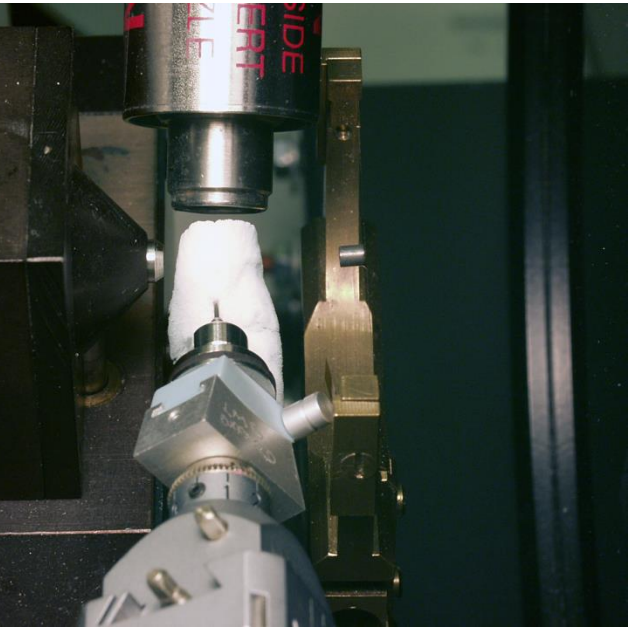
http://www.oxcryo.com/about/lmb-guide/

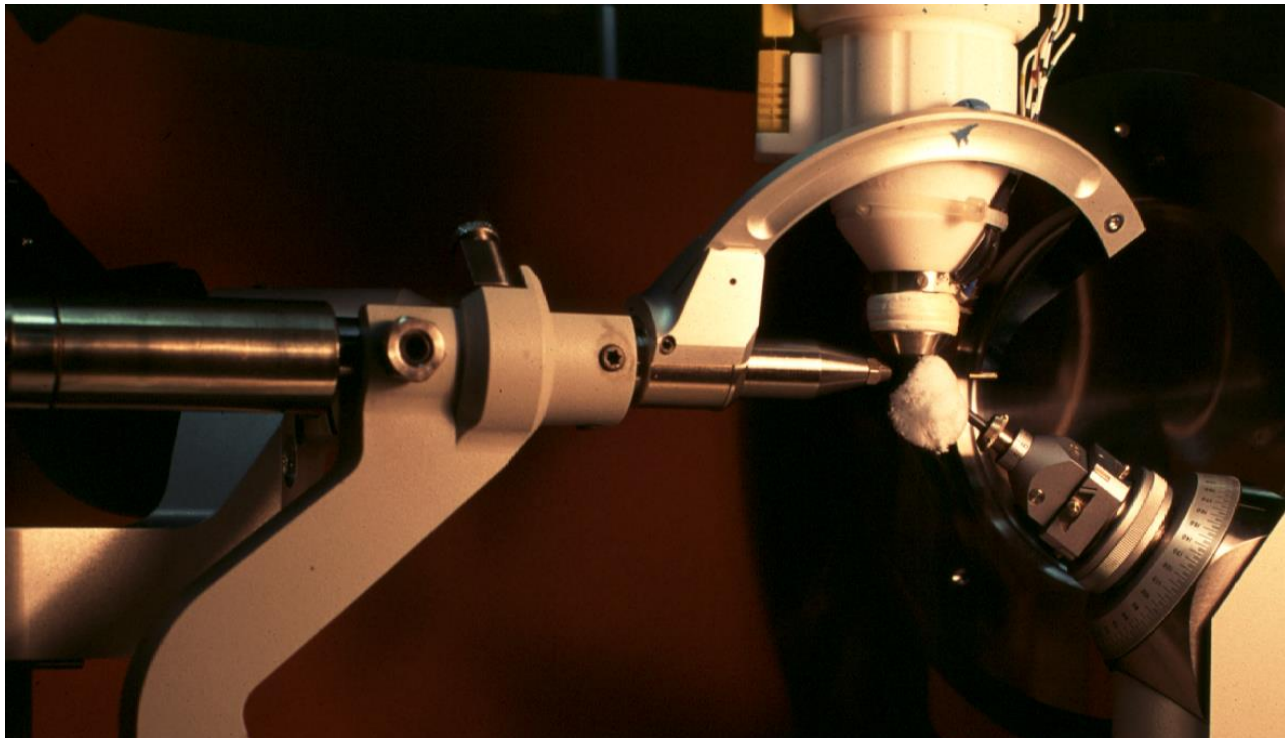
SAFETY: gloves and goggles

What am I doing wrong?



Absolutely NO ice of ANY sort.





CRYSTAL SOAKS:

- Use same cooling protocol
  [soak time, solution concentrations]
  BEST to use same human being too!

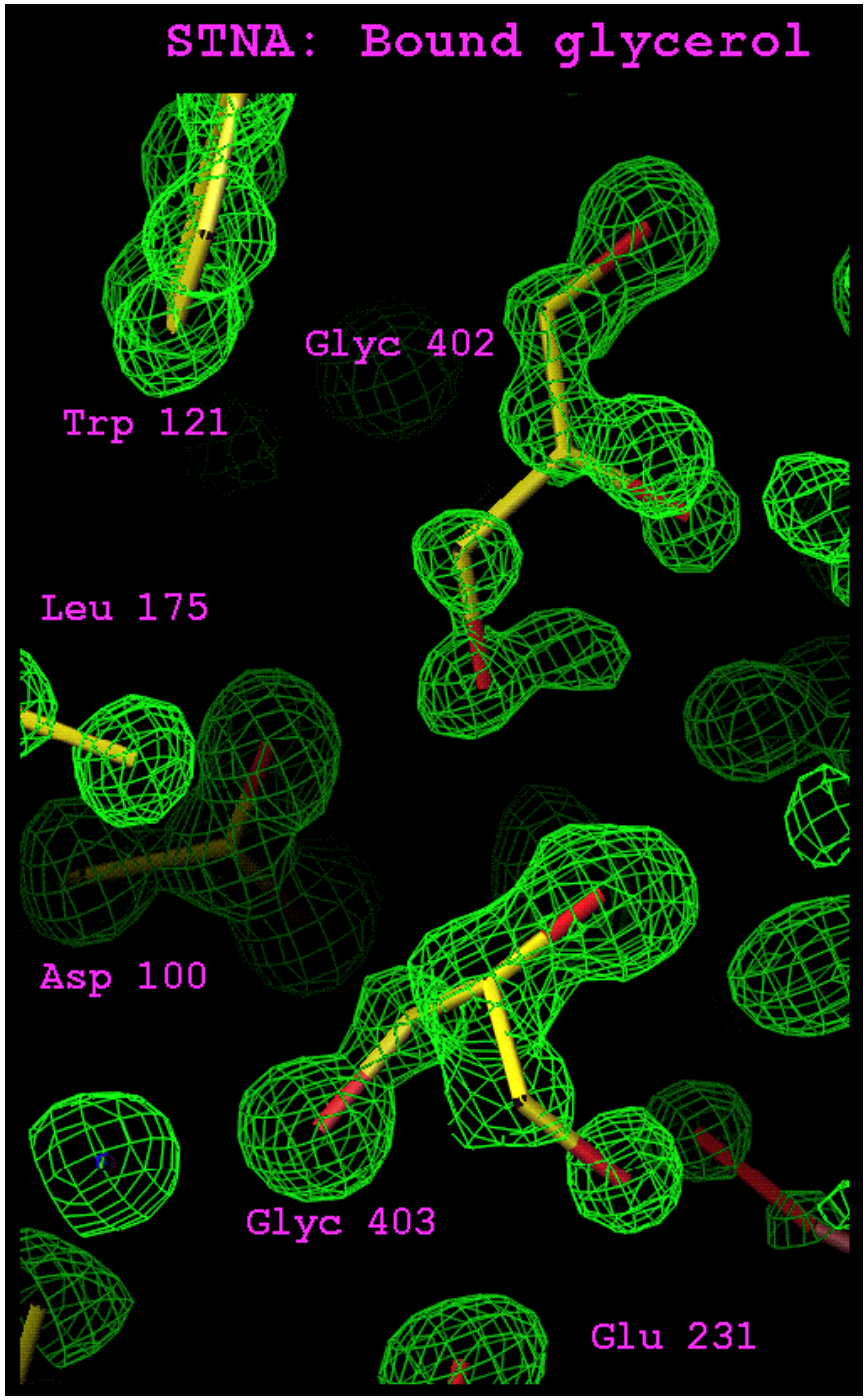
- MIR/substrate- put it in the cryo-buffer too:
  N.B.competitive inhibition with cryo-agent.

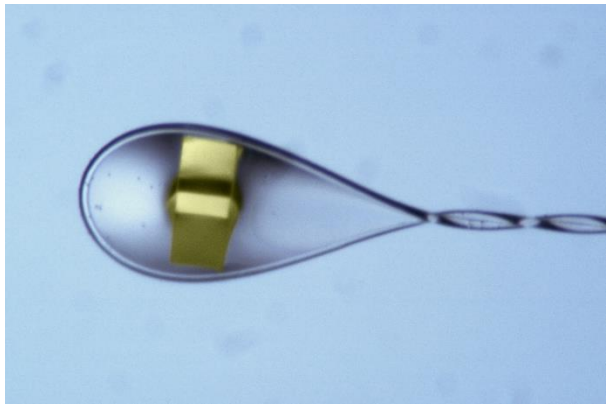
- Non-isomorphism: MR to native first

[Crick and Magdoff 1956]

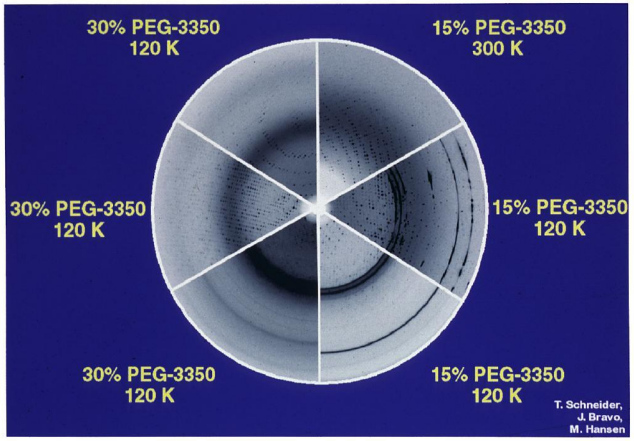
Bound cryoprotectant agent.

Beware competitive inhibition with wanted substrate. Put substrate in cryo-buffer.





The Plan:

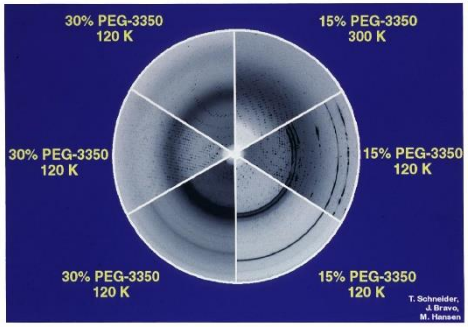


- Cryo techniques
  - Why cool? Radiation damage.
  - Optimising cryoprotection.
  - Testing at room temperature.
  - Storage and retrieval.
  - **If nothing seems to work….**

If nothing seems to work:

- **Diffraction at room temperature?**
- **Cryo-solutions**
- **Transfer/handling/soaking – vary time and temperature [e.g. try 4º overnight]**
- **Cryogen choice: nitrogen gas/liquid**
- Osmolarity matching
- Crystal annealing
- Swap buffer
- Dehydration
- Try more than once.

If nothing seems to work:



- Diffraction at room temperature?
- Cryo-solutions
- Transfer/handling/soaking – vary time and temperature [try 4º overnight]
- Cryogen choice
- **Osmolarity matching: Garman (1999) Acta D55, 1641-1653.**
- **Crystal annealing**
- Swap buffer
- Try more than once.

Copyrighted
Gary Larson
Cartoon ‘Slug
vacation disasters’.
Slugs on way to
Great Salt Lake,
Showing effect of
osmotic pressure
differentials across a
membrane…

Osmolarity matching:

1) Look up osmotic pressure of mother liquor in CRC Handbook of Physics and Chemistry, Section D-232. 11th column is O: Os/kg

2) Look up o.p. of cryoprotectant agent.

3) Modify conc. of mother liquor to minimise change in osmotic pressure.

Osmotic shock: compresses crystal $\Rightarrow$ cracks

$\Rightarrow$Mosaic spread increases $\Rightarrow$ resolution lower.

`Quick dips' give greatest osmotic shock but minimise time for cryoprotectant attack.

Crystal annealing: remove ice, reduce mosaicity and increase resolution

- Block stream temporarily (1-10 secs) OR
- Put crystal back in cryo-buffer solution.
- Then flash cool again.
- Worth a try (can repeat several times).
- Works sometimes.

  [misnomer: slow heat and fast cool is really `tempering']

Yeh and Hol (1998) *Acta Cryst*. D54, 479- 480.

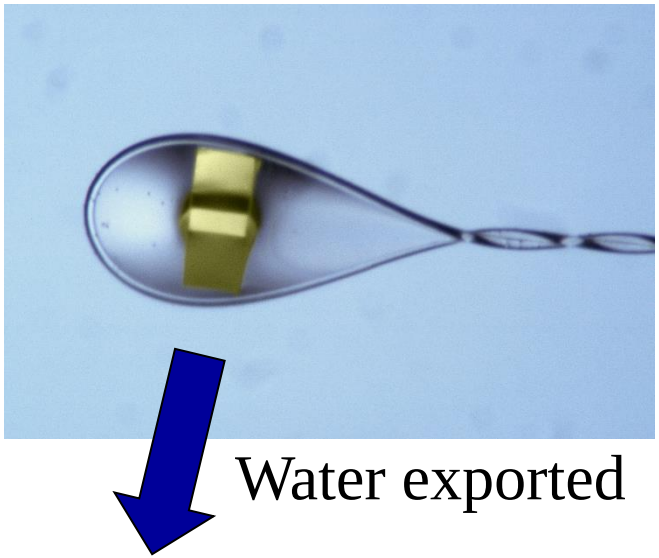
Harp *et al*. (1998) *Acta Cryst* D54, 622-628, and (1999) D55, 1129-1134.

Hanson *et al*. (2003) *Meth Enzym* 368, 217

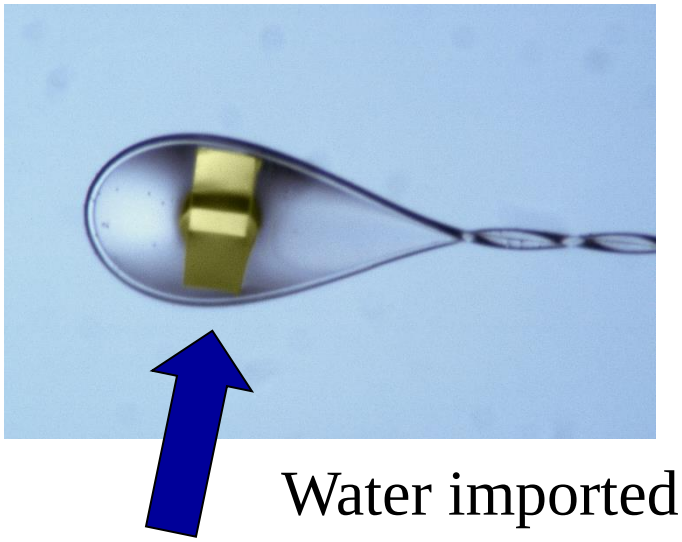
Understanding why annealing sometimes works.

Cryoprotectant agent concentration: match contraction of lattice to contraction of bulk solvent to avoid lattice distortion, higher mosaicity etc.

Not enough cryoprotectant



Too much cryoprotectant



Juers and Matthews, Acta Cryst (2004) 412-421.

If nothing seems to work:

- Diffraction at room temperature?
- Cryo-solutions
- Transfer/handling/soaking – vary time and temperature [4º]
- Cryogen choice
- Osmolarity matching
- Crystal annealing
- **Swap buffer**
- **Dehydration**
- **Try more than once**.

Dehydration (if desperate!)

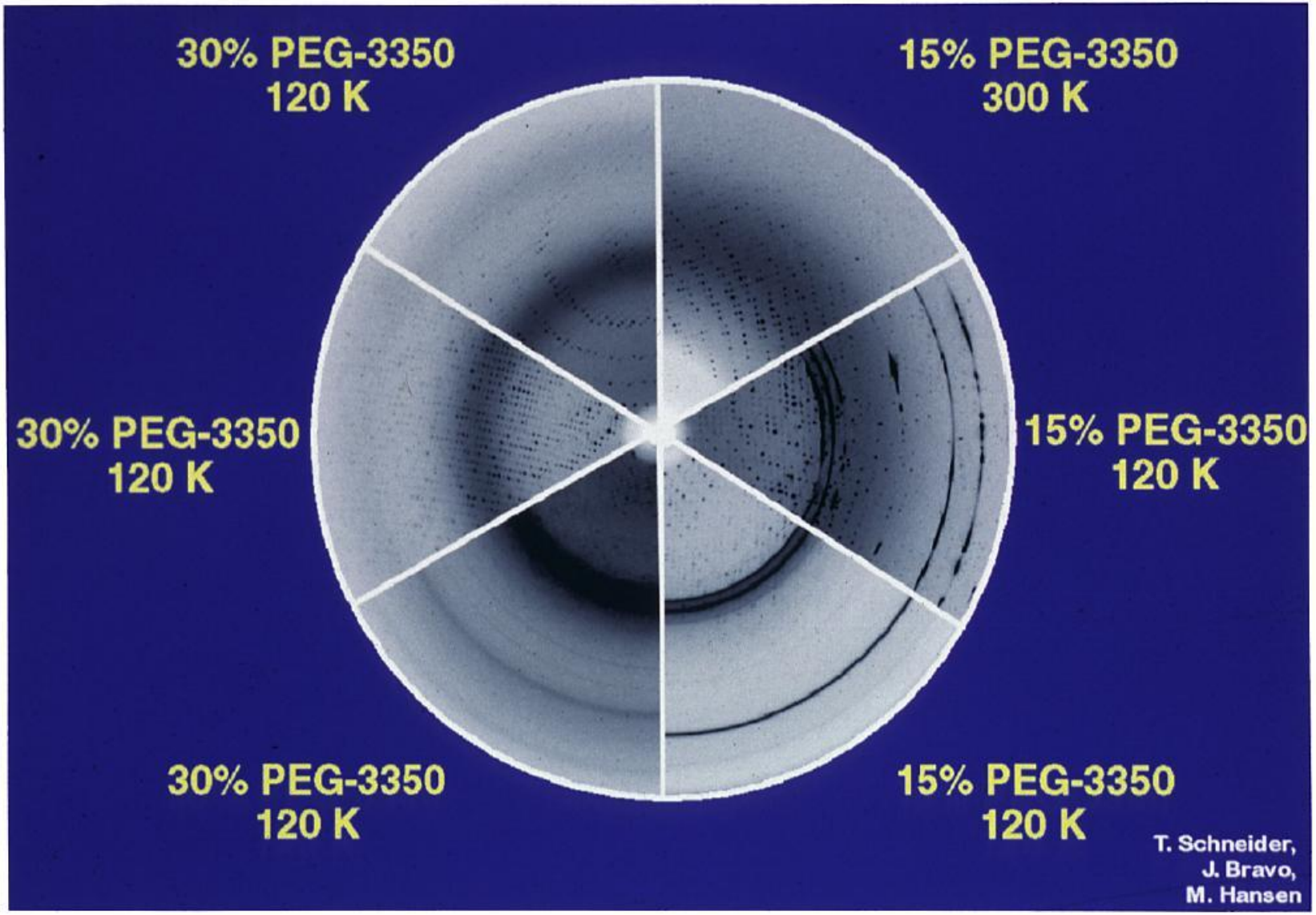
- Solvent channels effect:

https://www.researchgate.net/publication/51655454_Direct_cryocooling_of_naked_crystals_Are_cryoprotection_agents_always_necessary

- Combined dehydration and dry mounting results:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4346222/

Have at least 6 goes…



CRYO-COOLING:

Advantages

- Reduced radiation damage rate (~ factor 70).
- Gentler mounting
- Lower background
- Higher resolution
- Fewer crystals
- Can ship crystals
- Use crystals when ready.

Disadvantages

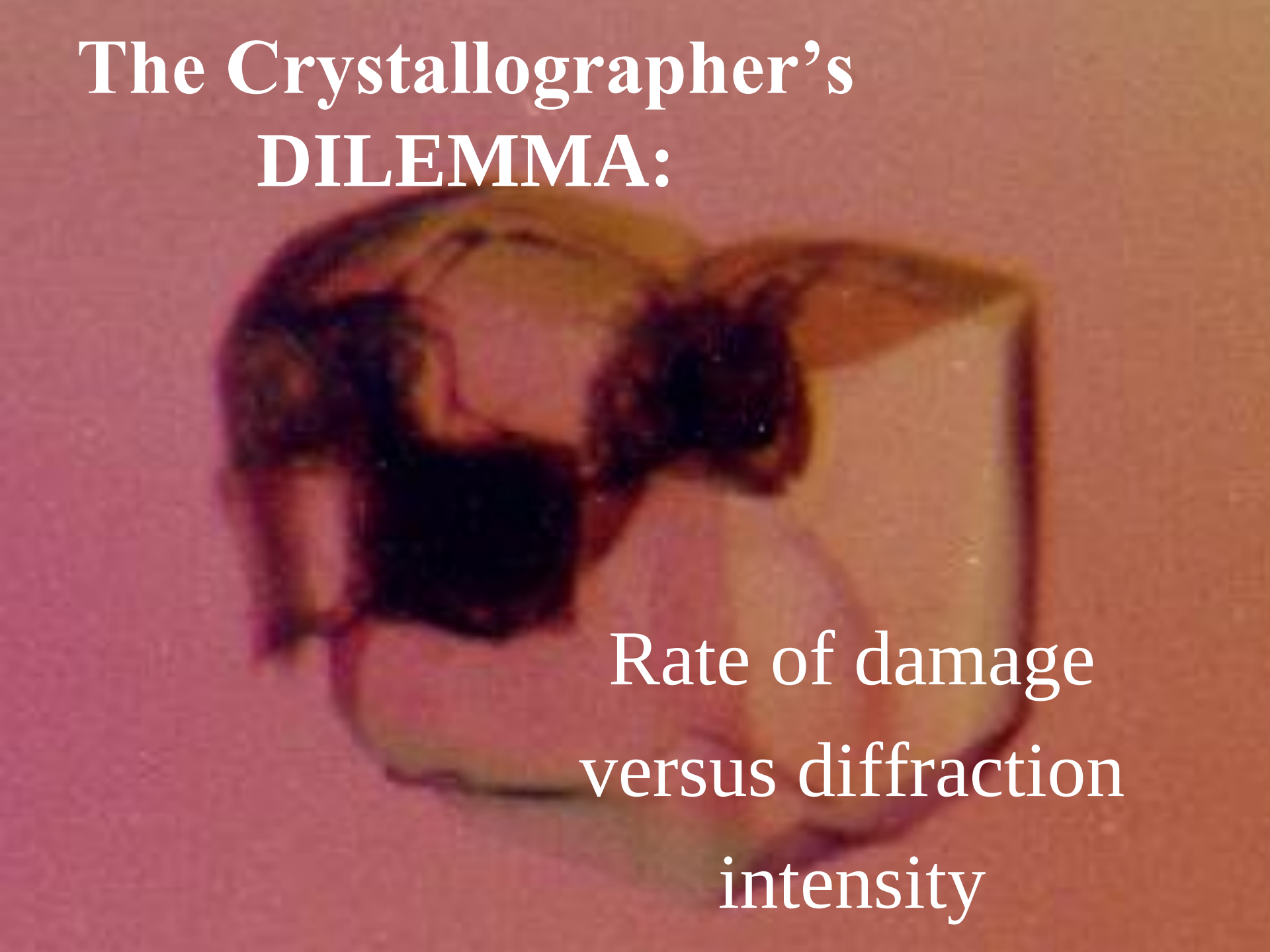
- Expensive equipment
- Increase in mosaic spread.
- Need to invest time and PRACTICE fishing!

DON'T just do what your colleagues say 'always works'. THINK about it!

Cryocrystallography: for more gory details see:

- Rodgers, D (1997) Methods in Enzymology 276, 183-203 and in International Tables of Crystallography Volume F (2001) 202-208.
- Hope, H in International Tables of Crystallography Volume F (2001) 197-201.
- Garman and Schneider (1997) J.Appl. Cryst, 30, 211-237.
- Parkin & Hope (1998) J.Appl. Cryst. 31, 945-953.
- Garman (1999) Acta D55, 1641-1653.
- Garman & Doublié (2003) Methods in Enzym. 396, 188-216.
- Garman (2003) Curr. Opin. Struct. Biol. 13, 545-551.
- Garman & Owen, (2006) *Acta Cryst. D* 62, 32-47.
- Pflugrath (2015) *Acta Cryst F* 71, 622-642.

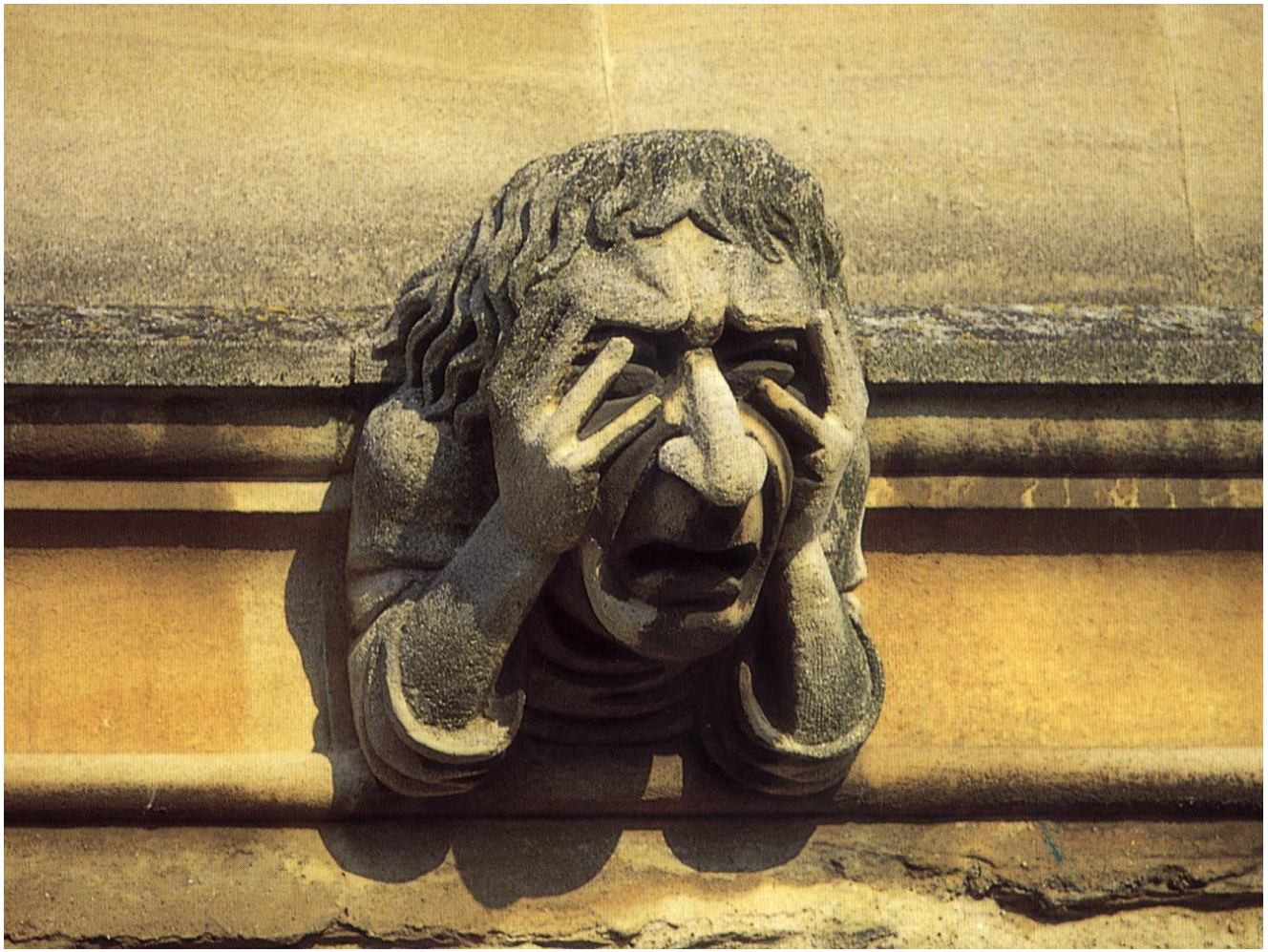
The Crystallographer's DILEMMA:



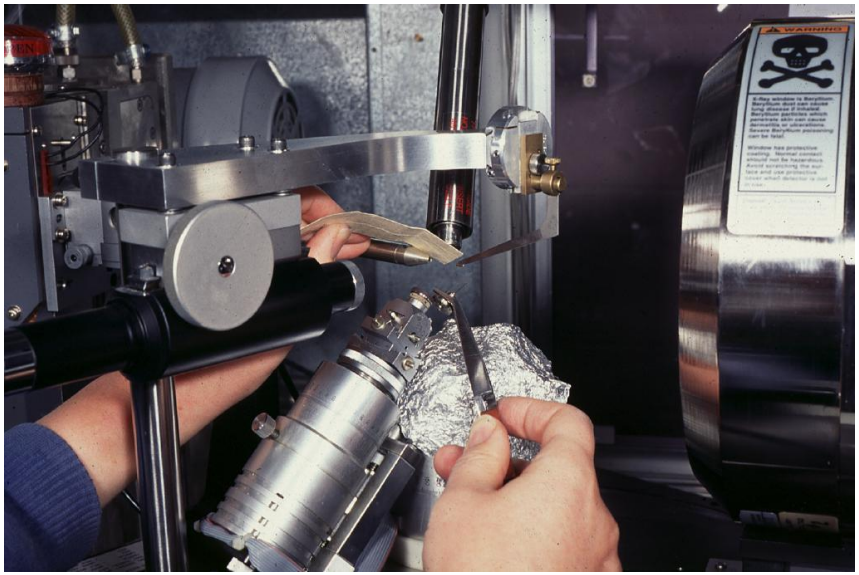
Rate of damage versus diffraction intensity

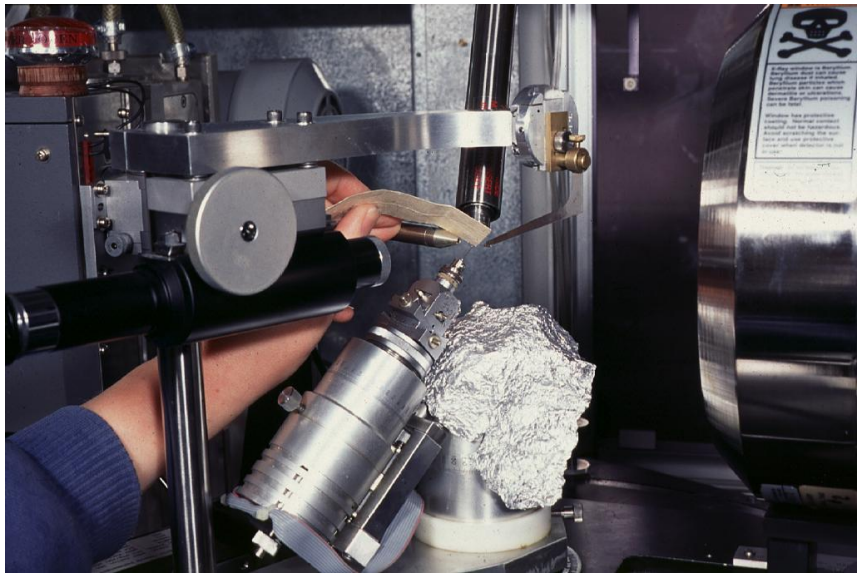
This is a Workshop so…

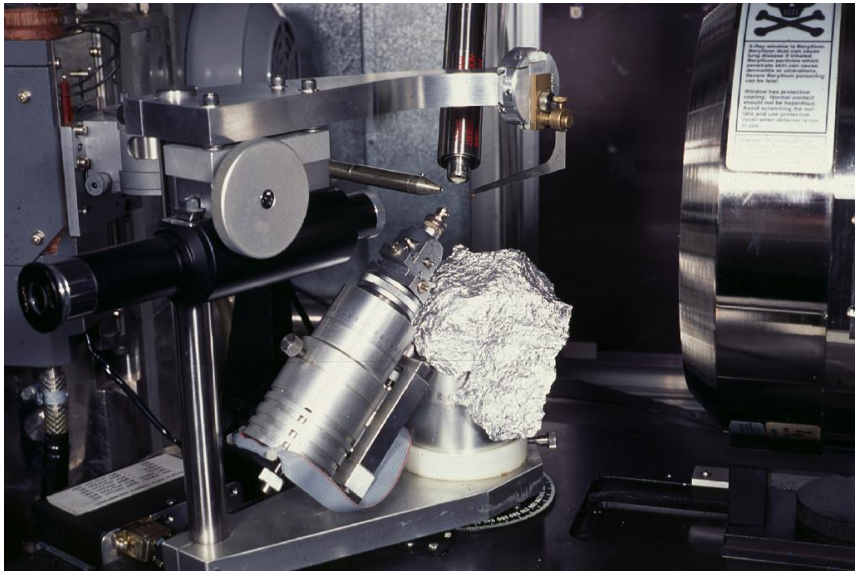
lots of questions expected and very welcome



Stream cooling







- Pre-centre loop.
- Speed of transfer from drop
- Block stream

[avoid dehydration]

- Speed of cooling

Why bother to cover the nitrogen stream?

- Avoid crystal being dehydrated by dry nitrogen/air.
- You may wave crystal in and out of stream while cooling it: slow cooling will give ice.
- Want to cool it FAST. Much easier to do that if you whip the cover away once crystal is in position.

Osmolarity matching:

1) Look up osmotic pressure of mother liquor in CRC Handbook of Physics and Chemistry, Section D-232. 11th column is O: Os/kg

2) Look up o.p. of cryoprotectant agent.

3) Modify conc. of mother liquor to minimise change in osmotic pressure.

Osmotic shock: compresses crystal $\Rightarrow$ cracks

$\Rightarrow$Mosaic spread increases $\Rightarrow$ resolution lower.

`Quick dips' give greatest osmotic shock but minimise time for cryoprotectant attack.

23 GLYCEROL, $CH_2OHCHOHCH_2OH$

MOLECULAR WEIGHT = 92.09
RELATIVE SPECIFIC REFRACTIVITY = 1.109

0.00 % by wt. d
For Values of 0

A % by wt.	ρ D_4^{20}	D_{20}^{20}	C_v g/l	M g-mol/l	C_w g/l	$(C_v - C_w)$ g/l	$(n - n_0) \times 10^4$	n	Δ °C	O Osm/kg	S g-mol/l	η/η_0
0.50	0.9994	1.0011	5.0	0.054	994.4	3.9	6	1.3336	0.072	0.039	0.020	1.009
1.00	1.0005	1.0023	10.0	0.109	990.5	7.7	12	1.3342	0.180	0.097	0.051	1.020
2.00	1.0028	1.0046	20.1	0.218	982.7	15.5	23	1.3353	0.411	0.221	0.119	1.046
3.00	1.0051	1.0069	30.2	0.327	974.9	23.3	35	1.3365	0.627	0.337	0.182	1.072
4.00	1.0074	1.0092	40.3	0.438	967.1	31.2	46	1.3376	0.849	0.456	0.247	1.098
5.00	1.0097	1.0115	50.5	0.548	959.2	39.0	58	1.3388	1.078	0.580	0.315	1.125
6.00	1.0120	1.0138	60.7	0.659	951.3	46.9	70	1.3400	1.316	0.708	0.385	1.155
7.00	1.0144	1.0162	71.0	0.771	943.4	54.9	82	1.3412	1.561	0.839	0.457	1.186
8.00	1.0167	1.0185	81.3	0.883	935.4	62.9	94	1.3424	1.811	0.974	0.530	1.218
9.00	1.0191	1.0209	91.7	0.996	927.4	70.9	106	1.3436	2.064	1.110	0.603	1.253
10.00	1.0215	1.0233	102.1	1.109	919.3	78.9	118	1.3448	2.323	1.249	0.678	1.288
12.00	1.0262	1.0281	123.1	1.337	903.1	95.1	142	1.3472	2.880	1.548	0.837	1.362
14.00	1.0311	1.0329	144.4	1.568	886.7	111.5	167	1.3496	3.469	1.865	1.004	1.442
16.00	1.0360	1.0378	165.8	1.800	870.2	128.0	191	1.3521	4.094	2.201	1.177	1.530
18.00	1.0409	1.0428	187.4	2.035	853.6	144.7	217	1.3547	4.756	2.557	1.359	1.627
20.00	1.0459	1.0478	209.2	2.272	836.8	161.5	242	1.3572	5.46	2.93	1.546	1.734
24.00	1.0561	1.0580	253.5	2.752	802.6	195.6	294	1.3624	7.01	3.77	1.944	1.984
28.00	1.0664	1.0683	298.6	3.243	767.8	230.4	347	1.3676	8.77	4.71	2.370	2.274
32.00	1.0770	1.0789	344.6	3.742	732.3	265.9	400	1.3730	10.74	5.78	2.814	2.632
36.00	1.0876	1.0896	391.5	4.252	696.1	302.2	455	1.3785	12.96	6.97	3.276	3.082
40.00	1.0984	1.1003	439.4	4.771	659.0	339.2	511	1.3841	15.50	8.33	3.757	3.646
44.00	1.1092	1.1112	488.1	5.300	621.2	377.1	567	1.3897				4.434
48.00	1.1200	1.1220	537.6	5.838	582.4	415.8	624	1.3954				5.402
52.00	1.1308	1.1328	588.0	6.385	542.8	455.4	681	1.4011				6.653
56.00	1.1419	1.1439	639.4	6.944	502.4	495.8	739	1.4069				8.332
60.00	1.1530	1.1551	691.8	7.513	461.2	537.0	799	1.4129				10.66
64.00	1.1643	1.1663	745.1	8.091	419.1	579.1	859	1.4189				13.63
68.00	1.1755	1.1775	799.3	8.680	376.1	622.1	919	1.4249				18.42
72.00	1.1866	1.1887	854.3	9.277	332.2	666.0	980	1.4310				27.57
76.00	1.1976	1.1997	910.2	9.883	287.4	710.8	1040	1.4370				40.49
80.00	1.2085	1.2106	966.8	10.498	241.7	756.5	1101	1.4431				59.78
84.00	1.2192	1.2214	1024.2	11.121	195.1	803.2	1162	1.4492				84.17
88.00	1.2299	1.2320	1082.3	11.752	147.6	850.7	1223	1.4553				147.2
92.00	1.2404	1.2426	1141.1	12.392	99.2	899.0	1284	1.4613				383.7
96.00	1.2508	1.2530	1200.7	13.039	50.0	948.2	1344	1.4674				778.9
100.00	1.2611	1.2633	1261.1	13.694	0.0	998.2	1405	1.4735				1487.0

24 HYDROCHLORIC ACID, HCl

Osmotic Pressure Matching: Example

Osmality: Os/kg

- 2.0M NaCl

  50mM pH 7.8 Tris HCl 3.95

- Need 20% glycerol 2.9

  1.05

From Tables, 0.55M NaCl has o.p. 1.05 Os/kg

$\Rightarrow$Try 0.55 M NaCl, 20% glycerol

50mM pH 7.8 Tris HCl

Theoretical study: starts to rationalise experimental practice.

Heat transfer study by a proper mechanical engineer!

Most to least important factors:

1) Crystal solvent content and solvent composition.
2) Crystal size and shape.
3) Amount of residual liquid around the crystal
4) Cooling method (liquid plunge *versus* gas stream).
5) Choice of gas/liquid.
6) Relative speed between cooling fluid and crystal.

[S. Kriminski, M. Kazmierczak and R.E. Thorne

`Heat transfer from protein crystals: implications for flash-cooling and X-ray beam heating. Acta Cryst. (2003) **D59**, 697-708.]