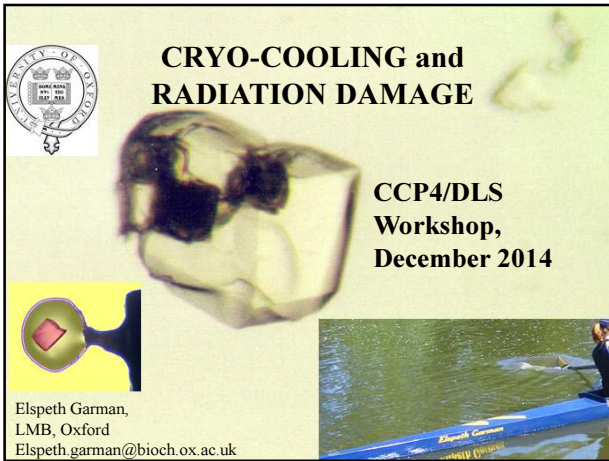


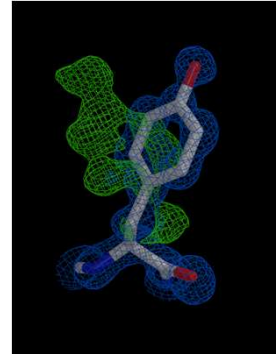
CRYO-COOLING and RADIATION DAMAGE

CCP4/DLS Workshop, December 2014

Elspeth Garman,
LMB, Oxford
Elspeth.garman@bioch.ox.ac.uk



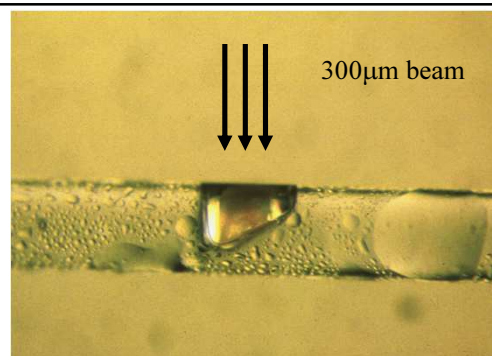
Data quality pivotally affects the amount of biological information we obtain.



Detailed biological information

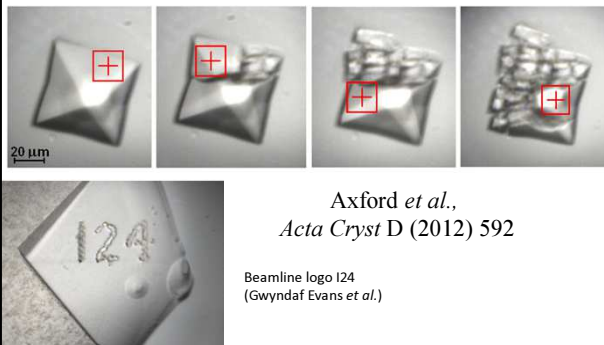
The Plan:

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

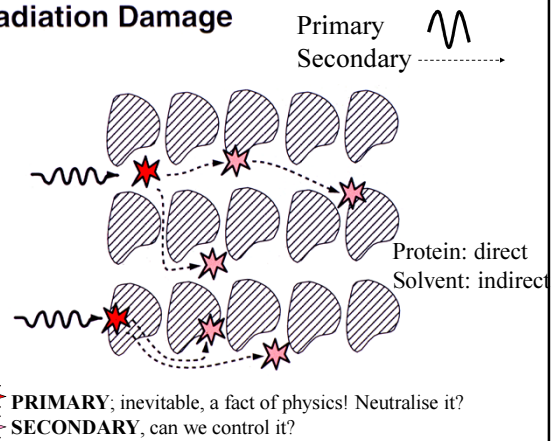


Room temperature: HEWL crystal after 3 hours in a 2nd generation synchrotron beam.

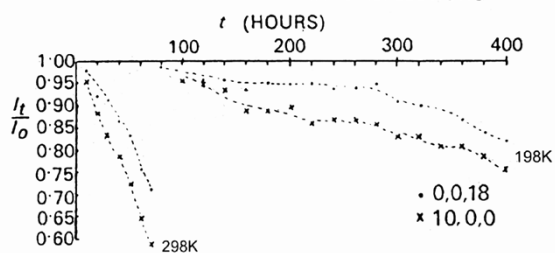
I24, Diamond, *in situ* data collection from a Bovine Enterovirus 2 crystal, room temperature, 0.5 s 20 μm x 20 μm beam



Radiation Damage



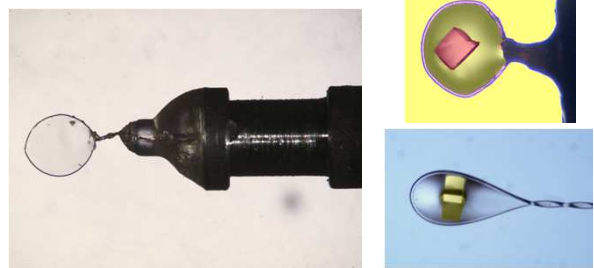
DECAY OF LDH REFERENCE REFLECTIONS



Haas and Rossmann 1970: lactate dehydrogenase
Acta Cryst B 26, 998-1004. ICE a major problem

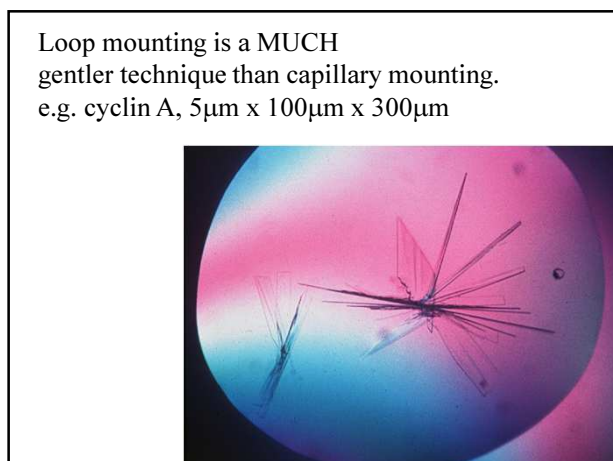
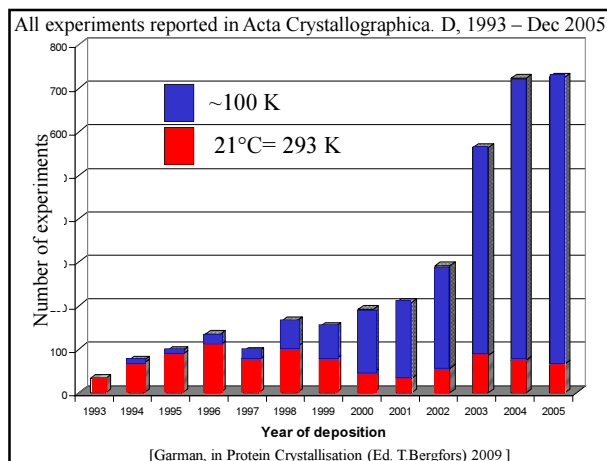
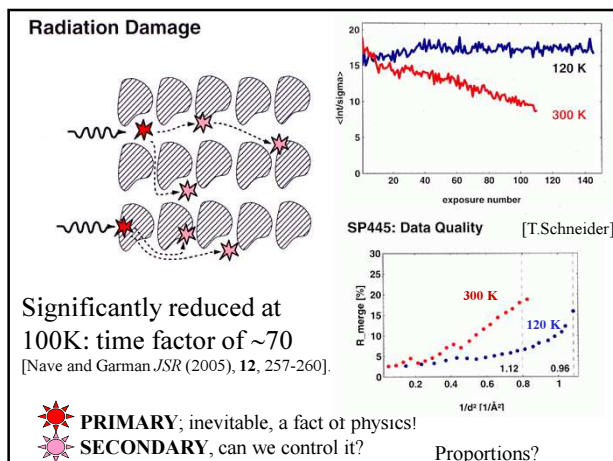
Loop mounting: T-Y.Teng (1990) *J. Appl. Cryst.* 23, 387-391.

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]



- 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.
 - No secondary radiation damage during storage.
 - New experiments are possible.

The Plan:

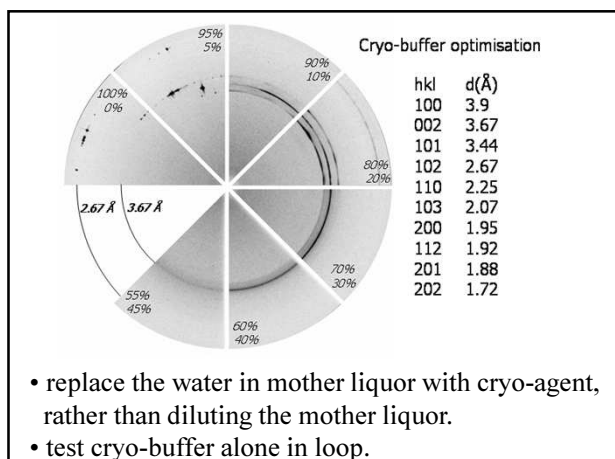
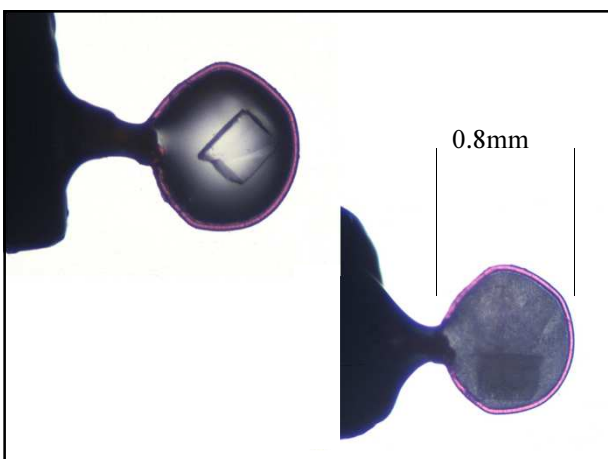
- Cryo techniques
 - Why cool? Radiation damage.
 - **Optimising cryoprotection.**
 - Testing at room temperature.
 - Storage and retrieval.
 - If nothing works...

Cryocooling: HOW?

- Cool the crystal so fast that the water is vitrified and does not form crystalline ice. [Pure water: $\sim 10^{-5}$ 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]]



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, +++

CRYSTAL MANIPULATION:



Acupuncture needles:
[free samples available from me]

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

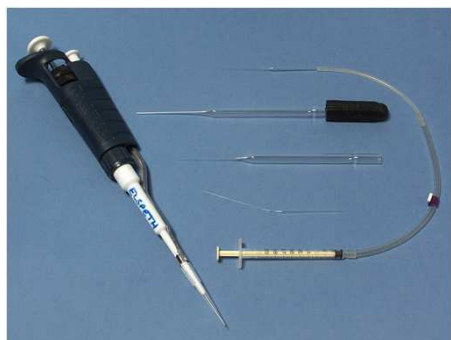
e.g. 'Skin' on protein drop:

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.



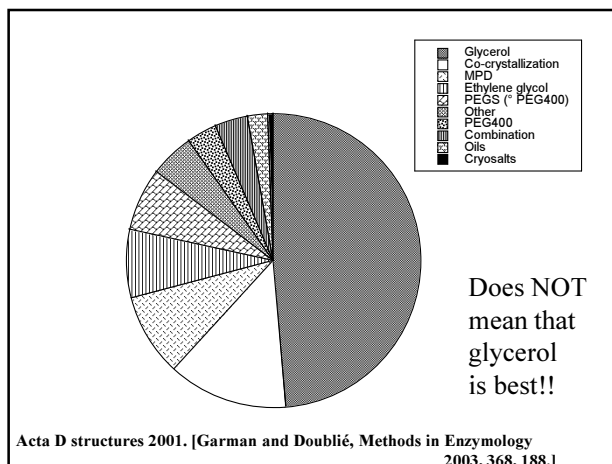
Illegible table of
minimum concentrations
of glycerol required
to cryoprotect
Hampton Screen I.

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]

TABLE 1 Minimum concentrations of glycerol to be added to solutions 1 to 10 of the Hampton Research Crystal Screen 200 (1) 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. %
1. 0.1M Mg Cl ₂	0.1M Na Acetate pH 4.5	0.1M Na ₂ S ₂ O ₈ 1.6-precipitated	0
2. None	None	0.1M Na ₂ S ₂ O ₈	20
3. None	None	0.1M Na ₂ S ₂ O ₈	20
4. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
5. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
6. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
7. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
8. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
9. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
10. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
11. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
12. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
13. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
14. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
15. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
16. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
17. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
18. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
19. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
20. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
21. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
22. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
23. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
24. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
25. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
26. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
27. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
28. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
29. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
30. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
31. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
32. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
33. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
34. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
35. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
36. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
37. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
38. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
39. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
40. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
41. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
42. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
43. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
44. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
45. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
46. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
47. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
48. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
49. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20
50. 0.1M Na Cl	0.1M Na ₂ SO ₄ pH 5.5	0.1M Na ₂ S ₂ O ₈	20



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 D* 51 (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....

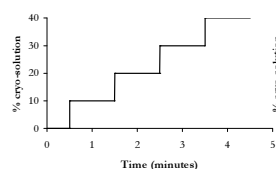
SOLUBILITY versus OSMOTIC shock

Crystal transfer optimisation:

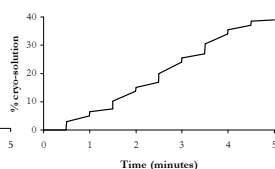
- balance osmotic shock and attack of crystal surface
- serial transfers: minimise handling and dehydration



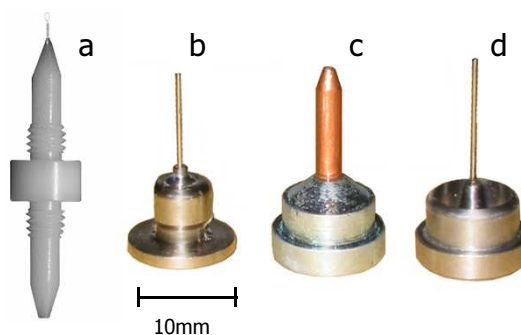
a) Move crystal between solutions

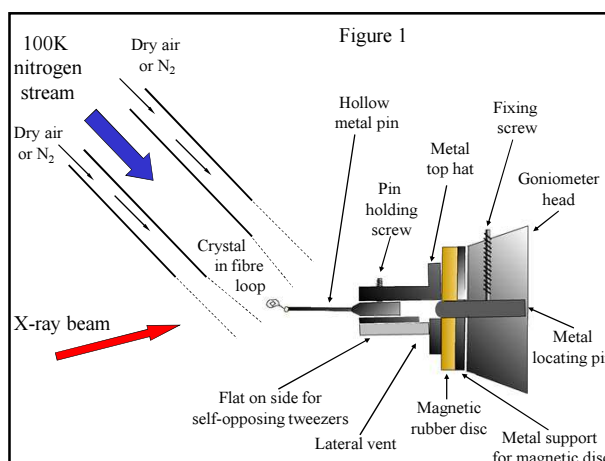
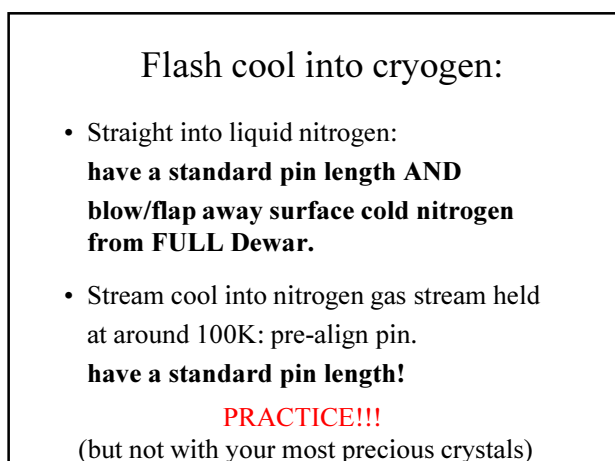
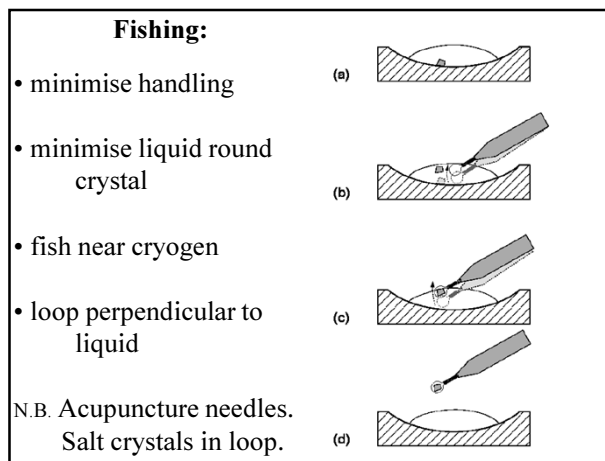
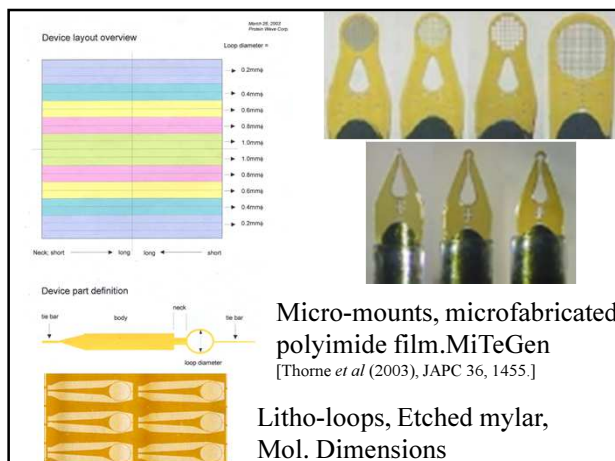


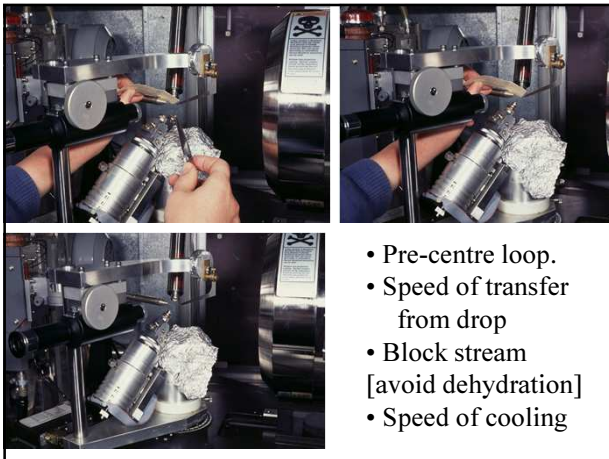
b) Solution pipetted onto crystal



Hardware development: standardisation...







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.

DIFFRACTION?

- NO ...

Does the crystal diffract at room temperature??

NO



Back to crystallisation trials

YES



Change cryo-protocol

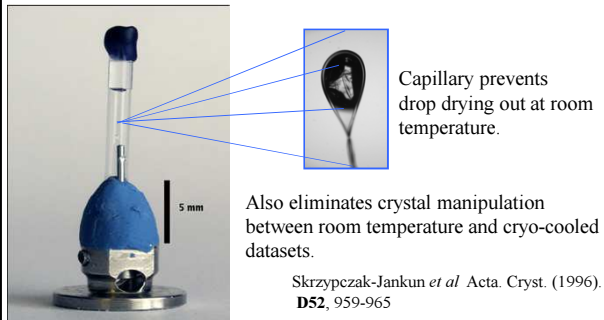
- YES... then take data and/or store it in a Dewar.

The Plan:

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

(Much) easier RT mounting method

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



Room temperature crystal mounting methods

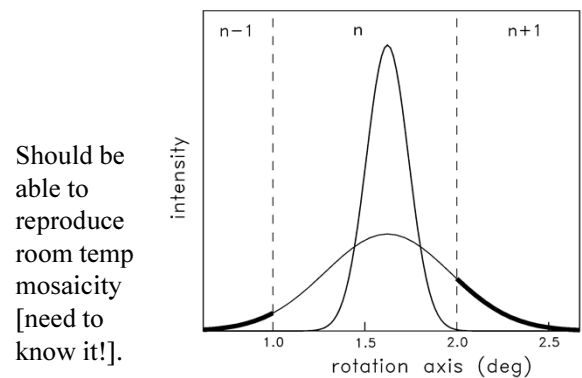


MOSAICITY:

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

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

Minimise mosaic spread to optimise data quality.



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

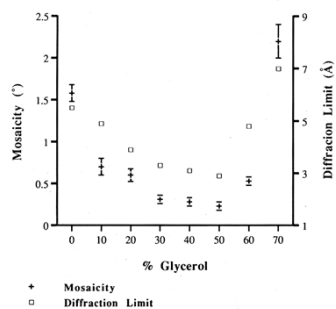


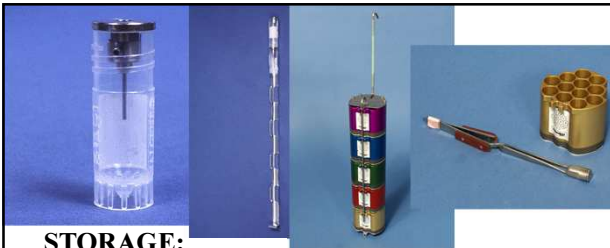
Fig. 2. Variation of mosaicity and diffraction limit of GpB crystals with percentage of glycerol in the buffer. The error bars represent statistical counting errors only.

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

The Plan:

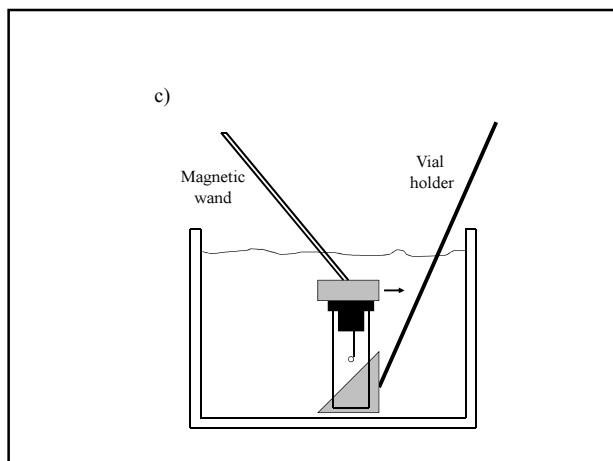
- Cryo techniques
 - Why cool? Radiation damage.
 - Optimising cryoprotection.
 - Testing at room temperature.
 - **Storage and retrieval [practical in groups].**
 - If nothing works...

Crystal storage and retrieval



STORAGE:

- Label the vials beforehand.
- Label the canes.
- Allow transport dewars to dry out after each trip.
- Change storage LN2 every 3 months.
- Make a vent hole in vial.



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?
[reward: a chocolate bar]

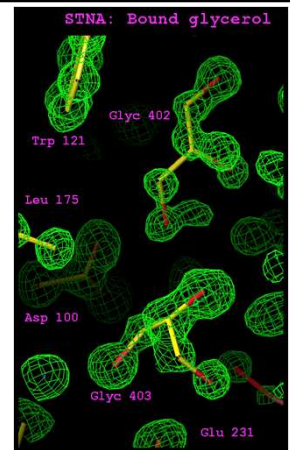


CRYSTAL SOAKS:

- 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 4° overnight]**
- **Cryogen choice: nitrogen gas/liquid**
 - Osmolarity matching
 - Crystal annealing
 - Swap buffer
 - Try more than once.

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.]

If nothing seems to work:

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

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, CH₂OHCHOHCH₂OH

MOLECULAR WEIGHT = 92.09
RELATIVE SPECIFIC REFRACTIVITY = 1.109

0.00%, by wt. d.
For Values of 0

A%	D ₂₀ ²⁰	D ₂₀ ²⁵	C ₂₀ ²⁰	M	C ₂₀ ²⁵	(C ₂₀ - C ₂₅)	ln - n'	n	Δ	O	S	ε%
by wt.	D ₂₀ ²⁰	D ₂₀ ²⁵	g/l	g-mol/l	g/l	g/l	× 10 ³		°C	Os/kg	g-mol/l	
0.50	0.9994	1.0011	5.0	0.054	984.4	2.9	6	1.3336	0.072	0.039	0.020	1.005
1.00	1.0005	1.0023	10.0	0.109	990.5	5.5	12	1.3342	0.140	0.087	0.051	1.025
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	25.3	35	1.3365	0.627	0.337	0.182	1.072
4.00	1.0074	1.0092	40.3	0.438	967.1	35.2	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	44.9	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	1.02625	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	1.358	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	1.3783	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				8.132
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	621.1	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				1487.6

24 HYDROCHLORIC ACID, HCl

Osmotic Pressure Matching: Example

Osmality: Os/kg

- 2.0M NaCl
- 50mM pH 7.8 Tris HCl
- Need 20% glycerol

3.95

2.9

1.05

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

⇒ Try 0.55 M NaCl, 20% glycerol

50mM pH 7.8 Tris HCl

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.

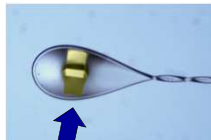
Cryoprotectant agent concentration: match contraction of lattice to contraction of bulk solvent to avoid lattice distortion, higher mosaicity etc.

Not enough cryoprotectant



Water exported

Too much cryoprotectant



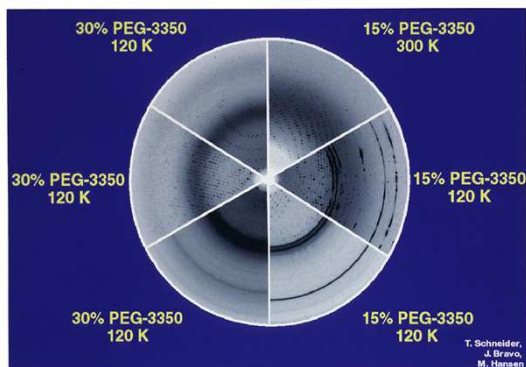
Water imported

Juergens 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 [4°]
- Cryogen choice
- Osmolarity matching
- Crystal annealing
- **Swap buffer**
- **Try more than once.**

Have at least 6 goes...



CRYO-COOLING:

Advantages

- Reduced radiation damage.
- Gentler mounting
- Lower background
- Higher resolution
- Fewer crystals
- Can ship crystals
- Use crystals when ready.

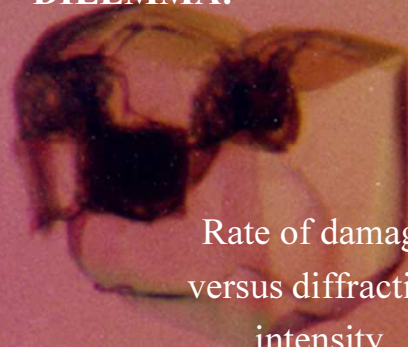
Disadvantages

- Expensive equipment
- Increase in mosaic spread.
- Need to invest time.

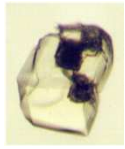
Cryocrystallography: for more gory details see:

- 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 and Hope (1998) *J. Appl. Cryst.* 31, 945-953.
- Garman (1999) *Acta D55*, 1641-1653.
- Garman and Doublé (2003) *Methods in Enzym.* 396, 188-216.
- Garman (2003) *Curr. Opin. Struct. Biol.* 13, 545-551.
- Garman and Owen, (2006) *Acta Cryst. D* 62, 32-47.

The Crystallographer's DILEMMA:



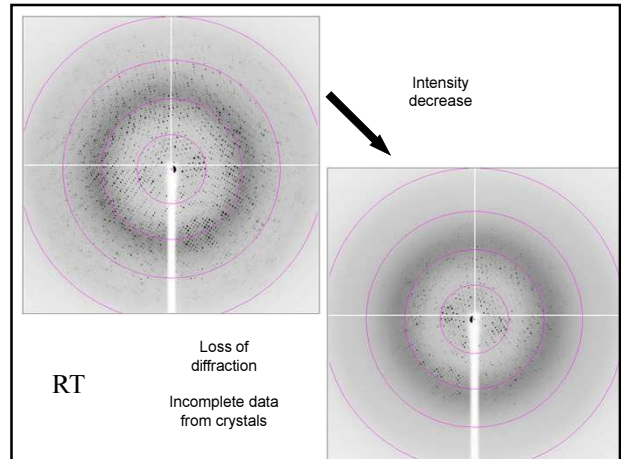
Rate of damage
versus diffraction
intensity



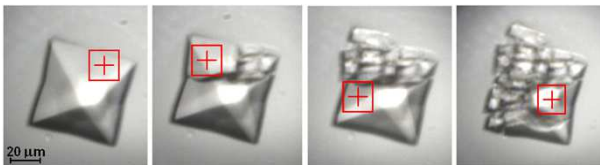
Radiation damage: The Plan:



- **What are the symptoms?**
- What is it?
- Why do we care? Effect on MAD/SAD.
- How do we calculate the Dose?
- What do we know/would like to know?



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
(Gwynhaf Evans *et al.*)

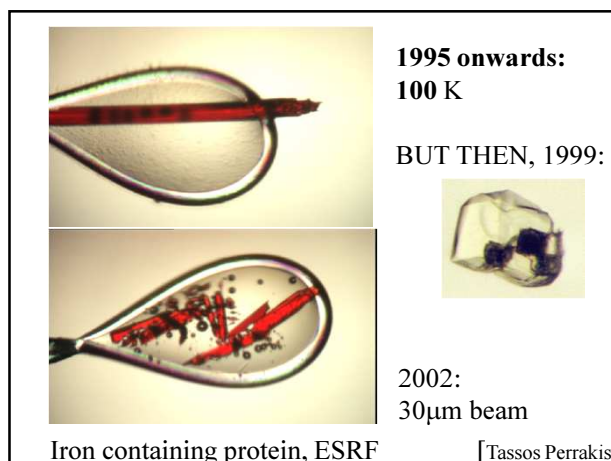
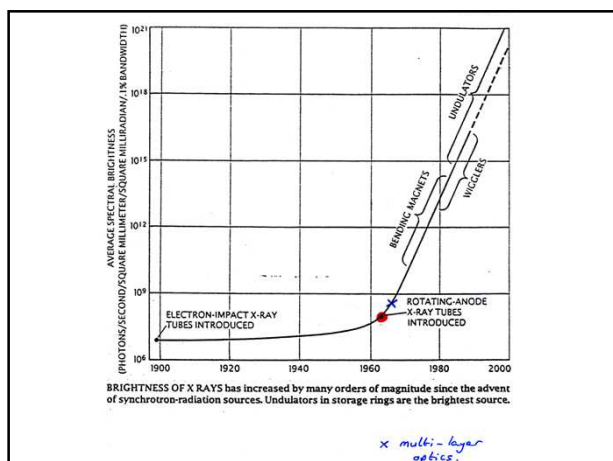
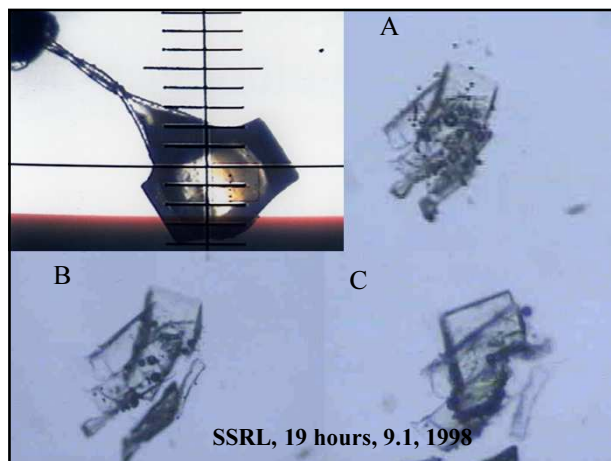
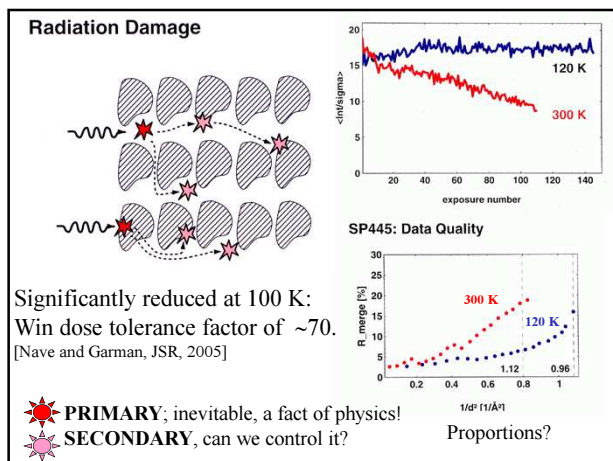
First systematic study of radiation damage in proteins (RT):

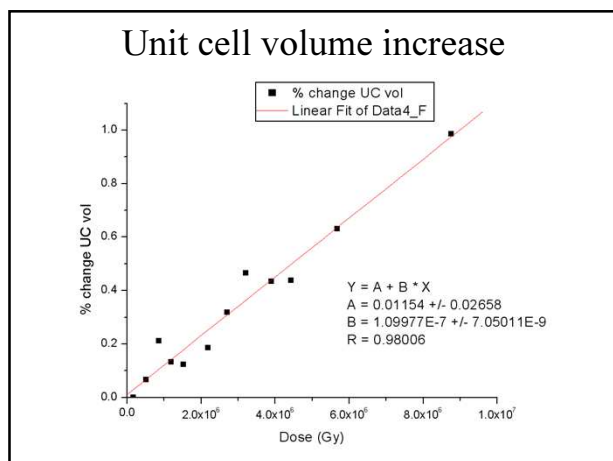
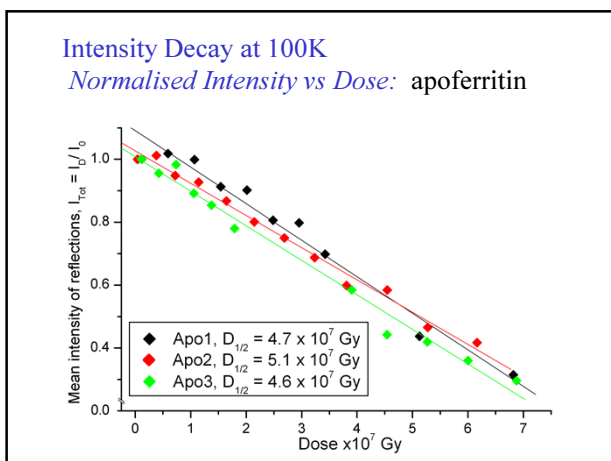
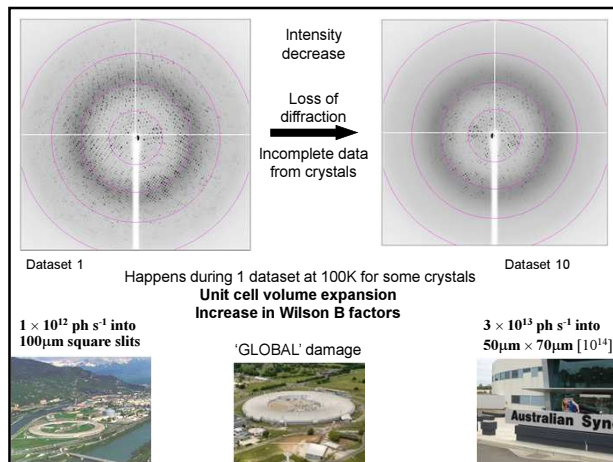
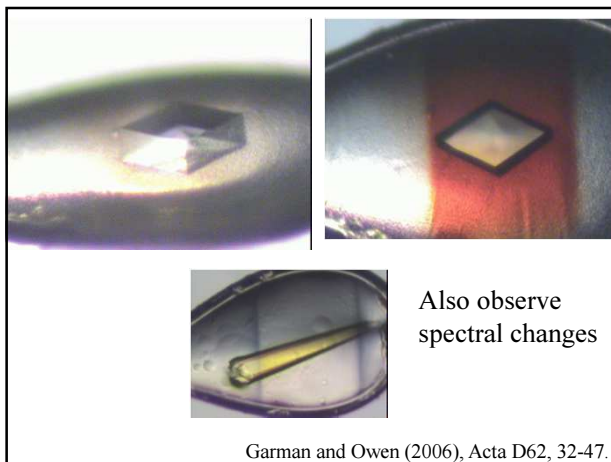
C.C.F.Blake and D.C.Phillips. 1962

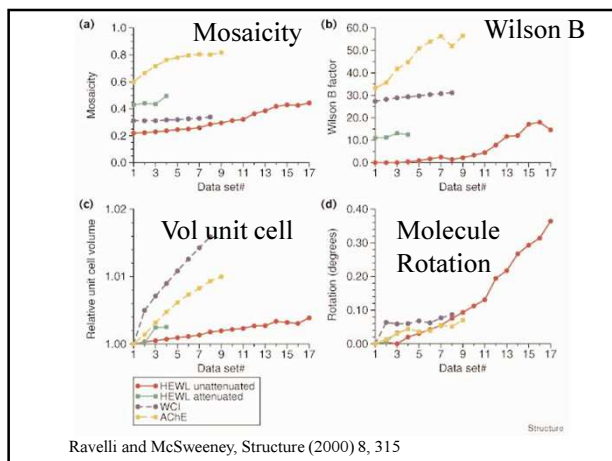
In 'Biological Effects of Ionising Radiation
at the Molecular Level'.

IAEA Symposium, Vienna, P183.

- Damage proportional to dose [Room temp].
- Each 8 keV photon absorbed disrupts ~ 70 molecules and somewhat disorders another 90.
- Damage may be structurally specific.
- [e-mail me for the pdf if interested]







Data Parameters affected by Radiation Damage

- $I/\sigma(I)$ or resolution limit ↓
- R_{merge} ↑
- Scaling B factors ↑
- Mosaicity ↑
- Unit Cell expansion a) function of dose ↑
b) function of cryogen temperature ↑

Could this be an on-line damage metric?

[Ravelli and McSweeney, (2000) Structure]

No!

[Murray and Garman (2002), JSR, Ravelli et al (2002) JSR]

What global damage metric should we use and against what should we plot it?

- I_n/I_0
- Not $I/\sigma(I)$
- B factors?
- An R_{meas} type measure?

Dose estimation (the x-axis!)

$$\text{Dose} = \frac{\text{energy absorbed}}{\text{unit mass}}$$

$$= \frac{\text{J}}{\text{kg}} = \text{Gy}$$

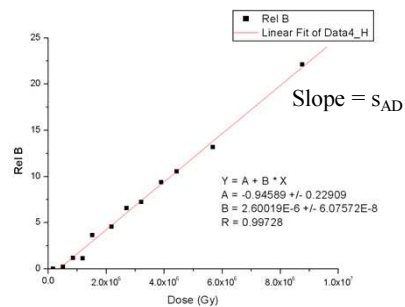
DOSE

- DOSE is the ENERGY lost per KILOGRAMME (!!)
- Measured in Joules/kg i.e. the absorbed energy per unit mass.
- Fundamental metric against which to measure damage.
- FLUX is in photons/second.
- Flux density is in photons/second/unit area.
- Dose takes care of the physics but NOT the chemistry.

Can define (to plot against dose):

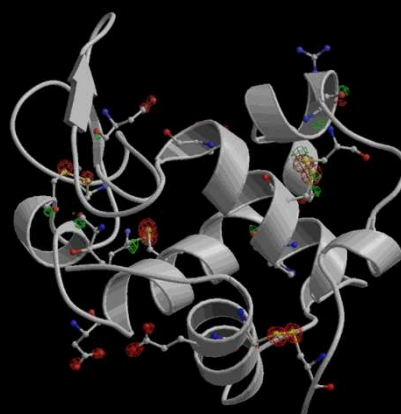
- Coefficient of sensitivity α change in relative isotropic B factor:
[Kmetko et al 2006, Acta D62, 1030]

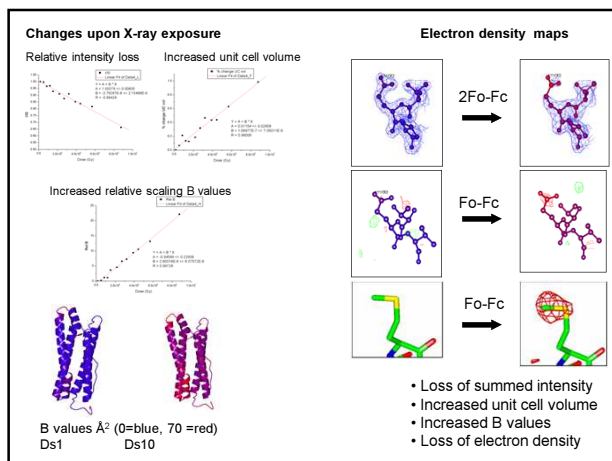
$$s_{AD} = \Delta B_{rel} / 8\pi^2 \Delta D \quad (\text{e.g. HEWL@100 K} = 0.012 \text{ \AA}^2/\text{Gy})$$



Global damage: summary

- Incomplete data.
- Causes non-isomorphism: MAD/SAD problems.
- No significant ($< \times 2$) dose rate effect at 100 K at current flux densities (10^{15} ph/s/mm²).
- No significant ($< \times 2$) temperature dependence, but weak minimum at around 50 K.
- Damage to lattice due to hydrogen abstraction and then build up?
- Heating not significant at current flux densities.
- For a particular system is predictable/can be modelled (using a sacrificial crystal)





Specific structural damage observed:

- Disulphide bridges broken
- Decarboxylation of glutamate and aspartate residues
- Tyrosine residues lose their hydroxyl group
- Methionines: carbon-sulphur bond cleaved

Weik *et al* (2000) PNAS 97, 623-628

Burmeister (2000), Acta Cryst D56, 328-341.

Ravelli and McSweeney, (2000) Structure 8, 315-328.

- Rupture of covalent bonds to heavier atoms:
C-Br, C-I, S-Hg

Note that if this were due to primary damage alone, damage would be in order of absorption cross sections of atoms, which it is not.

Specific Damage: summary.

- Can compromise biologically relevant observations (e.g. damage enzymatically important glutamates).
- Metallo-enzymes are reduced by X-ray beam.
- Perhaps weakly dose rate dependent ($< \times 2$)
- Perhaps weakly wavelength dependent ($< \times 2$)
- Weakly temperature dependent (varying results) ($< \times 2$)
- Can be reduced with certain scavengers: but very conflicting results (mainly $< \times 2$, benzoquinone RT $\times 9$)
- We DON'T understand pecking order of damage within an amino acid group pH? Solvent accessibility? Neighbouring amino acids?

Radiation damage:

The Plan:

- What are the symptoms?
- **What is it?**
- Why do we care? Effect on MAD/SAD.
- How do we calculate the Dose?
- What do we know/would like to know?

PHYSICS of the interaction of X-rays with crystals.

A) Diffraction

B) Absorption = Energy loss

N.B. > 90% of the beam does not interact at all,
but goes straight through.

A) Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering

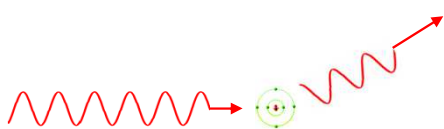


[8% at 1 Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering

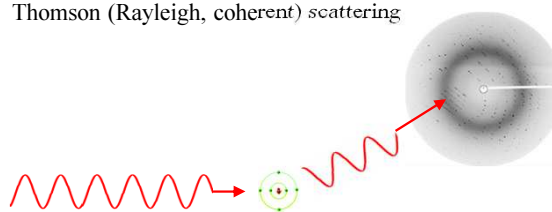


[8% at 1 Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering

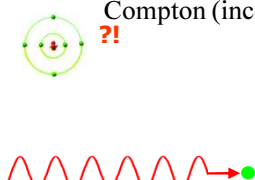


ELASTIC - no energy loss.

Coherent – adds vectorially and gives diffraction pattern.
Small proportion of total scattering: 8% at 1 Å

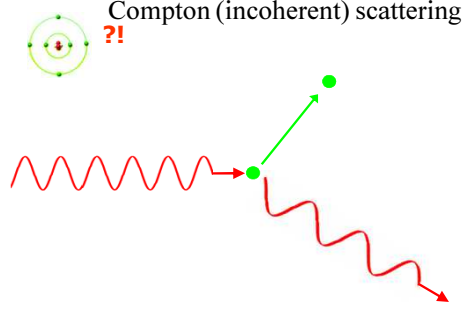
BUT IT IS THE BIT WE WANT!!

Compton (incoherent) scattering
?!



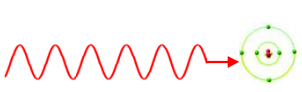
X-ray transfers some energy to atomic electron and thus has lower energy (higher wavelength).

Compton (incoherent) scattering
?!



X-ray transfers some energy to atomic electron and thus has lower energy (higher wavelength).
Incoherent – part of X-ray background in images.
Also a small proportion of total scattering: 8% at 1 Å

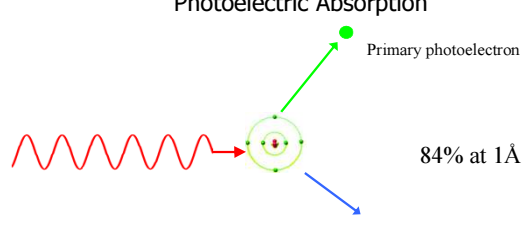
Photoelectric Absorption



84% at 1 Å

INELASTIC.

Photoelectric Absorption

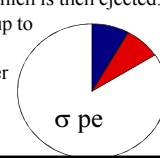


Primary photoelectron

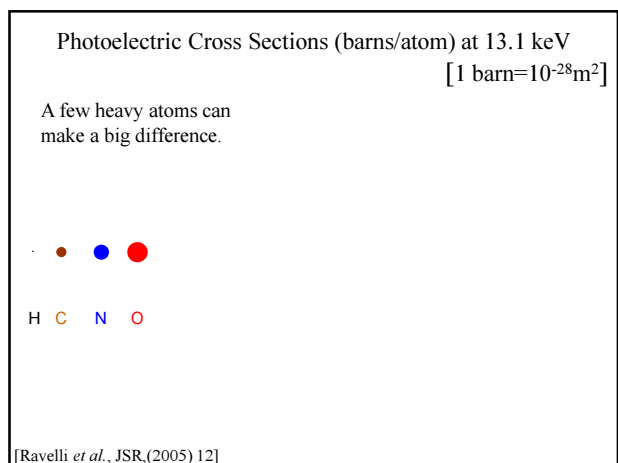
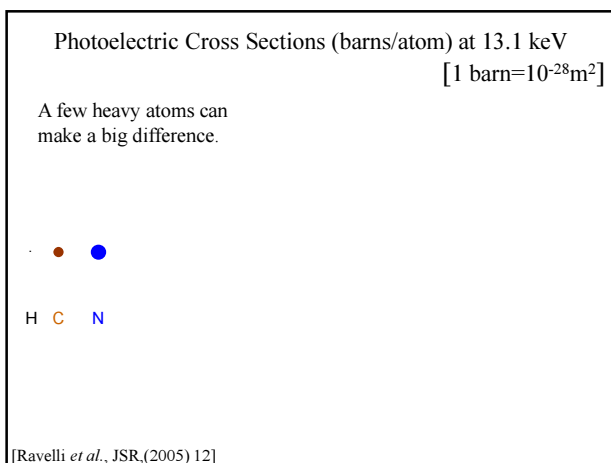
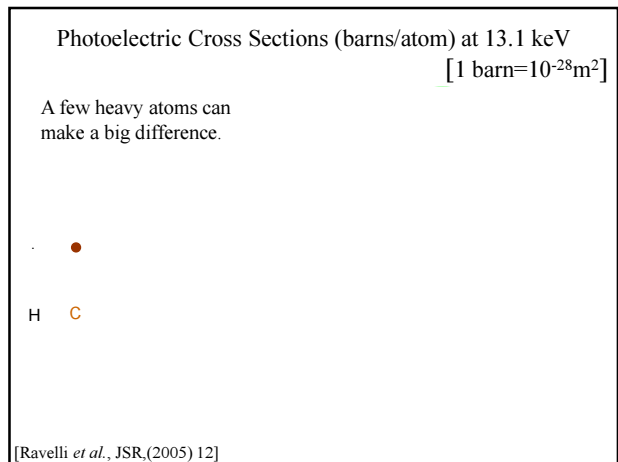
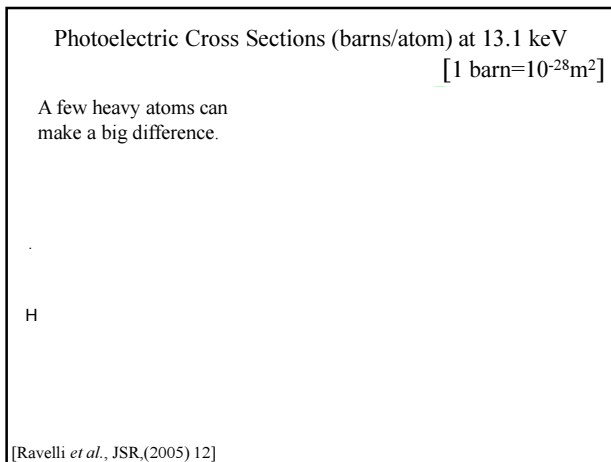
84% at 1 Å

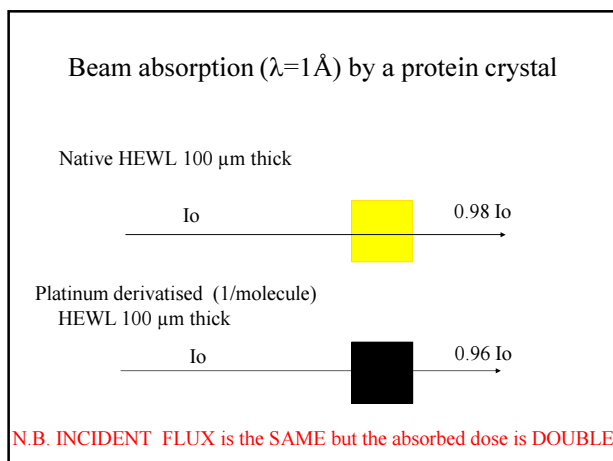
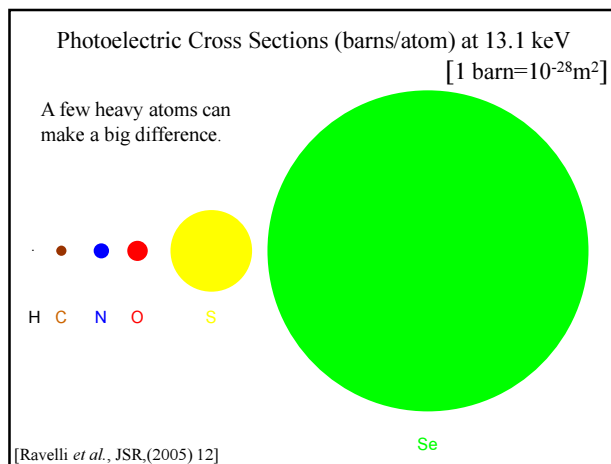
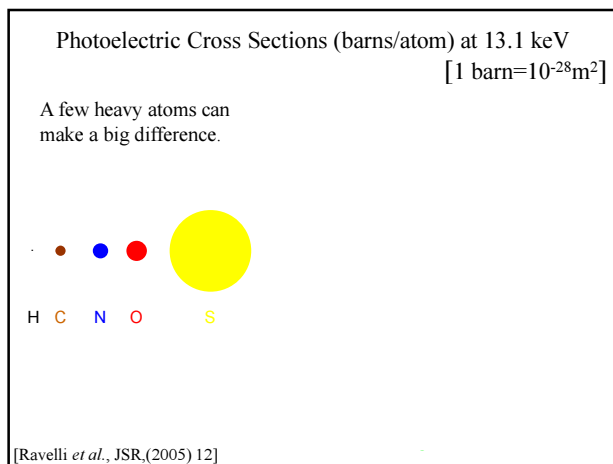
INELASTIC.
X-ray transfers all its energy to an atomic electron, which is then ejected.
Each 12 keV primary photoelectron can give rise to up to 500 ionisation events.
Atom can then emit a characteristic X-ray or an Auger electron to return to its ground state.

$$\sigma_{\text{tot}} = \sigma_{\text{pe}} + \sigma_{\text{inc}} + \sigma_{\text{coh}}$$

$$84\% + 8\% + 8\%$$


σ_{pe}





A few heavy atoms in the solvent can make a BIG difference to the absorption cross section and this the dose rate for the SAME flux.

e.g. Cacodylate buffer (arsenic, mass 75 cf selenium = 79)

**BACK SOAKING to REMOVE
Non-specifically bound heavy atoms**

e.g. a brominated DNA-protein complex will radiation damage much faster than a native crystal, and will de-brominate during data collection [Ennfar et al, Acta Cryst D (2002) 1263-1268].

Radiation damage:

The Plan:

- What are the symptoms?
- What is it?
- **Why do we care? Effect on MAD/SAD.**
- How do we calculate the Dose?
- What do we know/would like to know?

Effect on MAD/SAD.

- Failure of structure determination (Multi-wavelength anomalous dispersion MAD)
due to creeping non-isomorphism –
 - a) cell expansion and
 - b) movement of molecule in unit cell
 - c) structural changes DURING experiment.

Non-isomorphism: DISASTER!

Crick and Magdoff (1956) showed that for a 0.5% change in all three unit cell dimensions of 100Å, the intensity would change by

15% at 3Å

for general reflections

[Crick and Magdoff (1956) Acta Cryst **9**, 901-908]

i.e. **MAD/SAD phasing** signals (<5%) washed out completely.

Radiation damage:

The Plan:

- What are the symptoms?
- Why do we care? Effect on MAD/SAD.
- What is it?
- **How do we calculate the Dose?**
- What do we know/would like to know?

DOSE Postulate:

- There is a **MAXIMUM** dose (Joules/kg = Gy) which protein crystals can tolerate which depends only on the **PHYSICS** of the situation.
- Crystal might not reach that limit due to chemical factors, but it will not last **BEYOND** the limit.
- Need to be able to calculate the DOSE:
RADDOSE

V1: Murray, Garman & Ravelli (2004) JAPC, 37, 513-522
V2: Paithankar, Owen & Garman, (2009) JSR 16, 152-162
V3: Paithankar & Garman (2010), Acta D 66, 381-388

Typical MX experiment

A dose of 1 MGy will be absorbed in
10 s by a 100 μm cubed metal free
crystal in a 100 μm x 100 μm
12.4 keV (1 $\text{\AA}$) X-ray beam
flux 10^{12} photons s^{-1}

3 Gy

MX at 100 K: for above example,
The 30 MGy dose 'limit' is reached in
approx 5 mins



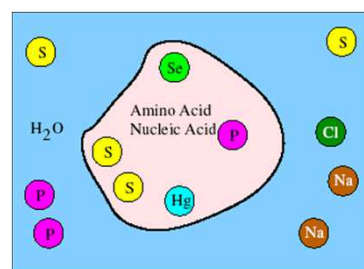
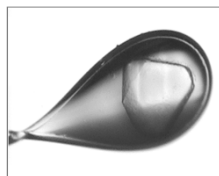
Dose calculation

To find the energy deposited per unit mass in the crystal, need to characterise two things:

The Beam



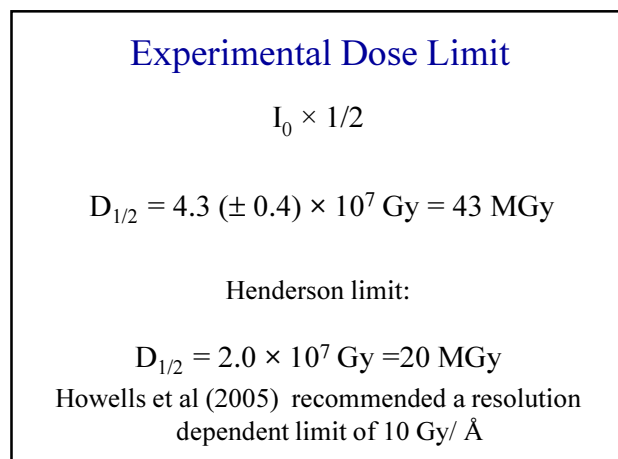
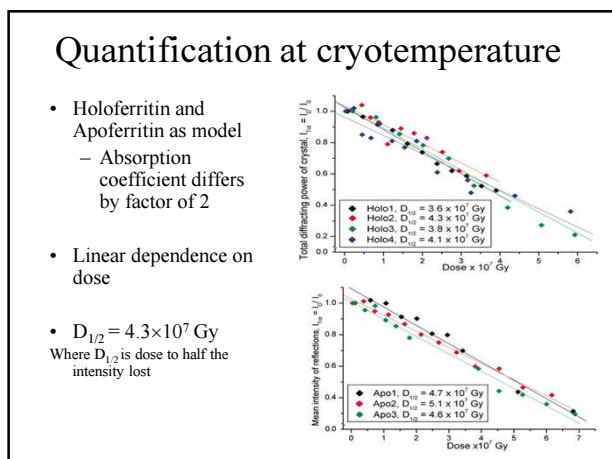
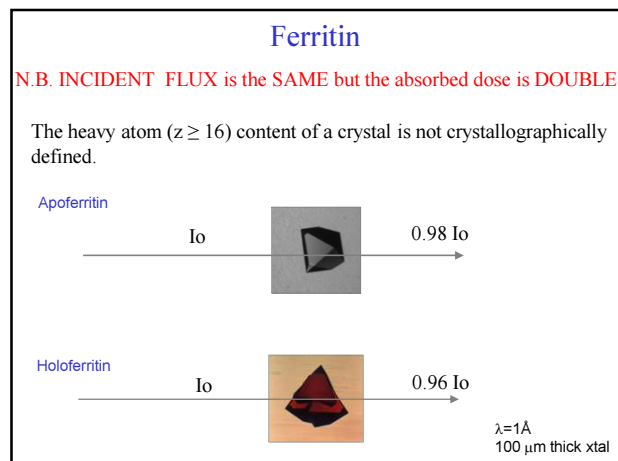
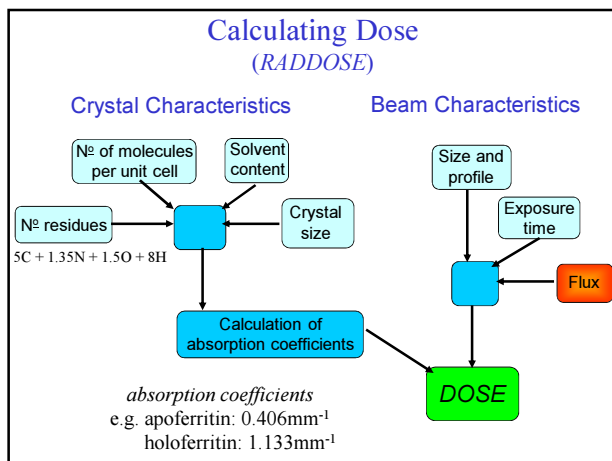
The crystal



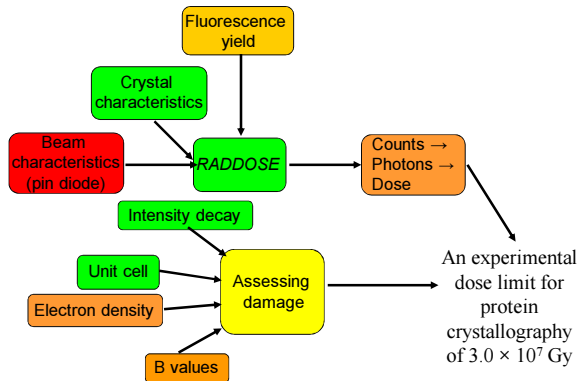
Number of Amino Acids

'HA' atoms per monomer, e.g. S, Se, Hg

Solvent - concentrations of components, e.g. Na^+ , Cl^-



Radiation damage to protein crystals



Experimental Dose Limit (100K)

For $I_0 \times 1/2$
 $D_{1/2} = 4.3 (\pm 0.4) \times 10^7 \text{ Gy} = \mathbf{43 \text{ MGy}}$

BUT

Suggested limit to retain biological 'fidelity'
 $I_0 \times 0.7$

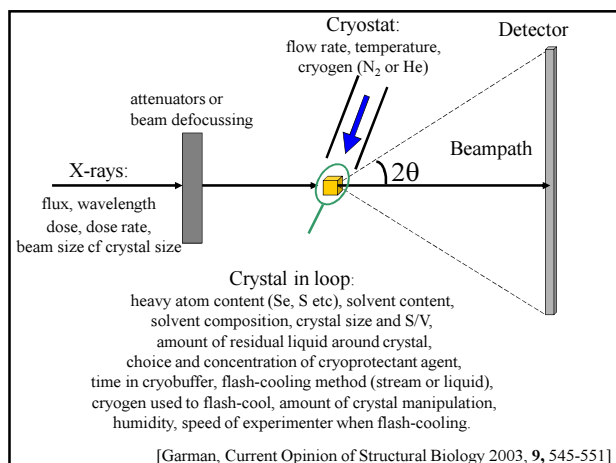
$D_{0.7} = 3.0 \times 10^7 \text{ Gy} = \mathbf{30 \text{ MGy}}$

$D_{0.7}$ for ferritin corresponds 107 photons/unit cell

Robin Leslie Owen, Enrique Rudíño-Piñera, Elspeth F. Garman.
 PNAS (2006) 103, 4912 - 4917.

Radiation damage: The Plan:

- What are the symptoms?
- Why do we care? Effect on MAD/SAD.
- What is it?
- How do we calculate the Dose?
- **What do we know/would like to know?**



PROBLEM: how do we know that we are making any difference?

- In order to investigate the effects of various parameters on the radiation damage process, we need a robust radiation damage METRIC which is preferably ON-LINE during the diffraction experiment.

No unanimous metric currently/ results from different metrics do not agree.

- Structural changes occur before degradation of diffraction quality is obvious.

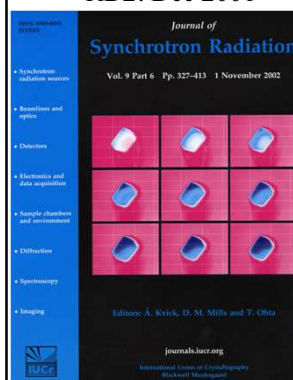
Work so far / ongoing:

- Lower the cryogen temperature? 40K? 50K? 16K? 140K (no!). **Less than a factor of 2 improvement.**
- Lower the wavelength? Lots of anecdote + now some systematic results: **no effect on damage rate.**
- Unit cell expansion as a metric? **No!**
- Change/ regulate the dose/dose rate regime? **No, not at current!**
- Effect on MAD/SAD? Order of data collection?
- Minimum crystal size? Several papers. (see Holton 2009)
- Beam heating. **Not a big factor at current flux**

Work so far / ongoing:

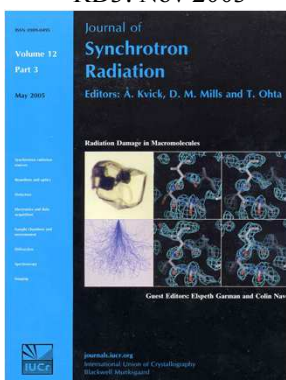
- X-ray absorption – **important parameters defined.**
- Remove oxygen? **Nothing yet.**
- Radiation damage Induced Phasing (RIP)
- Software developments – **big progress.**
- Add radical scavengers: **results disagree.**
- Biological implications/applications to mechanistic studies. **Now many.**
- Room temperature studies: dose rate effects? **Results disagree.**
- N.B. Need for **systematic statistically significant** experiments.
- **Series of Radiation Damage Workshops**

RD2: Dec 2001

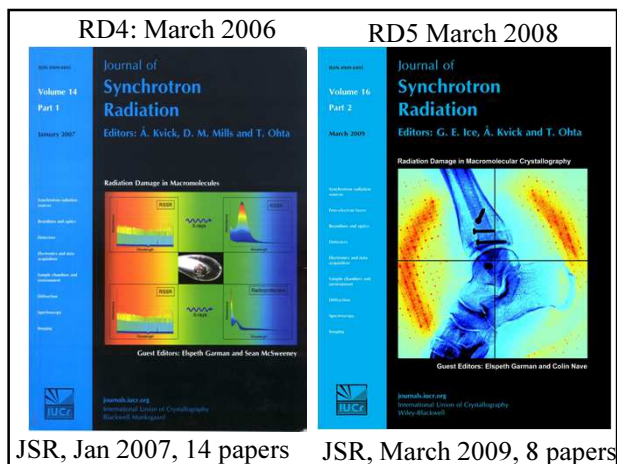


JSR, Nov 2002, 8 papers

RD3: Nov 2003



JSR, May 2005, 9 papers



RD8: April 2014

JSR, Jan 2015, 8 papers

General summary in:
Garman Acta D (2010)
66, 339-355

Also a Beginner's guide to
RD in Holton (2009) JSR 16,133-142

Summary 1: what can YOU, the experimenter do?

- Do not be afraid to merge data taken from different isomorphous crystals which all had lower doses.
- Back soak non-specifically bound heavier atoms out of your crystals.
- Be 'absorption aware' of the contents of your crystal (e.g. Se and buffer) and if possible, avoid cacodylate buffer (arsenic mass=75).
- Match beam size to crystal size

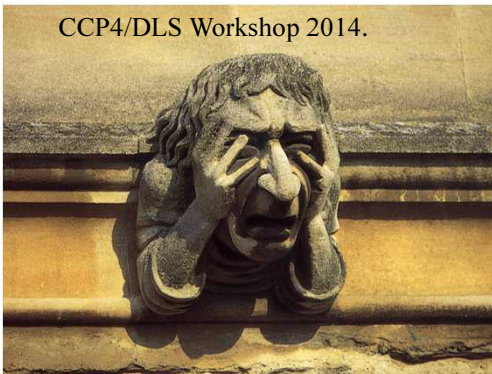
Summary 2: what can YOU, the experimenter do?

- Scavengers: try electron scavengers at 100 K (nitrate/ascorbate/benzoquinone).
- Dose 'spreading': use a tophat profile beam if possible. Consider Helical/Translational data collection.
- So you can estimate the dose, ASK at the beamline:
 - What is the flux today at this energy and with this slit size ('flux density')?
 - What is the beam profile today at this beam energy? FWHM in x and y ?

Current status: radiation damage in protein crystals

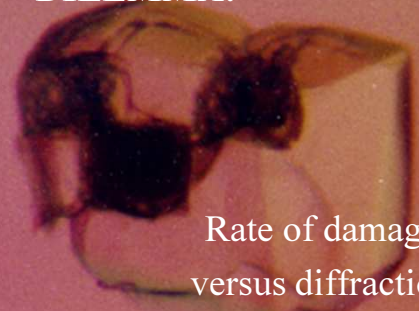
- Understand a lot more than twelve years ago, but still not nearly enough...
- Understand how to do experiments better.
- Research has prompted some very exciting new approaches.
- Many complementary methods now being used on the problem in concert with crystallography
- Experiments must involve more than one sample (!) to get statistically significant results: labour intensive and time consuming. Also MUST know incident FLUX density...
- **Radiation damage has DEFINITELY become a mainstream concern**

CCP4/DLS Workshop 2014.



Still the first day and my brain is already over full...

The Crystallographer's DILEMMA:



Rate of damage
versus diffraction
intensity