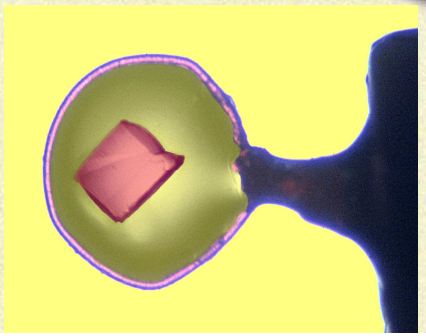
Why TotalCryst for Macromolecular Crystallography?

TotalCryst Workshop, ESRF, 1-3 April 2009.



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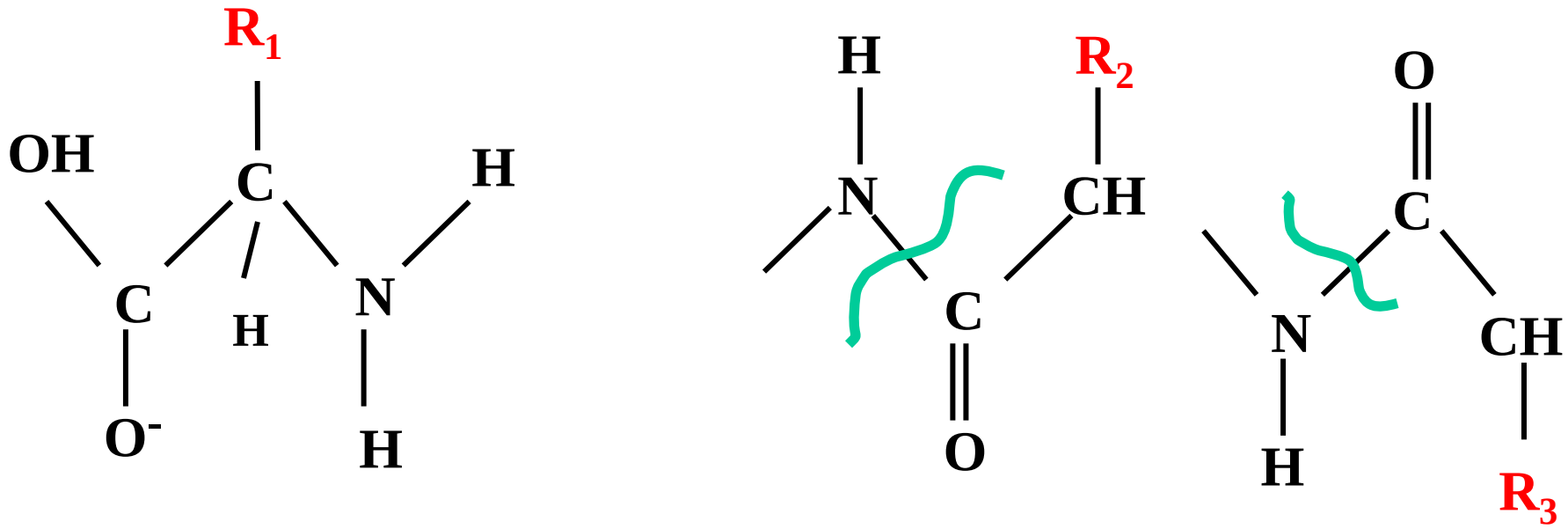
WHAT IS A PROTEIN?

Made of amino acids.

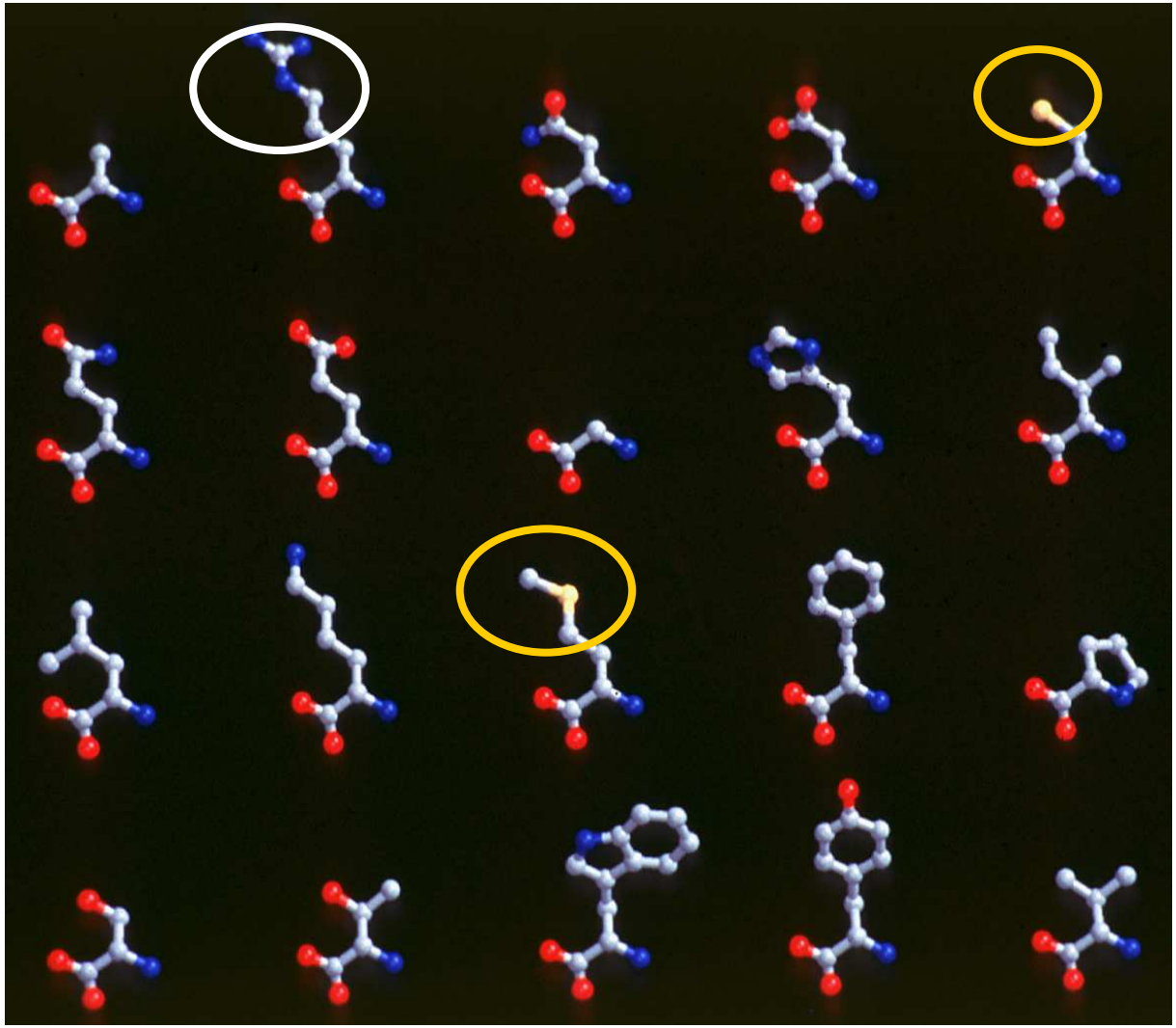
20 different `R' groups in Nature.



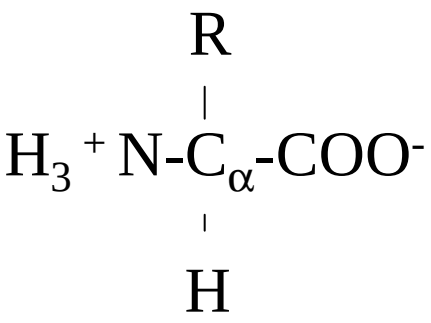
R can be H, CH_3, C_2H_5, C_3H_7, rings, CNH_4N, CSH_3……

Basic units join to form chains.

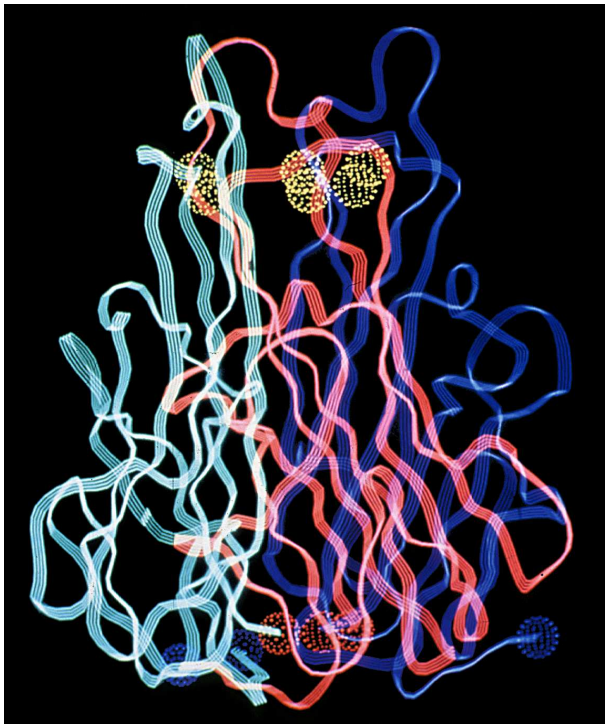
20 amino acids in Nature: methionine and cysteine contain sulphur

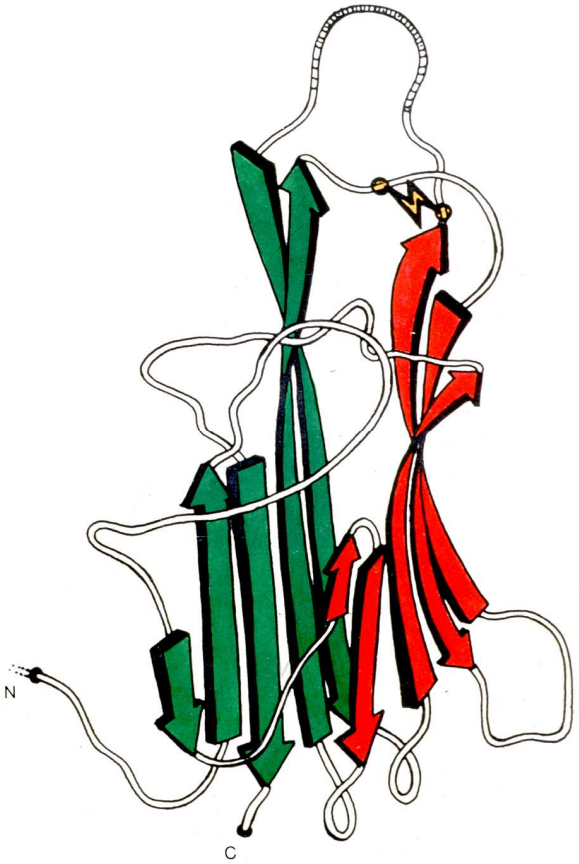


White: carbon
Red: oxygen
Blue: nitrogen
Yellow: sulphur

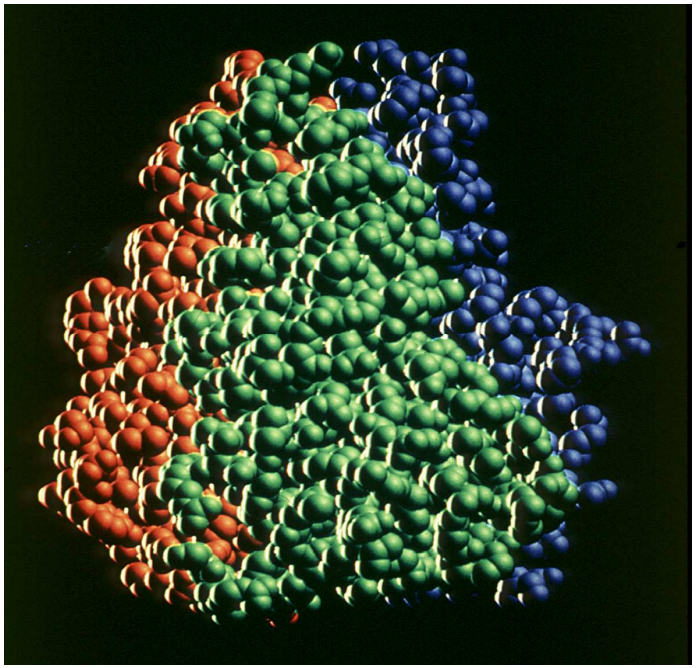


e.g. Tumour Necrosis Factor



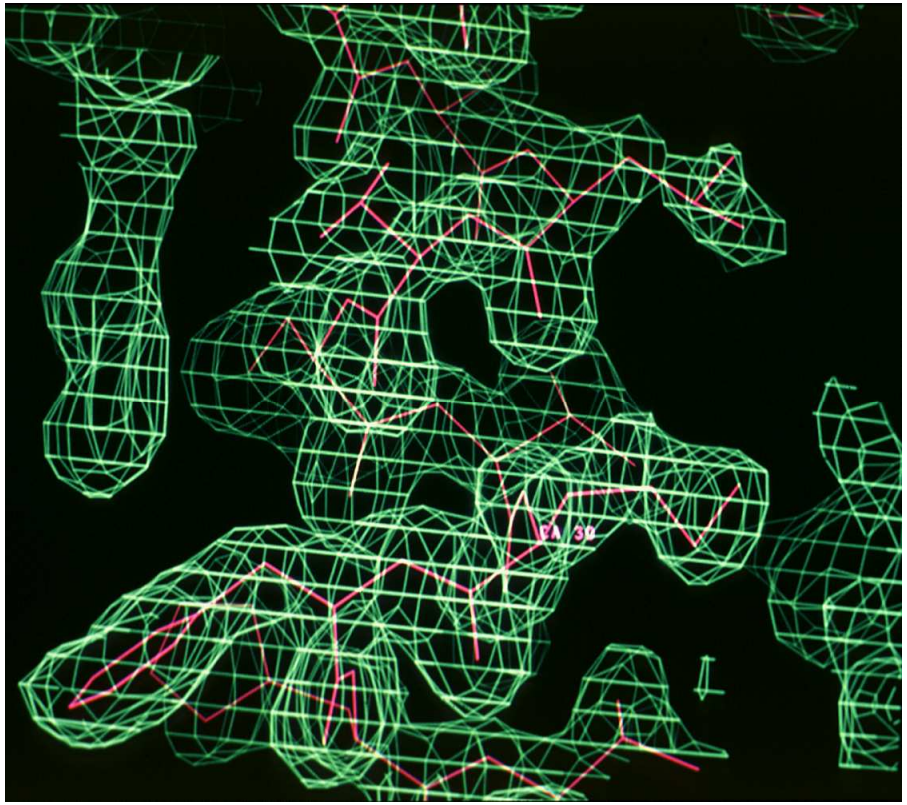


3-D shape of `string' linking beads

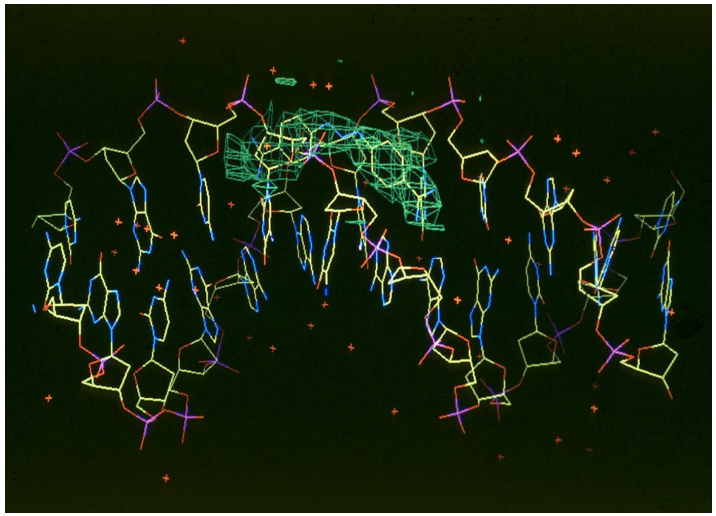


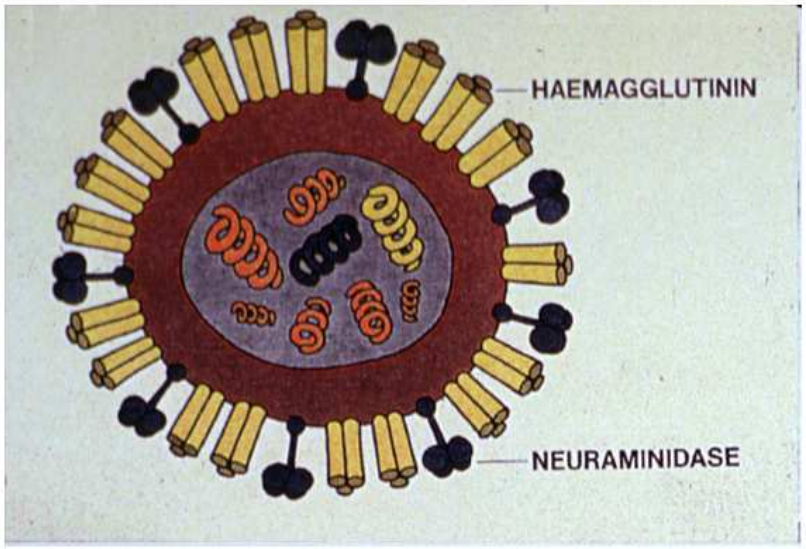
From X-ray diffraction, we get experimental electron density (green) and fit known sequence of amino acids to it.

Alpha helix

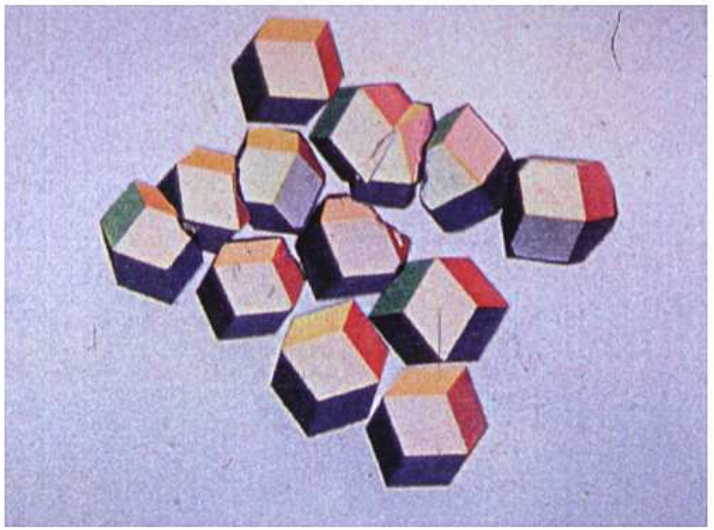


Difference map
DNA + berinil



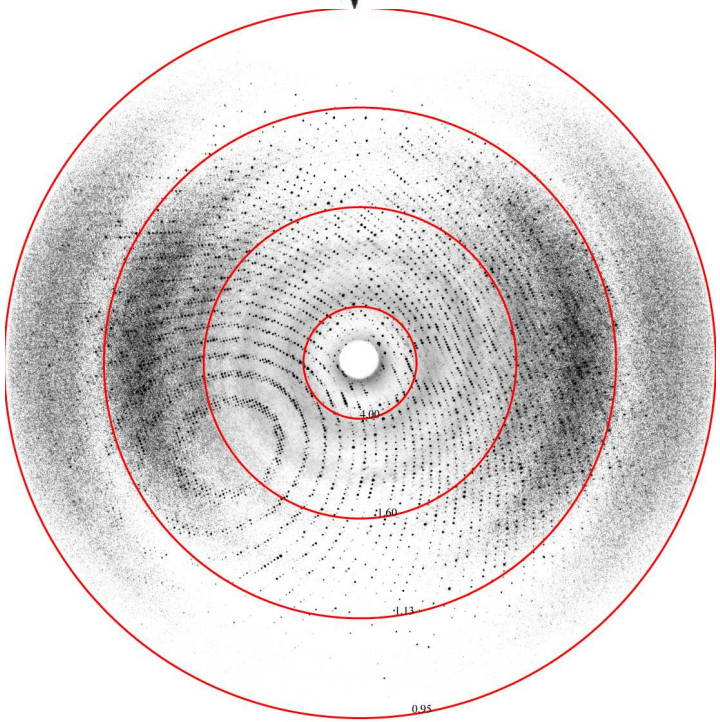


N9



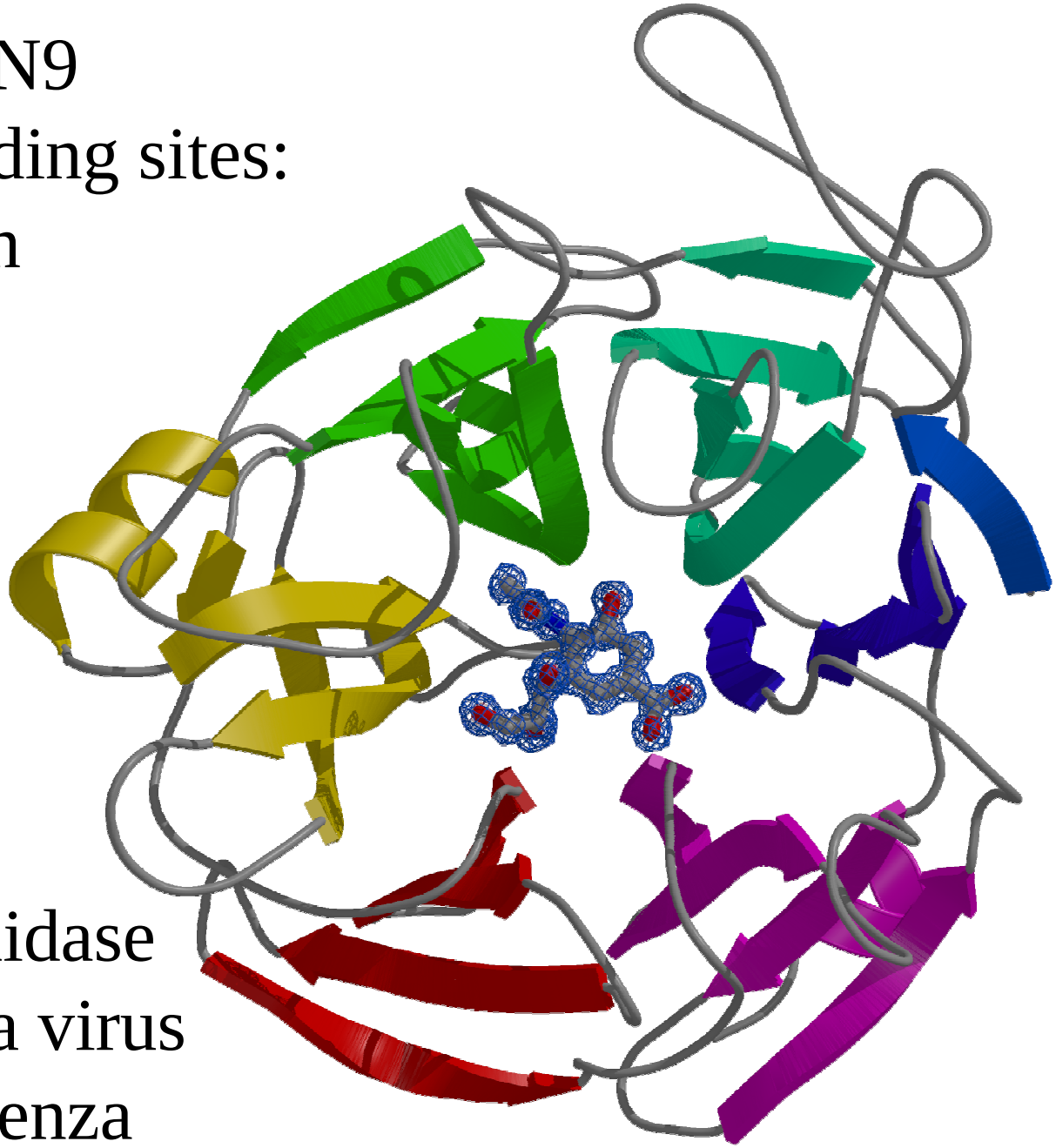
Protein crystal (0.020mm – 0.5mm in size)

e.g. H6N9 bird ‘flu



Sialic acid + N9
Substrate binding sites:
DRUG design

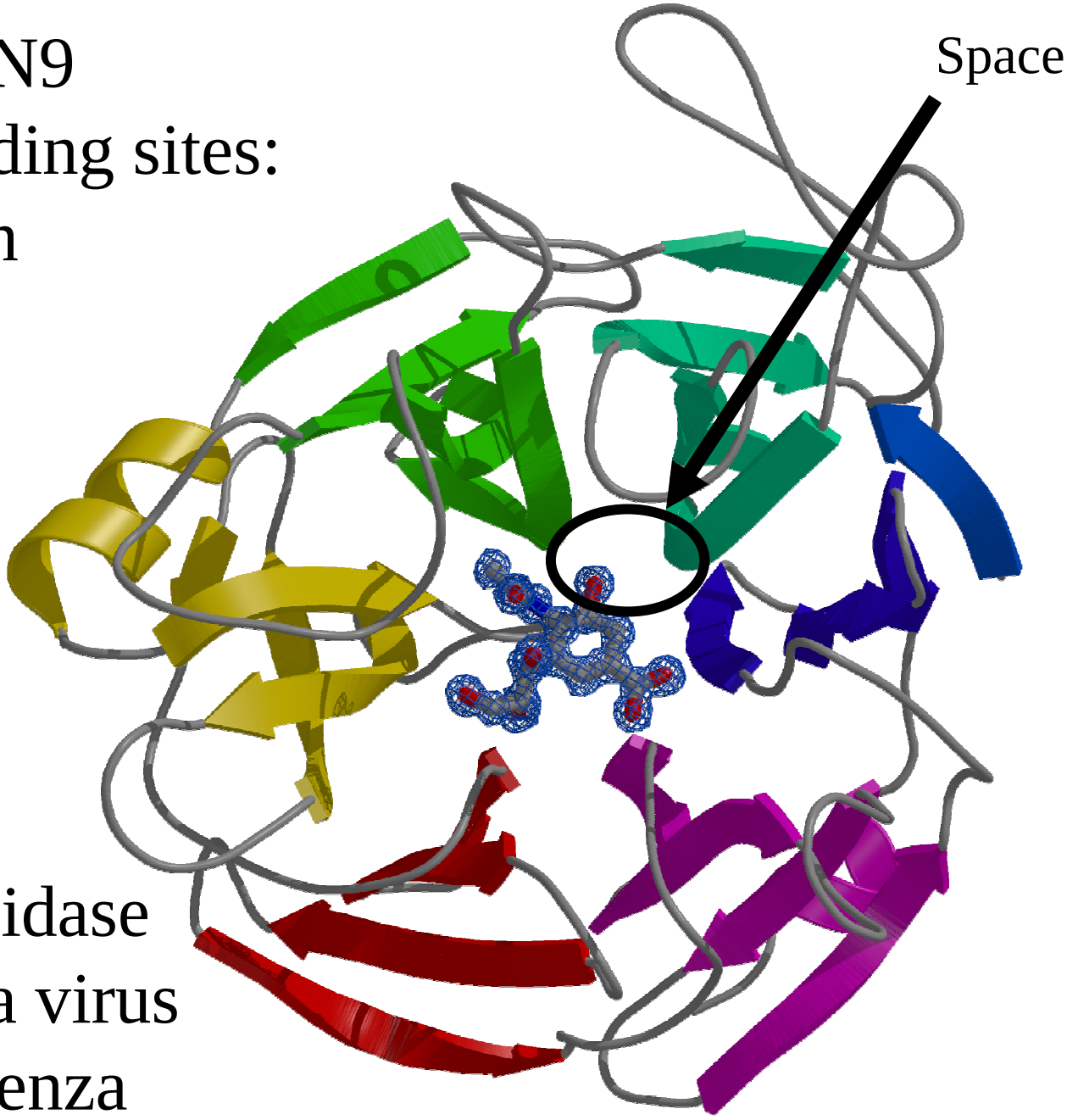
Needs
2.0Å- 2.8Å
(0.2nm)



e.g. Neuraminidase
from influenza virus
(tern N9); Relenza

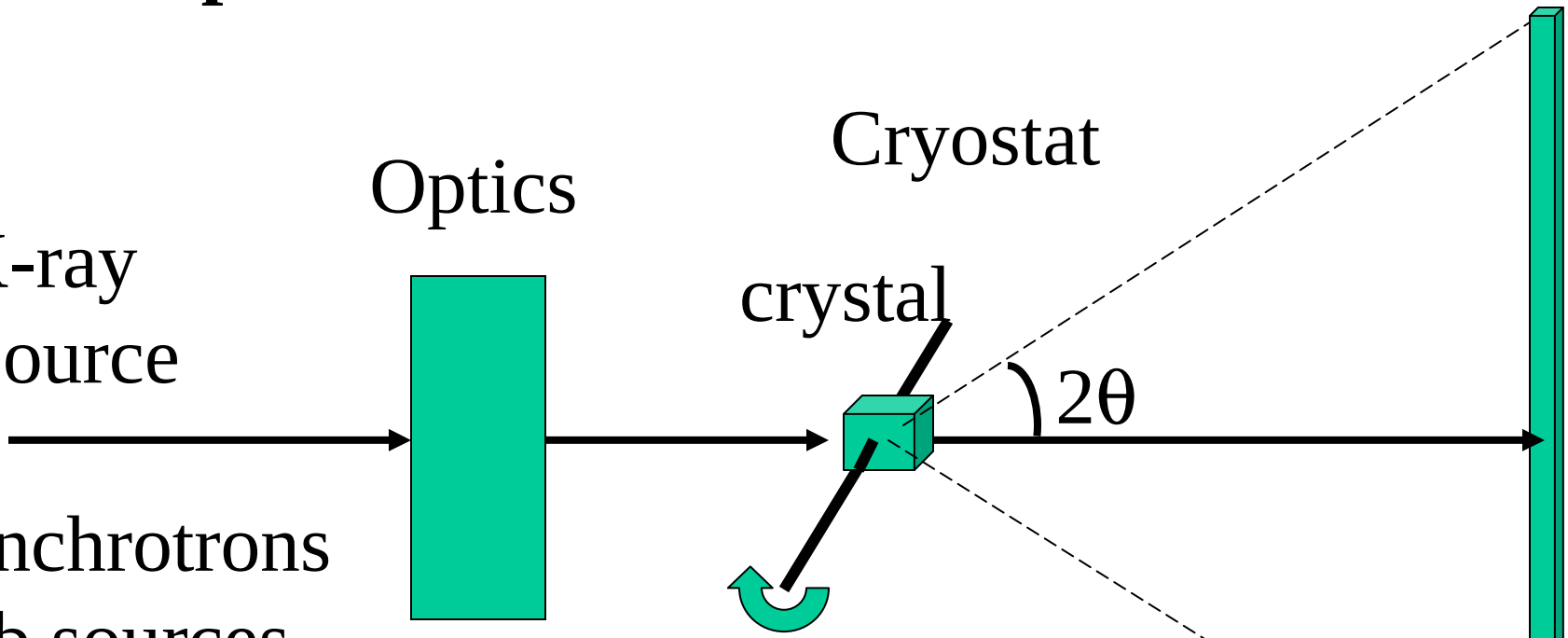
Sialic acid + N9
Substrate binding sites:
DRUG design

Needs
2.0Å- 2.8Å
(0.2nm)

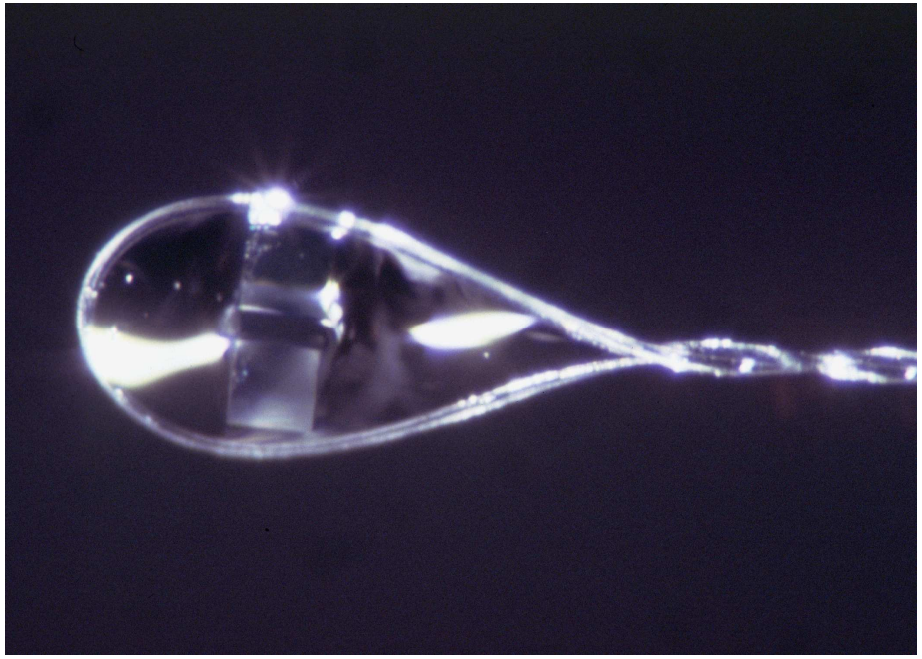


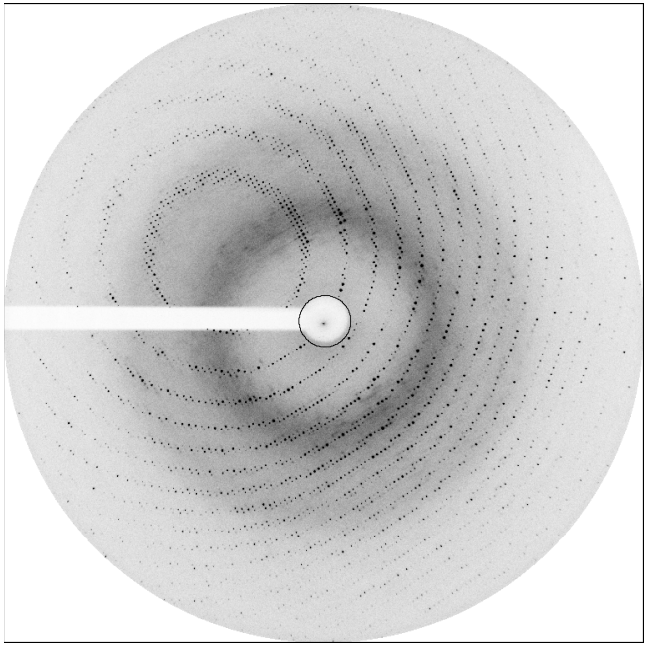
e.g. Neuraminidase
from influenza virus
(tern N9); Relenza

Basic Experimental:

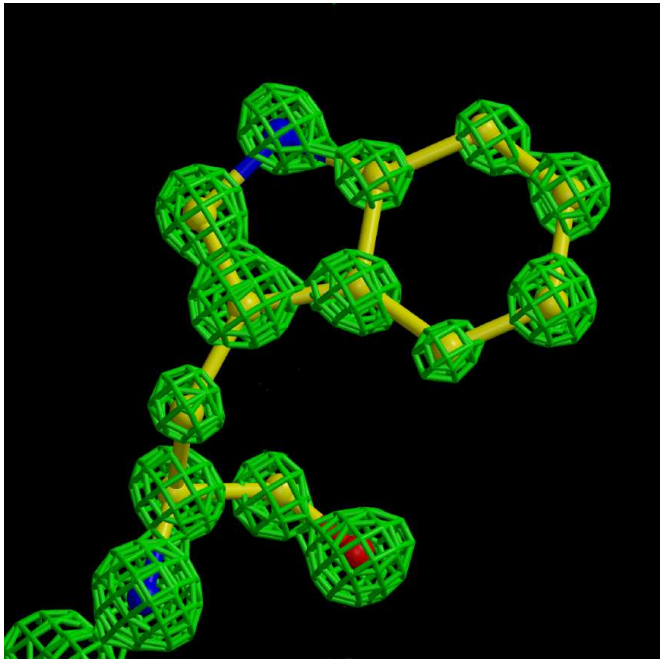


Crystal: > 10μm: synchrotron
> 40μm: home





Phases,
Fourier
Transform



Diffraction Images

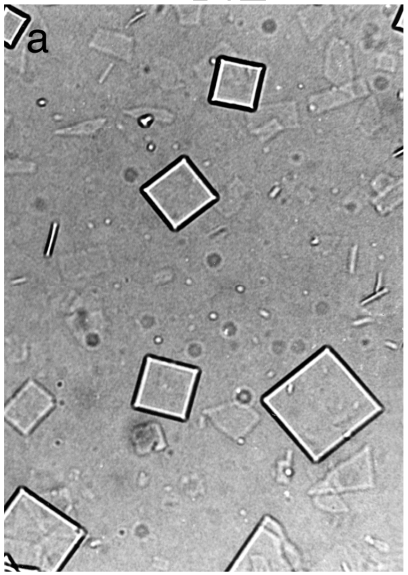
Electron Density + model

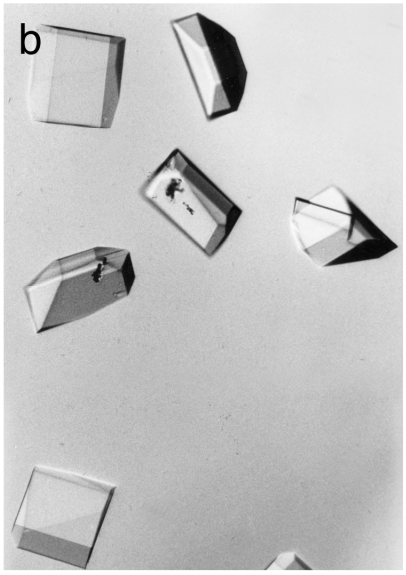
Why TotalCryst for Macromolecular Crystallography?

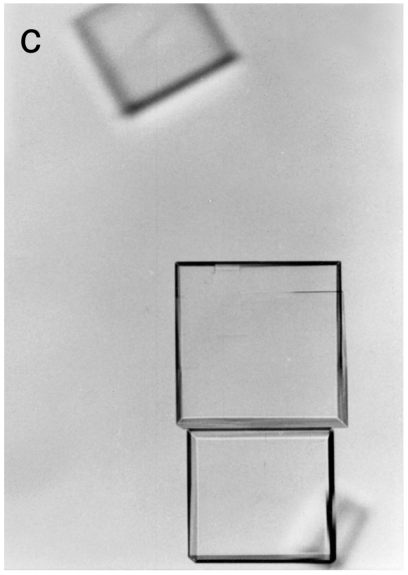
1) Crystals are sometimes multiple and inseparable, but still `single' enough to give information.

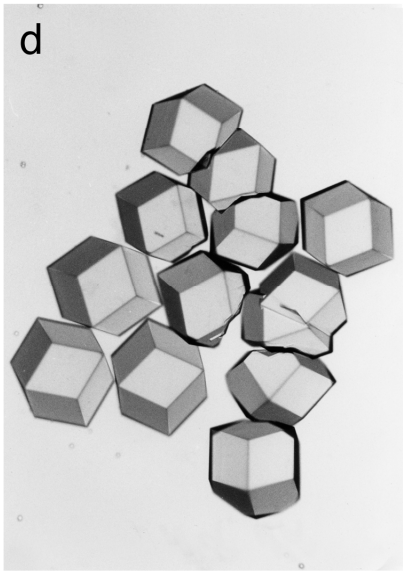
2) Radiation damage destroys crystal order during irradiation, and changes the structure DURING the experiment.

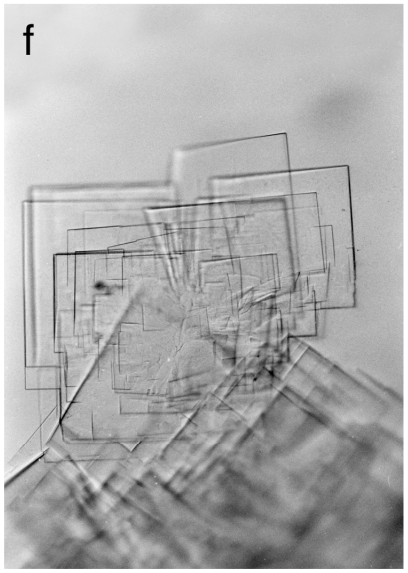
N2 N6 N8 N9

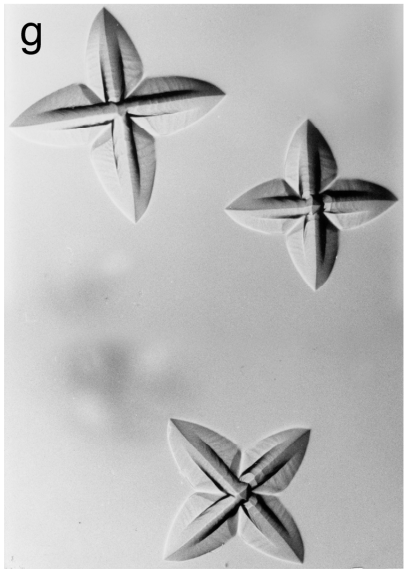


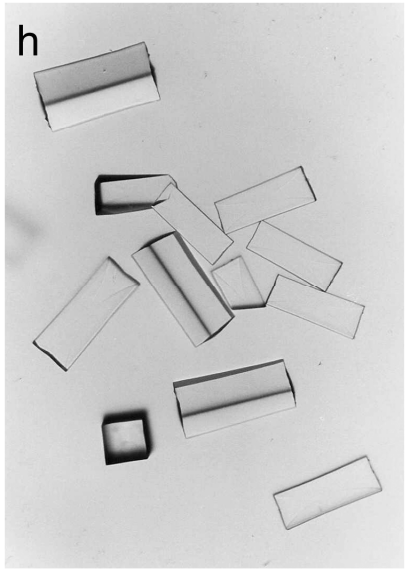












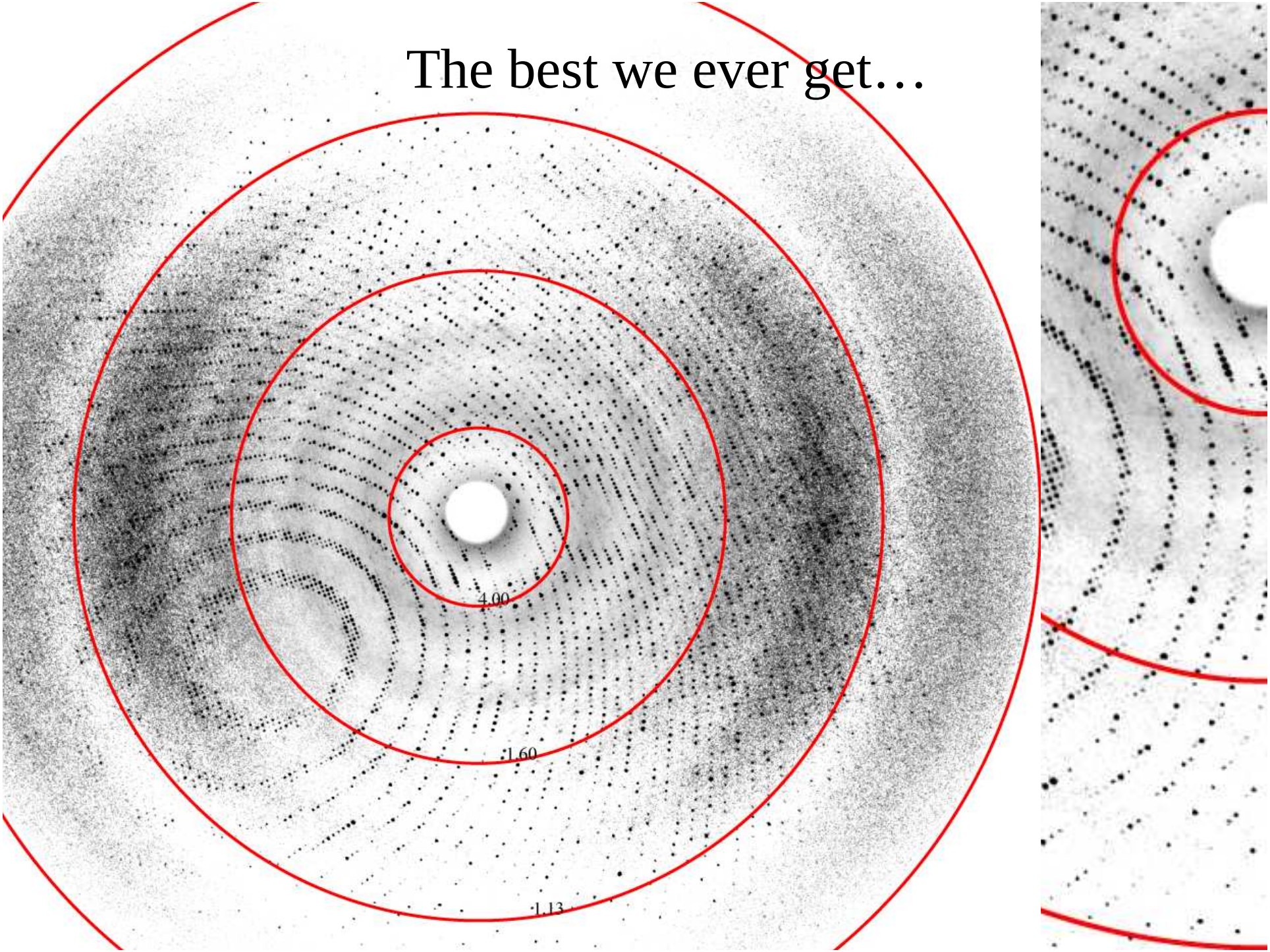
N1 N3 N5

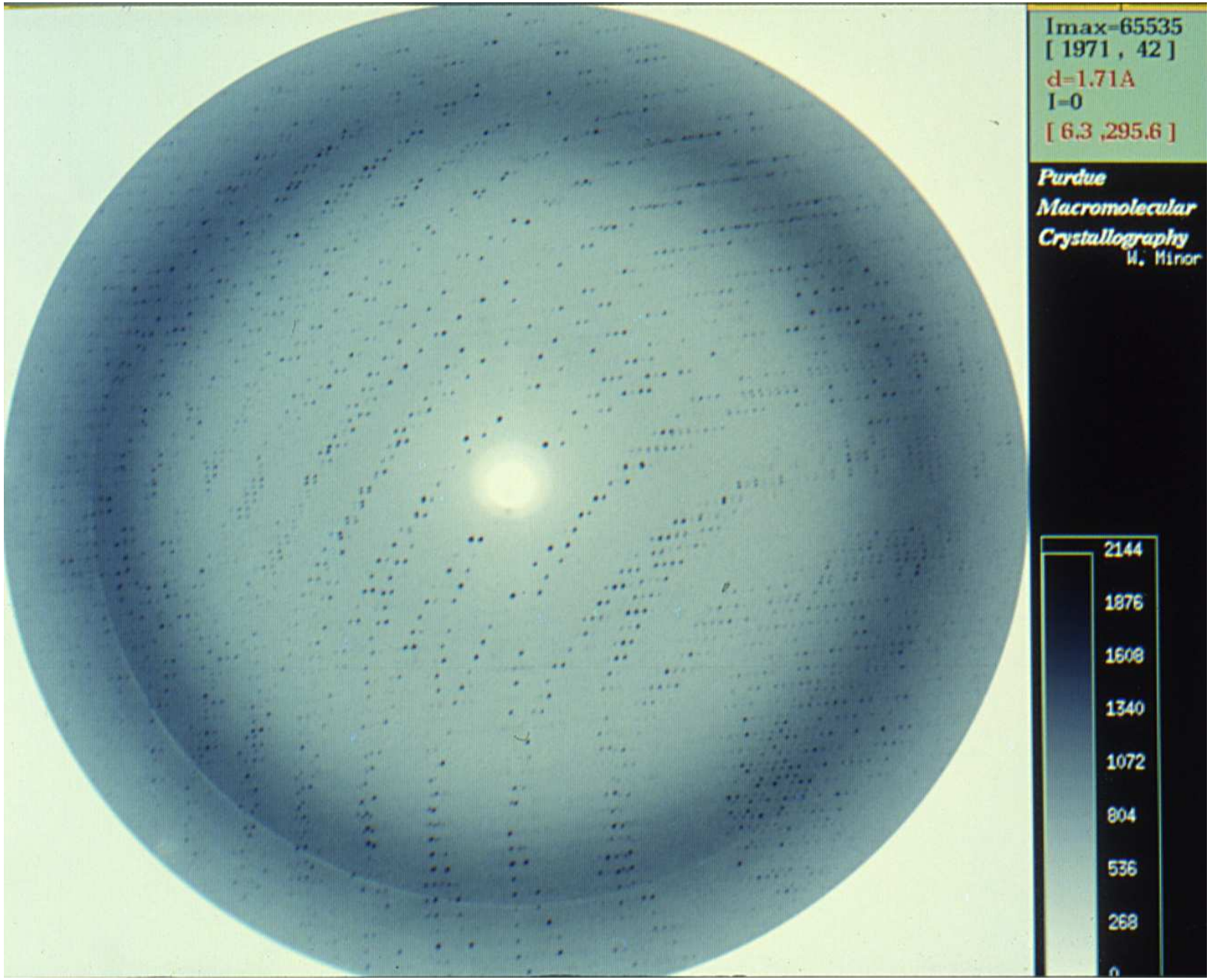
N9 (whale)+32/2
Antibody (FAB)

`Crystal' diffraction:

- Salt? Do a large $\Delta\phi$ image.
- Obviously twinned
- Internally twinned
- Disordered: high mosaic spread, disordered along one axis, statistical disorder.
- Diffraction weak.
- None…
- If good, what is resolution limit? Reassess crystal to detector distance.
- Reasonable mosaic spread
- Spots are resolved
- Spots are not overloaded

The best we ever get…



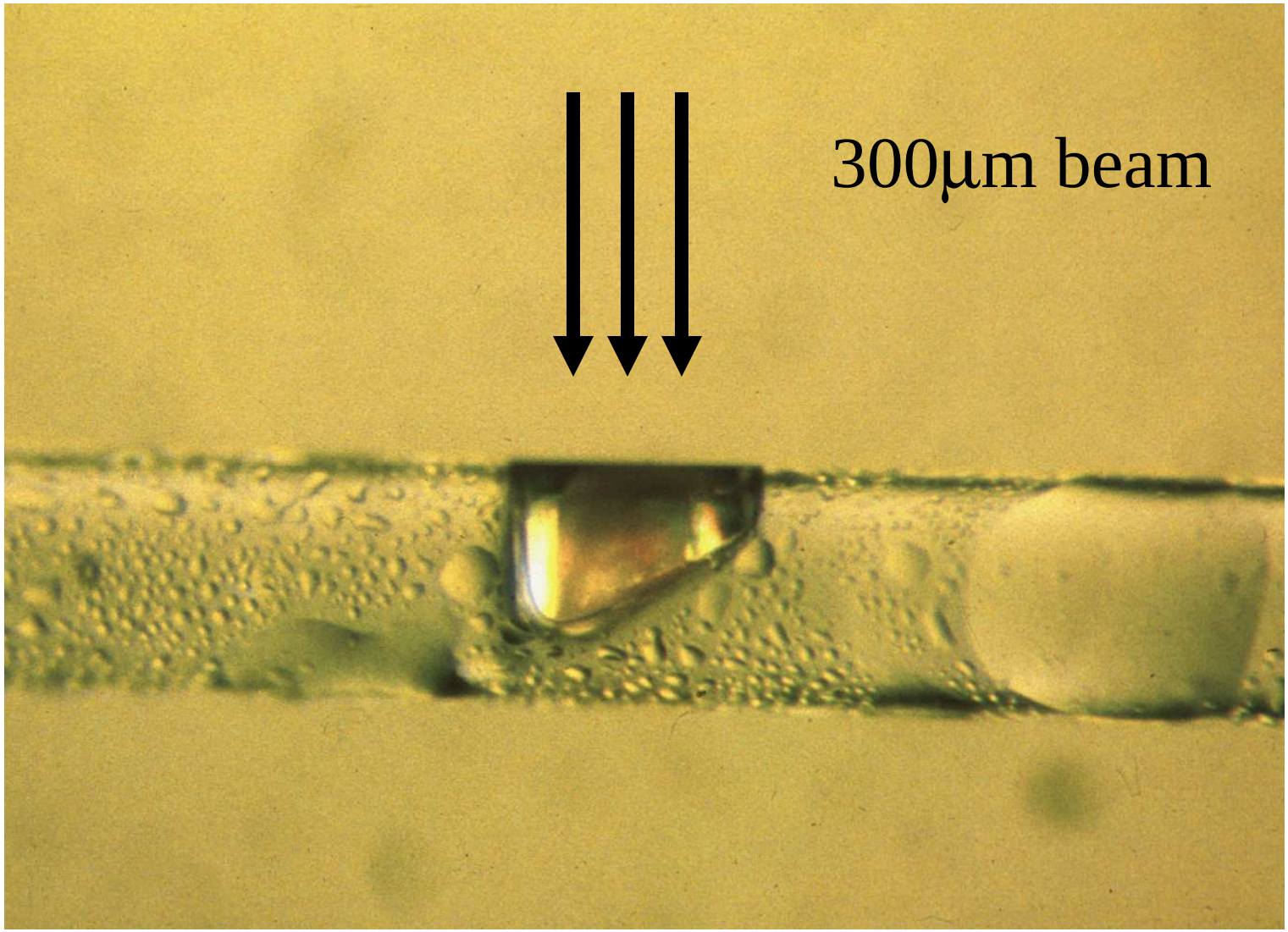


e.g Twinned crystal with 2 distinct lattices.

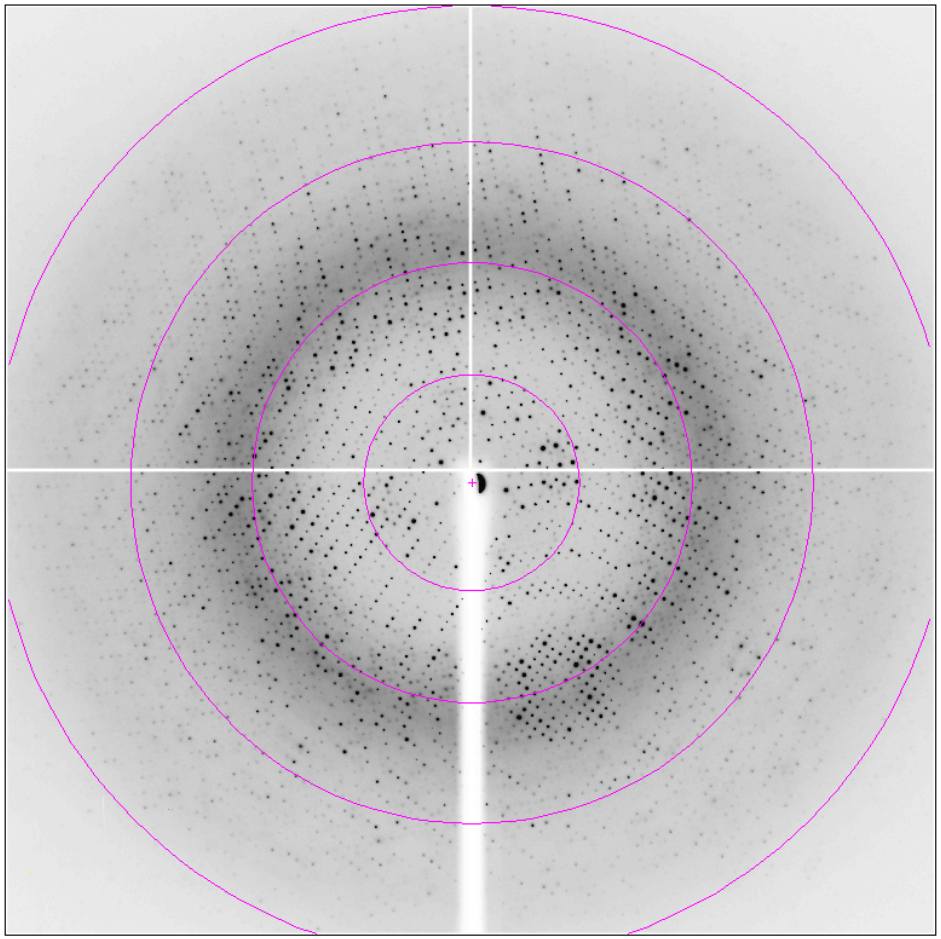
2. Radiation damage:

The Plan:

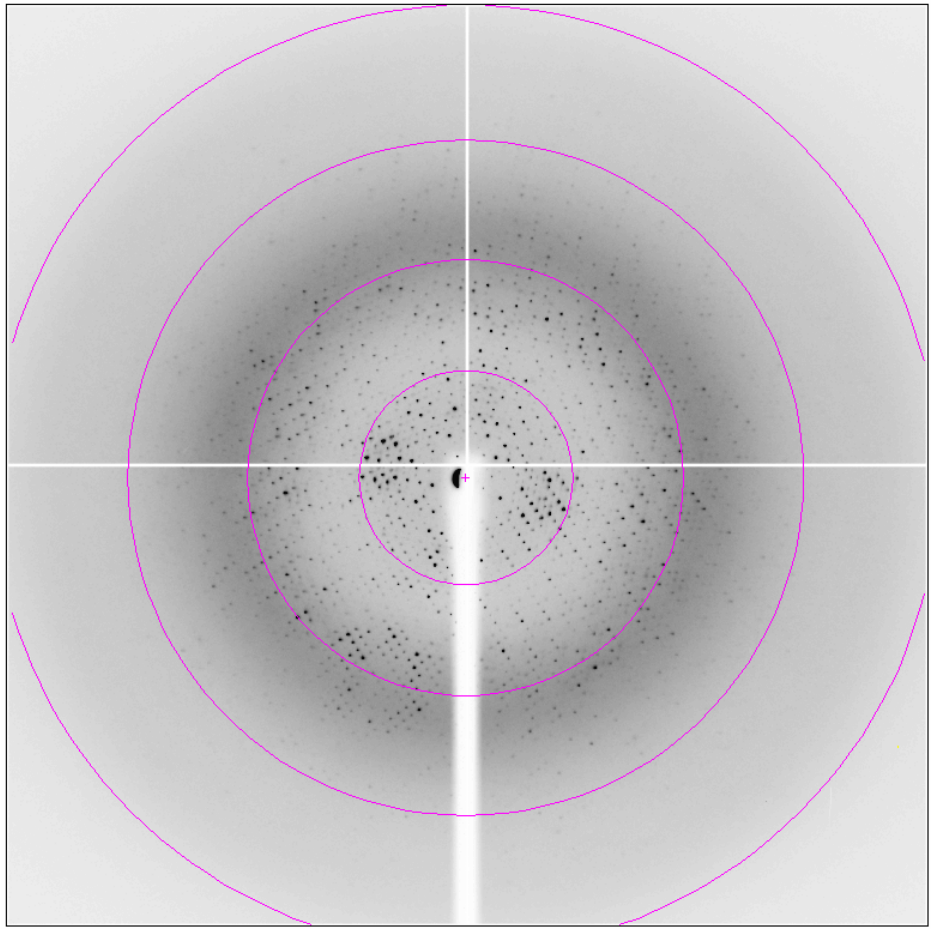
- **What are the symptoms?**
- Why do we care?
- What is it?
- What is `Dose'?
- How might TotalCryst help?



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



Intensity
decrease



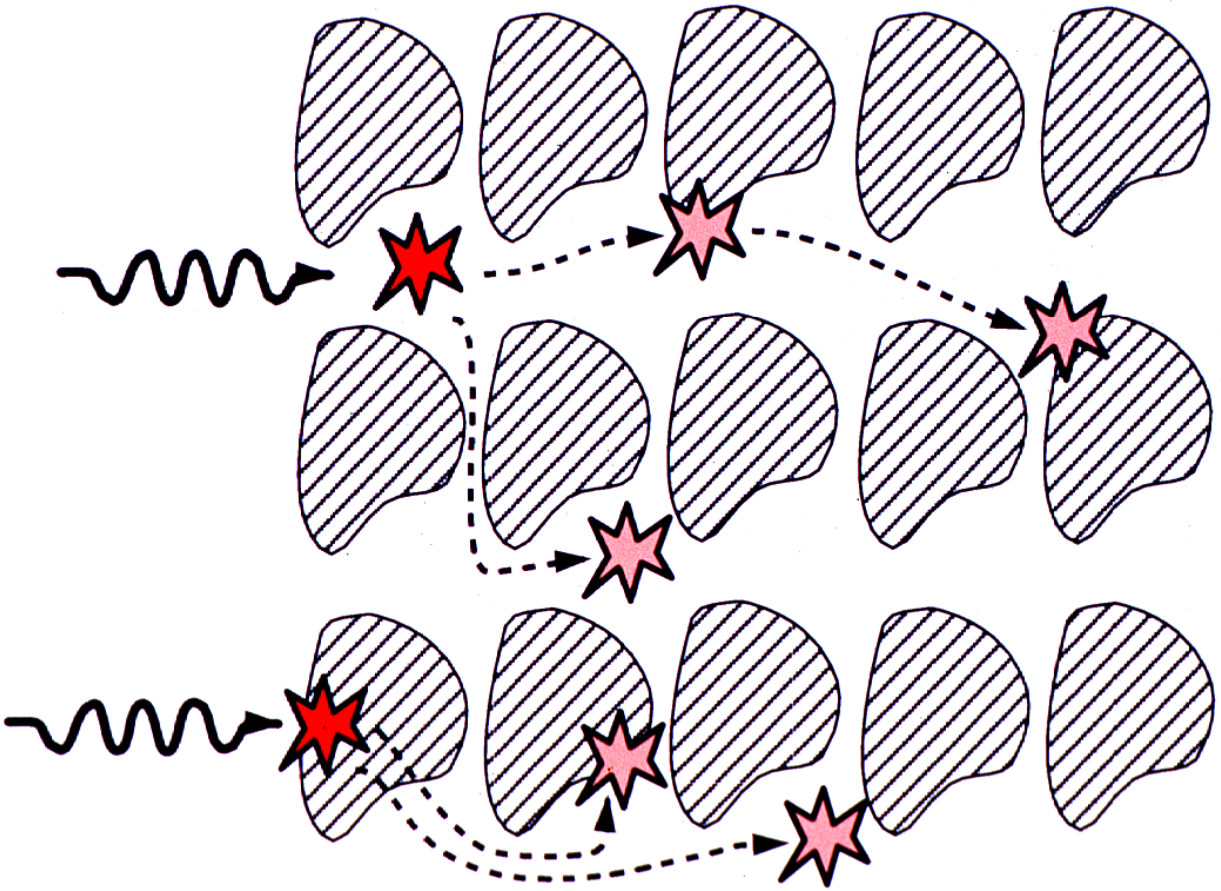
Loss of
diffraction

Incomplete data
from crystals

Radiation Damage

Primary: ∿

Secondary: ⇢



PRIMARY; inevitable, a fact of physics! Can we minimise it?

SECONDARY, can we control it?

First systematic study of radiation damage in protein crystals:

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

In 'Biological Effects of Ionising Radiation at the Molecular Level'. AEA Symposium, Vienna, P183.

- **Damage proportional to dose** [Room temp].

Dose= energy lost per kilogramme

This finding has become a basic assumption, only recently challenged (dose rate important at RT).

- Each 8 keV photon absorbed disrupts ~ 70 molecules and somewhat disorders another 90.

- Damage may be structurally specific.

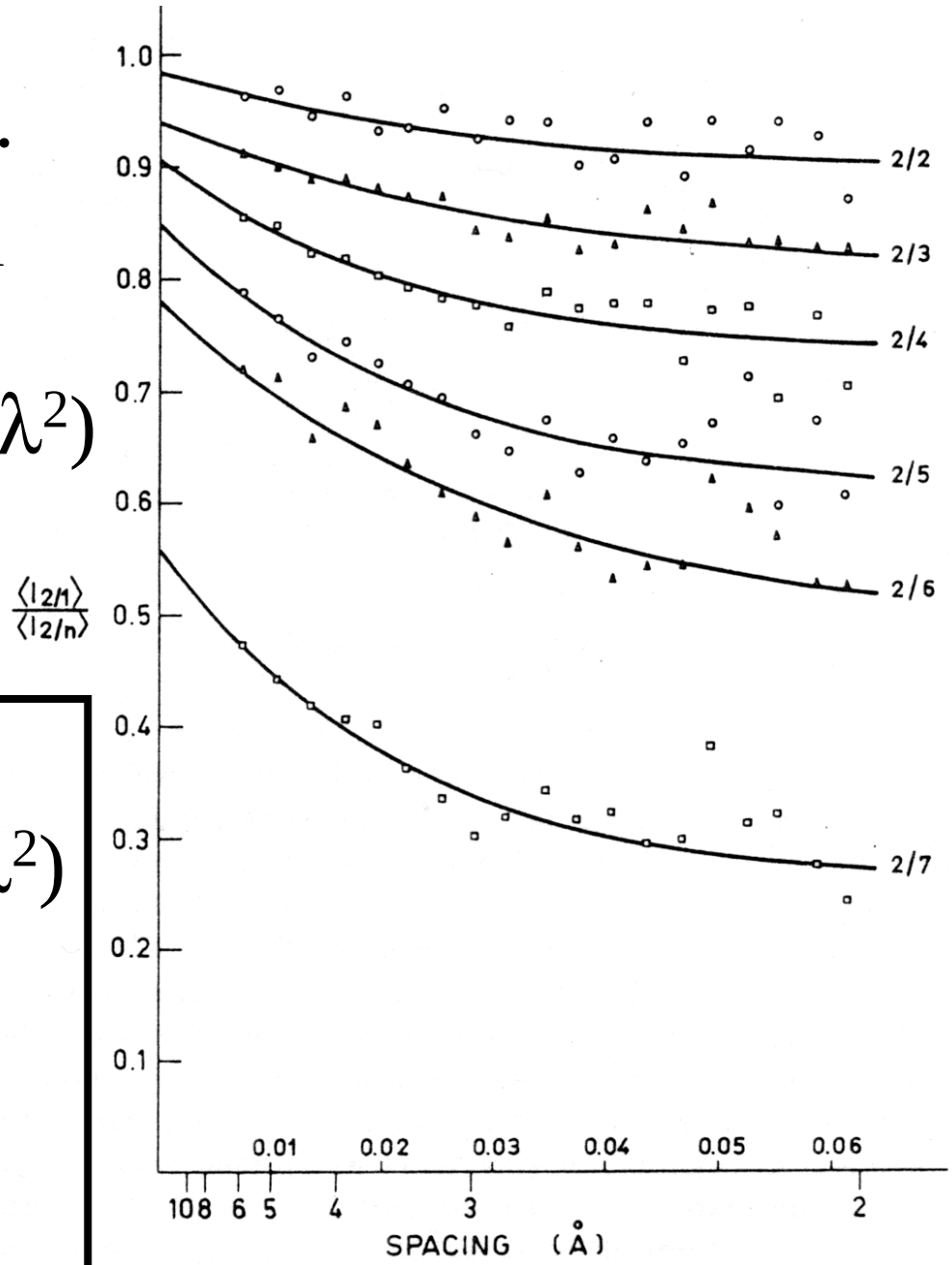
[Confirmed 38 years later at 100K…]

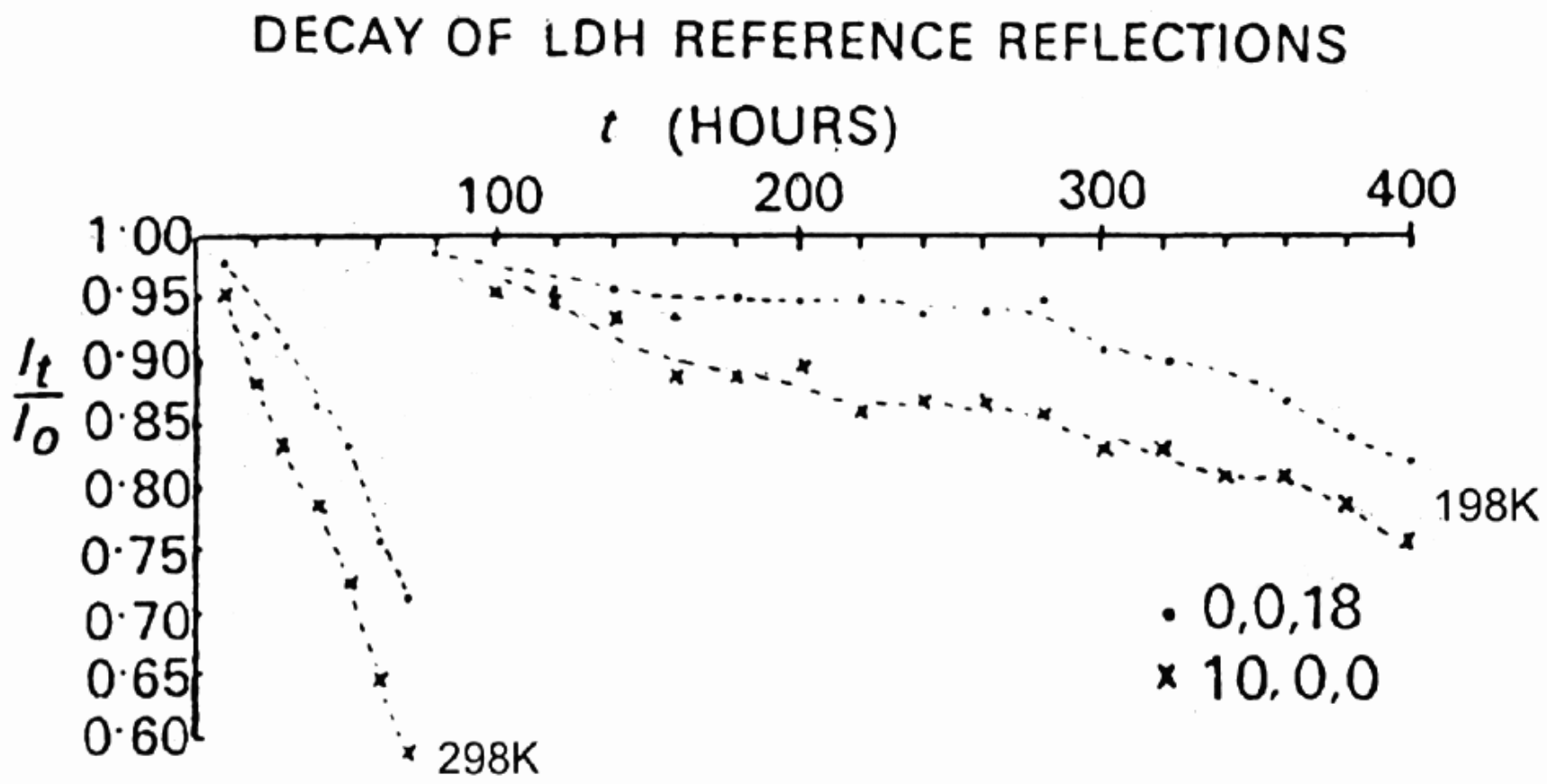
Top: $\langle I \rangle_{\text{Run 2}} / \langle I \rangle_{\text{Run 1}}$
as a function of resolution.
Bottom: $\langle I \rangle_{\text{Run 2-7}} / \langle I \rangle_{\text{Run 1}}$

$I(t)/I(0) = A_0 \exp(-B.\sin^2\theta/\lambda^2)$
did not fit.

$I(t)/I(0) = A_1(t) + A_2(t) \exp(-B_2.\sin^2\theta/\lambda^2)$

A_1 fraction unchanged
A_2 severely disordered
$1 - (A_1 + A_2)$ amorphous



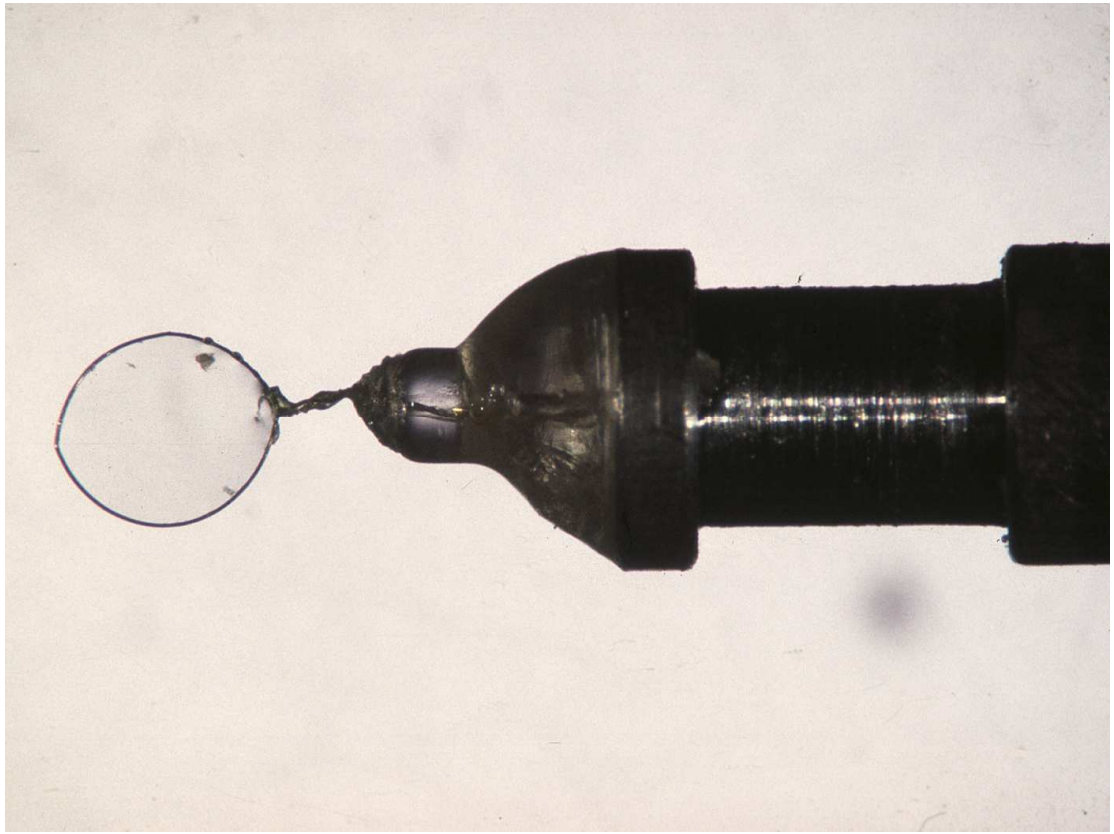


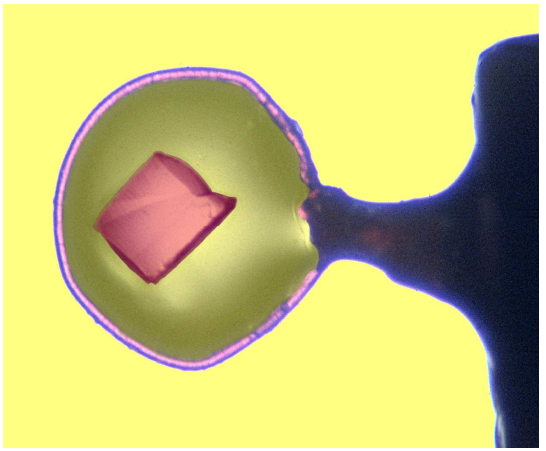
Haas and Rossmann 1970: lactate dehydrogenase

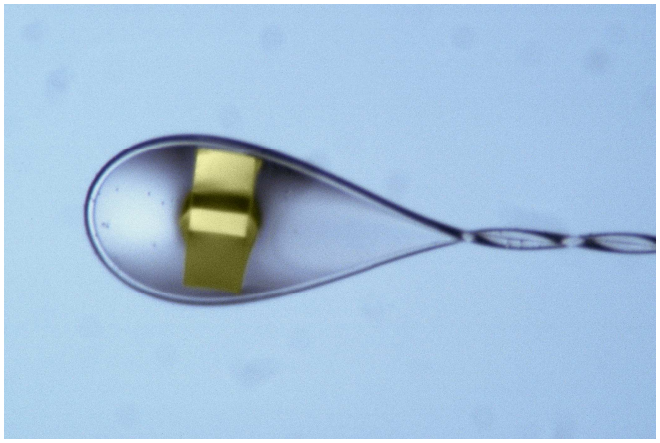
Acta Cryst B26, 998-1004.

ICE a major problem

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



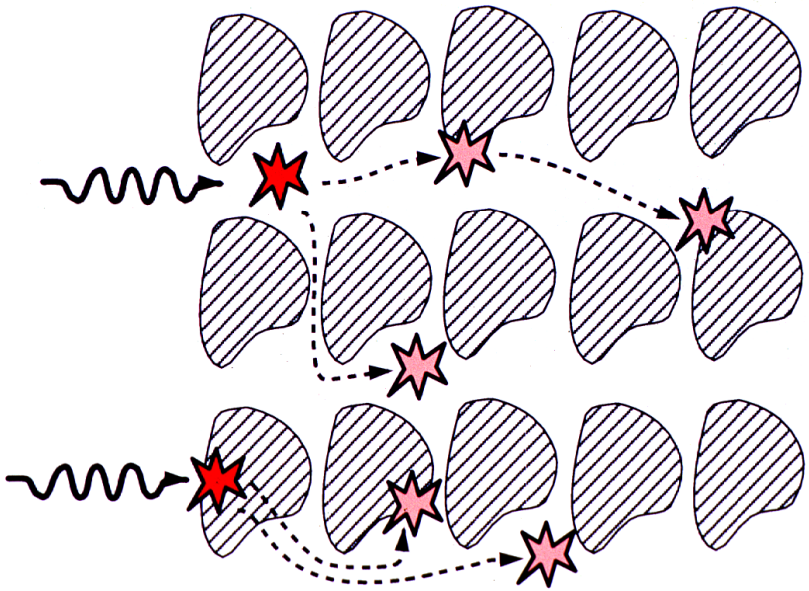


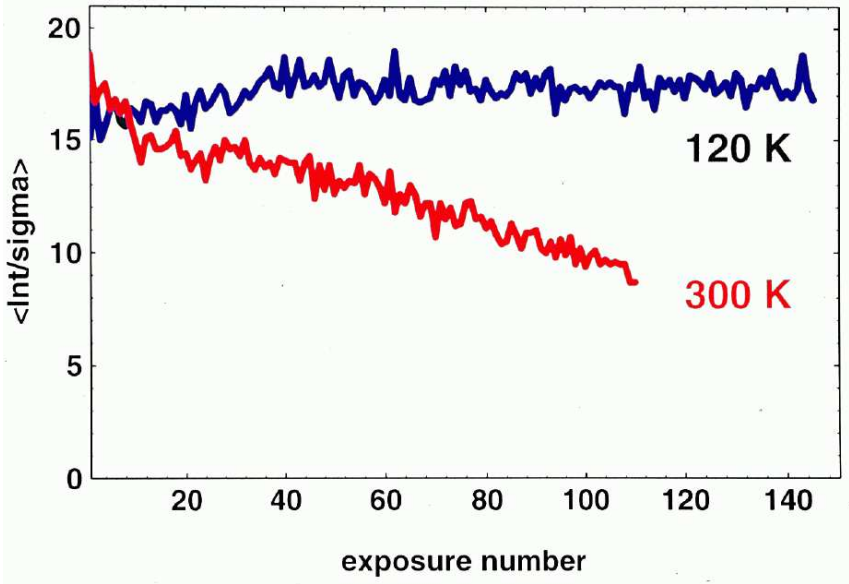


Also, a commercially available and easy to use cryostat (Cosier and Glazer 1986) made the technique accessible to many labs.

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

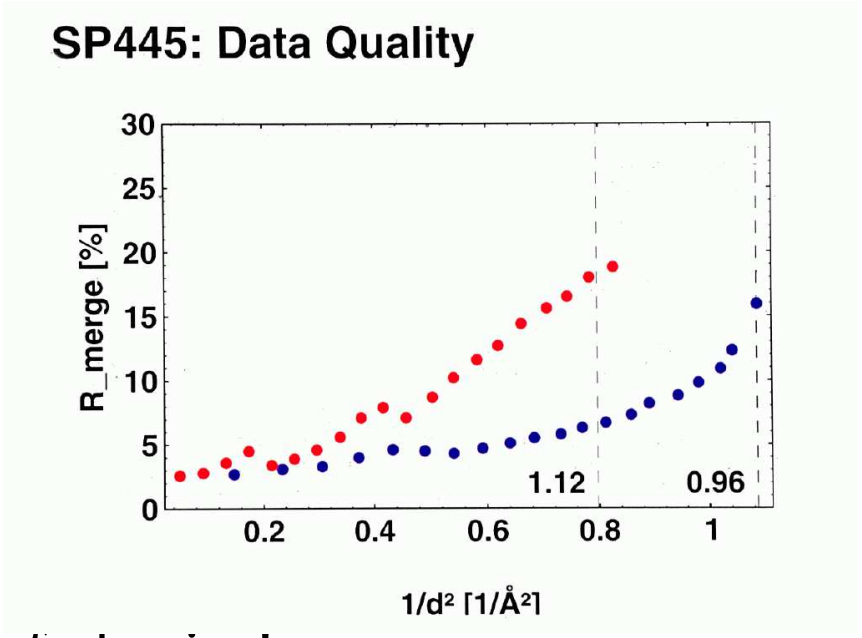
Radiation Damage





Significantly reduced at 100K: time factor of ~70.

[Nave and Garman, JSR, 2005]

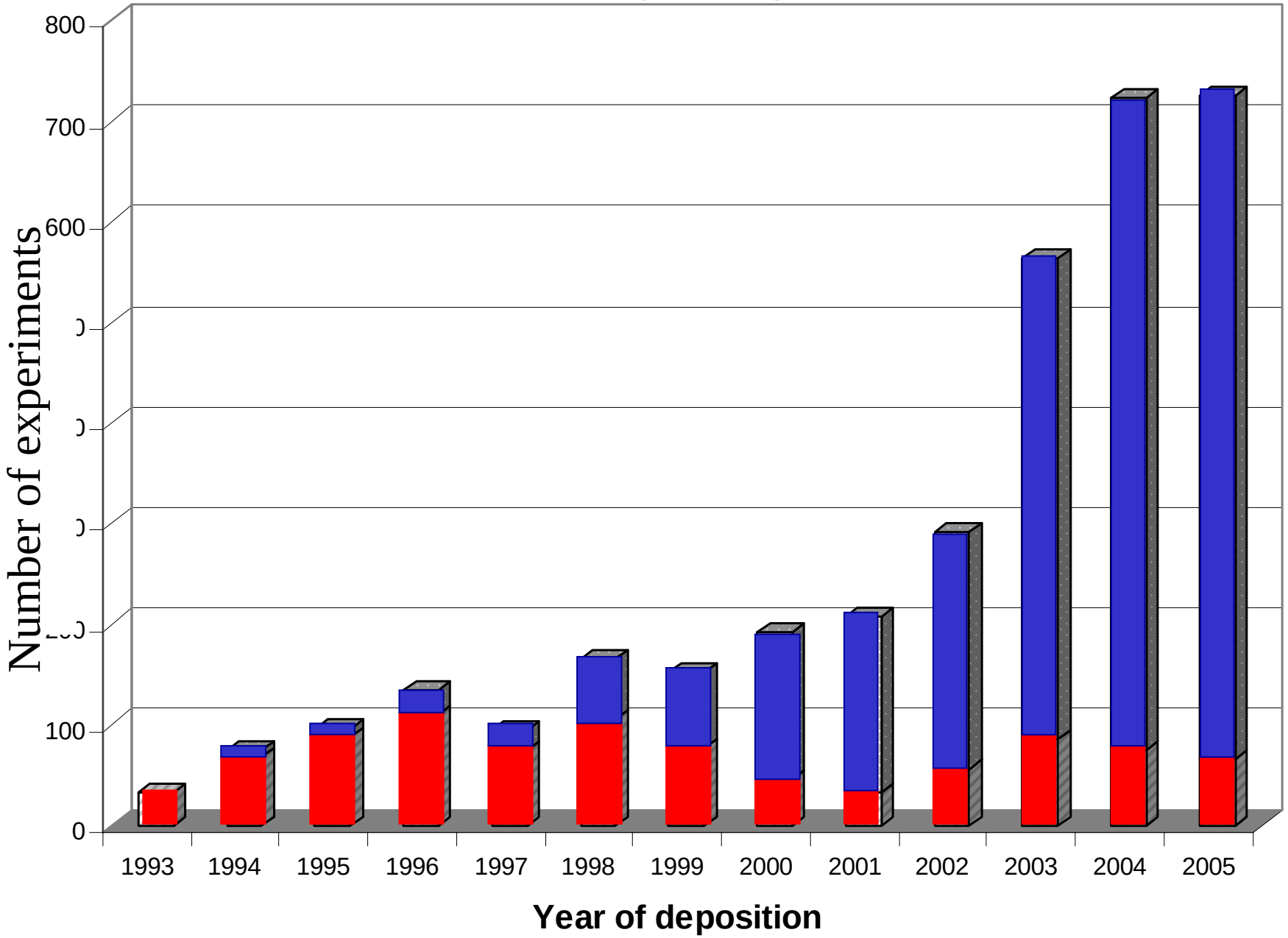


PRIMARY; inevitable, a fact of physics!

SECONDARY, can we control it?

Proportions?

All experiments reported in Acta Crystallographica. D, 1993 – Dec 2005



[Garman, Protein Crystallisation (Ed. T.Bergfors) 2009 (in press)]

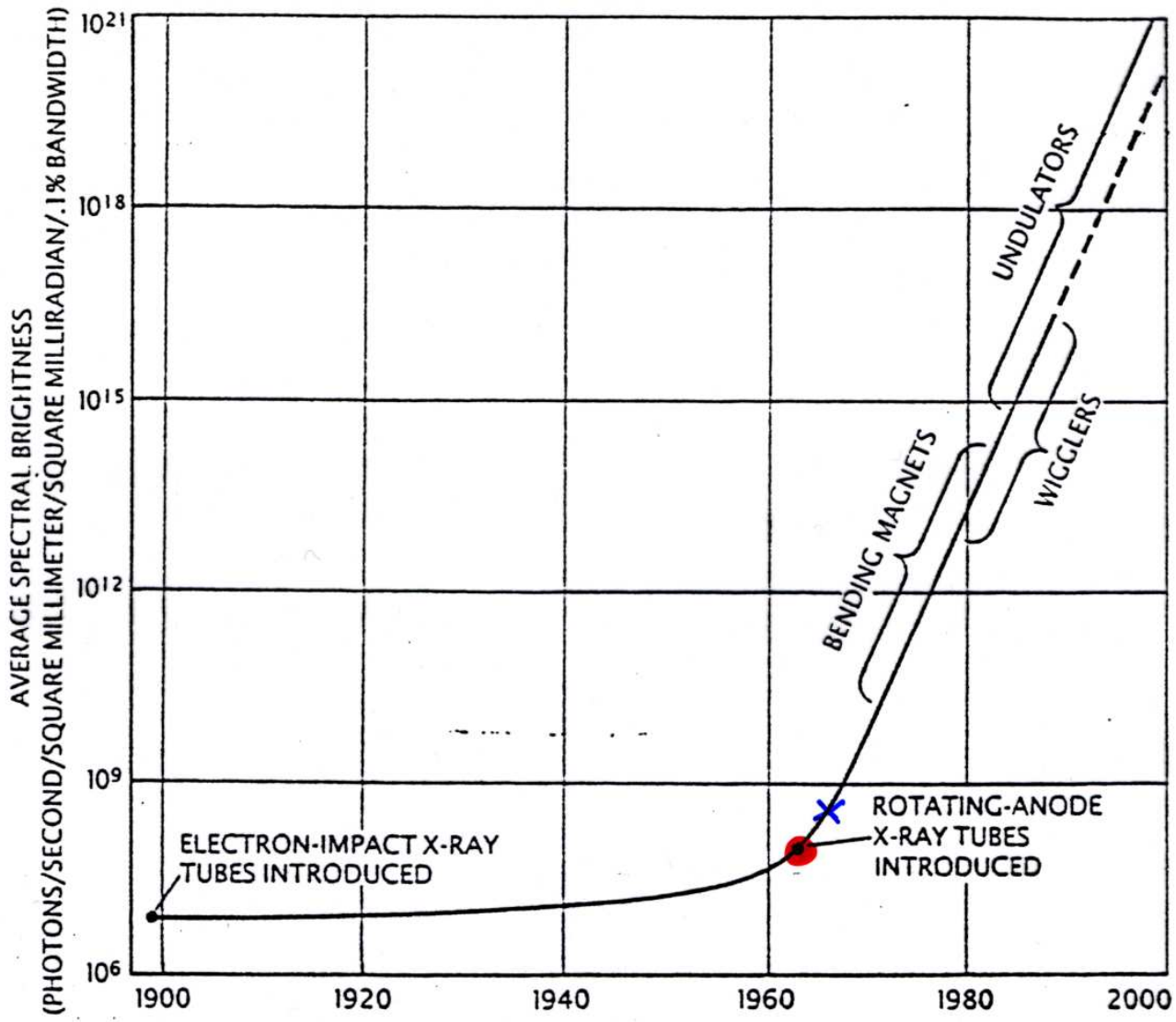
CRYO-COOLING:

Advantages

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

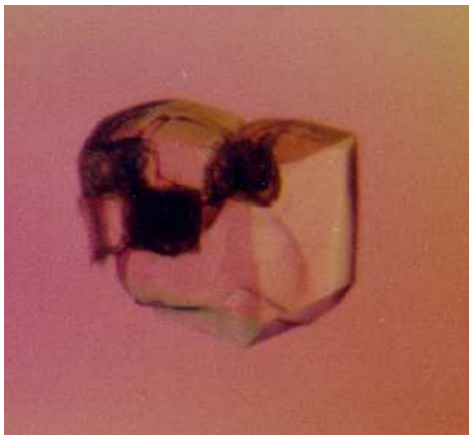
Disadvantages

- Expensive equipment
- Often an increase in mosaic spread.
- Need to invest time for optimisation.
- Waters not physiological.
- No foolproof protocols.



BRIGHTNESS OF X RAYS has increased by many orders of magnitude since the advent of synchrotron-radiation sources. Undulators in storage rings are the brightest source.

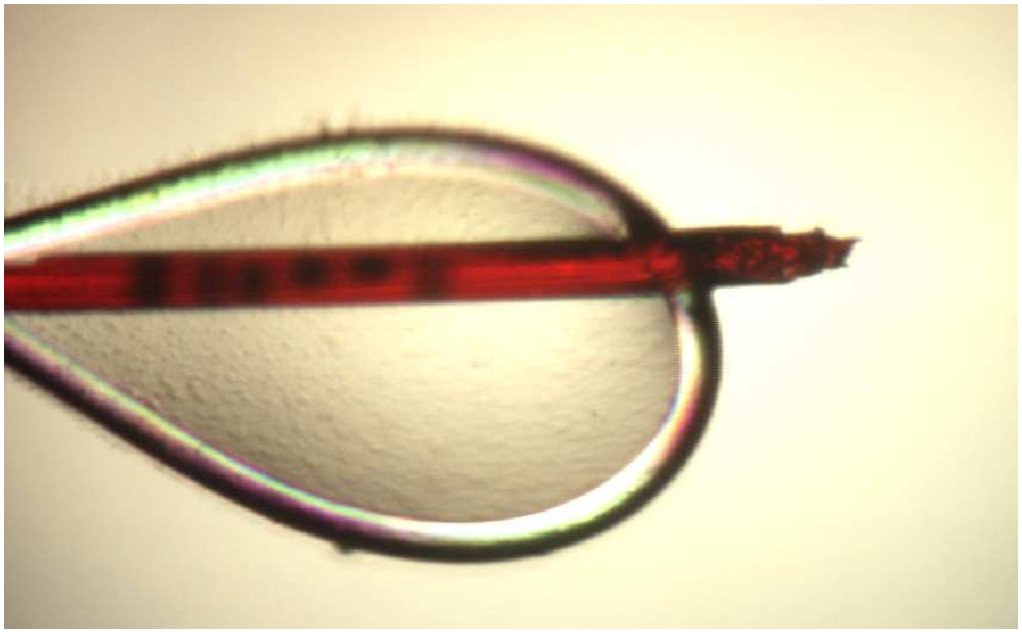
× multi-layer optics.

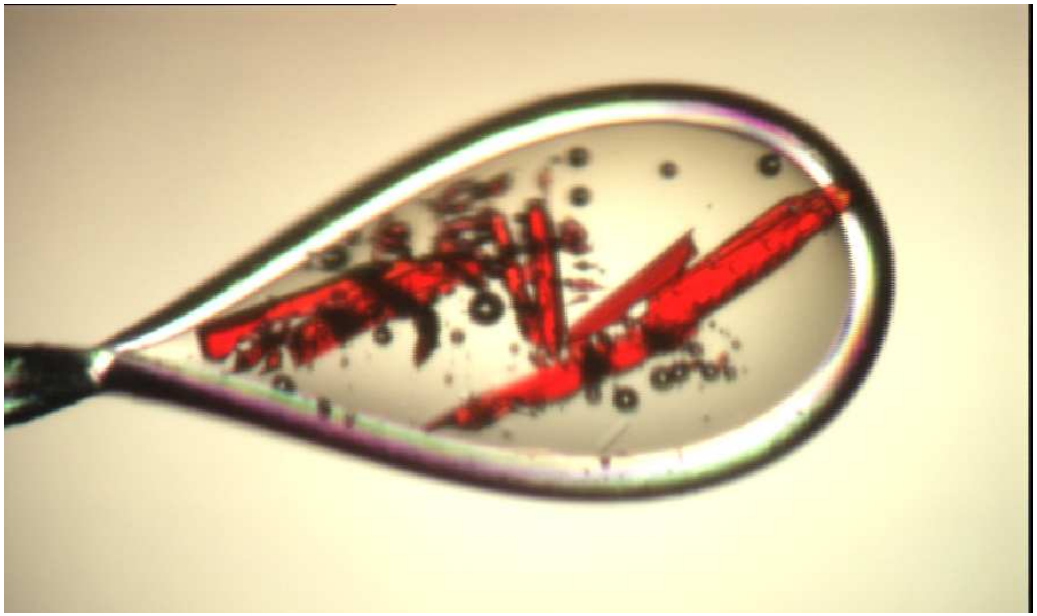


1995: 3rd generation synchrotron: ESRF, Grenoble.

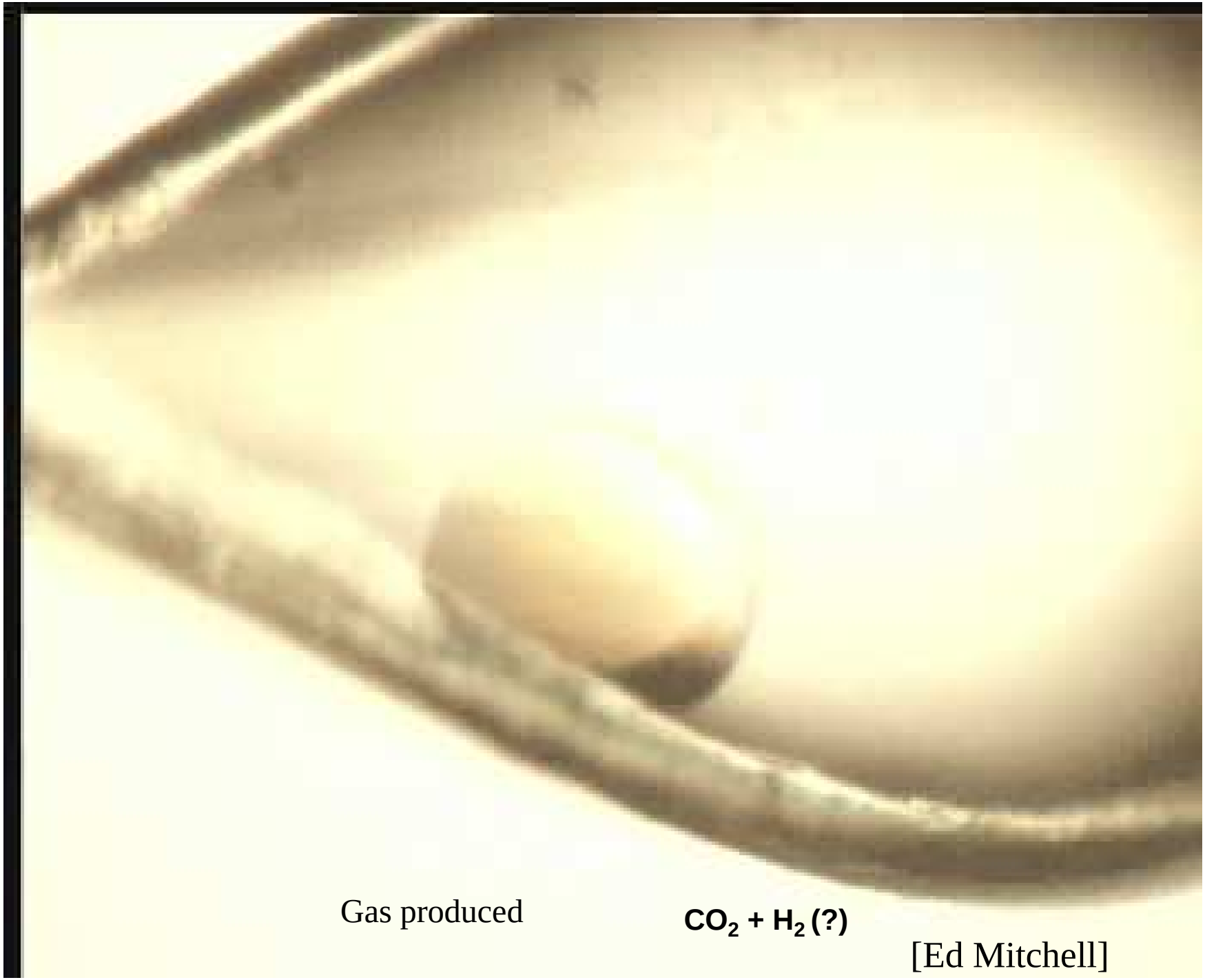
1999: ID14-4; **1×10^{12} photons s^{-1} into 100μm square slits**

2009: μMX, AS; **3×10^{13} photons s^{-1} into 50μm × 70μm**



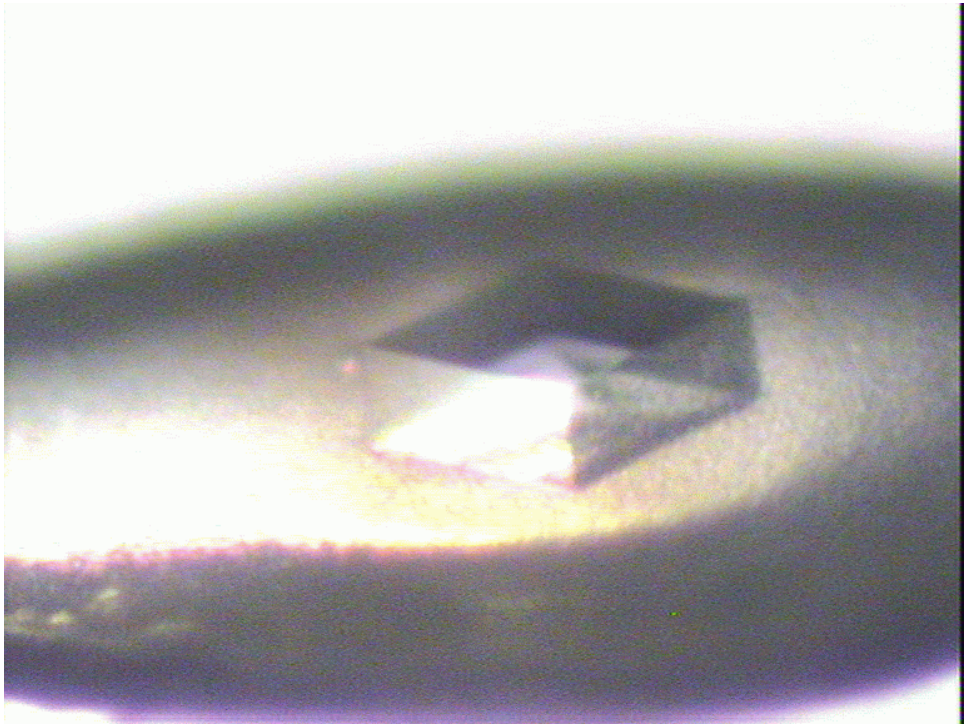


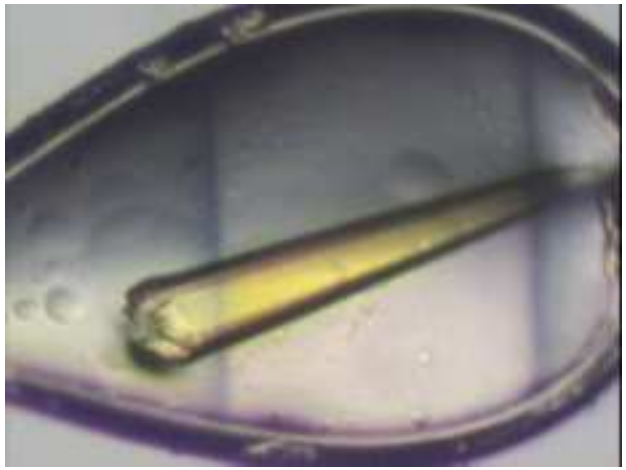
[Tassos Perakis]



Gas produced $CO_2 + H_2$ (?)

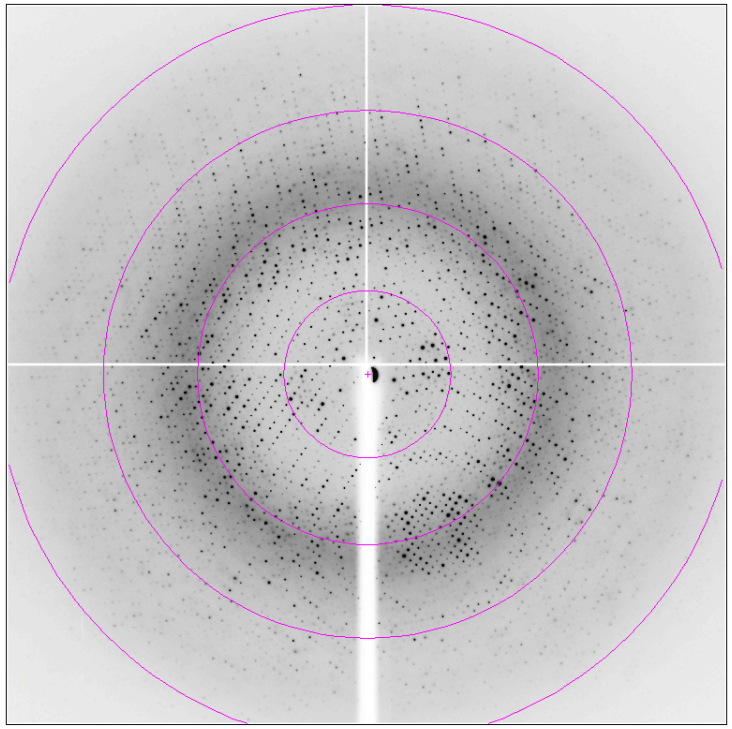
[Ed Mitchell]





Also observe spectral changes

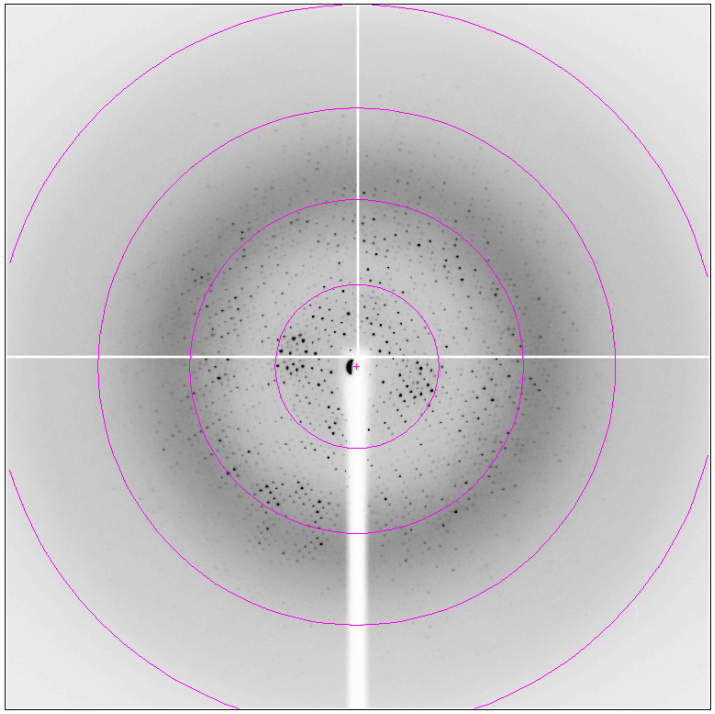
Garman and Owen (2006), Acta D62, 32-47.



Intensity decrease

Loss of diffraction

Incomplete data from crystals



Dataset 1

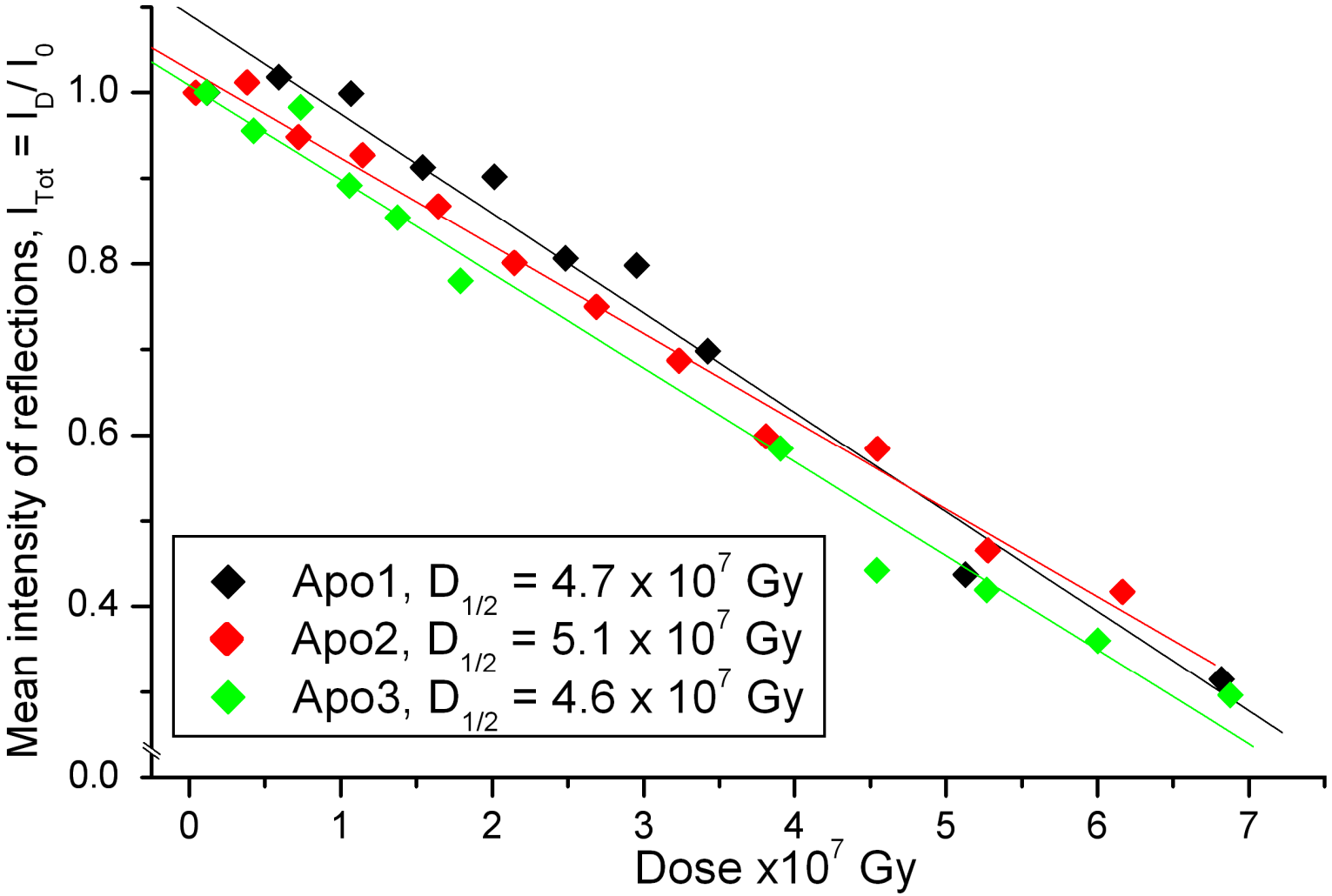
Dataset 10

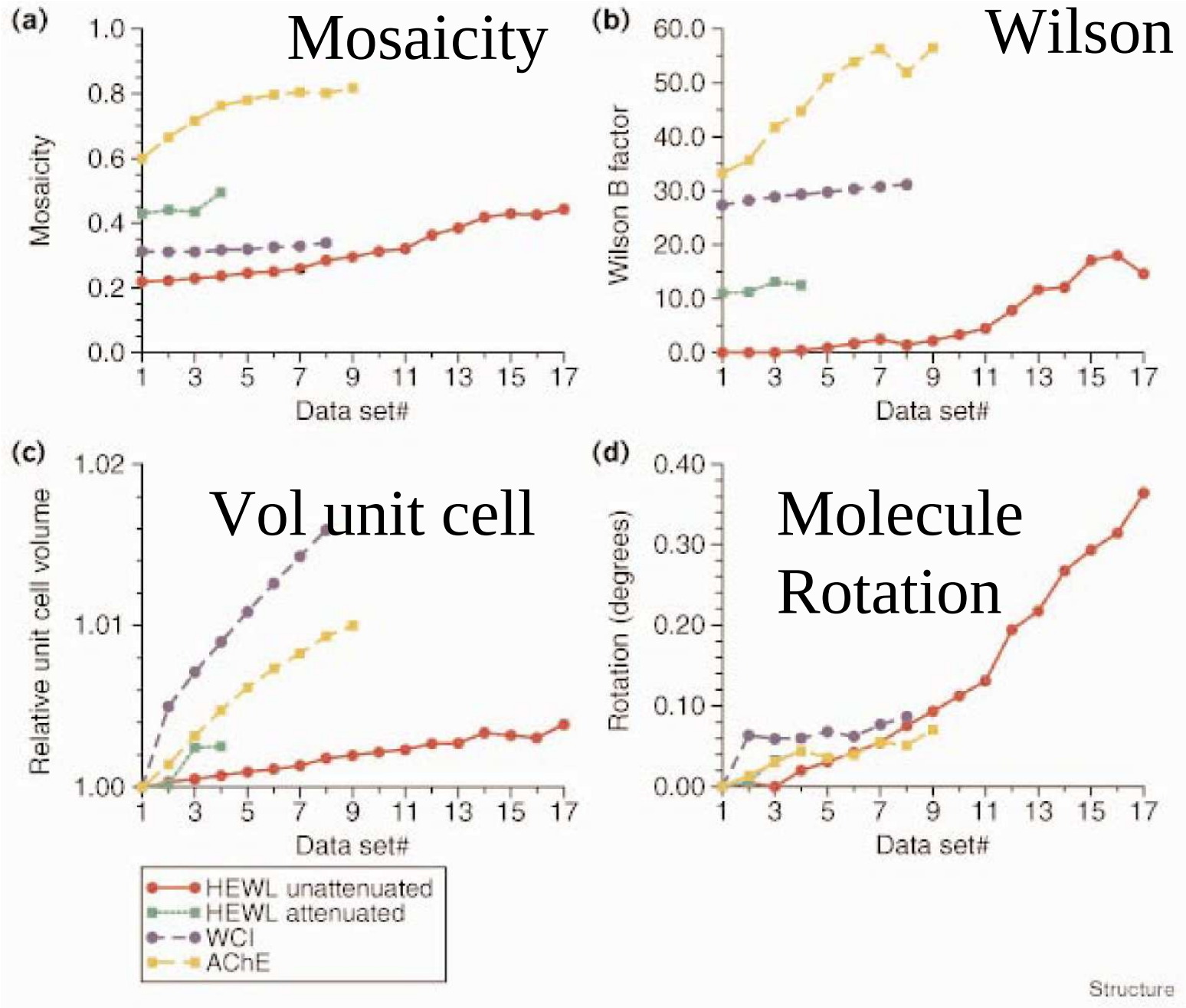
Happens during 1 dataset at 100K for some crystals

Unit cell volume expansion,
Wilson B factor increase.

Intensity Decay at 100K

Normalised Intensity vs Dose: apoferritin





Ravelli and McSweeney, Structure (2000) 8, 315

Data Parameters affected by Radiation Damage

- I / σ(I) or resolution limit
- R_{merge}
- Scaling B factors
- Mosaicity
- Unit Cell expansion a) function of dose

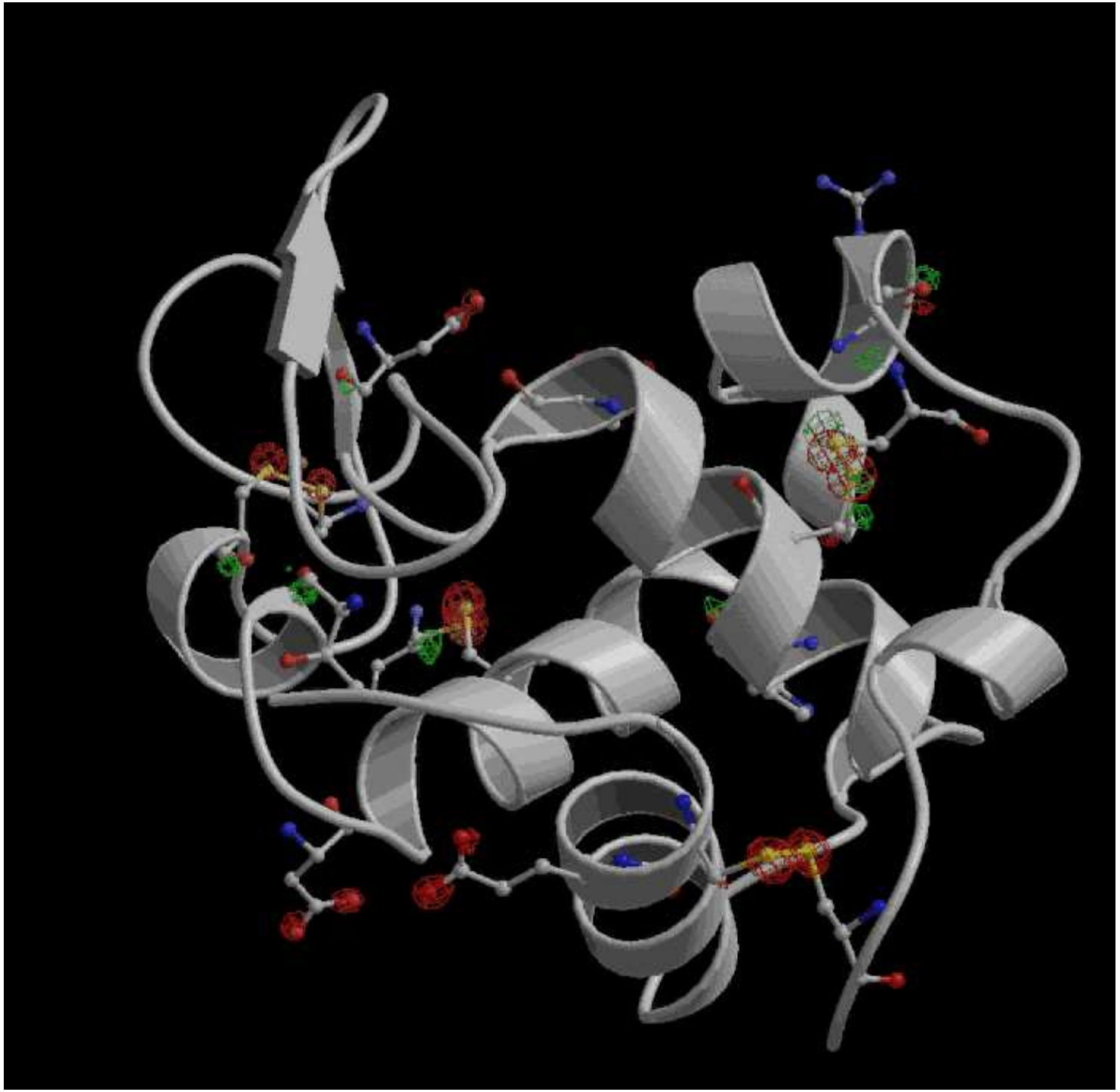
  b) function of cryogen temperature

Could this be an on-line damage metric?

[Ravelli and McSweeney, (2000) Structure]

No!

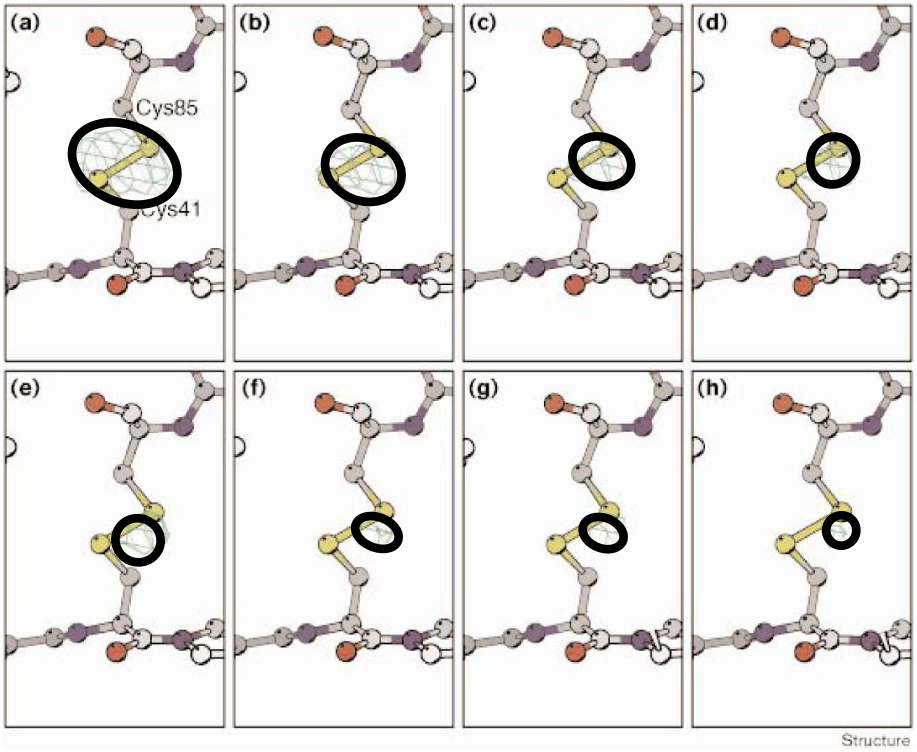
[Murray and Garman (2002), JSR, Ravelli et al (2002) JSR]



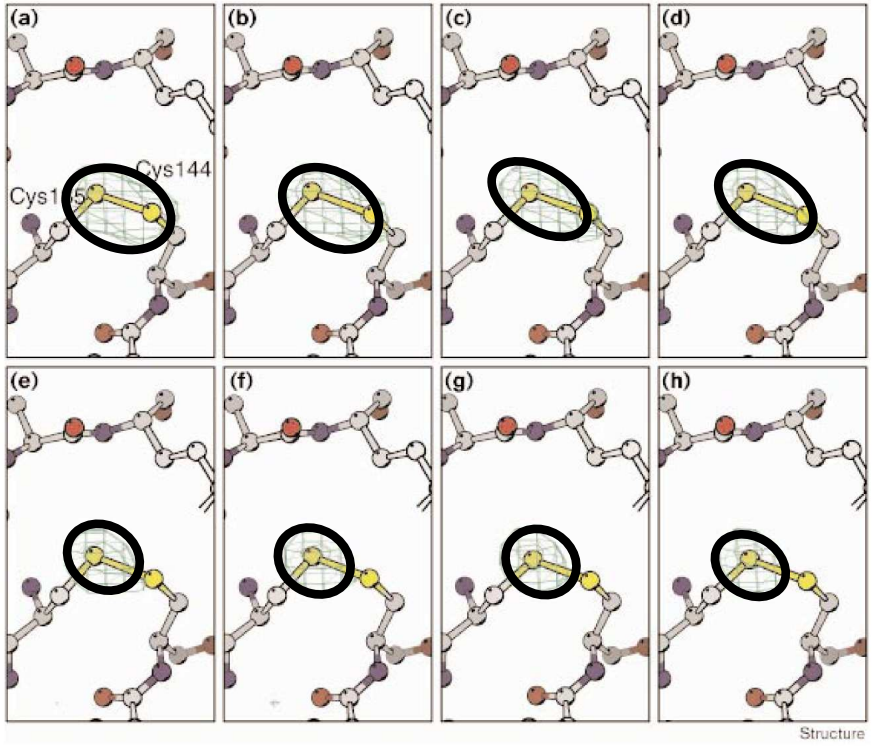
HEWL
4 S-S

Wing bean chymotrypsin inhibitor disulphides

Cys41-Cys85



Cys144-Cys135



Fo-Fc maps for successive data sets.
Fc with zero occupancy sulphurs.
[Ravelli and McSweeney (2000)]

Specific structural damage observed:

- Disulphide bridges broken: most electron affinic site
- 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.

2) Radiation damage:

The Plan:

- What are the symptoms?
- **Why do we care?**
- What is it?
- What is `Dose'?
- How might TotalCryst help?

Radiation damage affects our biological results

e.g.1 Decarboxylation of Glu may be part of the protein mechanism, but is indistinguishable from radiation damage at the synchrotron.

e.g.2. Metallo proteins often photo-reduced during the experiment [e.g. PSII, Yano et al, PNAS (2005)]

e.g.3. X-ray induced structural changes can be misleading in studies of intermediates

[Bacteriorhodopsin, Takeda et al, JMB (2004)]

Manifestations of Radiation Damage

- Loss of diffraction: incomplete data from crystals
- Specific Structural damage
- WRONG BIOLOGICAL INFORMATION
- `Pollutes' good ultra-high resolution data
- Failure of structure determination
  (Multi-wavelength anomalous dispersion MAD)

due to creeping non-isomorphism – cell expansion and structural changes DURING experiment.

2) Radiation damage:

The Plan:

- What are the symptoms?
- Why do we care?
- **What is it?**
- What is `Dose'?
- How might TotalCryst help?

PHYSICS of the interaction of X-rays with crystals.

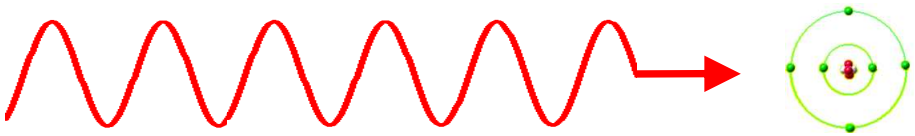
A) Diffraction

B) Absorption = Energy loss

N.B. > 90% of the beam does not interact at all.

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

Thomson (Rayleigh, coherent) scattering

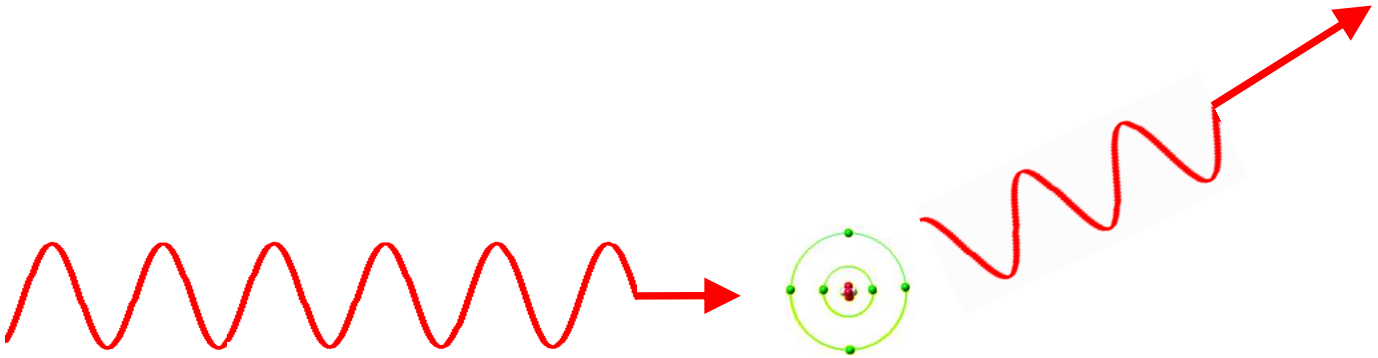


[8% at 1Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering

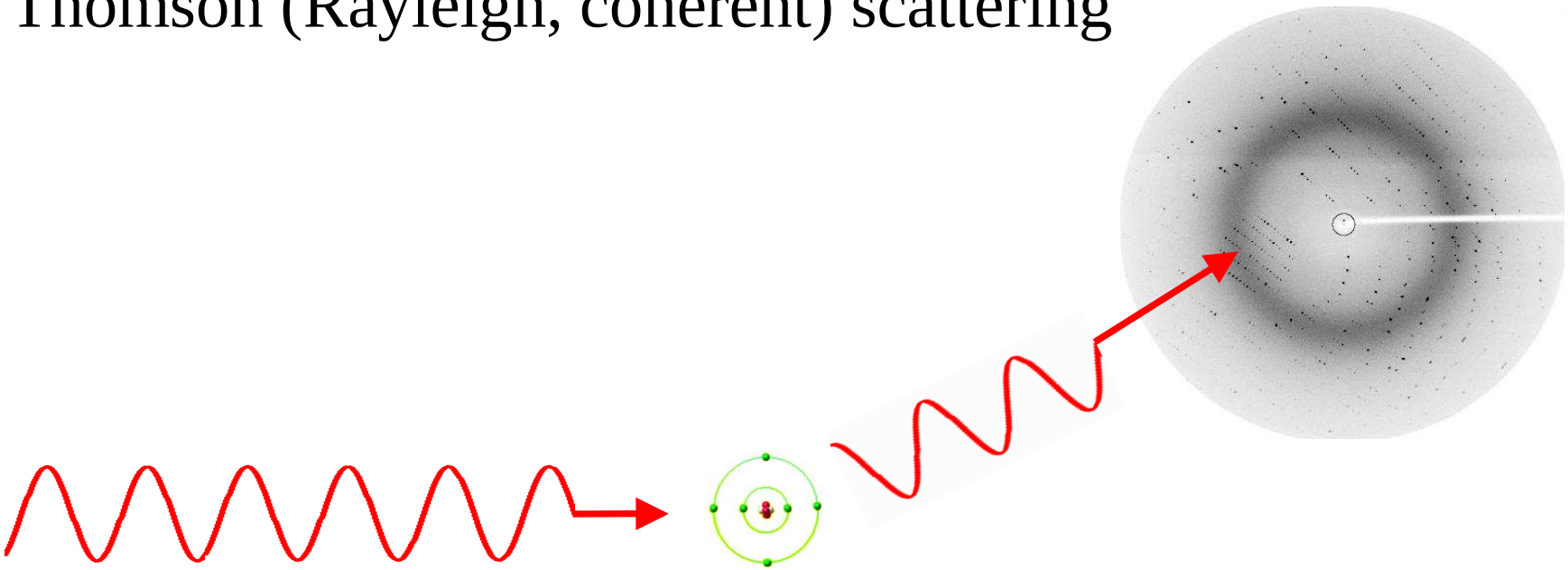


[8% at 1Å]

ELASTIC - no energy loss.

Primary X-ray interaction processes with crystal and solvent.

Thomson (Rayleigh, coherent) scattering



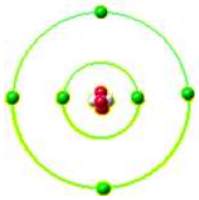
ELASTIC - no energy loss.

Coherent – adds vectorially and gives diffraction pattern.

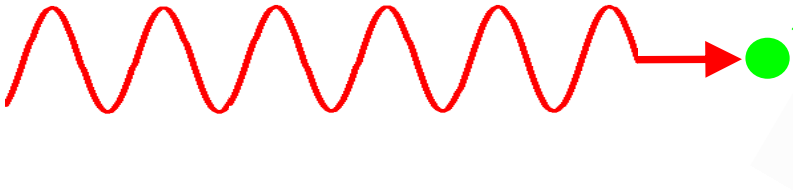
Small proportion of total scattering: 8% at 1Å

BUT IT IS THE BIT WE WANT!!

B) Compton (incoherent) scattering



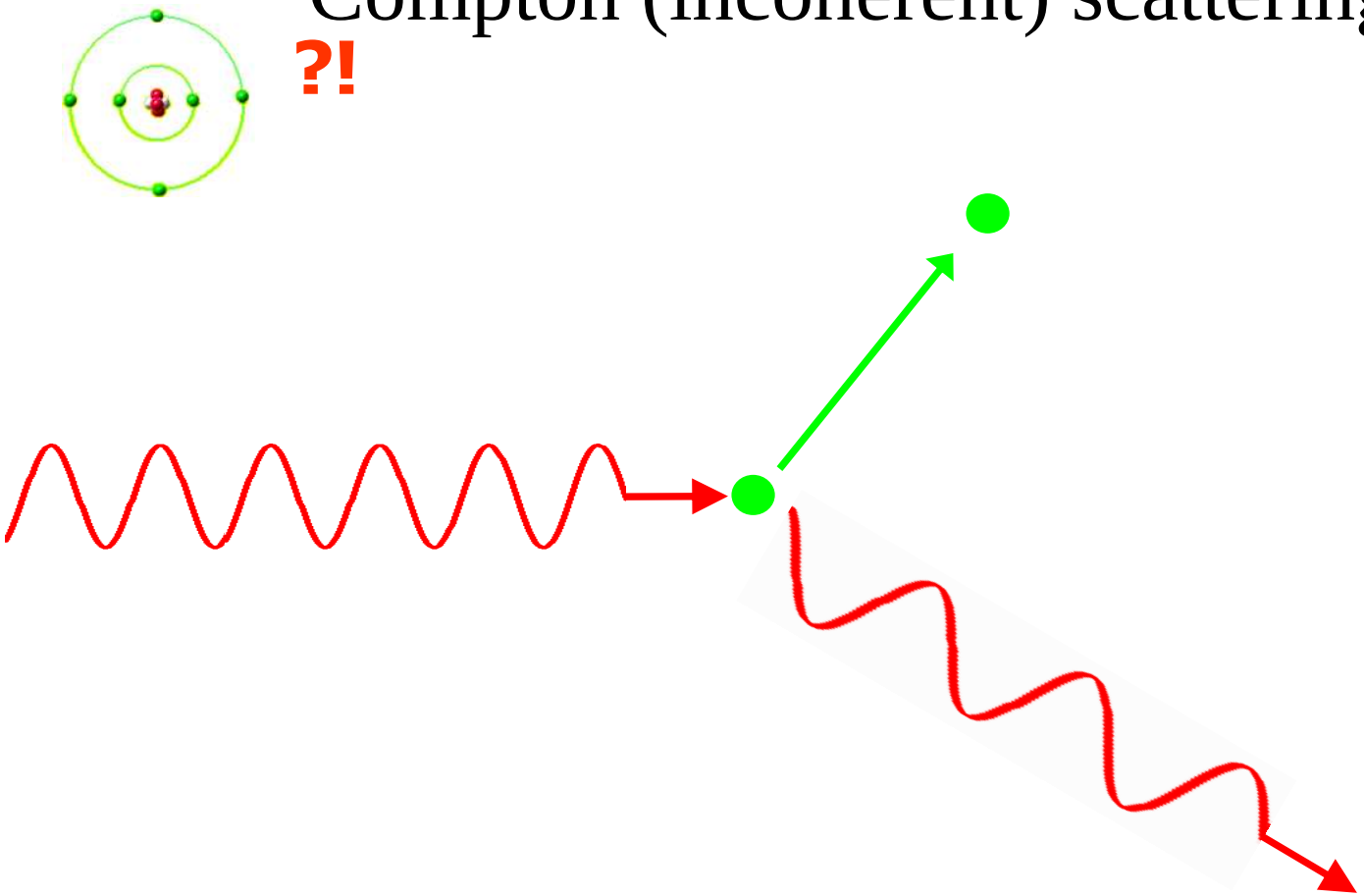
?!



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

Compton (incoherent) scattering

?!



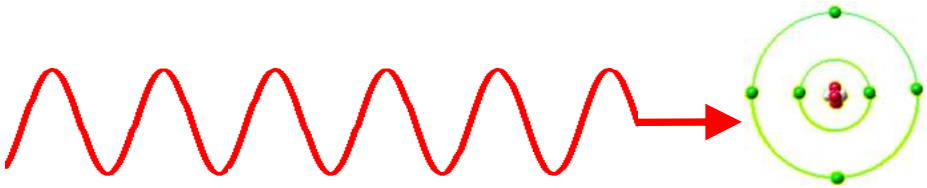
X-ray transfers some energy to atomic electron and thus has lower energy (higher wavelength).

Incoherent – part of X-ray background in images.

Also a small proportion of total scattering: 8% at 1Å

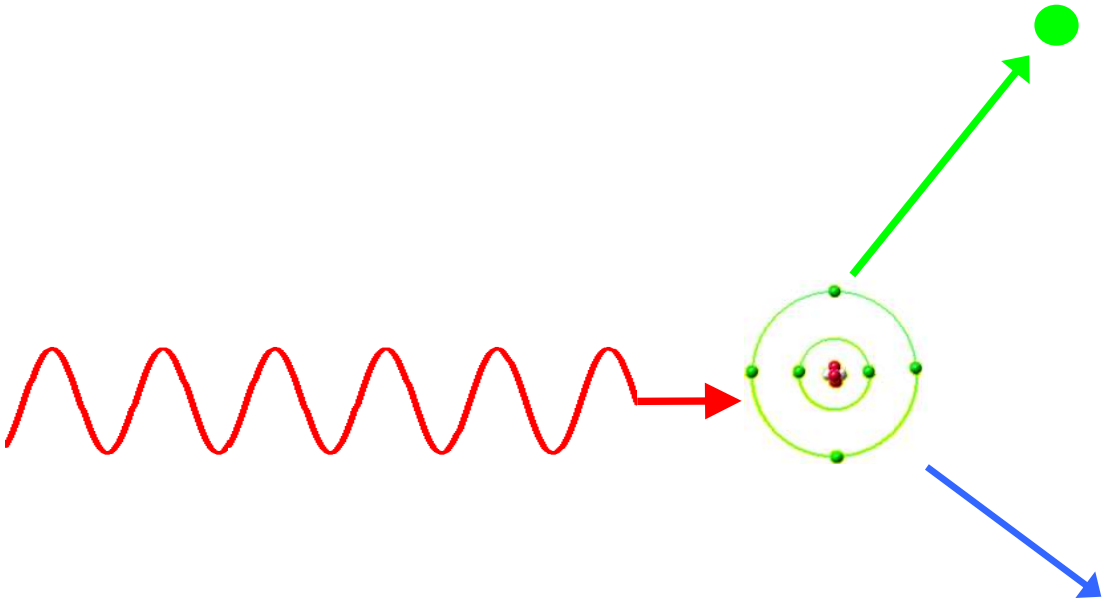
Photoelectric Absorption

84% at 1Å



INELASTIC.

Photoelectric Absorption



84% at 1Å

INELASTIC.

X-ray transfers all its energy to an atomic electron, which is then ejected.

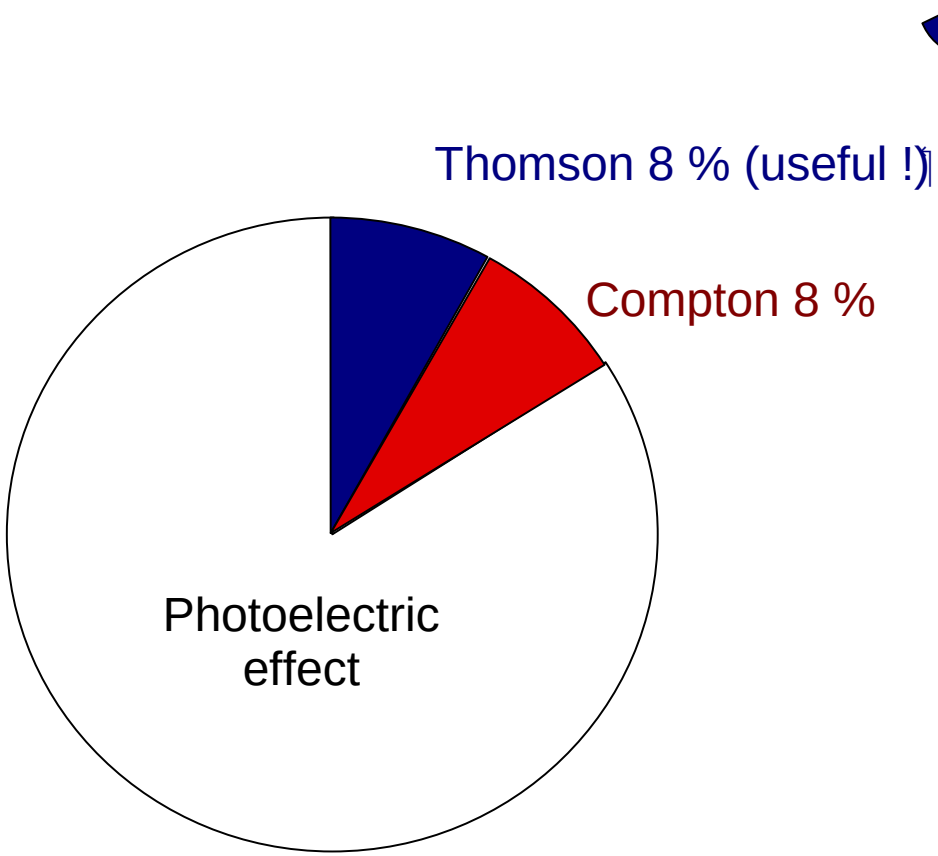
Each 12 keV primary photoelectron can give rise to up to 500 ionisation events.

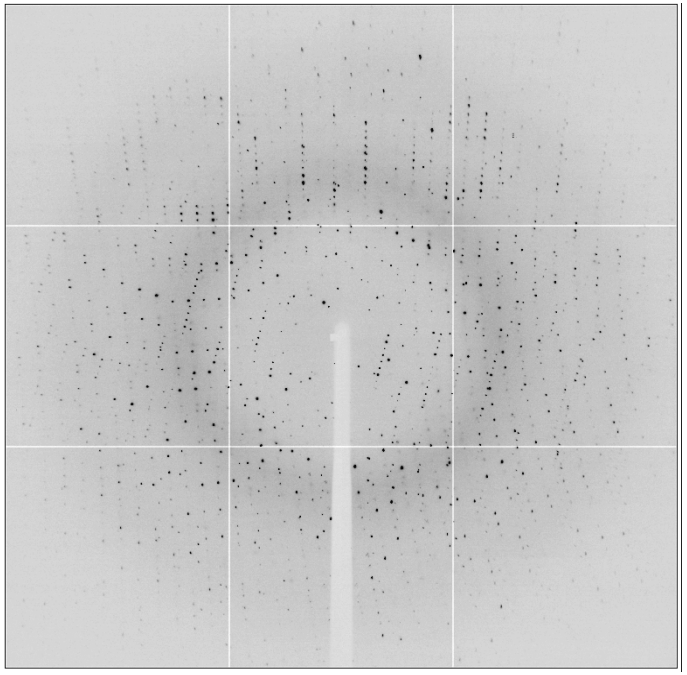
Atom can then emit a characteristic X-ray or an Auger electron to return to its ground state.

$$\sigma tot = \sigma\ pe + \sigma\ inc + \sigma\ coh$$

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

Summary: What really happens when X-ray photons hit the crystal ?





λ = 1 Å (at energy 12.4 keV) for a

100x100x100µm crystal

Photoelectric Cross Sections (barns/atom) at 13.1 keV

[1 barn=$10^{-28}m^2$]

A few heavy atoms can
make a big difference.

.

H

[Ravelli *et al.*, JSR,(2005) 12]

Photoelectric Cross Sections (barns/atom) at 13.1 keV

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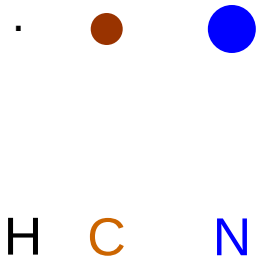

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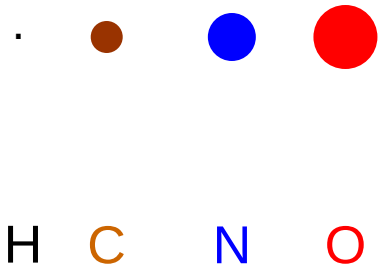

[Ravelli *et al.*, JSR,(2005) 12]

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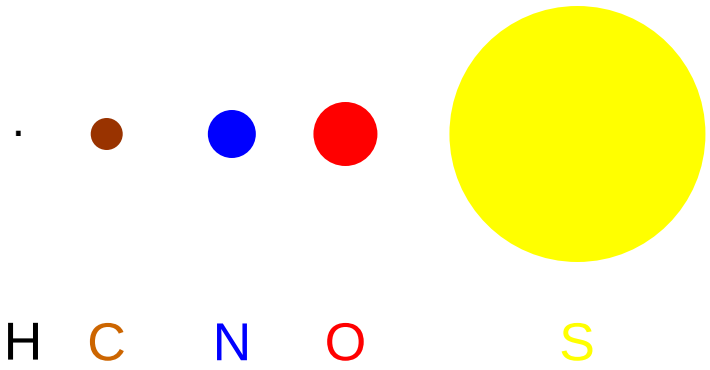

[Ravelli *et al.*, JSR,(2005) 12]

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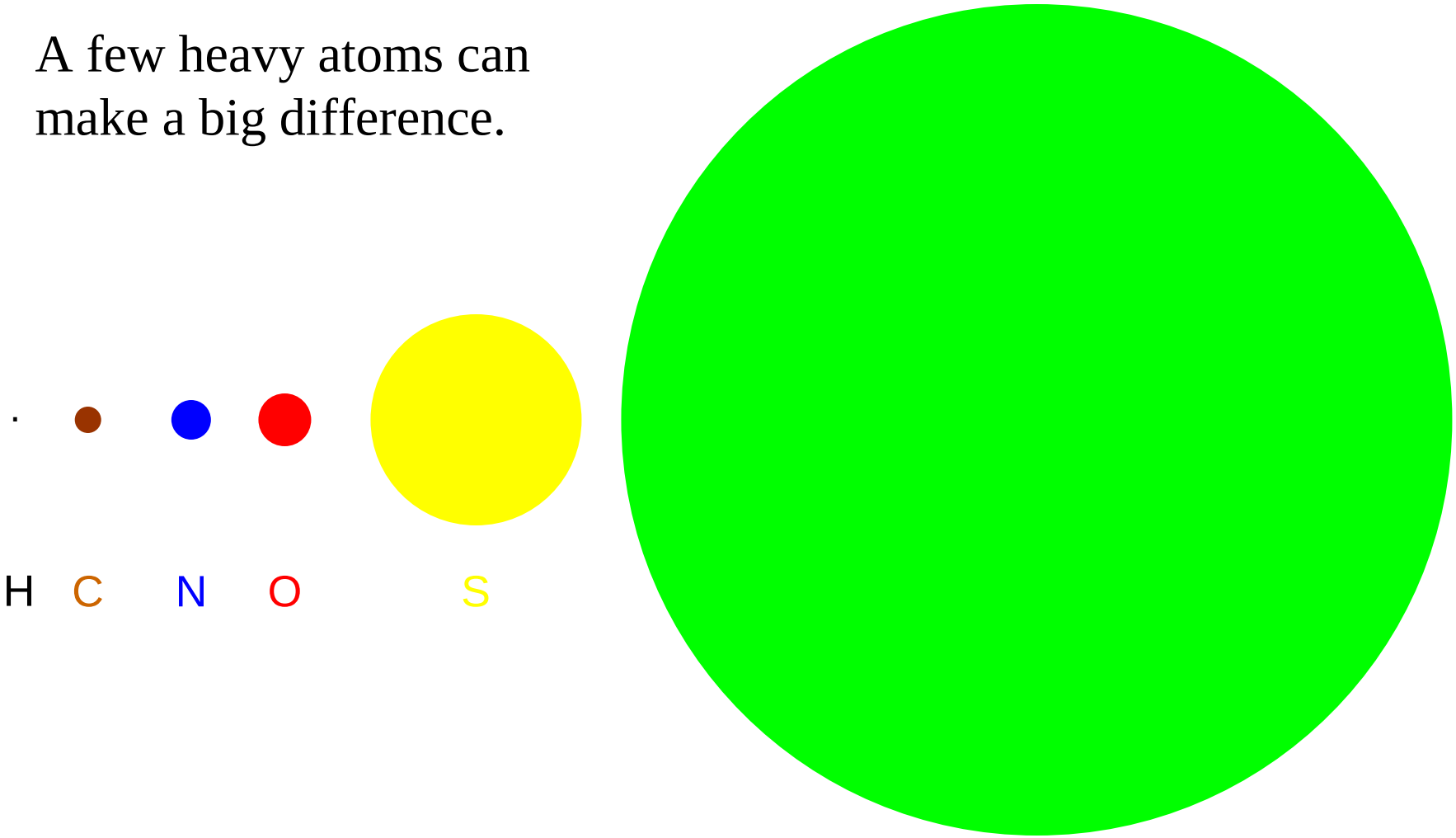
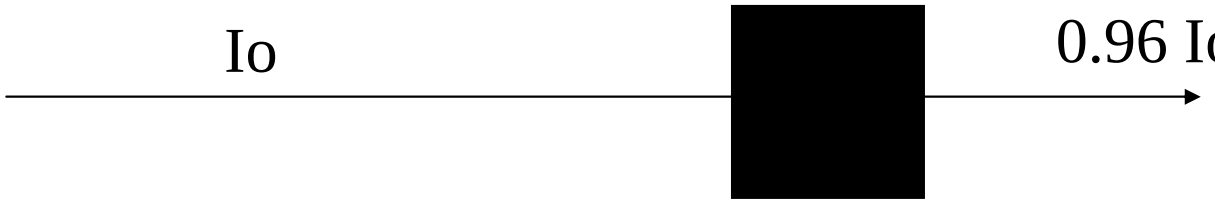


[Ravelli *et al.*, JSR,(2005) 12]

Beam absorption (λ=1Å) by a protein crystal

Native HEWL 100 µm thick

Platinum derivatised (1 site/molecule)
HEWL 100 µm thick



What about the CHEMISTRY?

Radiolysis of water:

$$H_2O \xrightarrow{\text{Ionizing radiation}} H_2O^{+\bullet} + e^- \quad \text{(ionization)}$$

$$H_2O \xrightarrow{\text{Ionizing radiation}} H_2O^* \quad \text{(electronic ionization)}$$

$$H_2O^{+\bullet} + H_2O \longrightarrow H_3O^+ + {}^{\bullet}OH$$

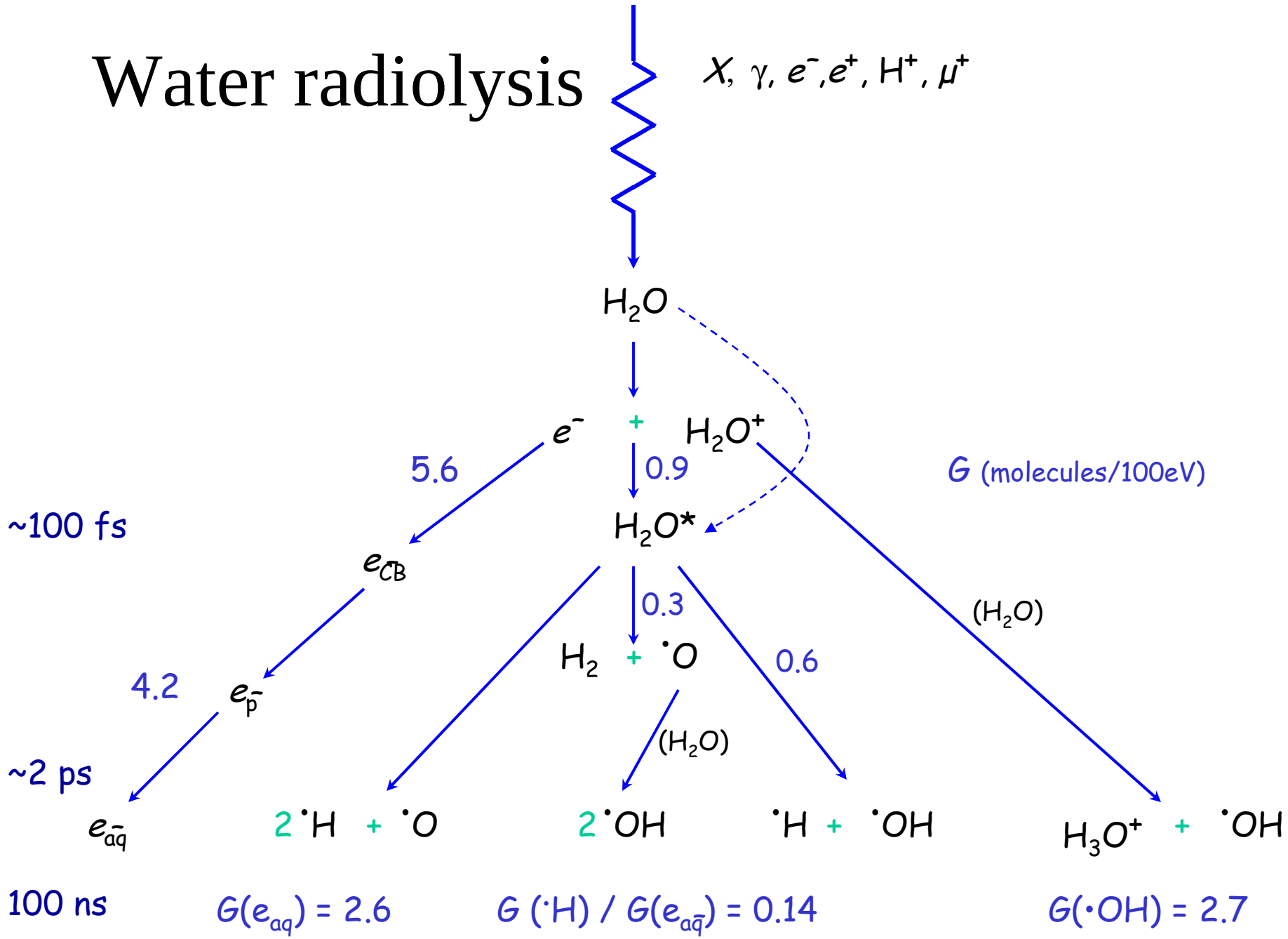
$$e^- + nH_2O \longrightarrow e^-_{aq}$$

$$H_2O^* \longrightarrow H^{\bullet} + {}^{\bullet}OH$$

$$e^-_{aq} + H^+ \longrightarrow H^{\bullet}$$

OH thought not to be mobile in glasses below 110K

Water radiolysis

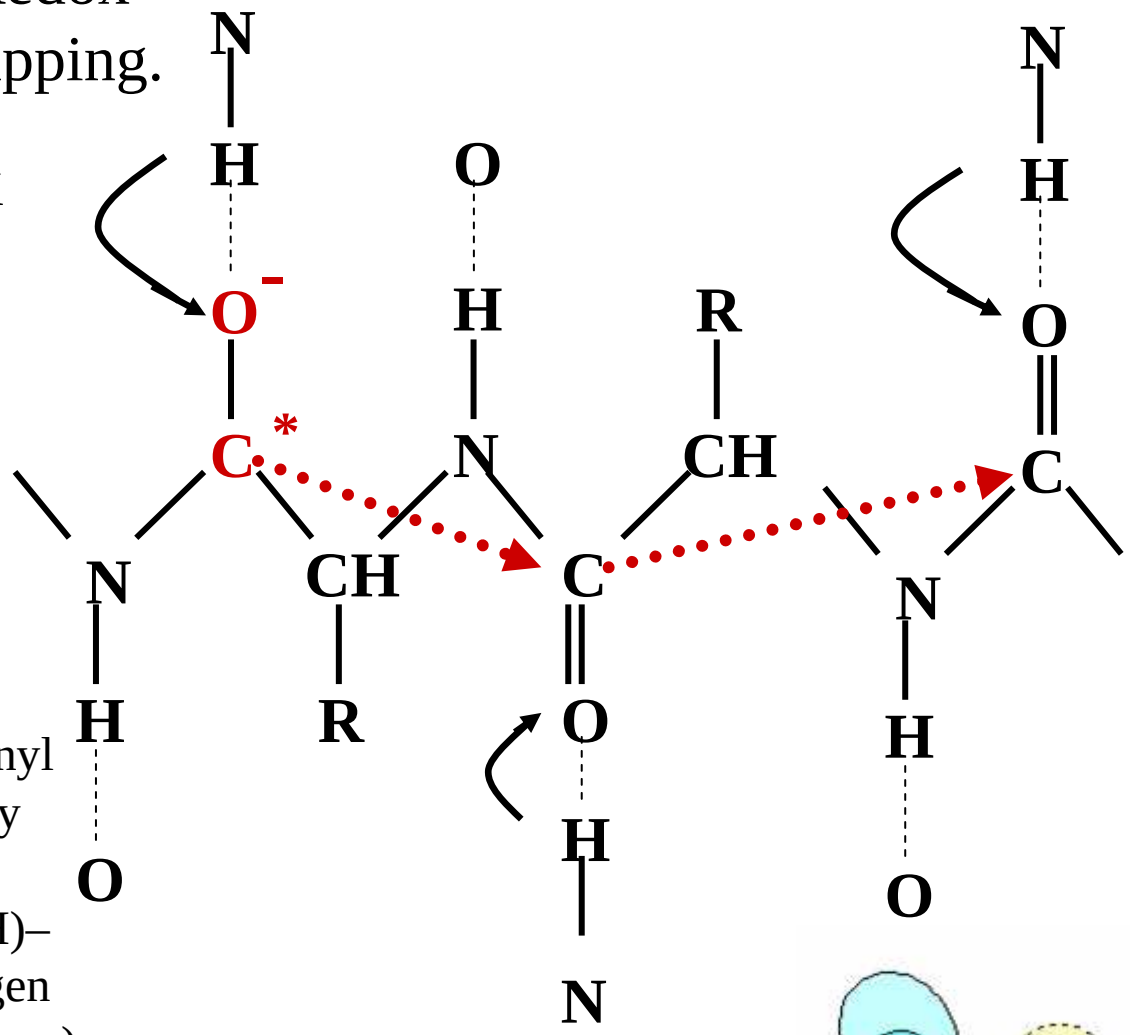


DIRECT RADIATION DAMAGE.

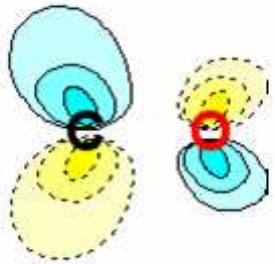
Reductive damage: protein Redox-
a) electron migration and trapping.

Excess electron migrates even at 100K (q.m.tunnelling) along backbone – trapped at a unique C=O

ESR: electrons mobile at 77K
Go to most electron affinic groups
M. Symons 1995, Radiat. Phys. Chem 45, 837-845.



Electrons typically attach at the carbonyl function where they may be trapped by protonation,
–(C=O)– –> –(•C–O–)– –> –(•C–OH)–
with the proton coming from a hydrogen bonded NH for example (the black arrow).
If it escapes this kind of trapping
it heads to the most electron affinic site.
e.g., the disulfide linkage.



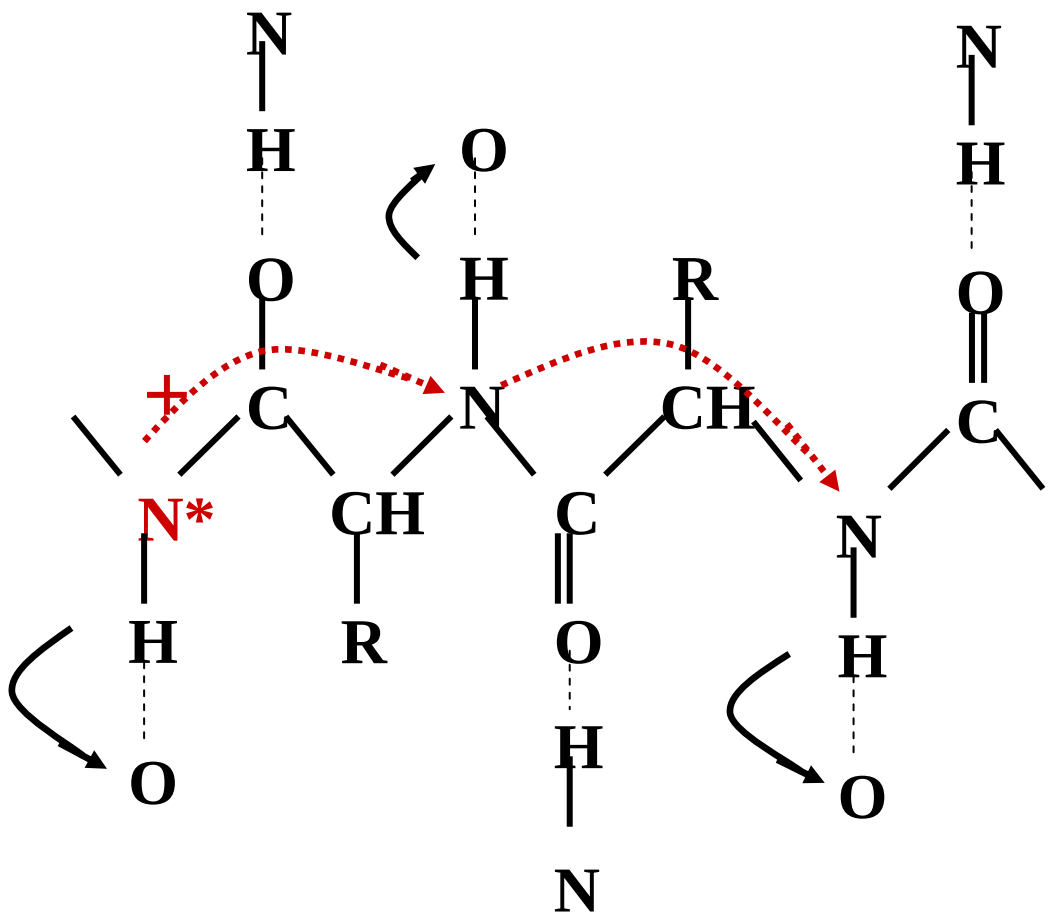
π* orbitals

DIRECT RADIATION DAMAGE. Protein Redox-

b) hole migration.

Loss of proton from the cationic site.

M. Symons 1995, Radiat. Phys. Chem 45, 837-845.



Positive holes are less mobile and are situated on the amide nitrogens.
They may be trapped there by deprotonation
(an H-bonded carbonyl picks up the proton for example)
and there is good epr evidence for the resulting amido radical
–NH– –> –•NH+– –> –•N–

2) Radiation damage:

The Plan:

- What are the symptoms?
- Why do we care?
- What is it?
- **What is `Dose'?**
- How might TotalCryst help?

DOSE

- DOSE is in Joules/kg

i.e. the absorbed energy per unit mass.

- FLUX is in photons/second.

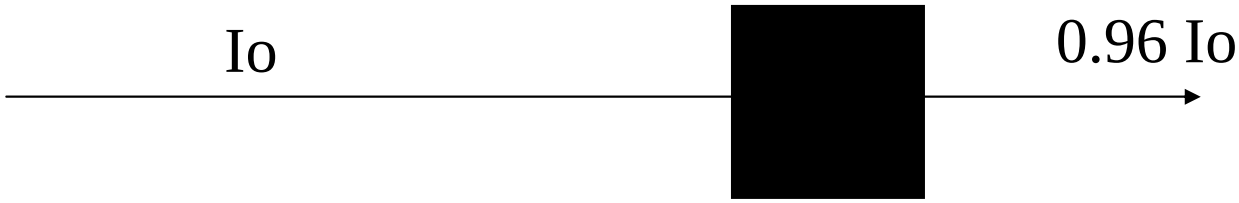
- Flux density is in photons/second/unit area.

- Takes care of the physics but NOT the chemistry.

Beam absorption (λ=1Å) by a protein crystal

Native HEWL 100 µm thick

Platinum derivatised (1 site/molecule)
HEWL 100 µm thick



N.B. INCIDENT FLUX is the SAME but the absorbed dose is DOUBLE

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: Murray, Garman & Ravelli, JAPC 2004

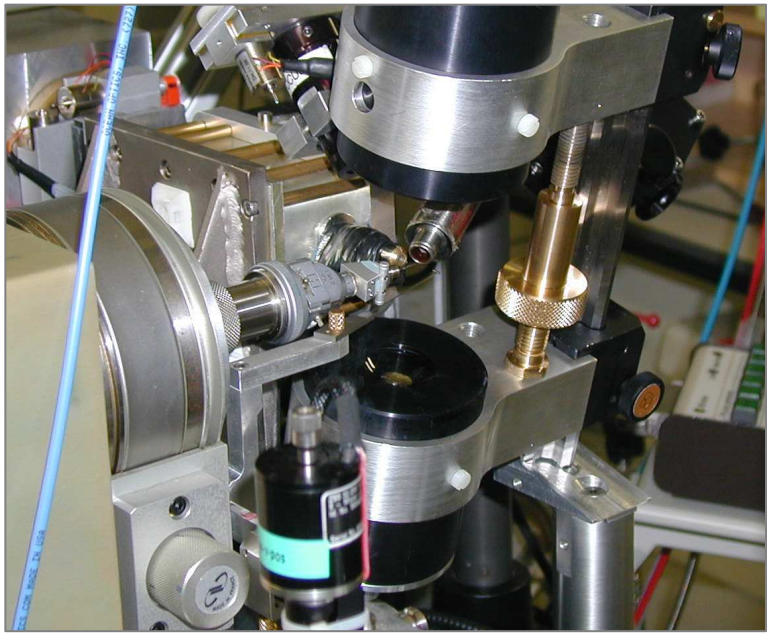
+ Karthik Paithankar work in TotalCryst:

Paithankar, Owen , & Garman. JSR (2009) 16, 152-162.]

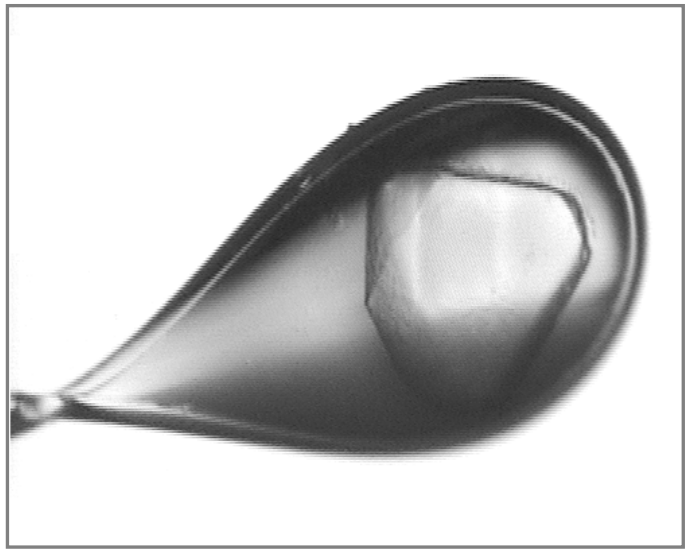
Dose calculation

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

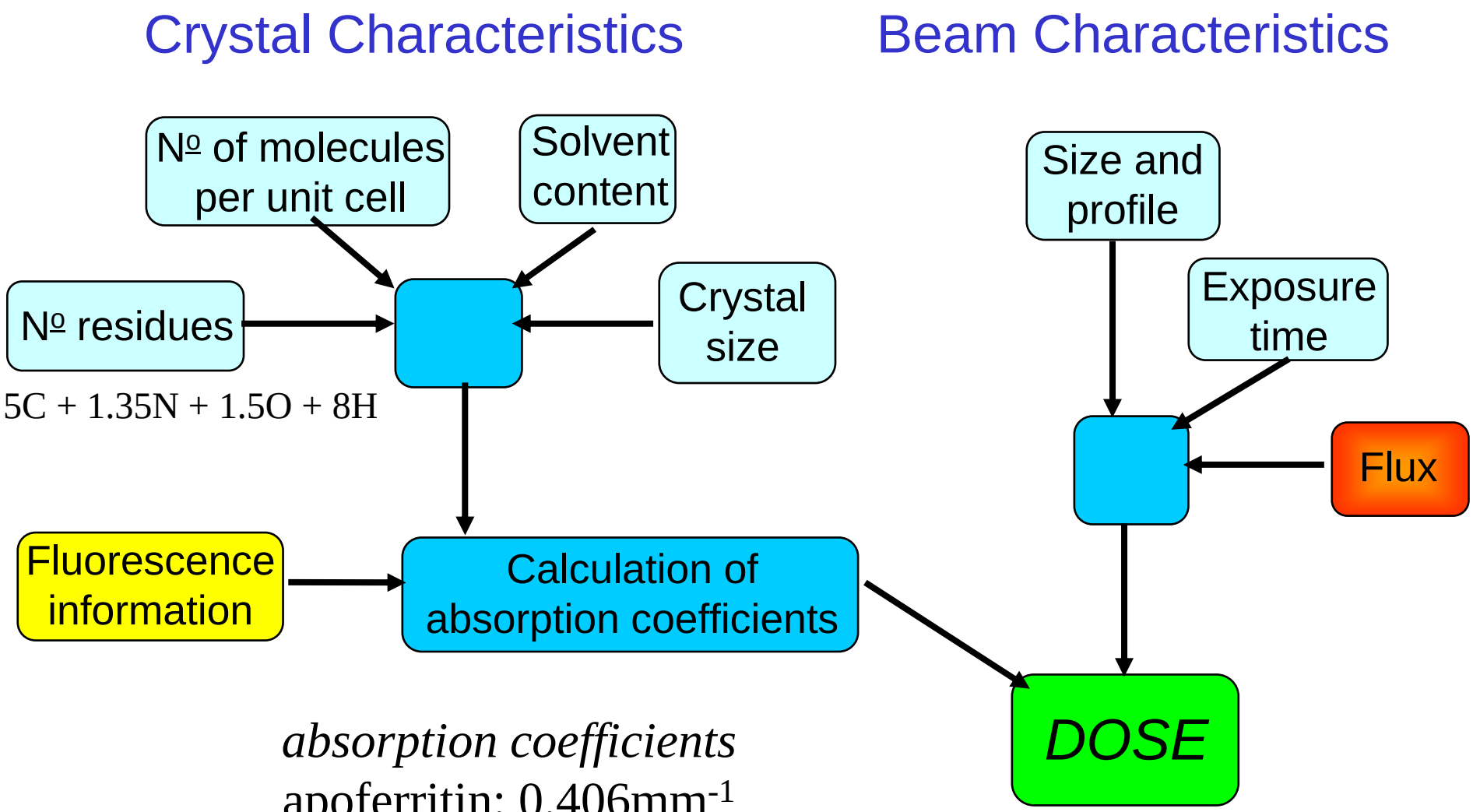
The Beam



The crystal



Calculating Dose
(*RADDOSE*)



absorption coefficients

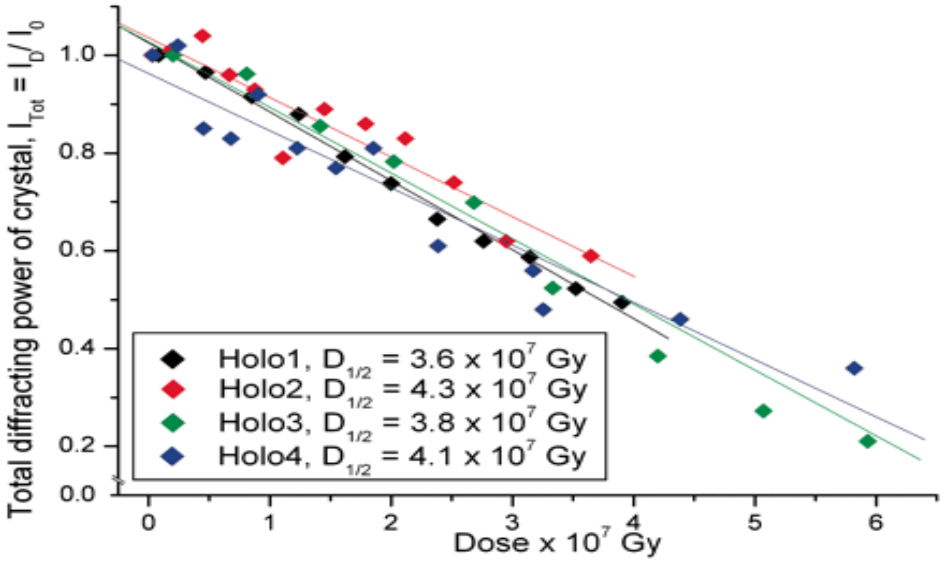
apoferritin: 0.406mm^{-1}

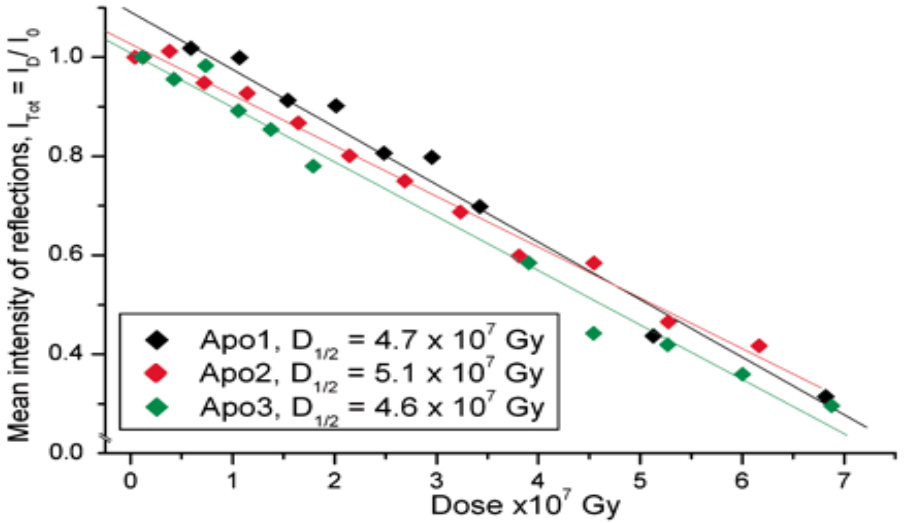
holoferritin: 1.133mm^{-1}

Quantification at cryotemperature

- Holoferritin and Apoferritin as model
  - Absorption coefficient differs by factor of 2
- Linear dependence on dose
- $D_{1/2} = 4.3 \times 10^7$ Gy

Where $D_{1/2}$ is dose to half the intensity lost





Experimental Dose Limit (100K)

For $I_0 \times 1/2$

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

(cf `Henderson limit' 20 MGy ≡ 5 electrons/Å^2
43 MGy ≅ 10 electrons/Å^2)

Suggested limit to retain biological `fidelity'

$I_0 \times 0.7 = I_0 \times \ln 2$

$D_{\ln 2} = 3.0 \times 10^7$ Gy = **30 MGy**

$D_{\ln 2}$ for ferritin corresponds 107 photons/unit cell

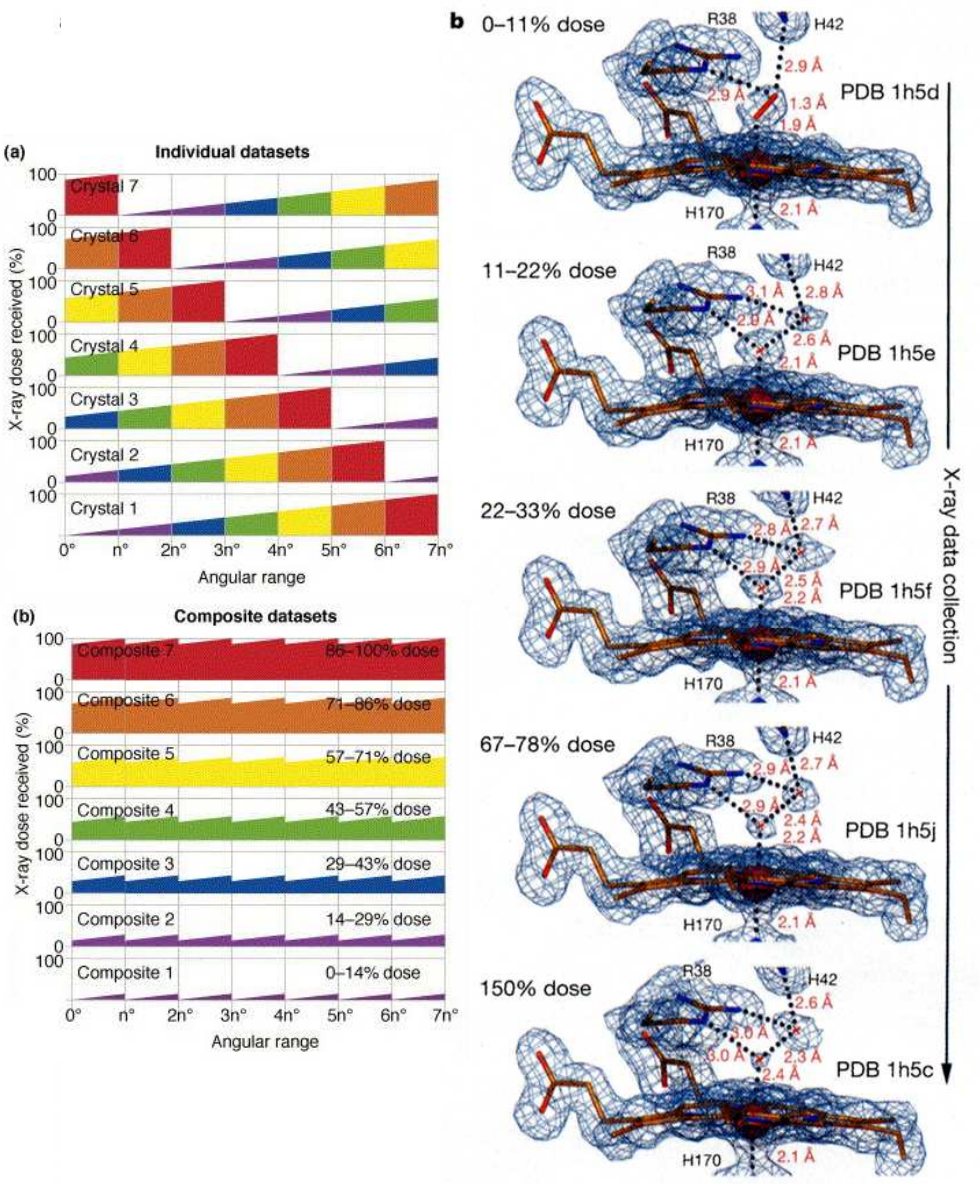
Robin Leslie Owen, Enrique Rudiño-Piñera, Elspeth F. Garman.
PNAS (2006) 103, 4912 - 4917.

Why TotalCryst for Macromolecular Crystallography?

1) Crystals are sometimes multiple and inseparable.

2) Radiation damage destroys crystal order during irradiation.

How to get maximum information for least damage.



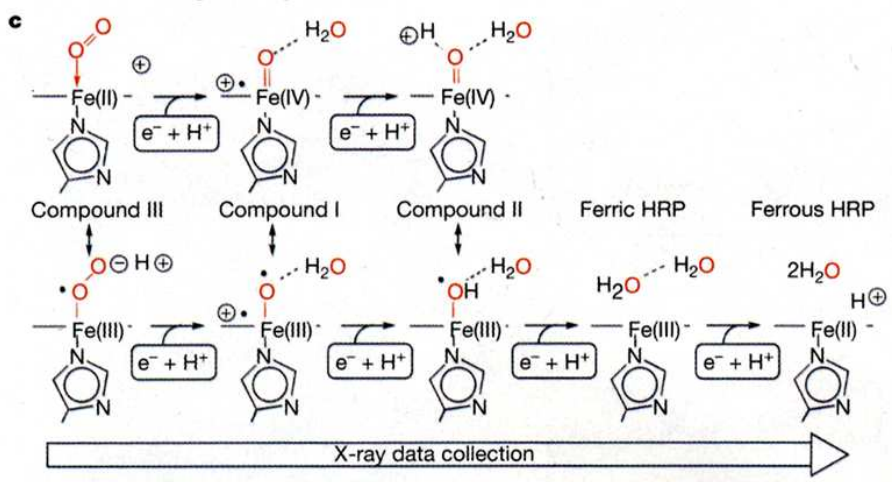


Figure 3 X-ray-driven catalytic conversion of a dioxygen species in horseradish peroxidase. **a**, The multicrystal data collection strategy, showing the distribution of the X-ray dose as a function of the rotation angle on individual (and spectrally uniform) crystals of HRP. The construction of composite data sets from small chunks of the individual data sets is shown at the bottom. Composite data sets represent structures that received different X-ray doses. This method permits experiments similar to redox titrations.

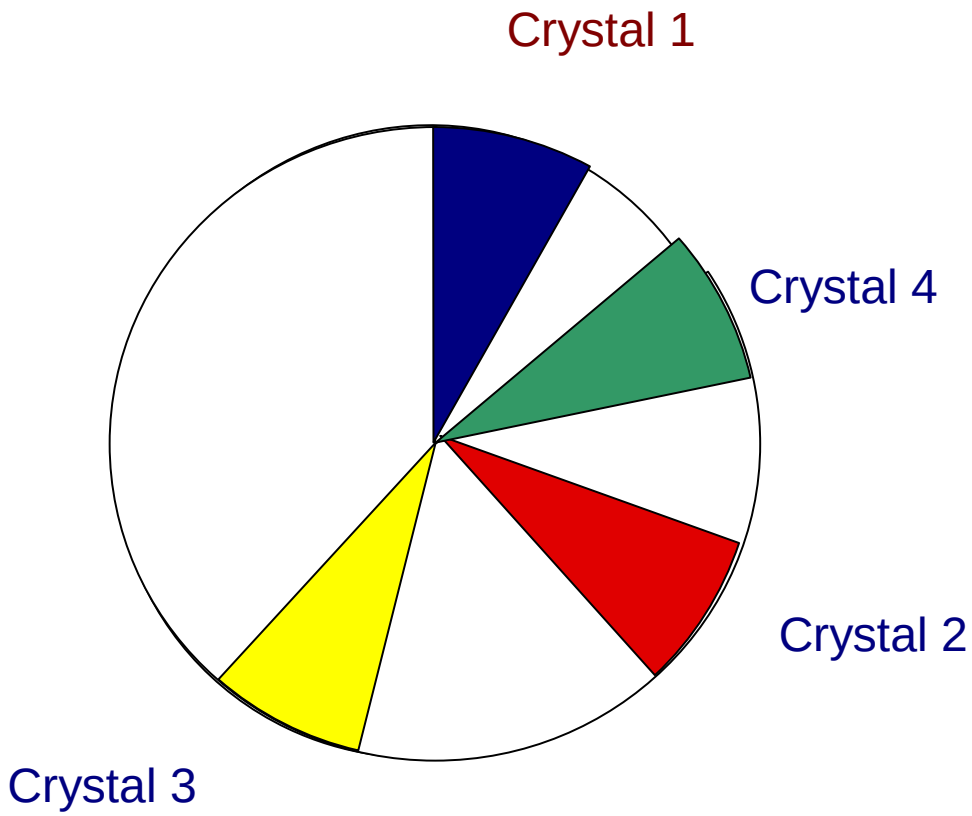
b, SigmaA-weighted[30] $2mF_{obs}–DF_{calc}$ maps contoured at 1σ showing X-ray-induced reduction of compound III. For the last structure, the crystal was pre-exposed to X-rays for 90° before another full X-ray data set was collected on it. Accession codes are shown. **c**, A possible mechanism for the reduction of the bound dioxygen species to two molecules of water. Structures linked by double arrows are isoelectronic with each other.

Horse radish peroxidase.
Berglund et al, Nature, 417: 463

Total Cryst

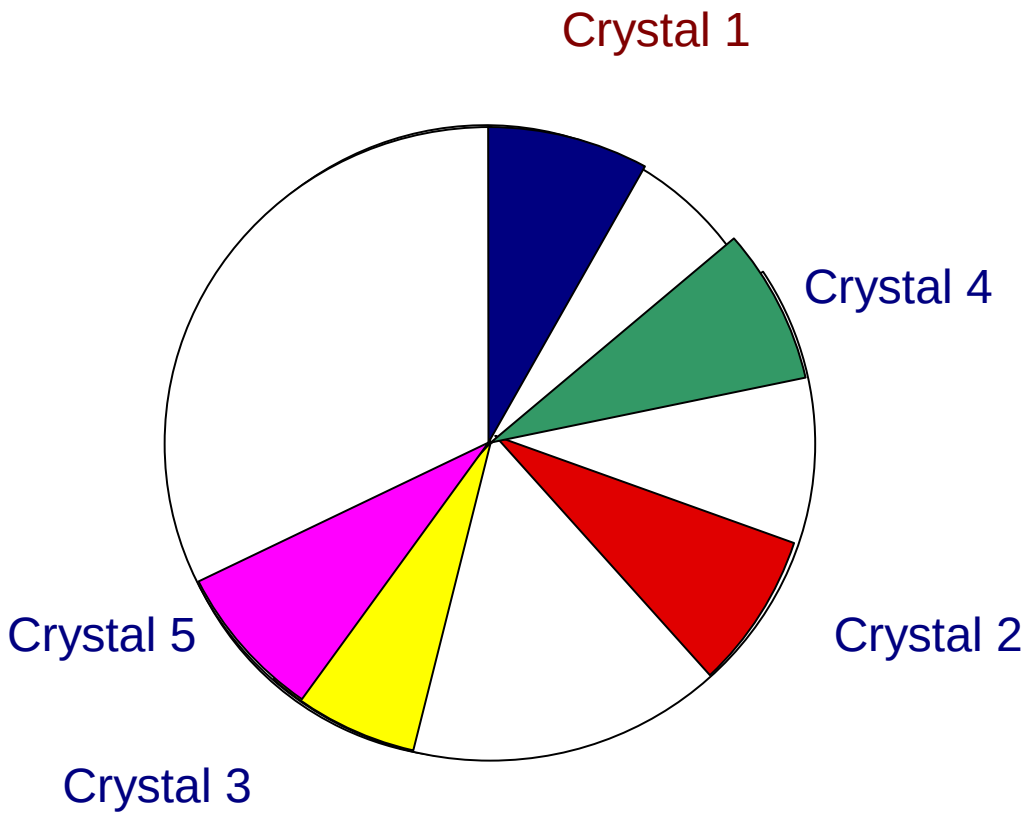
- Collect data from several crystals simultaneously.
- Take first 10° (or 15° etc) oscillation images for each crystal and integrate them all separately. Merge them to get a complete data set from which the structure at EARLY stage of radiation decay can be extracted.
- Take second wedge and treat similarly.

Building up a multi-crystal dataset using TotalCryst



Completeness

Building up a multi-crystal dataset using TotalCryst



Completeness

Thanks to:

My group, past and present:
James Murray (IC)
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Enrique Rudiño-Piñera (UNAM)
Robert Southworth-Davies (DLS)
Karthik Paithankar
Bill Bernhard (U of Rochester)
Ian Carmichael (Notre Dame)
John McGeehan (U Port)
Sean McSweeney (ESRF)
Raimond Ravelli (ULMC)
Martin Weik (IBS)
ESRF Grenoble for LS2047, MX-161, MX348, MX438, MX-666, MX-812

Raimond Ravelli

Martin Weik

Ian Carmichael

Funding:

bbsrc

OxfordCryosystems

diamond

- NEST **Adventure** Project….
- MX was the `blue skies' bit

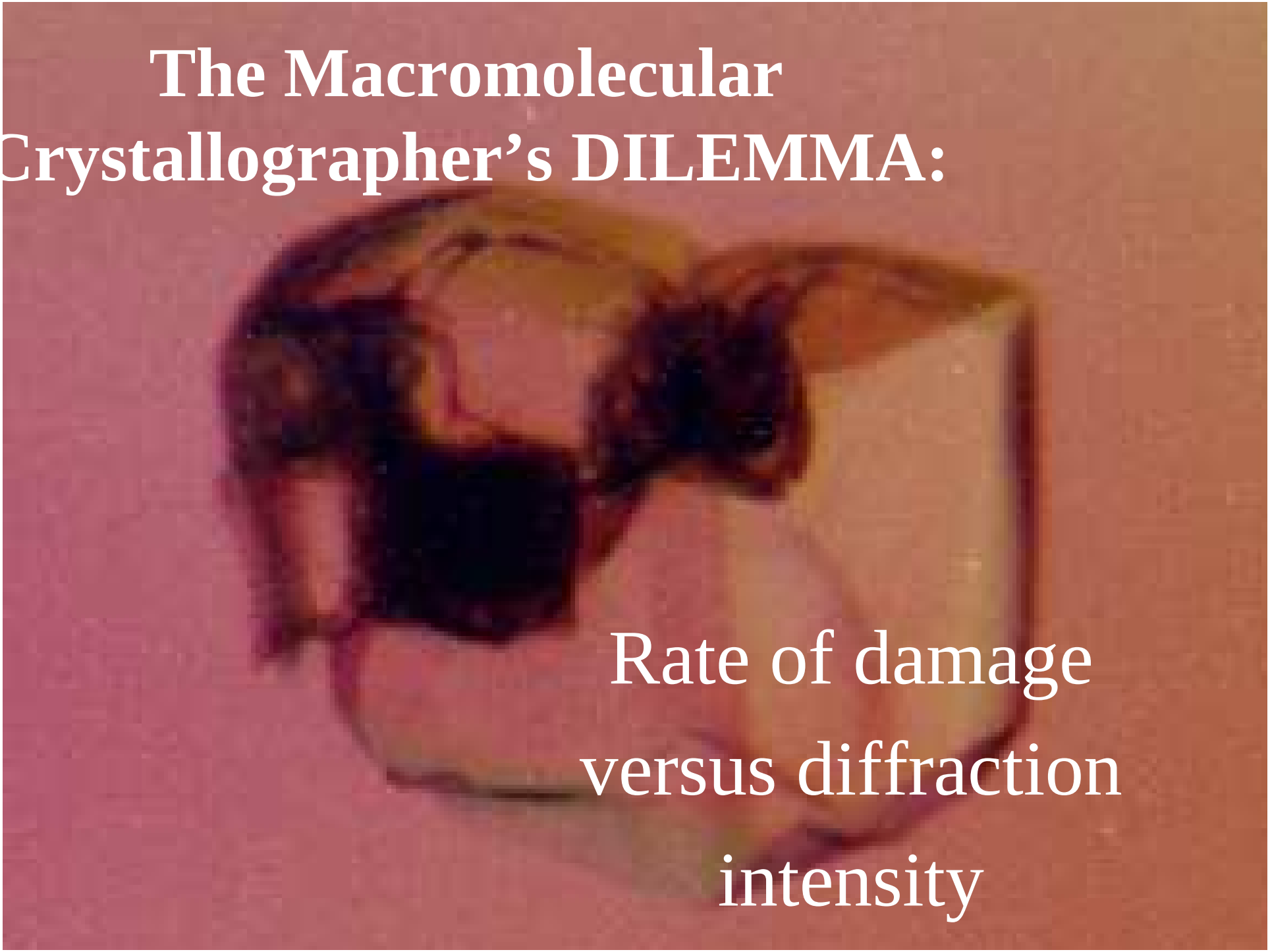
and has been

a true Adventure in research!

[jargon, language, orientation matrices etc]

With many thanks for the patience of all our partners!

The Macromolecular Crystallographer's DILEMMA:



Rate of damage versus diffraction intensity