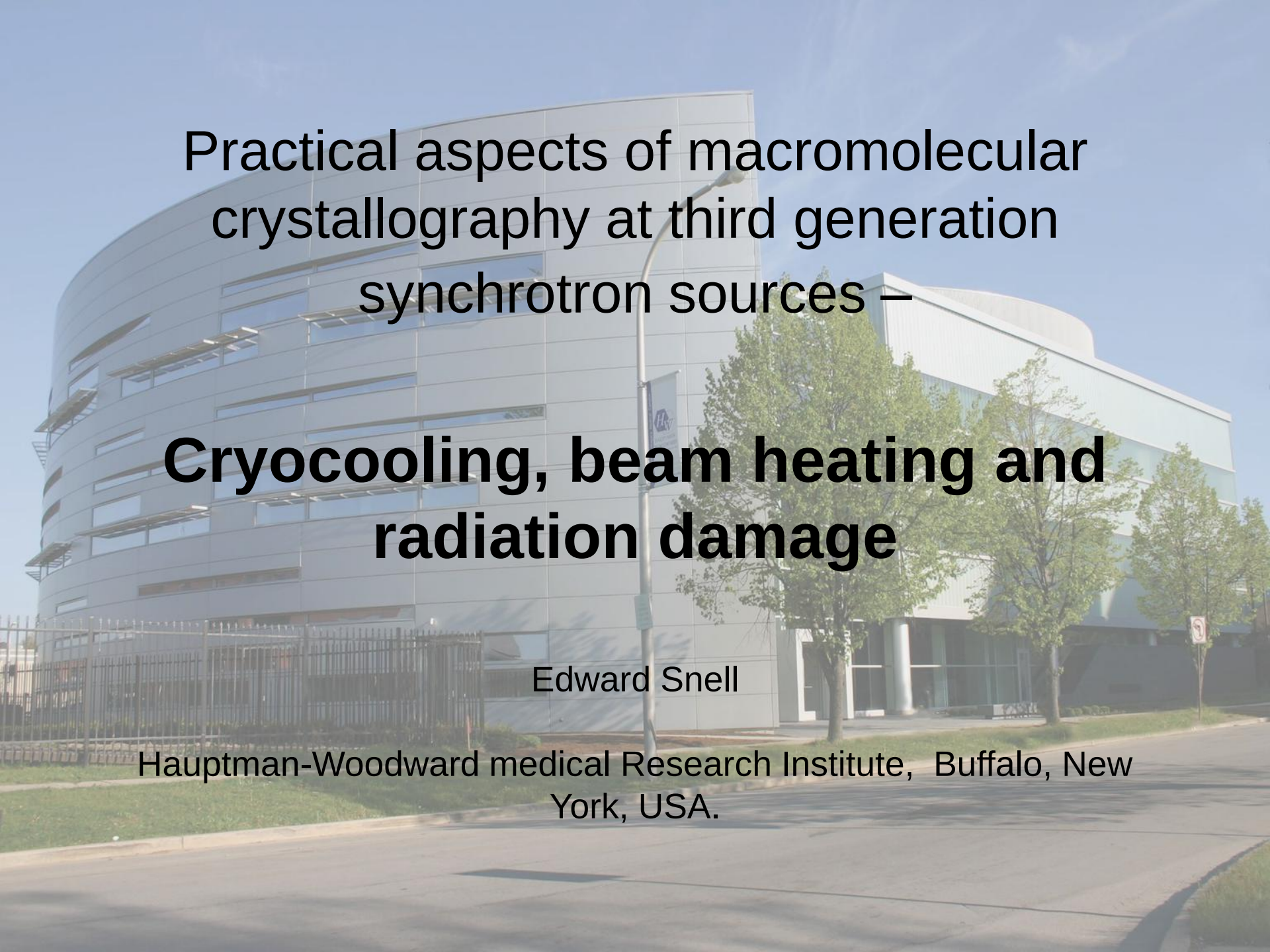
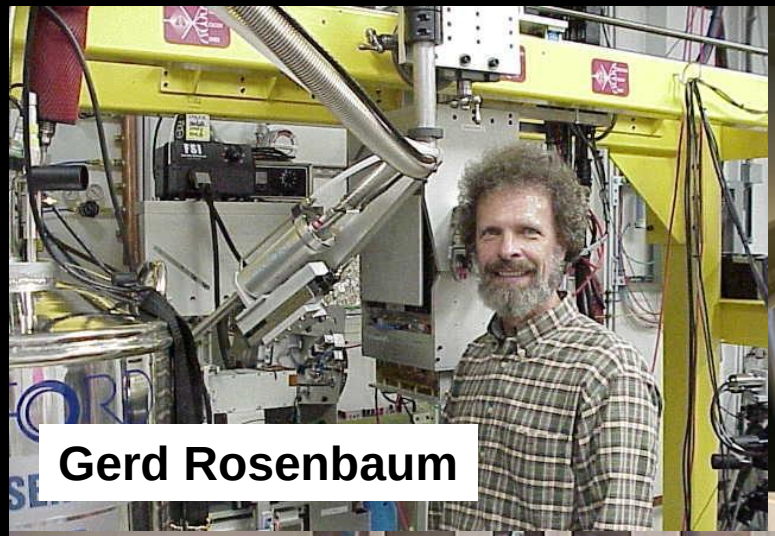


Practical aspects of macromolecular crystallography at third generation synchrotron sources –

Cryocooling, beam heating and radiation damage

Edward Snell

Hauptman-Woodward medical Research Institute, Buffalo, New York, USA.



The research team

Outline of the Snell Lab Research Interests

- Radiation damage in X-ray crystallography
  - What is it (knowledge) ?
    - What are the fingerprints of damage?
    - What are the short-scale and long-scale affects?
    - What produces these effects?
  - How can it be mitigated (panic) ?
    - Cryocooling (do we also need this to counteract beam heating?)
    - Free radical scavengers?
    - Alternatives
  - How can we make use of it (acceptance) ?
    - Free radical formation
    - Reducing active site
    - Following mechanism

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- What do intense X-rays produce?
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- Alternatives to X-rays
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Assumptions

- The audience is familiar with X-ray crystallography

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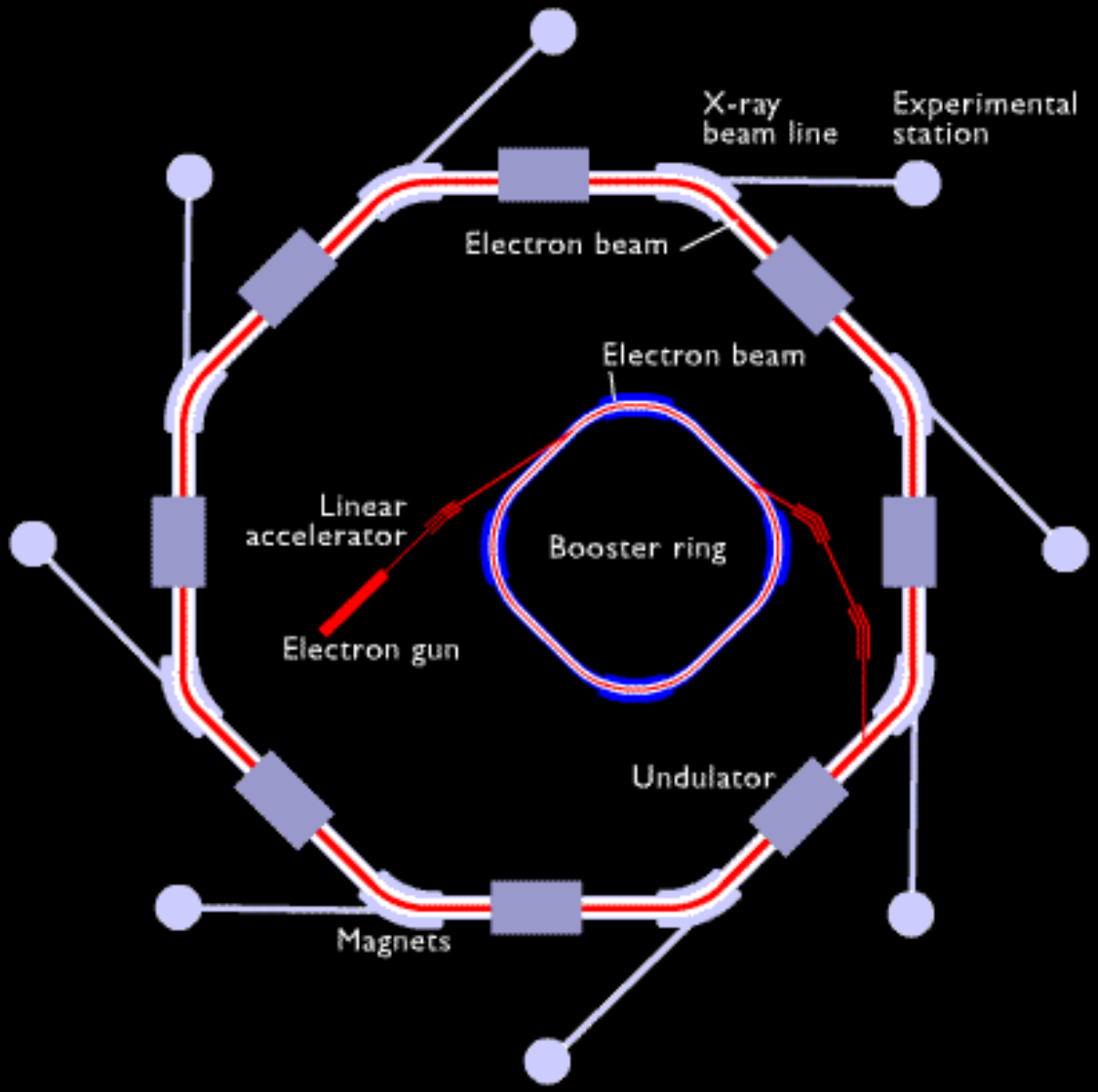
A synchrotron accelerates and stores particles (electrons or protons) moving at speeds close to that of light.

As the particles loose energy they give of electromagnetic radiation.

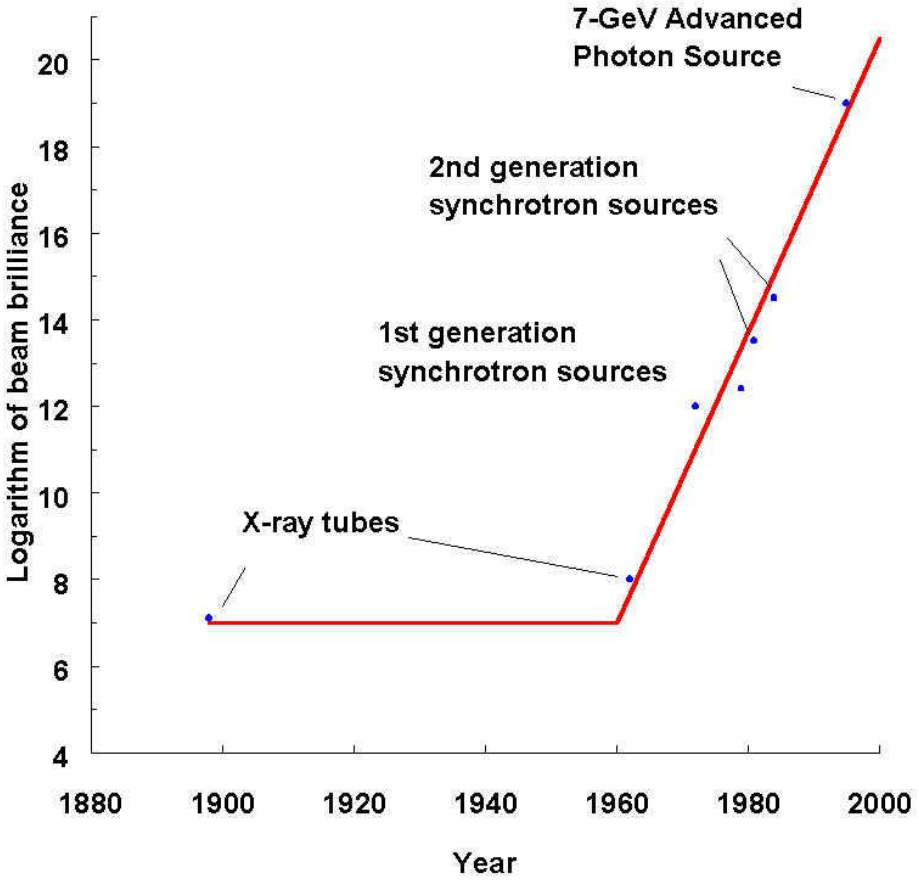
The particles are steered by magnetic fields.

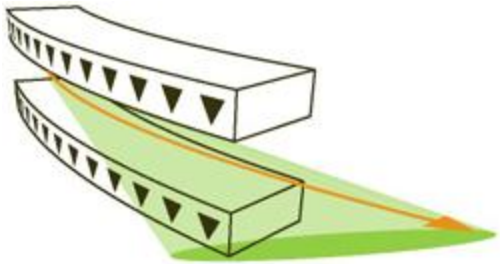
Electromagnetic radiation (photons) is not affected by these fields and is emitted at the tangent to the change in direction.

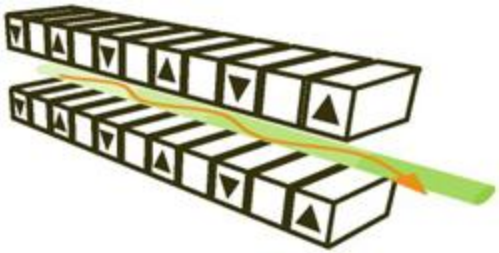
Insertion devices (undulators and wigglers) ‘amplify’ this radiation



- In the 1960s, physicists and chemists began to use the radiation from several of these accelerators in a "parasitic mode". The second generation of synchrotron radiation facilities, such as the Photon Factory in Japan, were constructed expressly to provide synchrotron X-rays for research.
- Recently a third generation of facilities is being completed, for example, the 7 GeV Advanced Photon Source in the USA, and are providing even higher brightness X-ray beams, about 10,000 times higher than those of the second generation





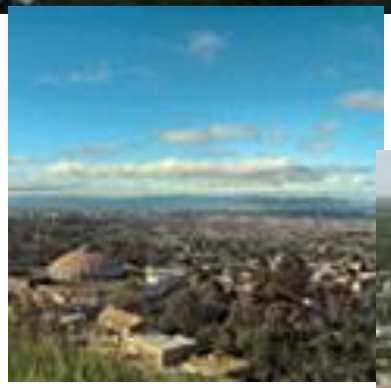






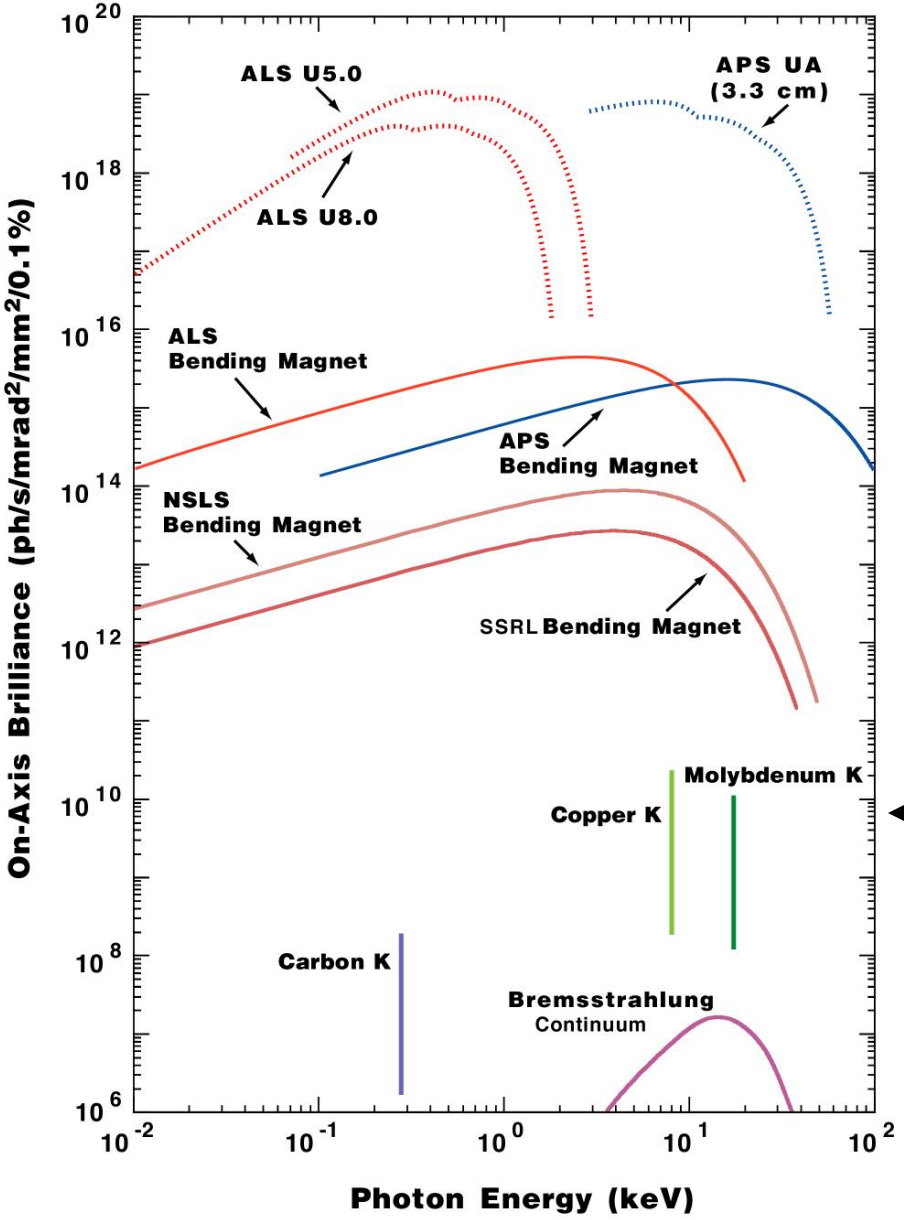




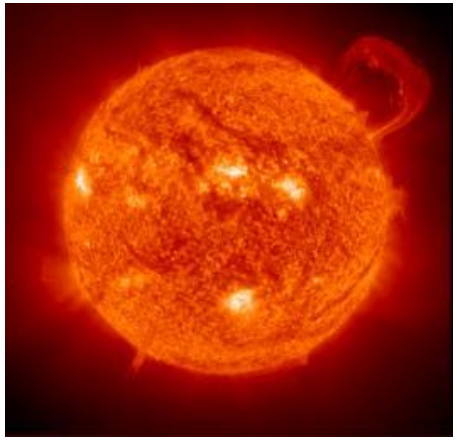








Synchrotron radiation is 10^9 times
More brilliant than the sun
and about 100 million miles closer



Why use a synchrotron?

- Roughly (very roughly) - The signal to noise in the data goes up by the log of the increase in brilliance.
- Brilliance has units of photons per second, per $mrad^2$ per mm^2 per 0.1% relative bandwidth.
- Laboratory X-ray source, brilliance $1.0x10^{10}$, synchrotron $1.0x10^{18}$, $Log(1.0x10^8)=8$ fold potential increase in signal to noise.

Another area of research saved for a later date – *i.e.* What will my crystal diffract to if it diffracts to X Å at home?

Potential pitfalls?

- Heat and radiation.
  - Is heat a problem that could be addressed to help improve data?
  - Radiation damage is known to be a problem, what causes the damage, how is it manifested, can we reduce it or even use it?

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X-ray Radiation effect on water

Ionizing radiation can remove an electron from water:

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + OH$$

And the ejected electron

$$e^- + H_2O \longrightarrow OH^- + OH$$

The simultaneous formation of H and OH free radicals gives further reactions

$$H + OH \longrightarrow H_2O$$

$$H + H \longrightarrow H_2$$

$$OH + OH \longrightarrow H_2O_2$$

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Processes of radiation damage

Primary, secondary, direct and indirect radiation-damage events in a protein crystal.

The incoming X-ray photons cause primary damage events, represented by darker stars. The paths of secondary radicals are shown by dotted arrows, and the damage events they induce are represented by lighter stars. Direct events occur on the protein molecules, and indirect events occur in the solvent region.

Primary effects are a fact of life, we cannot prevent them. Secondary effects are reduced by cryocooling.

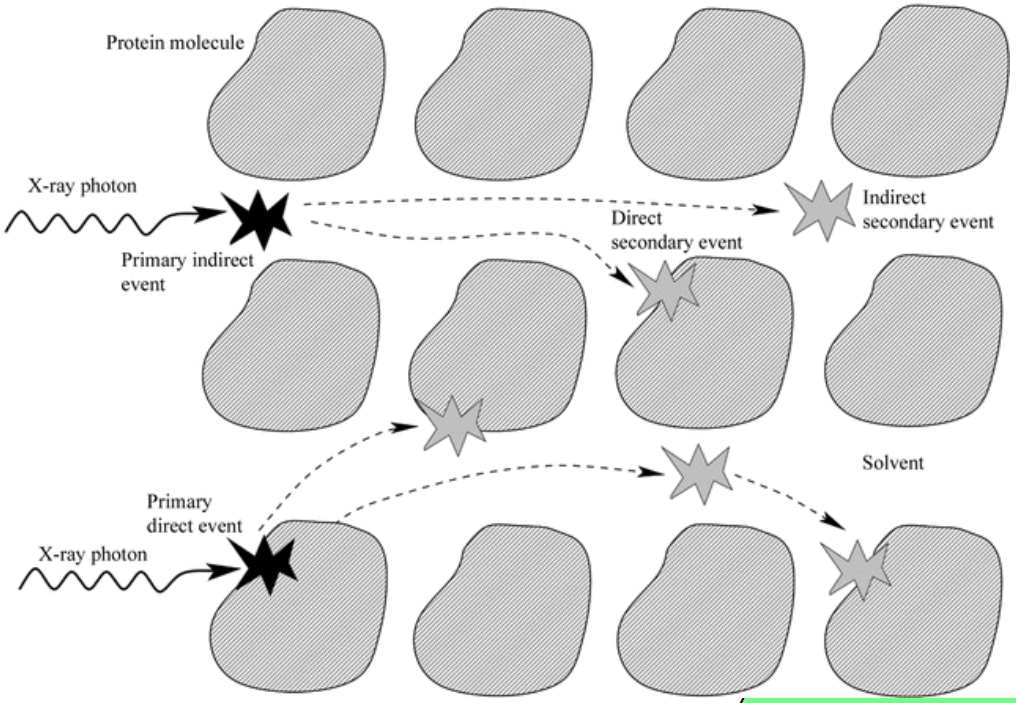
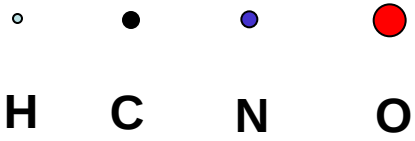
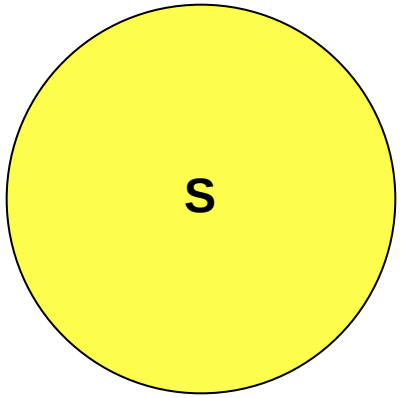
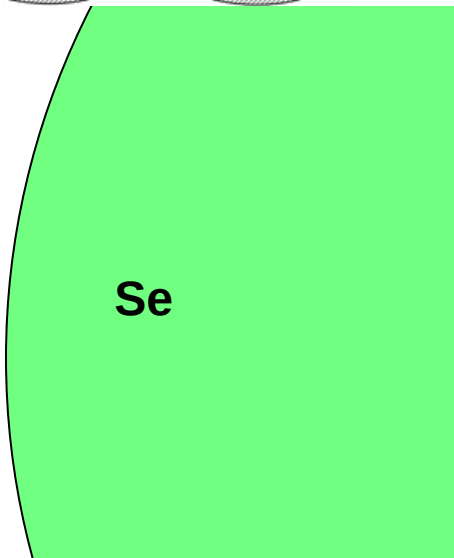


Image from Elspeth Garman

Effect on Other atoms







Radiation Damage

- 1 Å X-ray interaction in a crystal
  - 90% of the X-rays pass straight through (the reason for the beam stop).
  - 8.4% interact by the photoelectric effect. All the X-ray energy is transferred to an electron which is then ejected (main process of radiation damage).
  - 0.8% interact through Compton scattering. The X-ray transfers some of its energy to an atomic electron and a second lower energy photon is released. This forms the incoherent background.
  - 0.8% interact through Thomson (Rayleigh) scattering elastically with no energy loss. This is the X-ray that gives diffraction data.

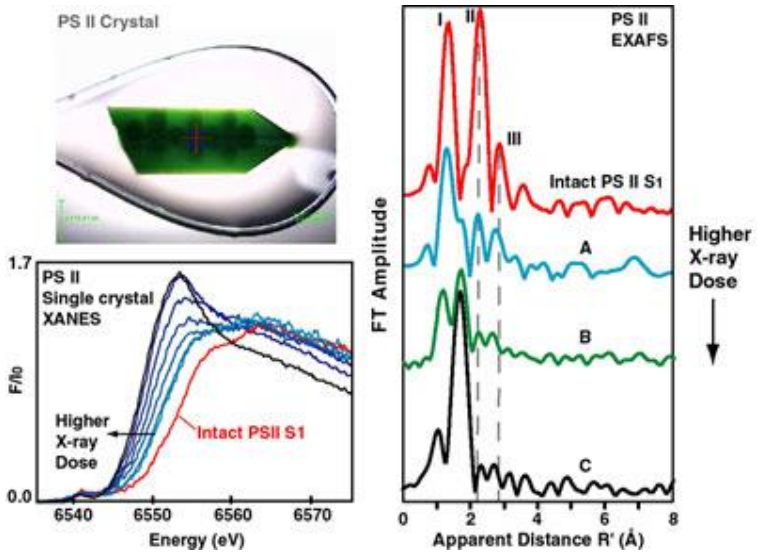
Henderson Limit

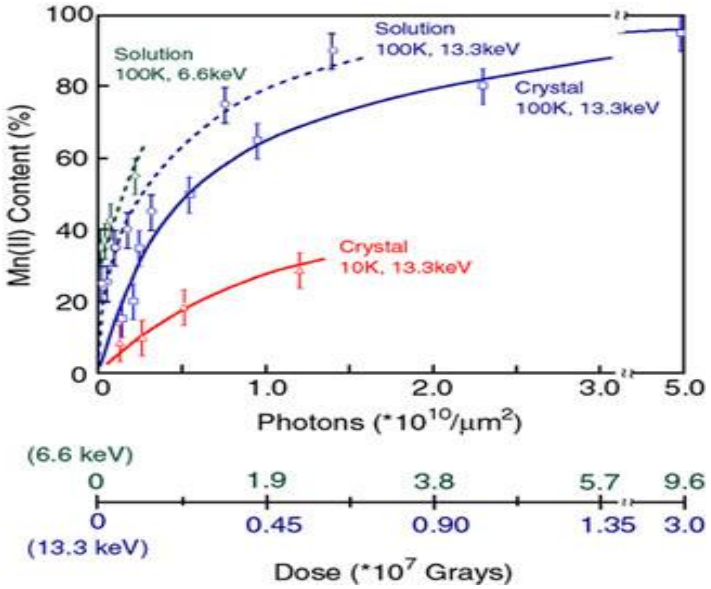
- Radiation damage by electrons and X-rays are comparable.
- Electron diffraction patterns fade to ½ their original intensity after 1 electron $Å^{-1}$at room temperature or 5 electron $Å^{-1}$at 77K.
- The amount of energy absorbed per unit weight is expressed in units of gray (Gy). One gray dose is equivalent to one joule radiation energy absorbed per kilogram. One gray is equivalent to 100 rads.
- 5 electrons $Å^{-1}$ is approx $5x10^{7}$ Gy.
- The depth dose curve (maximum dose at ~100 μm) reduces the energy deposition so the effective energy causing the damage is conservatively $2x10^{7}$ Gy.
- X-rays of 1.5 Å give $12x10^{-16}$ Gy per photon m^{-2}.
- The X-ray flux giving rise to $2x10^{7}$ Grays is $1.6x10^{16}$ photons mm^{-2}

(Henderson (1990) Proc. R. Soc. Lond. B. 241, 6-8).

What does it mean practically: Dead Crystals

- Remember,
  - The X-ray flux giving rise to 2×10^7 Grays (dead crystals) is 1.6×10^{16} photons mm^{-2}
- Lab source – crystals at 77K (close enough to 100K)
  - 1×10^8 photons s^{-1} mm^{-2}
    - Dead crystal in ~44,000 hours (5 years – in reality a lot less)
- Synchrotron - crystals at 77K (close enough to 100K)
  - Brookhaven $\sim 0.5 \times 10^{10}$ photons s^{-1} mm^{-2}
    - Dead crystal in ~ 1.5 days
  - Stanford $\sim 1.2 \times 10^{11}$ photons s^{-1} mm^{-2}
    - Dead crystal in ~ 1.5 hours
  - APS $\sim 1.3 \times 10^{13}$ photons s^{-1} mm^{-2}
    - Dead crystal in ~ 4 seconds





Case study - Photosystem II

Yano, J et al *Proc. Natl. Acad. Sci. USA* **2005**, *102* 12047-12052

As the X-ray dose increases, Mn normally present as Mn4(III2,IV2) is reduced to Mn(II) as seen by the changes in XANES spectra (left). The changes in the corresponding EXAFS spectra (right) show that the three Fourier peaks characteristic of Mn-bridging-oxo, Mn-terminal, and Mn-Mn/Ca interactions (dashed vertical line) are replaced by one Fourier peak characteristic of a Mn(II) environment.

Increasing Mn(II) content due to radiation damage. (Solid blue line) Mn(II) content in the crystals as a function of X-ray irradiation at 13.3 keV (0.933 Å) at 100 K - similar to those during x-ray diffraction data collection. At 66% of the dose (2.3x1010 photons/μm2) compared to the representative average dose of (3.5x1010 photons/μm2) used for crystallography, the crystals contain ~80% Mn(II). (Dashed blue line) The damage profile for solution samples is similar to that seen for crystals. (Dashed green line) The generation of Mn(II) is considerably greater when the x-ray irradiation is at 6.6 keV (1.89 Å) which is the energy at which the anomalous diffraction measurements were conducted. (Solid blue line)

Case Study: Xylose Isomerase

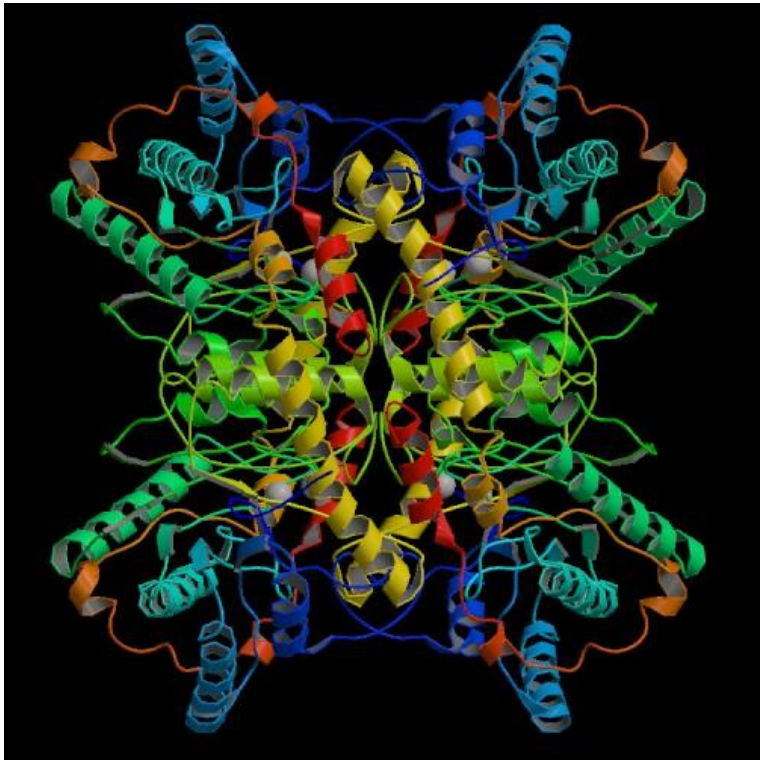
Enzymatic mechanism is a transfer of one H atom from one C atom of the substrate to an adjacent C atom.

Three mechanisms have been proposed – a base-catalyzed proton transfer, a simple hydride shift or a hydride shift mediated by a metal ion.

X-ray data, to date, has not revealed the exact mechanism.

Xylose isomerase is an important industrial catalyst for the production of fructose.

It has a molecular weight of ~ 160 KDa and can crystallize in the I222 space group – useful for Laue studies as every second reflection is systematically absent.

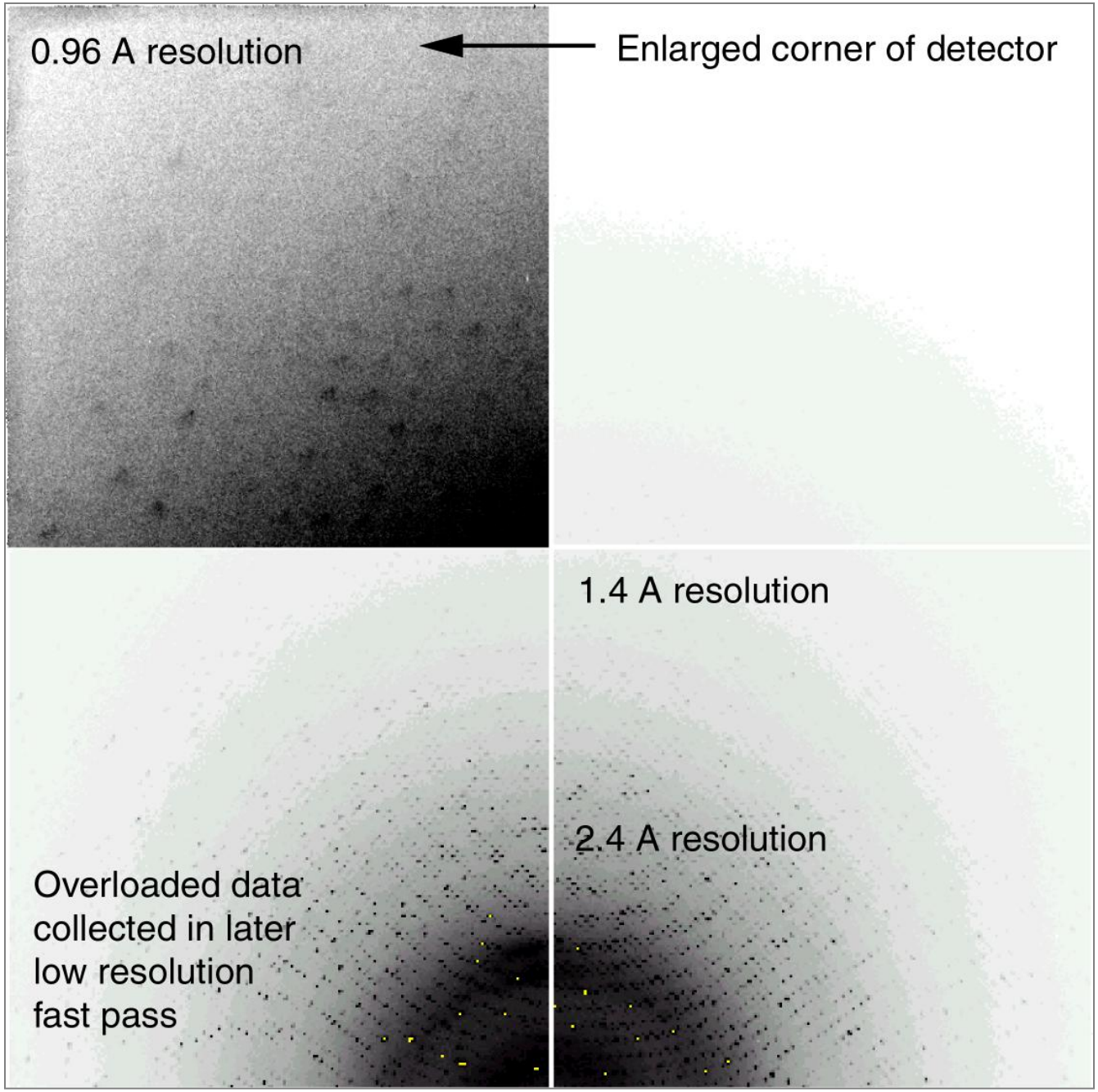


Good Short-range order.

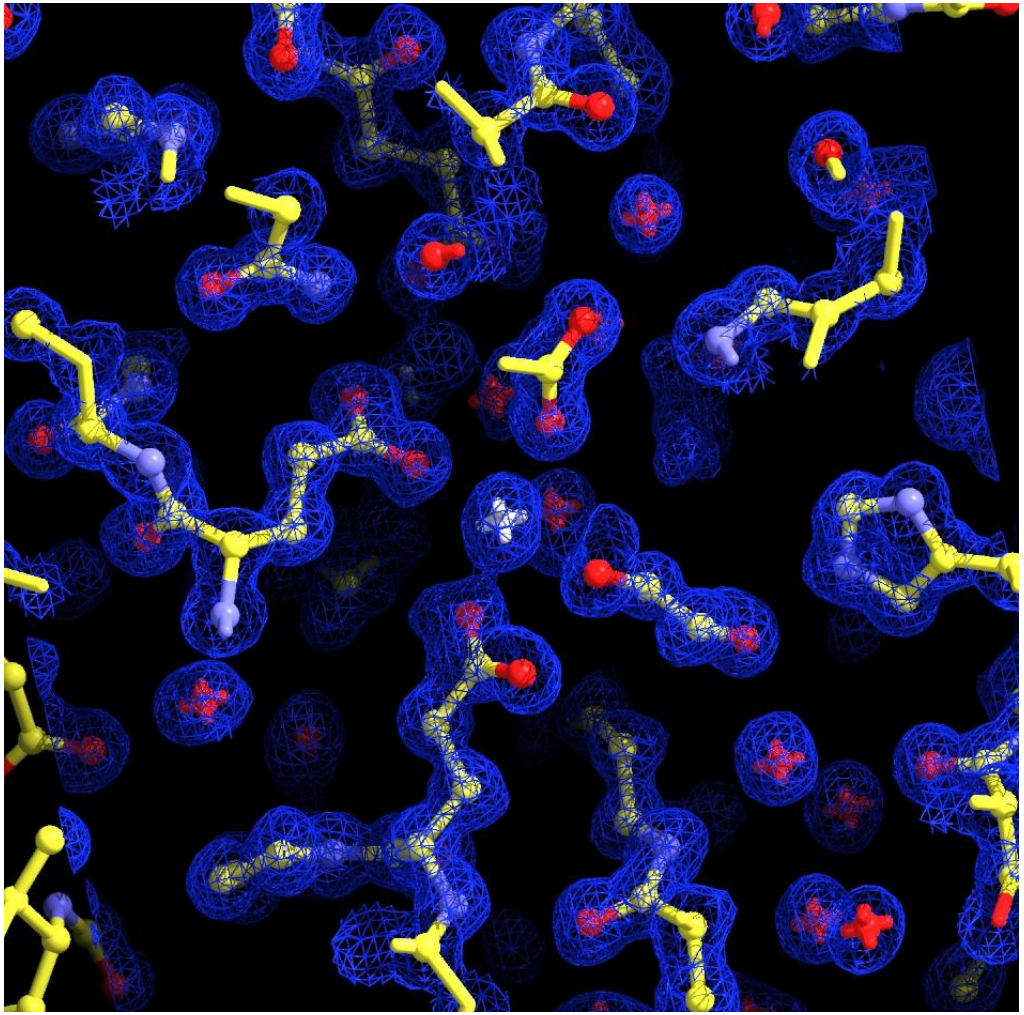
Mutated D-Xylose Isomerase X-ray data recorded at 100K.

Long-range order was poor with mosaicity of 0.2 degrees.

Data from beamline 9-2, SSRL, ADSC Quantum IV detector, 120 s exposure (dose mode equivalent at start).



Baseline High-Resolution Model



- I222 space group, a=92.69, b=97.87, c=102.24
- 40-0.87A resolution
- 3,989,654 reflections
- 376,419 unique
- 10.6 (9.3) multiplicity
- I/s(I)=19.61 (2.03)
- Rmerge 7.5% (82.1%)
- Rrim 7.9% (82.8%)
- Rpim 2.4% (26.1%)
- Average chi 0.954
- Completeness 99.5 (82.9)

- Structural refinement
- 8 cycles to date
- R factor 11.45
- Free R factor 12.60
- Many multiple conformations

2Fo-Fc map contoured at 2 sigma

Experimental – What is happening on the atomic scale

- Xylsoe isomerase grown in 3% isopropanol, 20% ethylene glycol, 50 mM $MgCl_2$ HEPES pH 7.0 (Ethylene glycol is a free radical scavanger and potentially useful for mitigating radiation damage as well as acting as a cryoprotectant).
- The crystal sizes were approximately 200 x 150 x 100 μm.
- Data was collected at beamline 11-1 of the Stanford Synchrotron Radiation Laboratory (SSRL) using an ADSC Quantum 310 detector
- Two crystals were used – one for a high-resolution base-line data set (low, medium and high resolution swathes in that order).
- An initial image was collected with l of 0.954 Å, crystal to detector distance of 150 mm, phi oscillation of 0.5º, and exposure time of 2 s. The dose was normalized to time at this point.
- The data were indexed and a strategy for optimum data collection calculated using Mosflm (Leslie, 1992).
- Following this the wavelength was changed to 0.855 Å and the beam optimized.
- A high-resolution swathe of reciprocal space was then collected with a total of 20 images, 30s equivalent dose exposure, crystal to detector distance of 100 mm and phi oscillation of 0.5º.
- The wavelength was than changed to 0.954 Å and again optimized.
- A complete data set of 180 images, 0.5º oscillation, 2s equivalent dose, and crystal to detector distance was then collected.
- Data collection continued alternating with experimentally identical high-resolution swathes and complete data sets to produce a total of 8 swathes and 7 complete data sets.
- Dose mode was used throughout to maintain a constant X-ray exposure in each case.

Experimental contd.

- The resulting data were indexed, integrated and reduced using Denzo and Scalepack (Otwinowski and Minor, 1997).
- The B_{factor} was calculated using the program Truncate in the CCP4 suite (Collaborative Computational Project, 1994).
- Normal probability plots (Abrahams and Keve, 1971) show whether data from two crystals are identical or differ systematically and provide information about individual pairs of measurements in addition to the overall agreement. Howell and Smith (Howell and Smith, 1992) made use of this technique to identify heavy atom derivatives.
- In this case we used the same technique, through the CCP4 program Scaleit, to look for differences that were manifest in structural changes rather than simple radiation decay

Current work underway

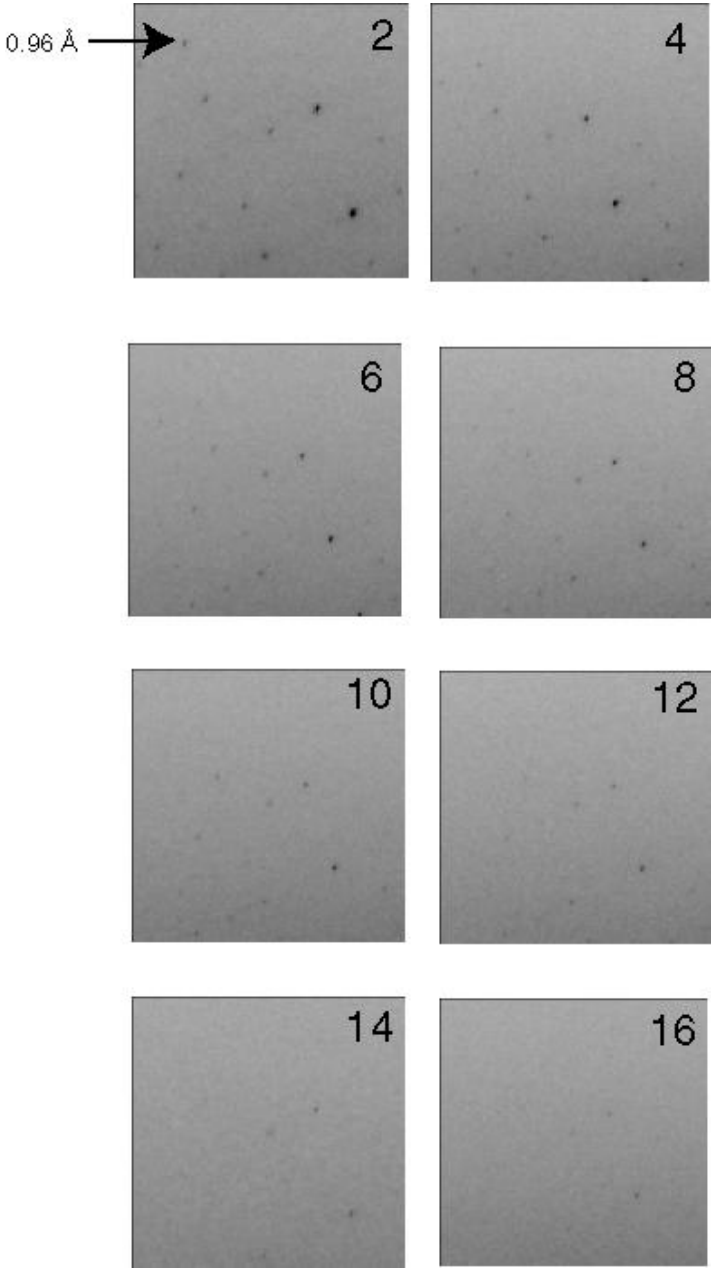
Structural refinement of each data set

- Arp/Warp to remove initial model bias
- Coot to fit model to density and model in cryoprotectant
- Refmac for further refinement with Warp used for water positions
- Procheck and Coot internal routines used to check result
- Iteration Coot, Refmac, Procheck

The Numbers – Radiation Damage Datasets

High resolution partial data set (0.9 Å)								
Data set	2	4	6	8	10	12	14	16
R_{factor}	6.7(45.8)	6.7(54.7)	6.9(57.5)	7.2(59.4)	7.5(85.2)	8.0(68.7)	8.0(73.5)	8.0(-)
I/σ(I)	8.9(1.6)	8.5(1.2)	8.6(1.0)	8.3(0.8)	8.3(0.7)	8.0(0.6)	7.8(0.6)	7.7(0.5)
Completeness (%)	24.8(24.8)	24.8(23.2)	24.5(19.6)	24.1(15.3)	23.6(10.9)	23.0(7.0)	22.2(3.1)	21.7(1.4)
Redundancy	1.4(1.4)	1.4(1.3)	1.4(1.2)	1.3(1.2)	1.3(1.1)	1.3(1.1)	1.3(1.0)	1.3(1.0)
Mosaicity (º)	0.17	0.17	0.17	0.16	0.16	0.16	0.16	0.16
B_{factor}	6.04	6.35	6.70	6.85	7.25	7.54	7.85	8.13
Medium resolution complete data set (1.2 Å)								
Data set	3	5	7	9	11	13	15	
R_{factor}	7.5(22.5)	7.5(24.7)	7.7(27.3)	7.6(30.1)	7.9(33.4)	7.9(37.3)	7.8(41.7)	
I/σ(I)	16.8(5.0)	16.6(4.7)	16.4(4.3)	16.6(3.9)	16.1(3.3)	15.4(2.8)	15.3(2.4)	
Completeness (%)	99.7(99.3)	99.7(99.4)	99.7(98.9)	99.7(99.1)	99.7(98.4)	99.6(96.8)	99.4(93.7)	
Redundancy	3.6(3.2)	3.6(3.3)	3.5(3.2)	3.5(3.1)	3.5(2.8)	3.5(3.0)	3.5(2.8)	
Mosaicity (º)	0.14	0.14	0.14	0.14	0.14	0.14	0.14	
B_{factor}	8.77	8.83	9.07	9.61	9.83	10.31	10.86	

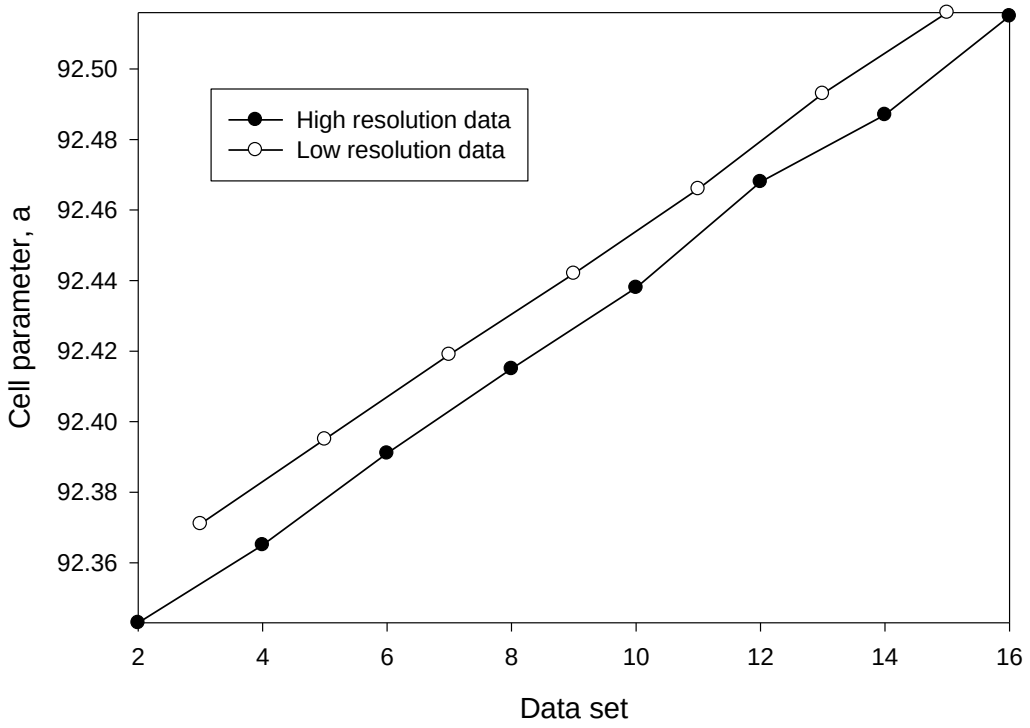
With each data set R_{factor} increases, signal-to-noise, completeness, and redundancy decreases. The mosaicity is unchanged, we are just seeing the beam contributions. The B_{factor} increases.

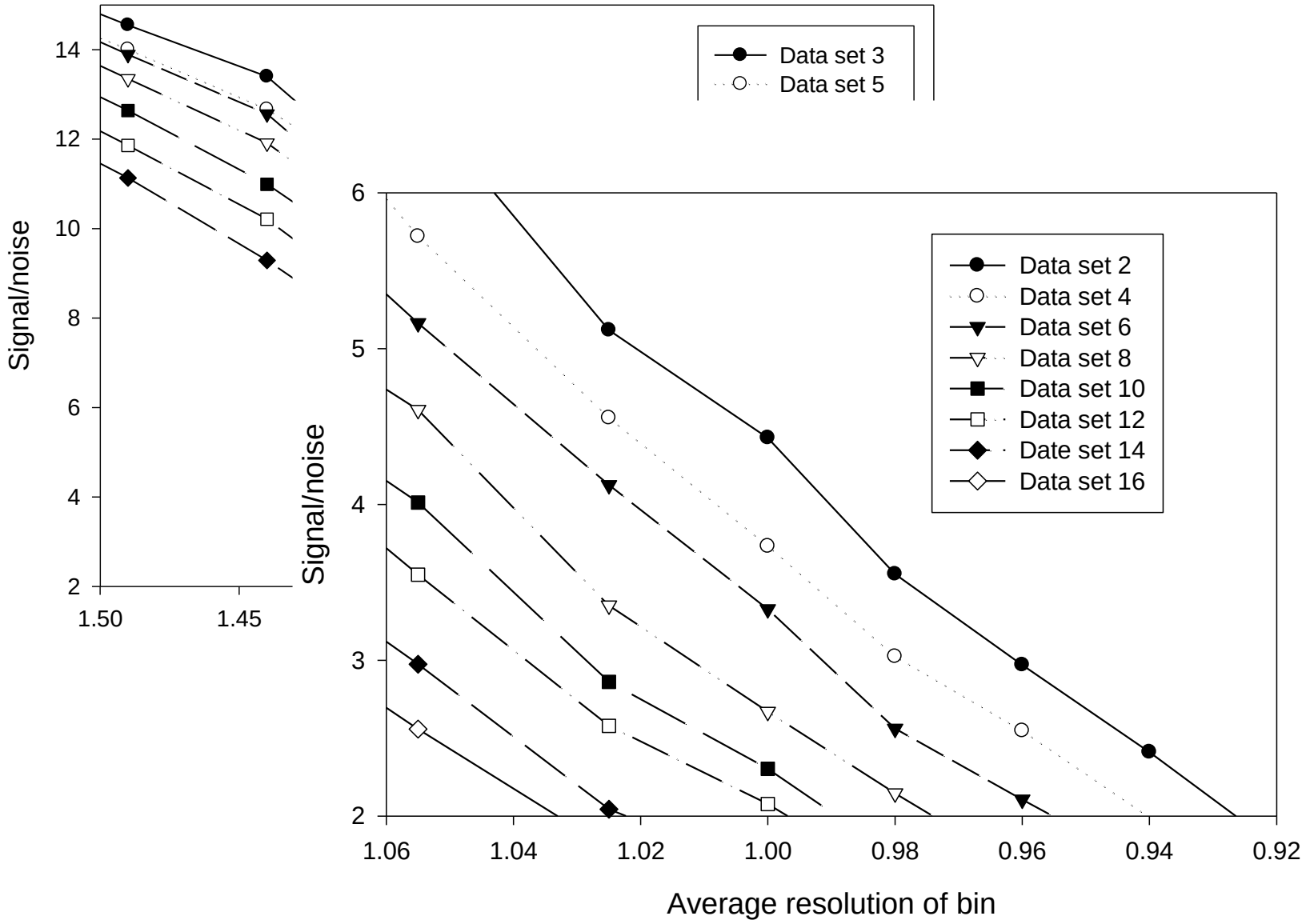


The Images

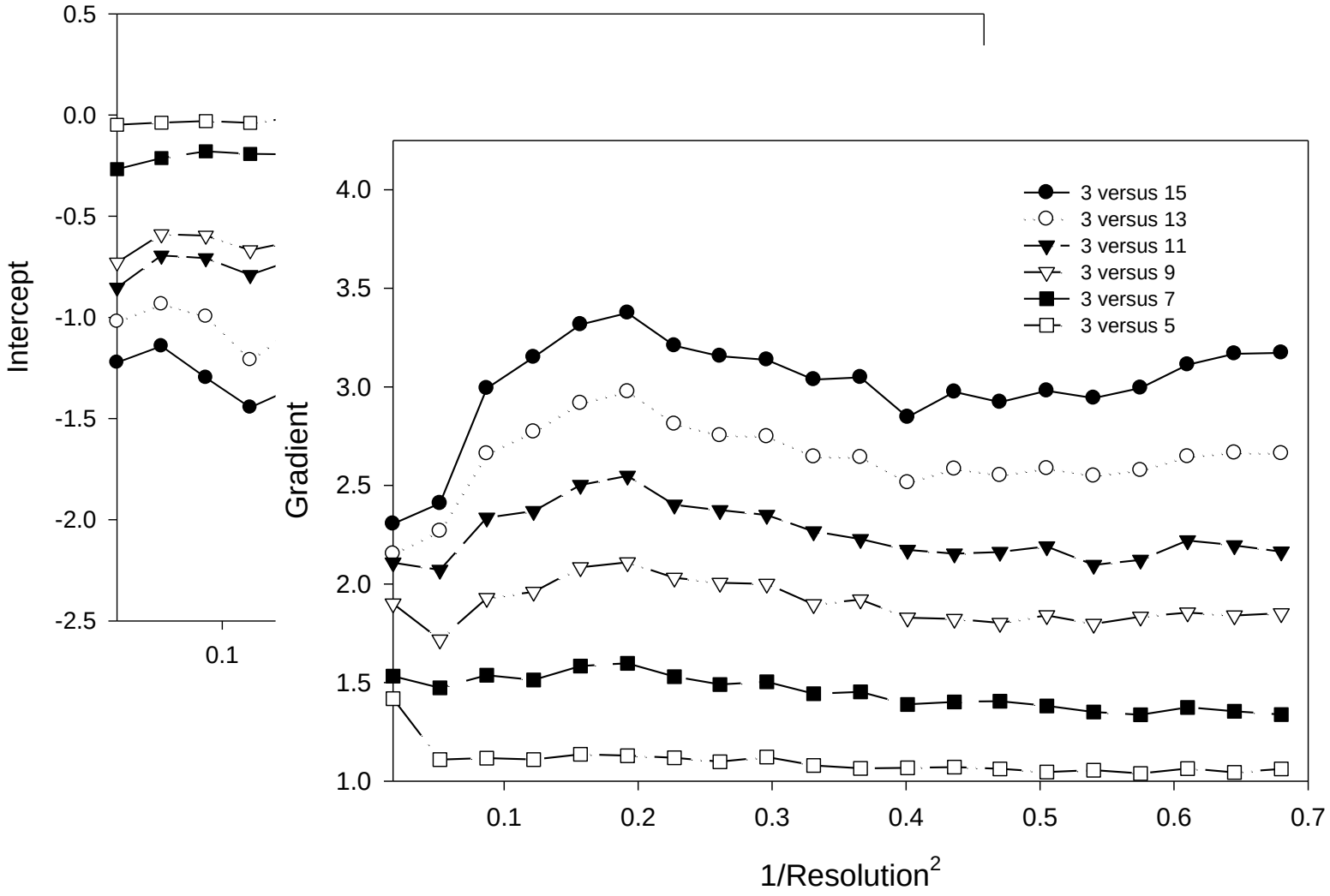
Same portion of high resolution data showing gradual decay of reflections.

Note that the background radiation remains constant



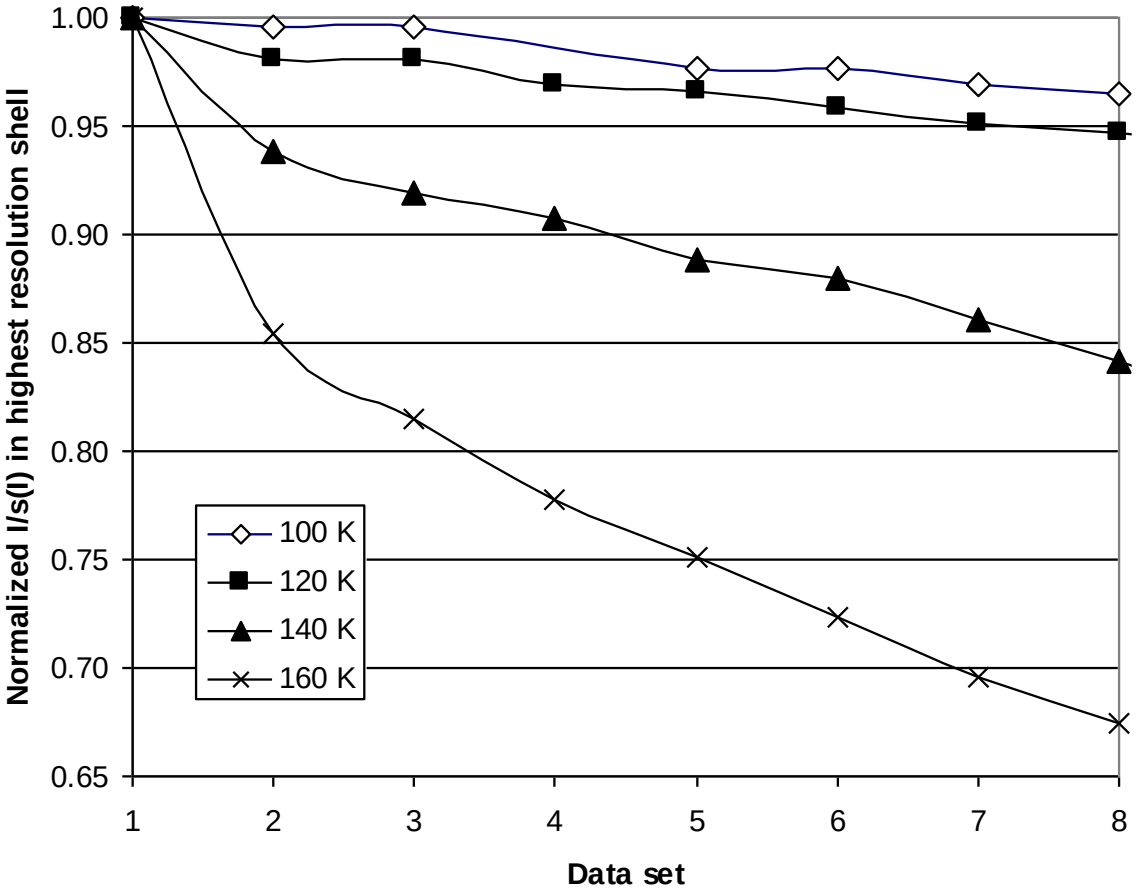

Data set 3
Data set 5
14
12
10
8
6
4
2
1.50
1.45
Signal/noise
Data set 2
Data set 4
Data set 6
Data set 8
Data set 10
Data set 12
Date set 14
Data set 16
6
5
4
3
2
1.06
1.04
1.02
1.00
0.98
0.96
0.94
0.92
Signal/noise
Average resolution of bin

Are there structural consequences?



Yes, but need the structural refinement before knowing what they are.

What effect does temperature have on radiation damage?



Repetitive identical sets of data 4 crystals of similar volume. Each crystal collected initially at 100K for baseline data point One crystal collected at 100K, The other 3 at 120, 140 and 160K

We know radiation damage occurs but what is actually happening?

Is gas CO_2, CO, H_2, O_2 …. A combination or something else. Under active investigation by a number of groups.

Status of research to date

- Clear metric in terms of cell parameter increase
- Similarly linear decrease in signal-to-noise
- Structural effects are present in the data.
- Structural refinement on each data set is beginning.
- Maintaining as low a temperature as possible is important.
- Structural refinement started.

Where is it heading?

- High resolution structural information on radiation damage process - current published studies at about 2 Å
- Data combined with neutron data for charge density studies.
- Complete knowledge about the enzymatic mechanism of xylsoe isomerase.

Questions to ask for the future

- Can we reduce, prevent or make use of the damage? (translate crystals, attenuate beam, scavangers, cryoprotectants)
- What is the process?
- What warning signs do we need to look for to differentiate from mechanism or radiation damage in our structure?

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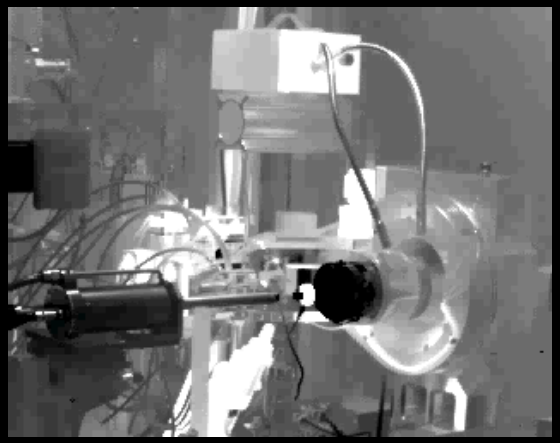
Infrared, another type of radiation



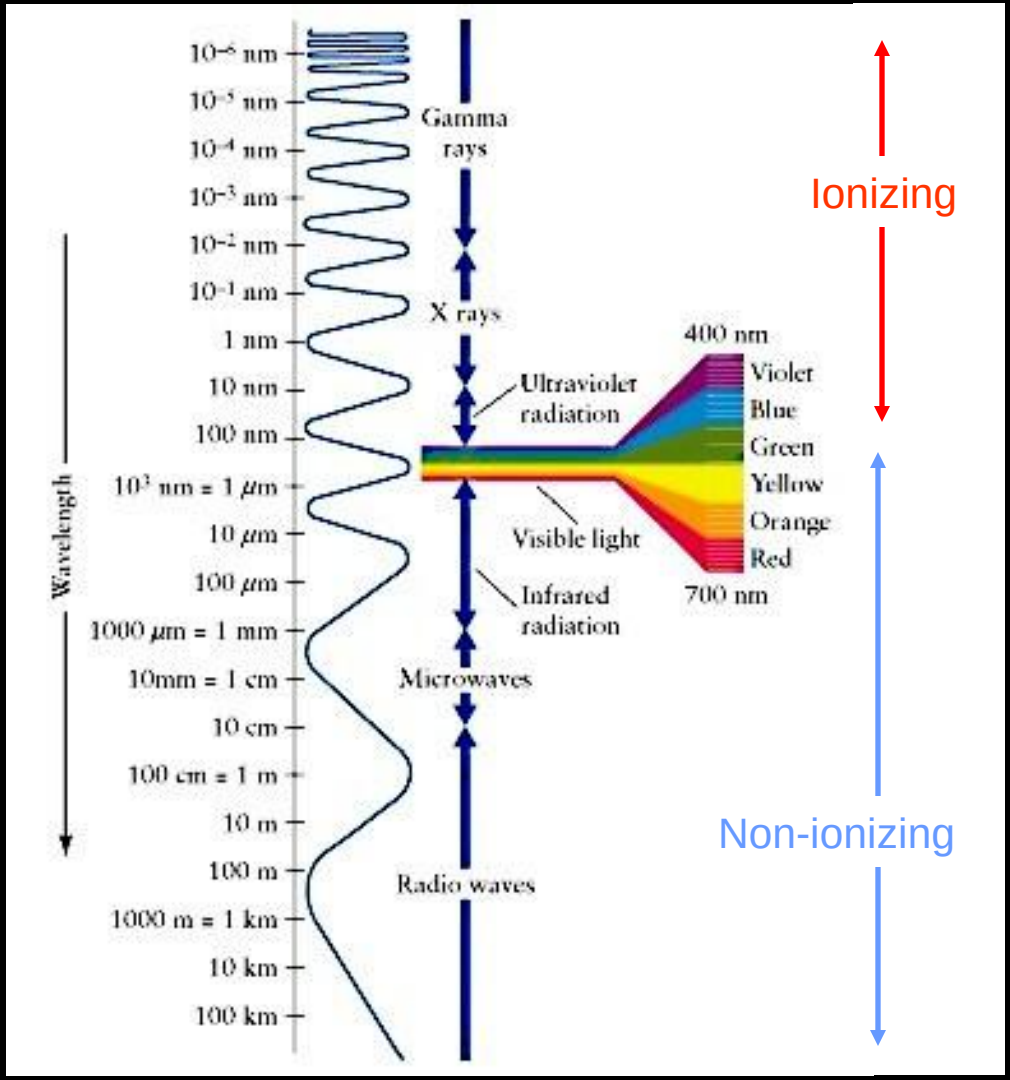
Thanks to Mitch Miller, Ashley Deacon and Mark van der Woerd

The Electromagnetic Spectrum

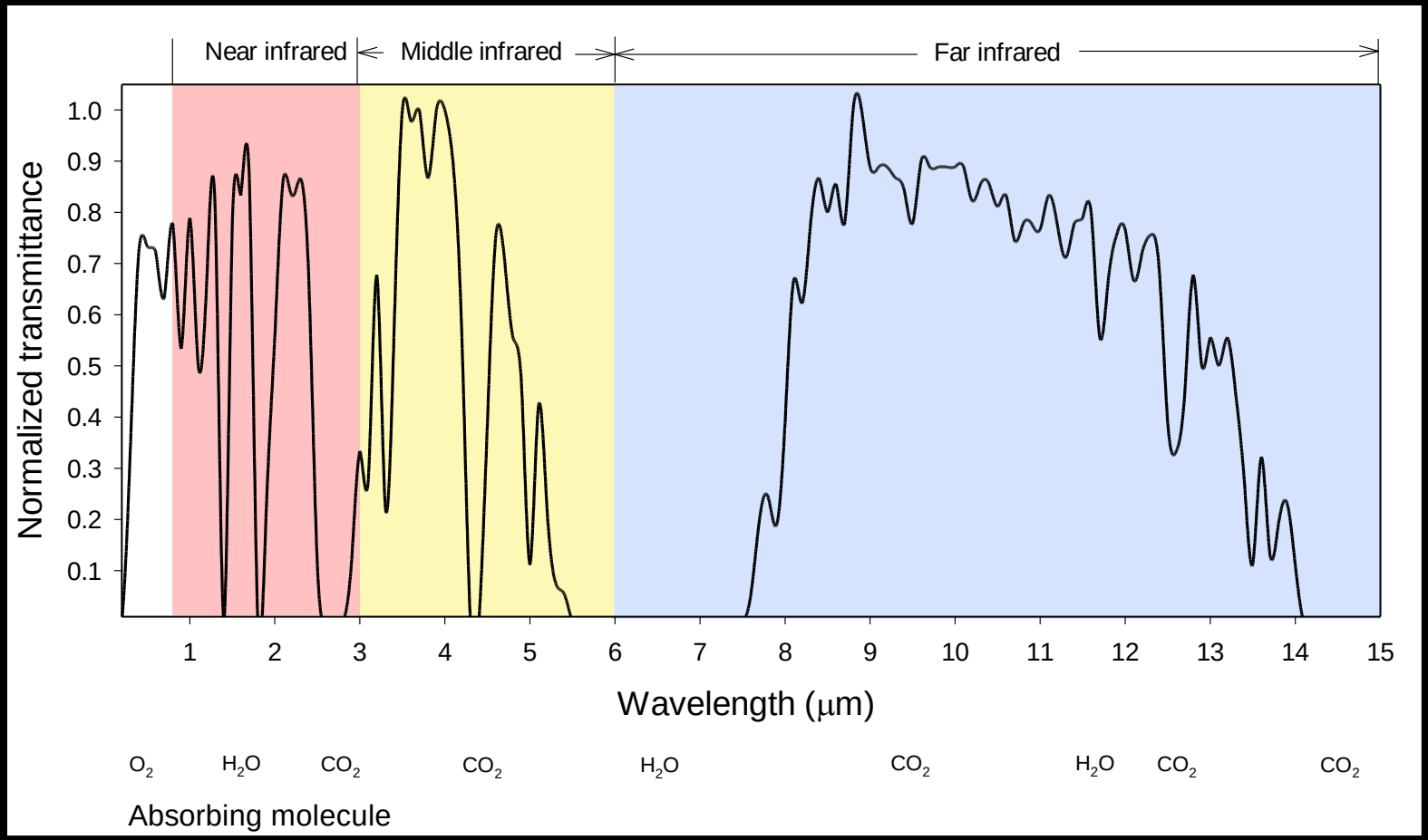
The electromagnetic spectrum can be divided into ionizing and non-ionizing radiation.

Ionizing, e.g. X-rays, high energy ultra violet *etc.* have enough energy to break chemical bonds – they are damaging to the molecules.

Non-ionizing radiation, *e.g.* visible and infrared does not have the energy required to break bonds. Observation with this type of radiation is non-invasive.



Infrared radiation is absorbed in the atmosphere.



There are three defined regions where absorption is minimized termed the far, mid and near infra-red. These “windows” in the atmosphere can be used for observation.

Black body radiation

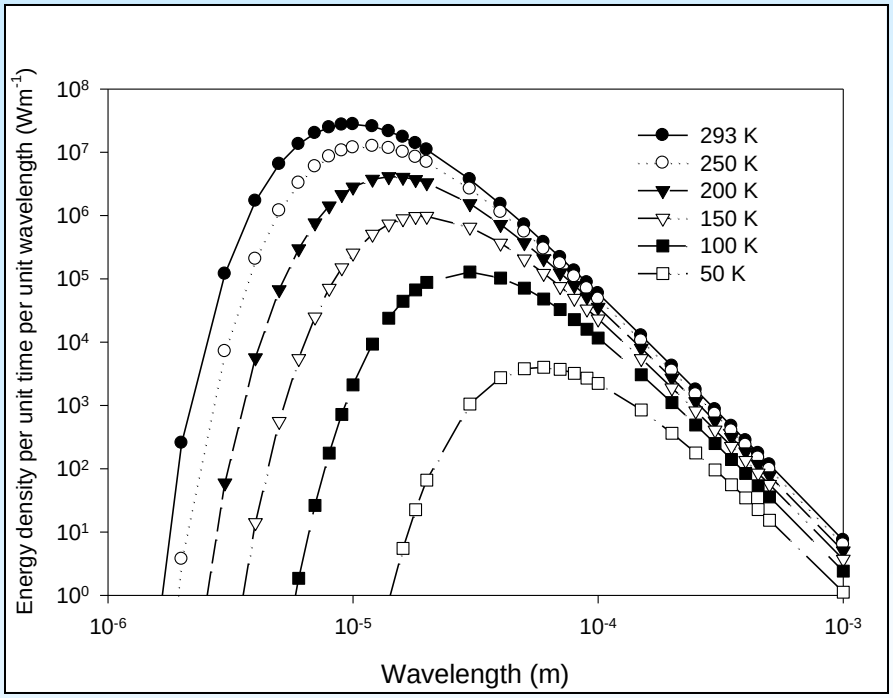
- All objects above 0K emit infrared energy as a function of their temperature.
- A black body perfectly emits and absorbs this thermal radiation.
- The energy spectrum for a black body is exactly given by Planck's radiation law;

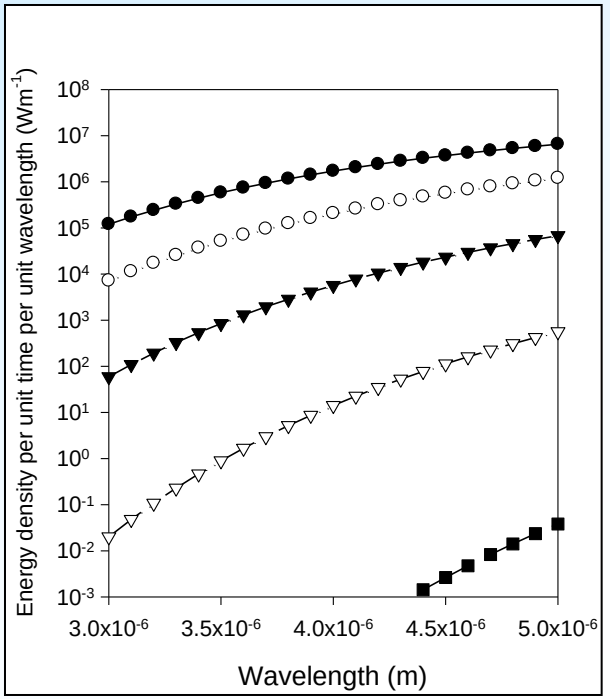
$$E(\lambda(T)) = \frac{2\pi hc^2}{\lambda^5 (e^{hc/\lambda kT} - 1)}$$

- Where λ is the wavelength, *c* is the speed of light, *k* is the Boltzmann constant, *h* is Plank's constant and *T* is the temperature in Kelvin.

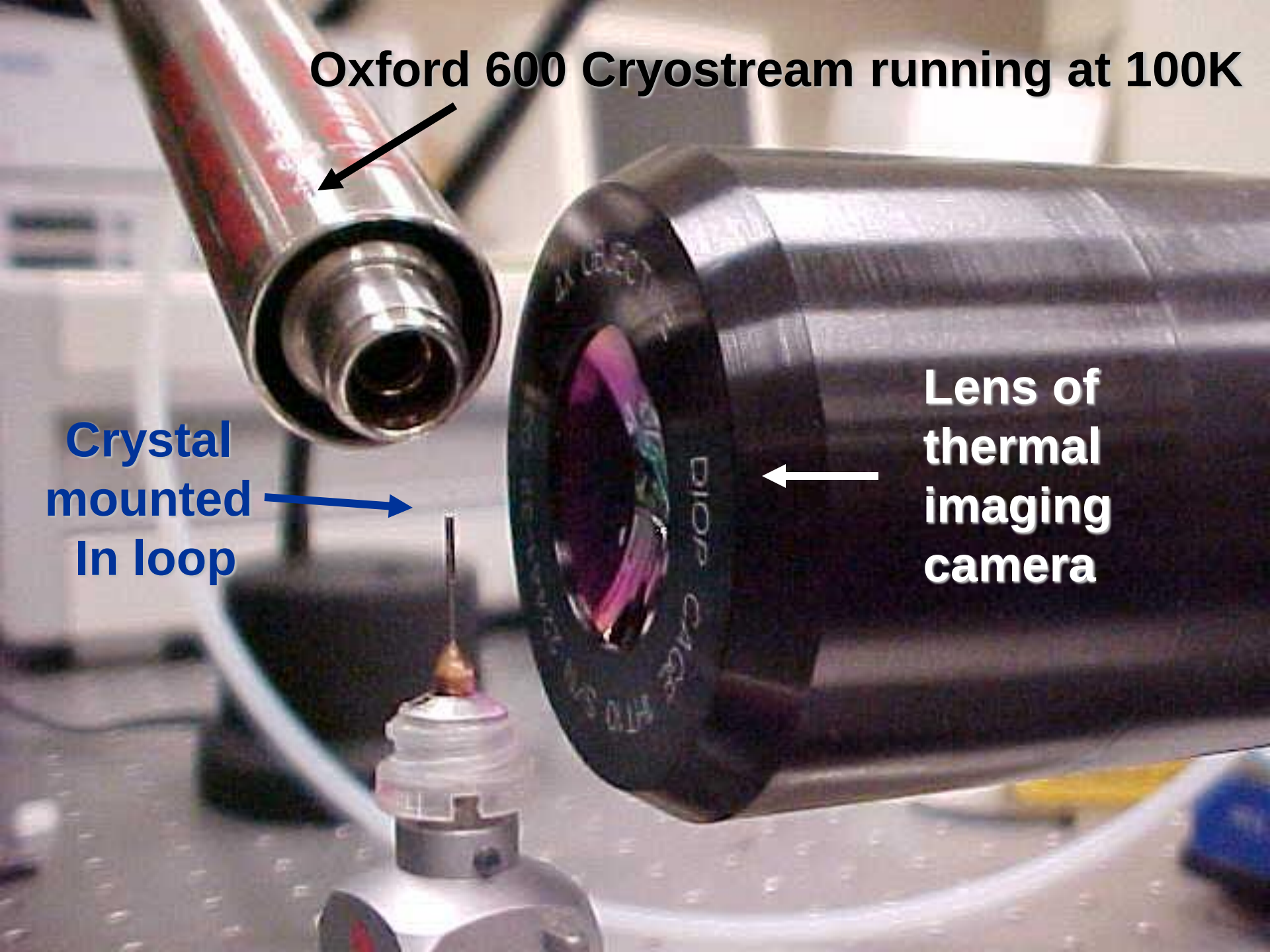
The energy spectrum for objects cooled below ambient conditions

The energy spectrum for the mid-range sensitivity of the infrared camera used

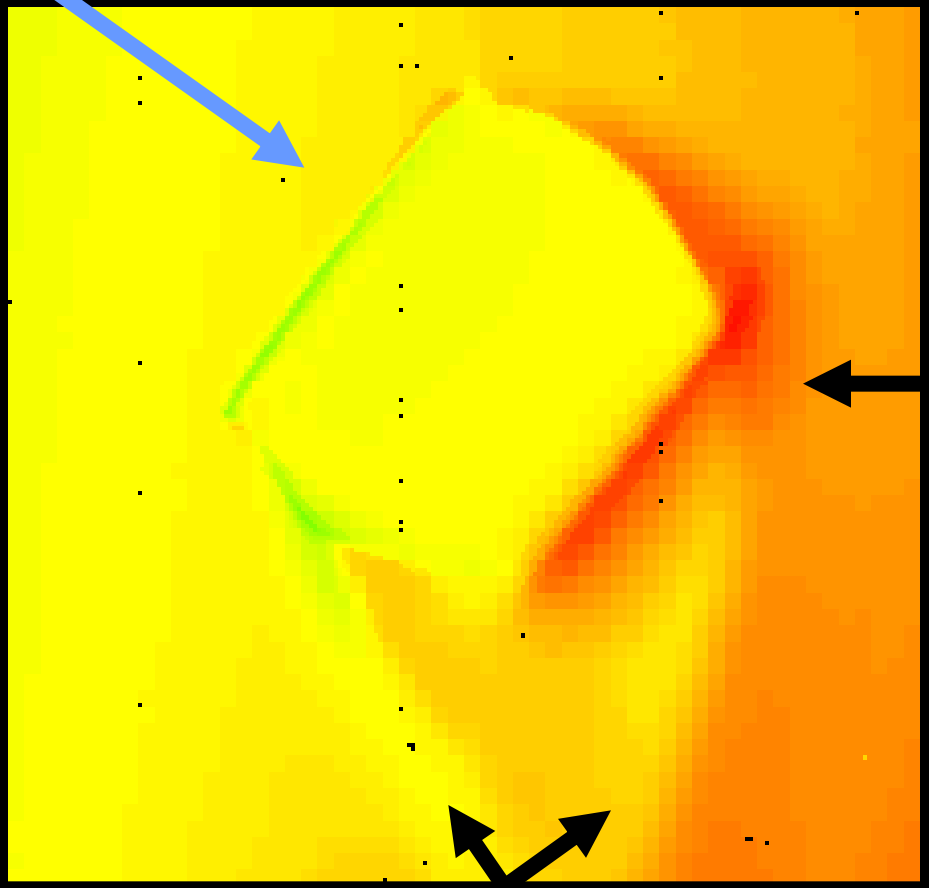




Imaging in the mid-range was chosen. This has a greater energy density and accuracy over the near-range and and greater response to temperature change over the far-range

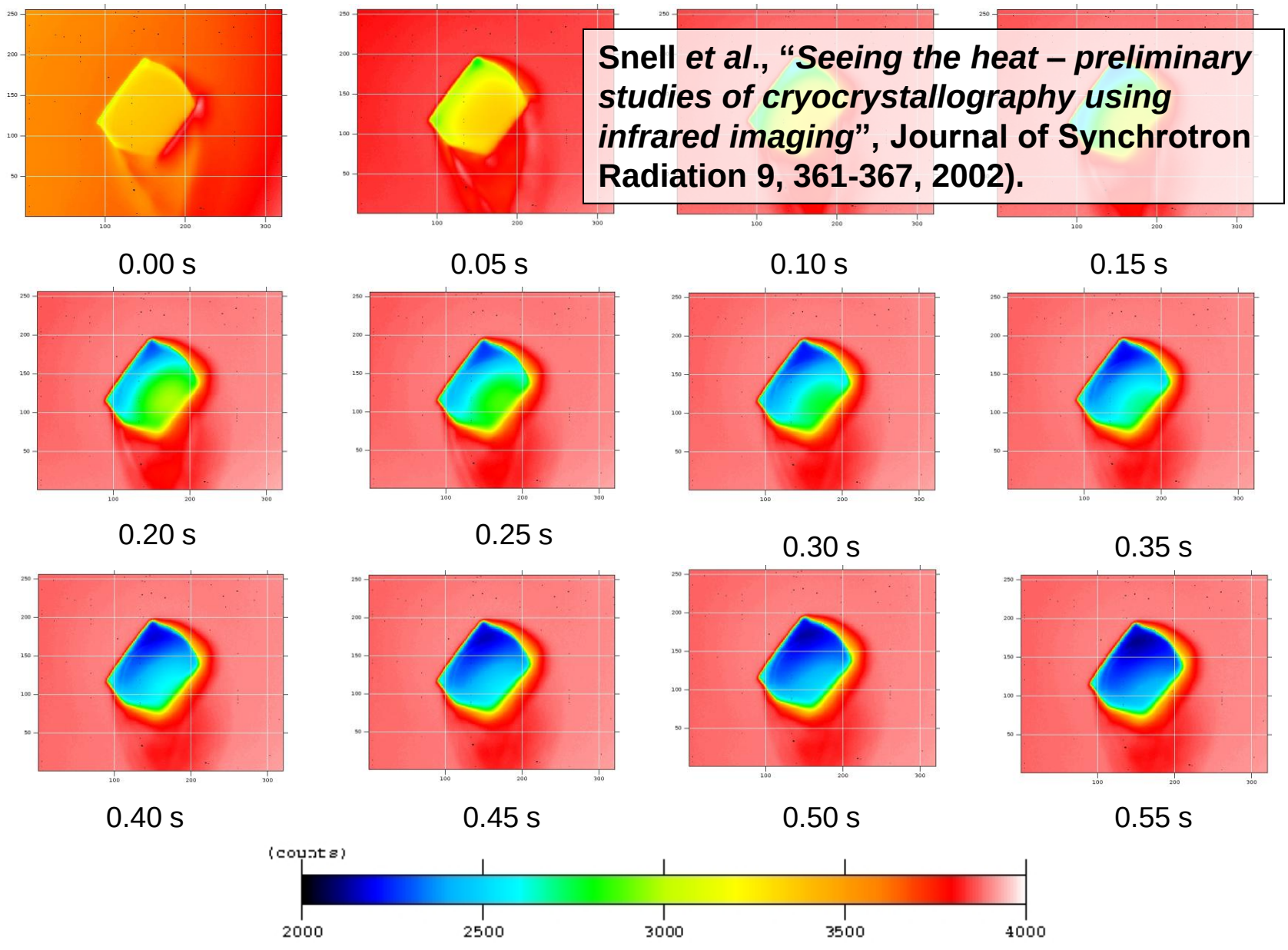

Oxford 600 Cryostream running at 100K
Crystal mounted In loop
Lens of thermal imaging camera

Cryostream at 100 K



Crystal

Nylon cryoloop


Snell *et al*., "*Seeing the heat – preliminary studies of cryocrystallography using infrared imaging*", Journal of Synchrotron Radiation 9, 361-367, 2002).
0.00 s
0.05 s
0.10 s
0.15 s
0.20 s
0.25 s
0.30 s
0.35 s
0.40 s
0.45 s
0.50 s
0.55 s
(counts)
2000
2500
3000
3500
4000

Properties of Infrared Radiation from a crystal (Why were the results qualitative?)

- Macromolecular crystals do not tend to be perfect black bodies:
  - They do not perfectly emit or absorb radiation
  - The spectral radiance is less than that predicted by Planck's law.

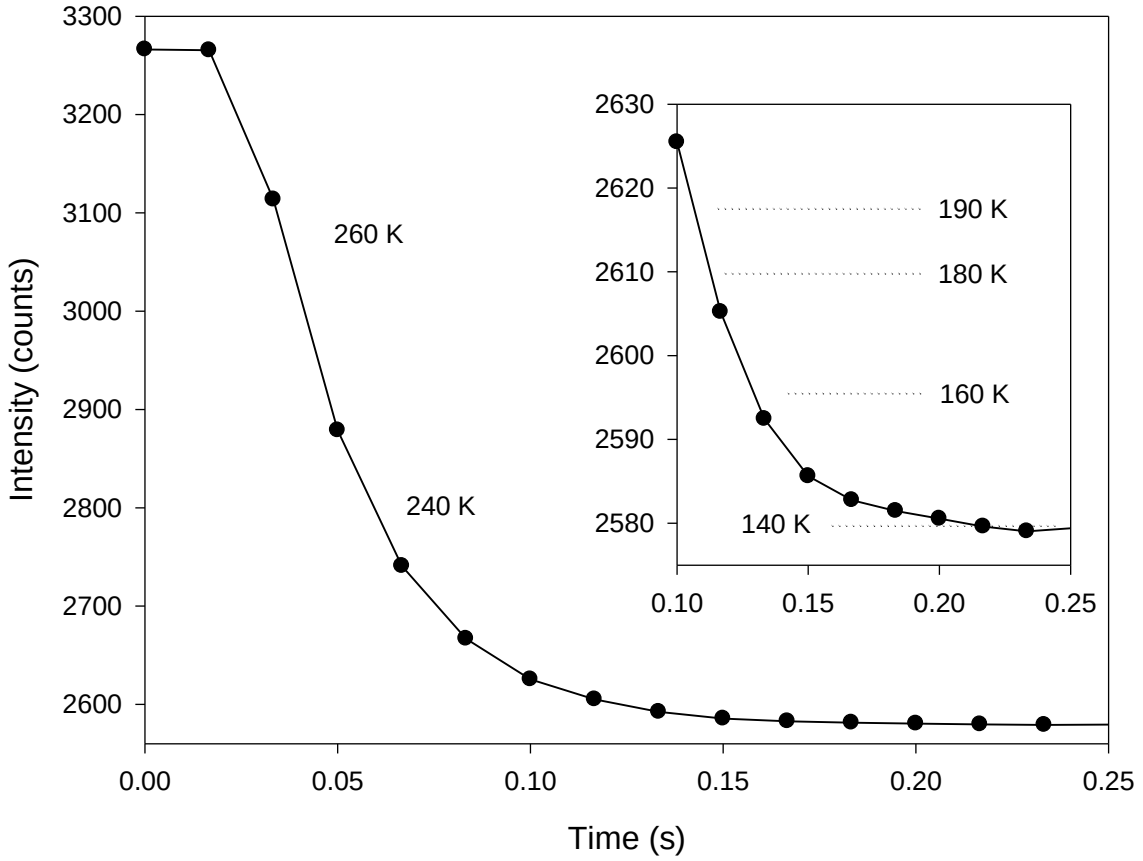
- Crystals tend to be illuminated by a number of infrared sources:
  - The ambient heat in the room
  - The experimenter
  - The illumination
  - The coldstream

- Crystals transmit and reflect heat
  - Heat is seen behind the object
  - Heat is seen reflected off the object

- Infrared properties of the crystal vary with wavelength and viewing angle

- Problem, it is not trivial to do quantitative studies

Lysozyme crystal cooling – Quantitating Intensity

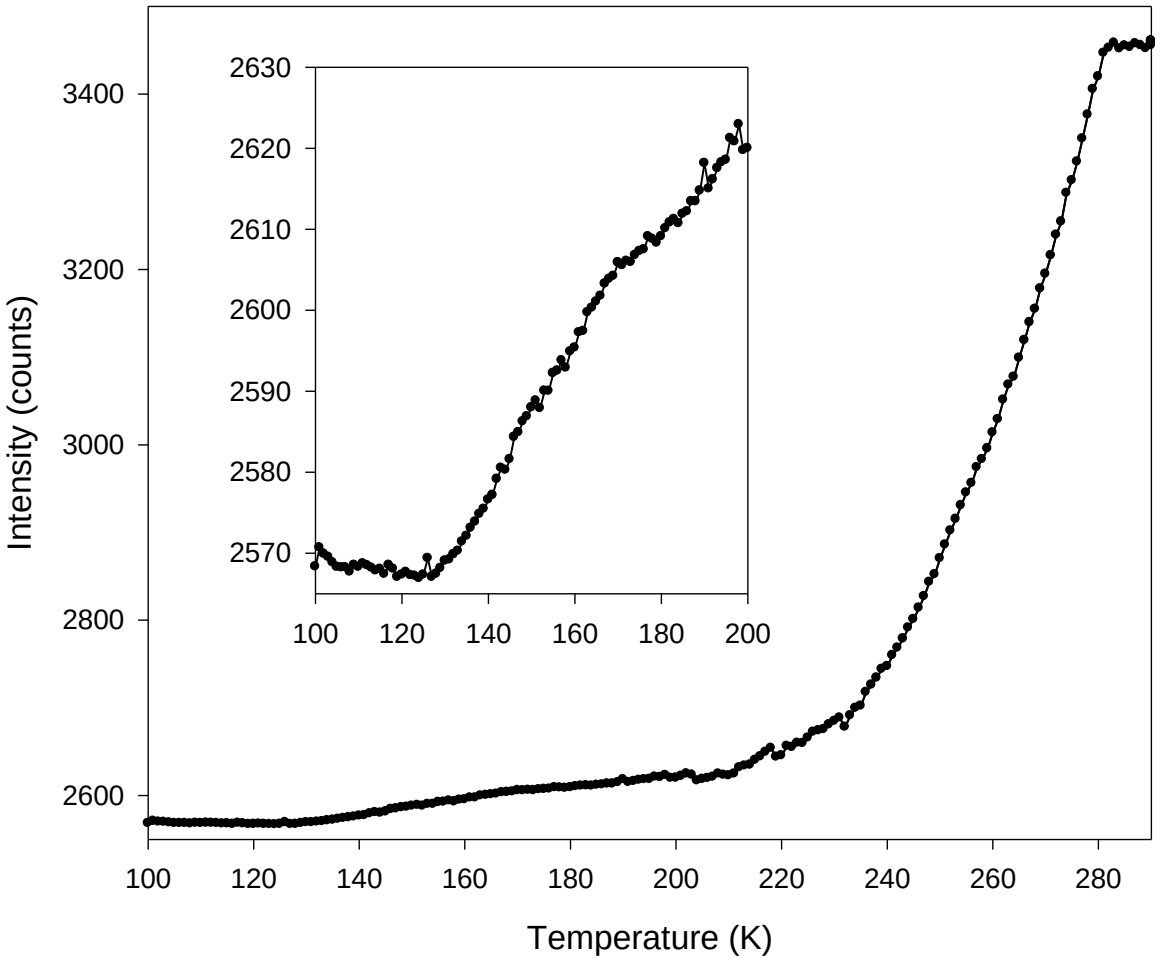


Experimental protocol similar to the glass bead:

1. Set stream to 100 K.
2. Block stream
3. Mount crystal
4. Focus
5. Start data collection (1000 images at 60 Hz)
6. Program stream to warm up at 2 K per minute once cooling data collection is complete.
7. Collect an image every 30s (1 K) from 100 K to 290 K

The cooling data is then calibrated from the warm up data. Due to background thermal radiation and crystal position each crystal has a unique value of intensity at a given temperature.

Lysozyme crystal calibration

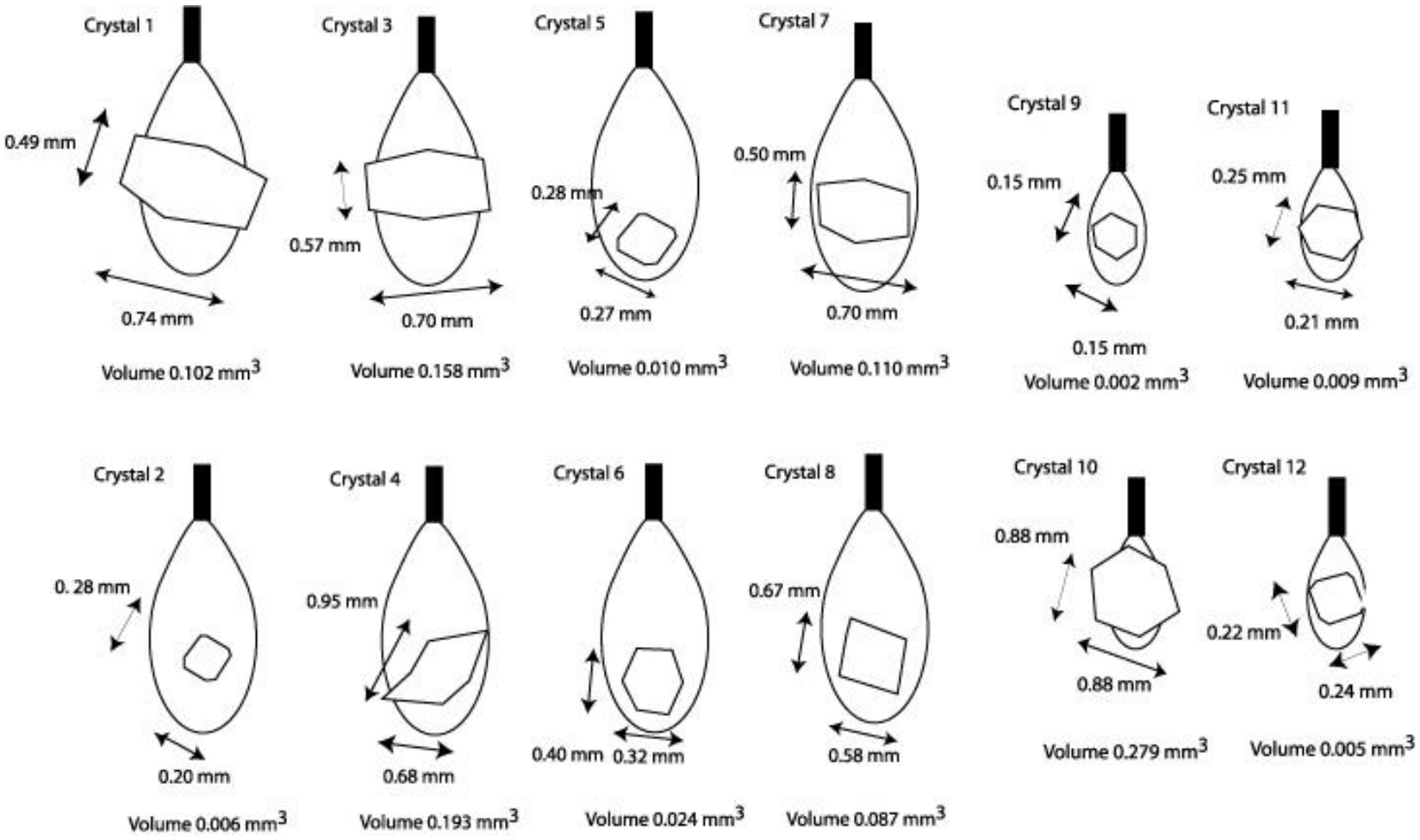


The calibration reveals that for lysozyme crystals the sensitivity of the camera is approximately 135 K.

This sensitivity is a property dependent on the non-ideality of the sample as a black body, i.e. emissivity. This is the ratio of the radiation emitted to that predicted by Plank's law.

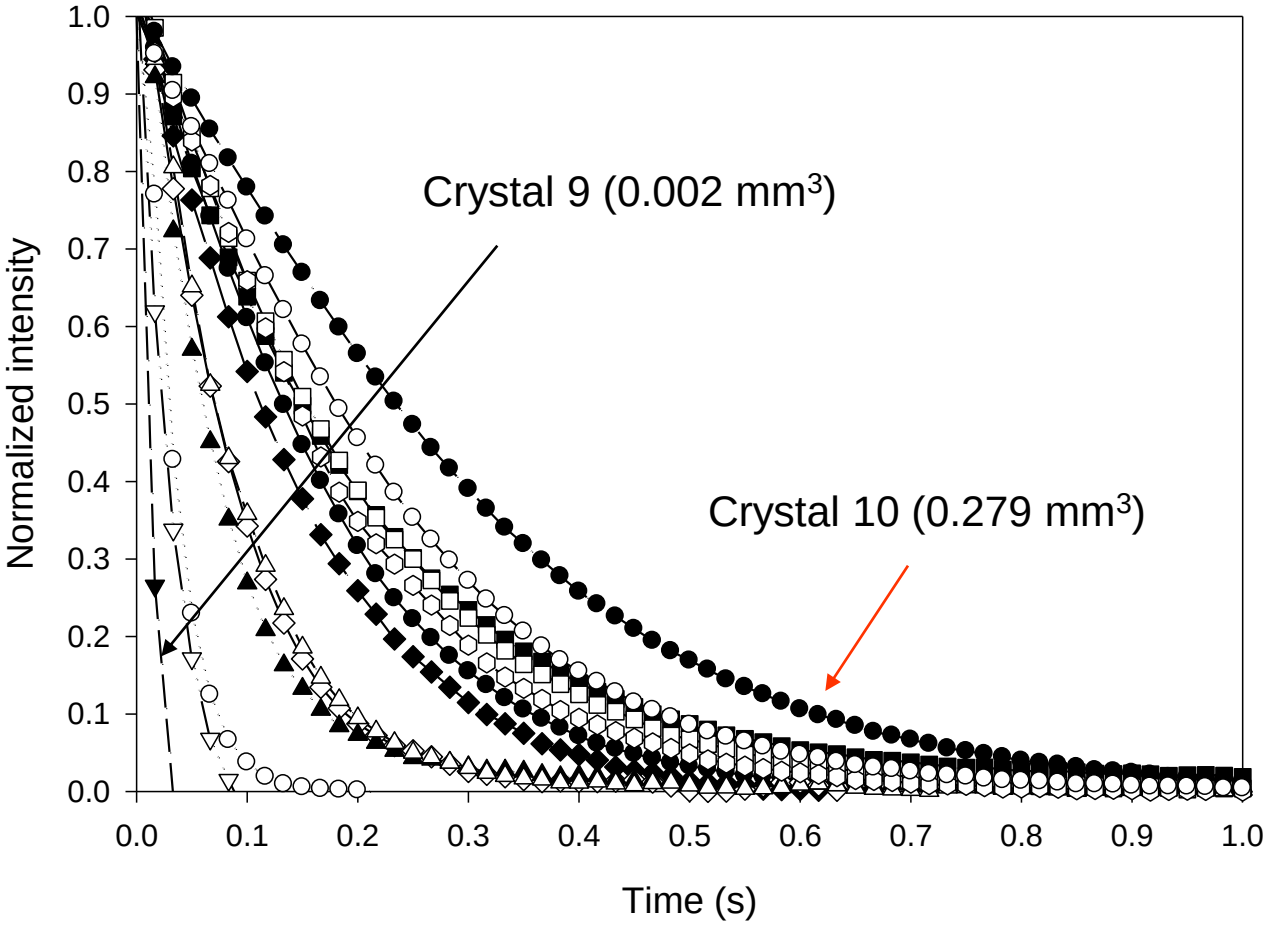
This emissivity and therefore camera sensitivity is sample dependent

Diagram of lysozyme crystal samples studied

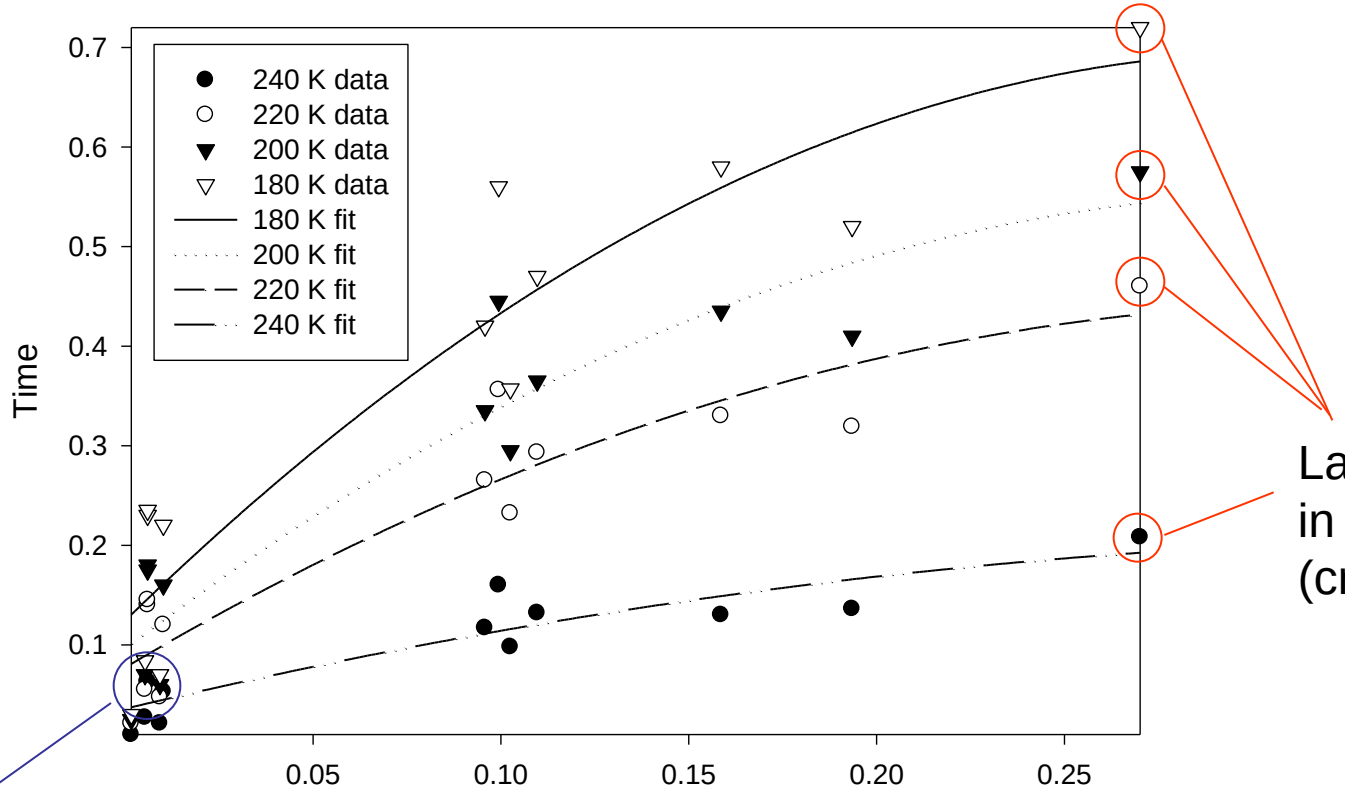


Two different loop sizes were used, 0.5 mm and 0.2 mm diameter.
Typically the smallest dimension is into the picture.

Normalized intensity profile of crystal samples during cooling.



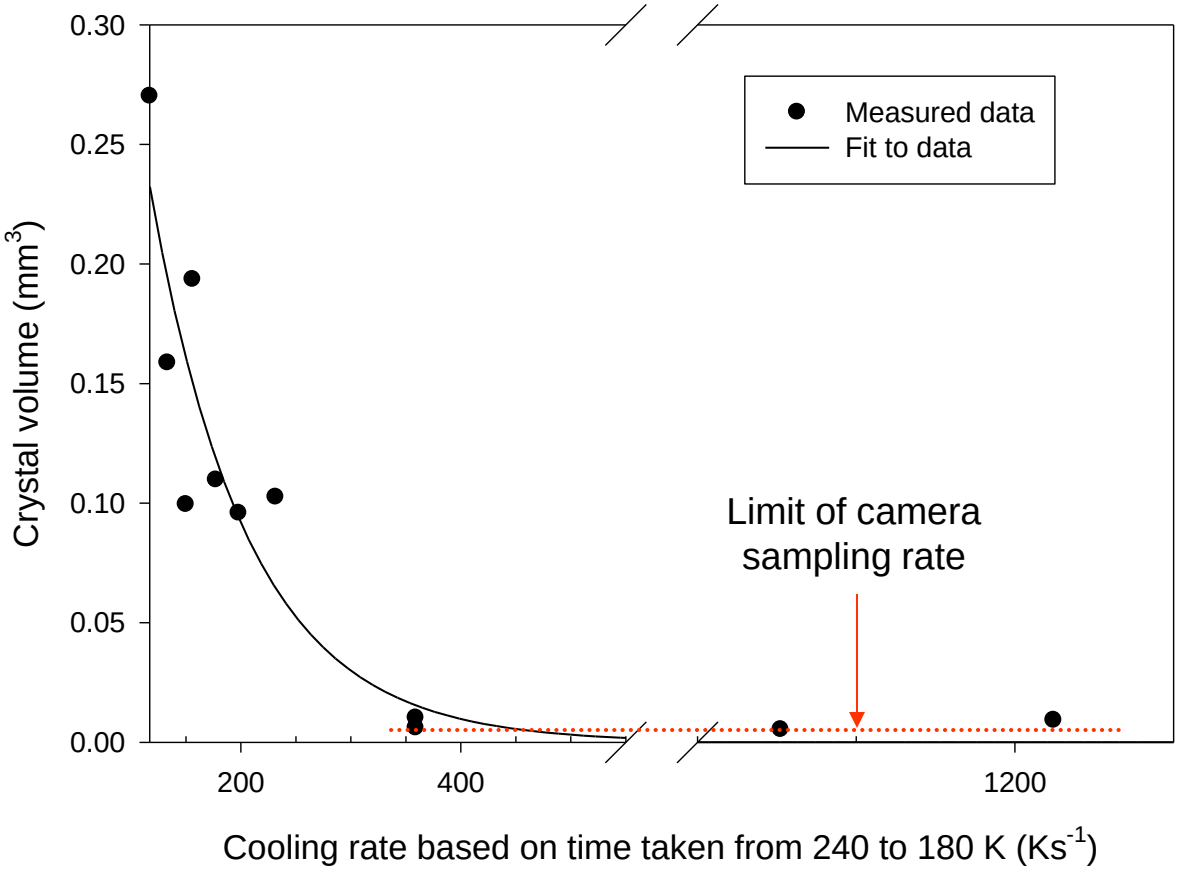
Cooling as a function of crystal volume



Large crystal in a small loop (crystal 10)

Small crystal in large drop (crystal 2)

The speed of cool….



Note that for the fastest cooled crystals the data is at the limit of the camera in the setting used.

The camera can image digitally at faster rates, full frame 150 Hz, half size frame 300 Hz (images every 0.0067 and 0.0033 seconds respectively) but the video output is unavailable. It is not possible to focus the crystal easily.

The smallest crystals await optics with improved depth of field.

Outline of talk

- Assumptions
- What are first second and third generation synchrotrons?
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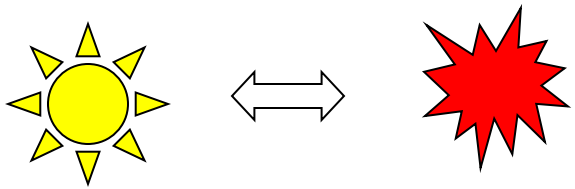
- Radiation damage
- Cryocooling
- Beam heating
- Alternatives to X-rays
- Summary

X-ray Heating of Biomaterials

•X-ray heating results in both **thermal** and **radiation** damage.



•**Cryocooling cools** samples & mitigates **radiation damage.**

•Energy deposited in sample is **conducted** through interior and then **convected** away by N_2 gas cooling stream.

•Sample temperatures have been previously **calculated**, but these predictions have never been validated **experimentally.**

Previous Thermal Studies: Experiments &Theory

Flash Cooling Experiments (without X-ray heating) **no convection**

- T. Teng and K. Moffat (1998)
- L.J. Walker, P.O. Moreno, H. Hope (1998)
- E.H. Snell, R.A. Judge, M. Larson, M.J. Woerd. (2002)
- etc.

Experiments measuring local sample temperature with X-ray heating were lacking

Thermal Modeling of Sample Heating by X-ray Beam

- Adiabatic Model (Helliwell,1992; and others) **no convection,** K/s, max rate of temp increase
- T.Kuzay, M.Kazmierczak, B. Hsieh (2001)
- J. Nicholson, C. Nave, K. Fayz, B.Fell, E. Garman (2001)
- G.Rosenbaum and M. Kazmierczak (2002)
- S.Kriminski, M. Kazmierczak, R.E Thorne (2003)
- A.Mahsiekar, M.Kazmierczak, R.K Banerjee (2003)

} **with convection**

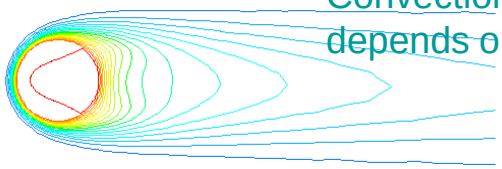
Thermal modeling predictions with experimental verification were lacking

Thermal modeling predictions with experimental verification

- Model using 3D Numerical Simulation with coupled CONDUCTION and CONVECTIVE heat transfer.

$$q''_{cond} = -k\nabla T \qquad q''_{conv} = h_x(T_s - T_{gas})$$

Convection coefficient depends on flow field



Fluid Mechanics Eqs

- Conjugate analysis solving N-S and Energy equations
- Surrounding flow and temperature fields
- Local hx and $\bar{h}$ are calculated
- Finite Volume Code

Initial studies

The South East Regional (SER) –CAT

Beamlines 22-BM (developing the method) and first tests on 22-ID

For the data presented here

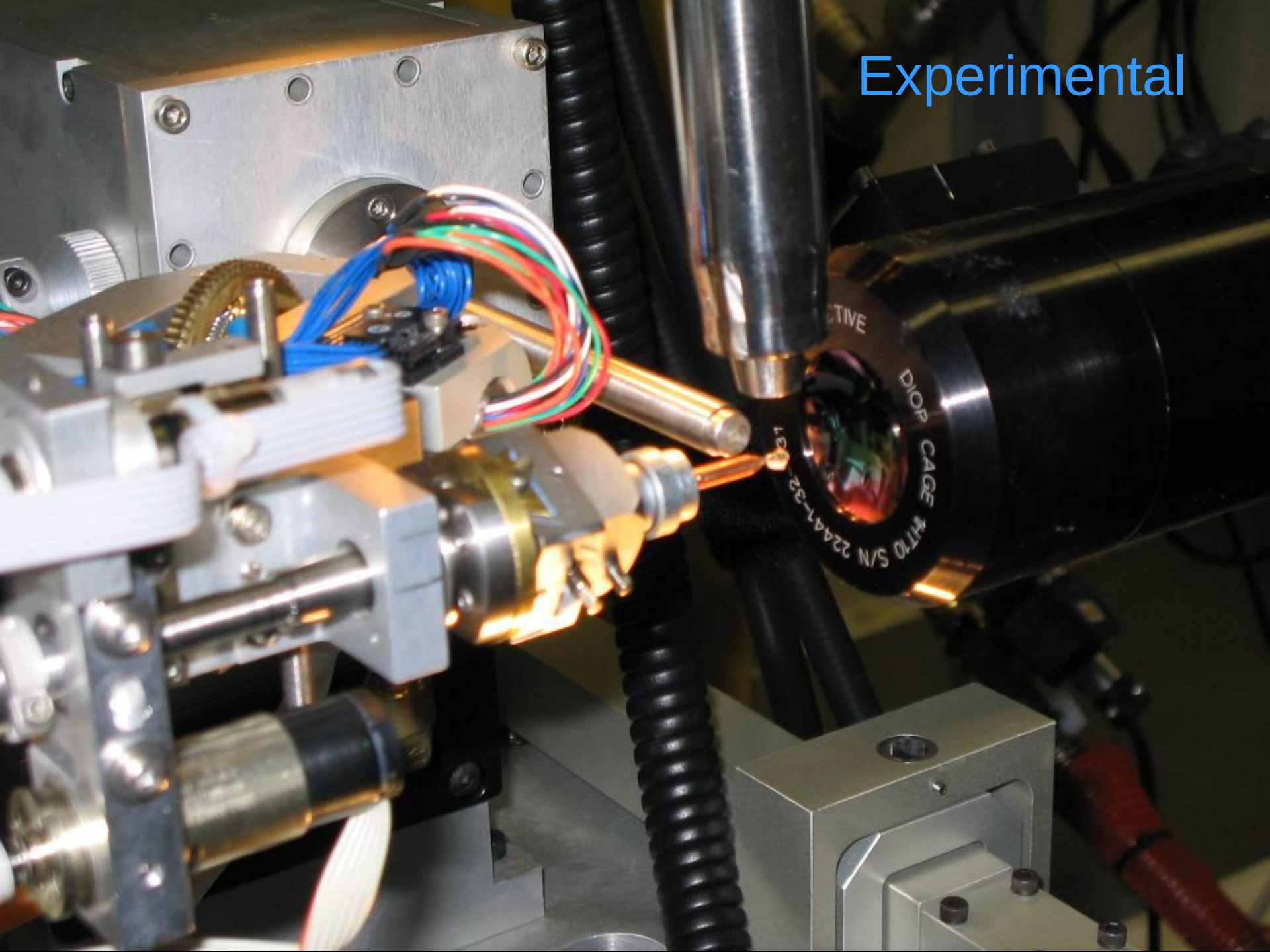
The Structural Biology Center (SBC) - CATBeamline 19-ID

Energy 6.5 Kev (1.9 Å)

Ring current ranging between 101 and 103 mA

Intensity of 3.24 x 10^{12} Ph/s.

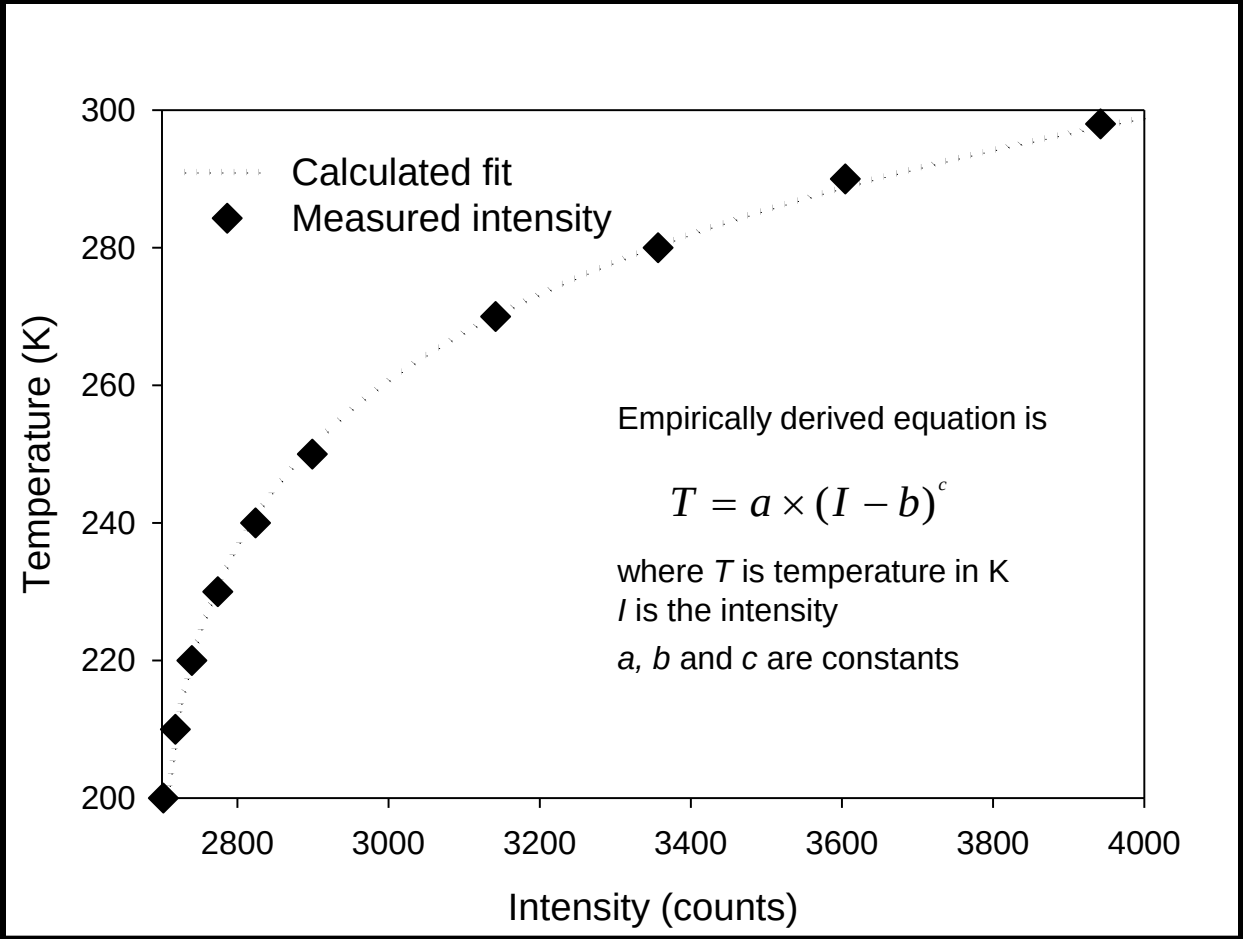
Experimental



Glass Bead Samples and Protocol

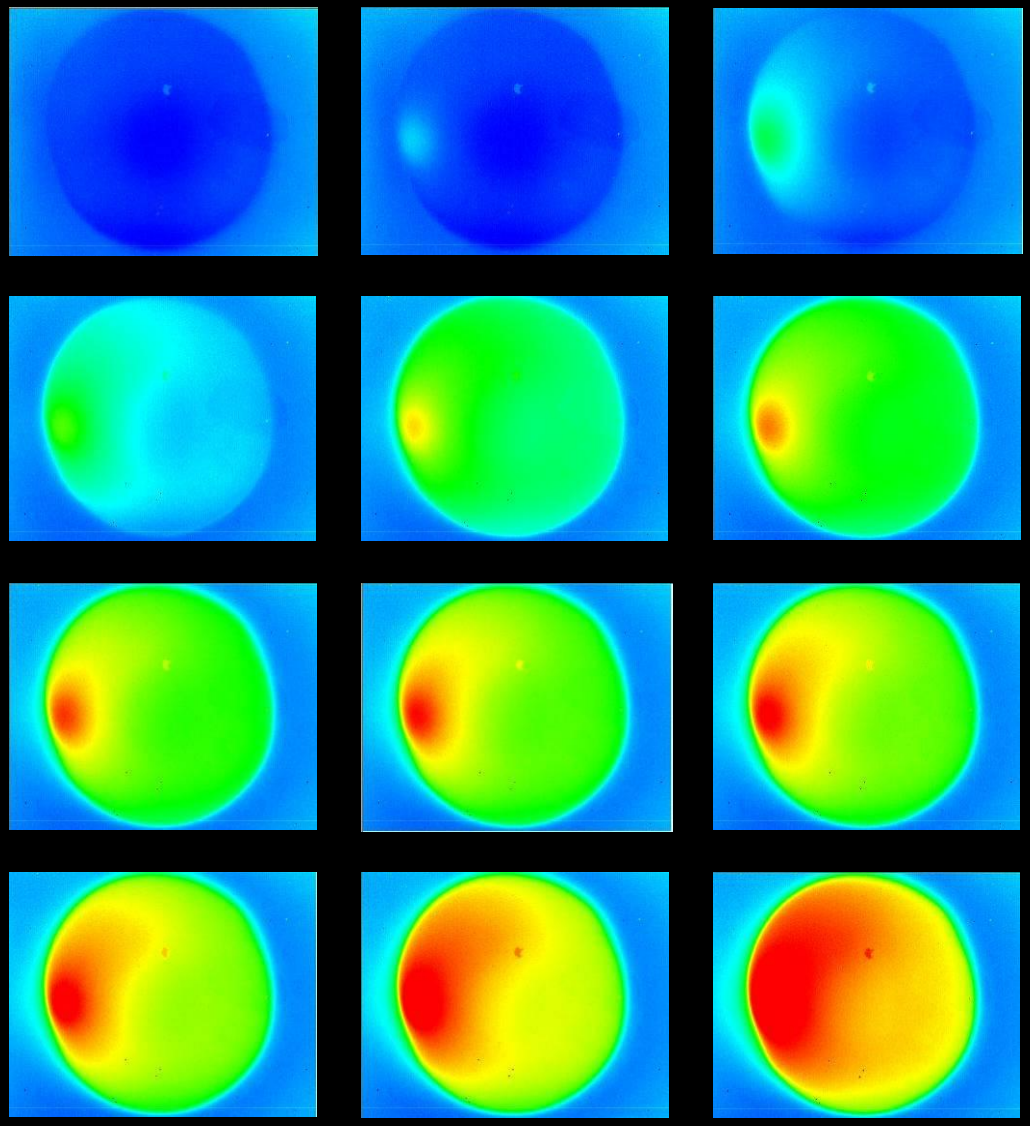
- Sample 1: 2mm diameter glass bead
  - imaged at 100K with no beam (steady state calibration point)
  - Imaged with shutter opening (time resolved)
  - Imaged after shutter had been open for 1 minute (steady state).
  - Measurements repeated in 10K steps up to 290K
  - Final measurement with the cryostream off.
- Sample 2: 1 mm diameter glass bead
  - Imaged from 290K down to 100K in reverse of 2mm case.

Temperature calibration



Bead at known temperature when shutter is closed. The intensity determined at this temperature and a calibration curve of intensity versus temperature calculated.

Shutter Opening



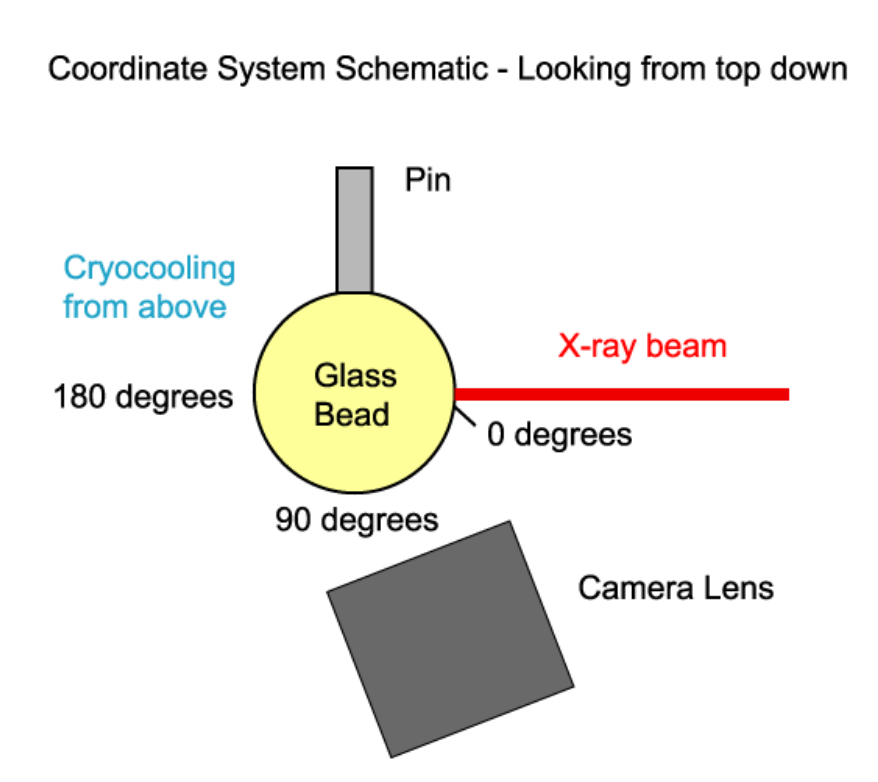
2mm glass bead at room temperature with no cryostream flow.

Beam hits bead from left, blue is cold, red is hot.

Temperature rise is 50K over 24 seconds.

Extreme case!

Experimental



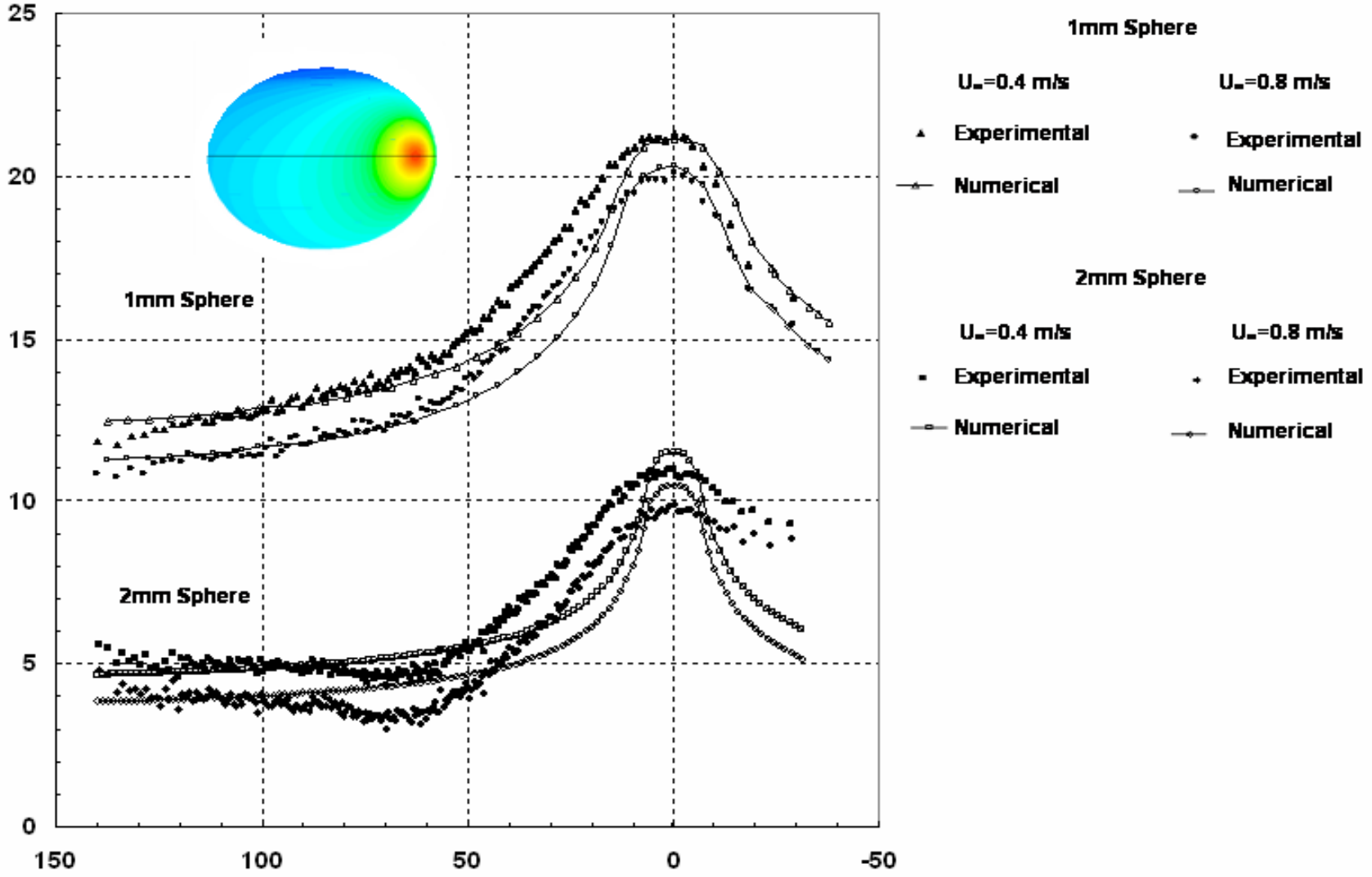
- For the 2mm glass bead the cryostream was set to 100K and a still image taken of the bead before the shutter was opened. A set of sequential images was then recorded as the shutter was opened. Finally a single image was taken with the shutter open when the bead had reached a steady state.

- The same protocol was repeated with cryostream settings in 20K increments until 200K and then 10K increments until 290K.

- At 290K an additional experiment was made with the cryostream at double flow. The cryostream was then switched off and the bead allowed to warm to ambient temperature, 298K, and the experimental measurement repeated.

- Following this the 2mm bead was replaced with a 1mm bead. Similar experiments were repeated.

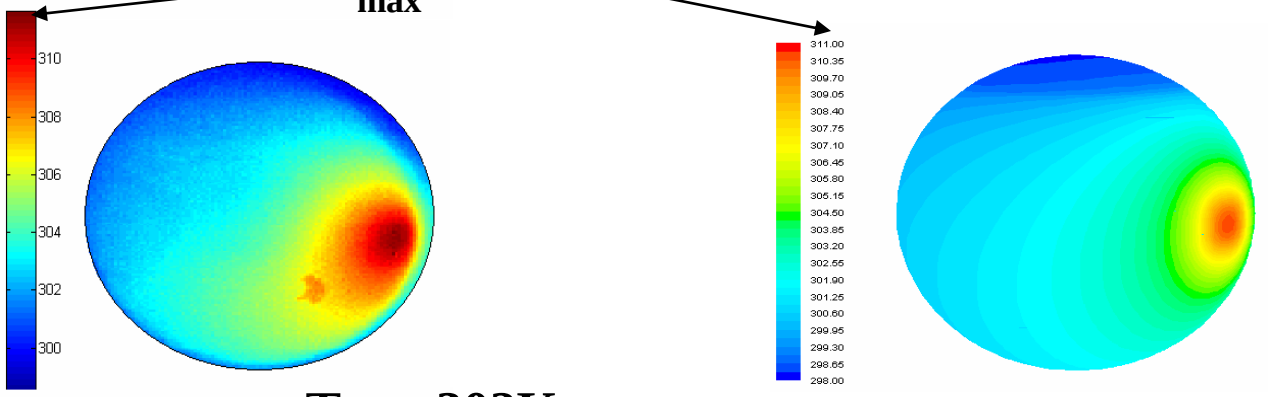
Steady State Results



Experimental **Numerical**

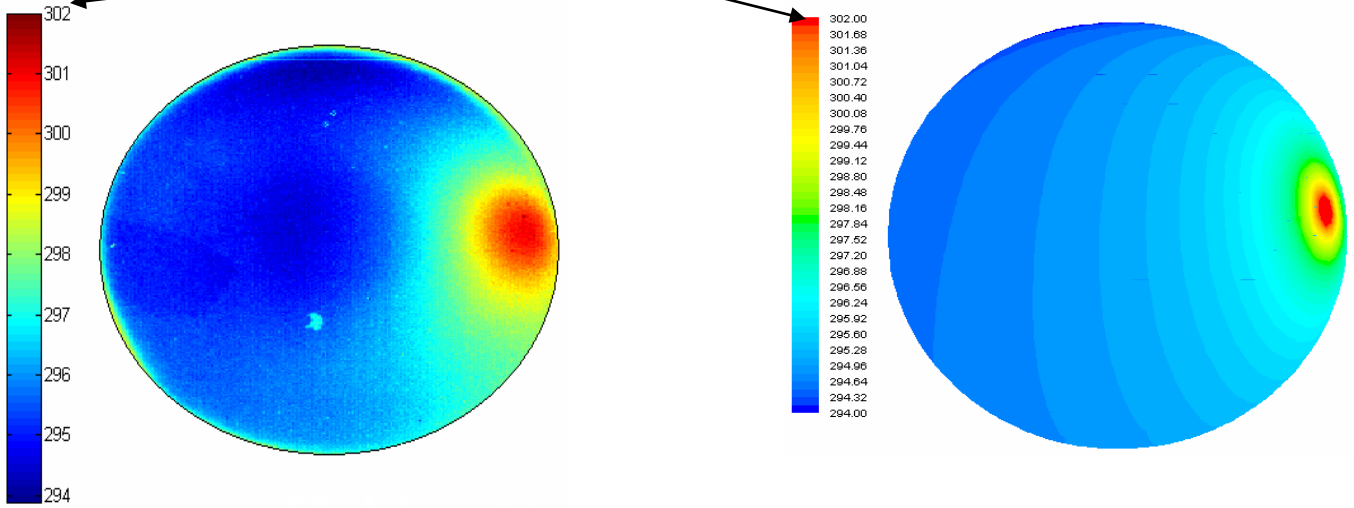
1mm Sphere

T_{max}=311K



2mm Sphere

T_{max}=302K



The 2mm sphere has lower temperatures

Results?

- Theory verified by experiment.
- Larger samples are better if beam heating is a problem.
- Higher cryostream flow rates are better (11% better cooling for doubling flow) – if you have an Oxford 700 keep it in Turbo mode
- The pin can extract heat from the system.
- Beam heating is not a serious problem with cryocooling, it is if no cryocooling is available.

Problems for the experiment?

- Real crystal were not as cooperative as glass beads …. As the theoretical physicist said, “consider a point crystal”, as the applied physicist said, “consider a spherical crystal”.

With real crystals?

- Radiation damage causes significant changes in the infrared properties of the crystal. Also seen in the visible spectrum as color changes.
- We have been unable to calibrate the temperature of the crystal to the intensity recorded by the camera.
- The next step is to use and infrared laser to put a heat load onto a real crystal and measure the thermal properties for modeling protein samples.

Dynamic modeling?

- We have presented steady state data. We also have dynamic data with a movie over time of the heating due to shutter opening.

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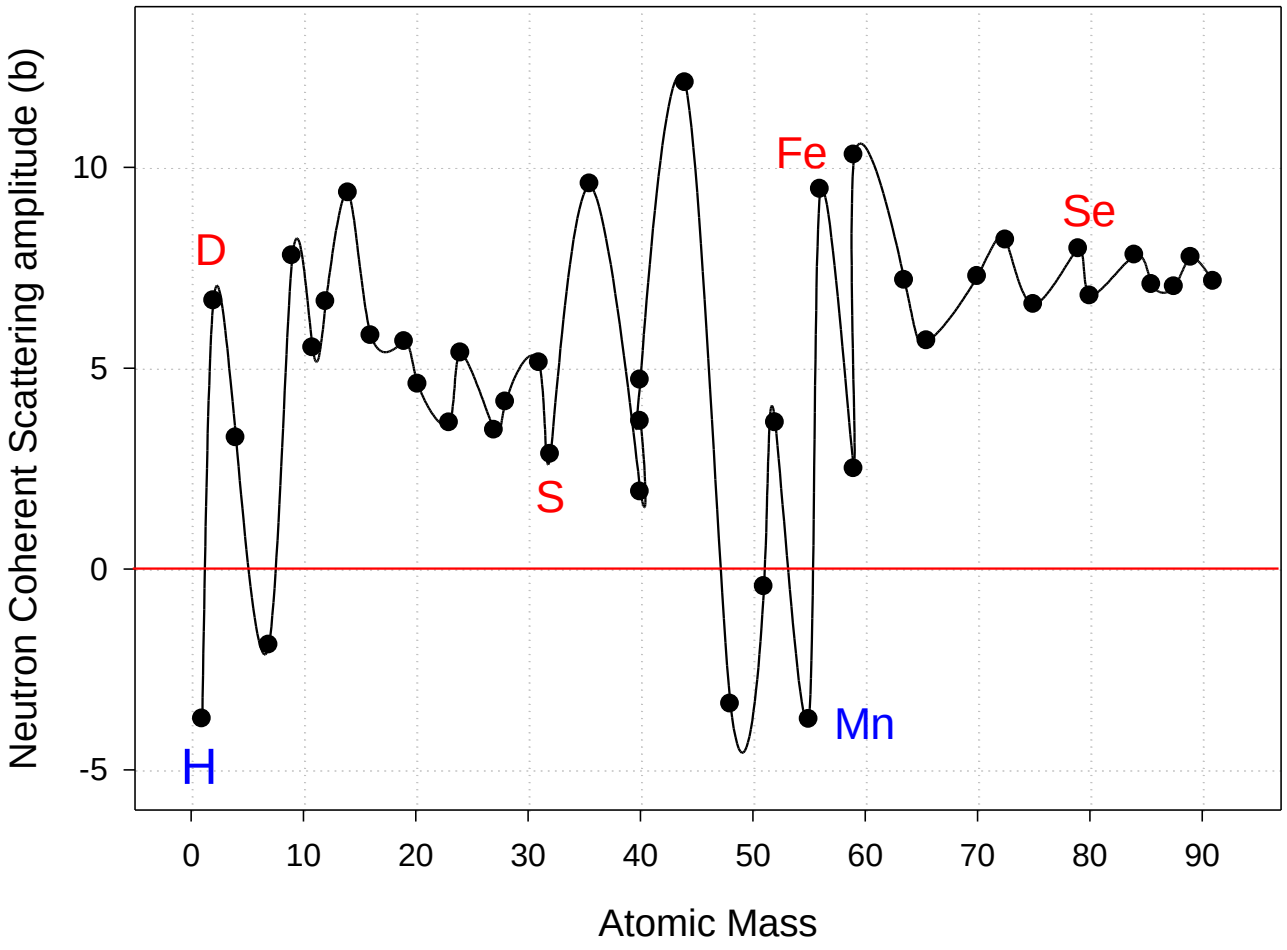
Neutron Diffraction Studies

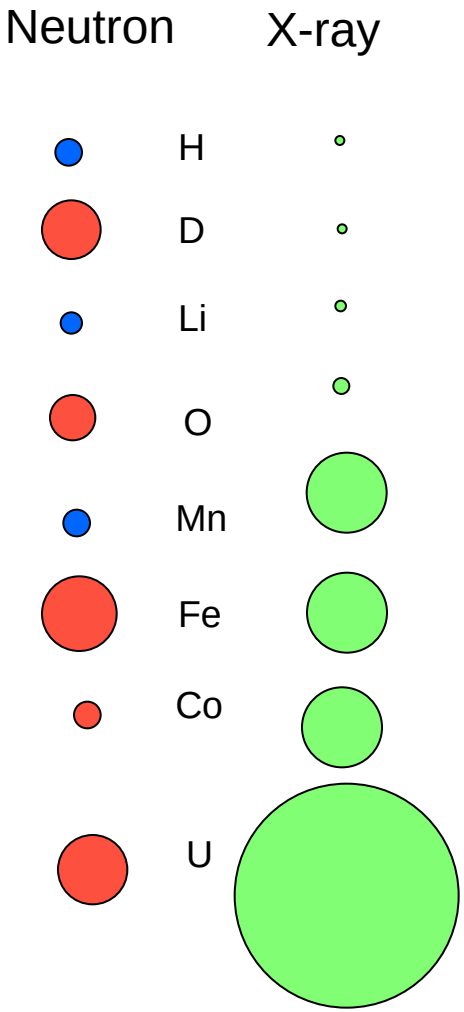
Or “12 hour exposures in a beautiful location with hiking, skiing, fine wine and dining opportunities”.

Why make use of neutrons?

- For neutron diffraction the scattering amplitudes vary from element to element in a non systematic way - atoms of similar atomic mass can be easily distinguished.
- The scattering amplitude of hydrogen is of the same order of magnitude as the amplitudes of other atoms typically found in biological molecules - hydrogen atoms can be seen thereby;
  - revealing whether a particular acidic group is dissociated or has a hydrogen atom bound to it,
  - discriminating between water and hydroxyl anion in the active site of an enzyme,
  - determining the orientation of a water molecule etc.
- Deuterium and hydrogen have opposite sign scattering amplitudes enabling contrast matching techniques.
- Radiation damage is not a concern.

Or, to answer it more graphically, what a neutron sees:





Statement of the problem:

Neutron sources have low fluxes:

- For example, the LADI (Laue Diffractometer) experimental station at Insitiute Laue Langevin has a flux of $3x10^7$ neutrons cm^{-2} s^{-1} for a partially monochromatised beam (l=3.5 A, $\delta\lambda/\lambda$=20%). A monochromatic beam from a wiggler source on a synchrotron has 10 orders of magnitude greater flux.

Neutrons are weakly scattered

- Neutrons are electrically neutral and interact weakly with matter, they are scattered by the nucleus and unpaired electrons.

Solutions to the problem

	Cost	Time	Probability of success
More neutrons			
Source intensity	Very expensive (new source or possibly use focusing optics)	Long-term	Certain
Source distance	Expensive (new station)	Medium-term	Certain
Exposure time	Inexpensive but reduces experimental throughput	Immediate	Certain
Better detection			
New and improved detector technlogy	Expensive	Medium-term	Good
Improve signal-to-noise			
Deuteration	Moderately expensive	Short-term	Good
Diffracting volume			
Grow larger crystals	Relatively inexpensive	Short-term	Good

Simplest solution: Bigger Crystals

The growth condition has a number of variables, e.g. protein concentration, precipitant concentration and temperature. Changing these variables changes the outcome of the experiment.

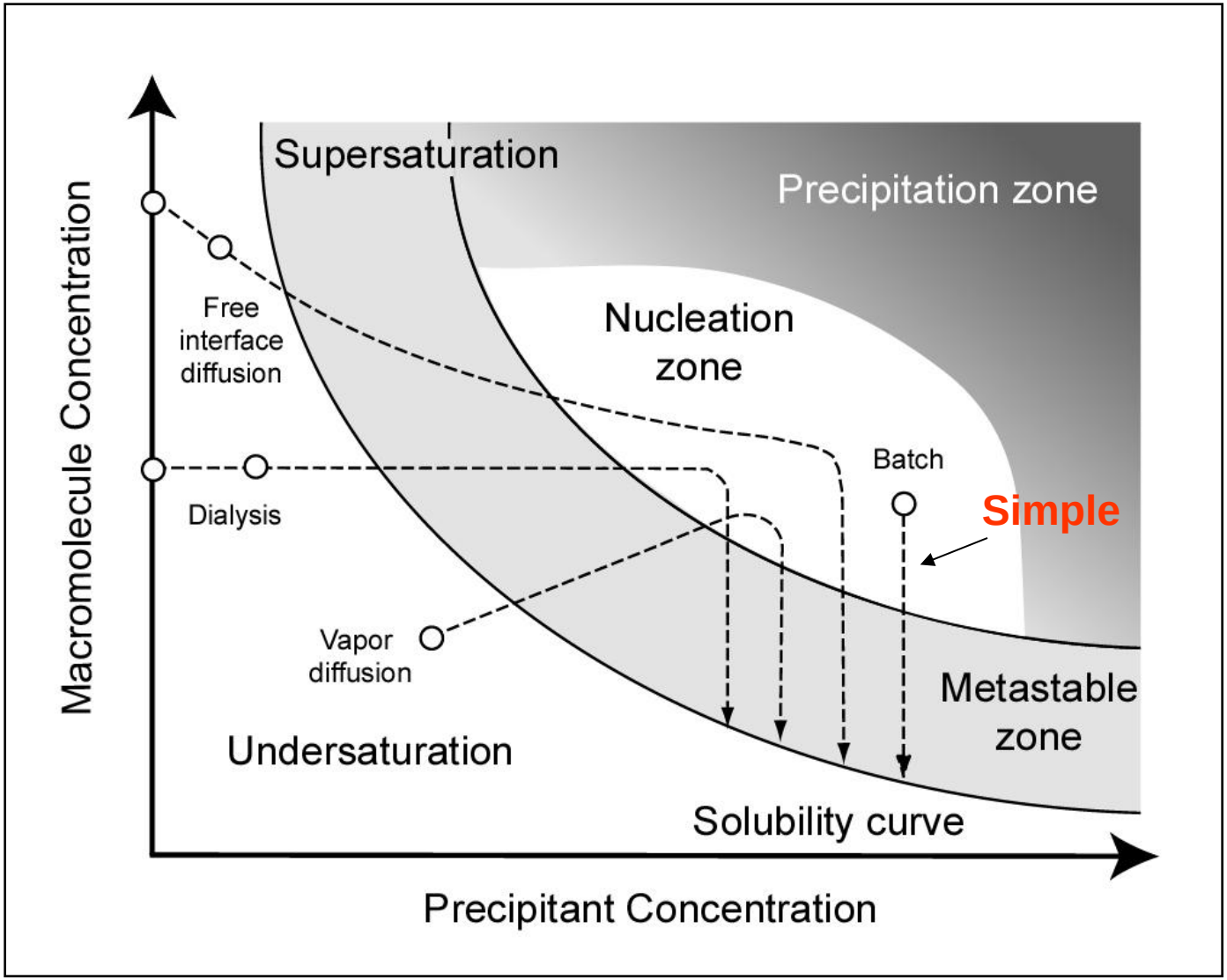
The initial condition is known, crystals already exist.

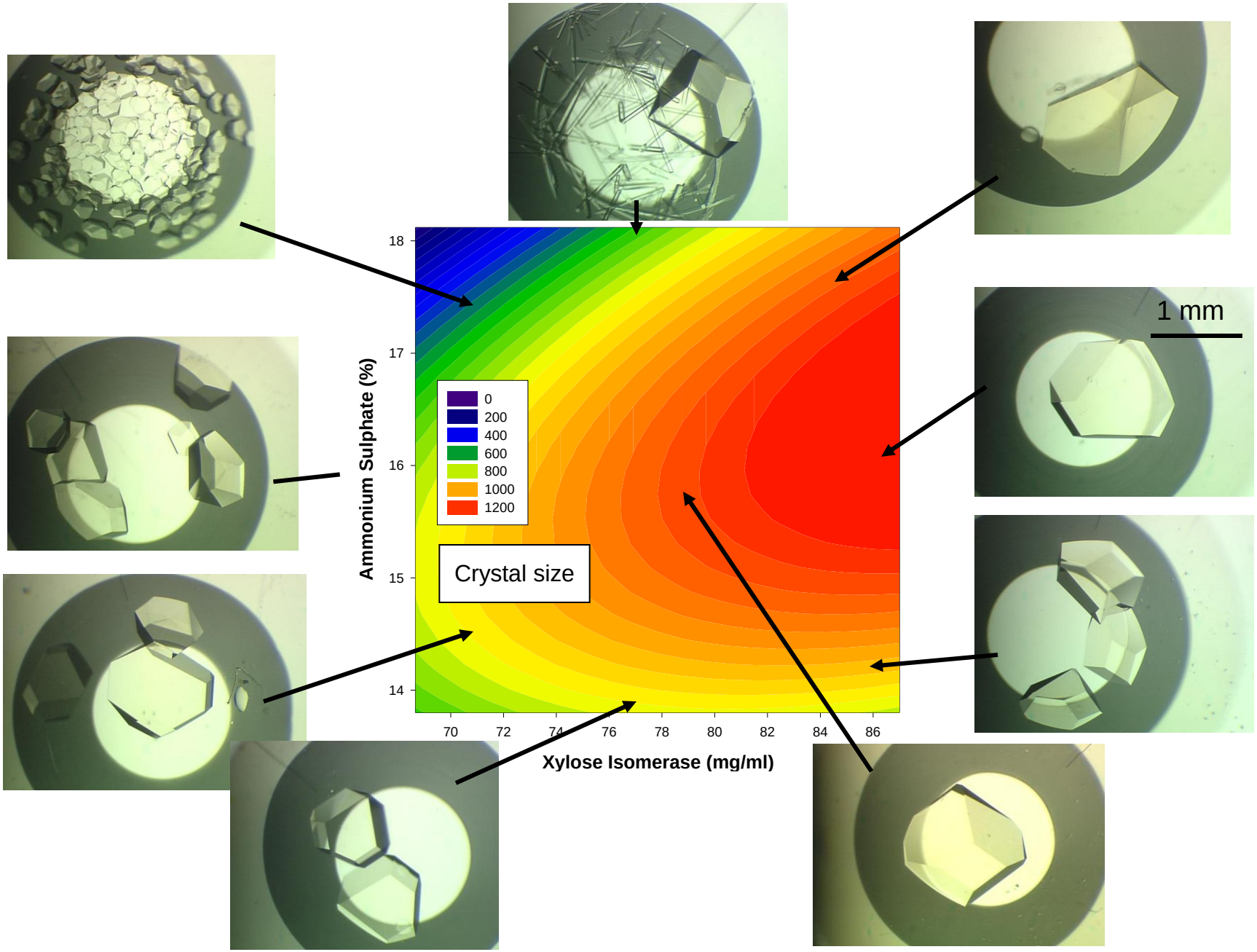
Conditions where no crystals are produced are known.

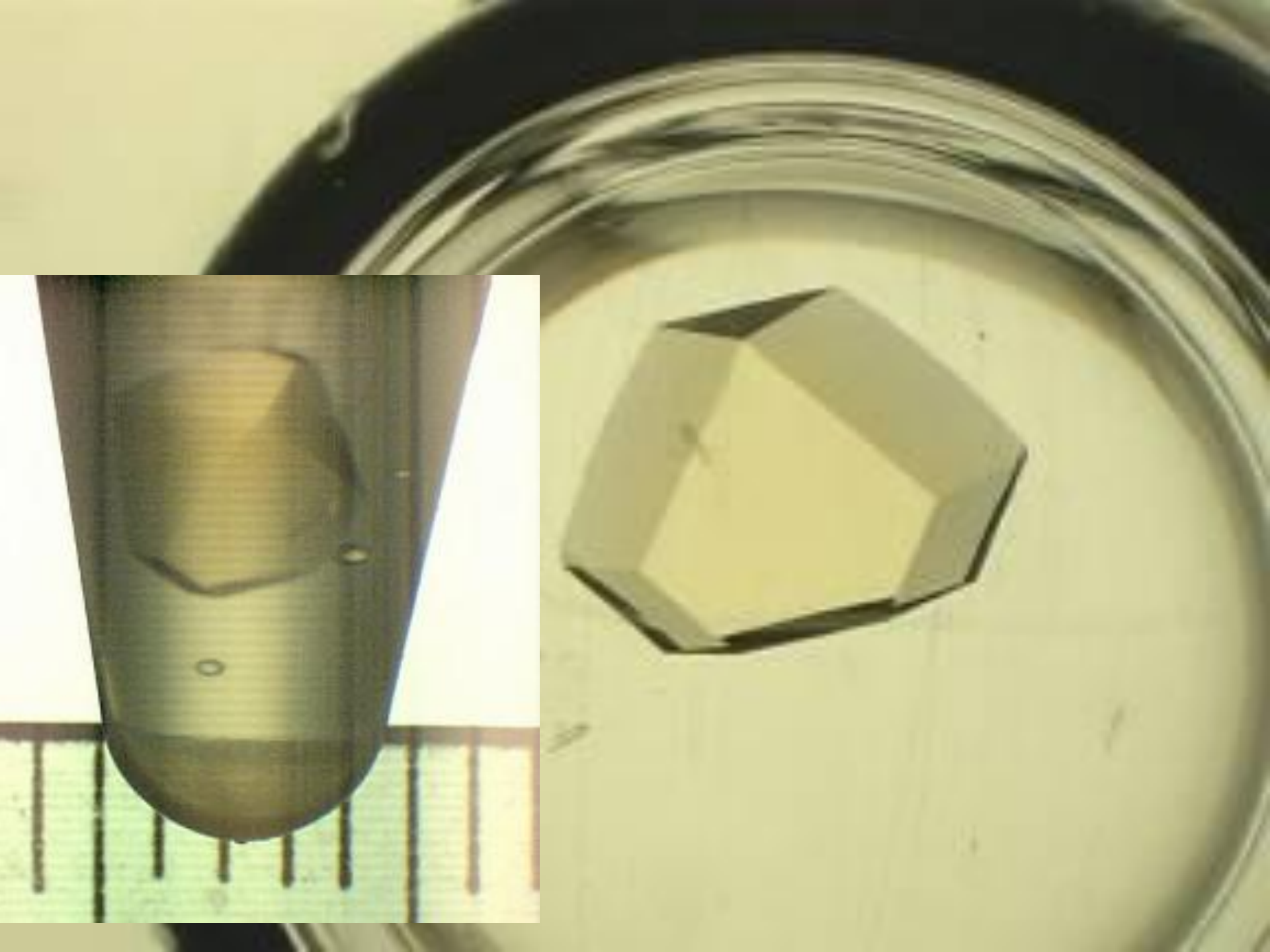
The goal is to grow a “few, large” crystals. This leads to two quantifiable metrics, crystal number and crystal size.

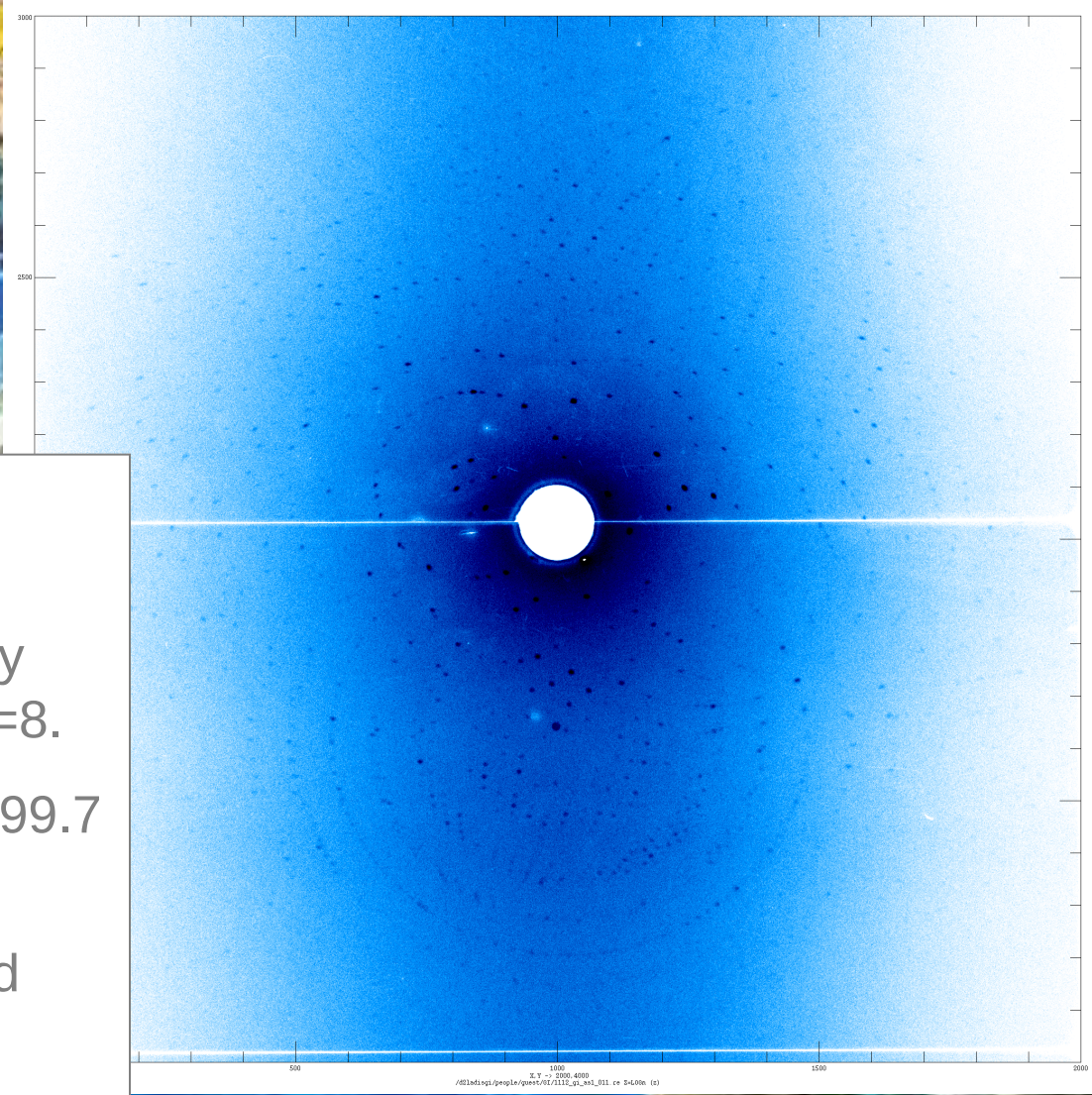
By changing the experimental variables within the known area of crystallization the experiment can be optimized to produce a few large crystals – the trick is doing this efficiently.

Method of Growth




Ammonium Sulphate (%)
18
17
16
15
14
0
200
400
600
800
1000
1200
Crystal size
70
72
74
76
78
80
82
84
86
Xylose Isomerase (mg/ml)
1 mm





Xylose Isomerase:

- Largest crystal sample successfully studied to date by neutron diffraction, 43 kDa, Z=8.
- Space group I222, cell 93.9 99.7 102.9, resolution 2.5Å
- Complete data sets collected from deuterated and non-deuterated crystals at the ILL, Grenoble, France using the Laue polychromatic technique.
- In press E. J. Biophysics, Meilleur, Snell et al (2006).

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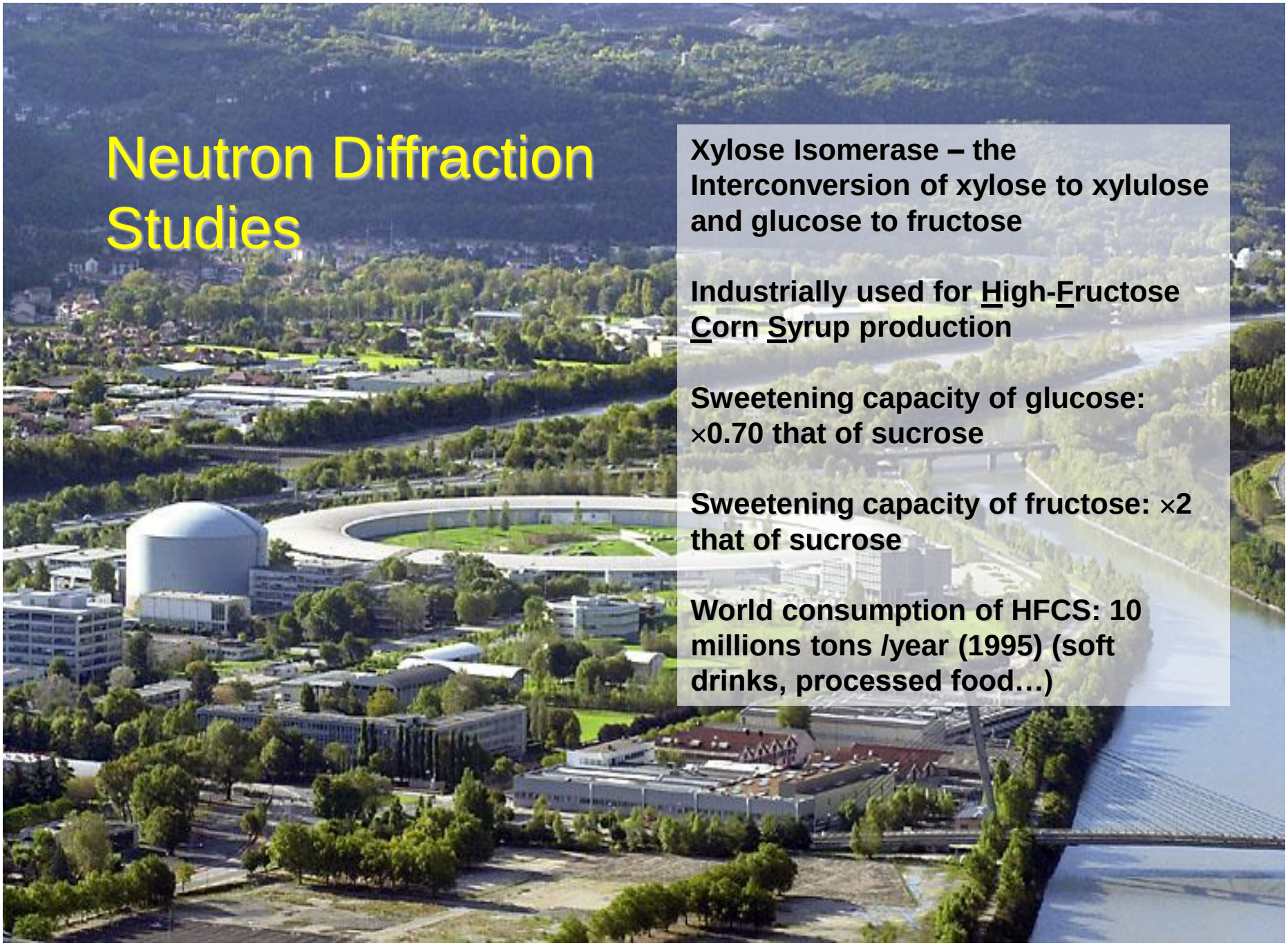
Experimental details

- Grown using the batch method
- 20% (w/w) ammonium sulphate, 50 mg/ml glucose isomerase, pH 7.7, 20°C
- 2.5 mm in longest dimension
- Genetically modified
- Small crystals used for X-ray data collection at SSRL
- Large crystals used for neutron data collection at ILL

Summary



Neutron Diffraction Studies

Xylose Isomerase – the Interconversion of xylose to xylulose and glucose to fructose

Industrially used for High-Fructose Corn Syrup production

Sweetening capacity of glucose: ×0.70 that of sucrose

Sweetening capacity of fructose: ×2 that of sucrose

World consumption of HFCS: 10 millions tons /year (1995) (soft drinks, processed food…)

Case Study: Xylose Isomerase

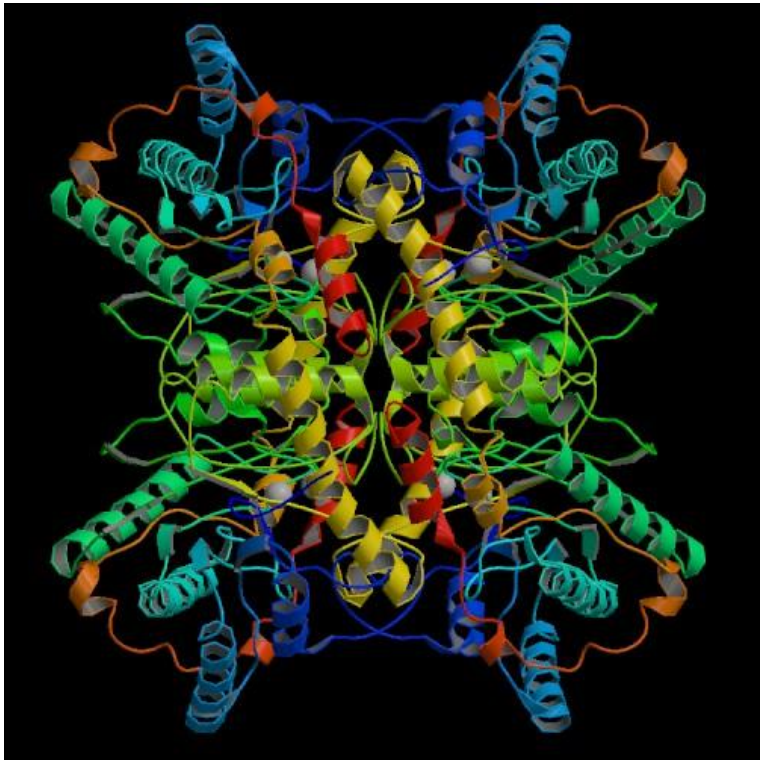
Enzymatic mechanism is a transfer of one H atom from one C atom of the substrate to an adjacent C atom.

Three mechanisms have been proposed – a base-catalyzed proton transfer, a simple hydride shift or a hydride shift mediated by a metal ion.

X-ray data, to date, has not revealed the exact mechanism.

Xylose isomerase is an important industrial catalyst for the production of fructose.

It has a molecular weight of ~ 160 KDa and can crystallize in the I222 space group – useful for Laue studies as every second reflection is systematically absent.

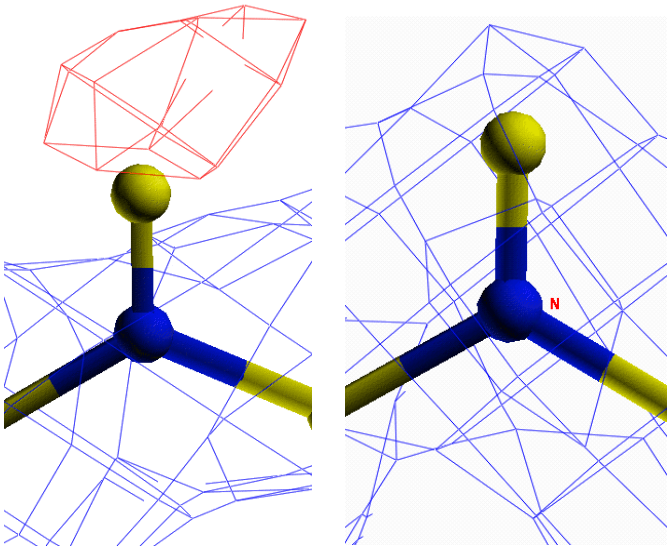


Neutron data – collected on the ILL LADI Line

- Making use of response surface methods large crystals were grown (>10 mm^3) with
  - No duteration
  - Exchange with D_2O
  - From solutions made with D_2O rather than H_2O.
- Laue date was collected from each type of crystal on the LADI diffractometer in a collaboration with Dean Myles and Flora Meilleur at the Institute Laue Langevin, Grenoble France.
  - A Ti/Ni multilayer filter selected a bandwidth of $\delta\lambda/\lambda$=20% centered on 3.5 Å
  - 16 Laue images were collected at 1 setting with a spindle rotation of 8° with a further 8 at an orthogonal setting to complete the blind region.
- X-ray data collection on cryocooled crystals was performed at Stanford Synchrotron Radiation Laboratory beamline 9-1 and 11-1.
  - Crystals diffract to 0.87 Å at an I/σ(I) of 2 but the cryoprotectant is present in the active site.

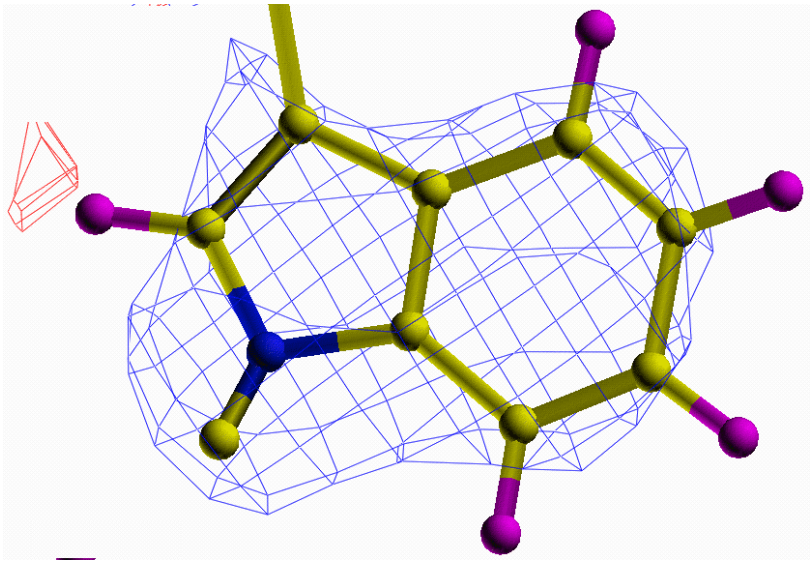
Preliminary neutron density results

All neutron density: Blue is 2fo-fc>0 Red is 2fo-fc<0



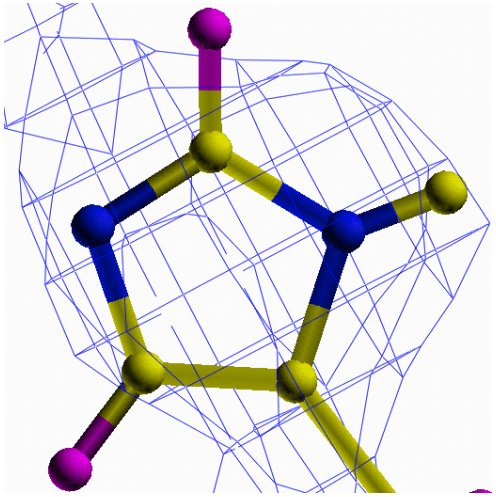
13 37

Deuterium exchange on the Ca backbone, 13 no exchange, 37 exchange likely

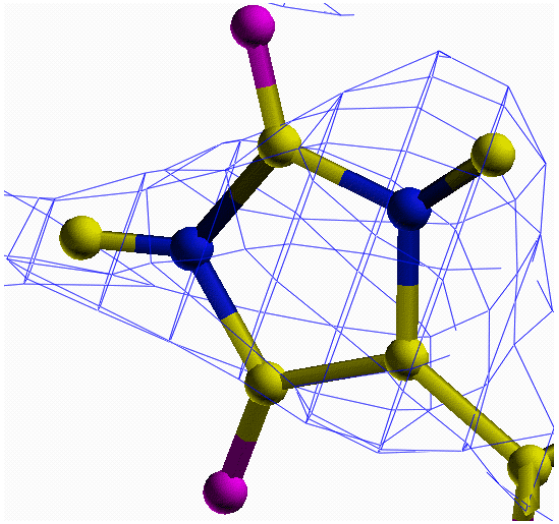


Trp 137 shows positive density for the nitrogen bound deuterium, negative for the carbon bound hydrogen

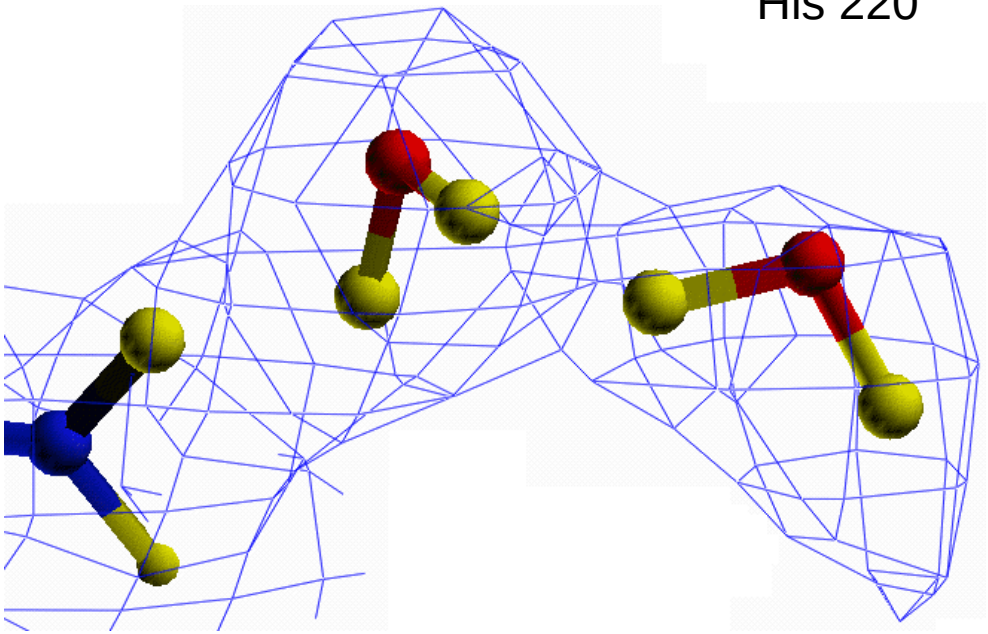
Histidines at the active site with different protonation states



His 220



His 54



Water molecules seen as D_2O

Refinement still under way.

Flora Meilleur, ILL.

Case Study: Xylose Isomerase

Enzymatic mechanism is a transfer of one H atom from one C atom of the substrate to an adjacent C atom.

Three mechanisms have been proposed – a base-catalyzed proton transfer, a simple hydride shift or a hydride shift mediated by a metal ion.

X-ray data, to date, has not revealed the exact mechanism.

Xylose isomerase is an important industrial catalyst for the production of fructose.

It has a molecular weight of ~ 160 KDa and can crystallize in the I222 space group – useful for Laue studies as every second reflection is systematically absent.