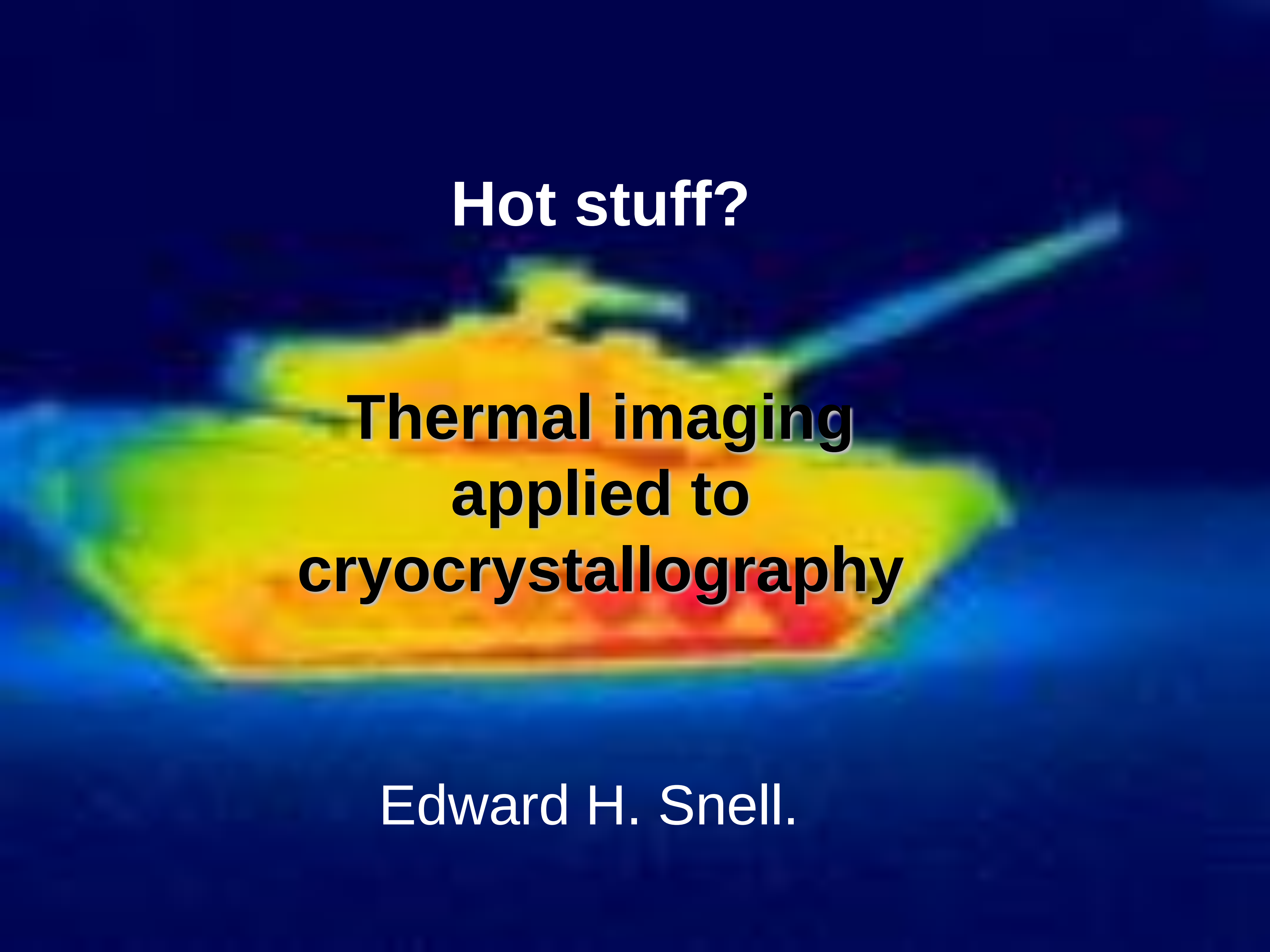


Hot stuff?

Thermal imaging applied to cryocrystallography

Edward H. Snell.

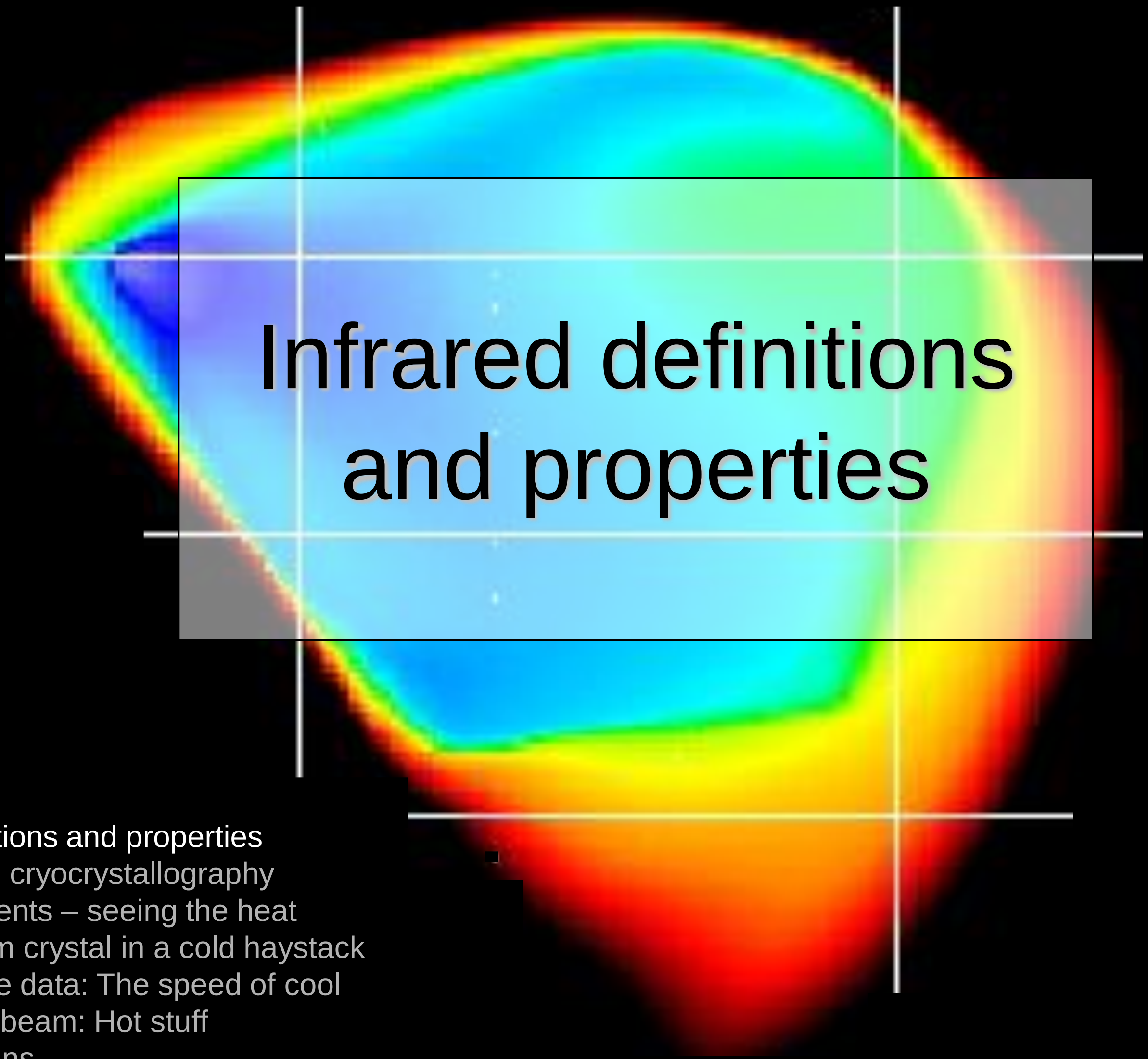
Acknowledgements

Ashley Deacon, Russell Judge, Henry Bellamy, Gerd Rosenbaum, Mike Kazmierczak, Michael Damon, the staff at Stanford Synchrotron Radiation Laboratory, the staff at the Advanced Photon Source SERCAT beamline, and funding support from NASA.

Outline of the talk

- Infrared definitions and properties
- Applications in cryocrystallography
- Initial experiments – seeing the heat
- Finding a warm crystal in a cold haystack
- Quantifying the data: The speed of cool
- Heat from the beam: Hot stuff
- Future directions
- Summary

Infrared definitions and properties



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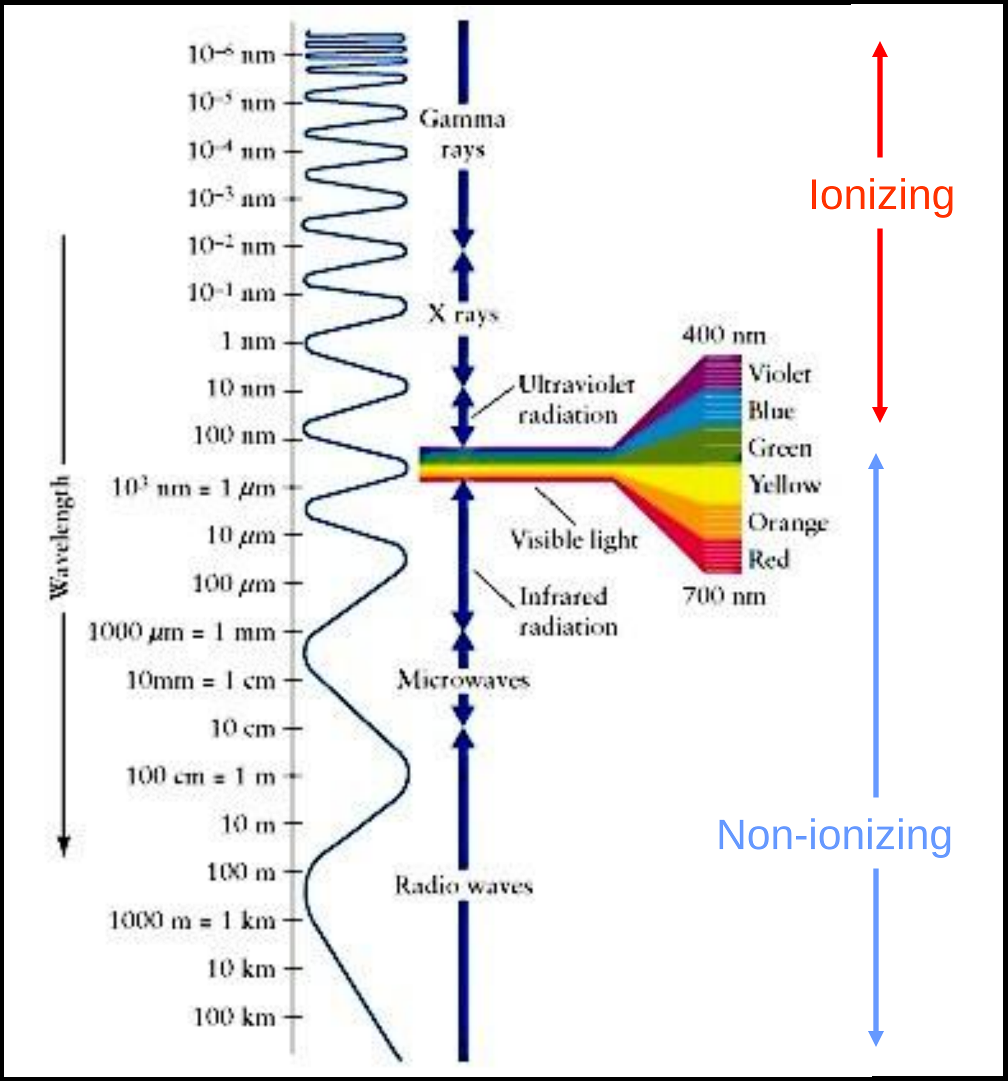
The Electromagnetic Spectrum

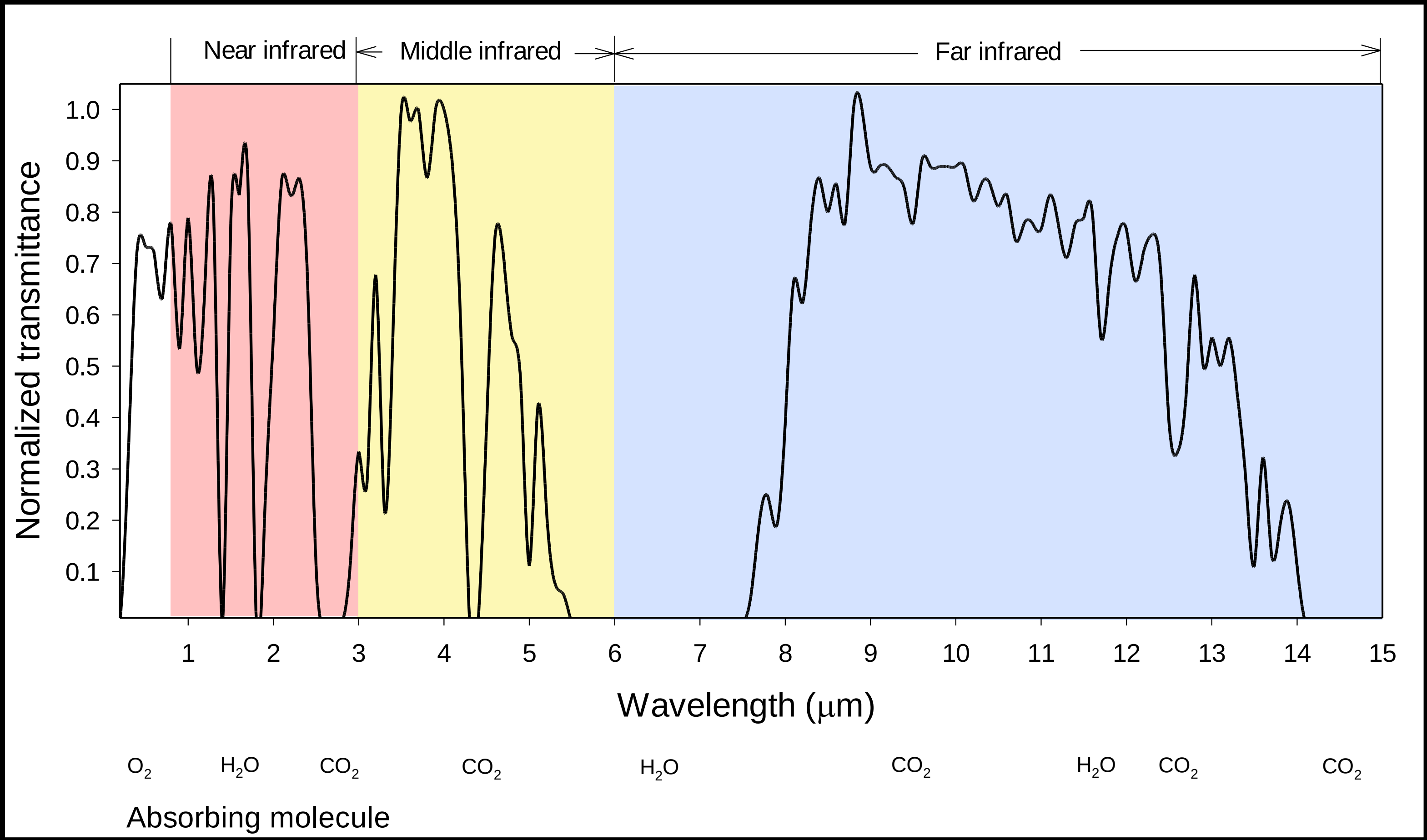
The electromagnetic spectrum can be divided into ionizing and non-ionizing radiation.

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

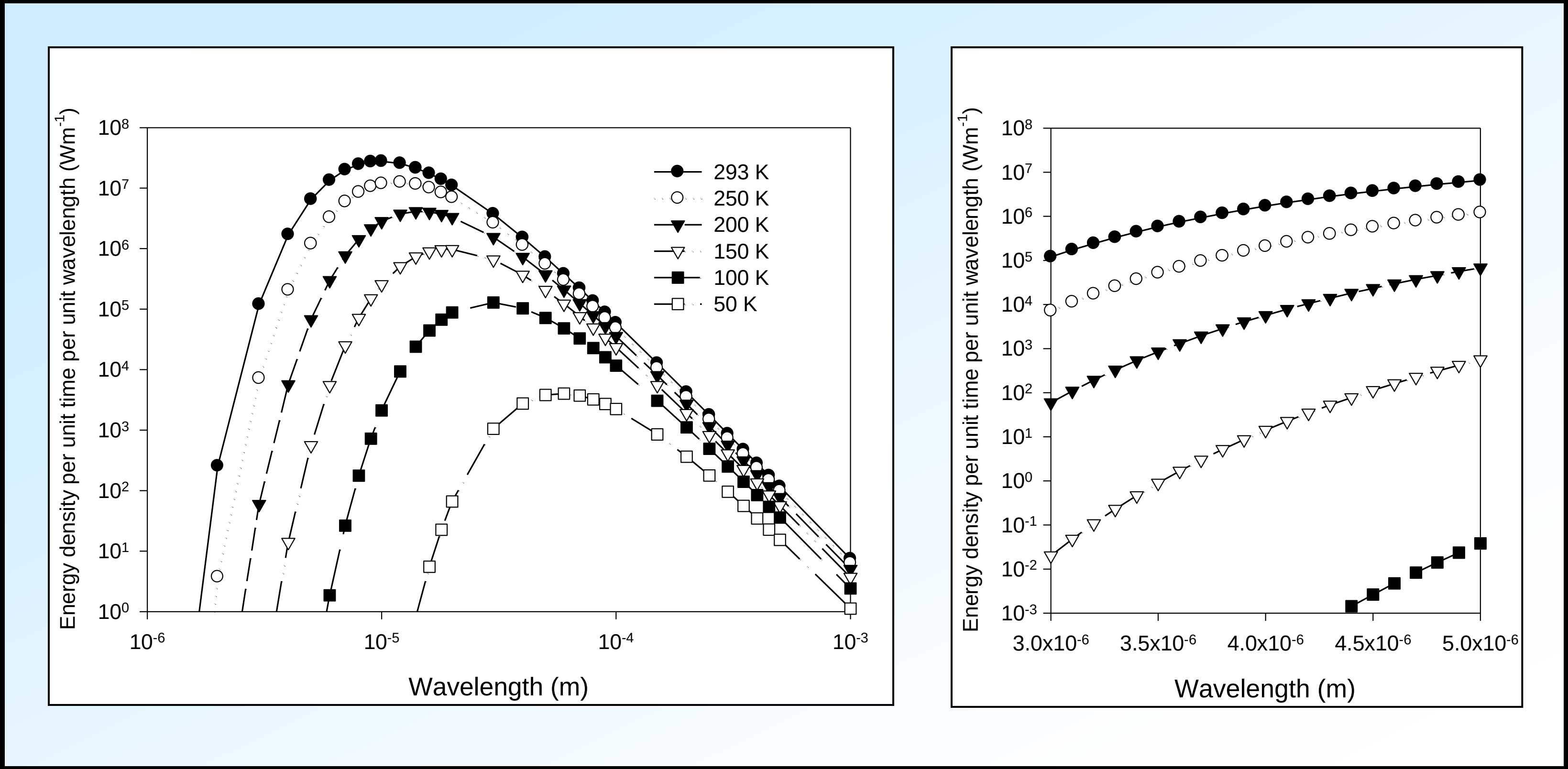
- All objects above 0K emit infrared energy as a function of their temperature.
- A black body perfectly emits and absorbs this thermal radiation.
- The energy spectrum for a black body is exactly given by Planck's radiation law;

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

- 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

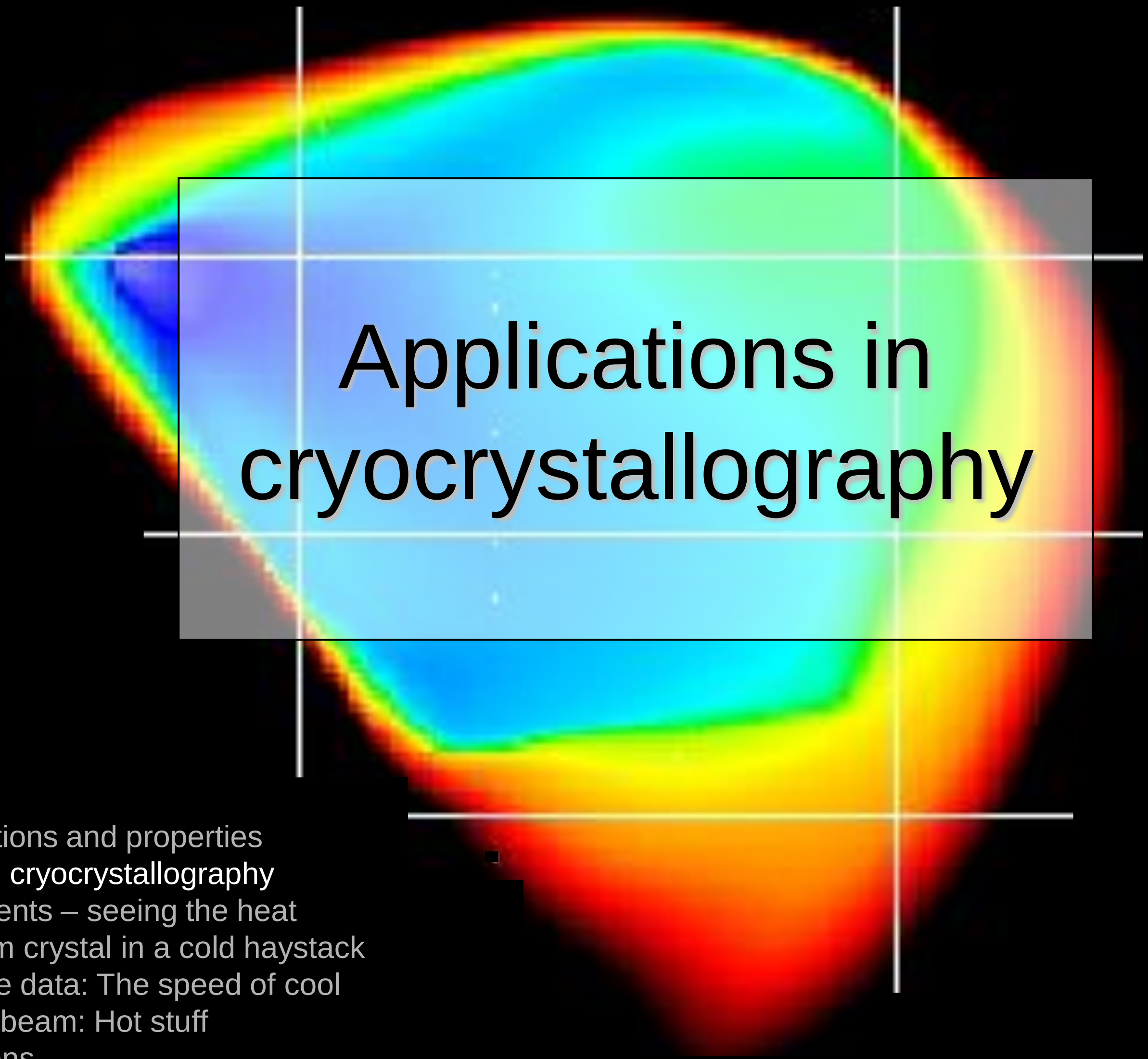


Imaging in the mid-range was chosen. This has a greater energy density and accuracy over the near-range and and greater response to temperature change over the far-range

Radiation from a real object

- Real objects 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.
- Real objects tend to be illuminated by a number of infrared sources:
  - The ambient heat in the room
  - The experimenter
  - The illumination
  - The coldstream
- Real objects transmit and reflect heat
  - Heat is seen behind the object
  - Heat is seen reflected off the object
- Infrared properties of the object vary with wavelength and viewing angle
- It is not trivial to do quantitative studies

Applications in cryocrystallography



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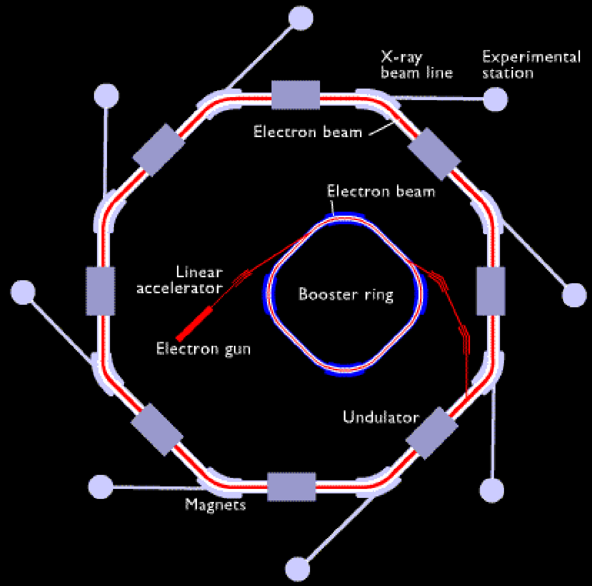
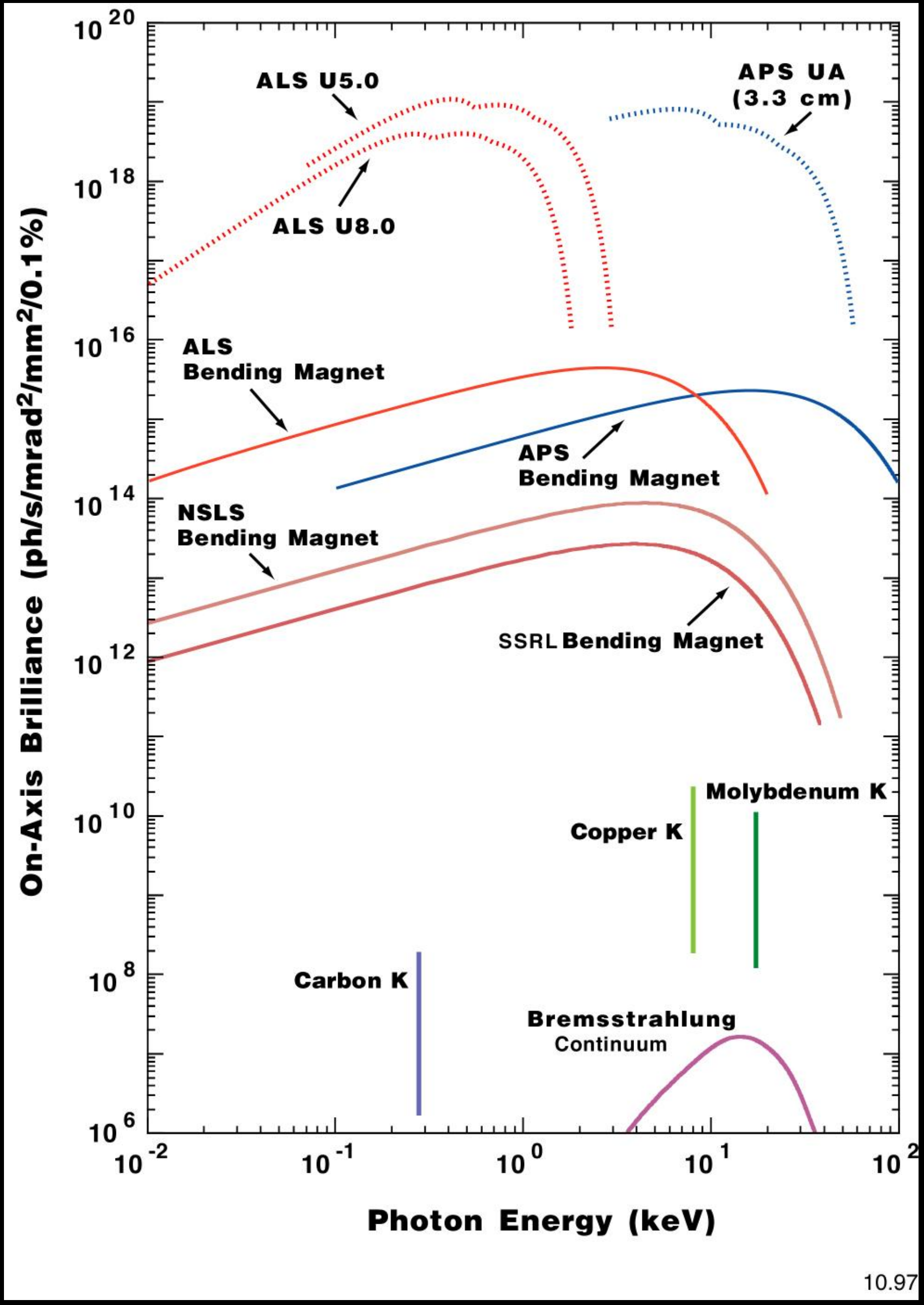
Why cryocooling of samples?

Synchrotrons are intense sources of electromagnetic radiation



Synchrotrons provide an intense source of X-rays producing significant radiation damage problems in macromolecular crystal samples.



Cryocrystallography is a key technique in structural crystallography.

1. It reduces X-ray radiation damage in crystal samples, particularly important at third generation synchrotron sources.

2. It reduces the thermal motion of atoms increasing the signal-to-noise and generally resulting in improved diffraction from the crystal.

3. Crystal handling and transport is relatively simple in a vitrified state. Important for high-throughput and individual crystal studies.

There are a number of empirical factors involved in successfully cryocooling a crystal.

Stating the obvious: Studying and understanding the empirical factors enable the optimization of the technique. One consistent factor independent of the technique used, the cryoprotectant, the sample etc. is that the crystal is cooled – a net temperature change occurs.

Thermal cameras are sensitive to temperature change and provide a non-invasive technique to study cryocooling.

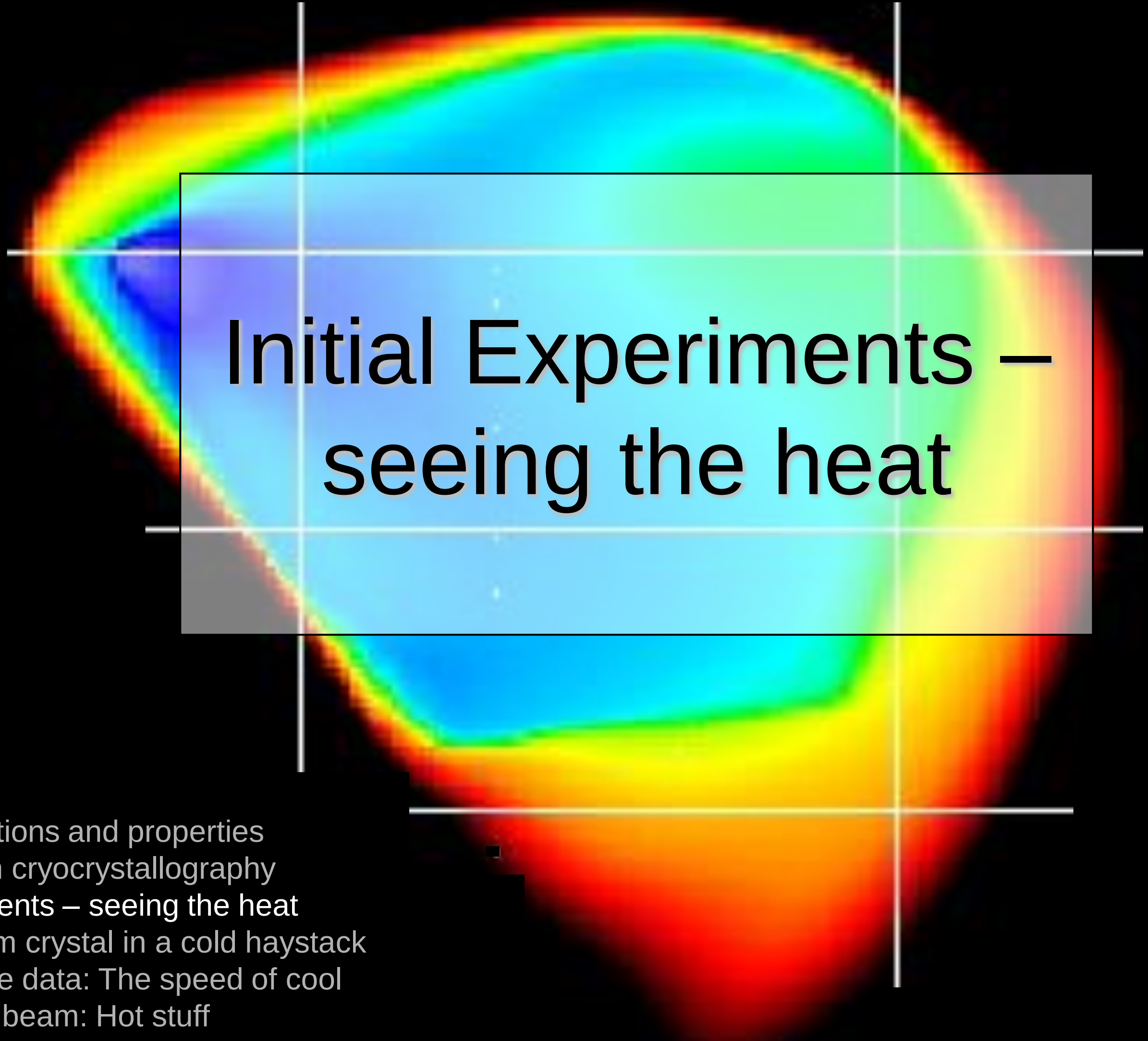
What processes are involved in cryocooling?

- Successful cryocooling rapidly cools a macromolecular crystal to a temperature of 100 K or lower without the formation of ice and maintaining the crystal in a good diffracting condition.

- Several steps are necessary for success;

  - Find cryoprotectant conditions that prevent ice formation
  - Manipulate the crystal
  - Rapidly cool the crystal
  - Locate the crystal
  - Check the diffraction properties
  - Keep the crystal cooled while collecting data.

- Thermal imaging can be used to study the cooling, locate the crystal and to ensure the crystal is maintained in a cool state.
- Thermal imaging can also be used to teat and validate models of the cryocooling process and beam heating effects.



Initial Experiments – seeing the heat

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Lights, Camera ….

Indium Antimonide detector.

3.0-5.0 μm bandpass.

320 x 240 30mm^2 pixels.

60 Hz frames.

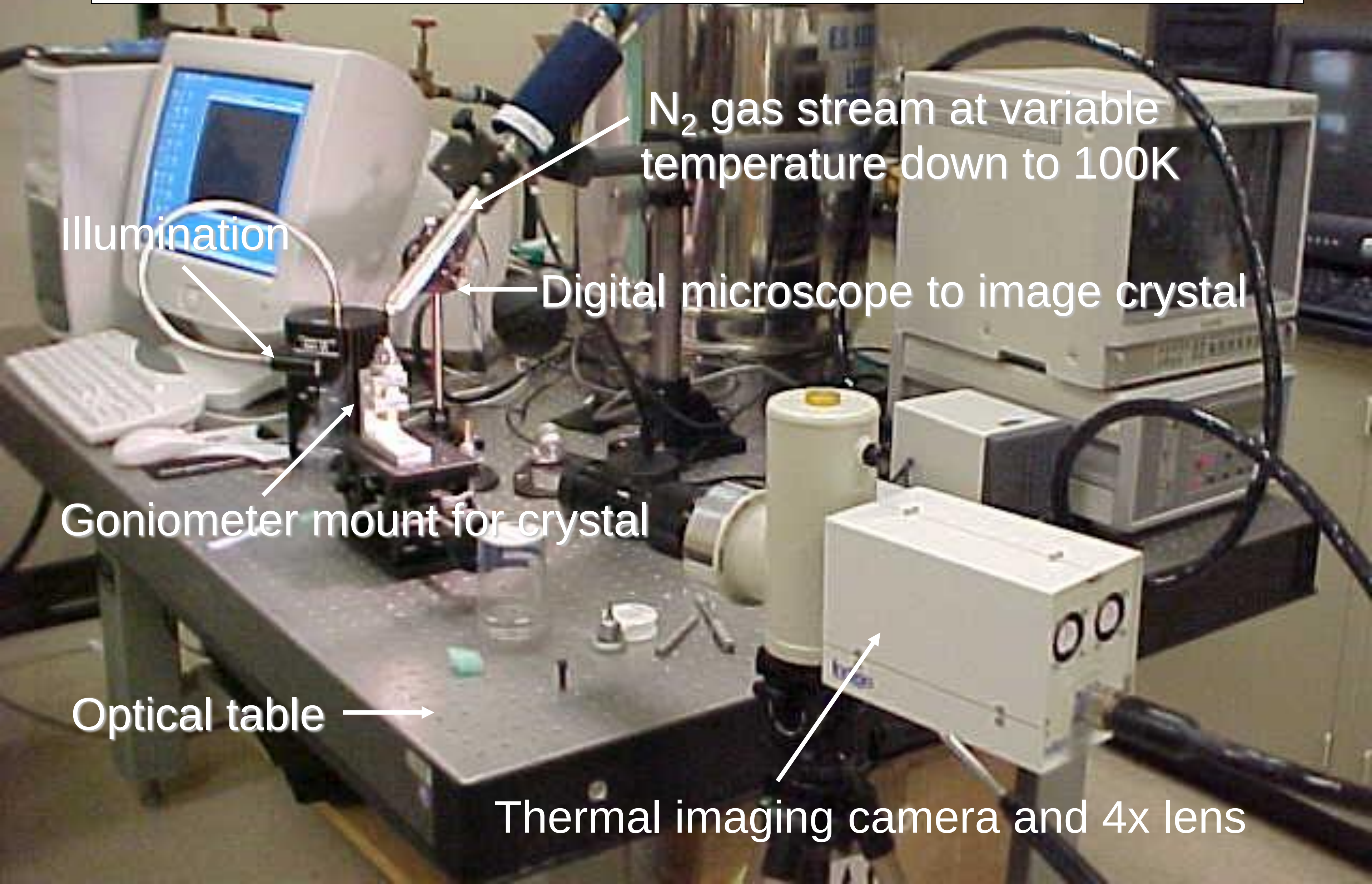
Integration time 10 μs to 17 ms.

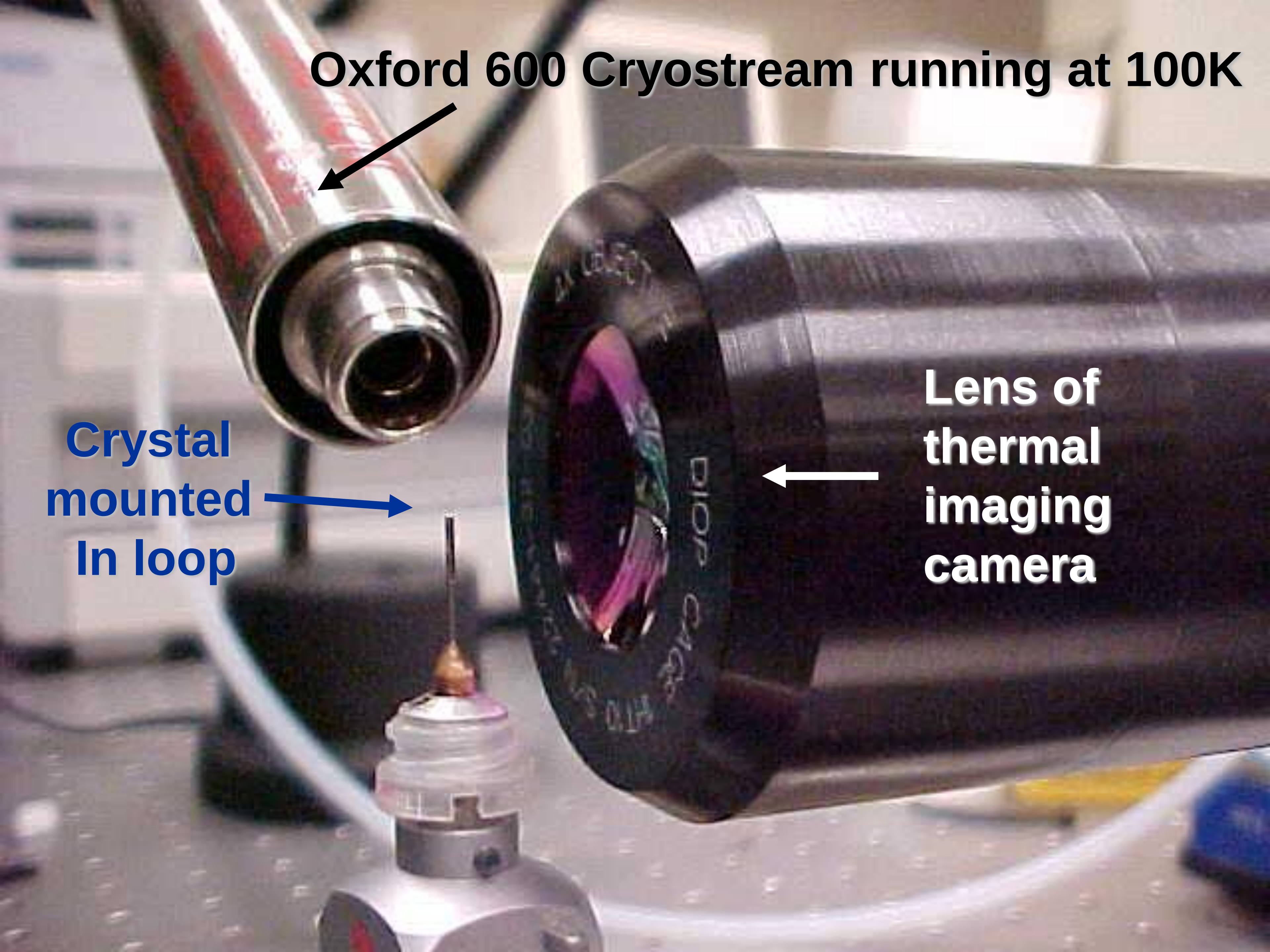
12 bit digital video output.

Cooling to 77K by integral Stirling system.

Size, 13 x 12 x 23 cm.

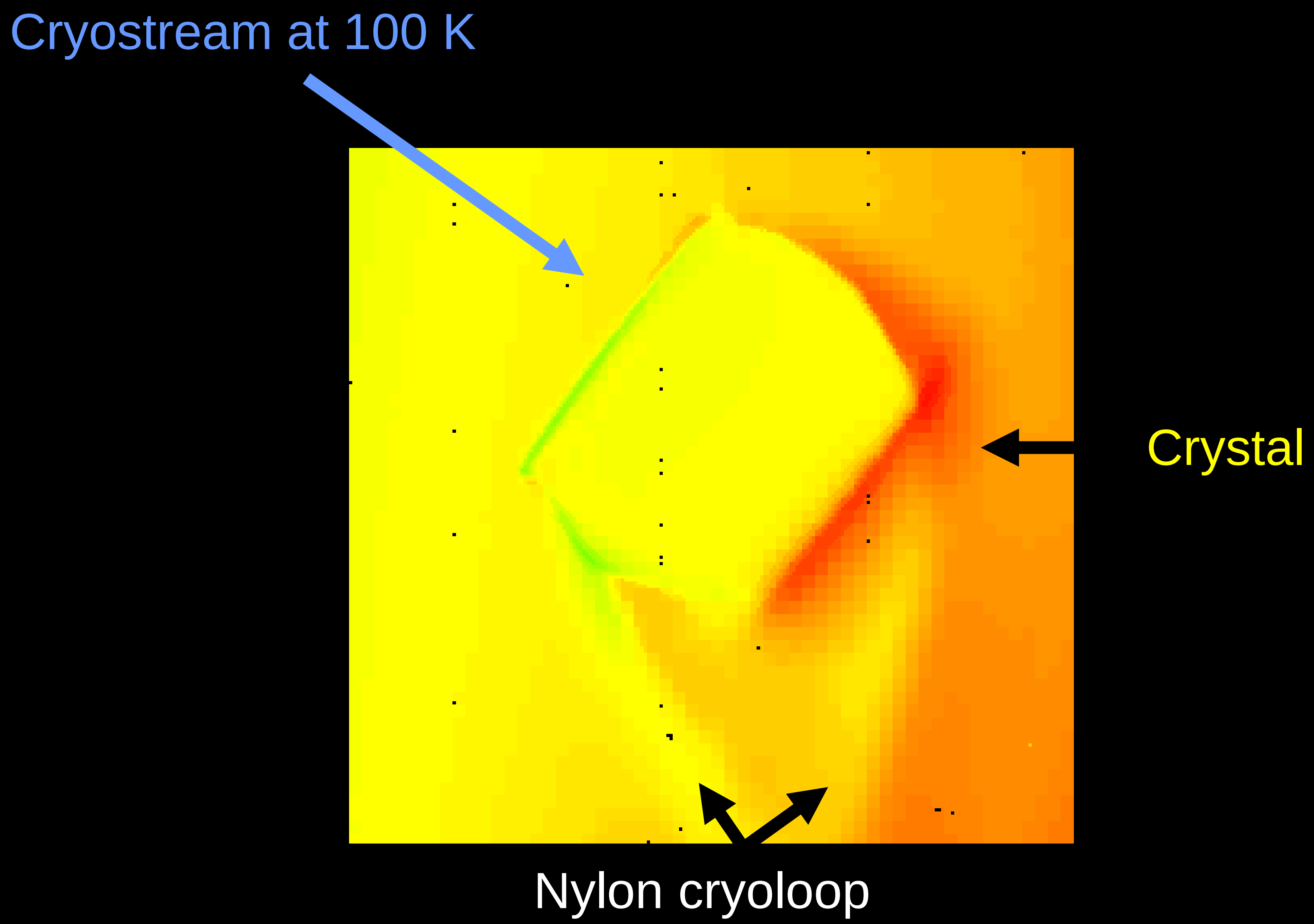
System setup in the Laboratory

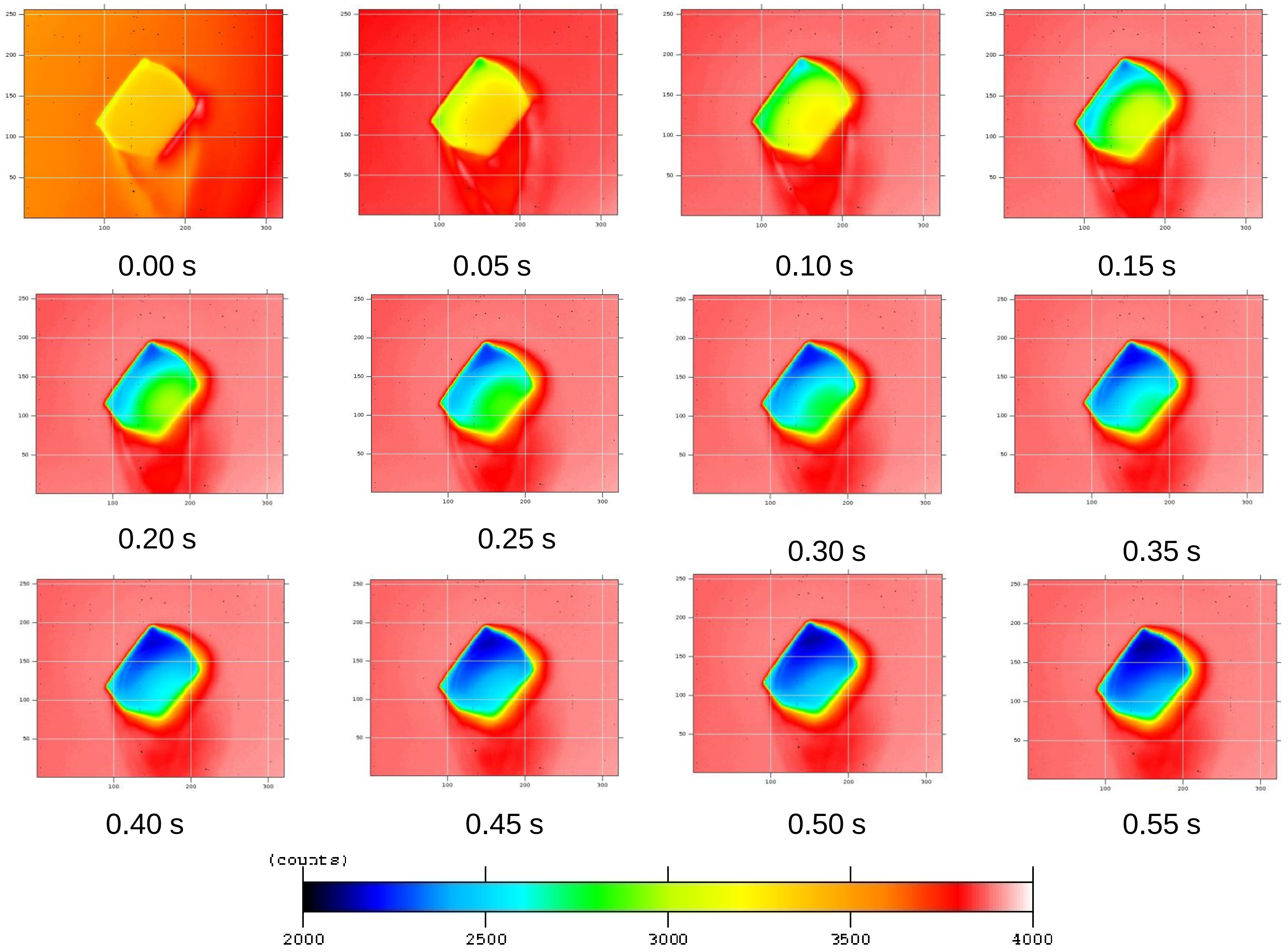


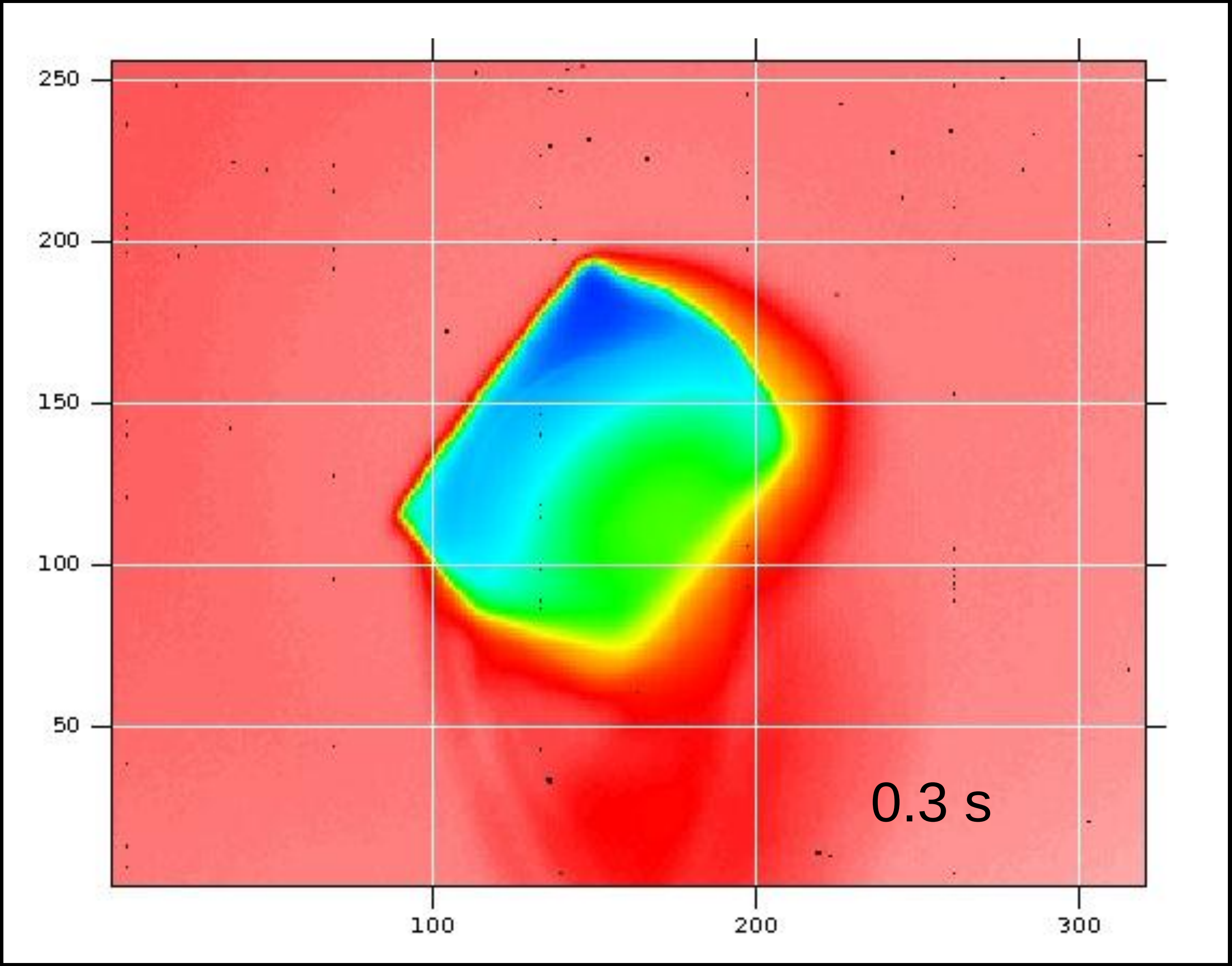

Oxford 600 Cryostream running at 100K
Crystal mounted In loop
Lens of thermal imaging camera

Example of one of the experiments

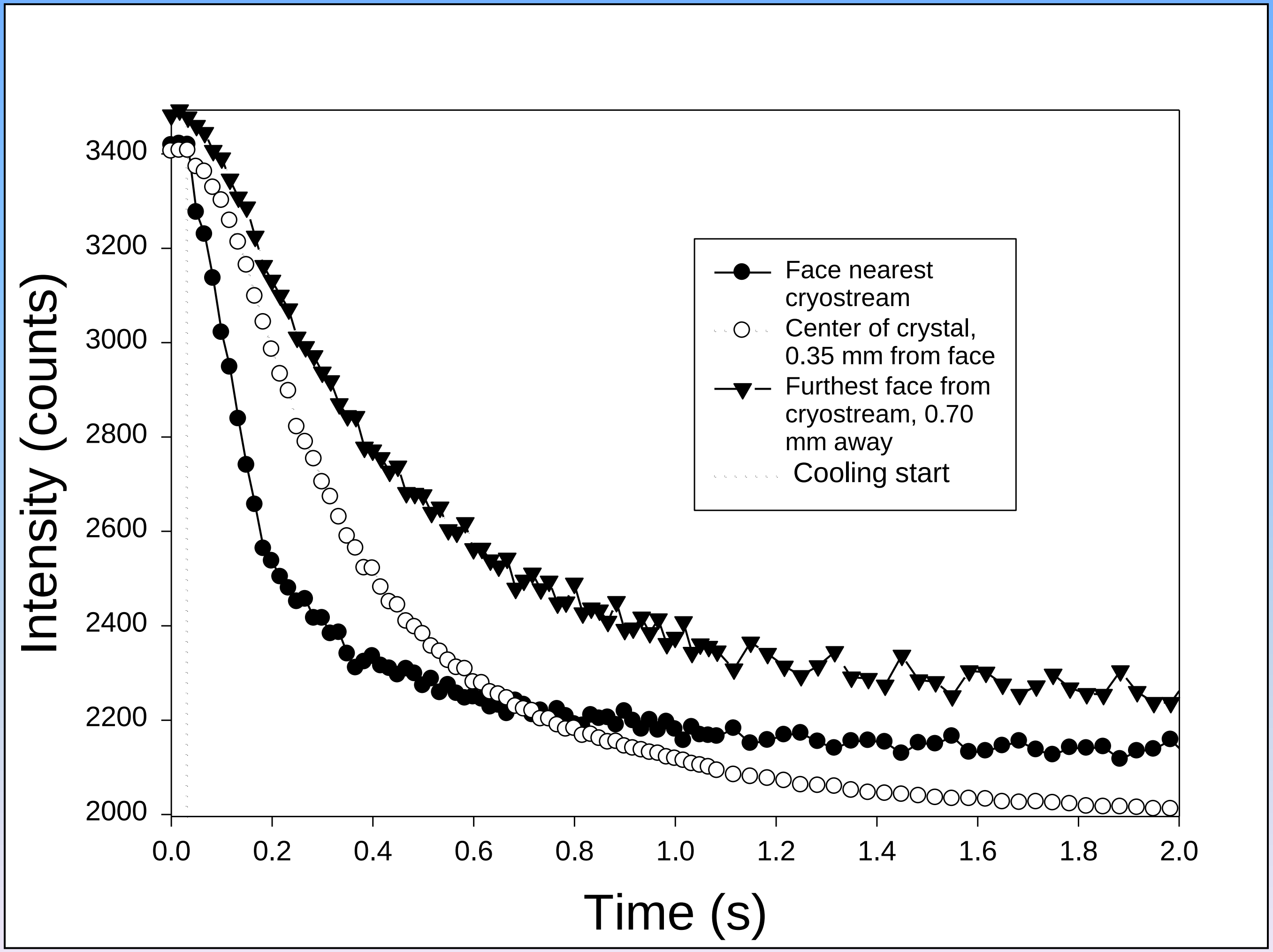
- Lysozyme
  - Lysozyme solution 75 mg/ml in 0.1 M, pH 4.5, Sodium Acetate
  - Reservoir solution 0.9 M Sodium Chloride with 25% Ethylene Glycol and 0.1 M, pH 4.5, Sodium Acetate
  - Drop solution, 6 μl of lysozyme and 4 μl of reservoir
  - Hanging drop crystallization
- Crystal size used for experiment
  - 1.00 x 0.72 x 0.24 mm
    (somewhat larger than typical)

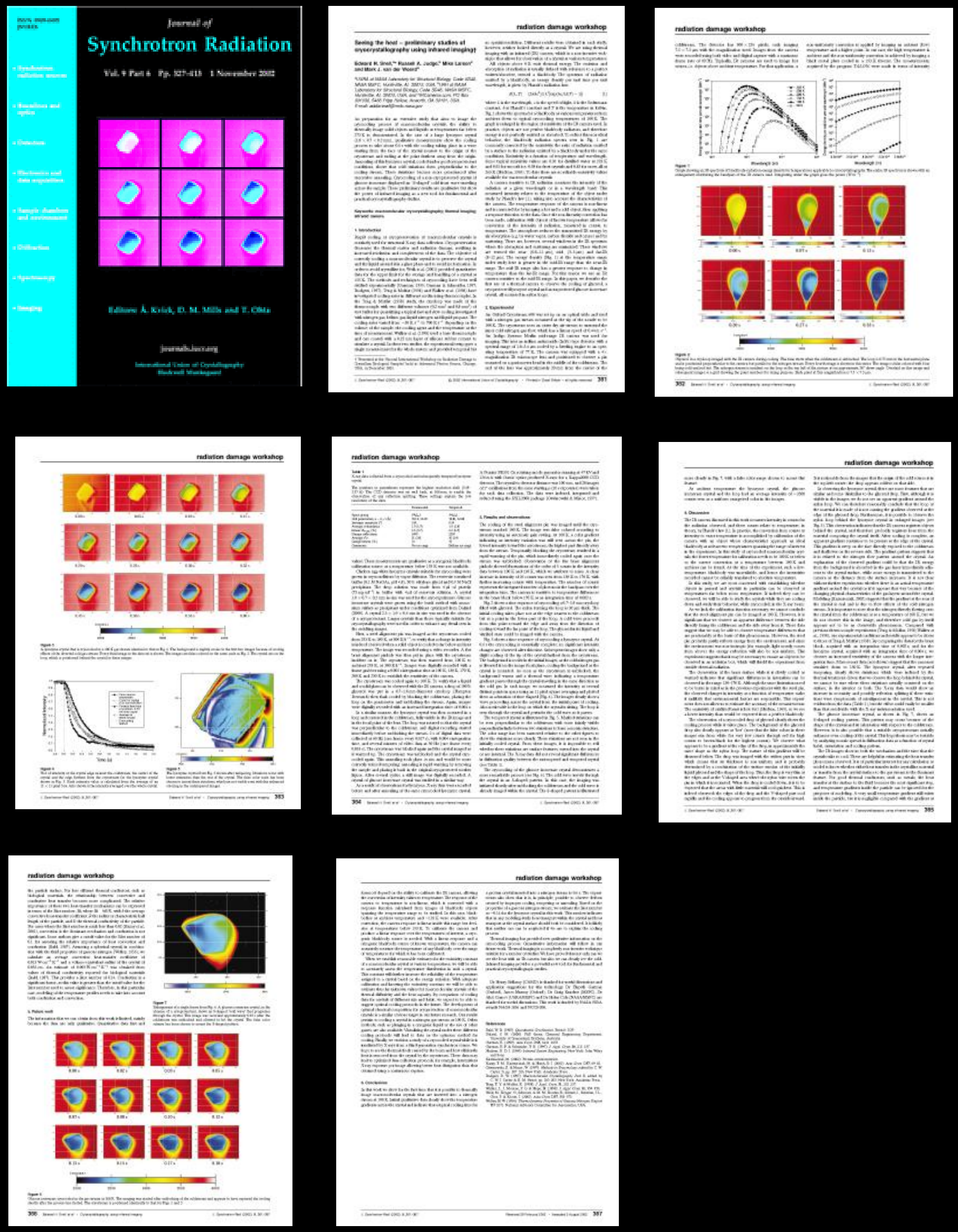

Cryostream at 100 K
Crystal
Nylon cryoloop


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


250
200
150
100
50
100
200
300
0.3 s

Intensity over time





Summary

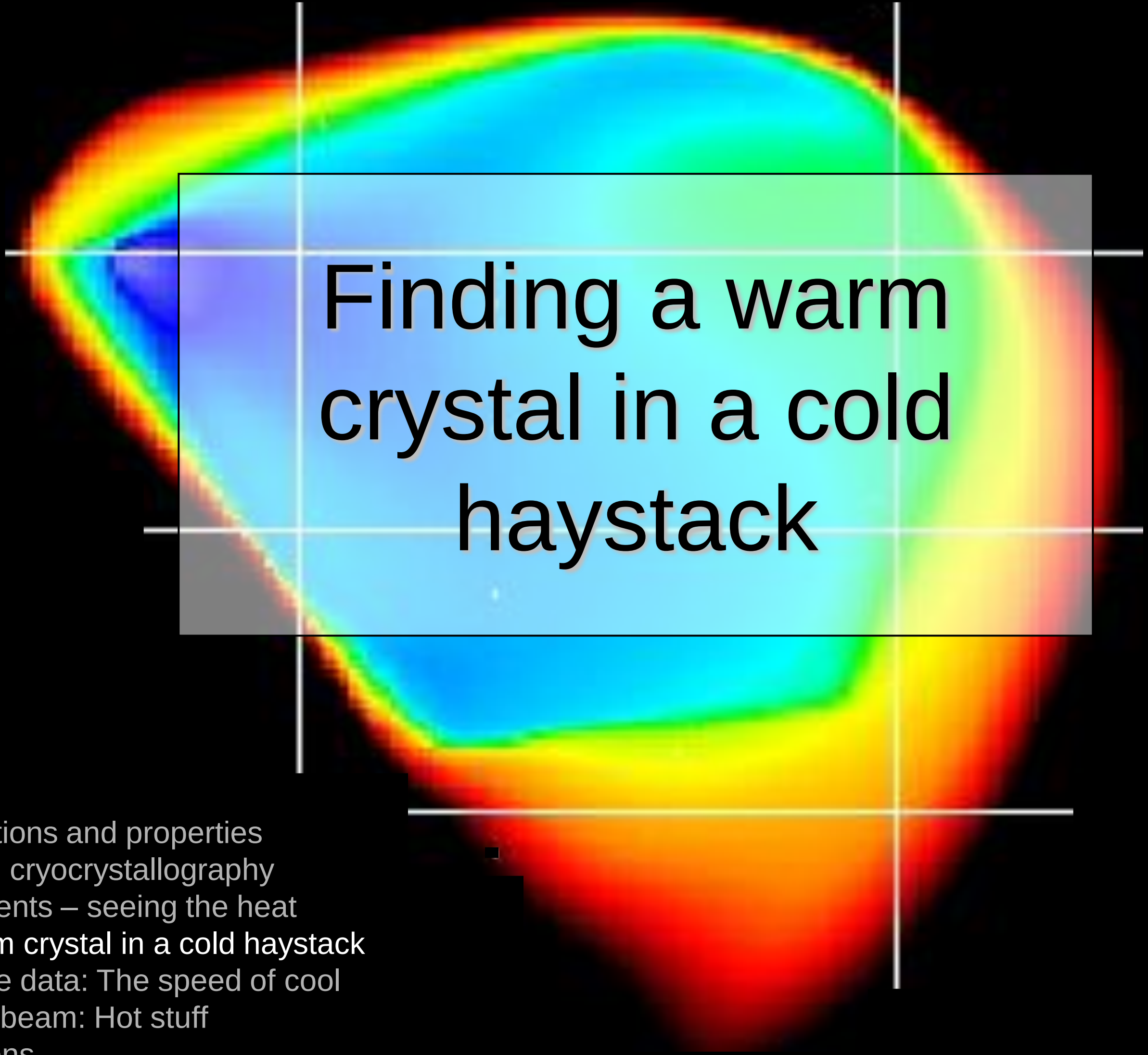
Thermal imaging captured the cryocooling process (Snell *et al*., Seeing the heat – preliminary studies of cryocrystallography using infrared imaging, Journal of Synchrotron Radiation 9, 361-367, 2002).

Crystals cooled in a wave from the point nearest the cryostream to the point furthest away.

The crystals studied were large to deliberately bring out the detail in the images.

Of note was that in each case the crystal was clearly distinguished from the loop.

Finding a warm crystal in a cold haystack



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Why would you want alternative methods to find a crystal in a loop?

- The first step of an X-ray experiment is positioning the crystal such that it’s rotation axis is completely centered in the X-ray beam
- For high-throughput automated systems over 50% of the non-data collection time is taken in centering the crystal (Abola et al., 2000, Nature Struct. Biol. 7, 973-977).
- Automated centering techniques are not always successful.

- Any process that can increase the success and speed of centering pushes high-throughput systems to high-output systems.

Why make use of thermal imaging?

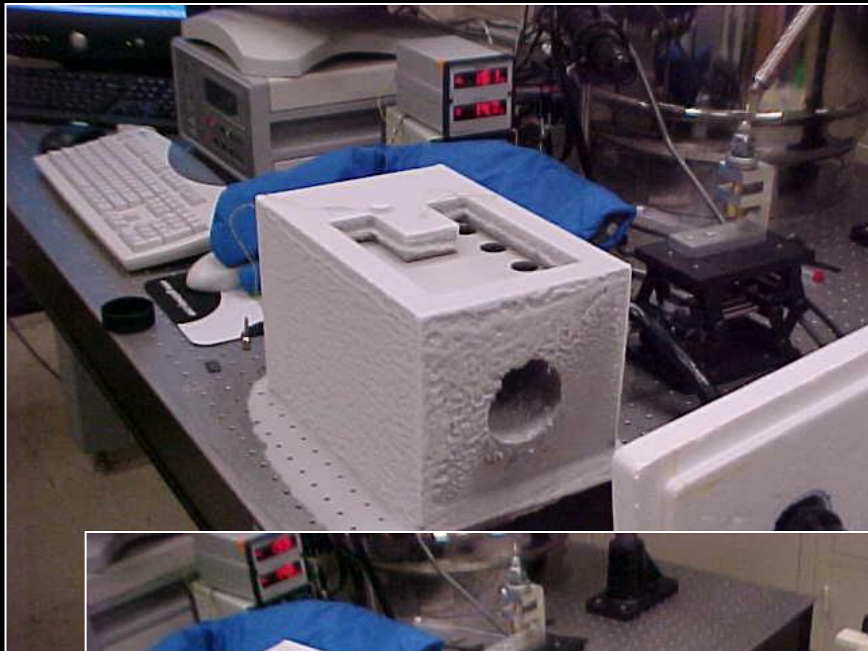
- In our initial experiments one aspect that became completely obvious is that in each case the crystals were clearly distinguished from the loop and vitrified mother liquor.
- Could thermal imaging be used as a standalone system for crystal positioning (or as an aid to visual imaging)?

Preliminaries.

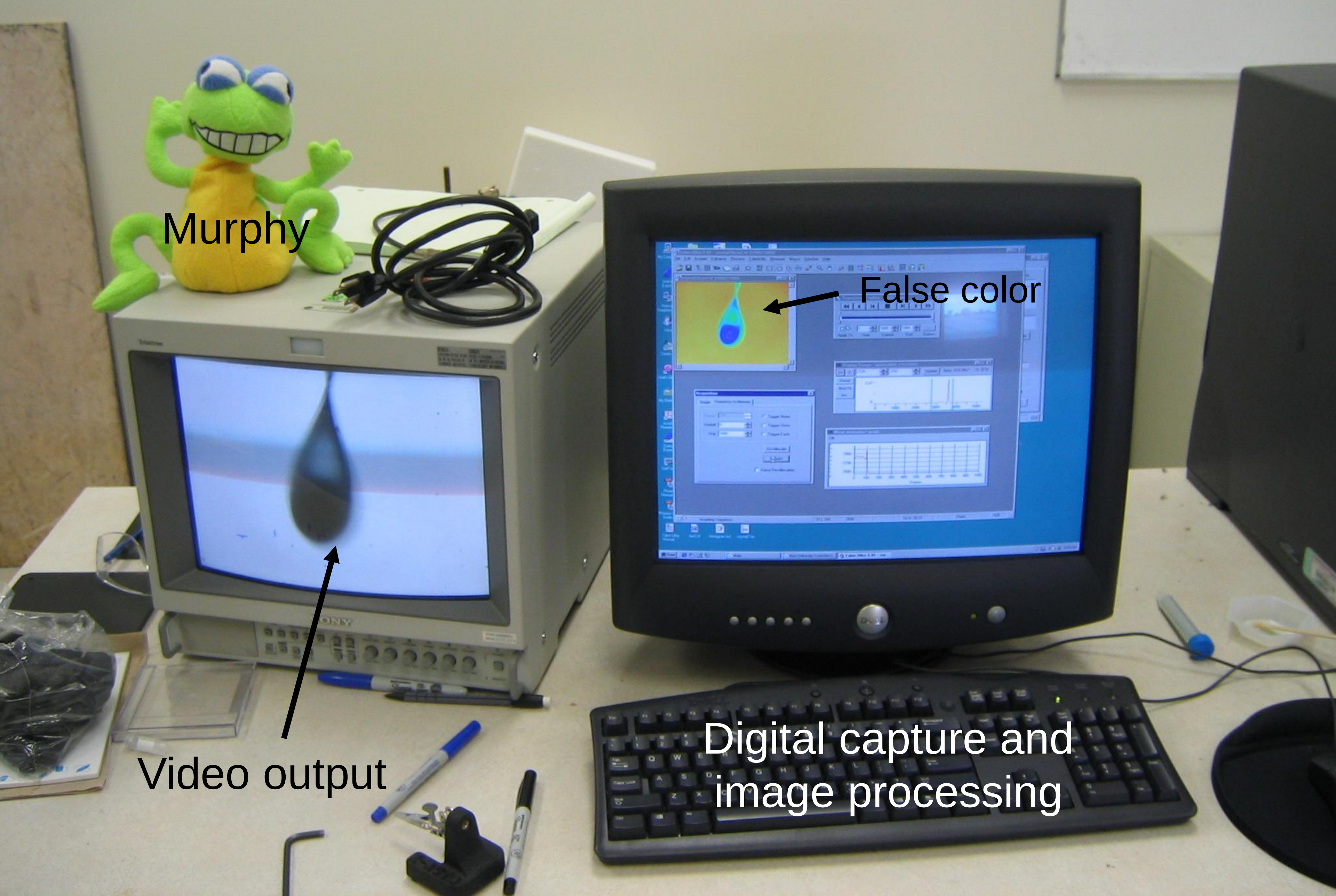
A blackbody source at 213 K is used for non-uniformity correction of the detector.

The infrared camera (cooled to 77 K) and digital microscope are then focused on the crystal position.

Measurements begin



Data collection system

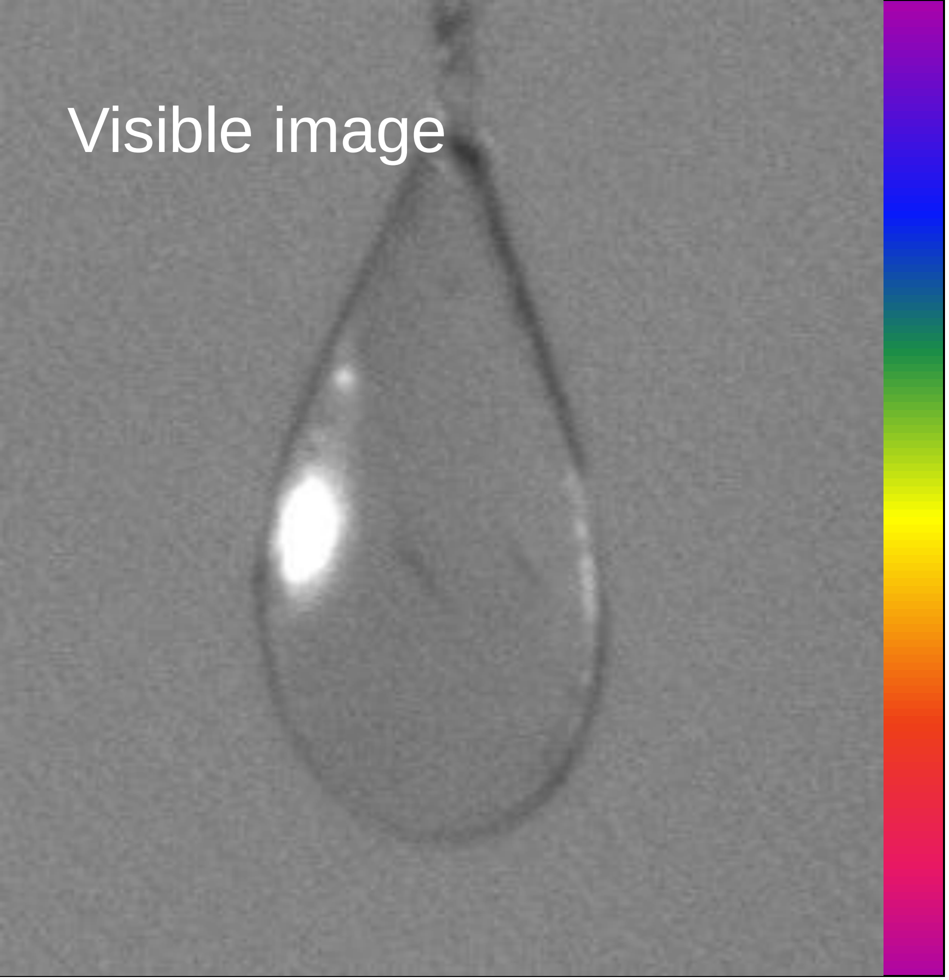


In the following images:

- The crystals are imaged after several minutes of cooling in the 100K nitrogen stream to ensure they have reached equilibrium.
- The loop used is 0.5 mm wide, <u>all images are inverted</u>.
- For <u>visible light</u> the best image of the crystal (illumination position and background are variables) is shown. No back illumination was available.
- For the infrared images the contrast and limits have been optimized to show the crystal. The dynamic range recorded is not visible without doing this.
- The infrared images are false colored (gray scale) according to heat. In this case white is hot, black cold.

Lysozyme crystal

($0.14 \times 0.11 \times 0.06\ mm^3$)

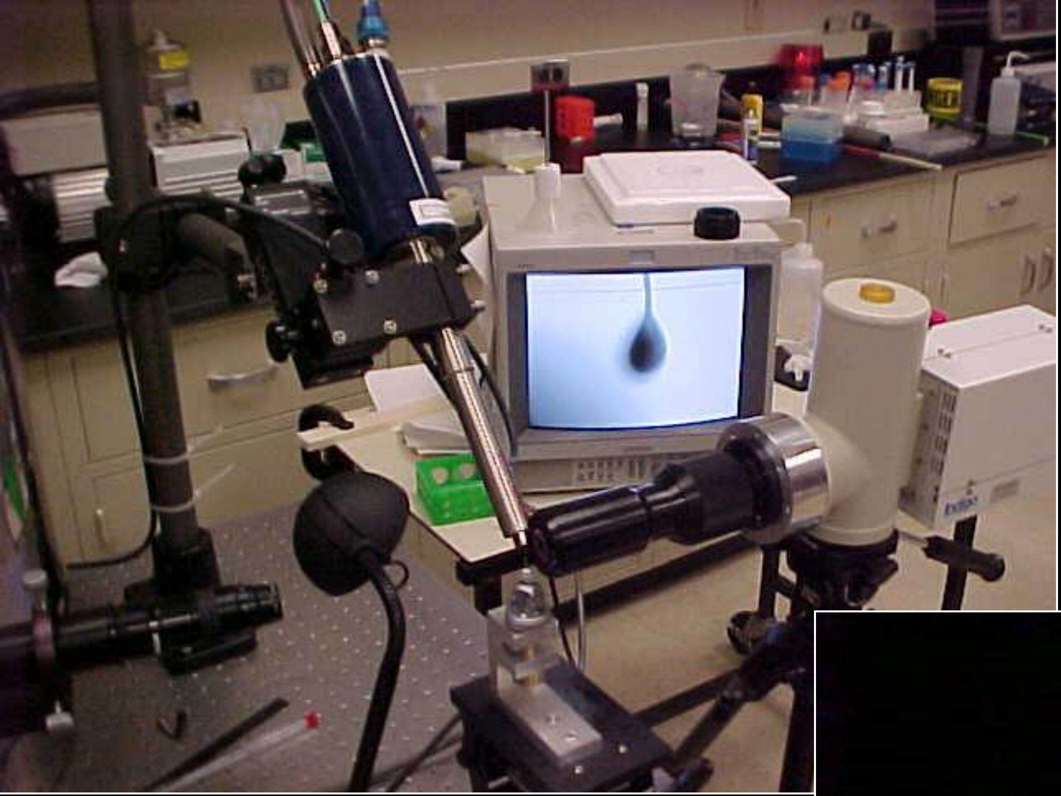


Cell parameters: $P4_32_12$ 78.5, 78.5, 37.8 Å

Solvent content: 40%

Cryoprotectant: Ethylene Glycol

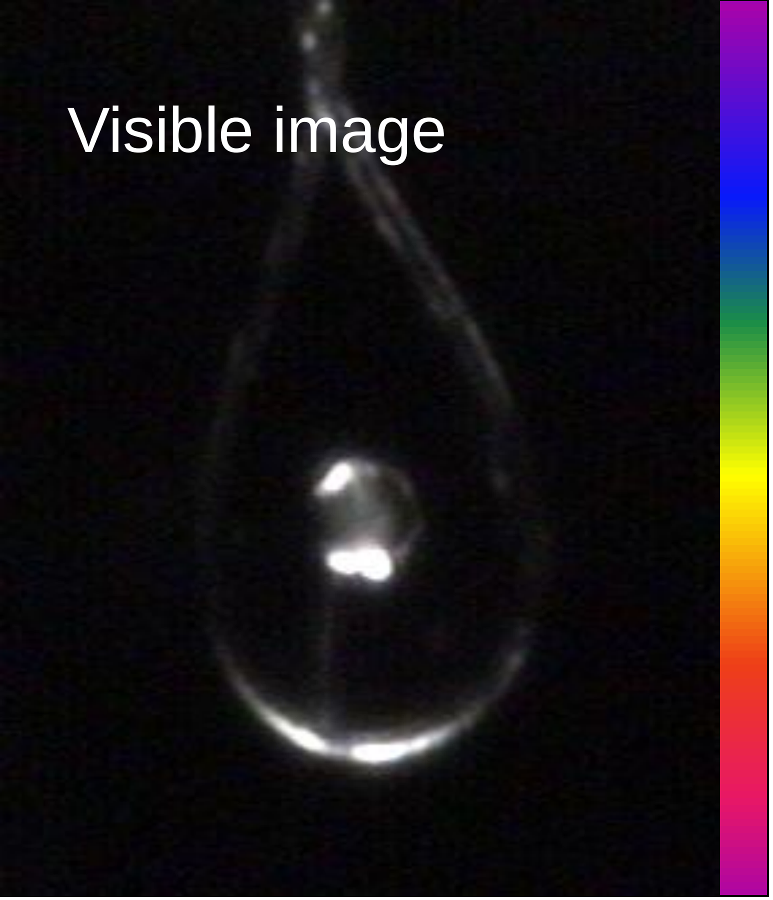
Infrared image (lamp illumination at 45°)



Lights on….

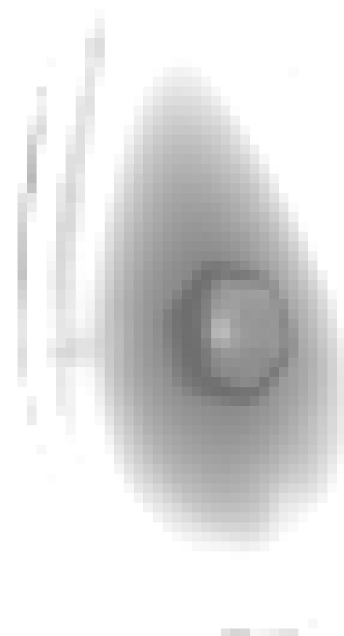
(note the lamp for visual illumination, off in this case)

Lights off….



Basic Fibroblastic Growth Factor/DNA complex crystal

($0.11 \times 0.17 \times 0.05$ mm^3)



Infrared image (lamp illumination at 45°)

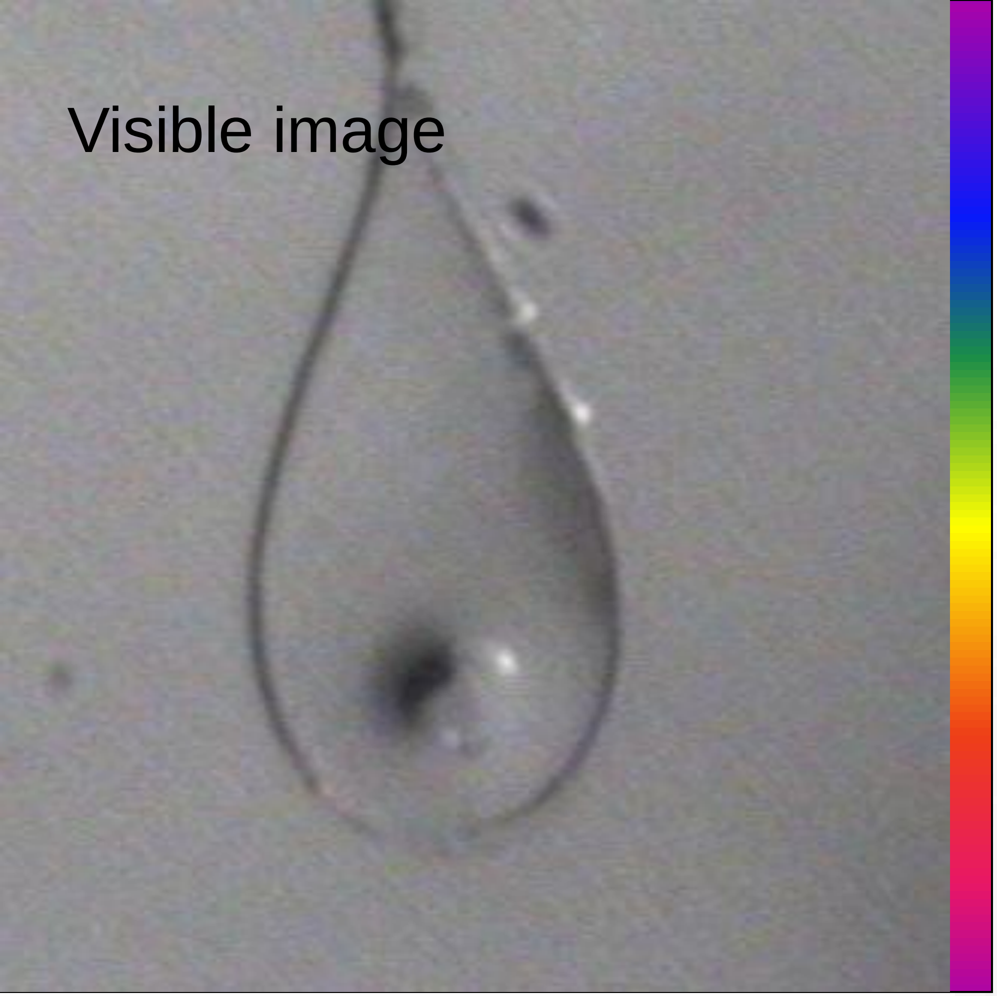
Cell parameters: P622, 112.8, 112.8, 450.2 Å

Solvent content: Unknown

Cryoprotectant: Butanediol

Glucose Isomerase crystal

(0.08 x 0.08 x 0.04 mm^3)

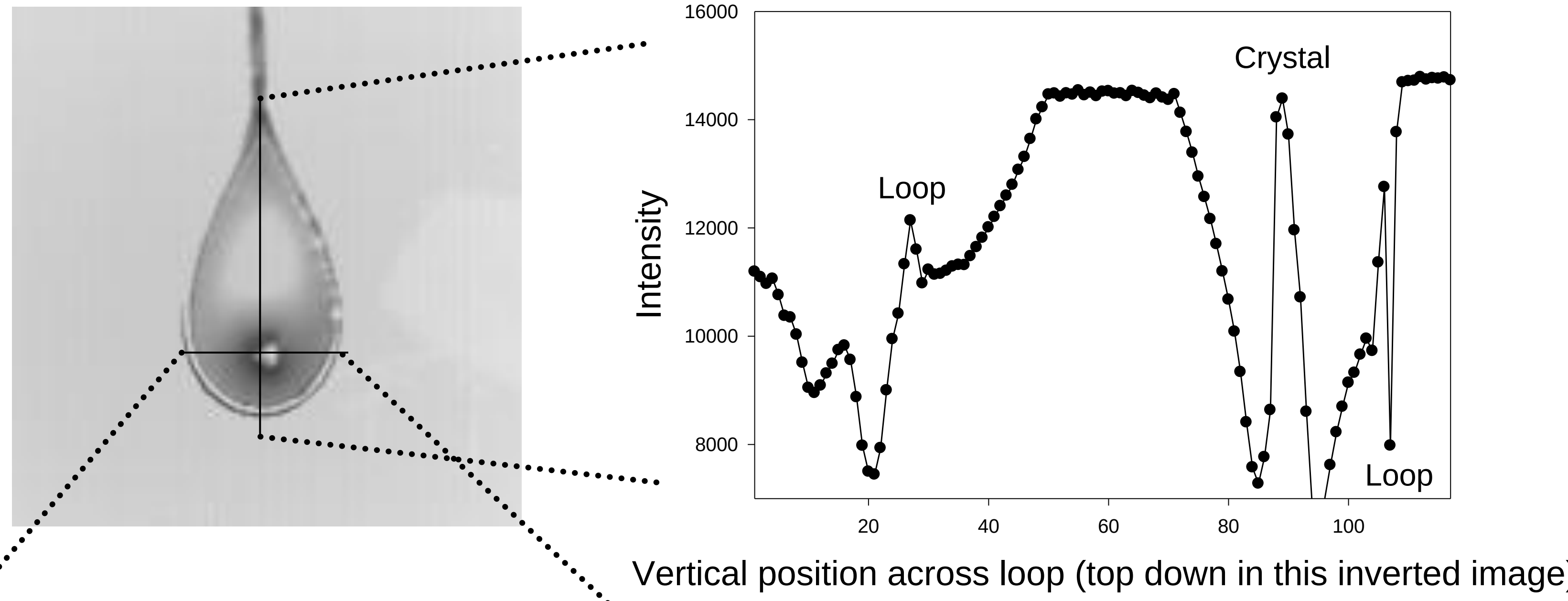


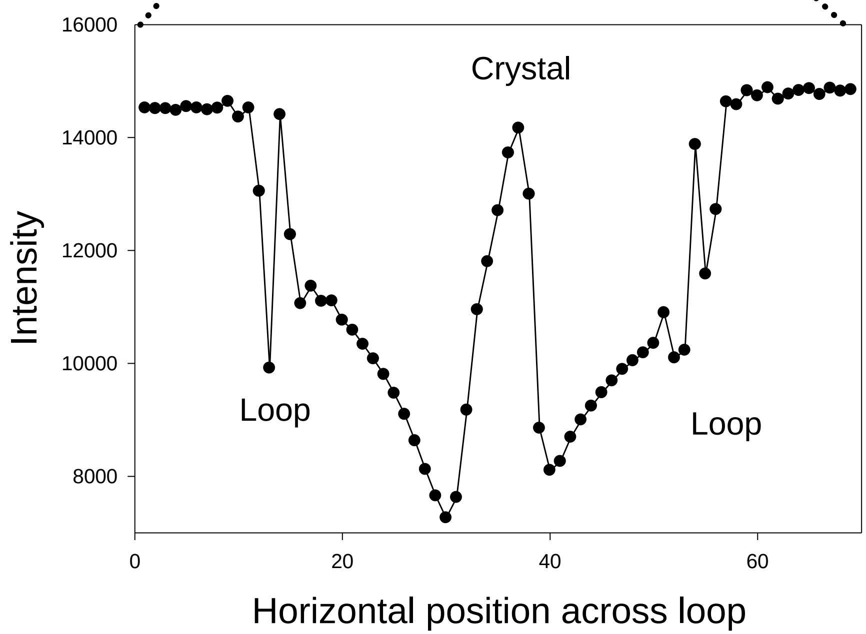
Infrared image (lamp illumination at 45°)

Cell parameters: I222, 92.5, 98.2, 102.2 Å

Solvent content: 50%

Cryoprotectant: Isopropanol, glycerol





In this case the crystal is a "warm" peak in a cold well – it is reflecting infrared radiation.

The bottom of the loop appears cold.

The effect of exposure time

Visible

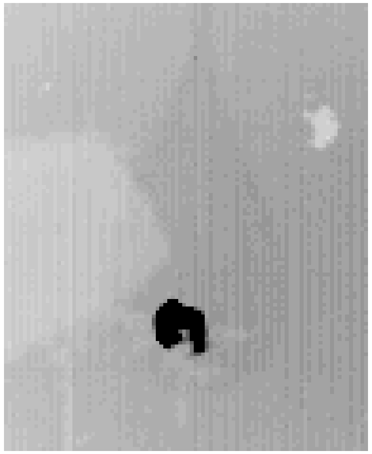
Glucose isomerase

White background

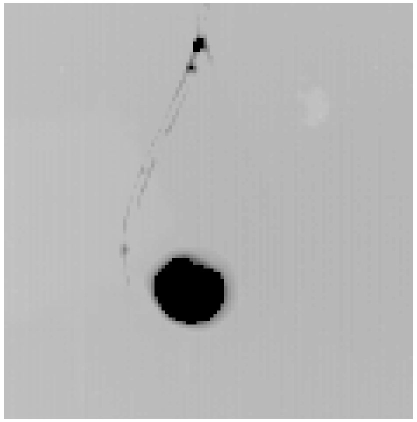
No background

Infrared (lamp illumination from behind)

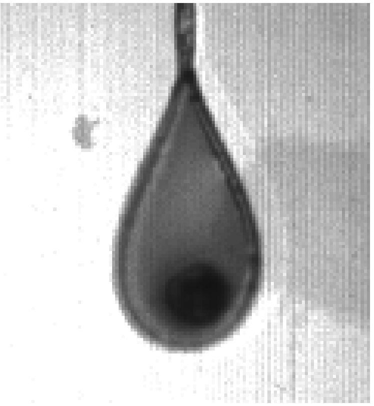
1.0 ms exposure



5.0 ms exposure

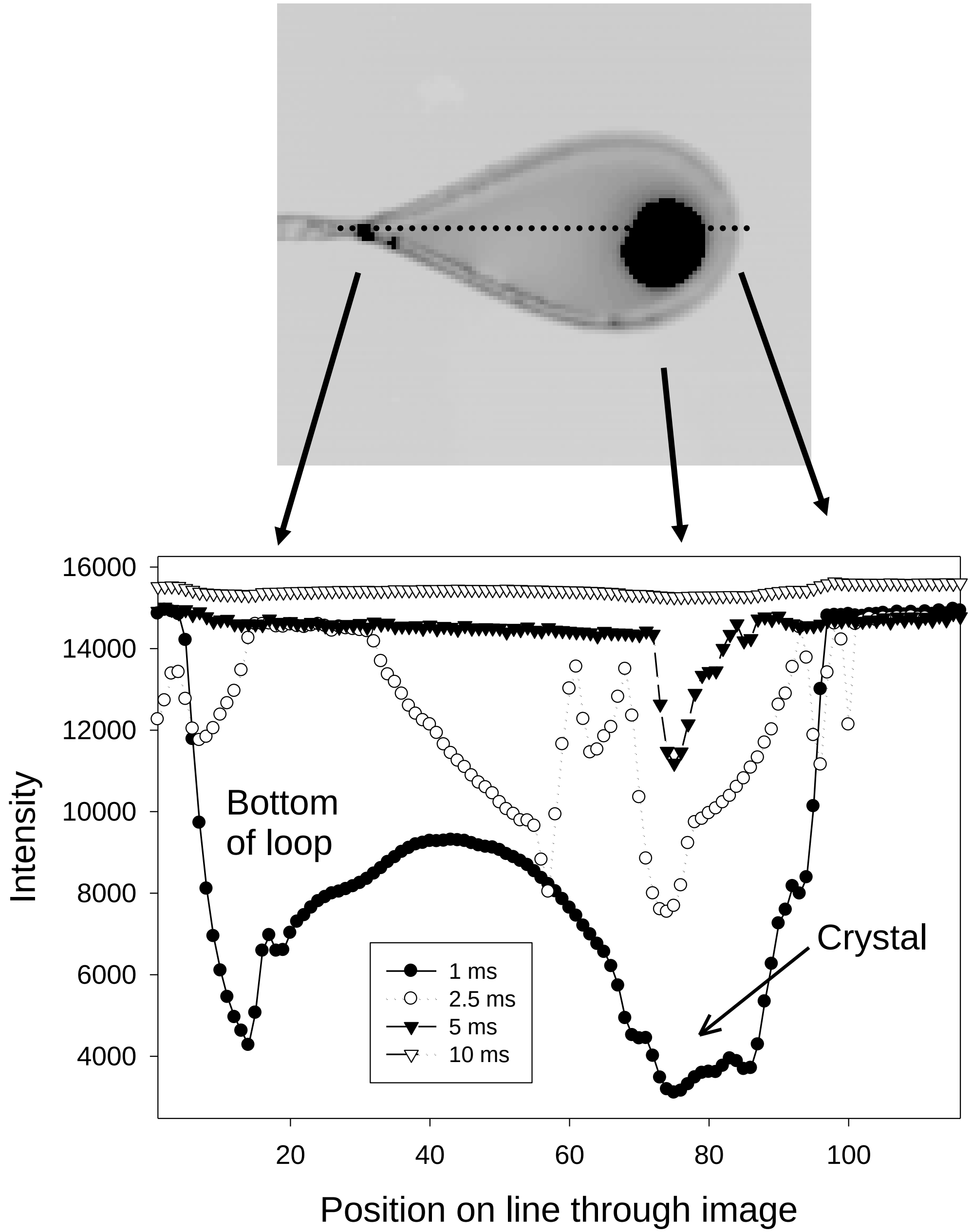


2.5 ms exposure



10.0 ms exposure

(0.22 x 0.15 x 0.06 mm)

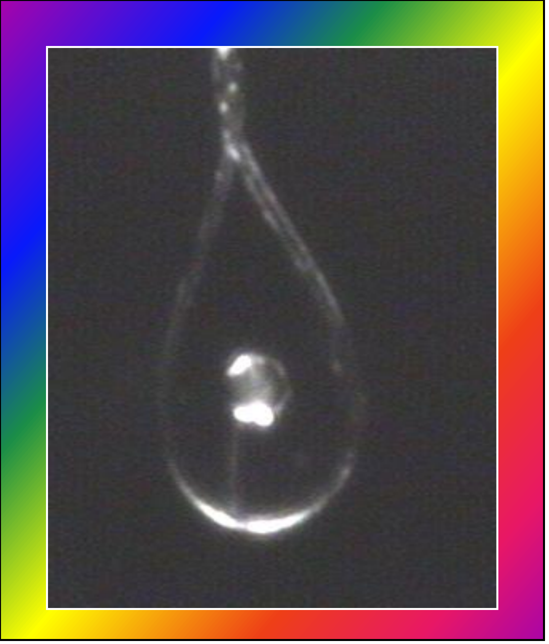


In this case the crystal (also glucose isomerase) is “cold” in comparison to the rest of the image – it is acts as an insulator to the infrared radiation from a lamp source behind.

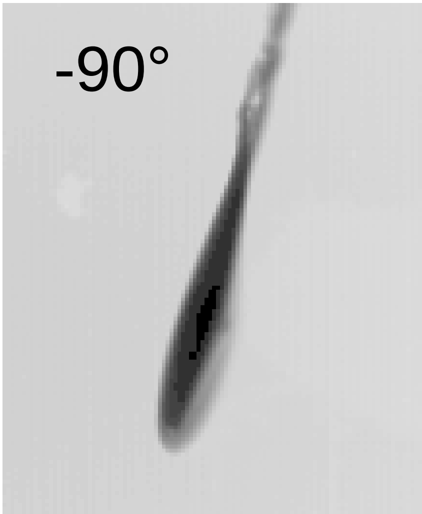
This lamp is barely warm to the touch but acts as an infrared illumination source without perceptibly heating the crystal.

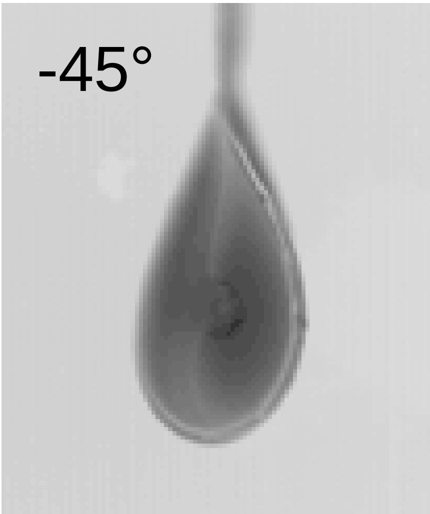
The bottom of the loop also acts as an insulator and appears cold.

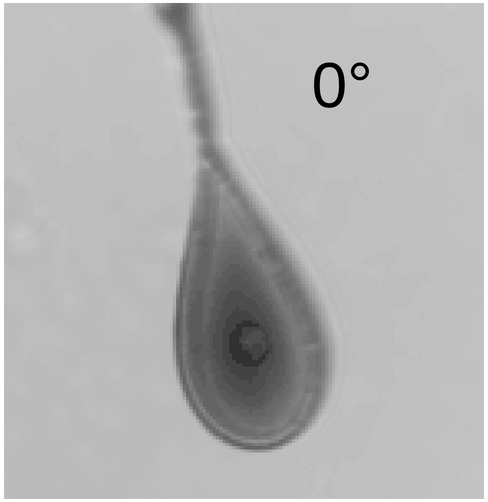
Angular sensitivity

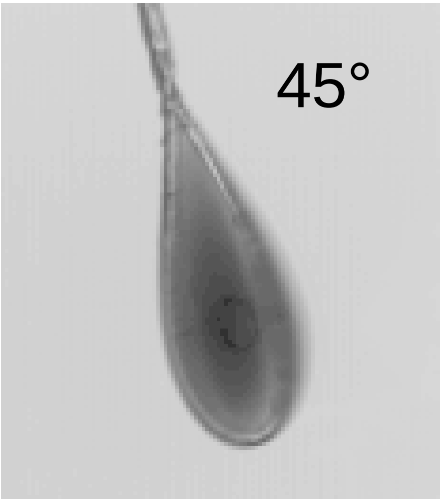


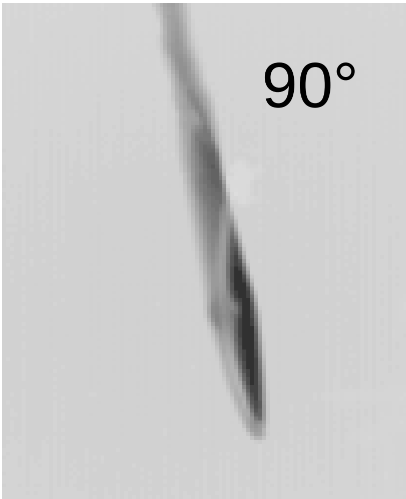
1.0 ms exposures





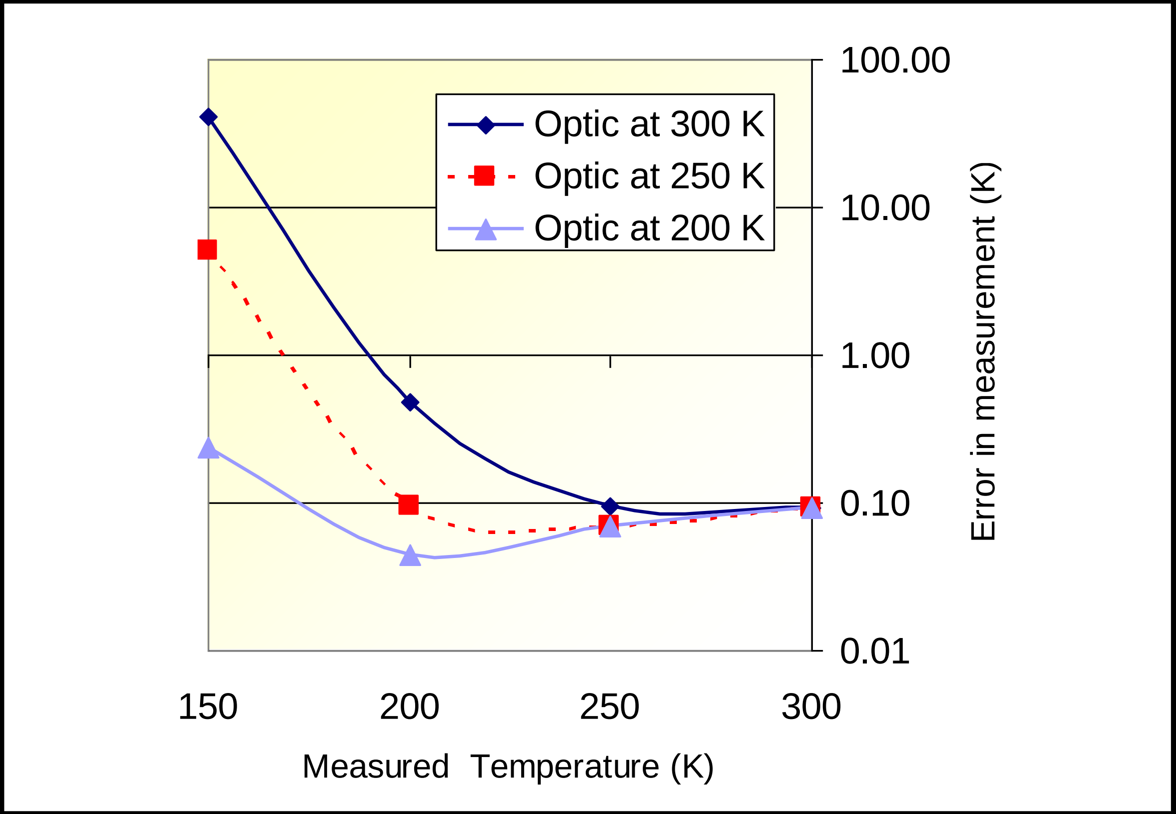






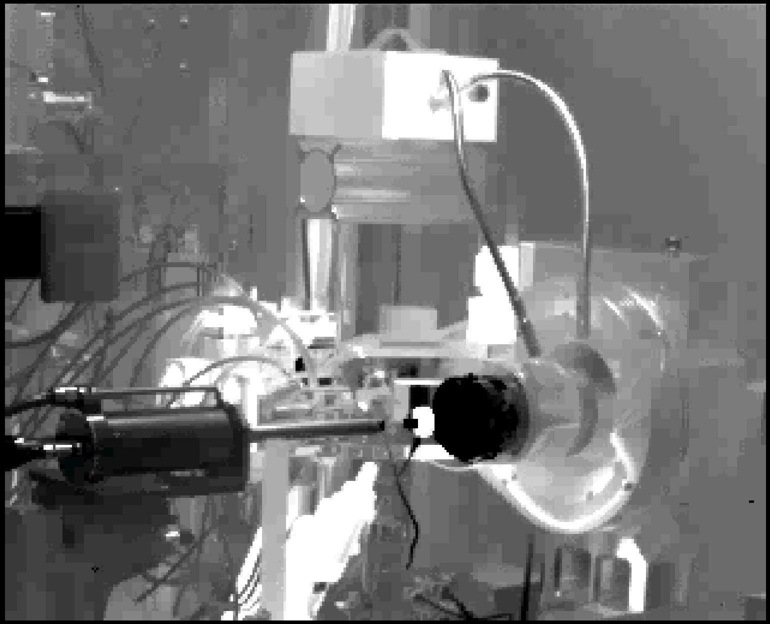
Why can we see the crystals?

- The system is at equilibrium at 100K.
- We do not see the temperature difference due to deviations from 100K.
- We see the crystals primarily due to the transmission and reflection of infrared radiation



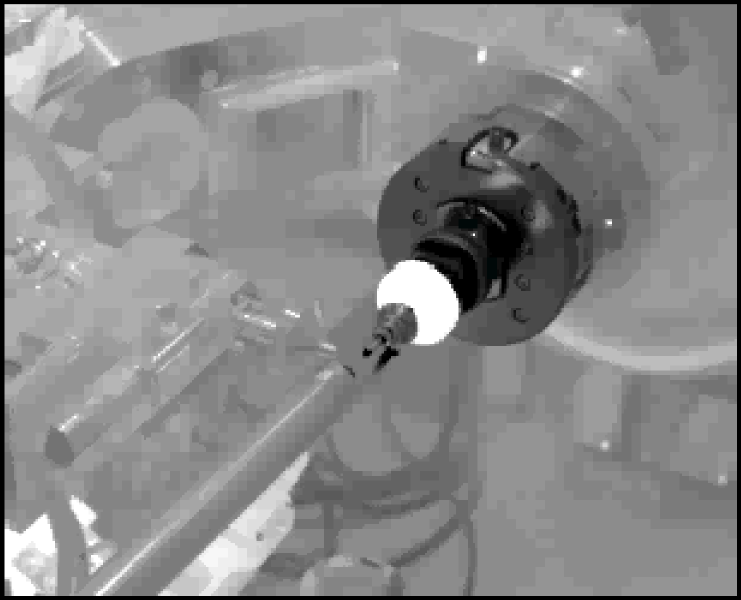
We know from our other studies that the camera in the configuration we are using is sensitive to 130 K (somewhat better than the calculated graph shown). By using it at maximum sensitivity we may also be able to measure the emissivity properties of the samples further enhancing the technique

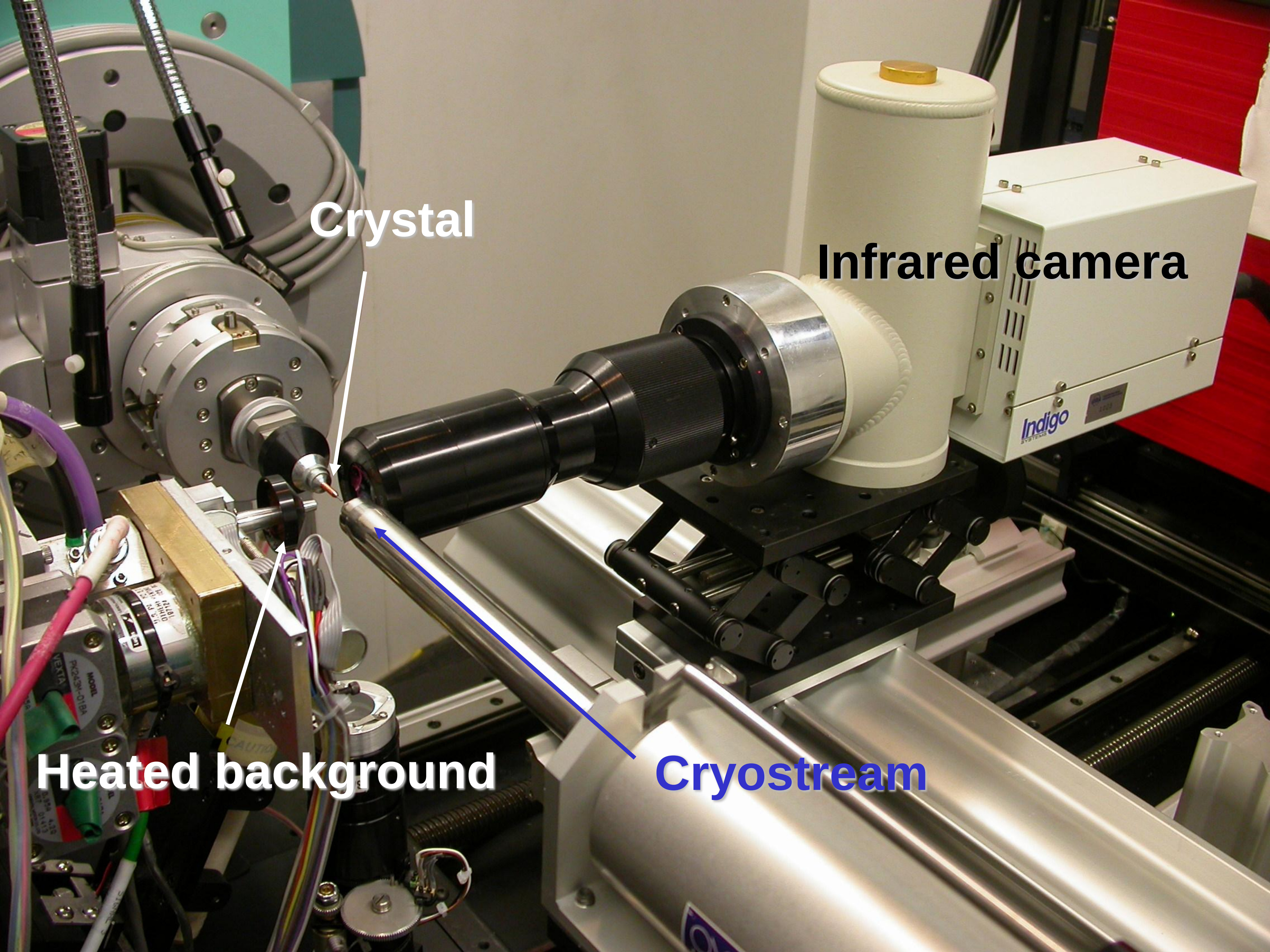
A real world case – SSRL Beamline 11-1



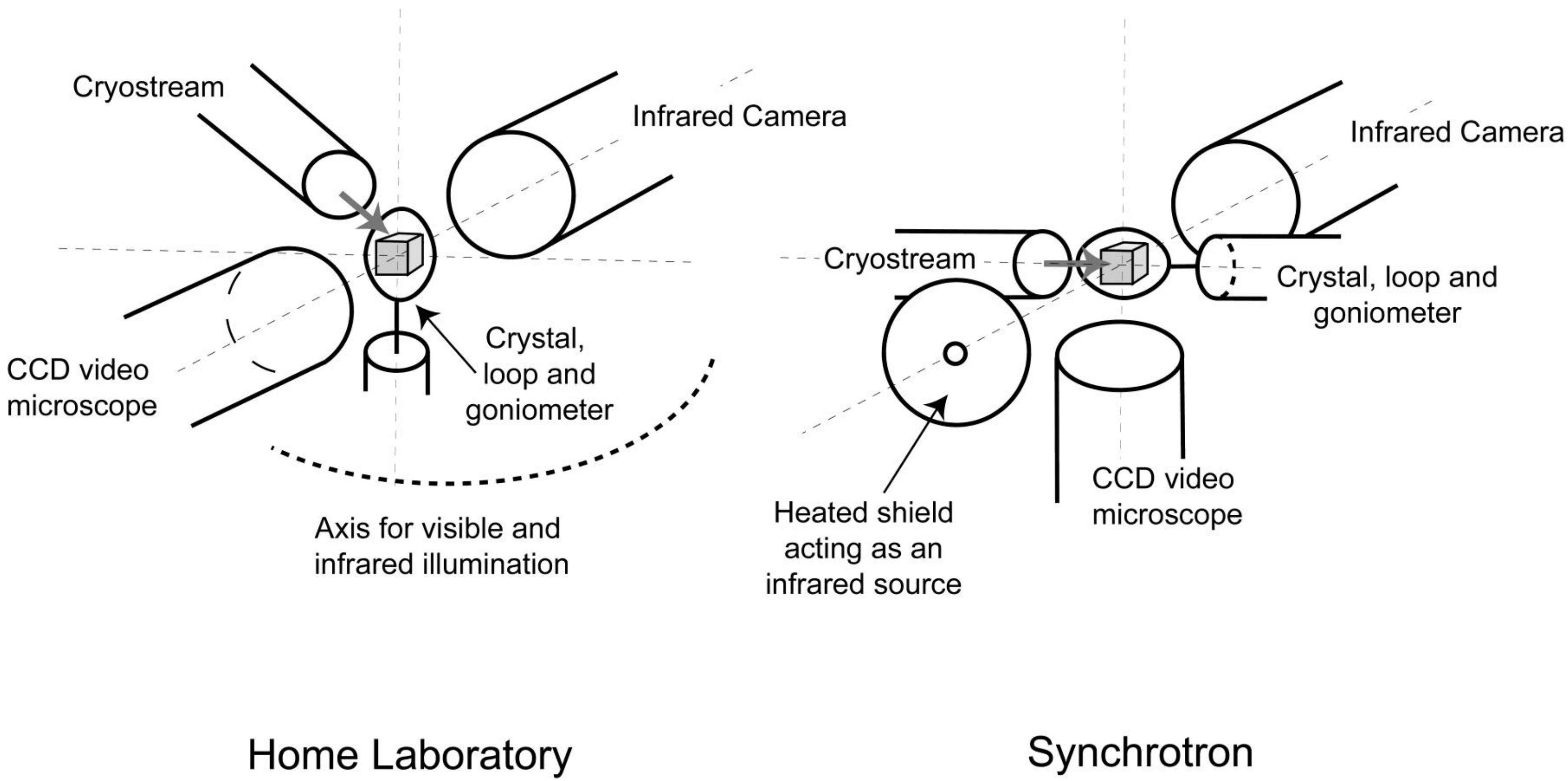
Mounting a crystal

Un-mounting a crystal

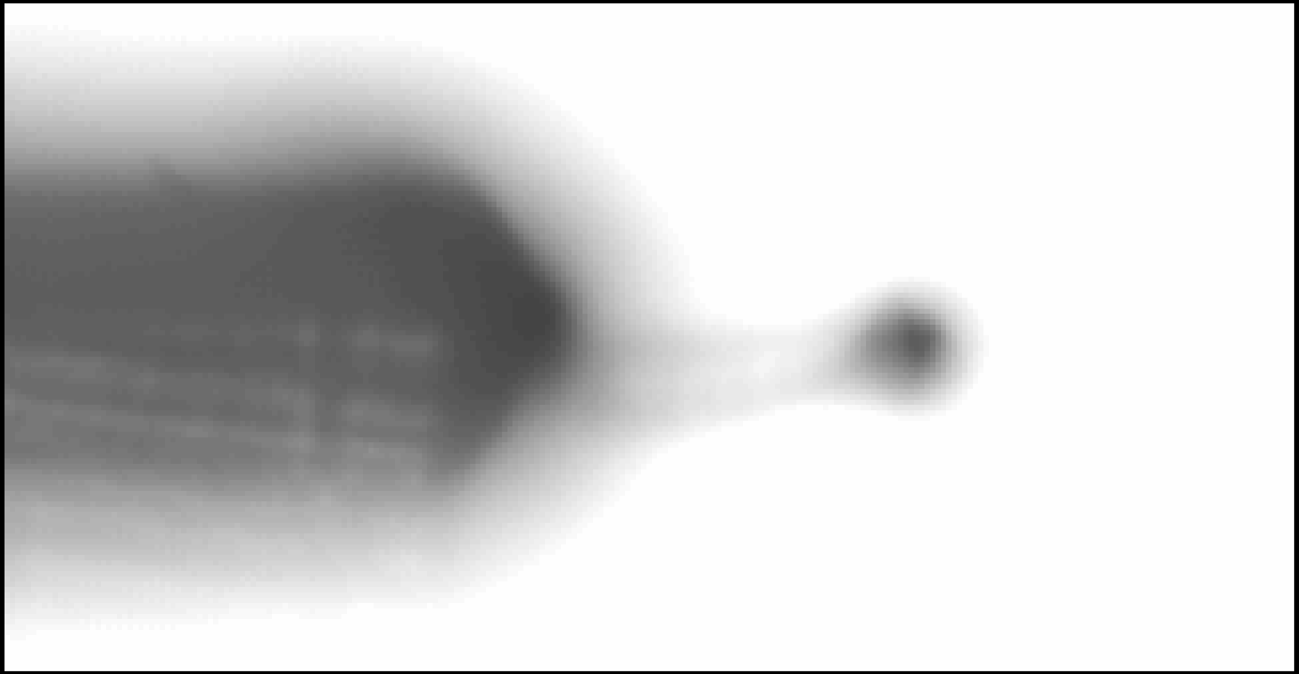



Crystal
Infrared camera
Indigo
Heated background
Cryostream

Difference between laboratory and synchrotron setup



Infrared Image with crystal being moved in 10 μm step size

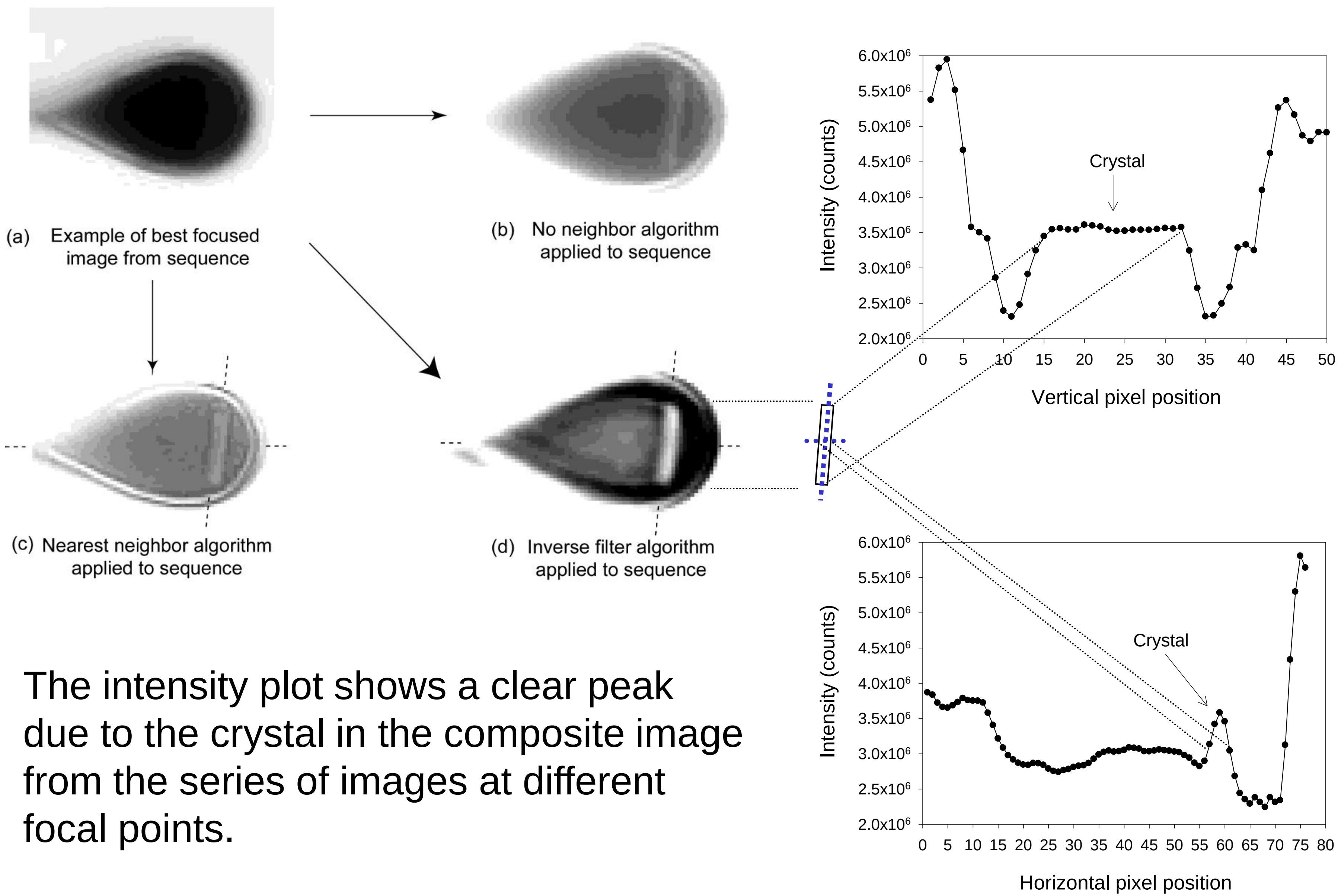


30% glycerol, real sample from structural genomics program.
Loop is 0.1 mm diameter.

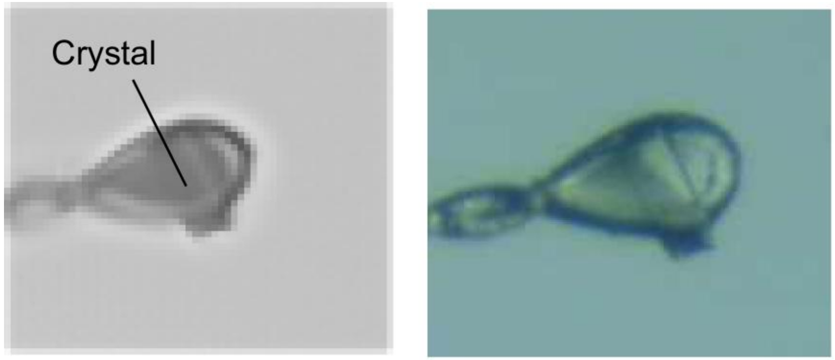
Depth of field of is small, less than 10μm.

Image Processing

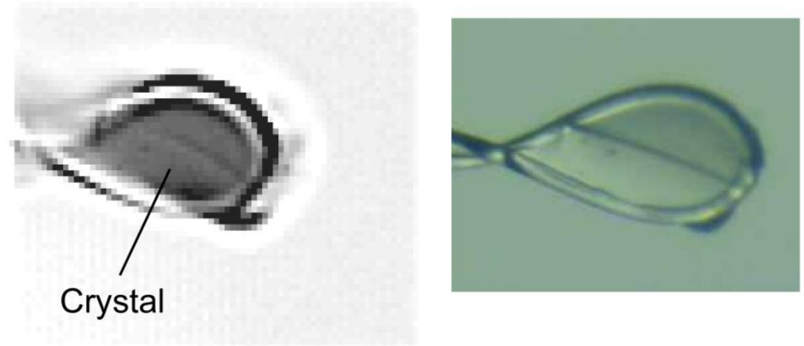
- Images take at successive depth of field were processed with ImageProPlus and the Sharpstack module.
- Four different image processing techniques were used:
  - Extended depth of field processing
  - Blind deconvolution
  - No neighbor deblurring
  - Nearest neighbor deblurring
- Extended depth of field processing makes use of each image having elements in its focal volume in focus. These focused areas are identified and integrated into a composite image.
- Deconvolution reassigns photons to their points of origin within the image volume while deblurring removes out of focus photons from the image stack.
- Blind deconvolution (inverse filter) uses a point spread function to determine blur in all three dimensions and deconvolve the blur to achieve a sharp image.
- No neighbor deblurring reverses a calculated blur from the image.
- Nearest neighbor analyzes a minimum of three neighboring slices to estimate blurring in the central image and reverse that blur.



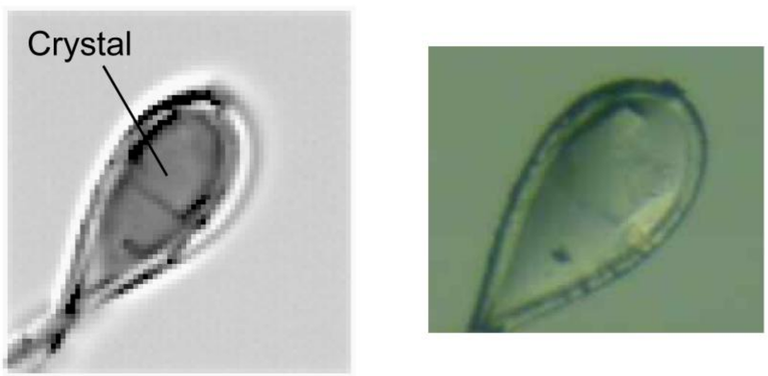
The intensity plot shows a clear peak due to the crystal in the composite image from the series of images at different focal points.



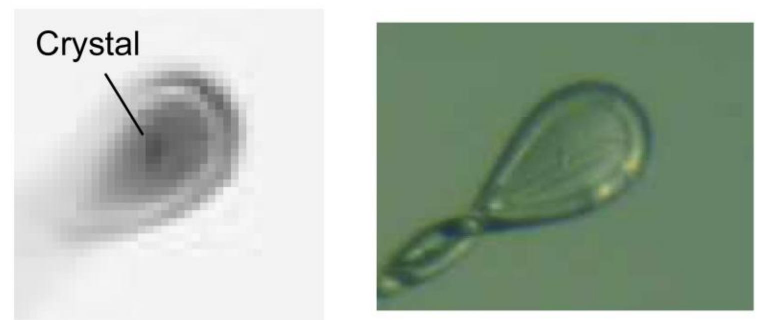
(a) Crystal 1



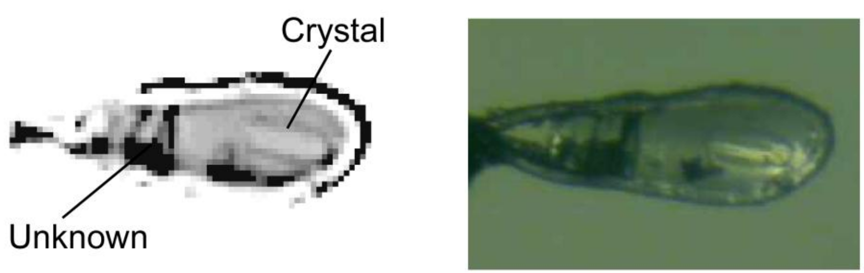
(b) Crystal 2



(c) Crystal 3



(d) Crystal 4

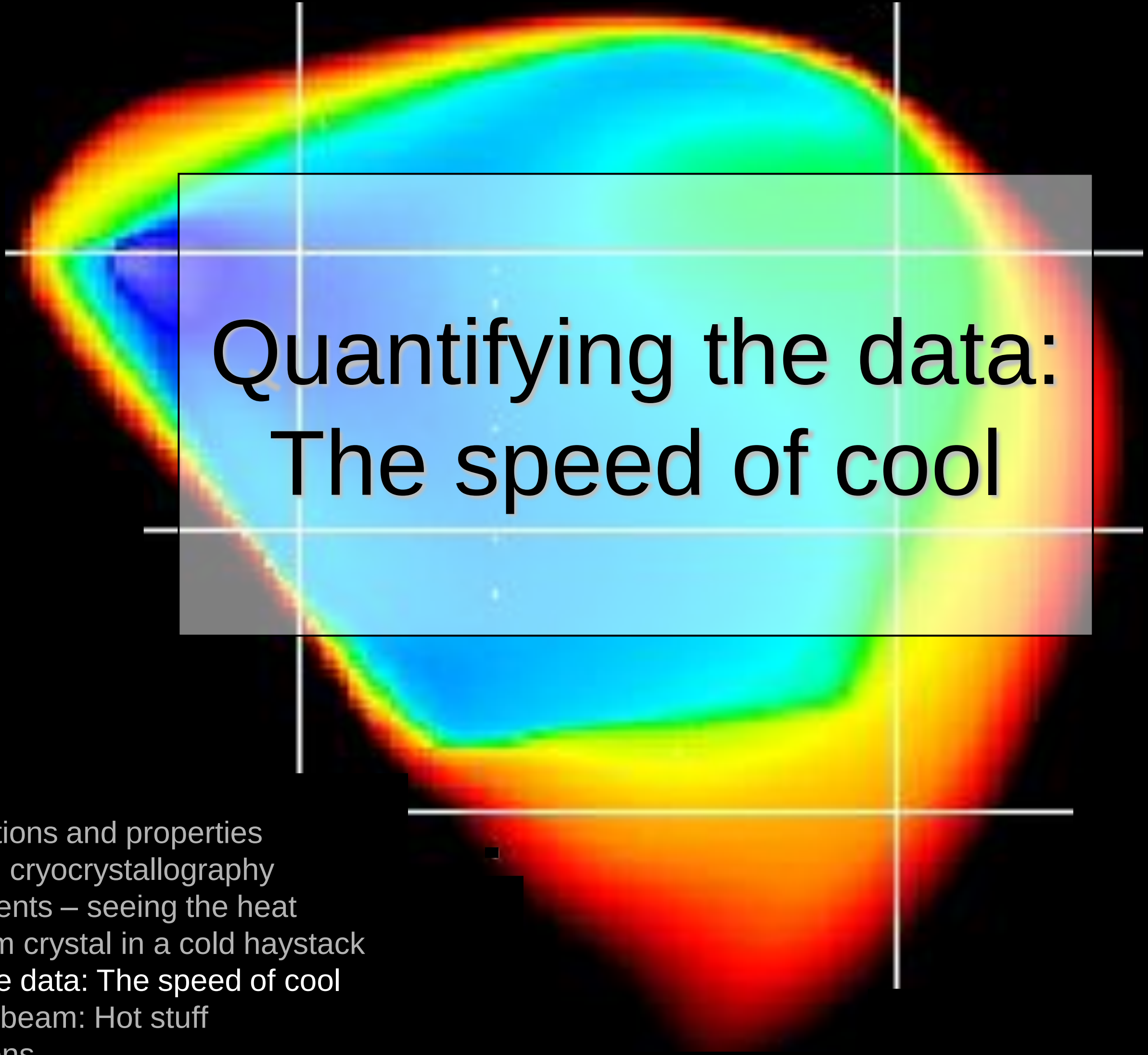


(e) Cystal 5

Observations

- The complete crystal was seen as the camera was translated so different parts of the loop were in focus – the optics have a small depth of field.
- The whole crystal was never completely in focus due to the small depth of field.
- A single focused image can be generated using image processing techniques from the sequence of images at 10μm focal points.
- The background illumination and shielding was not optimized in this case.
- The crystals were approximately the same size as the loop used.
- There is still a way to go to make the system of practical use at the synchrotron but the technique clearly shows promise.

Sex in Space



Quantifying the data: The speed of cool

- Infrared definitions and properties
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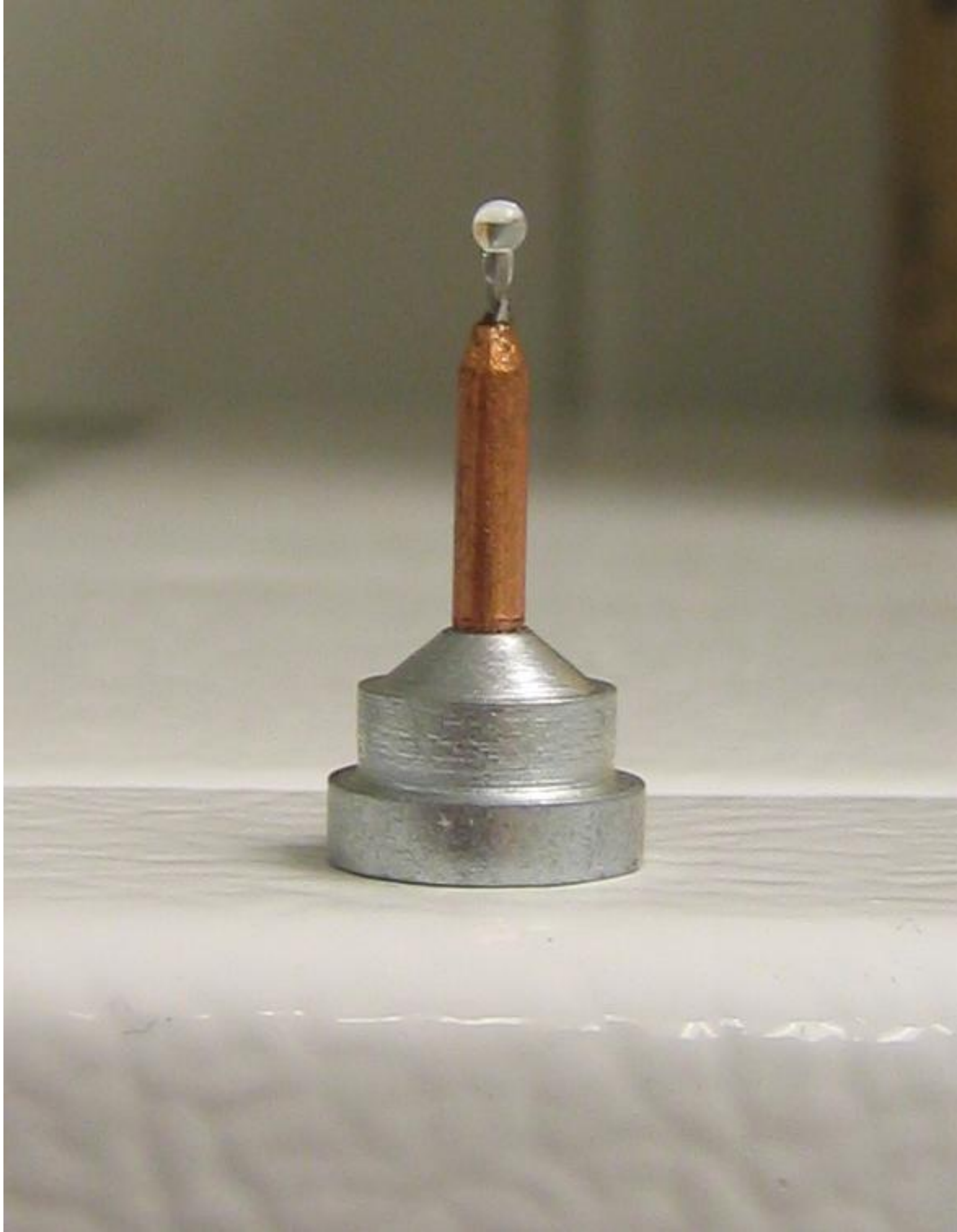
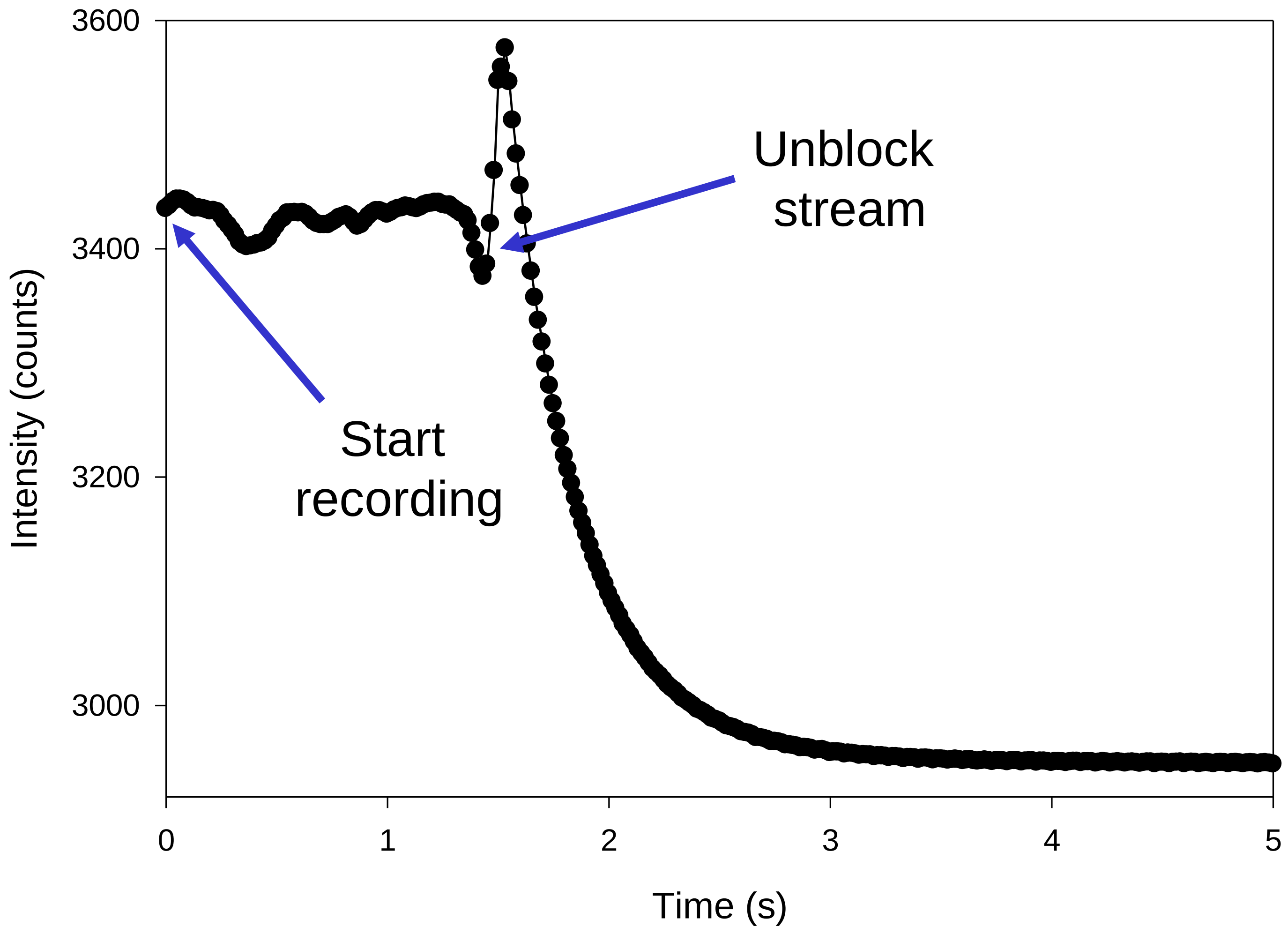
Test samples for model development

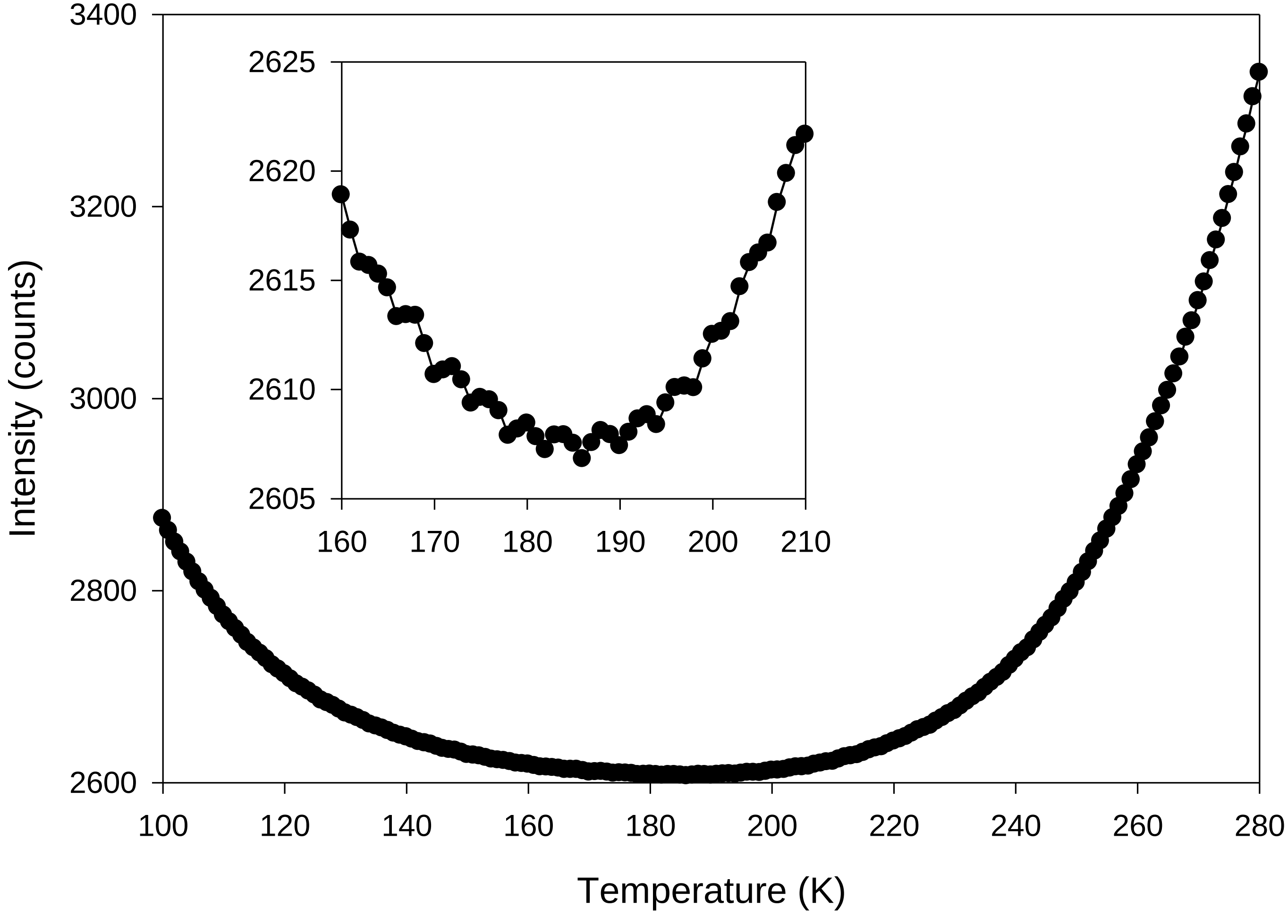
- Glass beads of 0.5, 1.0 and 2.0 mm diameter.
- Mounted on standard Hampton Research Cryomounts.
- Directly glued to the cryopin using Epoxy resin.
- Chosen as spherical samples allow for easy modeling.
- No special care needed to maintain glass at 100K – can study the system as a function of temperature.



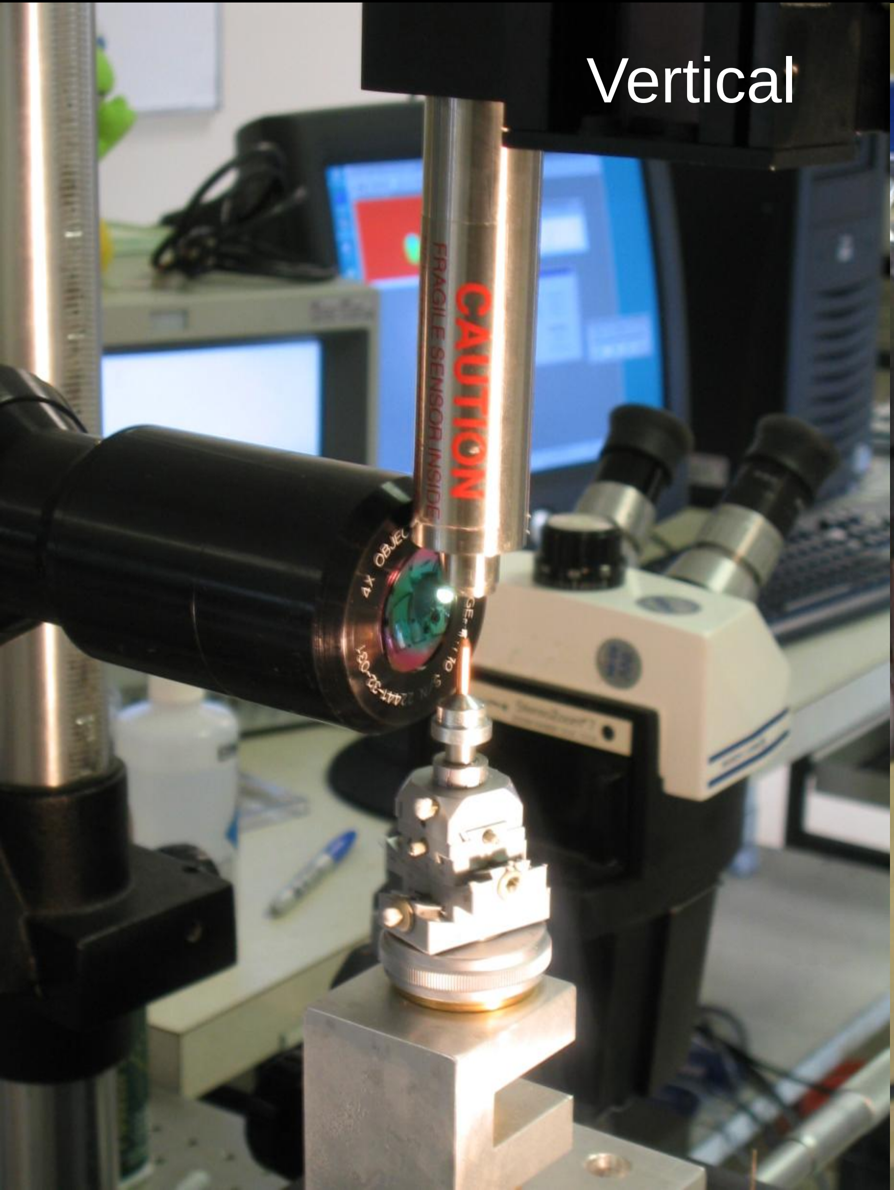
Data profile for 0.5 mm diameter glass bead flash cooled

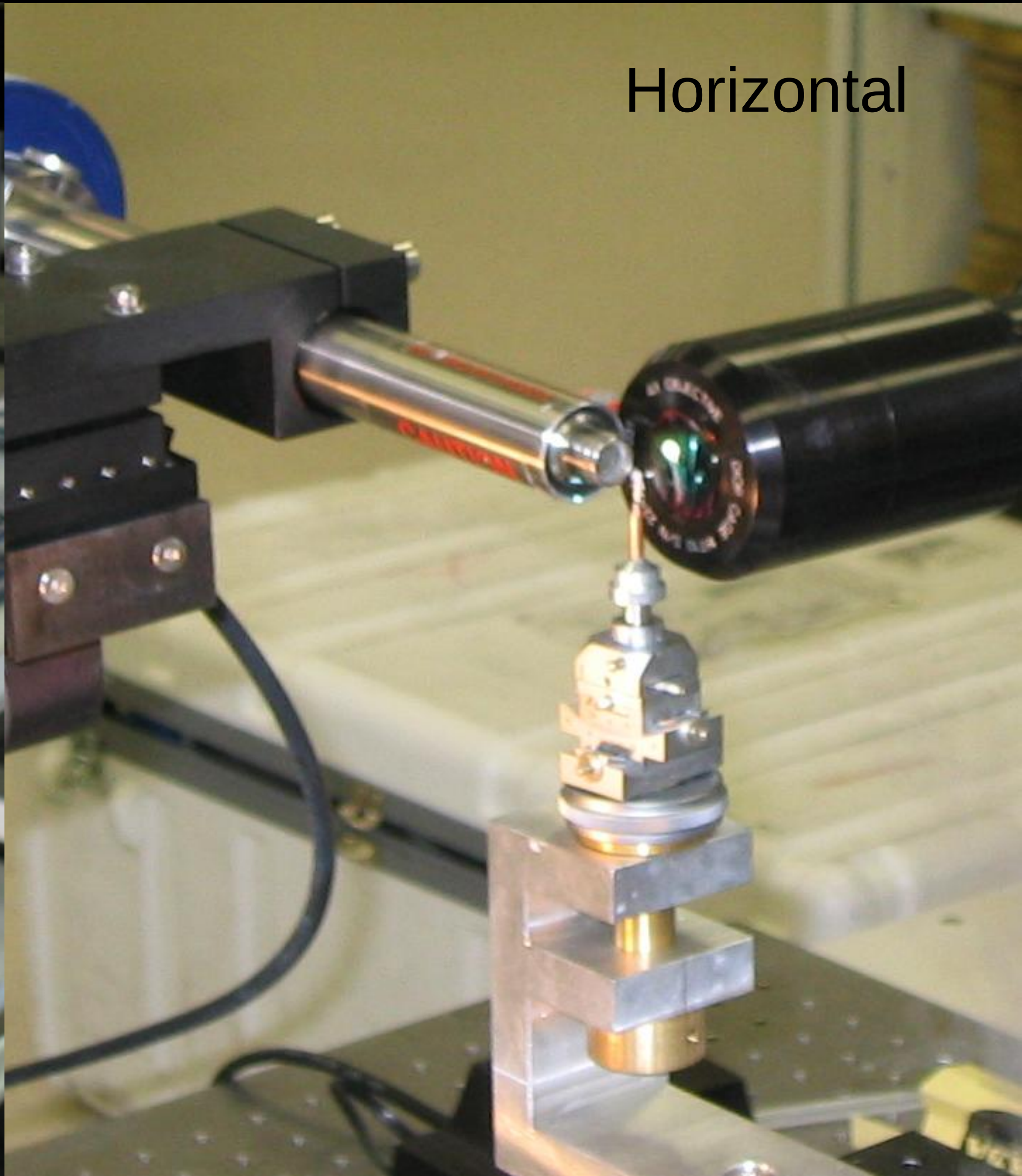


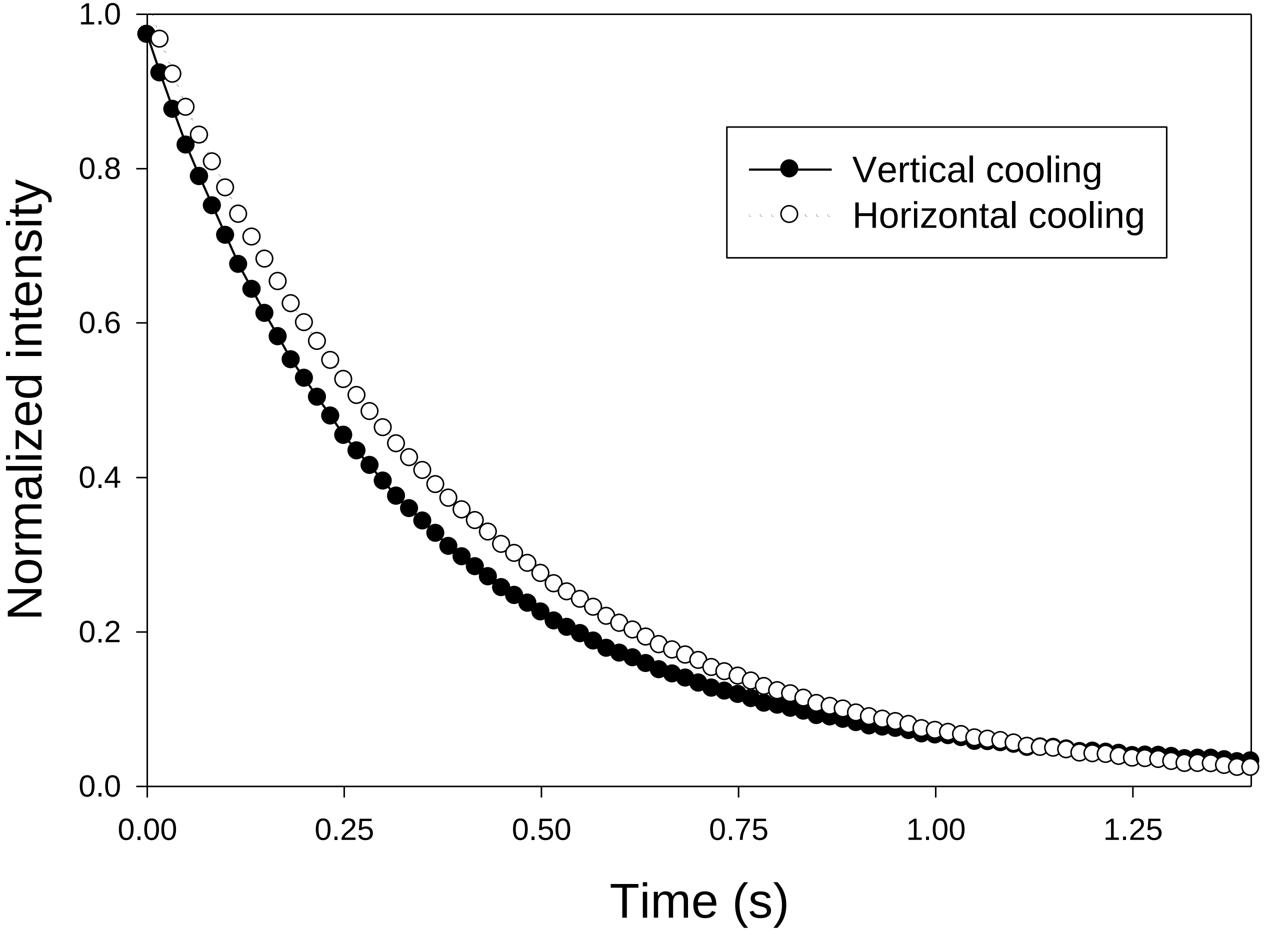
The bead is then warmed up at a known rate and the data collected for intensity to temperature calibration



Before we look at rate which direction of cooling is best?

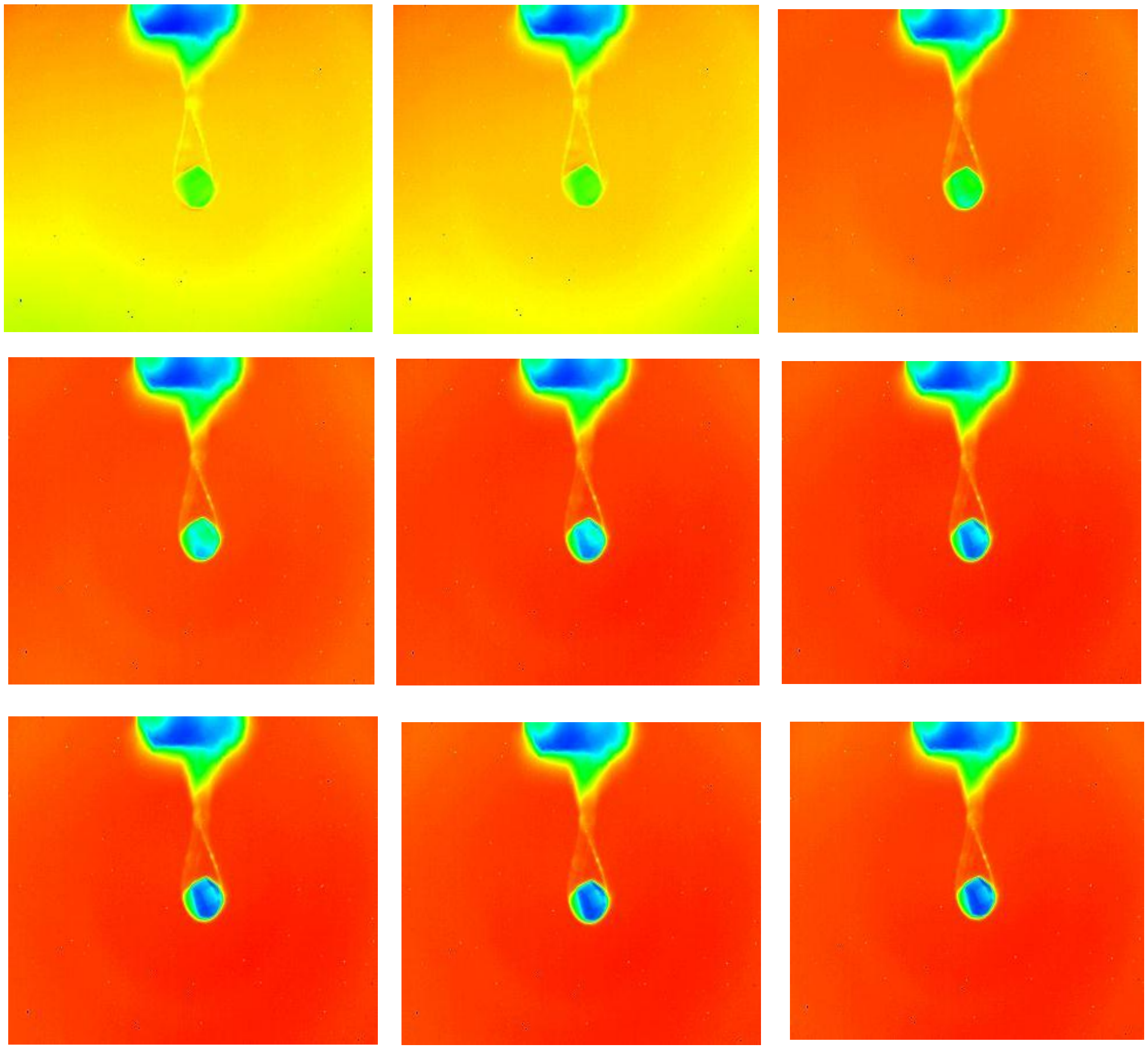






Little difference between vertical and horizontal cooling – direction is unimportant.

Example of a cryocooled lysozyme crystal



The crystal is 0.40 x 0.32 x 0.19 mm mounted in a 0.5 mm Hampton Research cryoloop.

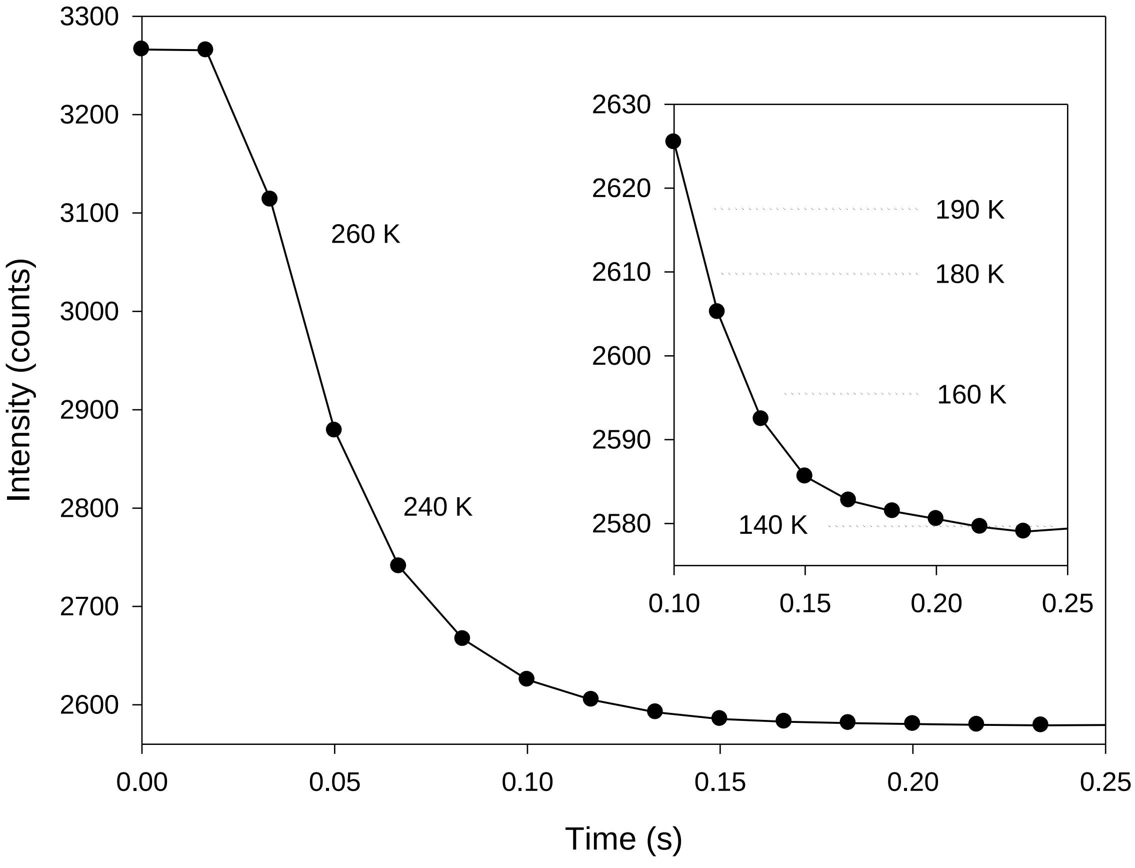
The images are shown inverted due to the microscope objective.

The images are false colored with blue (low intensity) being cold and red (high intensity) being hot.

Each image is taken 1/60 of a second apart.

The cryostream, at 100K, cools the crystal vertically from above (below in the image)

Lysozyme crystal cooling

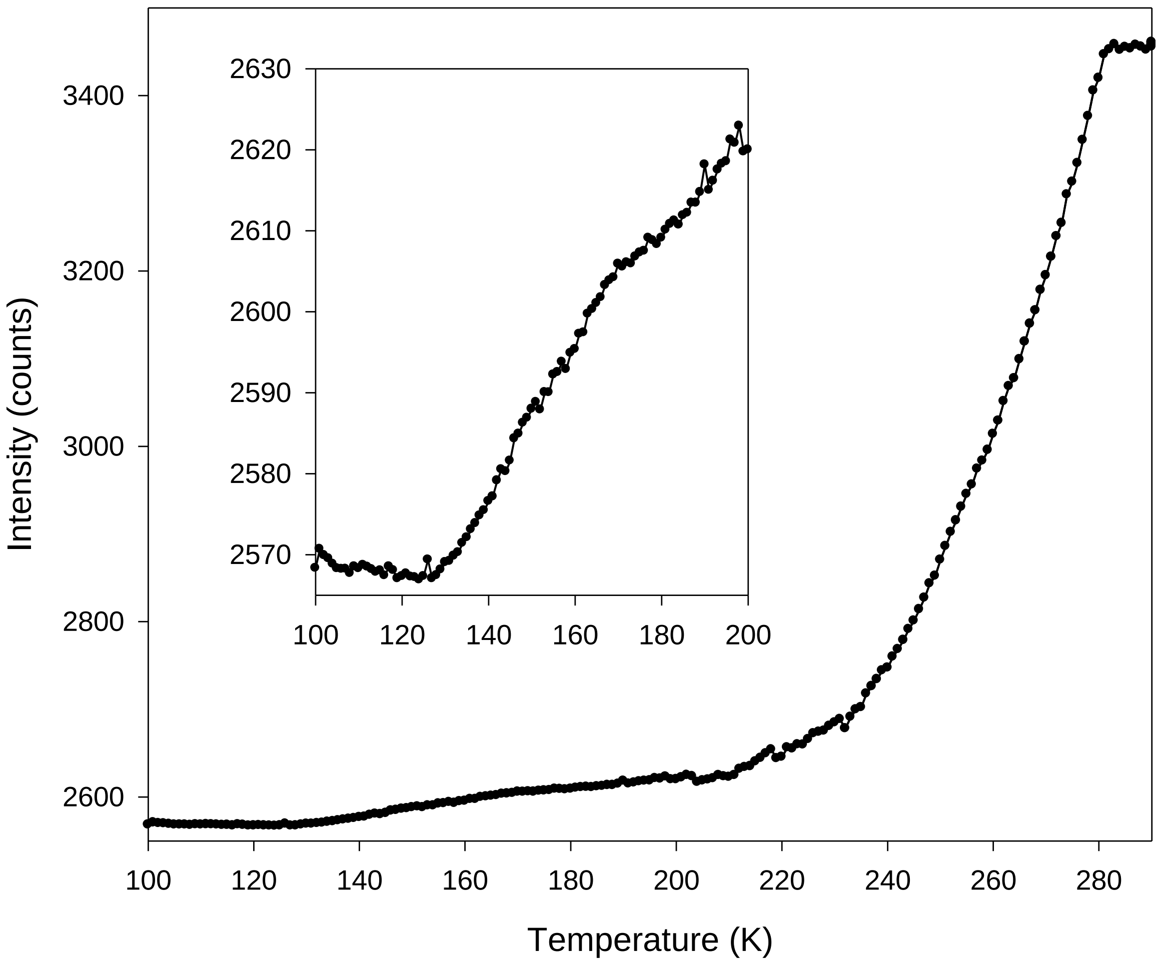


Experimental protocol similar to the glass bead:

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

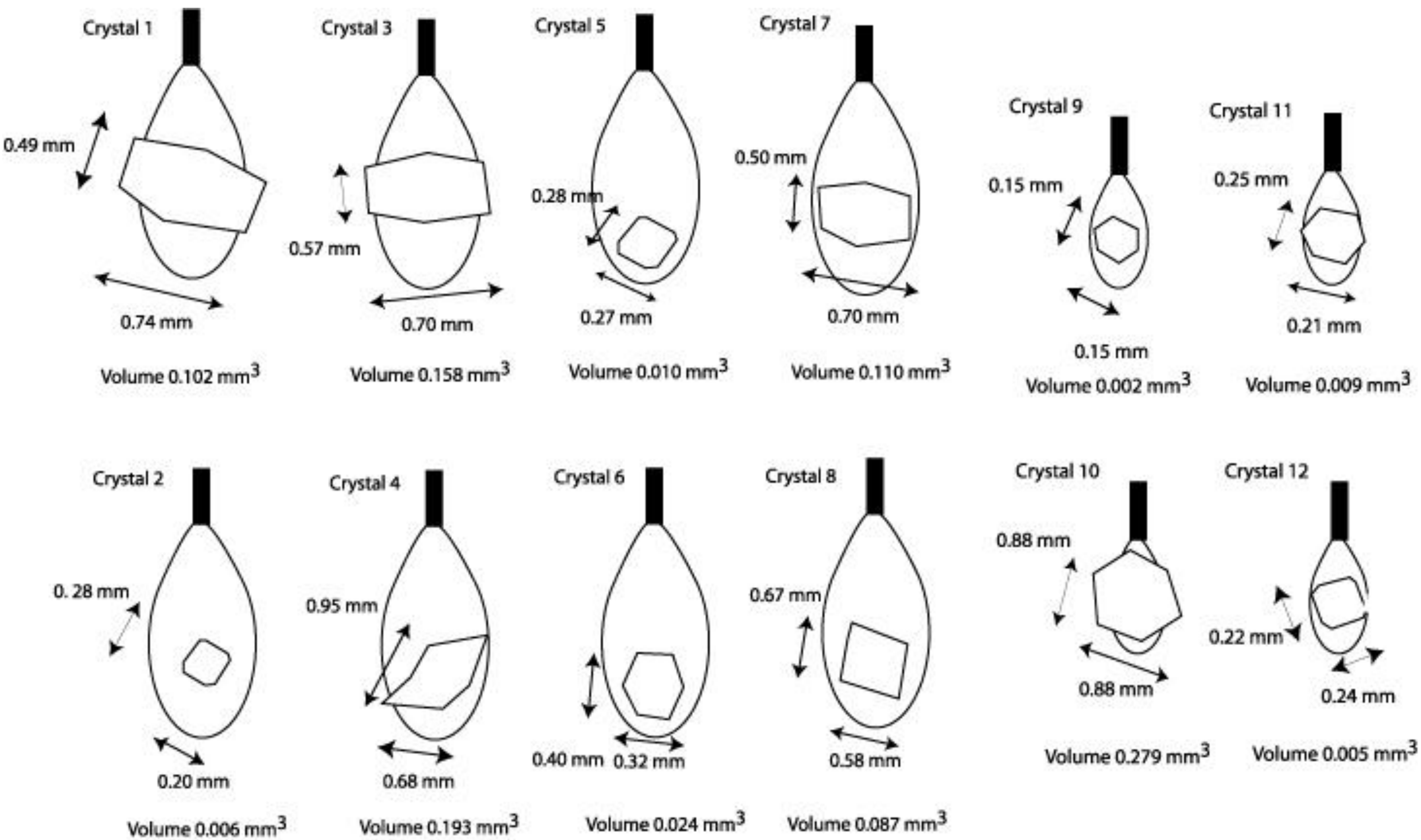


The calibration reveals that for lysozyme crystals the sensitivity of the camera is approximately 135 K.

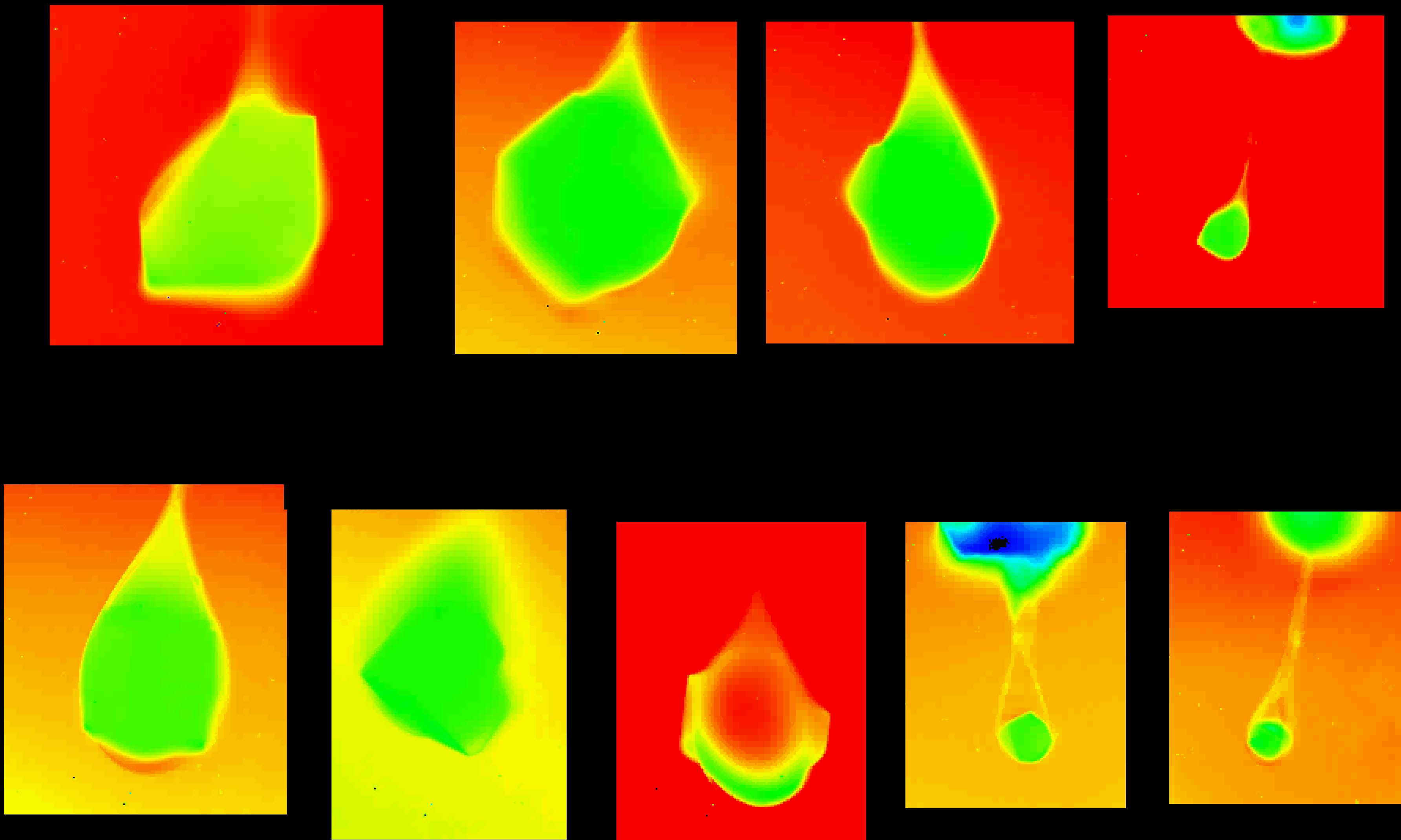
This sensitivity is a property dependent on the non-ideality of the sample as a black body, i.e. emissivity. This is the ratio of the radiation emitted to that predicted by Plank's law.

This emissivity and therefore camera sensitivity is sample dependent

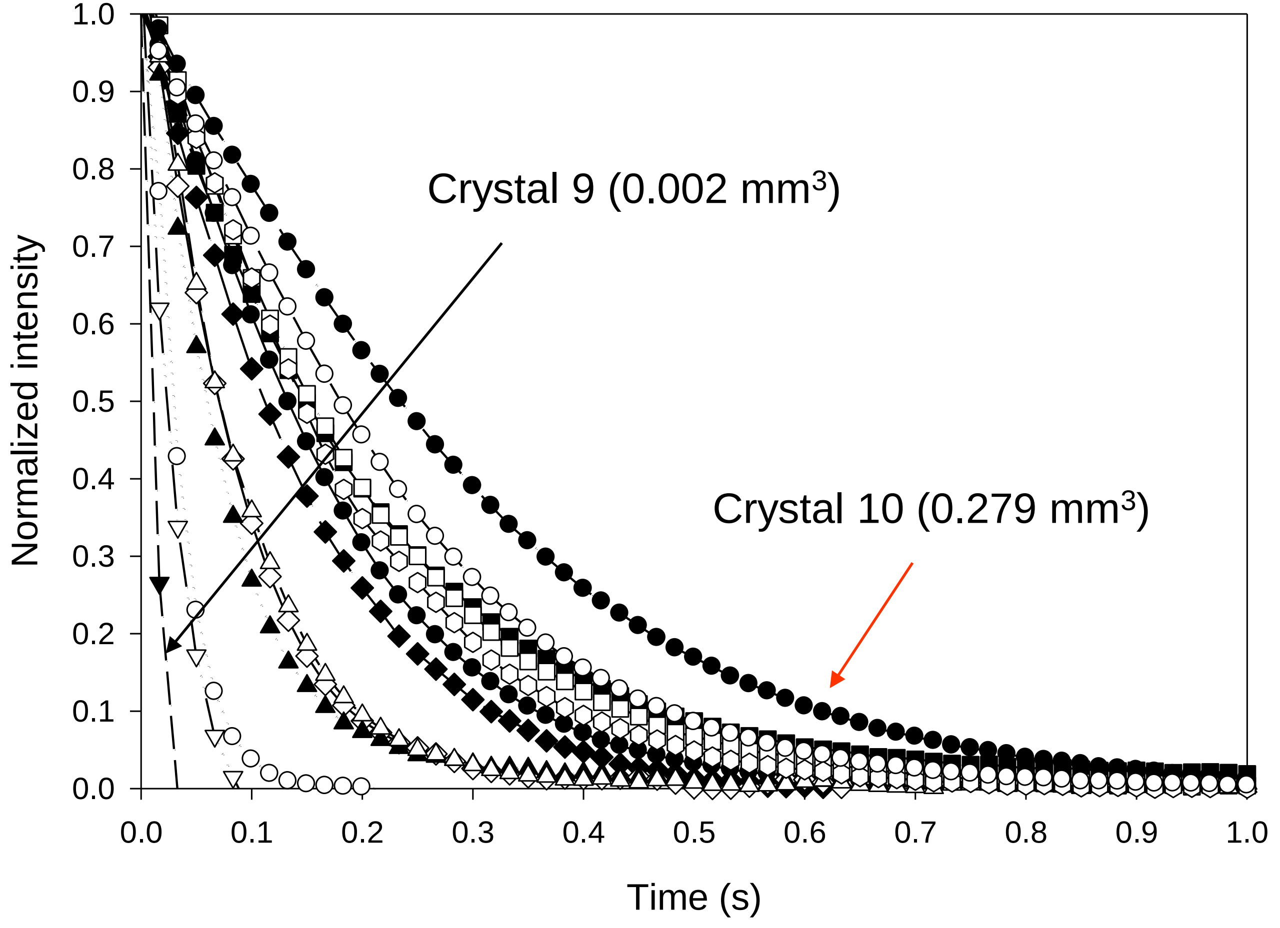
Diagram of lysozyme crystal samples studied



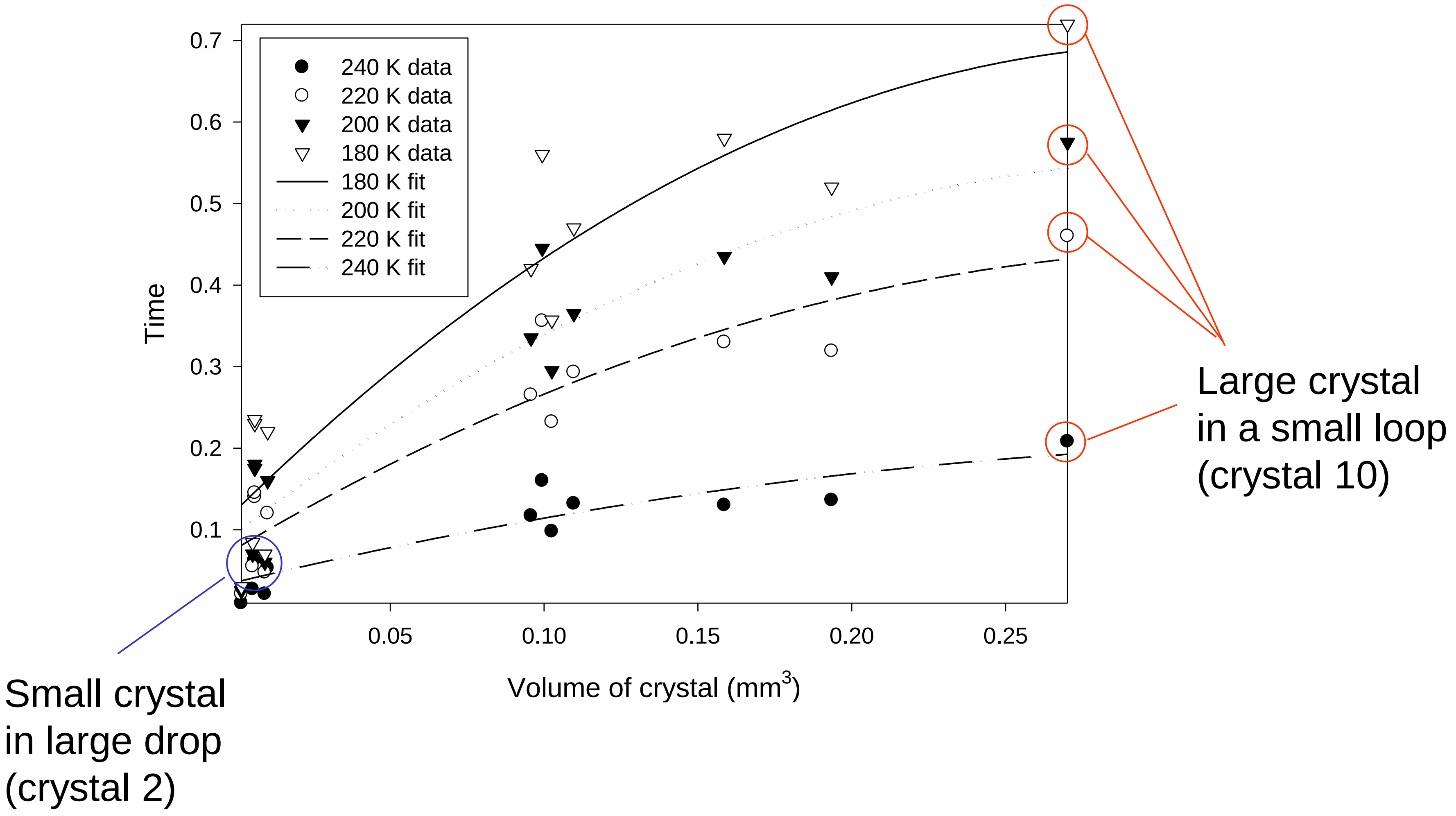
Two different loop sizes were used, 0.5 mm and 0.2 mm diameter.
Typically the smallest dimension is into the picture.



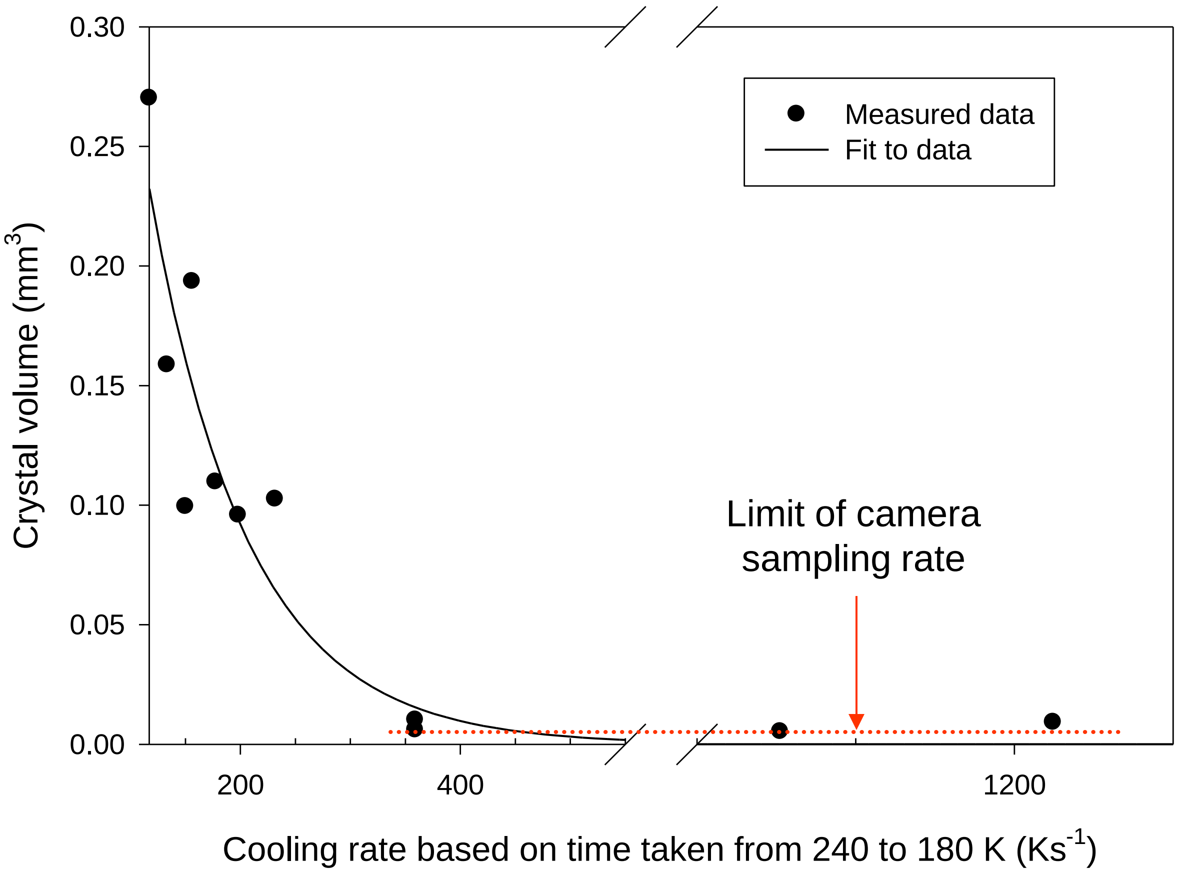
Normalized intensity profile of crystal samples during cooling.



Cooling as a function of crystal volume



The speed of cool….



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

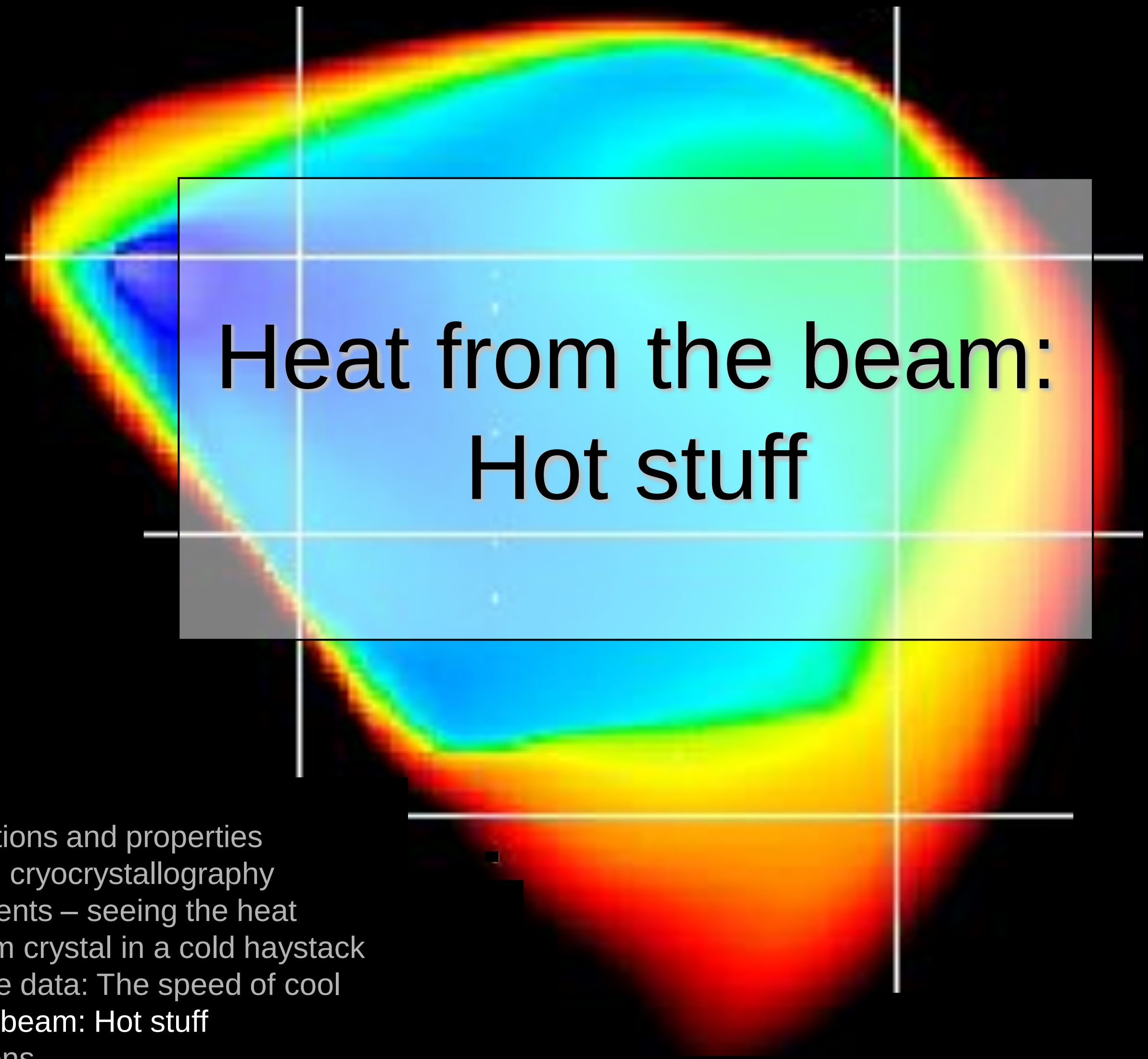
The camera can image digitally at faster rates, full frame 150 Hz, half size frame 300 Hz (images every 0.0067 and 0.0033 seconds respectively) but the video output is unavailable. It is not possible to focus the crystal easily.

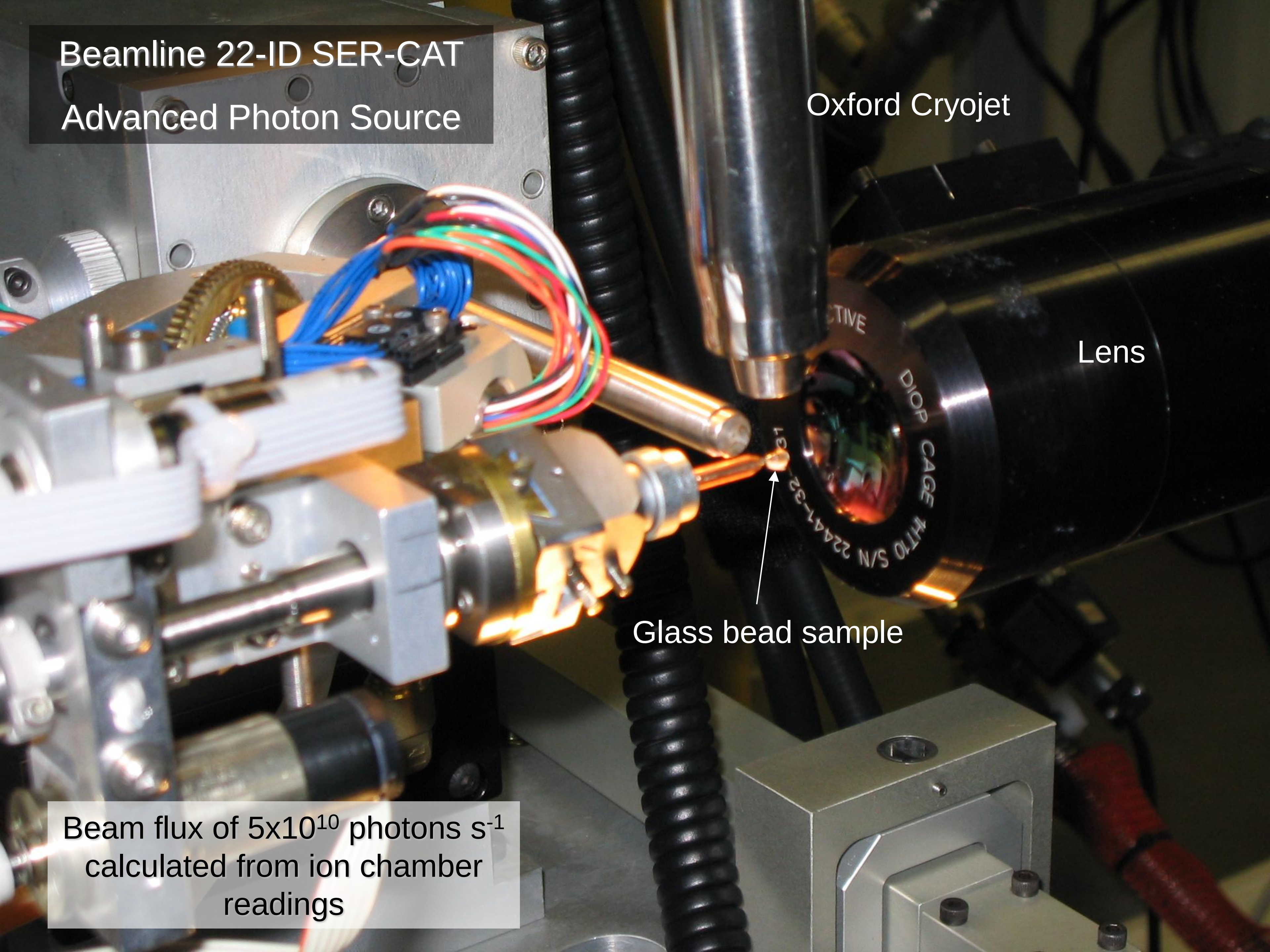
The smallest crystals await optics with improved depth of field.

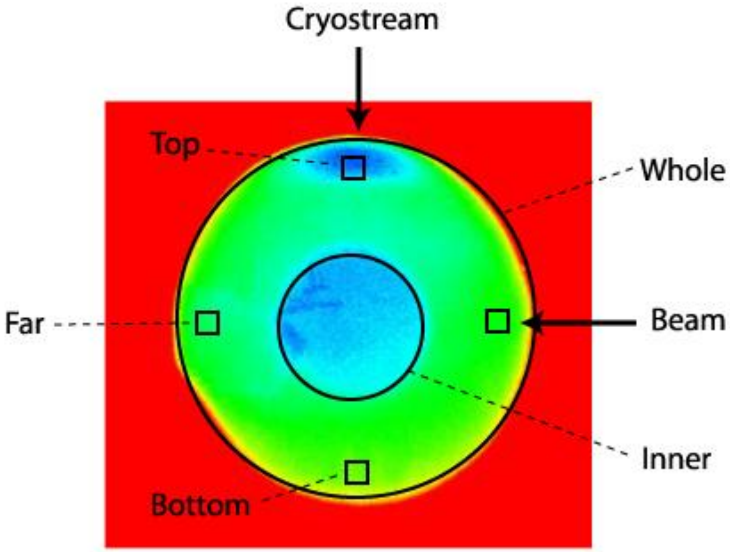
Observations – common sense?

- For the case studied:
  - Smaller crystals are cooled more rapidly.
  - Cooling rates on the order of 100’s of K per second are common.
  - The direction of cooling, in the limit from vertically downward to horizontally, has little influence on the rate.
  - Loop size (and therefore cryoprotectant in the loop) has little observable influence on the cooling rate.
  - The smallest samples will require improved optics (or a small depth of field visual camera focused at the same position as the thermal imaging camera.

Heat from the beam: Hot stuff

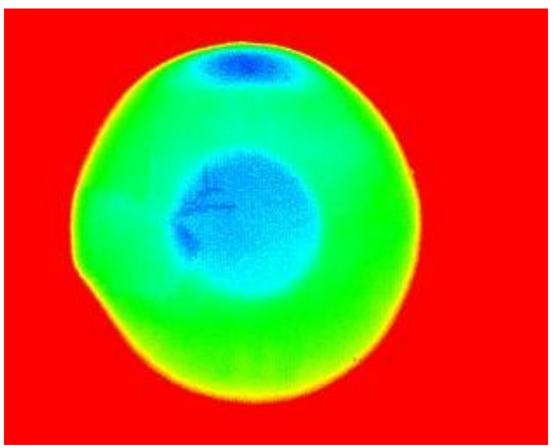



Beamline 22-ID SER-CAT
Advanced Photon Source
Oxford Cryojet
Lens
Glass bead sample
Beam flux of 5×10^{10} photons s^{-1} calculated from ion chamber readings

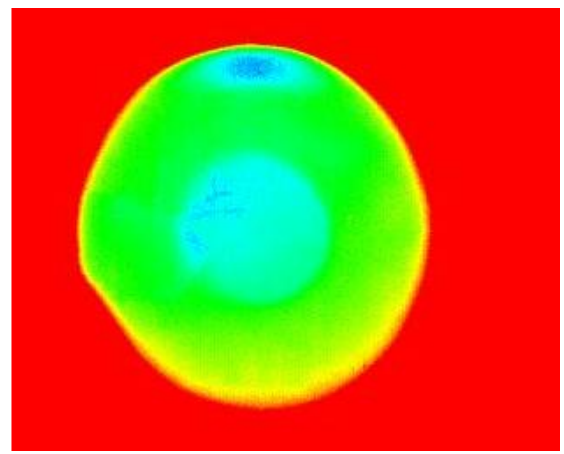


2 mm glass bead maintained at 250 K by the gas stream shown with the shutter closed and with the shutter open.

Bead calibrated at 300K, 250K and 200K.

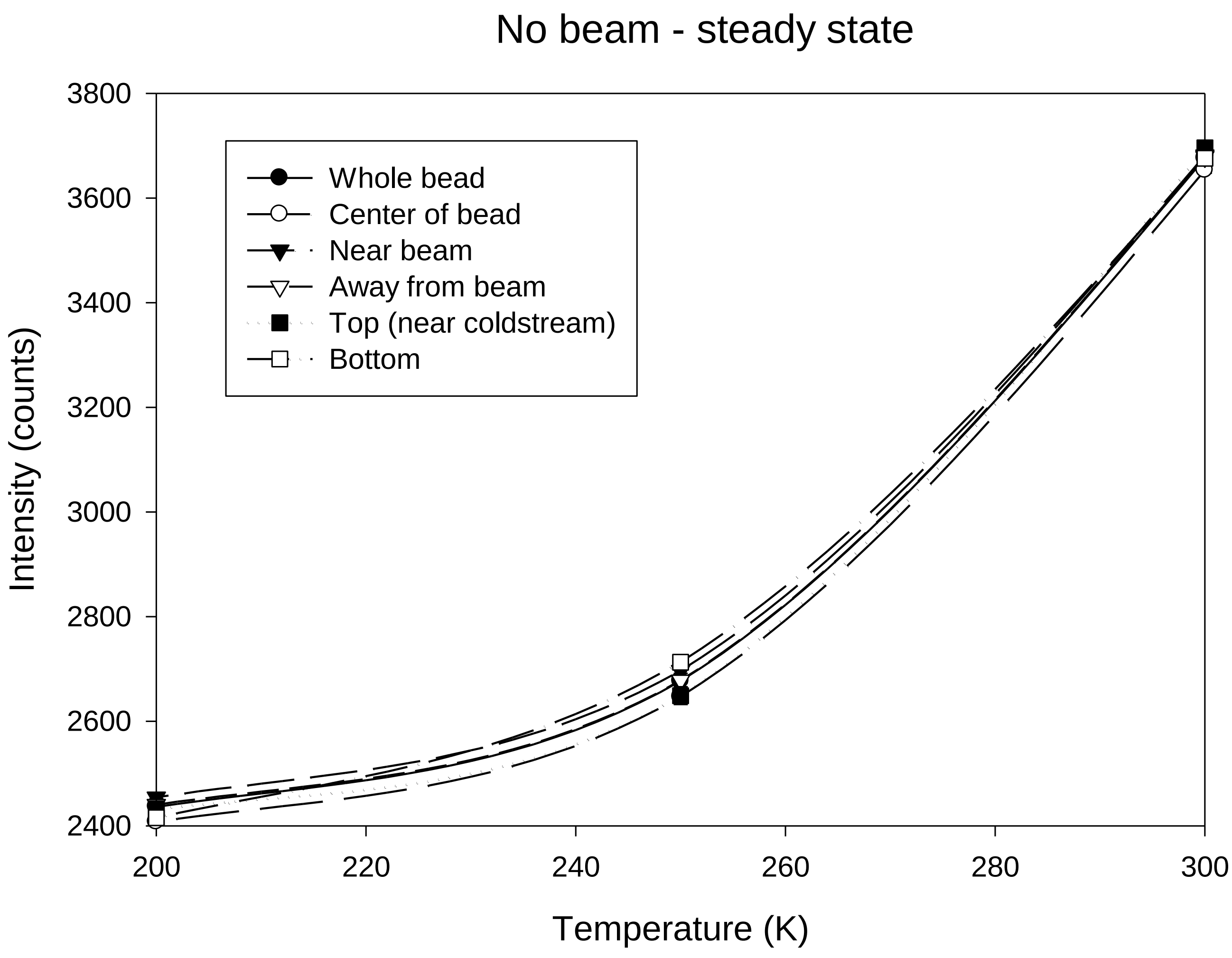


Beam off

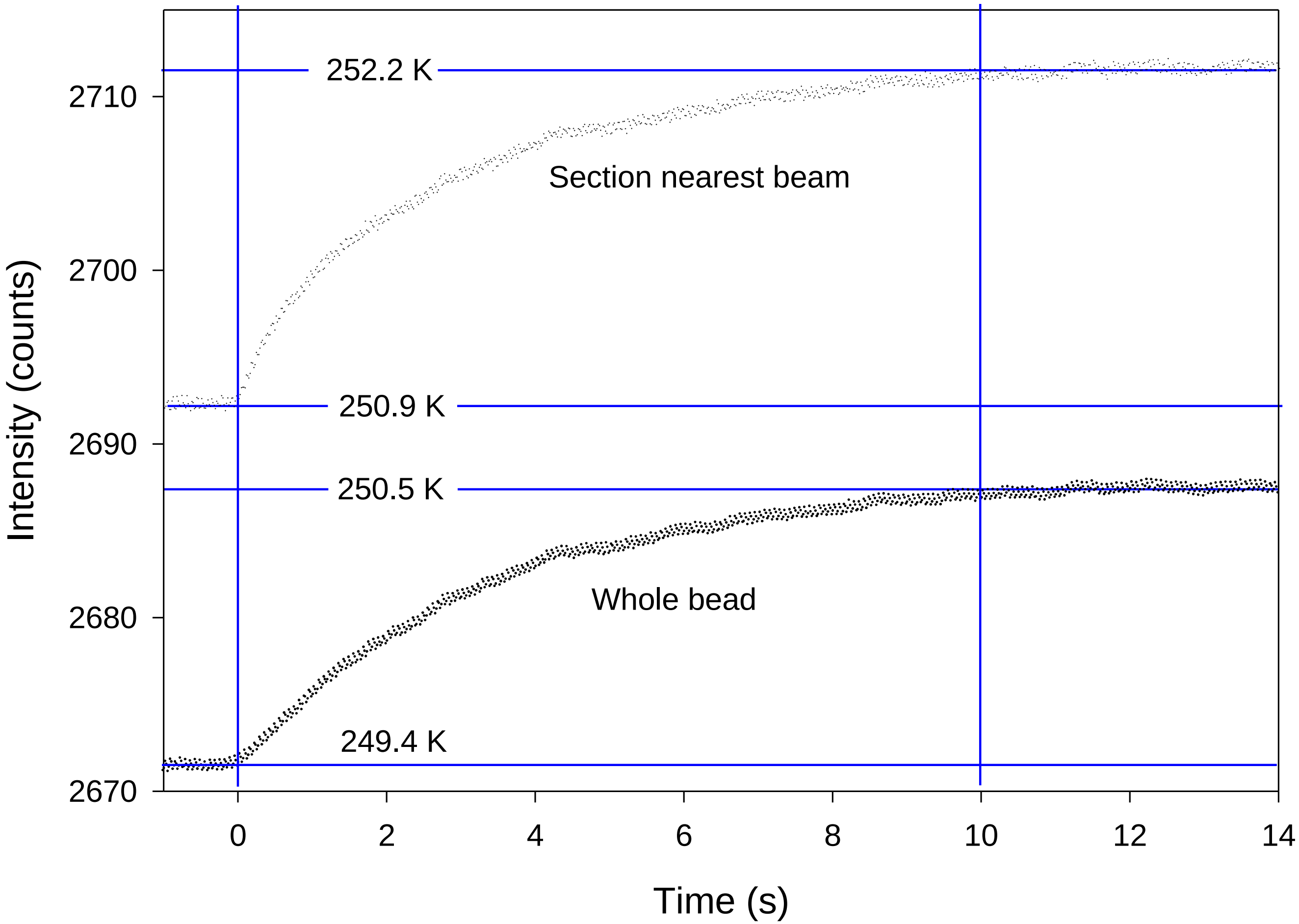


Beam on (steady state established)

Calibration curve



Beam heating at 250 K



Preliminary Results

- Glass beads were studied in 300, 250 and 200 K streams from the cryojet with beam and no-beam incident.
- The beads were studied as a whole and in sections closest to the beam, the cryostream jet and far from the beam and jet.

  – At 250 K it took about 10 s to reach steady state after the beam was incident on the bead.

  – The whole bead warmed up by 1 K.

  – The section nearest the beam was warmest overall but only warmed by 1K.

  – The section nearest the beam warmed marginally faster than the whole bead.

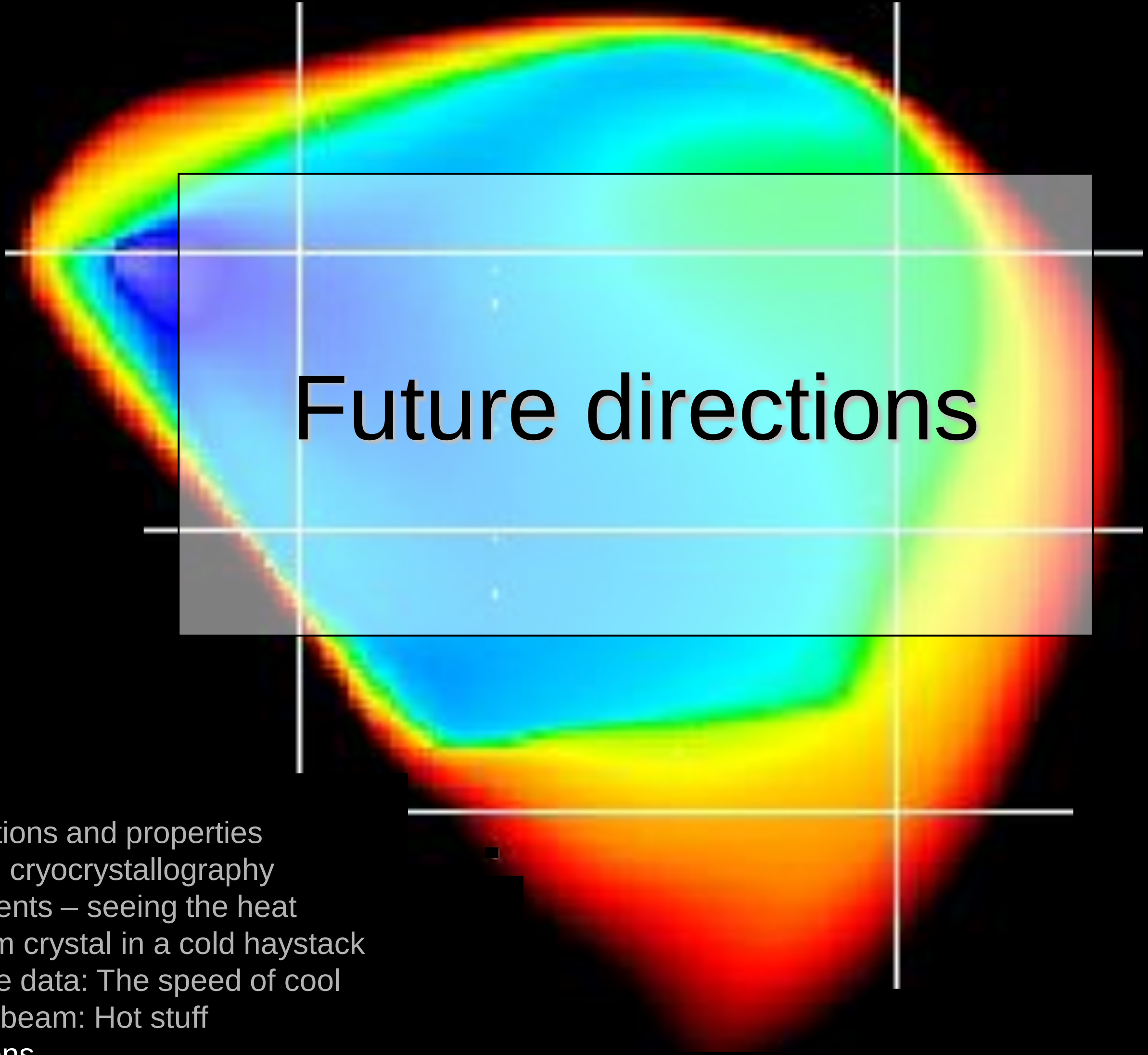
  – No data was recorded on the cool down once the shutter was closed.

  – There is a 2 K difference between the point near the beam and the point furthest away (data not shown).

Observations

- For the case studied:
  - Beam heating does not seem to be a significant problem.
  - It takes a surprisingly long time to reach steady state, it is not known if that time is the same for the sample to cool once the shutter is closed.
  - There is a temperature gradient across the sample.
- More data will have to be collected. This study represents the first of several experiments and has provided data on how to do the experiment well and more importantly, what experimental parameters will be used.

Future directions



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Very short term plans…..

- Fire Murphy
- More data
- Model development
- Publish

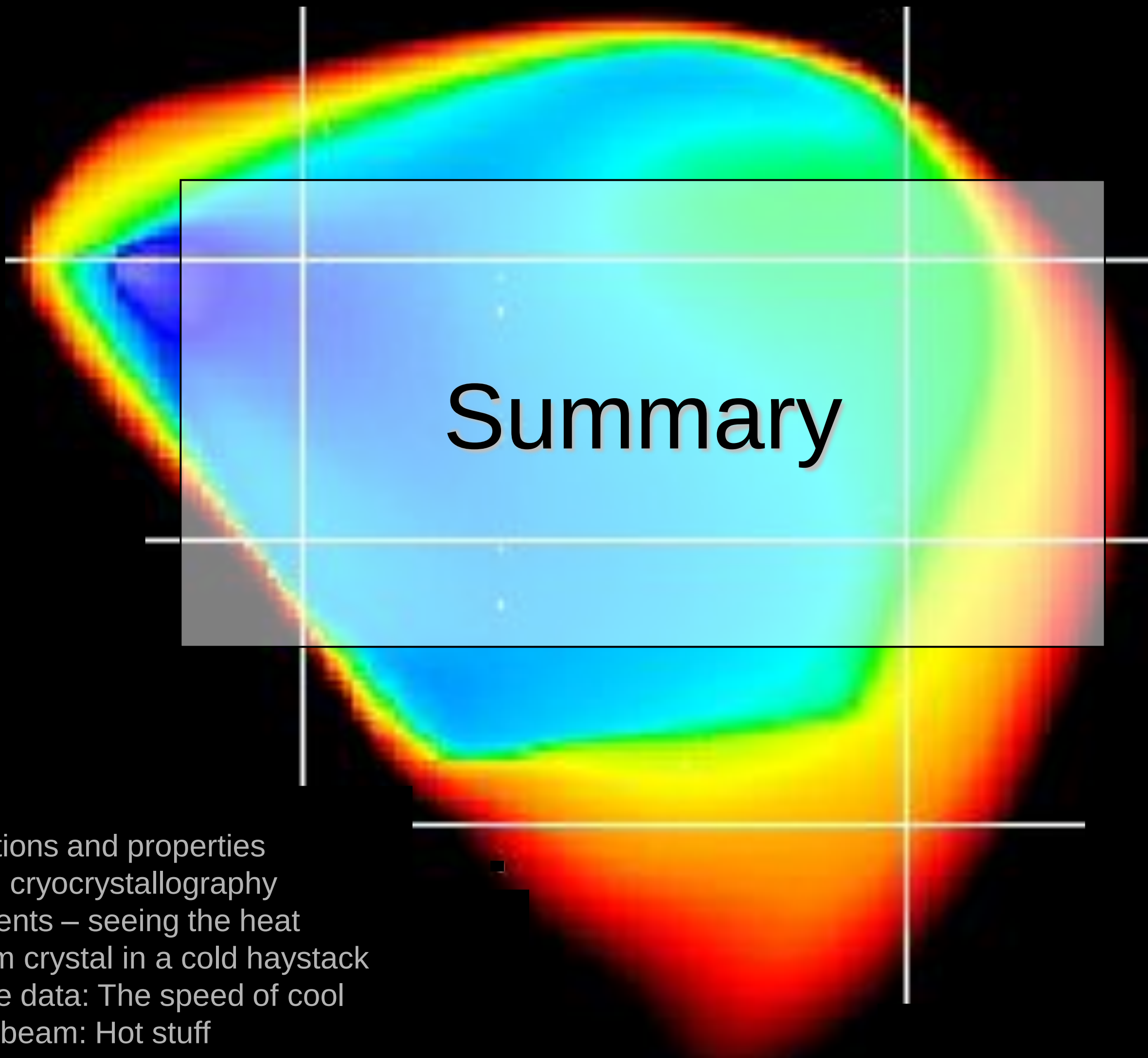
Short/medium-term plans

- Crystal cooling
  - Look at cooling as a function of sample, cryoprotectant, direction, shape, flow and cooling method.
  - Test model and develop it further..
- Crystal location
  - Design and produce optic with increased depth of field and cooling potential.
  - Develop a practical location station.
- Beam heating
  - Look at heating as function of exposure, cooling (e.g. flow, direction), sample and wavelength.
  - Test and develop model.
- Instrumentation/technique
  - Shielding stray infrared radiation
  - Increasing sensitivity through lens cooling

Long-term plans (pie in the sky)

- Crystallization
  - At ambient temperatures the IR camera used is sensitive to 15 mK temperature differences. Look at heat of crystallization.
  - Examine salt/protein crystals. Preliminary data shows that for the few salt crystals examined there is a dramatic difference in the emissivity for IR radiation.
- Crystal location and cooling
  - Build a stand alone robotic station to receive crystals, cool them efficiently, locate them, store them and pass on the position to a beamline that could automatically mount the samples.
- Heavy atom soaking, biochemical reactions
  - Use the technology to determine the state of biochemical reactions within crystals.
- Instrument/techniques
  - Stand alone calibration to temperature – understand the different infrared reflectance transmittance properties of crystals.

Summary



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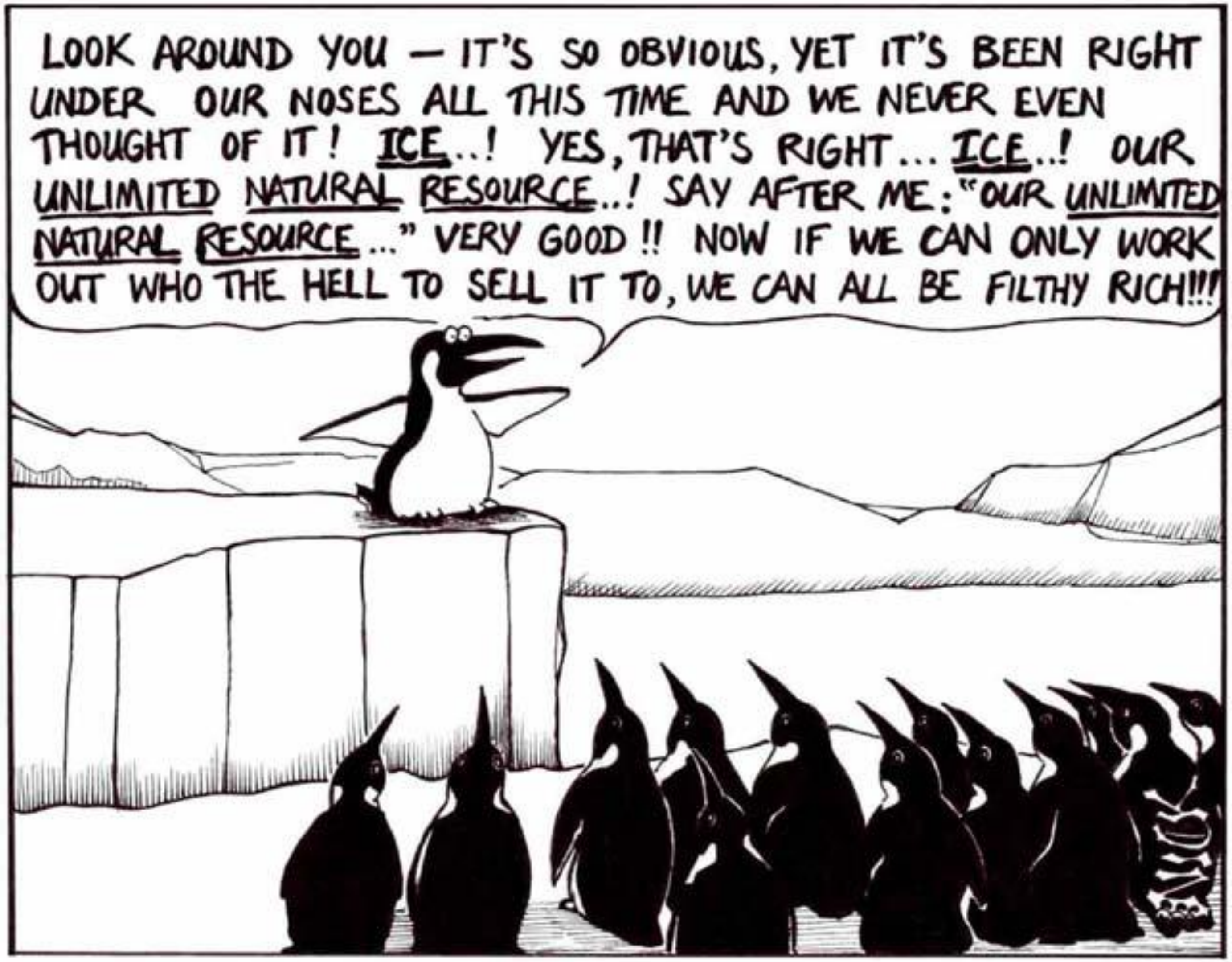
Summary

- Thermal imaging can be used to image cryogenic processes.
- Cooling of crystals takes place in a wave from the origin of the cold source to a point furthest from it – does this mean that cryocooling in liquid rather than a gas is better?.
- Thermal imaging can be used as a data collection tool, locating crystals, as well as a research tool.
- The cooling is independent of direction.
- Smaller crystals cool faster.
- Loop size is not a major factor.
- Beam heating is minimal.

…. For the samples studied.



Typical motivational speaker.