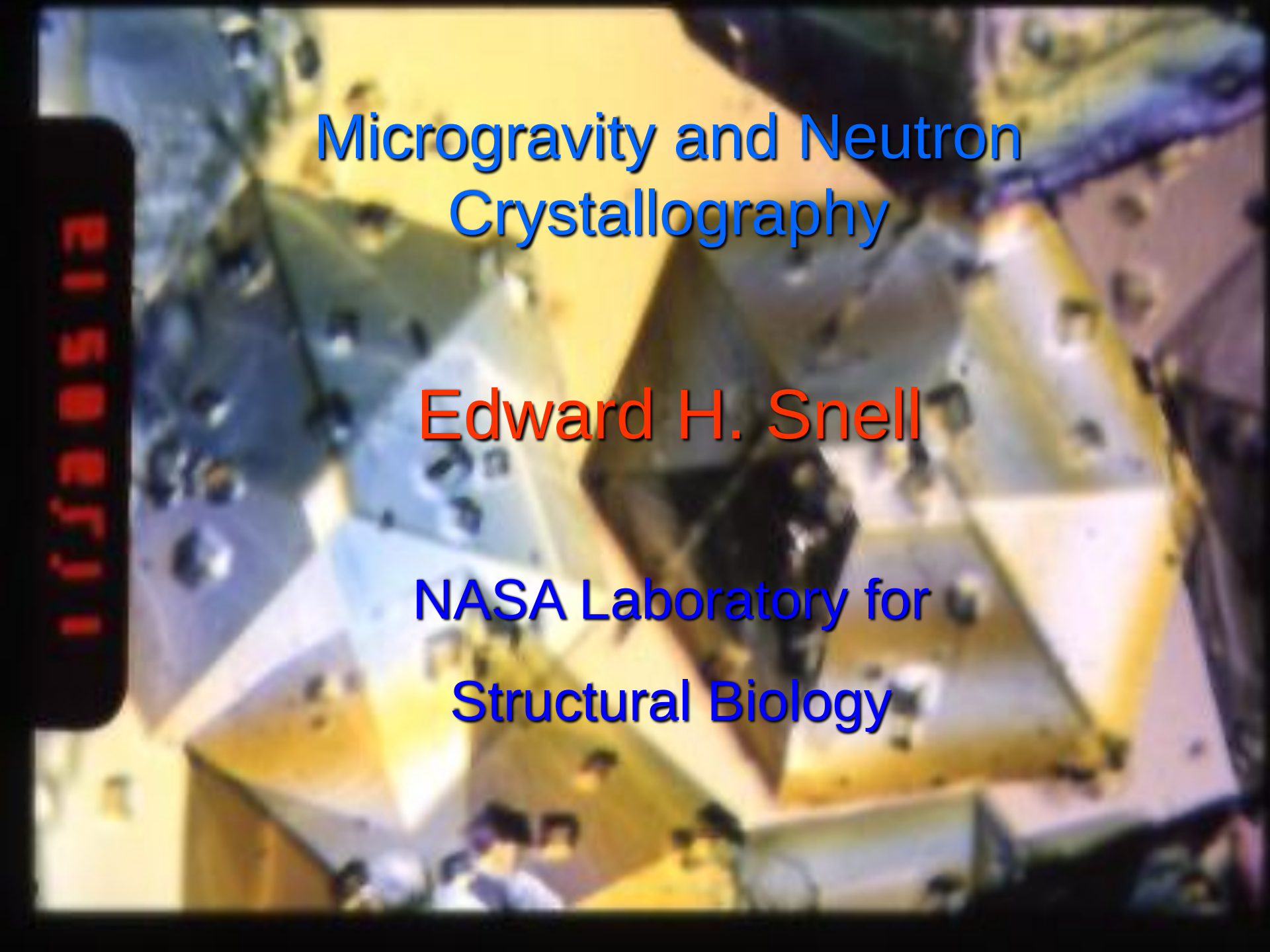
Microgravity and Neutron Crystallography
Edward H. Snell
NASA Laboratory for Structural Biology

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Summary of Talk

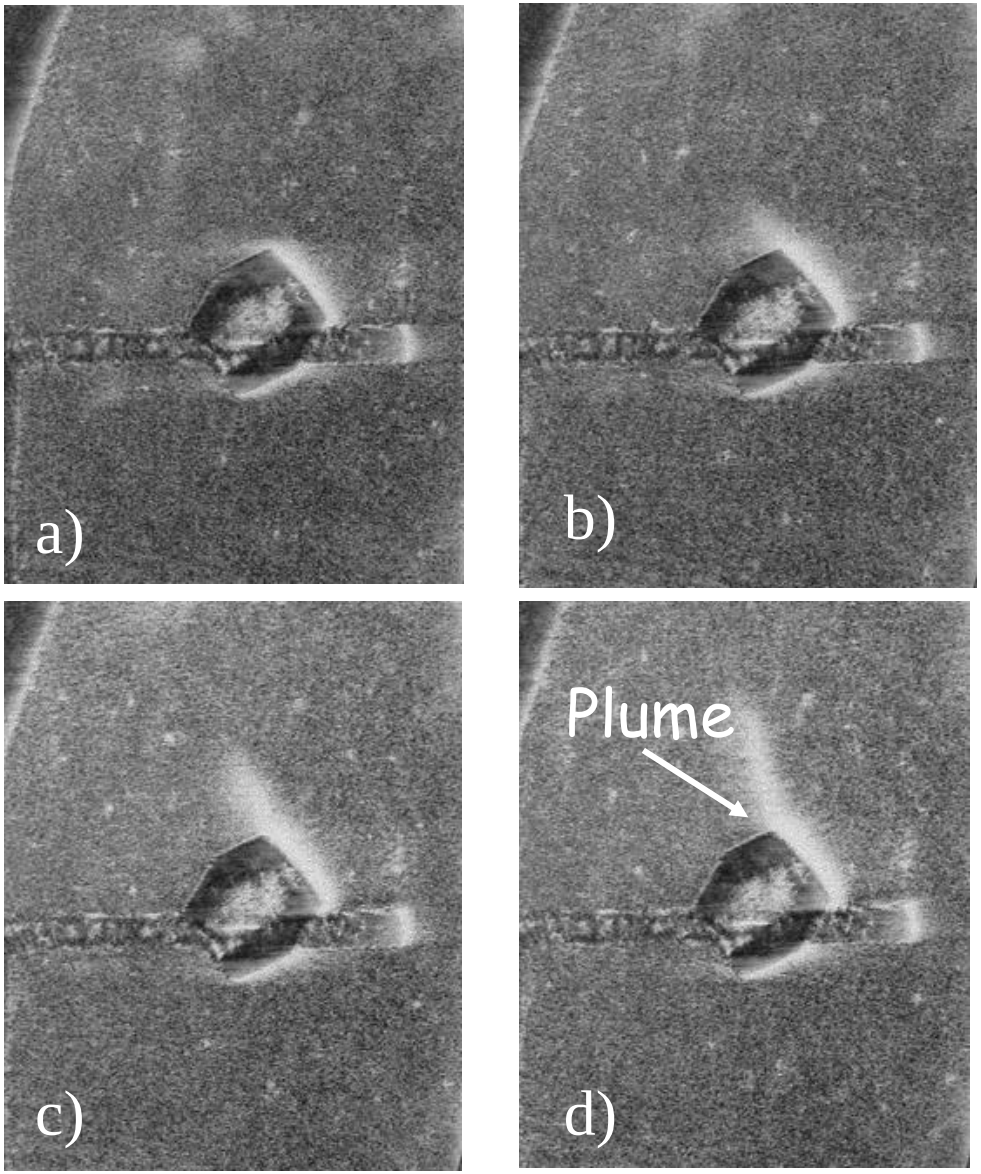
- Microgravity reduces sedimentation and buoyancy driven convection
- Larger volume crystals of reduced mosaicity result
- Neutrons scatter weakly and large crystals and large fluxes are needed
- The Laue method provides larger flux but needs low mosaicity crystals
- Microgravity can grow larger crystals enabling many neutron experiments


Why grow crystals in microgravity?

On the ground:

As the solution surrounding the crystal becomes depleted of the growing macromolecule the solution starts to rise due to density differences.

A convective growth plume of solution flows over the crystal face impeding growth and the quality of crystal packing.



Schileren photograph of a growth plume rising from a lysozyme crystal (pH 4.0, 0.1M sodium acetate, 5% NaCl at 15°C.

M.L. Pusey, J. Cryst. Growth, 122, 1-7, 1992

In microgravity:

Buoyancy driven convection effects are greatly reduced:

- A zone of depleted material is formed around the crystal. This is termed the depletion zone.
- Crystal growth is dominated by diffusion transport allowing more ordered growth producing a more perfect, higher quality crystal.

Sedimentation of the crystals to the bottom of the well does not occur:

- Crystals are suspended in nutrient for a longer time hence they grow to a larger volume.

Experiments are small volume, have low mass and have high potential scientific and commercial return:

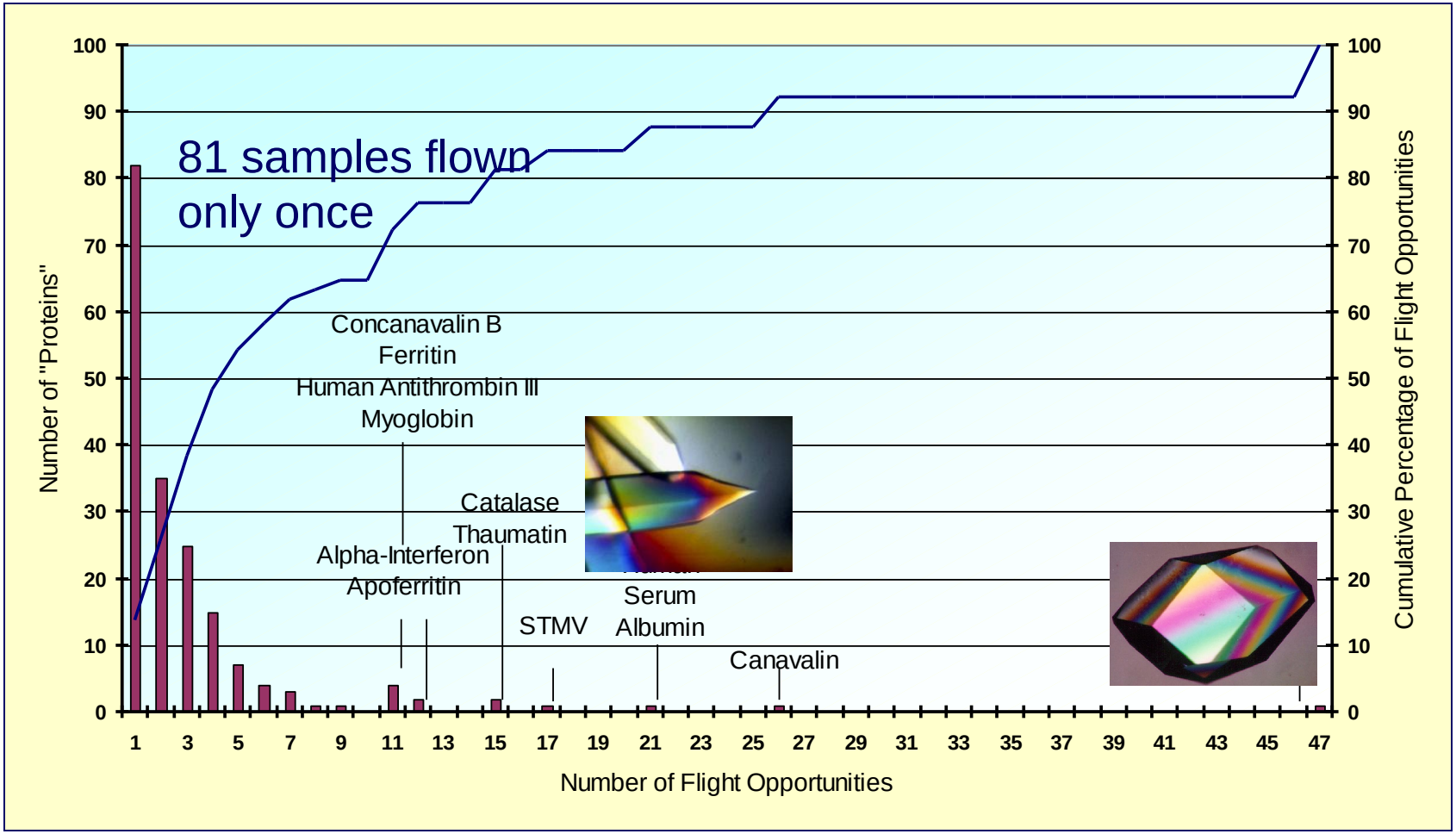
- Many experiments can be flown at one time and even a low success rate would still result in many successful results

Microgravity produces larger more perfect crystals

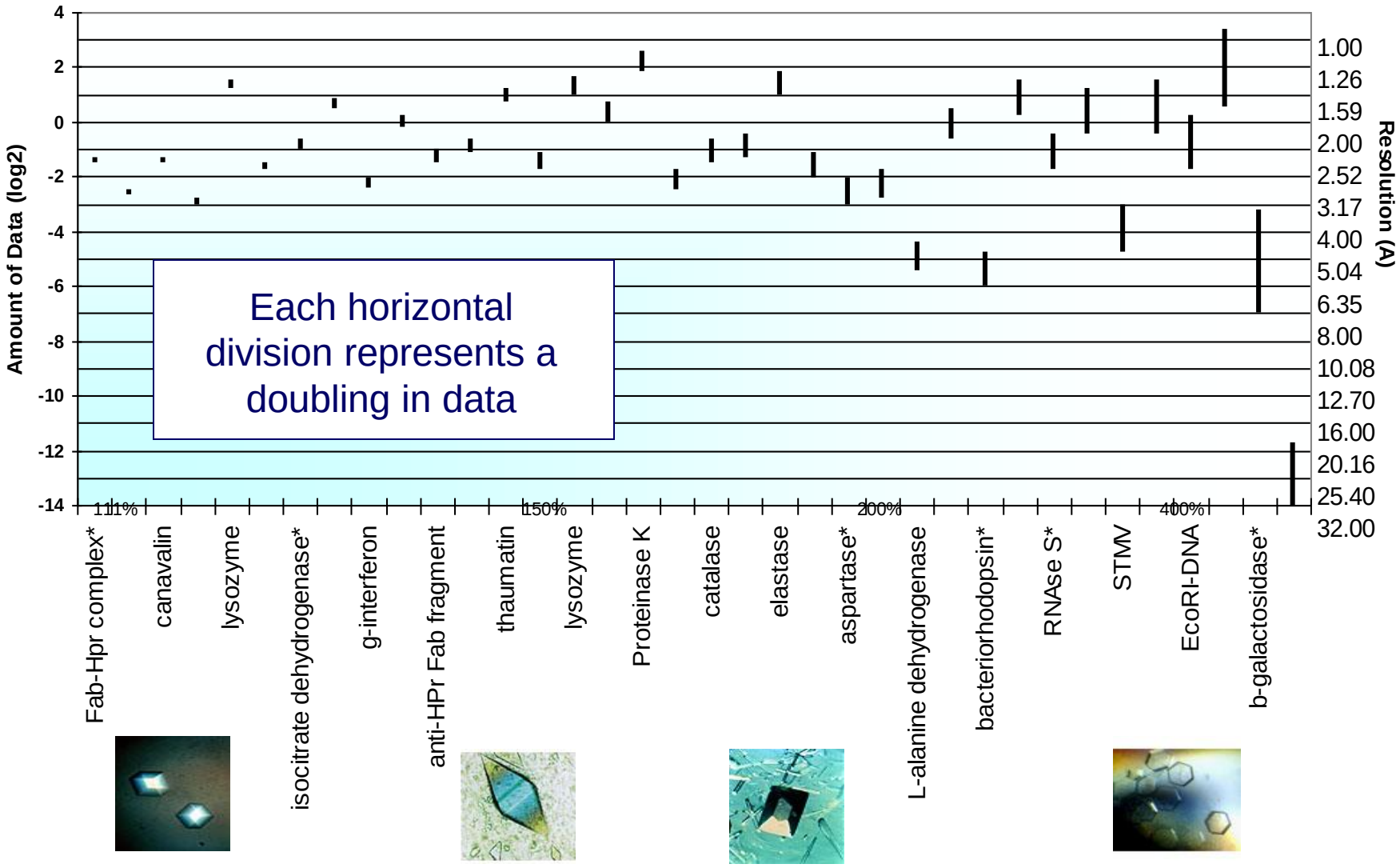

Experimental History

- First flight April 1981 using a sounding rocket
- Since then to the year 2000, NASA flew 45 flights on the Space Shuttle Orbiter with only 5 missions dedicated to microgravity
- The first 7 of these 45 missions had no temperature control
- 8 missions delivered experiments to the Mir space station which suffered from temperature variation (days above 30°C), extreme vibrations/accelerations and even the occasional fire!
- Currently the first experiments have been flown on the International Space Station

Over 185 different macromolecular samples have flown. Some are frequent flyers, others have flown only a limited number of times



Microgravity and Macromolecualr Crystallography: Kundrot, C.E., Judge, R.A., Pusey, M.L., & Snell, E.H. Crystal Growth and Design. Crystal Growth and Design, 1, 87-99 (2001).



Improvements seen from microgravity samples (same reference as previous figure)

Results from previous missions:

Up to the end of year 2000, 181 different macromolecules have flown:

- 81 flew only once
- 36 flew only twice
- 12 have flown over 10 times, all exhibiting improved results
- Lysozyme has flown 47 times

On average there are only 40 trials per macromolecule, allowing for duplication only 20 experimental conditions.

Success rates are:

- 20% improved overall,
- 35% for those that flew more than once
- up to 60% for those that have flown four times or more.

Specific examples of volume increase

Lysozyme STS-51F, 72, 89-M8

γ-interferon STS-26

Bovine insulin STS-37, 43, 49

Luciferase STS-42

Canavalin STS-42

2 domain CD4 STS-48

Factor D STS-50

α-interferon STS-50, 52, 68

Proline isomerase STS-50

Brain prolyl isomerase STS-50

Human insulin STS-60, STS-95

STMV STS-65

Apocructacyanin C1 STS-65

Catalase STS-73

Photosystem 1, STS-73

Pike Parvalbumin STS-94 (neutron study)

HIV Protease STS-85

Argumenter of liver regeneration, STS-73

raf kinase, STS-73

Concanavalin B, STS-77

alcohol dehydrogenase, STS-78

α-amylase, STS-79, M4

acid phospholrase a2, Chinese satellite

L-alanine dehydrogenase, STS-73

Thaumatin (ISS)

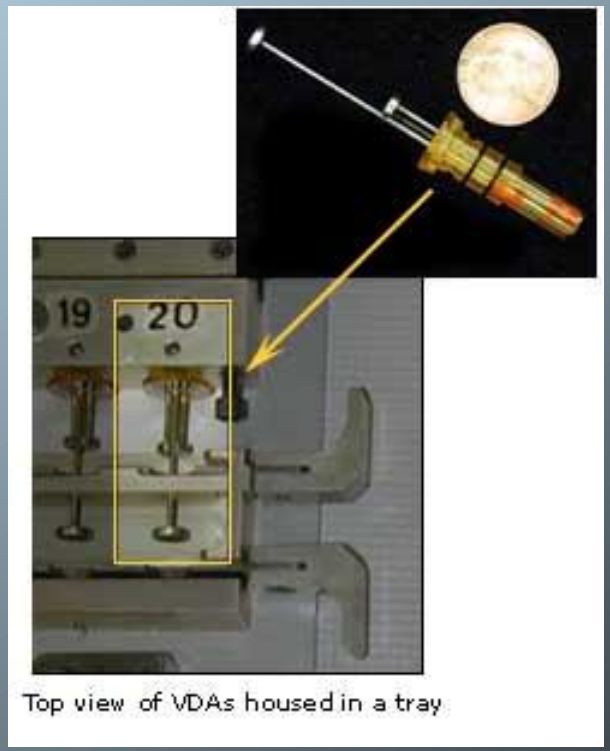
Types of Crystallization Hardware

PCAM trays with 7 experimental cells (top).

Nine trays are housed in one cylinder (center).

Six cylinders fit in a thermal carrier and are housed in an EXPRESS rack on ISS (bottom).

DCAM trays shown in Single-locker Thermal Enclosure System (STES)

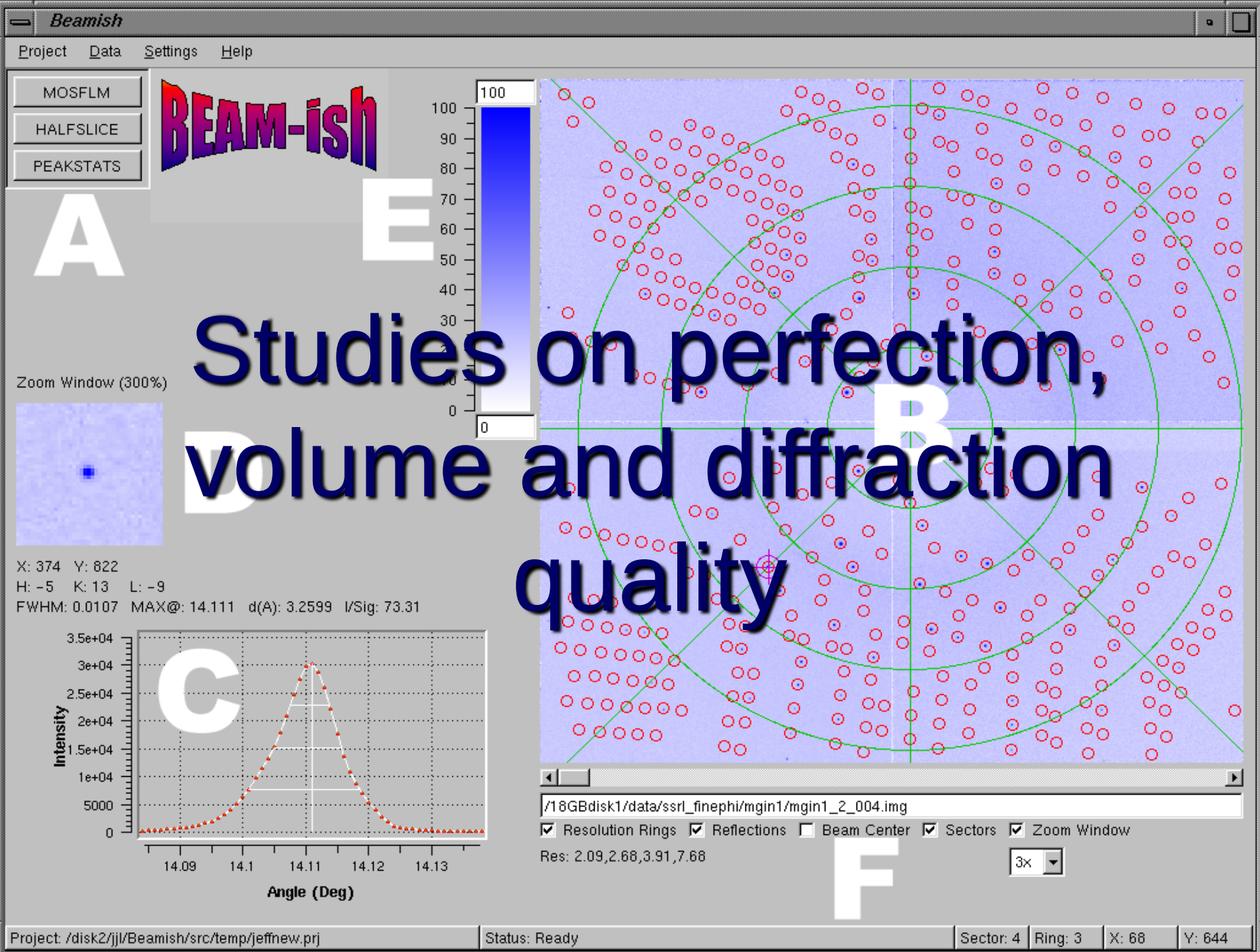


Top view of VDAs housed in a tray

See http://crystal.nasa.gov

The Laue Method

- Polychromatic incident wavelength
  - Exploits more of the available flux – used for neutron diffraction where scattering is poor and flux is much lower than an X-ray source.
- Limited number of exposures needed for a complete data set
  - A series of still exposures are taken with large ϕ steps between images.
- Reflections suffer from overlap
  - Many are illuminated at any one time due to the wide sampling of reciprocal space.
- Needs low mosaicity, high quality crystals
  - Reduce reflection overlap
- For neutrons, need large volume
  - >1mm^3 to achieve good signal to noise in the data.



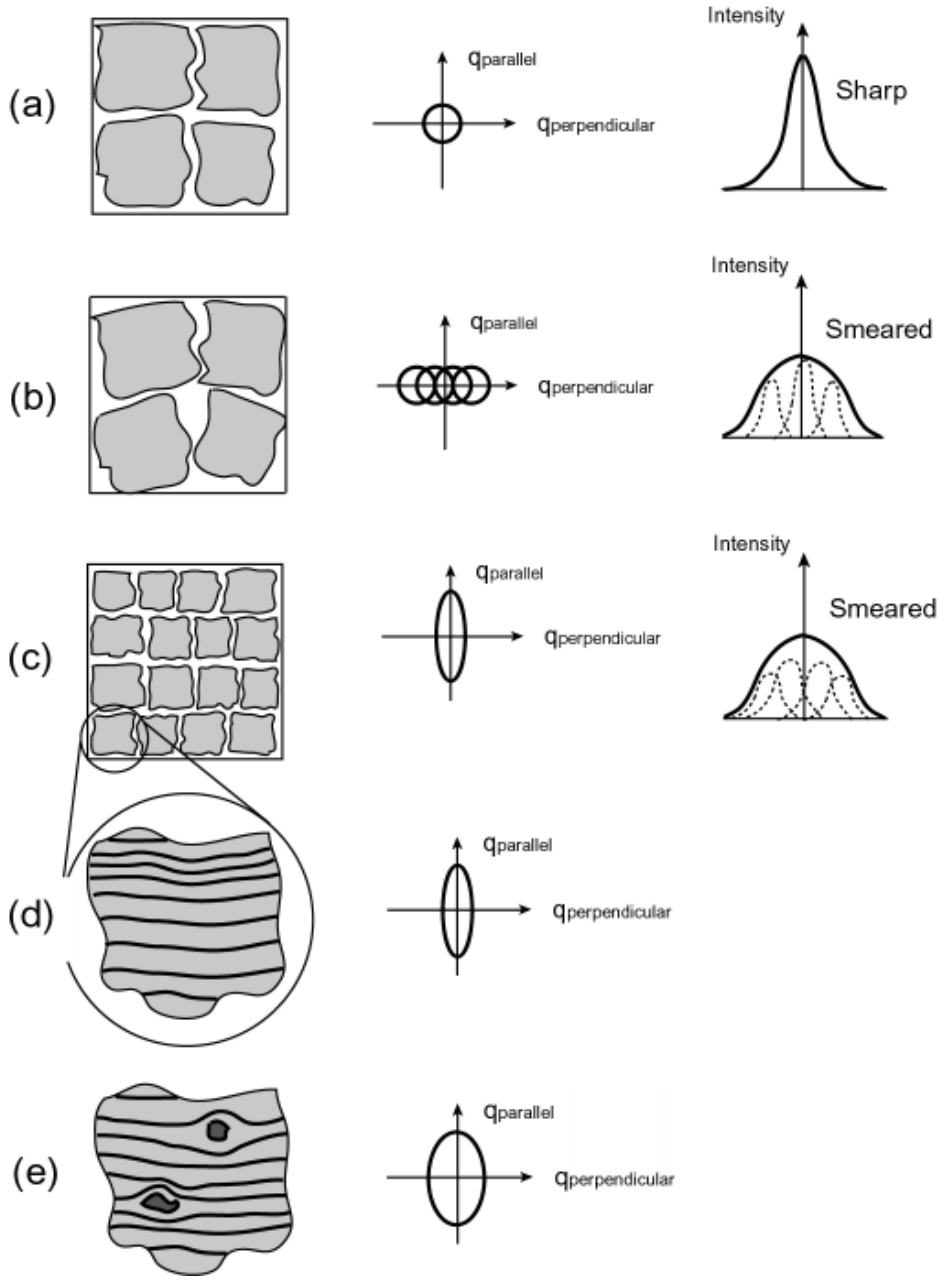
Studies on perfection, volume and diffraction quality


What is perfection?

Perfection is both good long-range and short-range order in the crystal

- Two types of order within the crystal
- Long-range – over many repeating units
  - Effects in real space large, small in reciprocal space. Increase results in reduced mosaicity and increased signal to noise.
- Short-range – molecule to molecule
  - Effects in real space small, global effects seen in reciprocal space. Increase results in increased resolution and reduced B-factors and diffuse scattering.
- The two types of order are not necessarily linked.


Long-range order -
Mosaicity



(a) Well ordered crystal

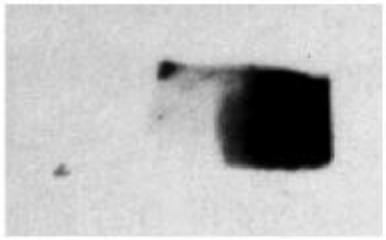
(b) Large domains are misaligned with respect to each other.

(c) Smaller domains give Fourier truncation

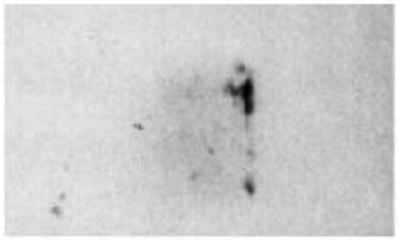
(d) Variation in a domain

(e) A number of effects combine

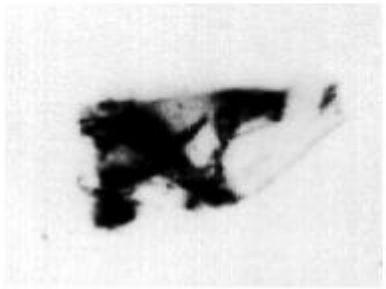
From Snell, Borgstahl and Bellamy, *"Methods in Enzymology, Macromolecular Crystallography Part C".* Edited by Charlie Carter and Robert Sweet – to be published



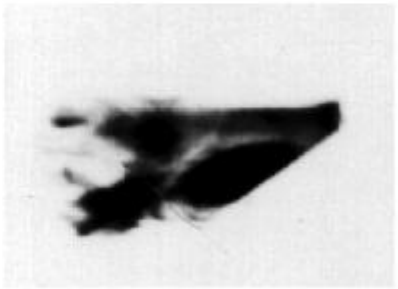
(a)



(b)



(c)



(d)

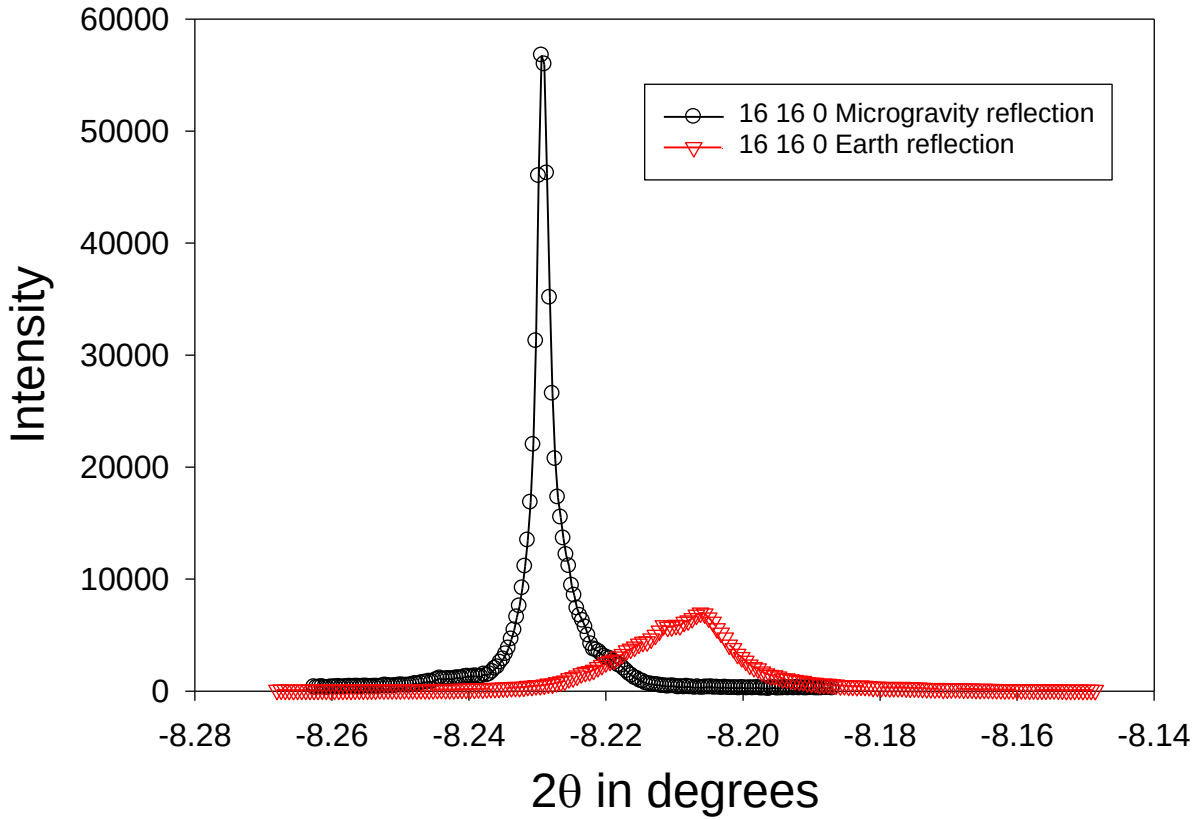
Evidence for the domain makeup of crystals.

X-ray topographs

From Snell, Borgstahl and Bellamy, *"Methods in Enzymology, Macromolecular Crystallography Part C".* Edited by Charlie Carter and Robert Sweet – to be published

Each topograph is a greatly magnified image of a reflection. In (a) and (b) the crystal is 1.1 mm by 0.9 mm in projection and defined regions are seen at the different reflections of (a) and (b). Some scattering is also seen on the crystal edges, probably due to mounting. In (c) and (d) the crystal is 1.5 mm by 1.1 mm in projection. In this case an array of domains is seen separated by a boundary layer. The different reflections (c) and (d) illustrate a region in the lower right of the crystal coming into the Bragg diffracting condition at the current orientation.

Original experiments using microgravity



Identical reflections from microgravity and ground grown lysozyme.

Eight times increase in signal to noise.

The larger illuminated volume only accounted for a doubling.

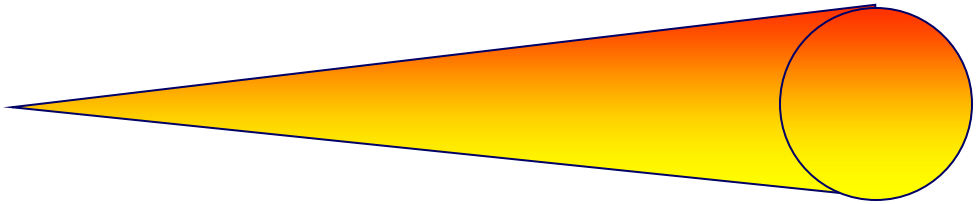
Microgravity 0.0023 degrees, ground 0.0130 degrees.

Snell et al., Acta Cryst. D51 099-1102 (1995)

How is mosaicity related to rocking width ?

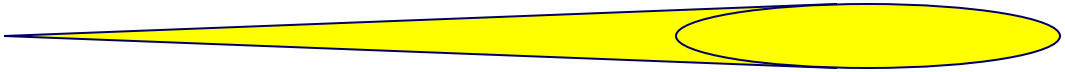
Three contributions from the X-ray beam

1



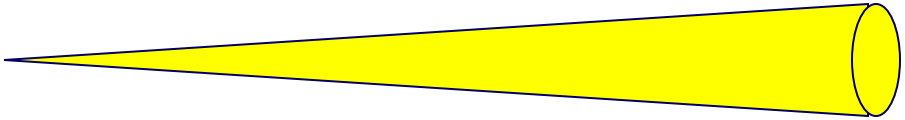
Spectral dispersion

2



Horizontal dispersion

3

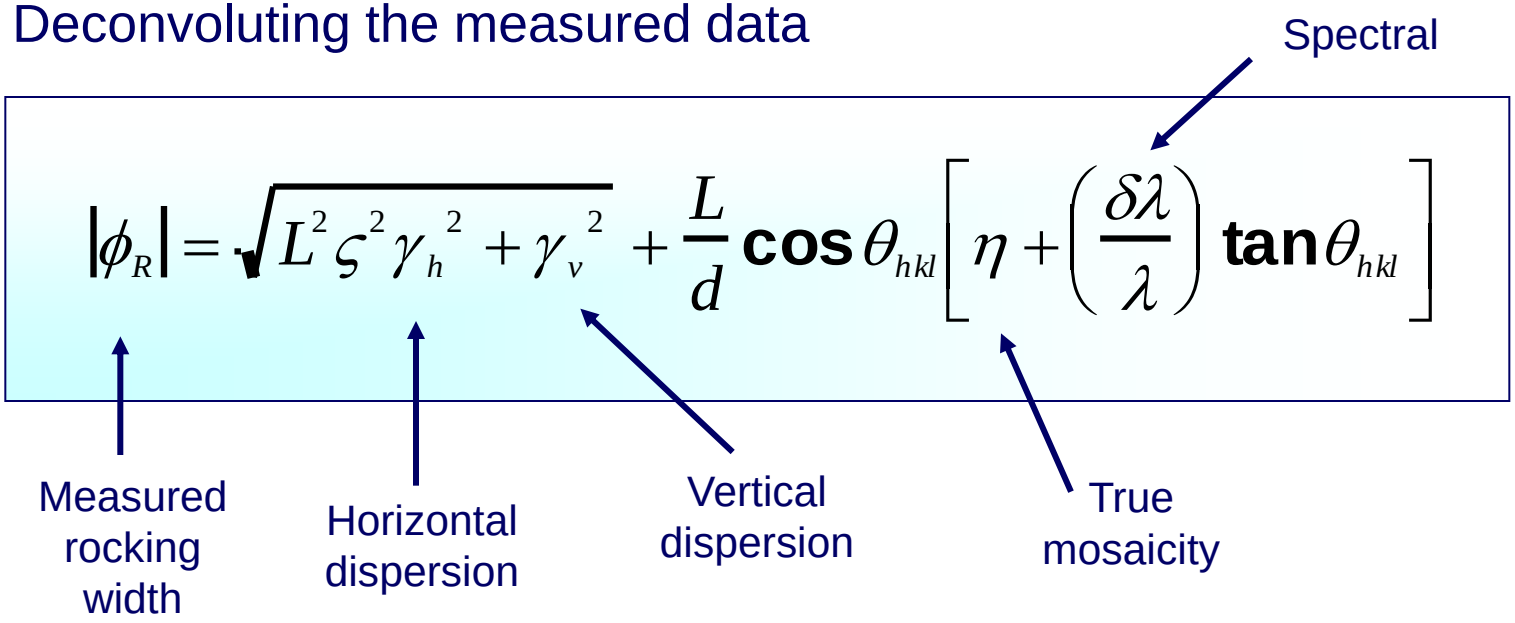


Vertical dispersion

Mosaicity is the reflection rocking width with these contributions deconvoluted.

From the theory to practice

Deconvoluting the measured data



To measure the mosaicity, η, record data in fine slices, 0.001 degree, minimize vertical and horizontal divergence (synchrotron radiation) and monochromate the beam.

See Bellamy, H. D., Snell, E. H., Lovelace, J., Pokross, M. and Borgstahl, G. E. O. "The High Mosaicity Illusion: Revealing the True Physical Characteristics of Macromolecular Crystals" Acta Cryst. D56, 986-995 (2000).

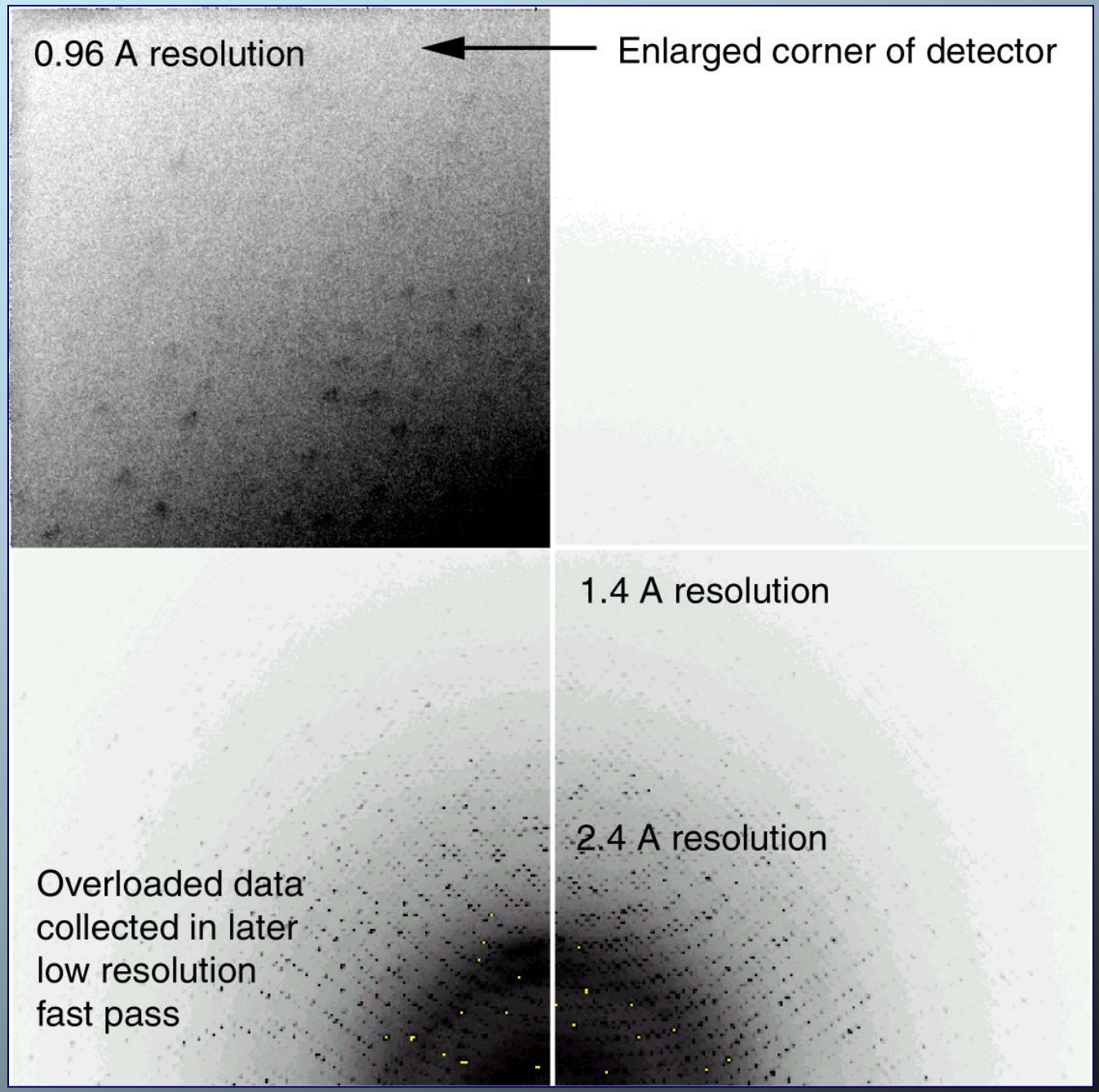
Short-range order - Resolution

Good Short-range order.

Mutated Glucose Isomerase X-ray data recorded at 100K.

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

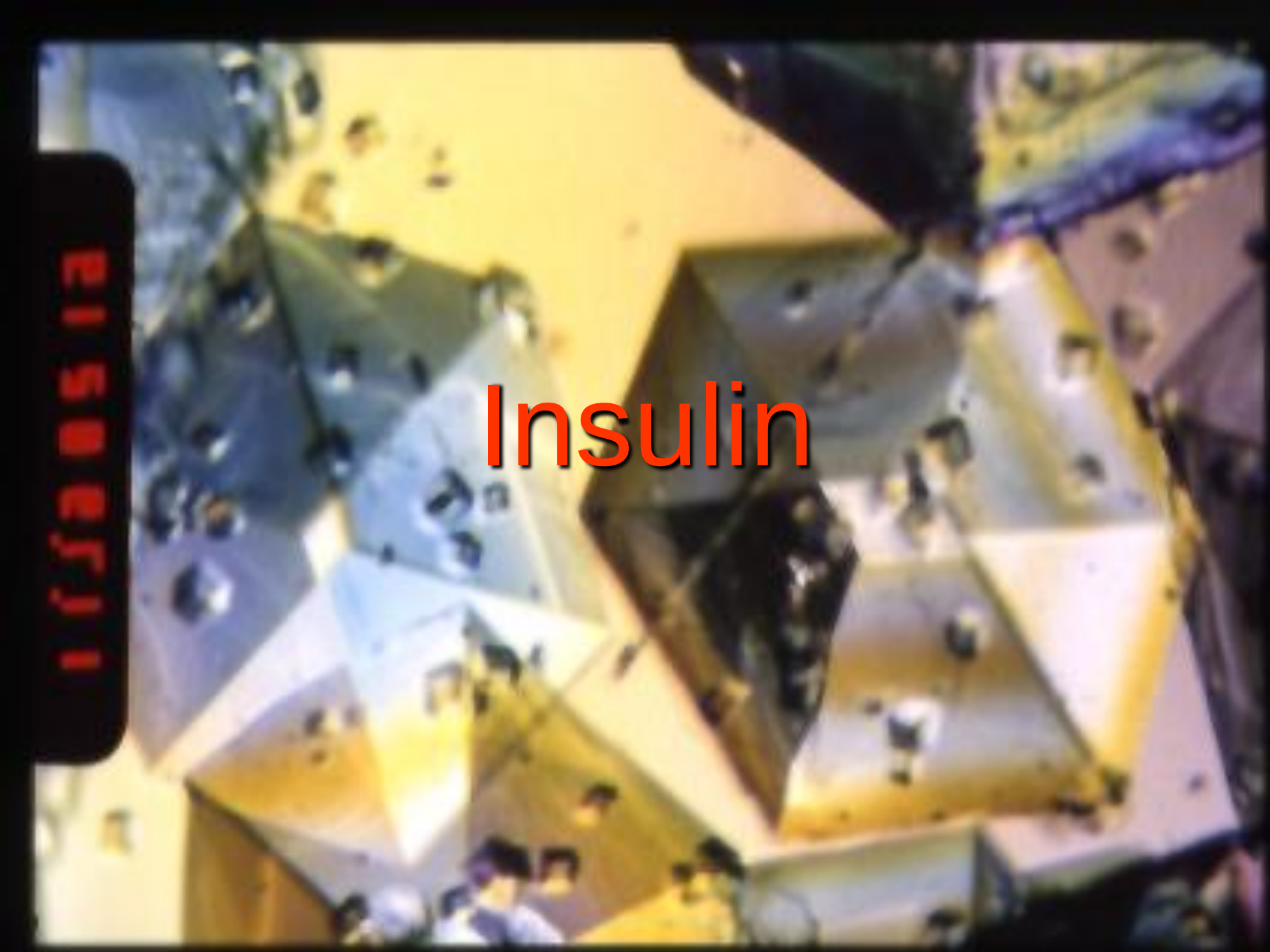
Data from beamline 9-2, SSRL, ADSC Quantum IV detector, 120 s exposure (dose mode equivalent at start).



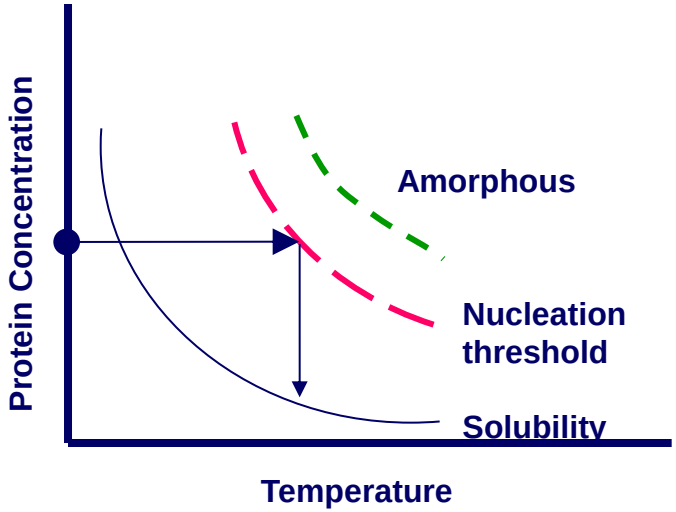
Our Experiments and Results

Experiments

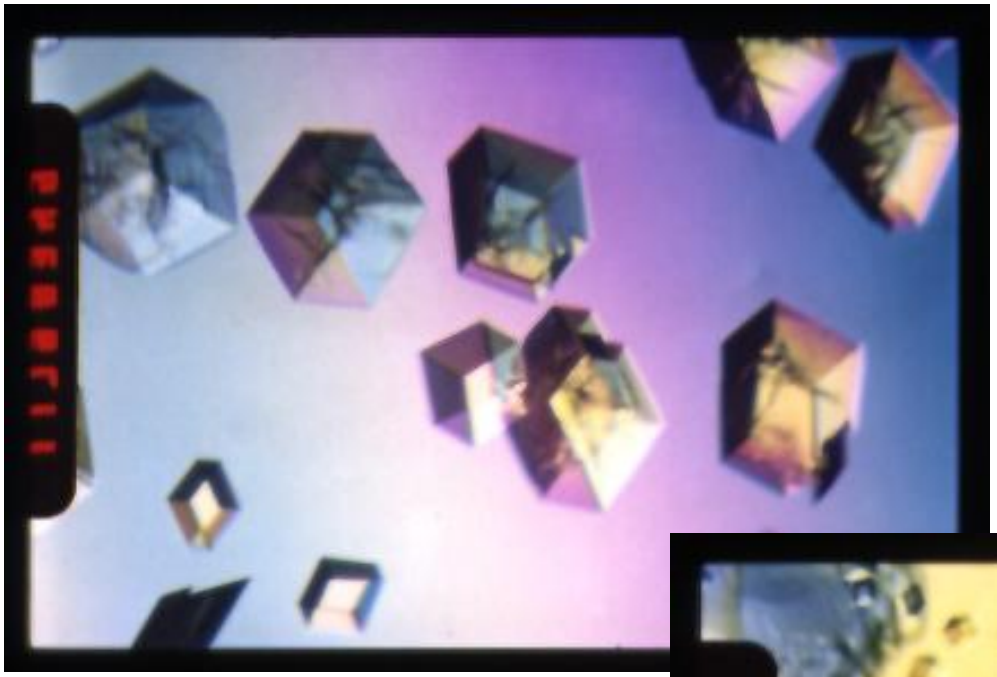
- Recombinant Human Insulin
  - Grown in the Protein Crystallization Facility (PCF) by temperature reduction on the STS-95 Space Shuttle mission (Launched October 29th, 1998).
- Chicken Egg-white Lysozyme
  - Grown by liquid-liquid diffusion on the STS-95 Space Shuttle mission
- Thaumatin
  - Grown by liquid-liquid diffusion in the Enhanced Gaseous Nitrogen dewar (EGN) on two successive missions to the International Space Station (Sep 11th-Oct 24th, 2000 and Feb 7th-Mar 21st 2001).
- Glucose Isomerase – to be launched
  - Grown on the ground by the batch method.


Insulin

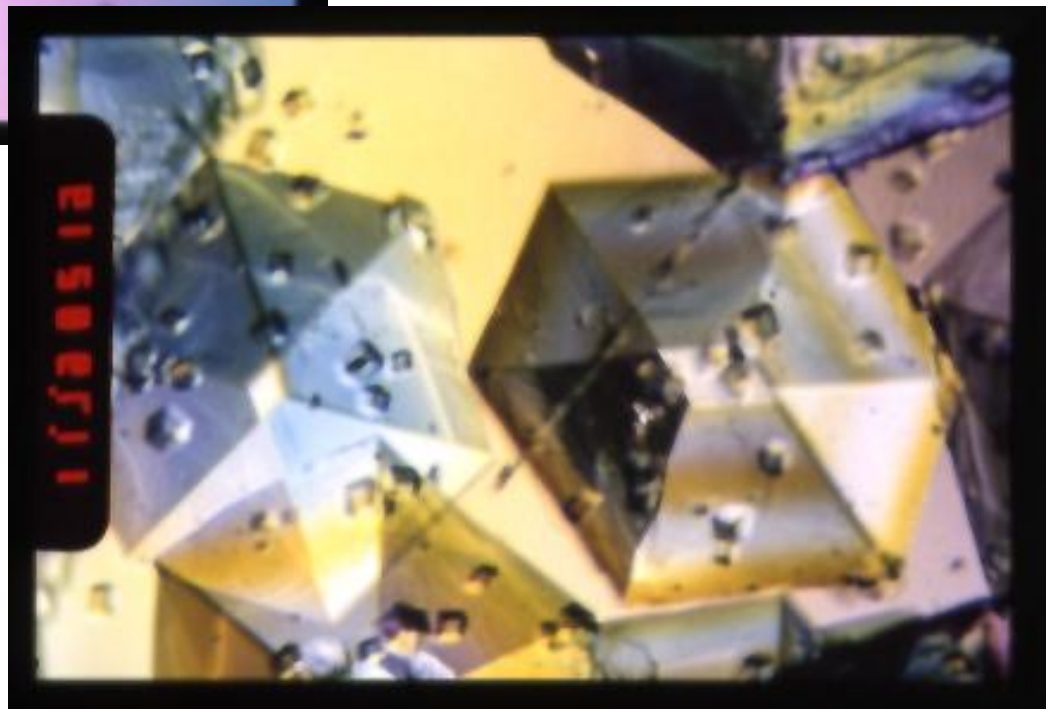
Temperature controlled method. Grown in Commercial Protein Crystallization Facility (CPCF)



Commercial production for pharmaceutical dosing purposes – uniform morphology and size of crystals from microgravity



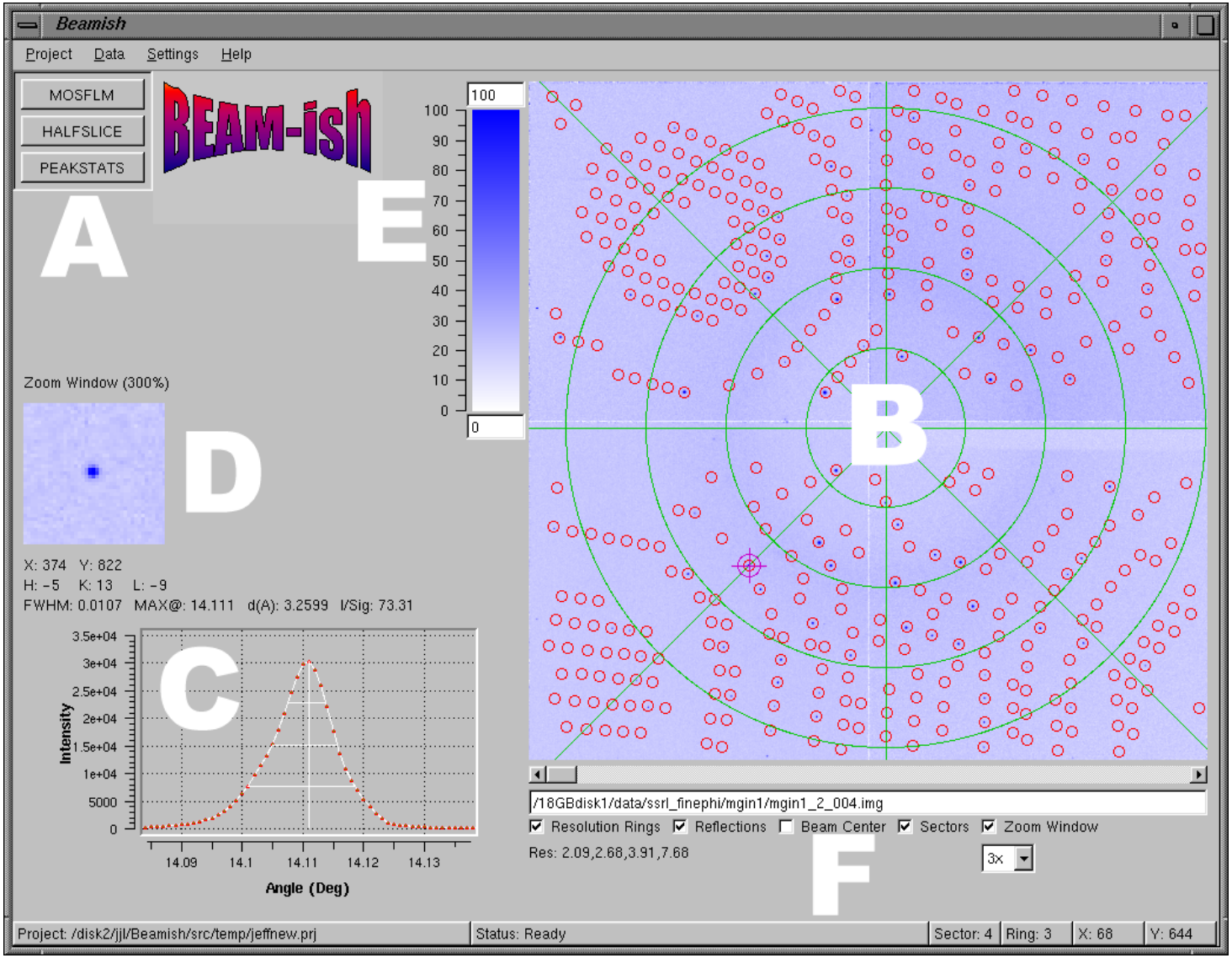
Images to same scale, sedimentation onto the bottom. Clumping of crystals.



Microgravity →

Many over 2 mm.
Free floating,
unsedimented.

Data Processing with BEAM-ish



Lovelace et al., J. Appl. Cryst. 33, 1187-1188, 2000

Data processing

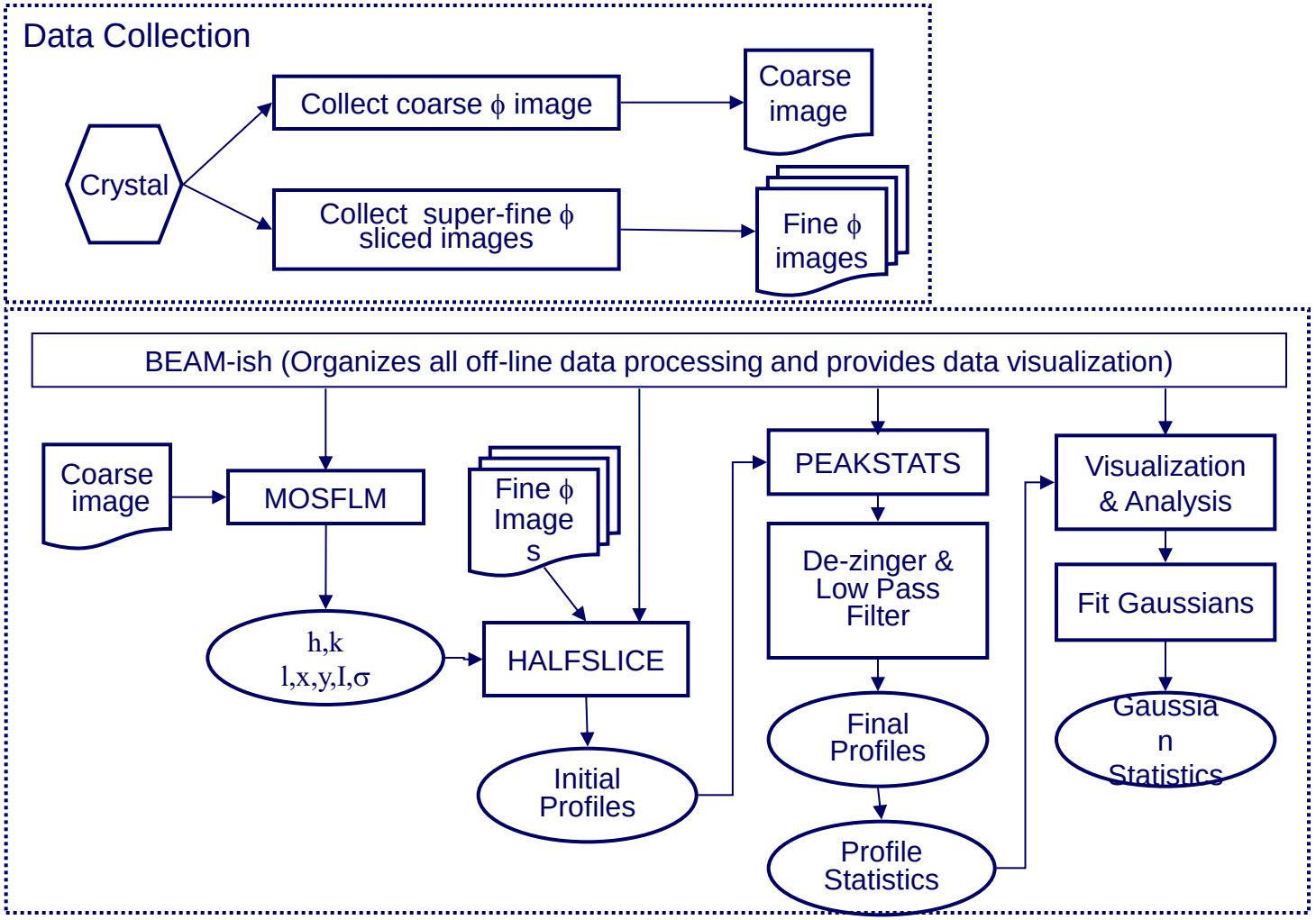
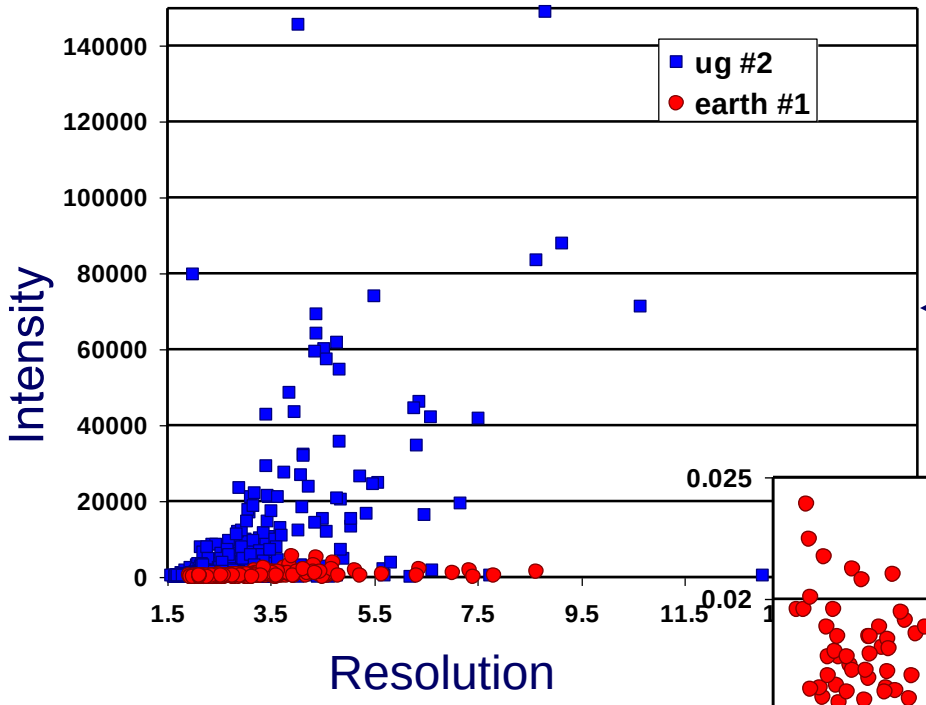


Table 1 Diffraction Statistics

Sample	Date[¶]	Orthogonal crystal dimensions (mm)	Crystal Volume (mm^3)	Avg. Max. Intensity (counts)	Avg. η[#] (degrees)	No. Refl.	No. data frames
earth-grown insulin crystals[†]							
earth-1	12/98	0.35X0.35X0.32	0.04	859	0.031 (0.017)	170	2000
earth-2	12/98	0.34X0.26X0.13	0.01	880	0.035 (0.015)	20	500
earth-3	12/98	0.40X0.27X0.19	0.02	914	0.017 (0.005)	174	2000
earth-4	7/99	0.43X0.34X0.19	0.03	202	0.038 (0.024)	14	2000
earth-5	7/99	0.39X0.24X0.22	0.02	590	0.013 (0.004)	172	1999
earth-6	7/99	0.39X0.24X0.17	0.02	431	0.023 (0.010)	72	2000
microgravity-grown insulin crystals[§]							
μg-1	12/98	0.96X0.88X0.37	0.31	18776	0.004 (0.002)	502	2000
μg-2	12/98	1.20X0.72X0.48	0.42	19528	0.006 (0.005)	241	1000
μg-3	12/98	0.90X0.88X0.32	0.25	8195	0.004 (0.004)	176	500
μg-4	7/99	1.29X0.84X0.43	0.47	12846	0.002 (0.001)	491	2000
μg-5	7/99	1.72X1.31X0.90	2.04	8362	0.004 (0.002)	489	2000
μg-6	7/99	1.59X1.59X0.50	1.25	7155	0.003 (0.001)	447	2000



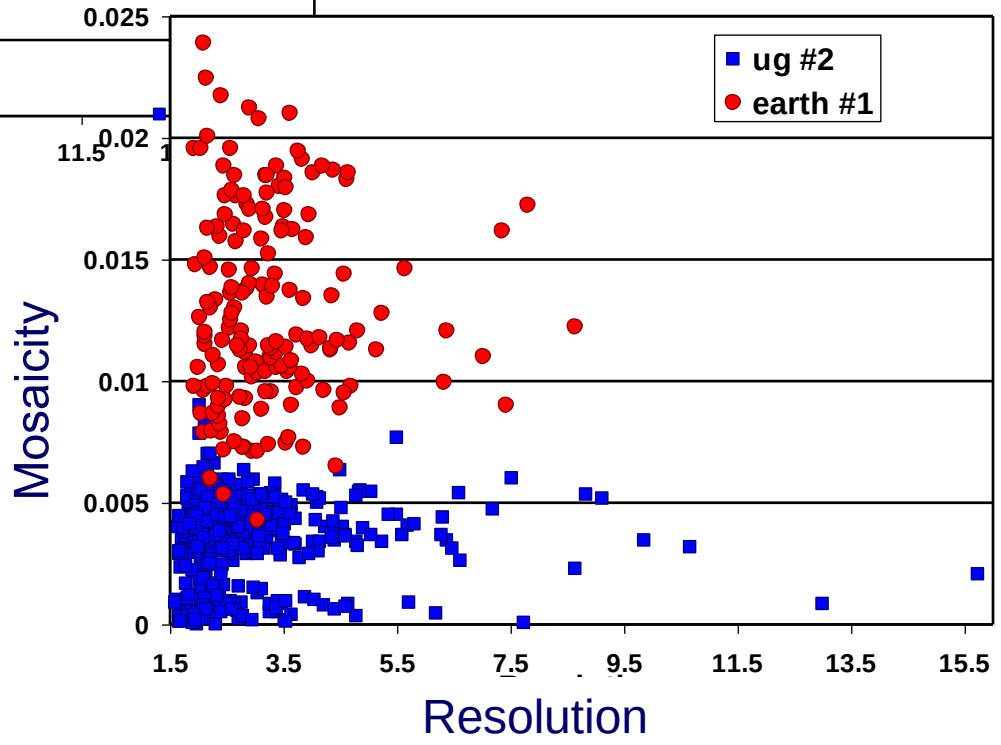
Microgravity Blue

Ground Red

← Structural, short range improvement

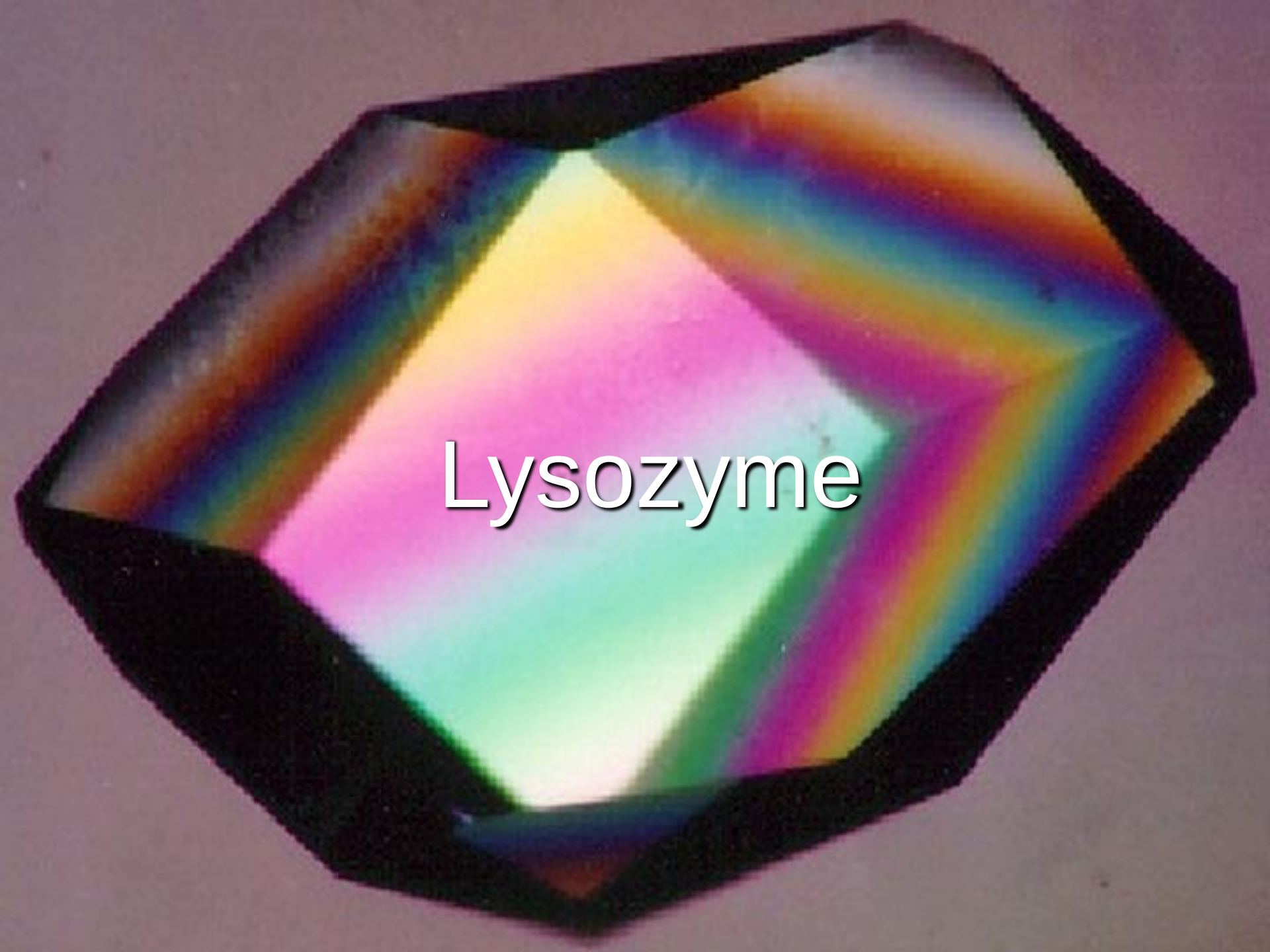
Physical, long range improvement.

The worst microgravity and best ground crystal data are shown.



Summary

- Microgravity crystals
  - Grown by thermal methods
  - had consistently larger diffracting volume > 2 mm in each dimension (34 times larger on average)
  - were more physically perfect (7 times lower mosaicity – 0.004° versus 0.026° on average)
  - (not covered in the talk) cryocooled extremely well for structural data collection
  - Showed a huge improvement in signal to noise


Lysozyme

Other partitioning results – *i.e.* why we did the experiment

Carter et al., 1999 “Lower dimer impurity incorporation may result in higher perfection of HEWL crystals grown in microgravity A case study”, *J. Crystal Growth* 196, 623-637, report:

A K_{eff} of 9 for ground

A K_{eff} of 2 for microgravity

for a lysozyme dimer impurity in crystallization of lysozyme.

Microgravity was seen to preferentially exclude the dimer – it seemed to act as an impurity filter.

Ground was seen to preferentially include the dimer. A significant result which can easily be tested.

Experimental

- Protein – Lysozyme extracted directly from fresh chicken eggs (Judge et al., Biotechnol. Bioeng. 1998, 59, 776-785).
- Dimer impurity – collected from late eluting fractions of lysozyme preparation on a cation exchange column.
- Crystallization:
  - STS-95, 9 day mission.
  - Microgravity – 0.0%, 0.5%, 0.9%, 1.8% and 3.6% impurities.
  - Ground – same conditions.
- Analysis
  - Size and axial ratios measured
  - Crystals washed and analyzed electrophoretically
  - X-ray data collected at Stanford SSRL beamline 1-5

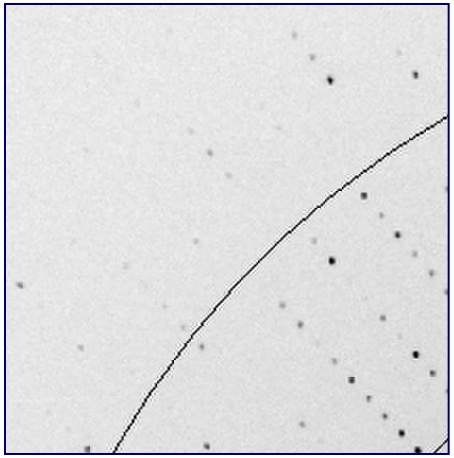
Experimental – X-ray analysis

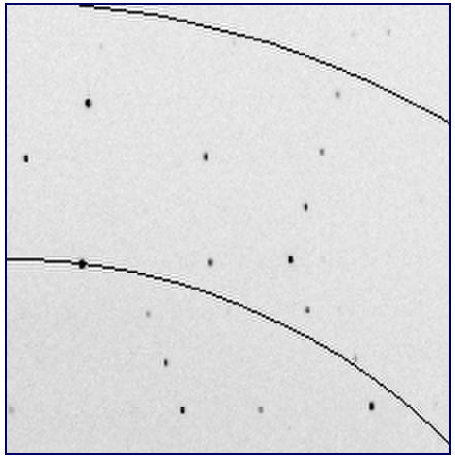
Crystal	Dimer	Size(mm)	Course Collection		Superfine Collection		Cell Parameters a=b, c (Å)	Date Collected
			Time	Images	Time	Images		
Microgravity	0%	1.12 x 0.96 x 0.72	20 sec	20	2 sec	2000	78.93, 38.02	7/99
	0.5%	0.62 x 0.61 x 0.37	120 sec	20	5 sec	2000	78.51, 37.50	11/98
	0.9%	0.40 x 0.26 x 0.19	60 sec	20	5 sec	1000	78.87,37.87	11/98
	1.8%	0.32 x 0.28 x 0.13	120 sec	20	5 sec	2000	79.09, 38.00	11/98
	3.6%	0.54 x 0.37 x 0.26	60 sec	20	5 sec	2000	78.89, 38.06	11/98
Ground	0%	0.86 x 0.40 x 0.40	20 sec	20	2 sec	2000	79.09, 38.09	7/99
	3.6%	0.48 x 0.35 x 0.32	15 sec	20	4 sec	2000	78.87, 38.07	7/99

For each crystal two 10° swathes of coarse data with $\Delta\phi$ =1° were collected 90° apart. Two 1° swathes of superfine f sliced data as 0.001° separated stills were then collected. For the ground 3.6% case swathes were collected 45° apart. The space group for all crystals was $P4_32_12$.

Results – Lysozyme, short-range improvement

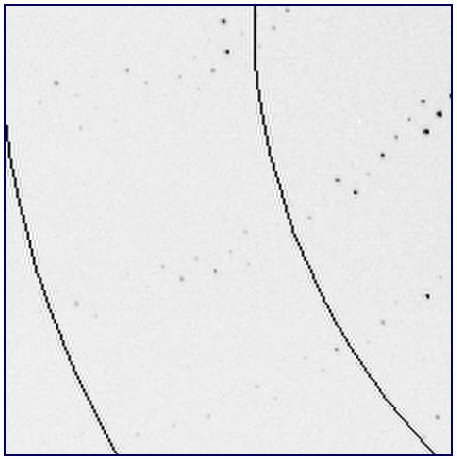
Microgravity, 20s exposure time

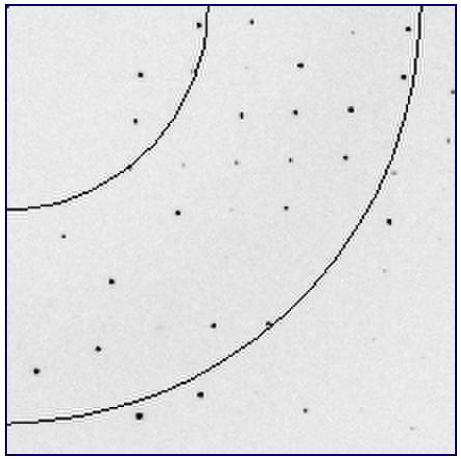




Ground, 20s exposure time

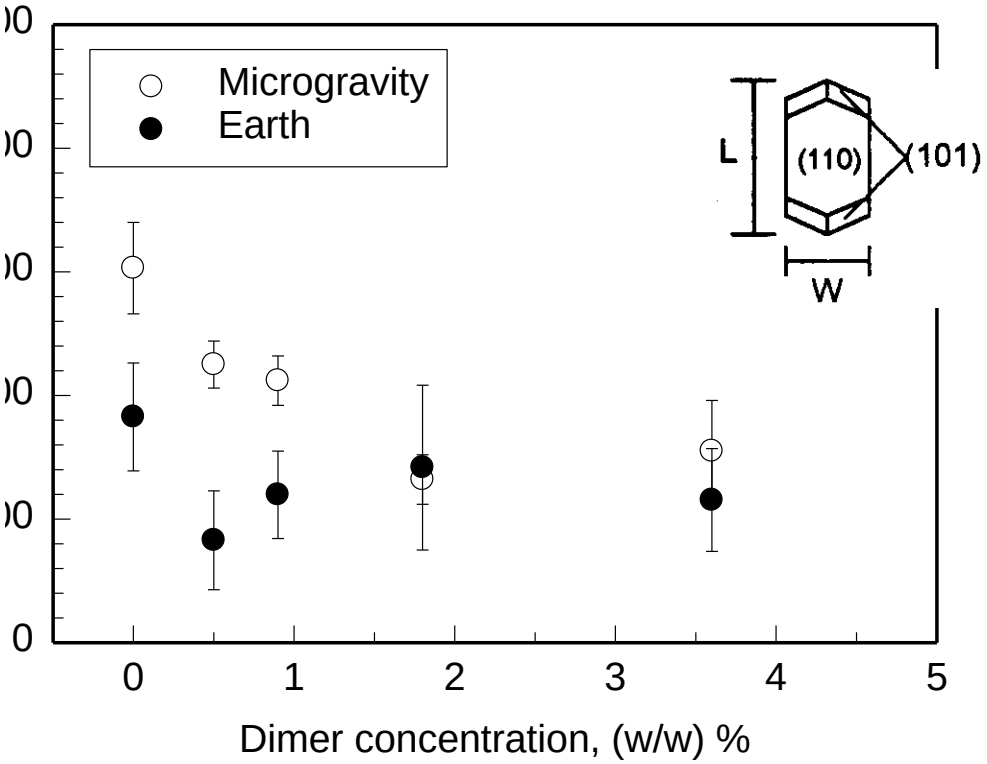
Microgravity, 0.9% impurity, 60s exposure time





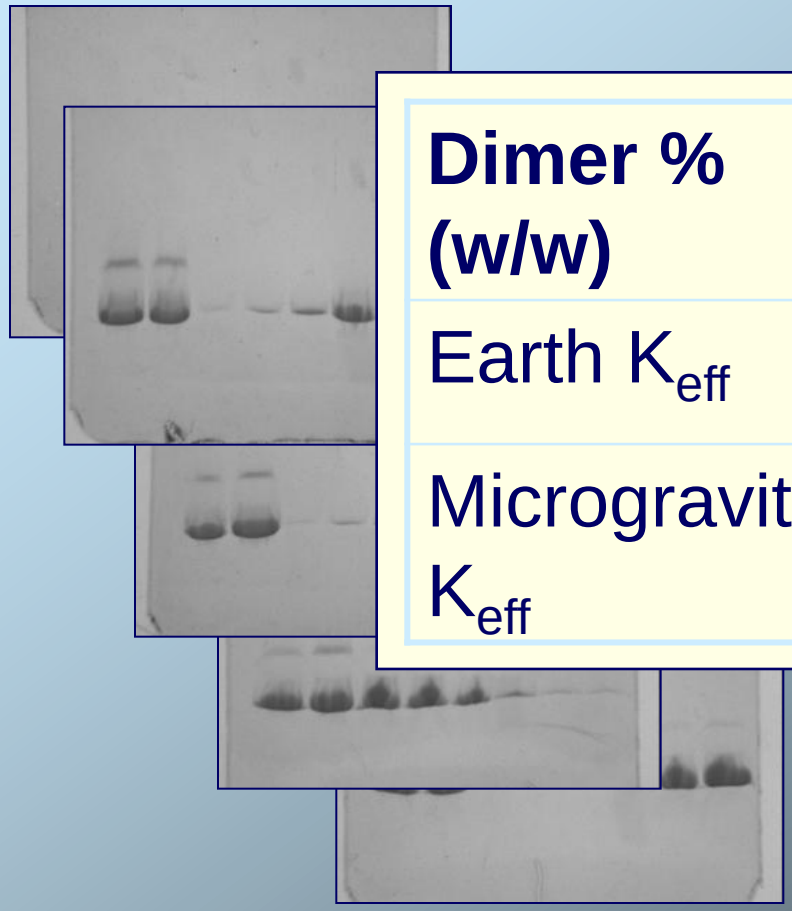
Microgravity, 3.6% impurity, 60s exposure time

Crystal Size



Microgravity increases volume, impurities decrease it.

Impurity partitioning



Dimer % (w/w)	**3.6**	**1.8**	**0.9**	**0.5**
Earth K_{eff}	**1.0**	**1.0**	**0.7**	**0.3**
Microgravity K_{eff}	**0.7**	**0.6**	**1.2**	**2.0**

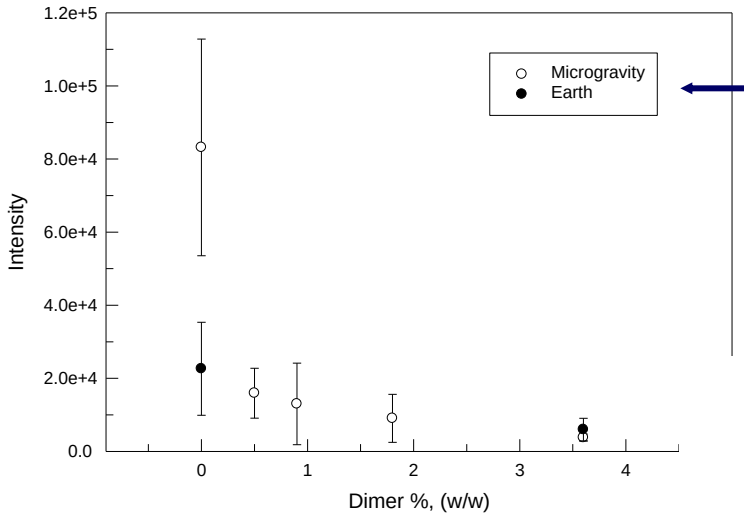
- Within the bounds of error there is no difference in partitioning for 3.6, 1.8 and 0.9% impurities.
- Microgravity preferentially incorporates the dimer at 0.5%

X-ray analysis

		Earth		Microgravity				
		0.0 %	3.6%	0.0 %	0.5 %	0.9 %	1.8 %	3.6 %
Earth	0.0 %	-	22 ref 4.9(0.6)	88 ref 9.9(0.9)	56 ref 9.8(0.4)	42 ref 9.8(0.4)	51 ref 10.0(0.5)	95 ref 9.8(1.0)
	3.6 %	22 ref 9.8(0.4)	-	45 ref 9.4(0.3)	28 ref 9.2(0.3)	13 ref 10.2(0.2)	23 ref 9.9(0.3)	30 ref 9.0(0.2)
Micro-gravity	0.0 %	88 ref 2.1(1.9)	45 ref 0.8(1.0)	-	94 ref 4.0(0.7)	70 ref 1.5(2.1)	102 ref 2.4(2.2)	135 ref 3.7(0.5)
	0.5 %	56 ref 2.1(0.8)	28 ref 2.2(0.8)	94 ref 6.1(0.3)	-	40 ref 1.9(1.0)	74 ref 4.5(0.6)	75 ref 5.1(0.2)
	0.9 %	42 ref 7.7(3.6)	13 ref 6.7(1.8)	70 ref 7.5(7.4)	40 ref 7.3(3.3)	-	51 ref 7.6(4.7)	101 ref 19.7(7.2)
	1.8 %	51 ref 19.6(2.7)	23 ref 5.3(3.8)	102 ref 20.3(5.7)	74 ref 20.2(3.9)	51 ref 20.9(4.2)	-	81 ref 7.6(7.5)
	3.6 %	95 ref 6.3(0.4)	30 ref 1.6(1.1)	135 ref 8.5(0.5)	75 ref 8.1(2)	81 ref 8.2(0.3)	101 ref 8.5(0.3)	-

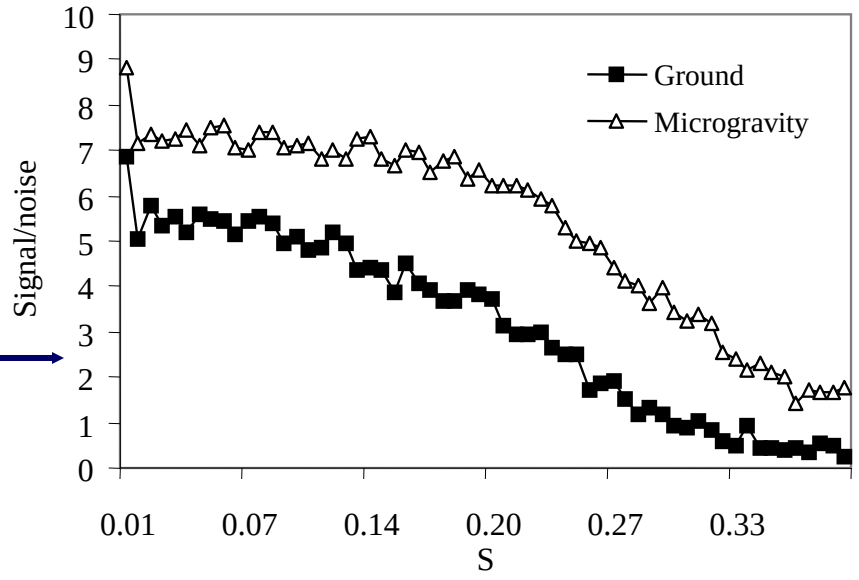
Symmetry related reflections are compared – Microgravity samples increase in mosaicity with increasing impurity reaching a peak at 1.8%. At 3.6% the mosaicity is lower but still higher than 0% and 5%. Earth showed increased mosaicity with impurity, greater than the equivalent microgravity with the exception of the 1.8% microgravity.

X-ray analysis



Intensity plotted against the initial dimer solution concentration for highest resolution bracket greater than 2.01 Å. Other resolution bins display similar trends. The error bars represent 95% confidence intervals

Signal to noise plot for 0% impurity microgravity and ground crystals.



- The best results (signal/noise, mosaicity and volume) come from using highly purified protein in microgravity.
- Microgravity should not be seen as a step to replace good biochemical practice but may be useful in situations where solution impurities are formed during the crystallization process.
- Microgravity seems to be more sensitive to impurities than the ground.

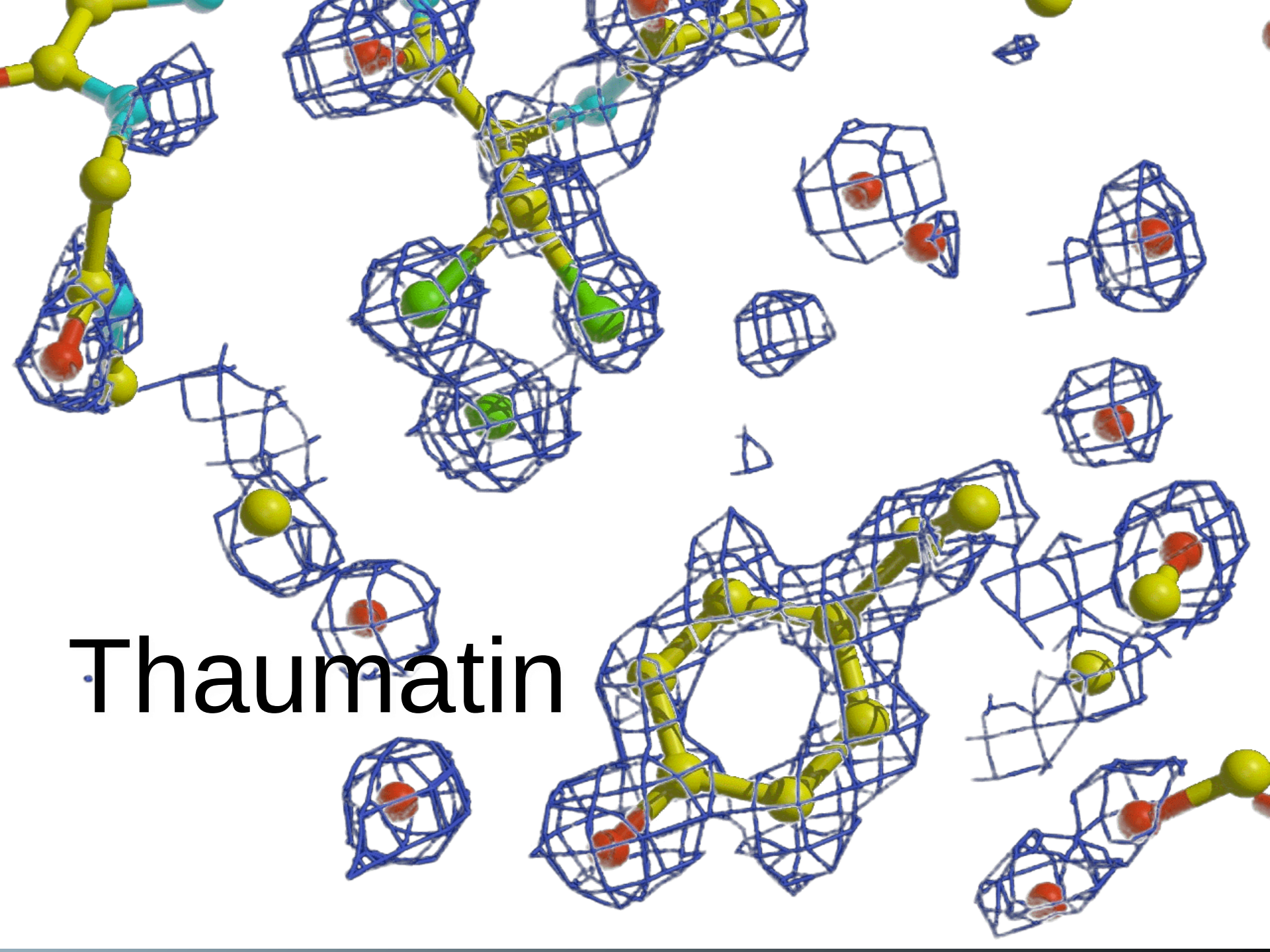
General notes of caution:

Bigger is not necessarily better

The lysozyme dimer may not be typical of a impurity found in general crystallization experiments.

Our results are the opposite of Carter et al.

Impurity partitioning electrophoretic and mosaicity analysis of microgravity and ground-grown crystals. Snell, E.H et al. Crystal Growth and Design, 1, 2, 151-158 (2001).


Thaumatin

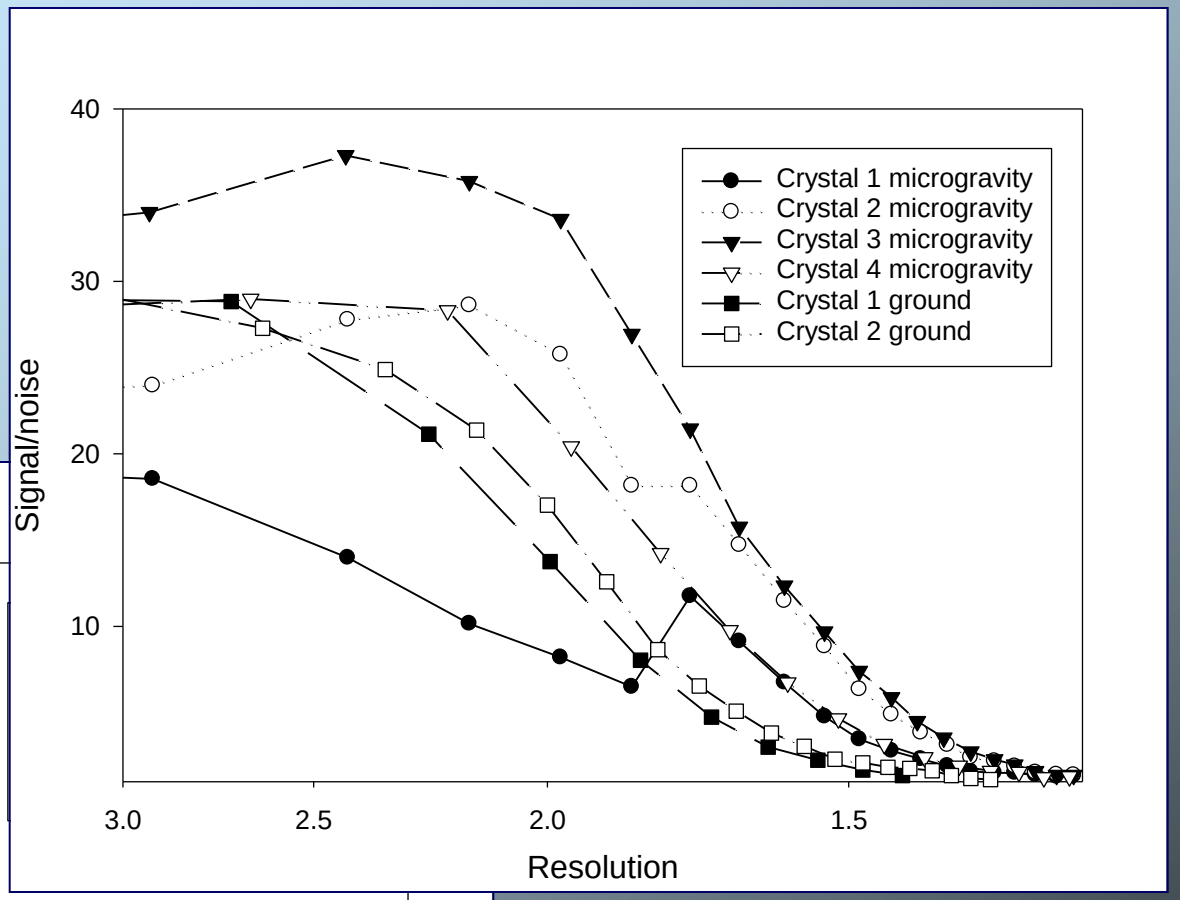
Experimental details

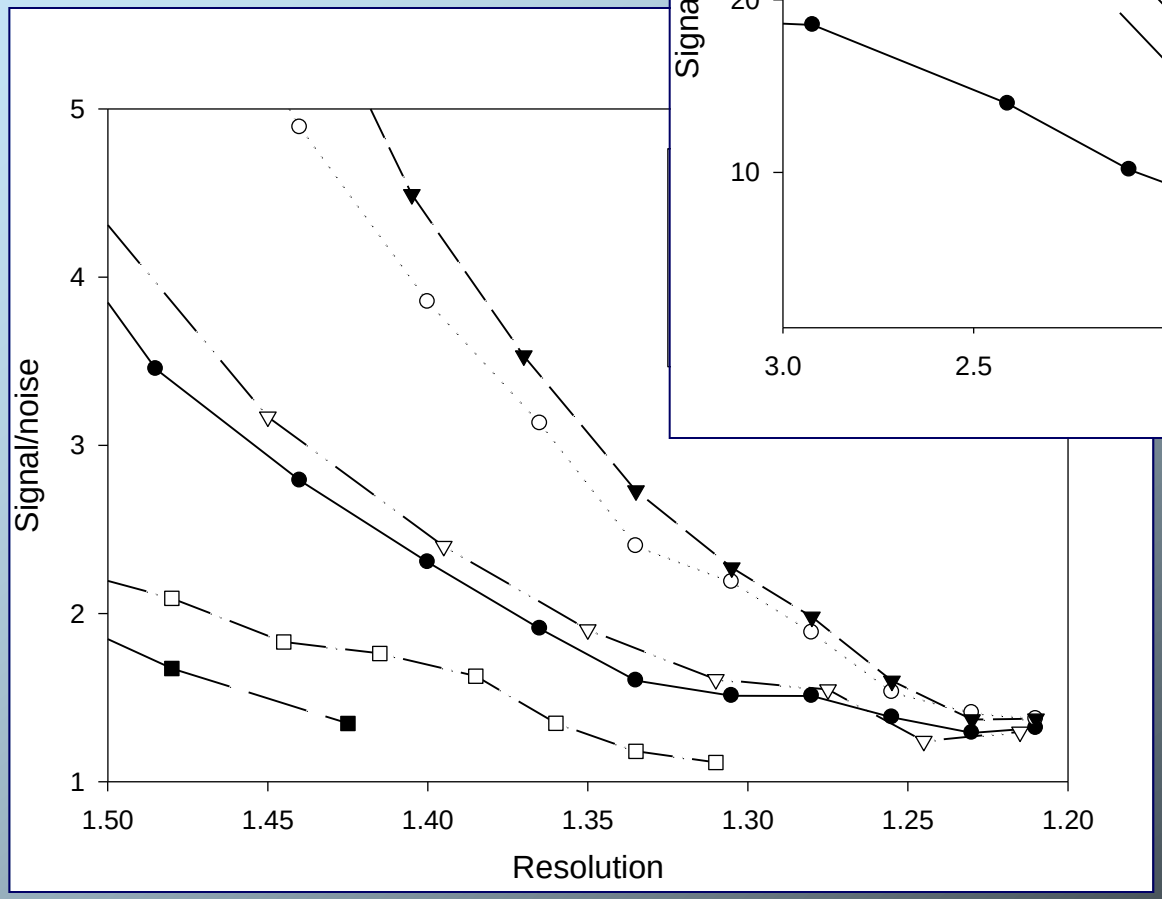
- 28 μl 87 mg/ml thaumatin from Sigma degassed and placed in tygon tube then frozen
- 112 μl of 1.25 M sodium potassium tartrate containing 100 mM ADA pH 6.5 was added to the tube and also frozen.
- The tube was sealed
- The tube was allowed to thaw over the duration of the microgravity mission
- X-ray data was collected from beamline 7-1 at SSRL
- Two equal 40 swathes were collected for each crystal studied, 4 microgravity and 2 ground.
- Data was collected in dose mode

	Crystal 1 (HOA) microgravity			Crystal 2 (HOA) microgravity			Crystal 3 (HOH) microgravity		
Size	0.97 x 0.57 x 0.46 mm			0.91 x 0.47 x 0.44 mm			1.97 x 1.00 x 1.00 mm		
Cell	a=b=58.56, c=151.58 Å			a=b=58.53, c=151.59 Å			a=b=58.50, c=151.63 Å		
	Slow	Fast	Combind	Slow	Fast	Combined	Slow	Fast	Combined
Res.	40-1.2	40-1.9	40-1.2	40-1.2	40-1.9	40-1.2	40-1.2	40-1.9	40-1.2
R-factor	9.8(60.1)	5.1(48.1)	7.4(60.1)	7.3(57.1)	5.7(13.0)	8.0(59.0)	6.8(39.0)	5.2(10.6)	6.9(39.0)
I/s	14.2(1.3)	10.7(2.2)	13.9(1.3)	17.3(1.5)	17.2(7.2)	17.5(1.4)	17.6(1.4)	23.9(11.2)	23.8(1.4)
Comp.	64.7(46.1)	42.7(35.6)	74.3(47.3)	85.2(74.5)	70.1(91.1)	89.3(78.2)	83.6(49.3)	86.5(98.8)	90.2(49.3)
Unique ref	54106	9270	61964	71094	15176	74394	69528	18758	75042
Mosaicity	0.122	1.633	0.170	0.106	0.113	0.105	0.090	0.097	0.091

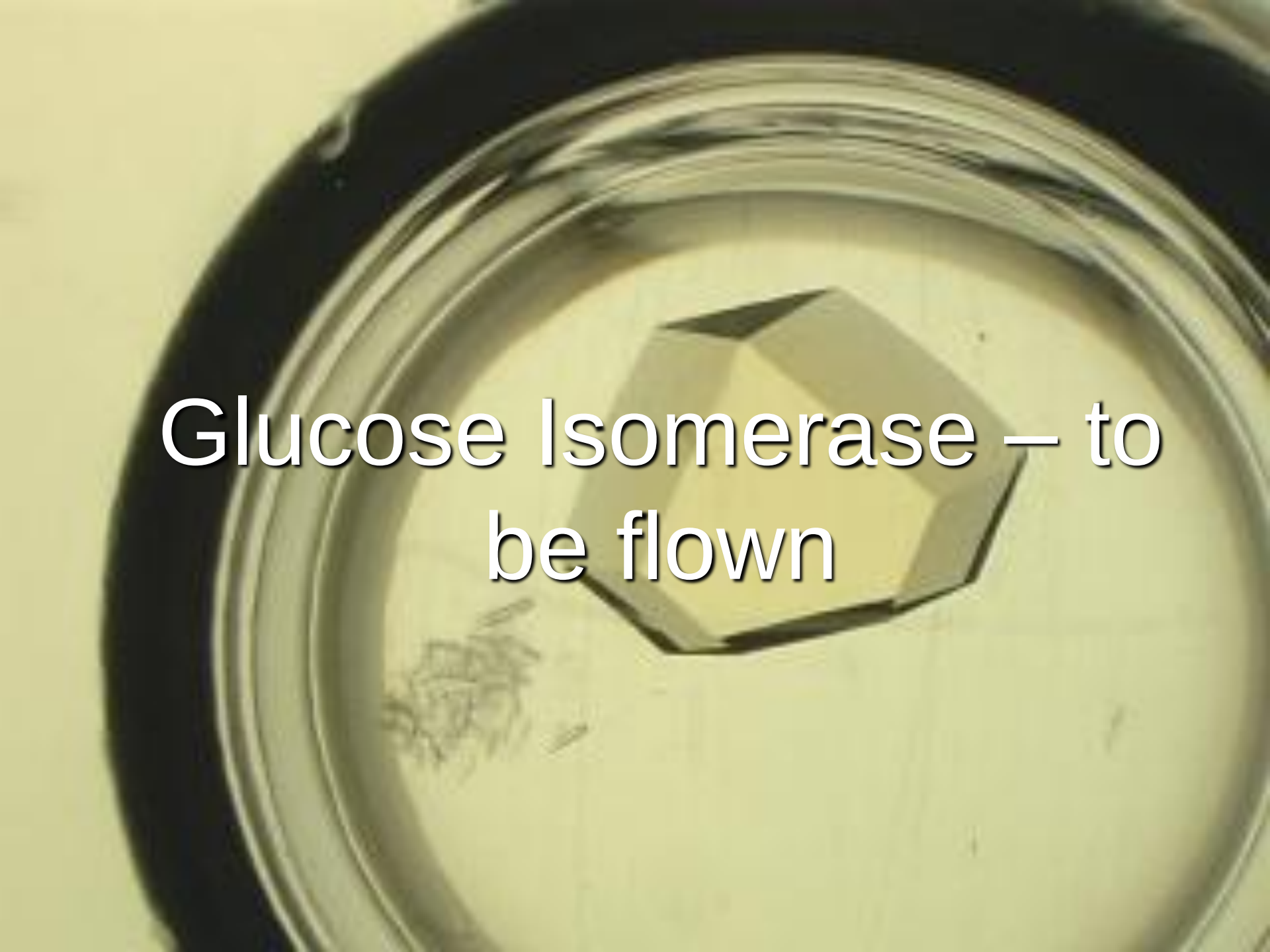
	Crystal 1 (HOO)- ground			Crystal 2 (HOO) - ground			Crystal 4 (IKG) microgravity		
Size	0.24 x 0.16 x 0.16 mm			0.34 x 0.18 x 0.18 mm			0.75 x 0.34 x 0.16 mm		
Cell	a=b=58.49, c=151.42 Å			a=b=58.52c=151.42 Å			a=b=58.56c=151.37 Å		
	Slow	Fast	Combind	Slow	Fast	Combined	Slow	Fast	Combined
Res.	40-1.4	40-1.9	20-1.4	40-1.3	40-1.9	40-1.3	40-1.2	40-1.9	40-1.2
R-factor	5.5(53.3)	10.0(85.3)	13.9(55.6)	7.4(62.9)	11.5(61.7)	15.0(62.6)	4.7(39.4)	3.5(8.6)	5.9(39.3)
I/s	16.8(1.2)	8.7(1.1)	18.6(1.3)	17.1(1.1)	11.2(1.8)	17.5(1.1)	17.9(1.4)	18.4(7.2)	17.8(1.3)
Comp.	89.8(62.5)	87.7(86.4)	90.7(67.3)	85.7(37.8)	91.5(98.1)	86.6(37.7)	72.3(24.3)	55.0(76.6)	72.8(26.1)
Unique ref	47499	18987	47897	56326	196843	56926	60201	11916	60585
Mosaicity	0.102	0.199	0.111	0.118	0.317	0.137	0.148	0.201	0.157

Plots of signal/noise against resolution





← Enlarged plot


Glucose Isomerase – to be flown

Experimental details

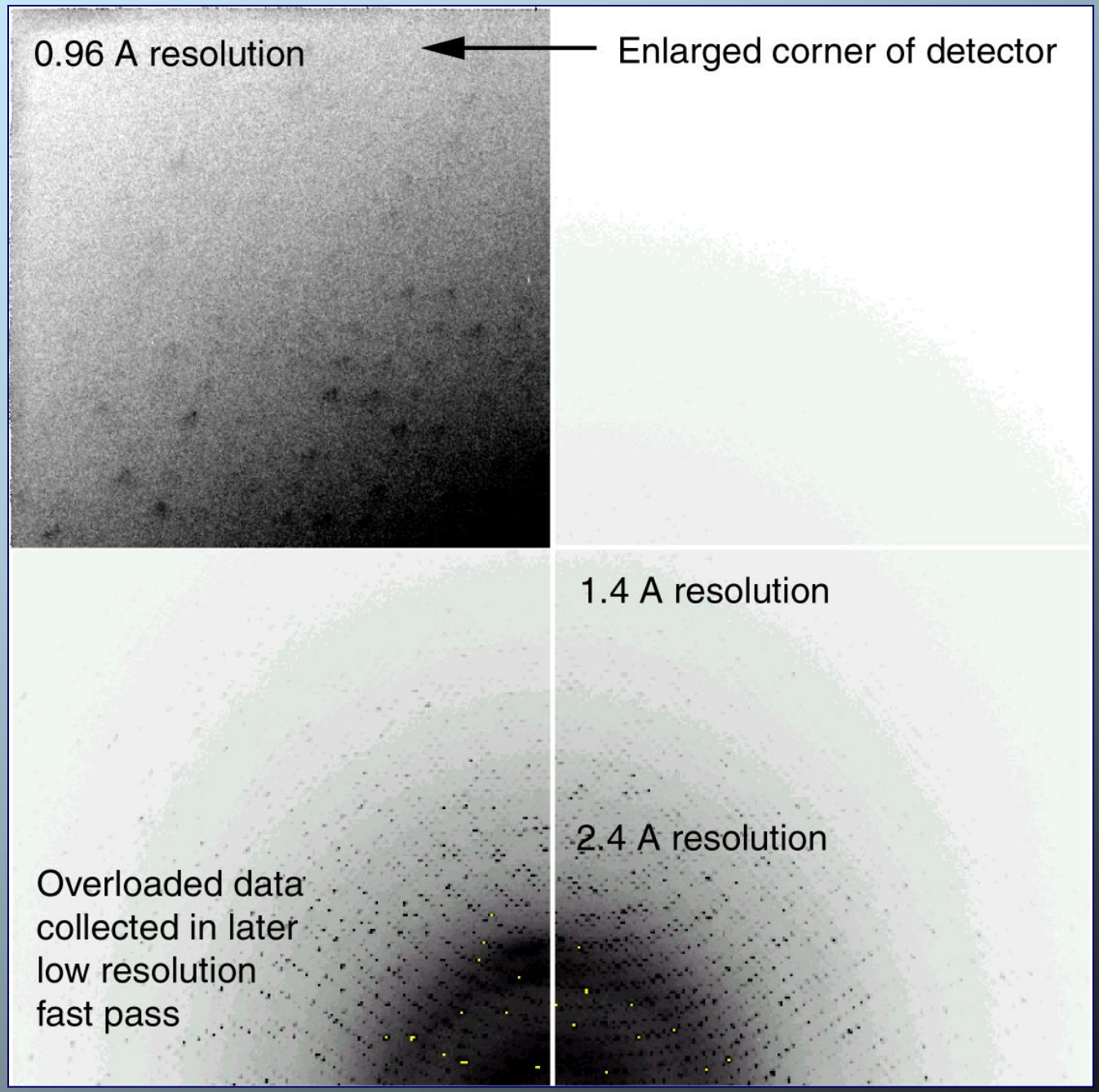
- Grown using the batch method
- 20% (w/w) ammonium sulphate, 50 mg/ml glucose isomerase, pH 7.7, 20°C
- 2.5 mm in longest dimension
- Genetically modified
- Small crystals used for X-ray data collection at SSRL
- Large crystals used for neutron data collection at ILL

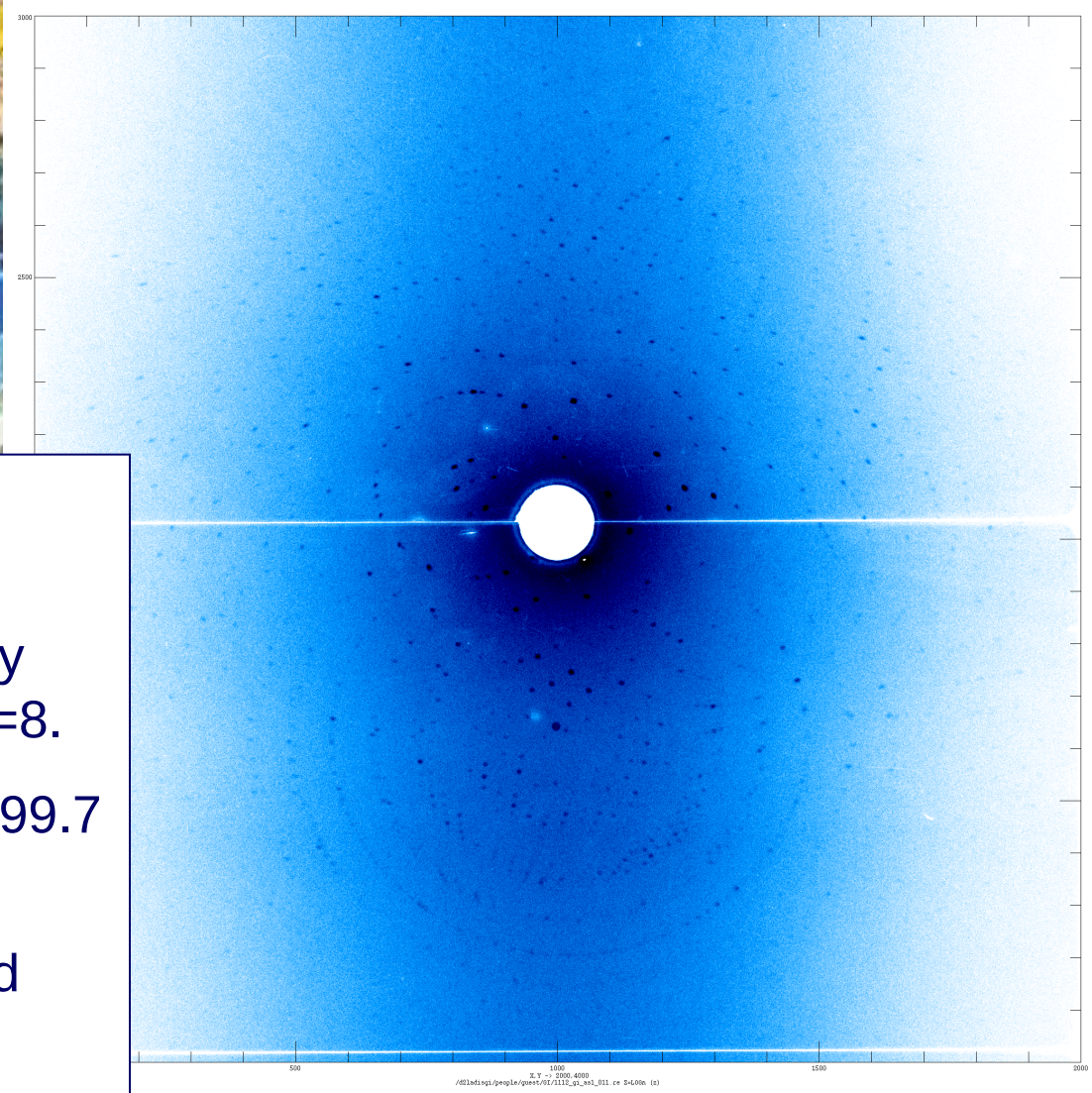
Good Short-range order.

Mutated Glucose 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).





Glucose 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.
- Judge, Snell, van der Woerd and Myles, work in progress.


Summary

Summary

- Our experiments in microgravity produced consistently larger volume crystals of insulin, lysozyme and thaumatin.
- Other experiments show the same trend
- Our microgravity experiments showed a consistent reduction in mosaicity
- Our microgravity experiments showed a large increase in signal to noise
- Long range order has been dramatically improved by microgravity. We do not know if short range order is being significantly improved.
- Microgravity can make a big impact on neutron diffraction.

Summary of Talk

- Microgravity reduces sedimentation and buoyancy driven convection
- Larger volume crystals with reduced mosaicity result from microgravity growth
- Neutron scatter weakly and large crystals and large fluxes are needed
- The Laue method provides larger flux but needs low mosaicity
- Microgravity can enable many neutron experiments

- However:
  - Neutron diffraction should not be justified just because large perfect crystals are available.
  - Ideally, experiments designed to use the potential of seeing hydrogen's, protonation state or using contrast matching should be aimed for.

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Naomi Chayen, Imperial College, UK

Dean Myles, EMBL, Grenoble, France

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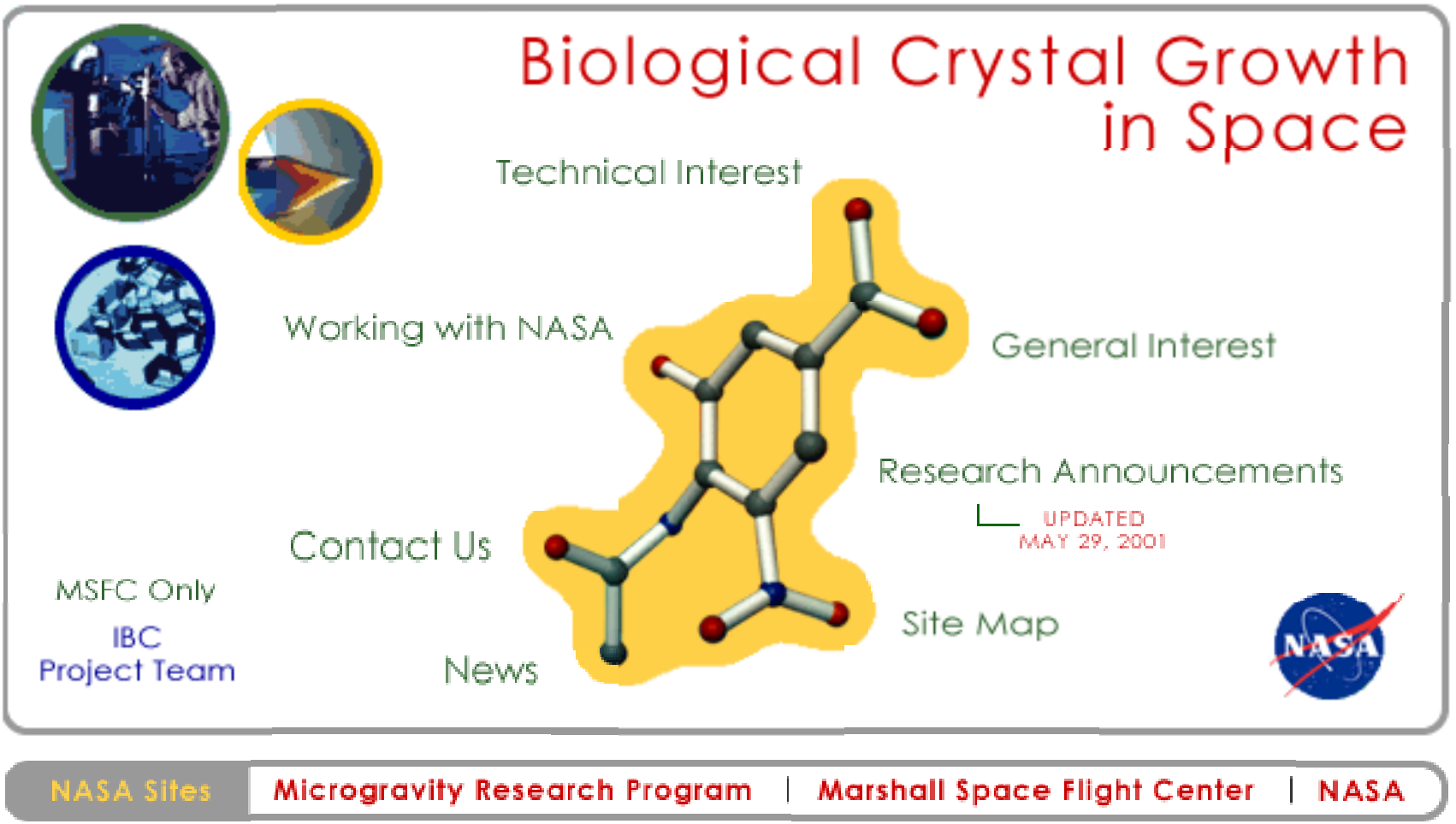
- Snell, E.H., et al.. Improvements in lysozyme protein crystal perfection through microgravity growth. *Acta Cryst*. D51, 1099-1102 (1995).

In Preparation

- Macromolecular Crystal Quality, Snell, E.H. et al., Methods in Enzymology, Macromolecular Crystallography Part C edited by Carter and Sweet – in preparation
- Thaumatin crystallization aboard the International Space Station using liquid-liquid diffusion in the enhanced gaseous nitrogen dewar (EGN). Barnes, C.L. et al., Submitted to Acta Cryst D.



To fly samples go to **http://crystal.nasa.gov** - an application form is in the technical interest section