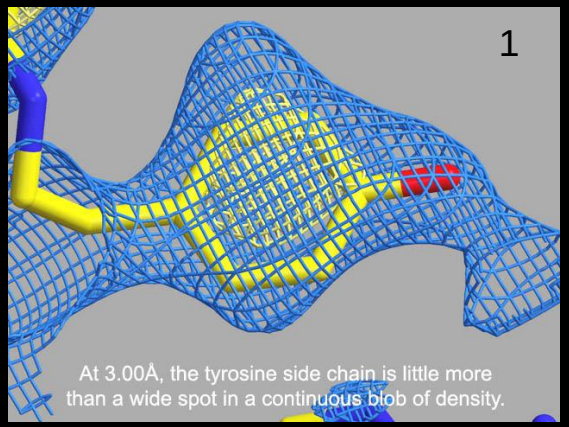
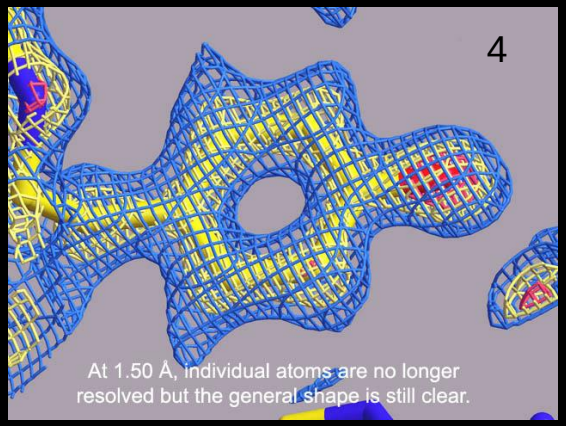
Exploiting On-Orbit Crystal Properties for Structural Studies of Medically and Economically Important Proteins

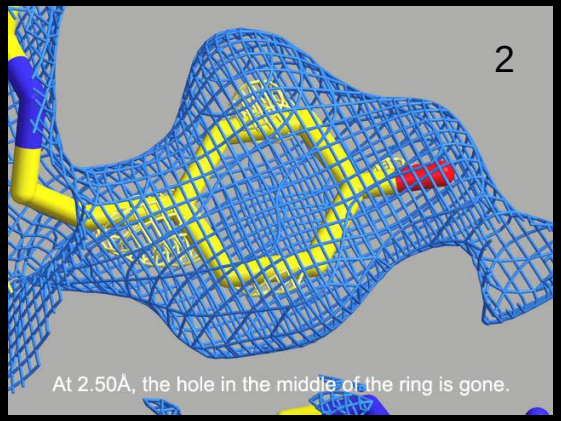
Edward H. Snell

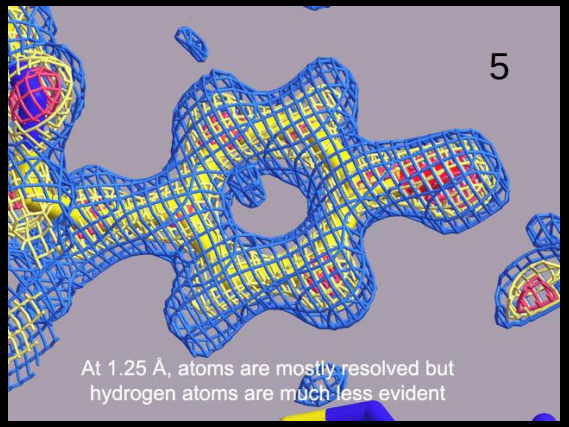
Hauptman Woodward Medical Research Institute, Buffalo, NY 14203, USA.

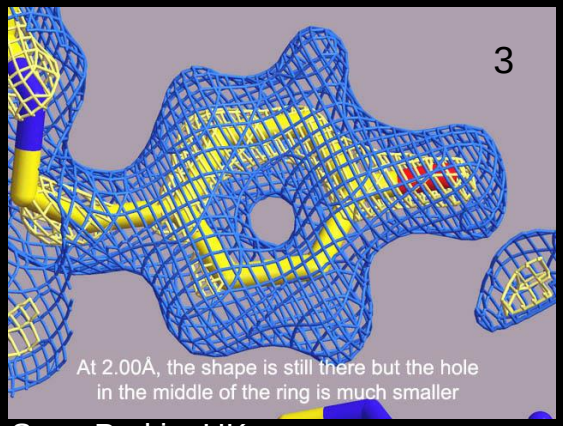
Why grow biological crystals in microgravity?

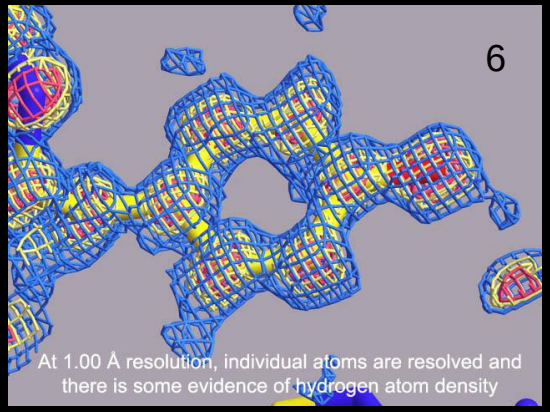












Sean Parkin. UK

Better quality crystals result in improved structural information (resolution).

Improved structural information aids in understanding of the mechanism.

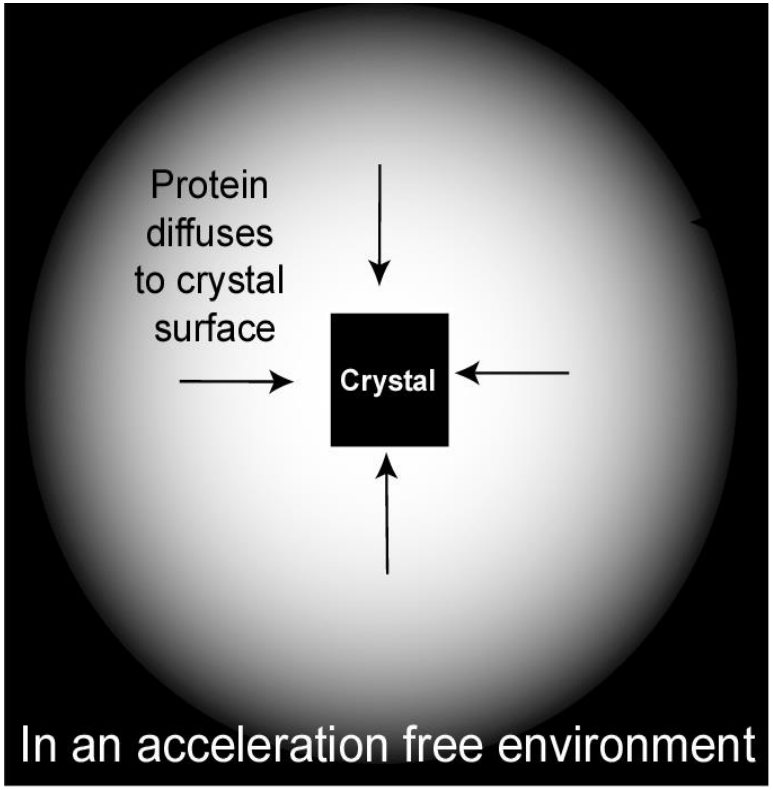
Understanding mechanism aids pharmaceutical design cutting years of development.

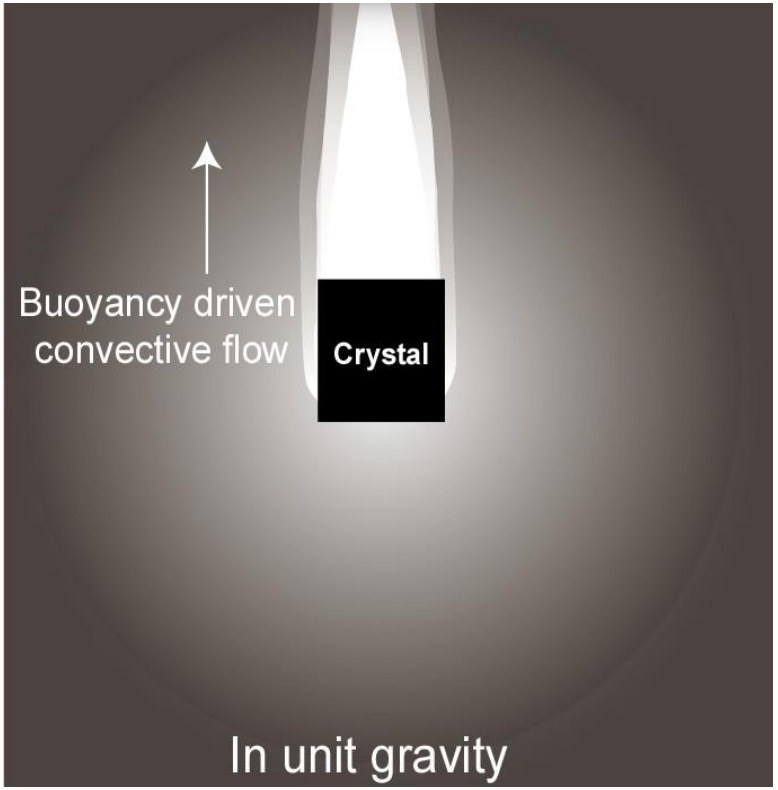
But microgravity does not directly enhance resolution….

- At the molecular length-scale reducing the effective gravitational forces will have no direct influence on short-range order.
    - Self assembly is unaffected. Sun et al., *Adv. Space Research* 24, 1341-1345, (1999) showed that coagulation of polystyrene spheres in a density matched liquid was not influenced by gravity for 0.1 μm particles and showed only a weak influence for 1.0 μm particles (a protein molecule is on the order of 0.01μm in dimension).
    - Brownian motion dominates. Prodi et al., *Atmospheric Research* 82, 379-384 (2006) showed that the displacement of particles in microgravity, due to Brownian motion, follows a Gaussian distribution like that at 1g.
    - Gravitational forces do not affect bond energies at the molecular level. Physical properties such as boiling and freezing points, enzyme kinetics etc. have not been observed to change, Giachetti et al., *Microgravity Sci. Technol*, 12, 36-40 (1999).
- Brownian motion dominates at the short-range level – that level that fundamentally determines the diffraction limit (amount of detail seen).
- Physically, for a well prepared sample microgravity growth has no direct effect on resolution.

So, why grow biological crystals in microgravity?

Microgravity conditions can be diffusion limited



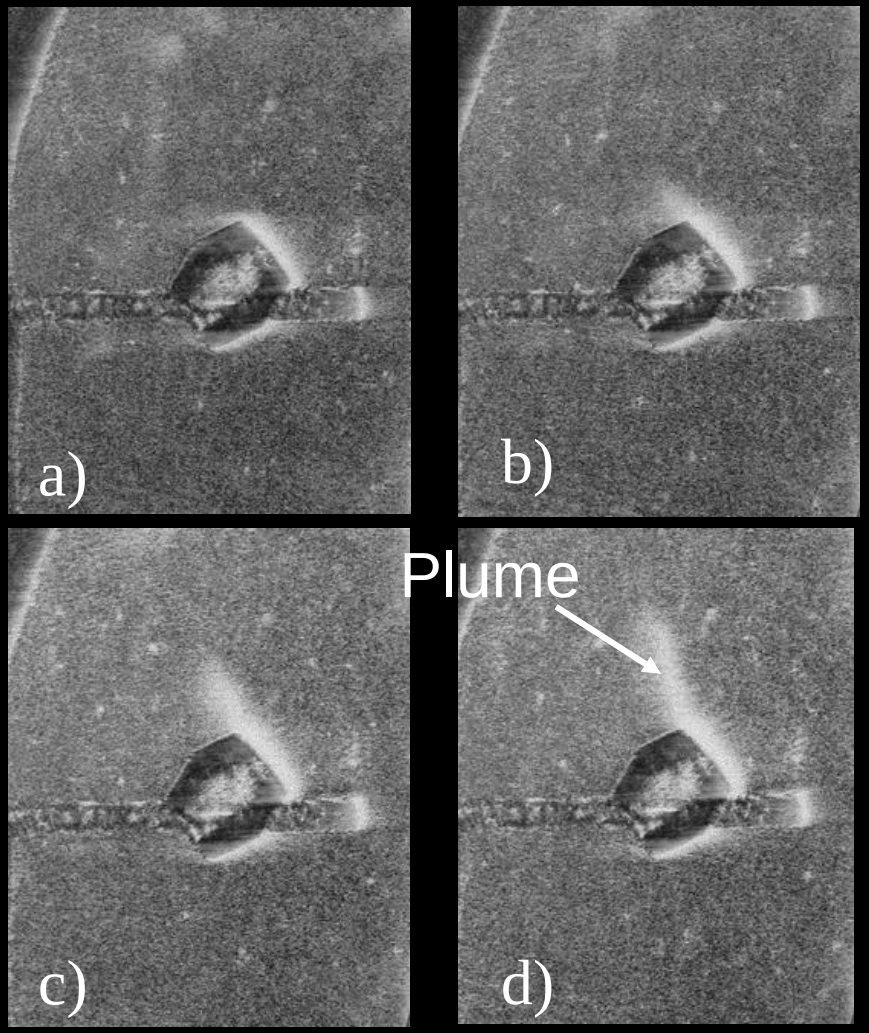


This can be observed experimentally (it's not a theory)

On the ground:

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

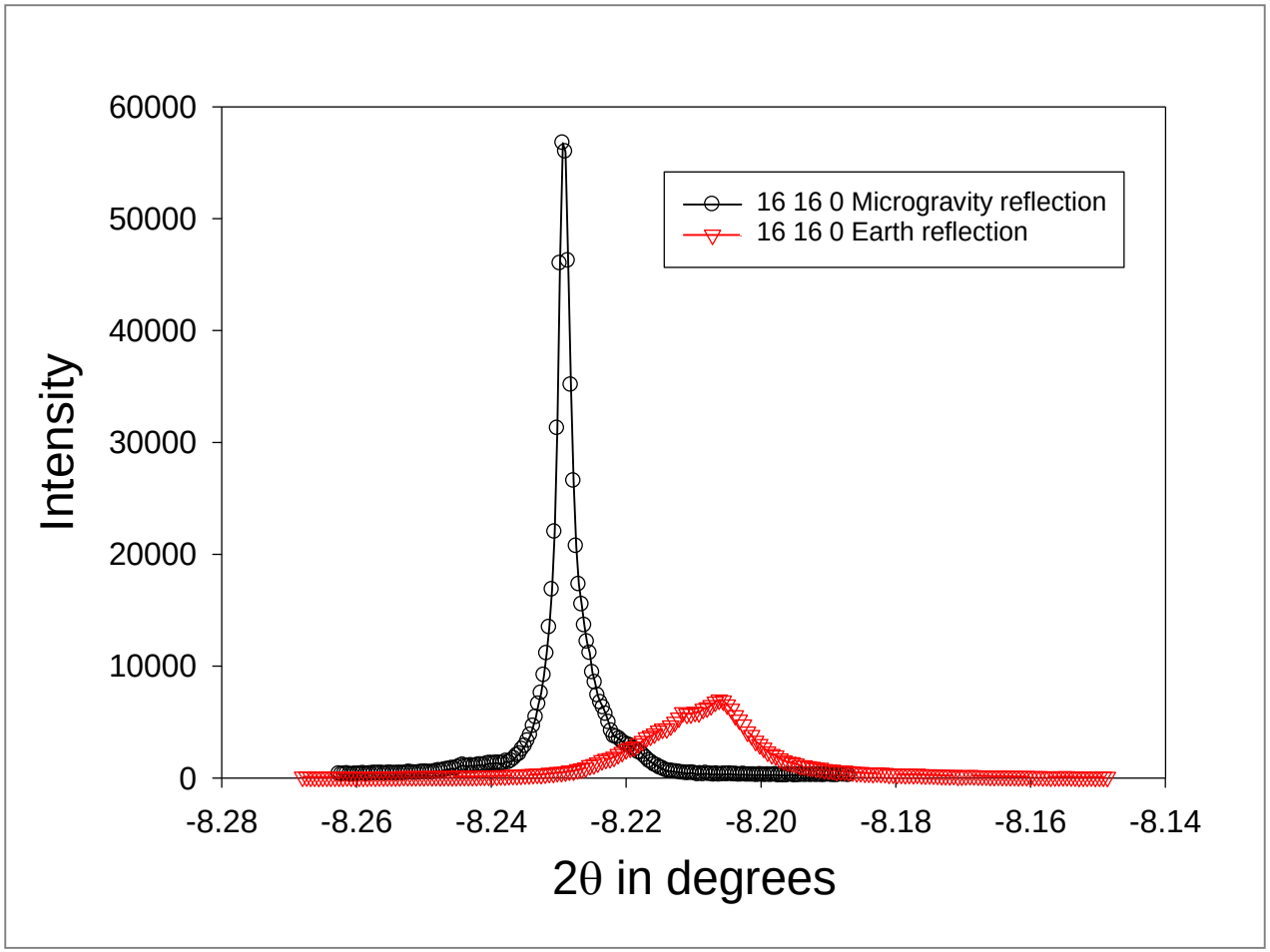
A convective growth plume of solution flows over the crystal face impeding growth and the quality of crystal packing.



Schlieren 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) .

Long range order (length scales of many proteins in the crystal) can be improved.

Original experiments investigating microgravity crystal growth (mosaicity)



Identical reflections from microgravity and ground grown lysozyme.

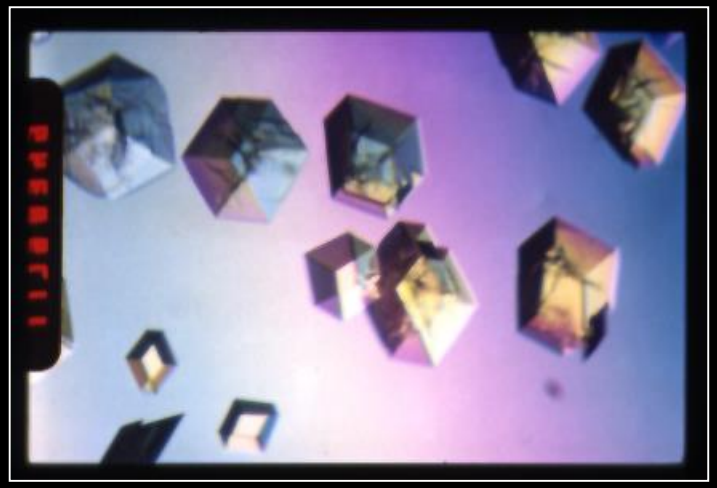
Eight times increase in signal to noise.

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

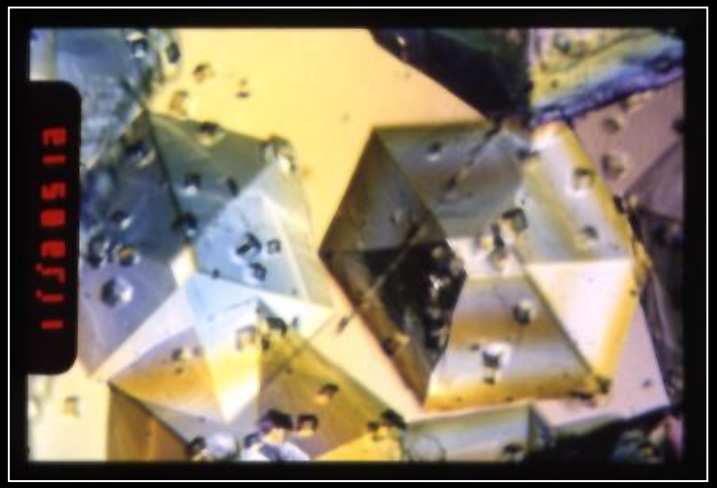
Previous studies on insulin



Images to same scale.

Ground:

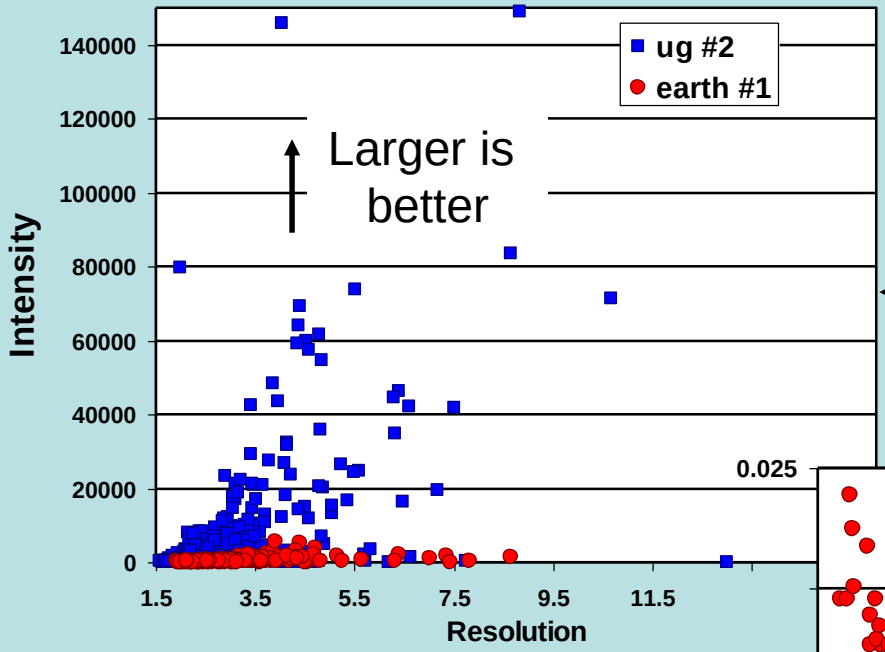
Sedimentation onto the bottom. Clumping of crystals.



Microgravity:

Free floating, unsedimented. had consistently larger diffracting volume > 2 mm in each dimension (34 times larger on average)

From STS-95. Borgstahl, G.E.O., Vahedi-Fardi, A., Lovelace, J., Bellamy, H. & Snell, E.H. Acta Cryst, D57, 1204-1207 (2001).



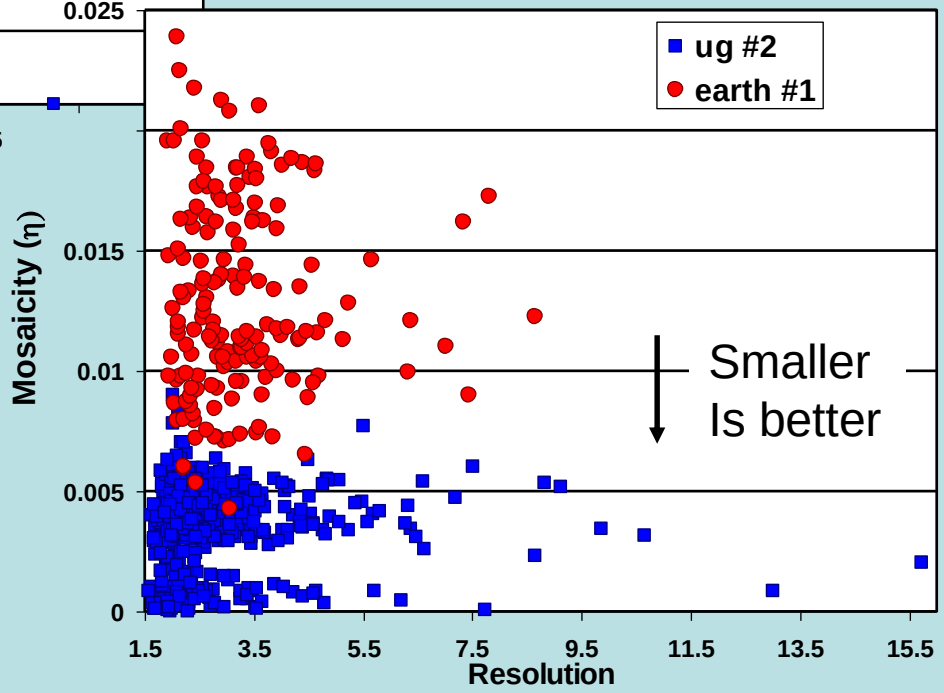
Microgravity Blue

Ground Red

Structural, short range improvement

Physical, long range improvement.

The worst microgravity and best ground crystal data are shown.



Effects of microgravity on crystallization

- Increase in volume (due to suspension in crystallization solution)
  - Leads to higher signal-to-noise, can be exploited to reduce radiation damage. Harder to cryocool (protect from radiation damage, 3Gy to kill a hamster, 30 Mgy used in crystallography)

- Reduction in mosaicity due to improved physical perfection.
  - Mosaicity reduced to a fraction of a degree. Each reflection extends over this angle and if you record in these angular steps you can optimize the signal. Destroyed by cryocooling.

Cryocooling is required to mitigate radiation damage

Cryocooling decreases long-range order

- Cryocooling to minimize radiation damage destroys much of the order imparted by growth in microgravity,.Vahedi-Fardi et al., Acta Cryst. D59, 2169-2182 (2003).

Cryocooling misses information

- “cryocooling remodels the conformational distributions of more than 35% of side chains and eliminates packing defects necessary for functional motions”, Fraser et al., PNAS, 108, 16247-16252 (2011).

Applying this hypothesis to four targets (status report)

Don’t be disappointed as there are no results yet, we’ve not flown!

Hypothesis

1. Microgravity does not directly enhance short-range order
2. Microgravity enhances long-range order
3. Cryocooling reduces order that is produced (but does not completely destroy it).
4. If we can match the experiment to take advantage of the improved long-range order we can improve the signal and therefore detail.
5. If we can make use of the improved volume we can record data without cryocooling while still minimizing radiation damage.

Our Biological macromolecules of Interest

All our proteins of interesthave a common theme, they are part of a project to understand the body's natural defense against brain and lung-damage (caused by blast or inhalation hazards on the battlefield), visualize the pathways on a molecular scale, and thereby provide functional knowledge that can be used to enhance, and supplement this response through pharmacological intervention.

All are involved with oxidative stress.

They are part of a bigger project identifying the interactome of oxidative stress and focusing on structural studies of those components where structure is unavailable.

Each is also medically relevant in its own right so success in any single study provides important information.

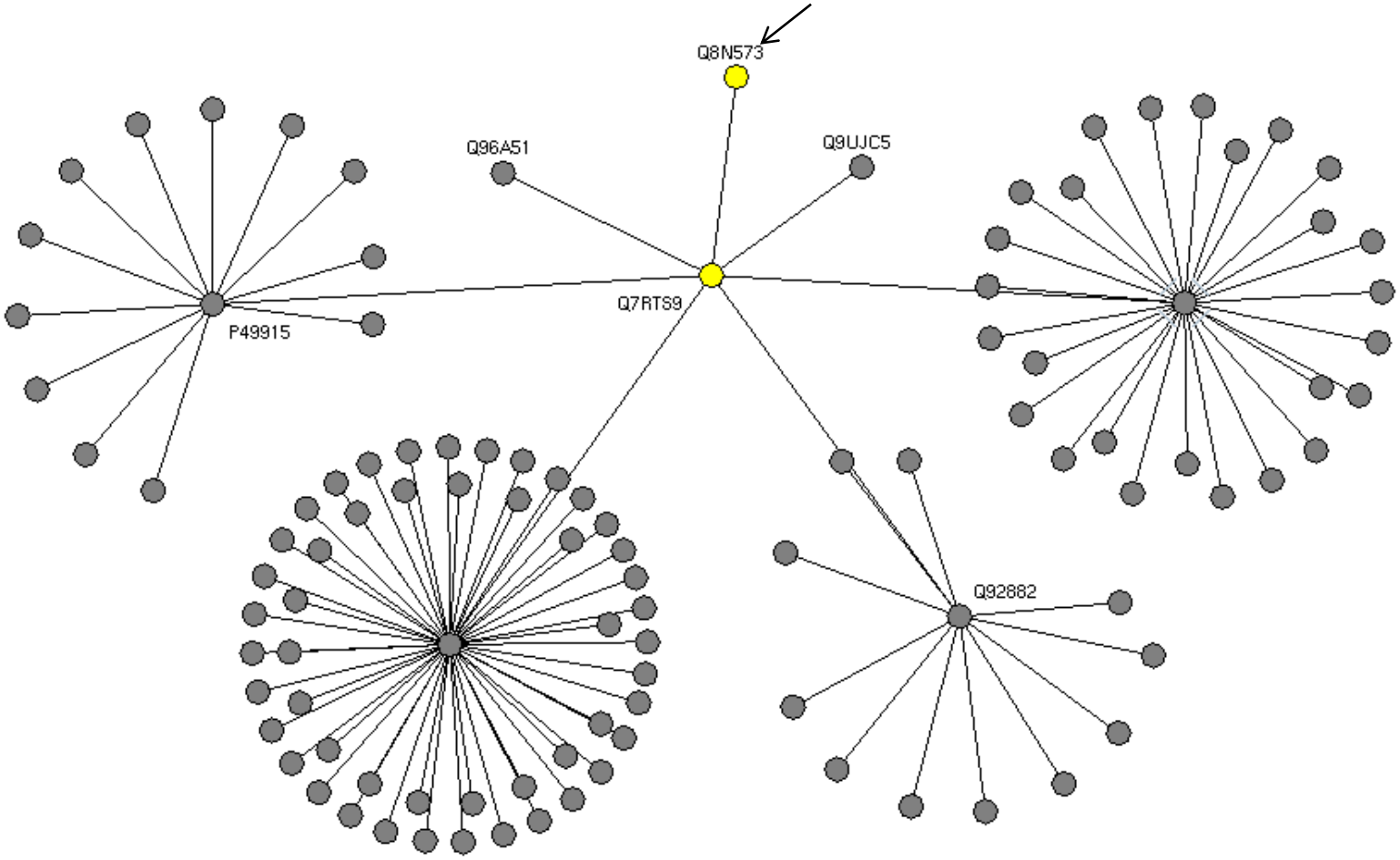
Common Link: Brain and Lung Injuries

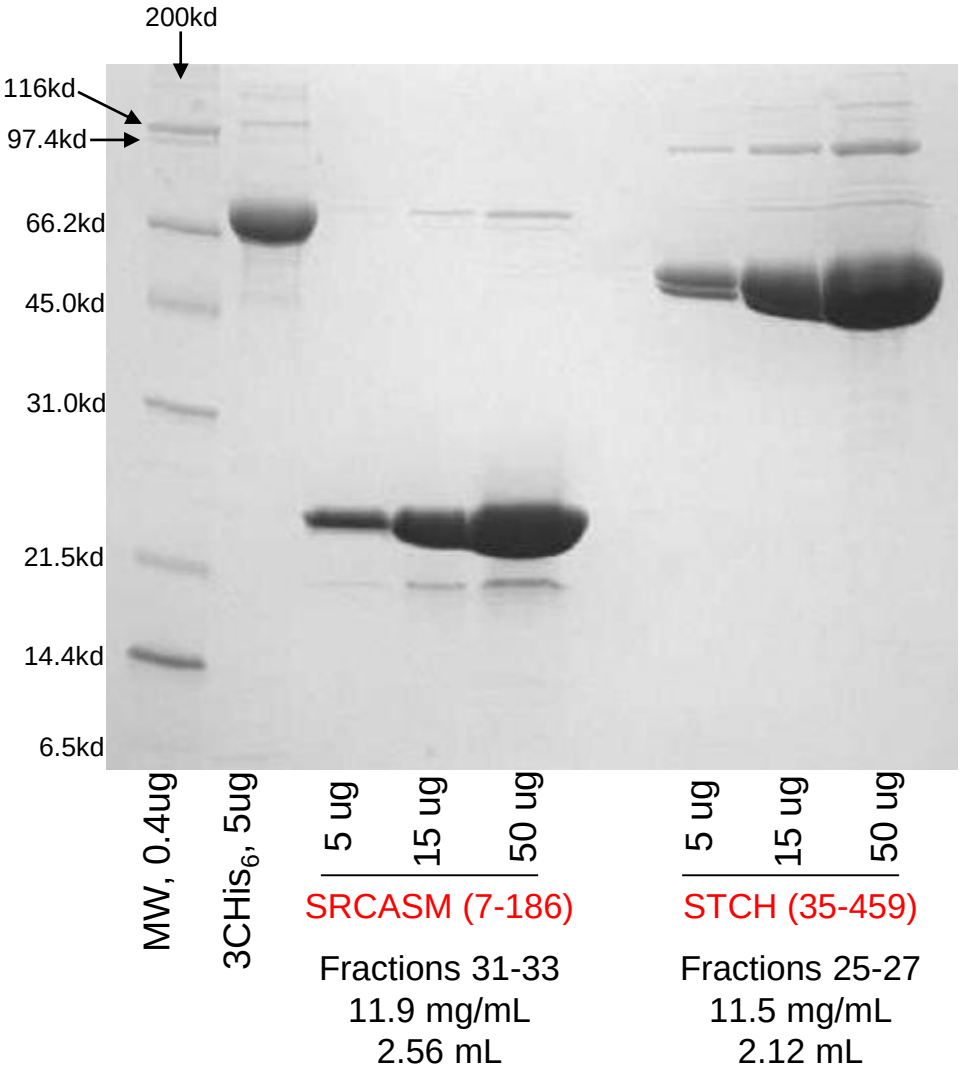
“Bombs' hidden impact: The brain war” Sharon Weinberger Nature 477, 390-393 (2011)

- IEDs have killed more than 3,000 US and allied troops, and wounded roughly ten times that number. But many more troops have been exposed to multiple blasts and not suffered any visible physical injuries … they often report an array of symptoms, ranging from sleep disturbance to problems concentrating. And an increasing body of evidence suggests that the repeated concussions have left them with an invisible, subcellular-level form of traumatic brain injury (TBI) that not only impairs their day-to-day functioning, but also increases their long-term risk of developing neurodegenerative diseases.
- “*The risk that these guys are going to get a disease like Alzheimer's or Parkinson's is soaring*.”
- The number of troops affected by this kind of silent TBI has already topped 200,000, according to the Defense and Veterans Brain Injury Center in Washington DC. A survey done by the Rand Corporation, a not-for-profit research firm in Santa Monica, California, suggests it could be as high as 320,000. The Pentagon and the US Department of Veterans Affairs, which are responsible for the health care of current and former troops, respectively, are getting worried about a potential epidemic of disability and dementia.

- TBI is a significant current and long-term problem for the DoD
- Similar problems seen in young athletes.
- Related to diseases associated with ageing.

Q8N573 (oxidation resistance protein): May be involved in protection from oxidative damage.

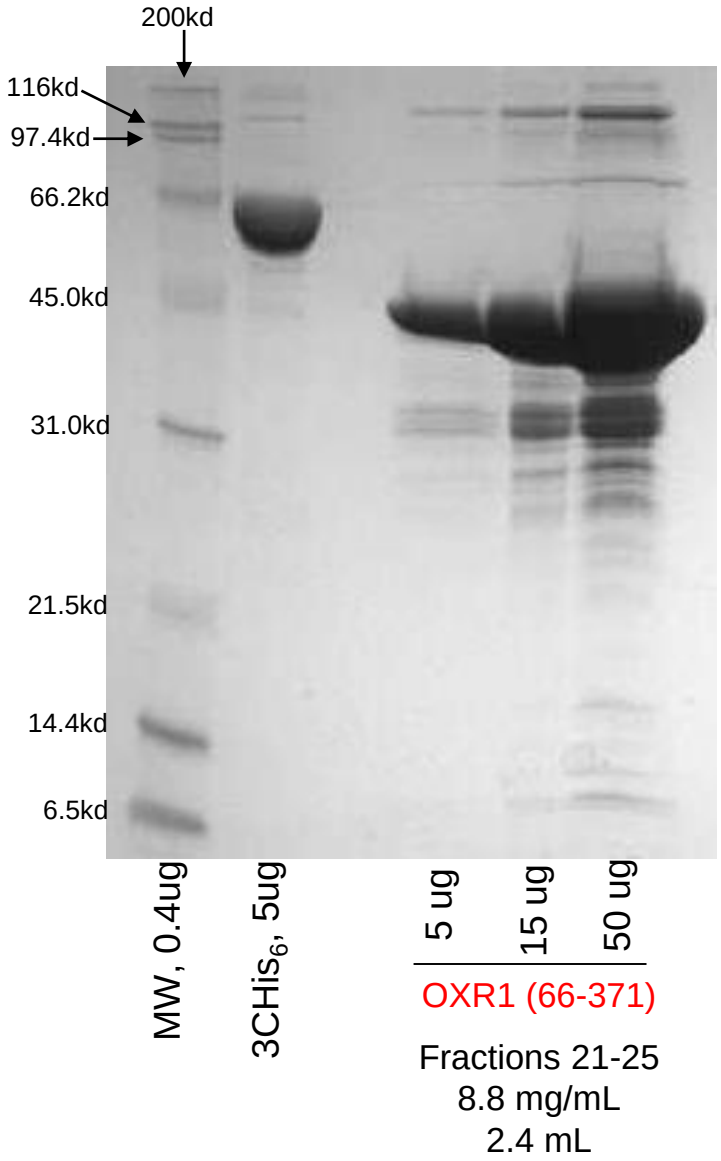




Targets are being expressed in large and pure quantities suitable for crystallization

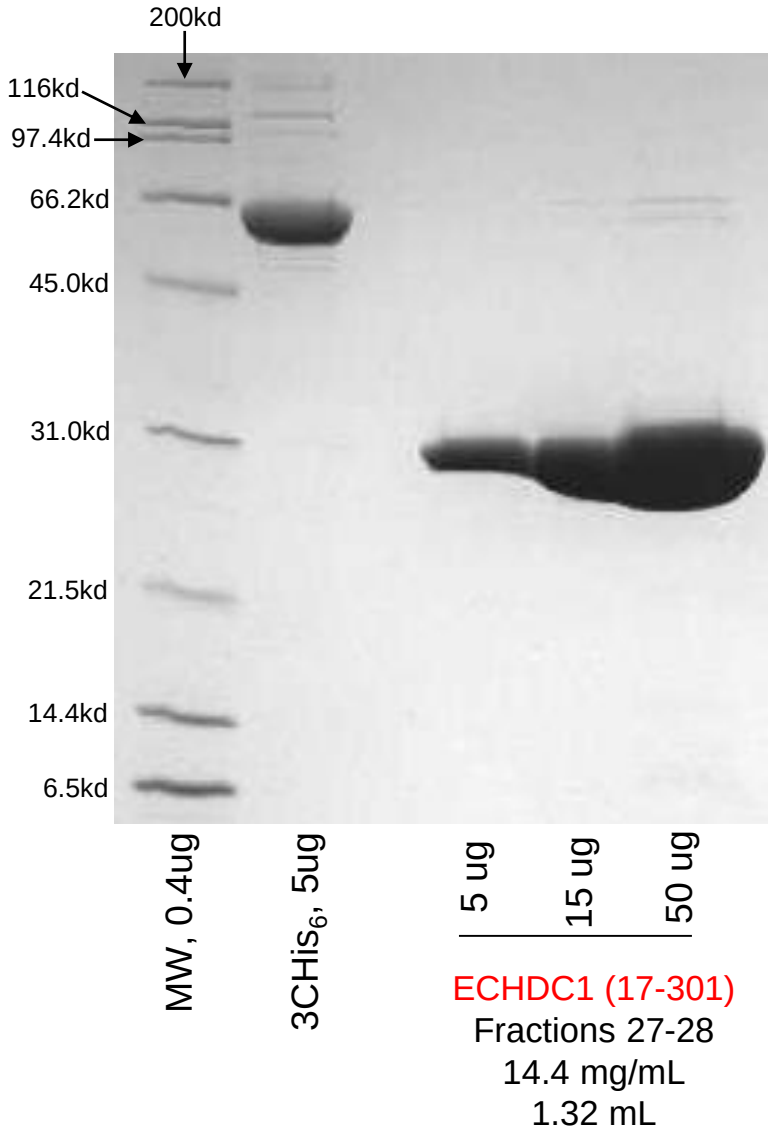
SRCASM is associated with cutaneous squamous cell carcinoma (the second most common form of cancer in the United States with over 700,000 cases diagnosed each year in the US.

STCH has been shown to be involved in prion disease but the precise role is undetermined. Prion diseases are fatal neurodegenerative diseases usually with a long clinically silent incubation period. Overexpression of STCH decreases this incubation period and its structure may provide a clue to mechanism and possible inhibition.



OXR1 is vital for the protection of neuronal cells against this oxidative stress. The brain is particularly susceptible to oxidative stress, and ROS–induced damage is a common feature of all major neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). The Center for Disease Control rated complications from Parkinson's disease as the 14th leading cause of death in the United States with the prevalence expected to increase substantially in the next 20 years due to the aging population. The economic burden of Parkinson's disease in the US alone is estimated to be $6 billion annually.

Targets are being expressed in large and pure quantities suitable for crystallization



ECHDC1 Ethylmalonyl-CoA Decarboxylase is part of a family of enoyl-CoA hydratases. Oxidative stress and free radical production leads to damage in metabolites. ECHDC1 expression has recently been identified as a novel metabolite proofreading enzyme. There are few known enzymes that perform proof reading of metabolites in an analogous way to DNA proofreading. It may be involved in the development of certain forms of ethylmalonic aciduria (nevorus system problems and delayed development). Mutations in ECHDC1 have been detected in high risk, cancer affected Jewish Ashkenazi women although the nature of the link, if present, is unclear.

Targets are being expressed in large and pure quantities suitable for crystallization

Crystallization/X-ray diffraction status

Ethylmalonyl-CoA Decarboxylase (ECHDC1)

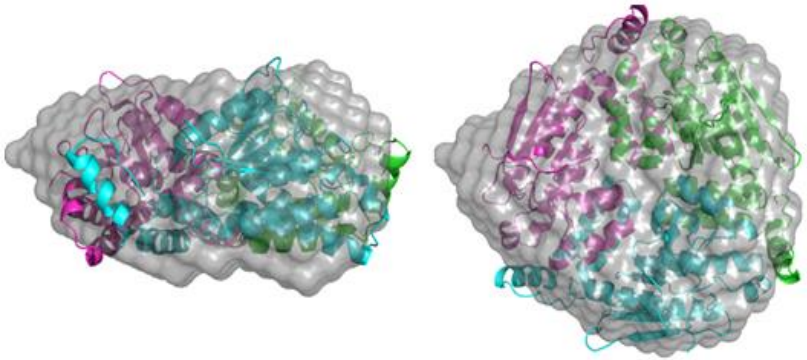
	Overall	Inner Shell	Outer Shell
Resolution limit	39.6-3.4	39.6-10.8	3.59-3.40
R_{merge}	0.086	0.034	0.313
R_{meas} (within I+/I-)	0.089	0.036	0.325
R_{pim} (within I+/I-)	0.024	0.011	0.086
Total number of observations	647173	17172	97978
Total number unique	48950	1679	7017
Mean((I)/sd(I))	22.9	52.1	8.0
Completeness	99.8	98.0	99.2
Multiplicity	13.2	10.2	14.0
Anomalous completeness	98.5	81.2	98.9
Anomalous multiplicity	6.9	5.7	7.2

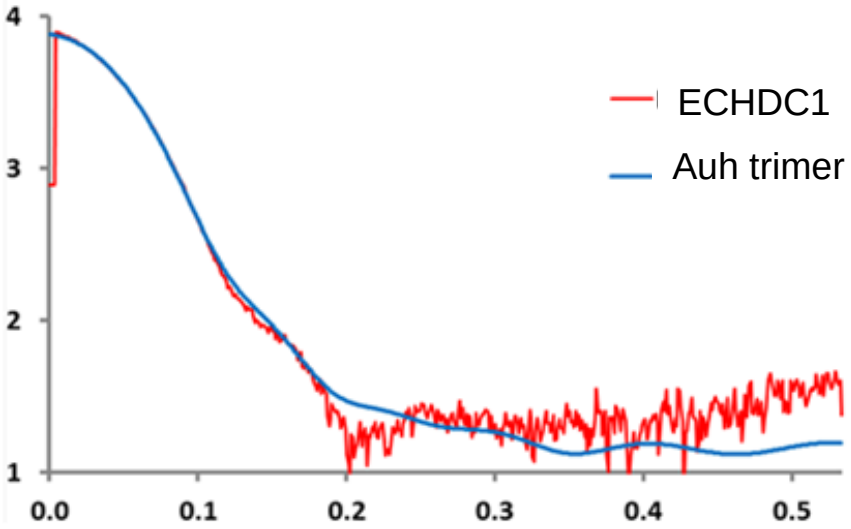
Average unit cell: 130.62 149.17 180.11 90.00 90.00 90.00

Space group: $P2_12_12_1$ Average mosaicity: 0.06

SAXS analysis

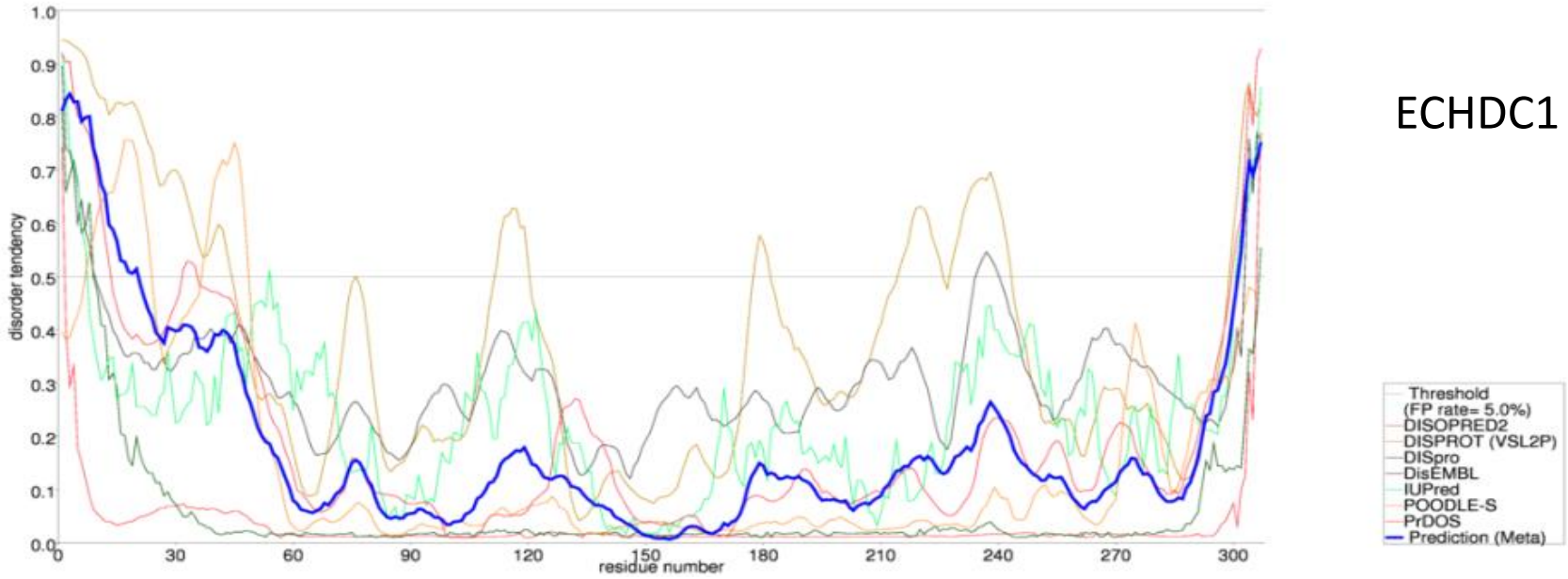
- From sequence analysis and mass spec The 17-301 construct has a molecular weight of 31.7 kDa
- Small Angle X-ray Scattering (SAXS) data collected by us suggests a molecular weight of ~90 kDa indicating a probable trimer
- Compared to a trimer of the nearest homologue (human Auh, max identity 29%)





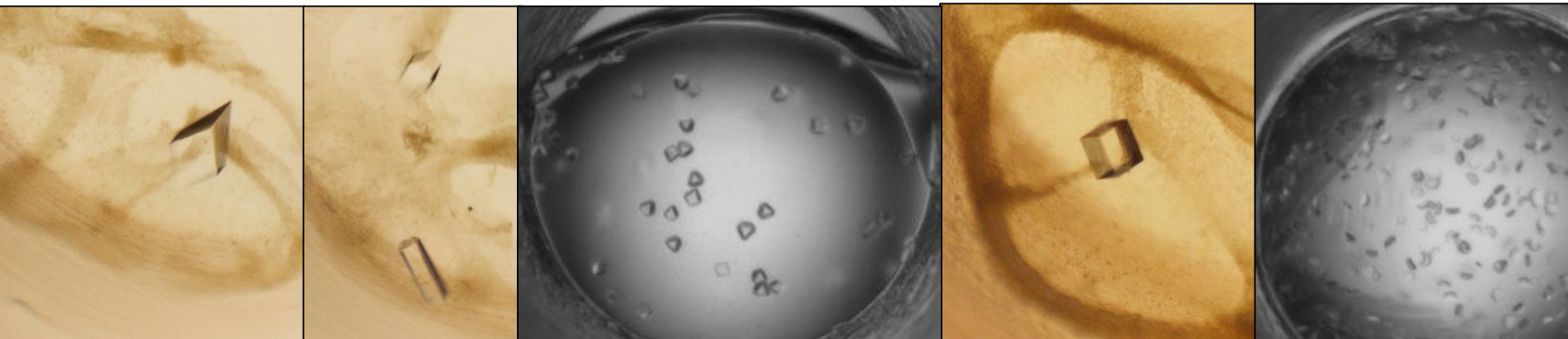
But, 11 Seliniums per protein and 12-18 proteins in the assymetric unit – non trivial

Not solved to date



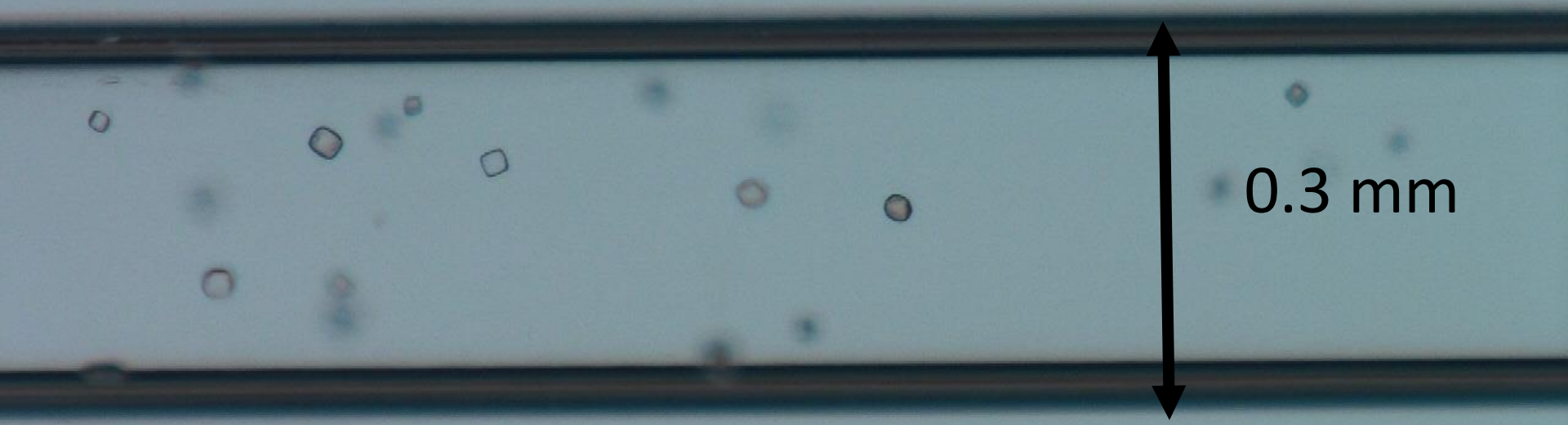
ECHDC1

Note: Our target proteins are human and contain disordered residues. This disorder is potentially key to mechanism and part of a joint crystallography, spectroscopy (THz) and small angle X-ray scattering investigation. ECHDC1 is the easiest case possibly related to the large number of successful crystallization conditions.



5 micron beam or smaller now routinely available

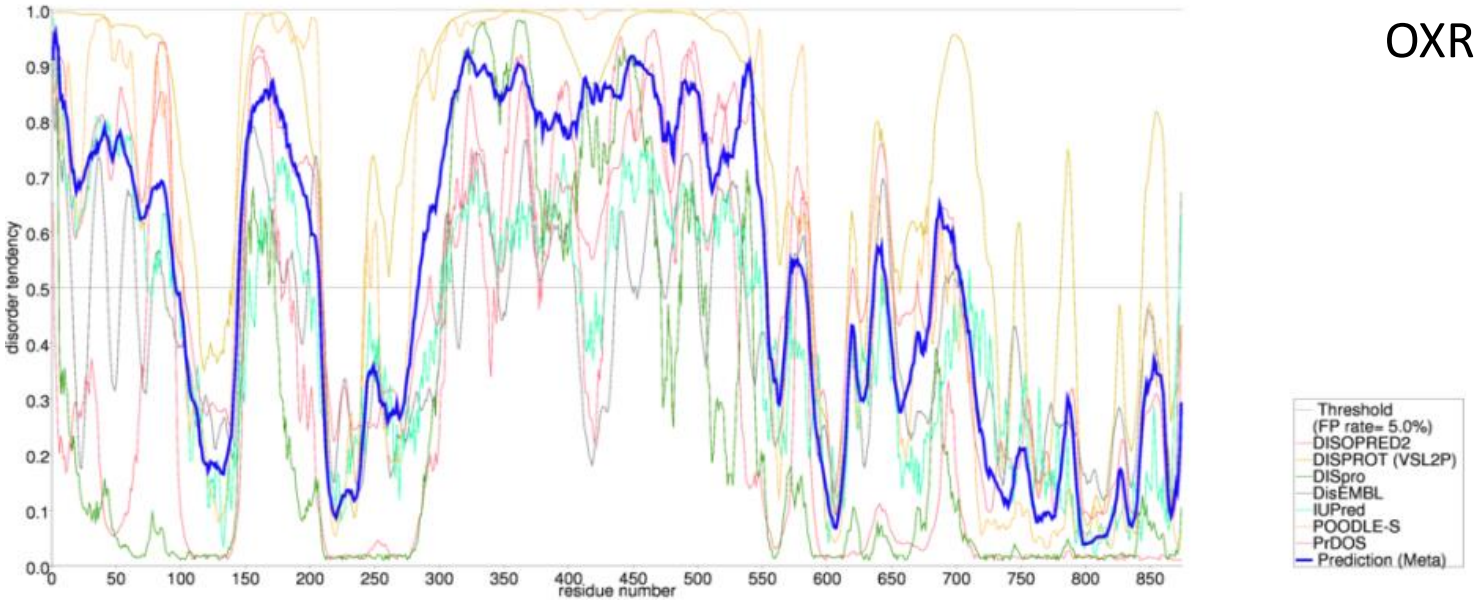
(Active proposals at Stanford Synchrotron Radiation Lightsource, Diamond Light Source, European Synchrotron Radiation Facility)

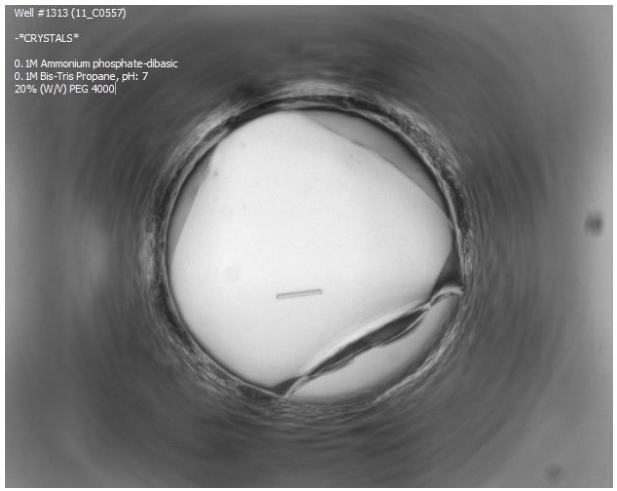


We operate the IMCA beamline at the Advanced Photon Source and have an active collaboration with MacCHESS (Cornell High Energy Synchrotron Source).

No problem getting beamtime when crystals return

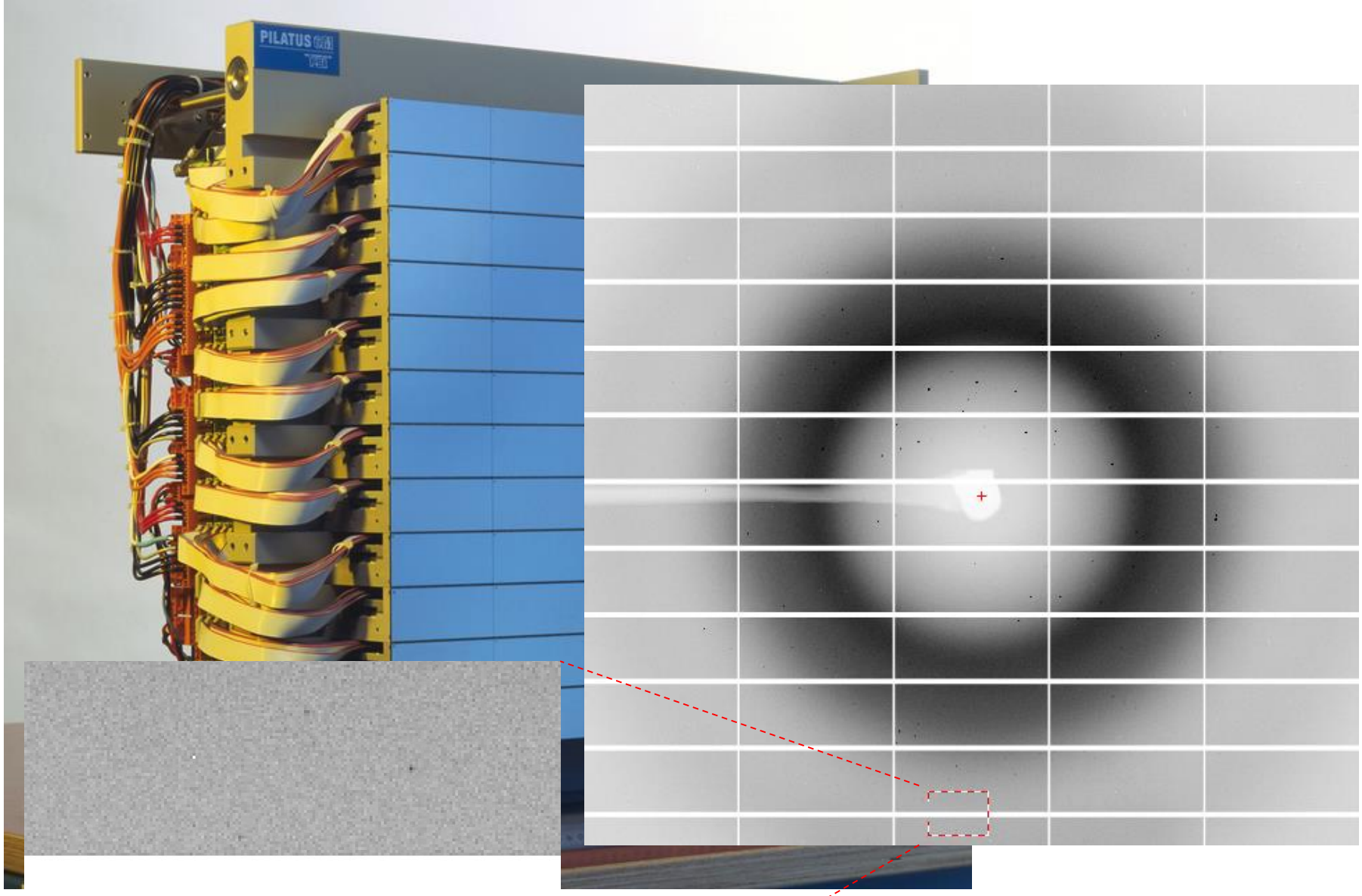
OXR1





Even disordered proteins crystallize. OXR1 has highly disordered regions presumably becoming ordered on complexing or a signature of multiple domains linked by flexible regions – both important for understanding mechanism

Why study these in microgravity now?



nable us to exploit microgravity grown
crystal properties

The technology is **now** available to exploit the improvements we have observed in the past.

Use the best facilities in space with the best on the earth

The Ground Control Issue ….

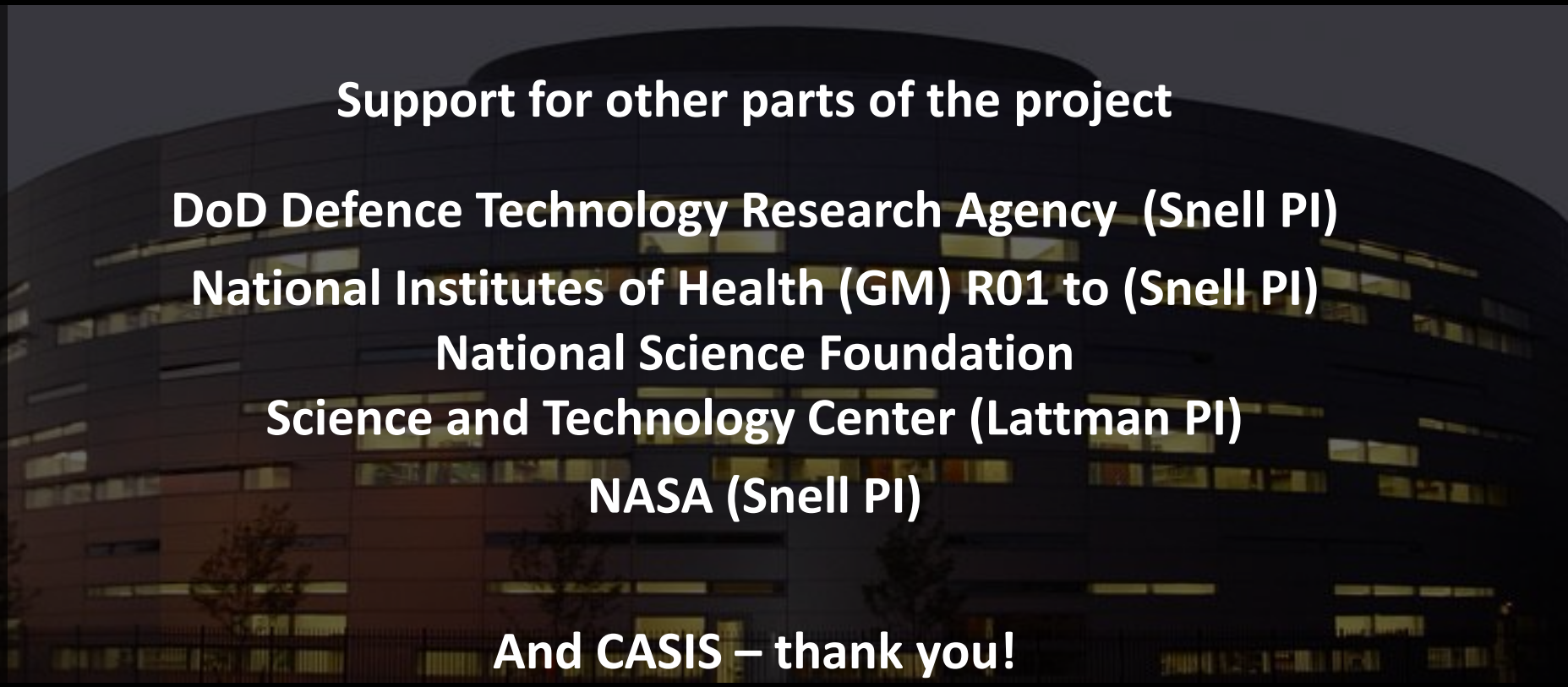
- Microgravity experiments are artificially handicapped by flight requirements.
  - They are chemically static: The chemical ingredients have to be finalized a significant time before the flight.
  - They are physically dynamic: Temperature control is non-ideal.
- On the ground research always moves forward.
  - Iteration is possible.
  - Better conditions become available.
- What is a ground control?
  - Identical to the microgravity experiment but with gravity?
  - The best possible ground versus microgravity?
  - Both?

BioXFEL

Eight post-doctoral positions available at Buffalo, Phoenix, Cornell, Stanford and Milwaukee in the US plus one at Hamburg

(Full details embargoed till Nov 7th)

Leveraging Other Support



Support for other parts of the project

DoD Defence Technology Research Agency (Snell PI)

National Institutes of Health (GM) R01 to (Snell PI)

National Science Foundation
Science and Technology Center (Lattman PI)

NASA (Snell PI)

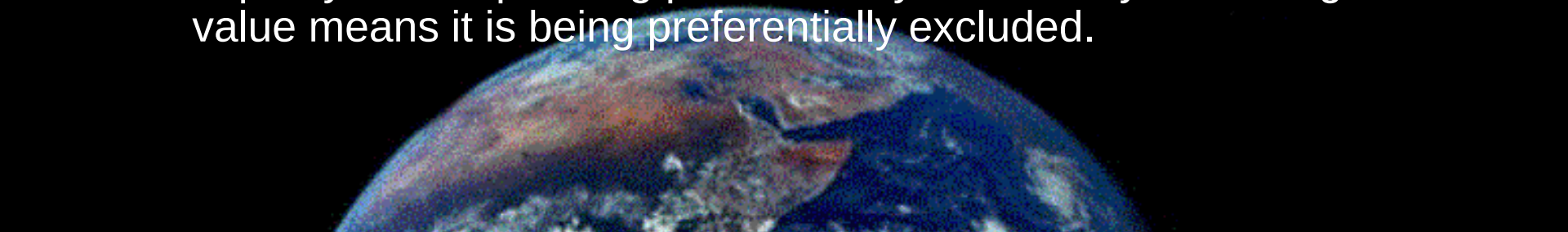
And CASIS – thank you!

Thank you and questions?

esnell@hwi.buffalo.edu

Impurity partitioning calculation

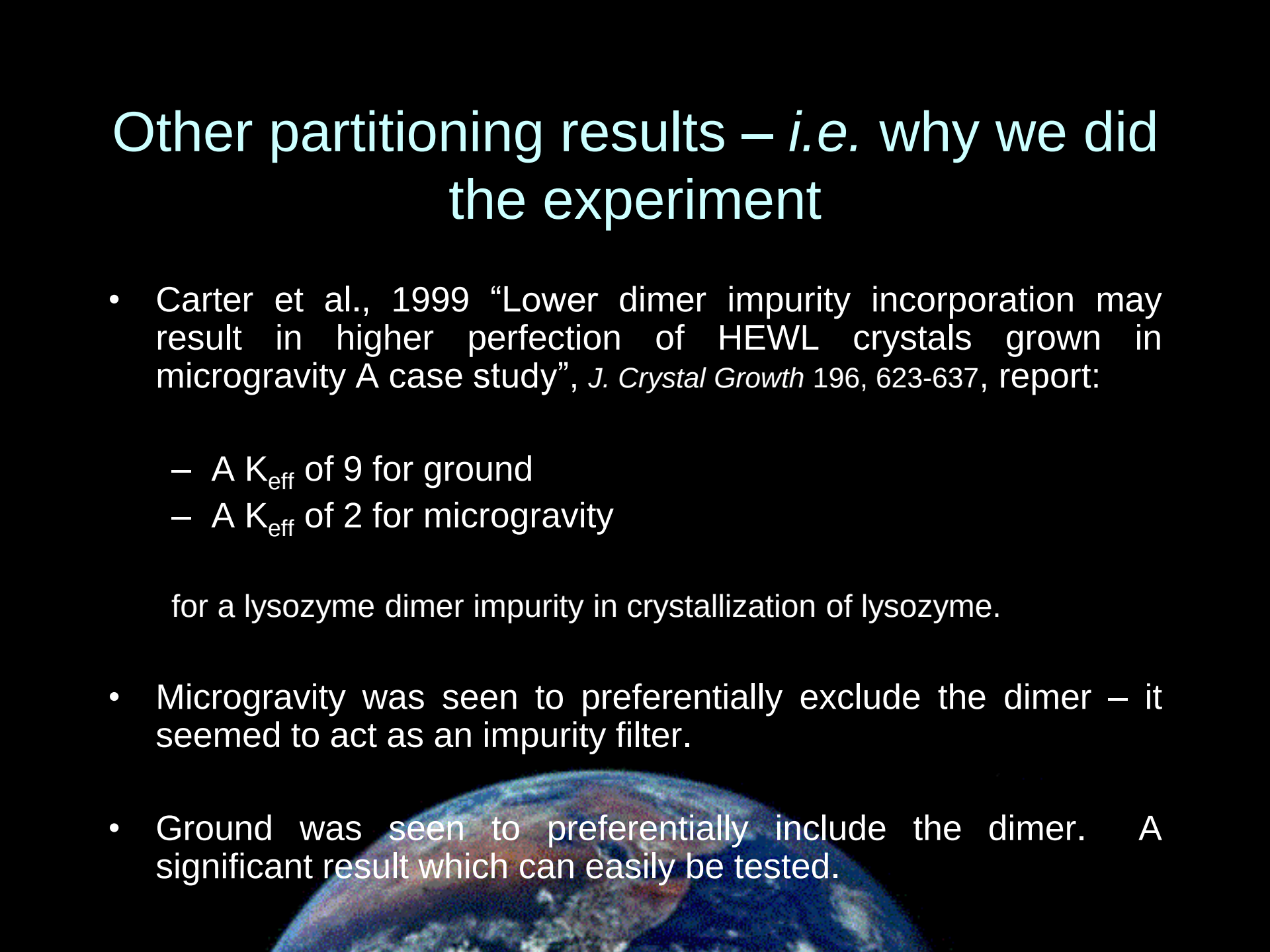
- Impurity partitioning was calculated according to Carter et al., 1999 J. Cryst. Growth 196, 623-627;
  - $K_{eff} = (C_{iS}/C_{pS})/(C_{iL}/C_{pL})$
- Where C_{iS}, is the concentration of impurity in the solid crystal, C_{pS}, is the concentration of the major protein in the crystal, C_{iL}, is the concentration of impurity in the initial solution and $C_{pL,}$ is the concentration of the major protein in the initial solution.
- What does it mean – A positive value of K_{eff} means that the impurity is incorporating preferentially into the crystal, a negative value means it is being preferentially excluded.



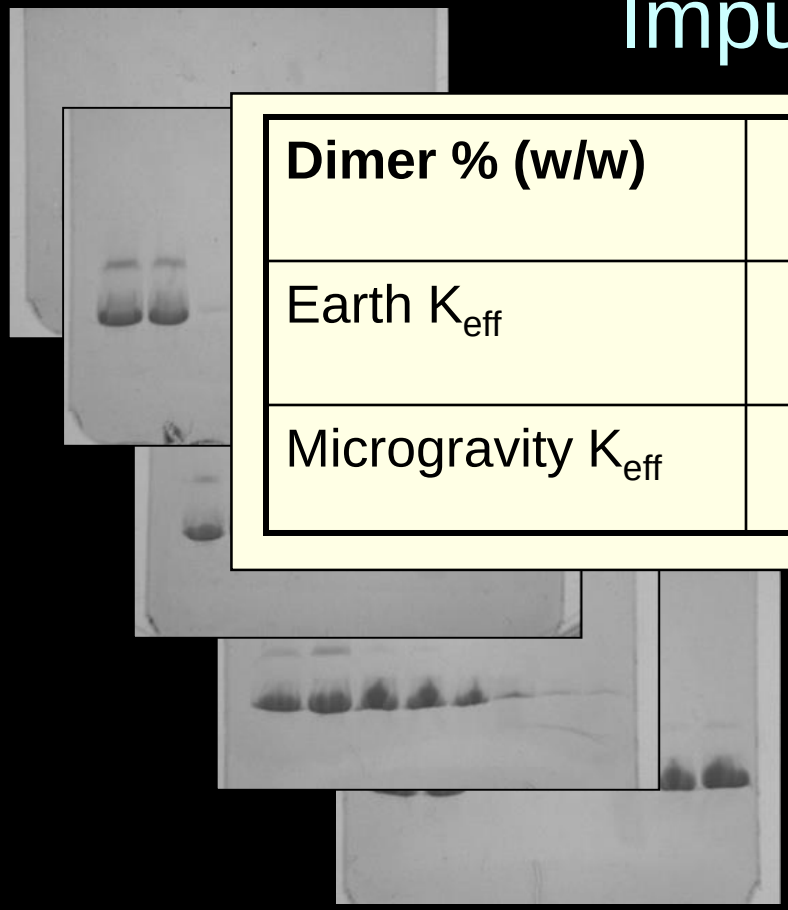
Other partitioning results – *i.e.* why we did the experiment

- 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.



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%

Snell et al., Investigating the Effect of Impurities on Macromolecule Crystal Growth in Microgravity, *Crystal Growth and Design*, 1, 151-158, (2001)