CASIS™

Microgravity Protein Crystal Growth Workshop

FINAL REPORT

Workshop conducted at HudsonAlpha Center for Biotechnology

HUNTSVILLE, ALABAMA
OCTOBER 22-23, 2015

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U.S. NATIONAL LABORATORY

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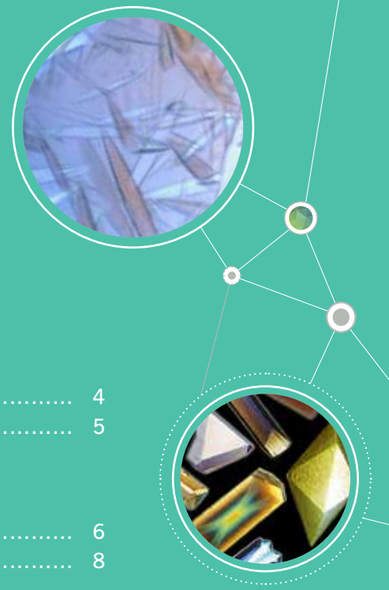

Report Introduction

Microgravity has been used for more than 30 years to improve outcomes of crystal analyses through production of higher quality and larger crystals. To examine the readiness and requirements of this marketplace for a more commercial approach to crystallization on the International Space Station (ISS) U.S. National Laboratory, the Center for the Advancement of Science in Space (CASIS) held a subject matter expert workshop in October 2015 with experts across the field of crystallography.

The mission of this workshop was to outline the basic science requirements for a long-term protein crystallization program onboard the ISS National Lab, including accessibility and timing, flight and ground resources, education, and funding. This report outlines the participants and their presentations, discussions, and recommendations. CASIS intends to harness the information gained from the workshop and the ongoing support of this group to outline an ISS National Lab program for repetitive, low-cost crystallization in microgravity. Such a program will provide a platform for discovery to users across many disciplines—commercial, other government agencies, academia, and private research. In addition, a key component of the program will be inspiring the next generation through experiential learning opportunities.

ABOUT THE ORGANIZERS

About CASIS: The Center for the Advancement of Science in Space (CASIS) was selected by NASA in July 2011 to maximize use of the International Space Station (ISS) U.S. National Laboratory through 2020. CASIS is dedicated to supporting and accelerating innovations and new discoveries that will enhance the health and wellbeing of people and our planet. For more information, visit www.iss-casis.org.

About the ISS National Lab: In 2005, Congress designated the U.S. portion of the International Space Station as the nation's newest national laboratory to maximize its use for improving life on Earth, promoting collaboration among diverse users, and advancing STEM education. This unique laboratory environment is available for use by other U.S. government agencies and by academic and private institutions, providing access to the permanent microgravity setting, vantage point in low Earth orbit, and varied environments of space.

ABOUT GOOD HEALTH

GOOD HEALTH is a CASIS research initiative in partnership with NASA to improve human health by enabling biomedical discovery in space for the benefit of life on Earth. Good Health capitalizes on the unique environment of the ISS National Lab to study, in humans and model organisms, transitions from health to disease that are accelerated by microgravity, including osteoporosis, muscle wasting, and immune dysfunction.

Workshop Guide

INVITEE/ATTENDEE LIST

Tim Allen
Teledyne Brown Engineering

Stephen Aller
University of Alabama at Birmingham

Benjamin Apker
MiTeGen

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JAMSS America, Inc. (JAI)

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Workshop Agenda

Center for the Advancement of Science in Space (CASIS)
Hudson Alpha Center for Biotechnology Technical Interchange Workshop
Implementation of a Low Cost, Robust Protein Crystallization Program aboard the International Space Station National Lab
October 22-23, 2015

DAY 1, OCTOBER 22

Coffee/Pastries/Fruit ..... 8:00A CDT

Welcome/Introduction – CASIS
- » *Debbie Wells/Ken Shields – Who we are and why microgravity crystallization is important* ..... 8:30 – 8:40A

Historical Perspective
- » *Alex McPherson/Laurel Karr* ..... 8:45 – 9:15A

Current Microgravity Investigational Lessons Learned ..... 9:15 – 10:30A
- » *Larry DeLucas – Double Blind Study*
- » *Joe Ng – Crystals for Neutron Crystallography*
- » *Paul Reichert – Merck Pharma Program*
- » *Ken Savin – Eli Lilly Pharma Program*

Break (Coffee/Soda) ..... 10:30 – 10:45A

Theoretical Prediction
- » *Jim Baird, Professor of Chemistry UAH* ..... 10:45 – 11:15A

Panel 1: Molecules of Interest ..... 11:15 – 12:00P
- » *Roberts, Moderator*
- » *Reichert (Merck), Johnson (Pfizer), Eli Lilly (Savin), Tomchick (ACA), DeLucas (UAB), Ng (UAH)*
- ▸ What molecules or classes of molecules should be the focus of the program?
- ▸ Are proteins the only molecules that should be considered?

Lunch Seminar (Box Lunches Provided) ..... 12:15 – 1:00P
- » *Debbie Wells, CASIS – Mysteries of Microgravity Fluid Dynamics*

Panel 2: State-of-the-Art Imaging and Analyses ..... 1:00 – 2:00P
- » *Spinale, Moderator*
- » *Coates (ORNL), Moeck (Microscopy Society), Reibenspies (TAMU), Kovalevskyi (ORNL)*
- ▸ Light microscopy including confocal microscopy
- ▸ All types of electron microscopy
- ▸ X-ray and neutron diffraction
- ▸ Nuclear magnetic resonance
- ▸ Scanning force microscopy, scanning probe microscopy, and tunneling microscopy
- ▸ Digital image processing
- ▸ Computational insights into structure

Panel 3: Laboratory-Based Crystallography ..... 2:00 – 3:00P
- » *Wells, Moderator*
- » *Clemente (Robbins), Pryor (Microlytic), Apker (MiTeGen), Isaac (Molecular Dimensions)*
- ▸ Tools and Techniques
- ▸ Commercial crystallography

Break (Coffee/Sodas) ..... 3:00 – 3:15P

Panel 4: Space-based Crystallography Capabilities ..... 3:15 – 4:30P
- » *Spinale, Moderator*
- » *O'Connor (UAB), Arias/Tanaka (JAMSS), Carter (PCAM), Pusey/Gorti (MSFC), Jenkins (Granada), Boland (Techshot), Murphy (Nanoracks), Allen (TBE)*
- ▸ Flight approved hardware – organic and inorganic molecules
- ▸ Other items not

Day 1 Wrap-up ..... 4:30 – 4:45P

Depart for Hotel 5:00P

DAY 2, OCTOBER 23

Coffee/Pastries/Fruit ..... 8:00A CDT

Summary of Day 1 Panel Points/Break-Out Prep ..... 8:30 – 9:00A
- » *Debbie Wells*

Break-Out Session: Crystallization to a Set of Program Science Requirements ..... 9:00 – 10:30A
- » *IP Reps/CASIS Reps*
- ▸ Break-out teams with representatives from distributed background to narrow down the set of "requirements" for the spaceflight program
- ▸ One CASIS person/one CASIS IP person will lead each break-out team
- ▸ Notes from Day 1 Sessions provided

Break (Coffee/Soda) ..... 10:30 – 10:45A

Break-Out Presentations ..... 10:45A – 12:15P
- » *Wells/Break-Out Teams*
- ▸ Each break-out group presents their requirement concepts
- ▸ Note-taking/grouping of ideas

Lunch (Full Meal) ..... 12:15 – 1:15P

Funding Strategies – Translation of Lab to National Lab ..... 1:15 – 2:15P
- » *Wells*
- ▸ Commercial
- ▸ Academic
- ▸ Other Government Agencies
- ▸ Foundations
- ▸ Research Organizations
- ▸ Venture Capital

STEM Education/Outreach ..... 2:15 – 3:00P
- » *Ng/DeLucas/Shields*
- ▸ Year of Crystallography Competition
- ▸ IUCr Growing Competition 2015

Consolidation of Requirements and Group Concurrence ..... 3:00 – 3:45P
- » *Wells*

Next steps and Closing ..... 3:45 – 4:00P
- » *Wells/Shields – Report to be distributed and reviewed during breakout at ASGSR.*

Recommendations from Workshop

GENERAL RECOMMENDATIONS:

- The comparative advantage of doing PCG in microgravity should be documented/published, and focus should turn to the state-of-the-art.
- Fundamental research on how to do crystallization in space for any protein remains critical.
- There is a consensus that outreach (backed up by literature) remains critical in the PCG community about the how/when there are advantages of PCG in microgravity.
  - » *In addition to traditional publications, social media (YouTube videos/tweets/Facebook page) may be a good way to target new audiences.*
  - » *Relationships with international societies should be explored to bridge structural biologists and chemists with the available capabilities (a comprehensive overview of hardware capabilities needs to be created to communicate various options).*
  - » *A workshop/session and/or exhibit could be held at the American Crystallographic Association's annual meeting, but planning would need to be started for the 2017 meeting.*
- Information for new investigators including previous flight information, relevant publications, lessons learned, detailed experimental design/operations plans, etc. should be consolidated in an accessible location. Provide website or brochure where dedicated data for microgravity PCG can be referenced.

MOLECULES OF INTEREST:

- Molecules of interest include any protein of high biological significance with an indication of scientific, medical, or commercial interest and that shows the need for improved crystalline order, diffraction, or an improved electron density map or where good diffraction or a good electron density map cannot be obtained on Earth.
- Specific molecules of interest include:
  - **ORGANIC MOLECULES:**
  - » *Membrane proteins*
  - » *Protein-protein and multiprotein complexes*
  - » *Protein-ligand interactions*
  - » *Enzymes*
  - » *Ion channel proteins*
  - » *Side chain configuration of proteins*
  - » *Small molecules*
  - **INORGANIC MOLECULES:**
  - » *Semi conductor/liquid crystals*
  - » *Zeolites*
  - **NANOCRYSTALS (VERY TINY STRUCTURES)**
- There is a need to grow both large crystals as well as small uniform crystals. Hardware and processes must be available to support both.
  - » *By 2017, 50% of proprietary novel therapeutics will be biologics (monoclonal antibodies and vaccines). There is a need for advances in the drug delivery and manufacturing of these therapeutics. Crystalline suspensions are being investigated to fulfill these unmet needs.*
  - » *There is a need for uniform crystalline suspensions for structural studies using FEI Company's nano-crystallography experiments.*
- Check-list for a good candidate protein to crystallize in microgravity:

1. *The protein should be able to achieve nucleation on Earth preferably in the flight hardware.*
2. *The protein should be able to be produced in enough quantity and with enough uniformity to support experiment/operational design and improve chances for successful crystallization.*
   - » *Material for at least 3 scrub refurbishments should be planned*
   - » *Contingency plans in the event of additional delays—availability of alternative protein?*
3. *If the investigator has a crystal of the protein, there should be a demonstrated need for improvement (the need for a larger or more uniformly produced crystal, a crystal with a better diffraction resolution, improvement of diffraction data/electron density).*
   - » *Current crystal resolution is less than 4.0 angstroms*
   - » *Current crystals that diffract at 2.5 angstroms but higher quality is needed for different analyses to provide additional/new data*
4. *The investigator should have some characteristics of the protein's stability (such as temperature range and time-dependent deterioration). Sodium dodecyl sulfate (SDS) gel electrophoresis and/or size exclusion chromatography (SEC) may provide helpful data in preparation of flight experiment.*

- Partnerships should be sought to identify and validate models that predict crystallization success. Once these models are validated, the PCG community could determine the best role of the models to identify good candidate proteins.

IMAGING AND ANALYSIS:

- Imaging is not required for the science, but it would be very useful for both the science (knowing when nucleation took place or when the crystal grew and then degraded) as well as educational outreach. Imaging is difficult to obtain for crystals requiring temperature control. Best option may be to provide for room temperature samples only.
- There is a need for vapor diffusion, liquid-liquid diffusion, and batch capabilities for x-ray and neutron diffraction.
- X-ray diffraction and neutron diffraction are the main tools used for primary structure determination (although there are some other options available for nanocrystals).
- For analysis, investigators need to be able to remove the crystal from the hardware and mount it, so access to the crystal and ease of crystal removal needs to be one of the top characteristics of hardware. It can be difficult to remove the crystal from the hardware without damaging the crystal (this often happens when the crystal adheres to the hardware), so if hardware could also be compatible with in situ data collection, this would be a desired feature.
- Hardware options are important because some proteins may crystalize better in one set of hardware versus the other.
- Hardware material, type of plastic or glass, is important for any type of in situ data collection.
- Different options for hardware should be made available for investigators to test in their lab to determine what works best for their protein.
  - » *Investigators may be hesitant to try new hardware/methods, which may explain why there are so many types of plates and ways to grow crystals (it is sometimes dependent on the protein and/or the expertise of the crystallographer).*
  - » *Testing with available hardware allows the PI to evaluate the potential for microgravity success—both those interested in microgravity research and those not primarily interested in it.*

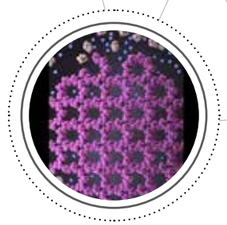
LABORATORY COMPATIBILITY:

- Optimization for spaceflight improves lab procedures that can be translated to standard ground crystallization.
- When investigators connect with CASIS for a flight experiment, CASIS should provide consulting options to investigators for reliable expertise to assist with this selection for their proteins of interest.
- CASIS should focus on down-selecting to a small number of hardware pieces (maybe 2 to 5 pieces) that work best, and make sure those are fully enabled to fly experiments quickly—there could be a sustainable placeholder for PCG experiments on a fixed fly schedule that would just need to be filled with users.
  - » *Instead of an on-demand service for PCG in microgravity, it would be a laboratory service model already in place for users to utilize.*
  - » *This would not preclude a one-off PCG experiment that does not fit into the model.*
- Some investigators want an end-to-end solution—they would like someone else to go through the optimization process and hand-load or auto-load the protein for them.
- Investigators should know the type of analysis they will be doing before they decide which hardware to use.
- Over the last decade protein crystallization for X-ray has become a very systematic, automated, high throughput process. Any efforts to make PCG compatible with the industry standard Society For Laboratory Automation And Screening (SLAS), formerly Society of Biomolecular Screening (SBS) crystallization plates and robots used in these workflows would benefit both CASIS and the investigators.

Recommendations from Workshop

FLIGHT HARDWARE:

- Current PCG Systems options include:
  - *Depression Plates (Hanging drop) (Vapor diffusion) or COTS 96 well plates (Vapor Diffusion)*
  - *Capillaries (Liquid-to-Liquid)*
  - *Vial/Bottle (Batch Crystallization)*
- Must-have:
  - *A way to keep the protein and precipitant separated for controlled release through use of physical barriers or freezing/cooling (it would be ideal to keep the two components physically separated in the hardware so there is not a need to cool it or reload due to a flight delay or mission scrub).*
  - *High-fidelity replicates in sufficient quantity for ground testing and optimization.*
  - *Ease of crystal removal and minimization of crystal handling.*
  - *Size that is scalable to crystal size (from 100 nanoliters up to 200 microliters, or up to 1 mL in some cases).*
  - *Disposable wells for the protein (cleaning wells should be avoided due to potential damage to the wells).*
  - *Ability to use temperature to activate and arrest crystallization*
  - *Materials need to be non-permeable and stable.*
- Nice-to-have:
  - *SBS compliance (investigators would like the capability to fly what is already being used in the lab so they do not have to adapt their science to different flight hardware).*
  - *The ability to image through the hardware (the trade-offs would need to be examined).*
  - *A way to address evaporation issues.*



Additional Comments from Advisory Panel:

- The concept of “Quality Control” is important. Checking and recording every status during the experimental processes are necessary to find the reasons for good or bad results. In some cases, unsuccessful results are not dependent on the space crystallization but on the protein sample and /or the unsuitable crystallization condition.
- There are three optimization steps of the protein sample and the crystallization condition for the space experiment. The first one is the reproducibility of the crystallization, the second is the optimization to the crystallization device and the third is the optimization to enhance micro-gravity effect. To launch protein samples without any optimization would likely lead to poor results.
- In the future, the duration of the flight can be selected for the target protein. For example, a membrane protein that does not produce a stable crystal is preferable for a short flight, i.e. one to two weeks. While experiments designed to ripen a crystal, may require a longer flight, i.e. more than 6 month.
- “Ripening” is a very interesting issue (J.D.Ng et al., Acta Cryst. F71, 358-370, 2015), however it is necessary to further investigate the phenomena in order to determine its usefulness.

Day 1 Session Summaries

HISTORICAL REVIEW

Historical Perspective – Alex McPherson

The first protein crystallization experiments in microgravity were carried out on the Space Shuttle. In 1984, NASA flew a hanging drop experiment in an apparatus strapped to the side of the main deck of the Space Shuttle. Although it was a crude experiment, it produced interesting results—some crystals appeared to benefit from the microgravity environment, while others did not. The results were not definitive, yet the experiment generated much interest within the crystallography community regarding the idea that macromolecular crystals may grow better in microgravity.

In the early 1990s, scientists in Germany flew a liquid-liquid diffusion device called CRYOSTAT on the Space Shuttle. The experiment yielded satellite tobacco mosaic virus (STMV) crystals with a volume about 40 times greater than had ever been achieved for STMV. The crystals allowed researchers to refine the structure of STMV to a 1.8-angstrom resolution—this remains the virus particle refined to the greatest resolution of any to date.

European scientists also constructed the Advanced Protein Crystallization Facility (APCF), a much more sophisticated facility with 36 cells for liquid-liquid diffusion activated automatically in microgravity. The experiment was carefully designed to reveal any differences in crystal growth that occurred as a result of the microgravity environment; and the results demonstrated that microgravity does, indeed, appear to affect crystal growth.

In the 1990s, U.S. scientists designed a PCG experiment to fly on Russia's space station Mir. For the experiment, one end of each Tygon tube was filled with protein solution and frozen, and the other end was filled with precipitant and frozen. The frozen tubes were packed into a dewar and flown to Mir. Once on the space station, the tubes were allowed to defrost, permitting liquid-liquid diffusion. The experiment was cheap, easy, and successful, and it flew several more times on Mir and on the ISS. The experiment yielded numerous crystals; and, in many cases, the mosaic spread of the diffraction of the crystals grown in space showed enormous improvement. Researchers hypothesized that this improvement may occur because the molecules are presented in the crystal in a much more ordered way in microgravity, thus producing fewer defects. They further hypothesized that crystals grown in microgravity may exclude impurities because impurities are generally larger aggregates of the target molecule and, therefore, diffuse more slowly.

In 1992, NASA funded the development of the Observable Protein Crystal Growth Apparatus (OPCGA)—a solid-state Mach-Zehnder interferometer with 96 cells—to fly on the ISS. Completed in 2002, the OPCGA was designed to monitor the exact concentration gradients around growing crystals. Scientists hoped the experiment would produce solid scientific data on PCG in microgravity. Unfortunately, the mission prior to its scheduled flight resulted in the tragic loss of Space Shuttle Columbia, and the OPCGA was never sent to the ISS.

Research on PCG in microgravity lay fallow until about three years ago, when NASA revived interest in PCG research on the ISS. Recently, experiments have been conducted on the ISS National Lab to test the growth of additional proteins in microgravity. Although the experiments have yielded positive results, they do not appear to be as good as those from the experiments of the early 1990s. Providing researchers with very high-quality hardware, similar to the hardware available in the early 1990s, may help to achieve higher quality results.

Historical Perspective—Laurel Karr

During the Space Shuttle era, NASA sponsored more than 60 PCG experiments on more than 200 different macromolecules (in addition to the many PCG experiments sponsored by ESA, JAXA, and Roscosmos). Several NASA-sponsored experiments were also flown on the ISS until 2003. After 2003, ESA, JAXA, and Roscosmos continued flying PCG experiments to the ISS in their own hardware.

The "Researchers Guide to Macromolecular Crystal Growth on the ISS," published by the NASA ISS Program Science Office, is available at http://www.nasa.gov/mission_pages/station/research/researcher_guide.

CURRENT MICROGRAVITY LESSONS LEARNED

Double Blind Study—Dr. Larry DeLucas

This study aimed to demonstrate the singular effect of microgravity on crystal quality for a variety of high-value proteins that are not easy to crystallize on Earth. The analysis was performed as a "double-blind" experiment—the investigators did not know which samples were flown in microgravity and which were from ground controls. The study included 360 chambers at 20°C for vapor diffusion, 360 chambers at 4°C for vapor diffusion, 900 capillaries at 20°C for liquid diffusion, and 840 capillaries at 4°C for liquid diffusion.

The proteins were mixed with the precipitant but were not activated until they reached the ISS. The samples were originally loaded on 4/12/14; however, the launch was delayed. It did not occur until 4/18/14, and the samples were activated on 4/21/14. Some, but not all, of the samples were reloaded on 4/16/14.

A total of 96 proteins were flown, and 61 proteins (63 percent) yielded crystals in both microgravity and ground controls. Several of the vapor diffusion experiments exhibited significant precipitation without evidence of crystals. This could be due to the launch delay, as the vapor diffusion experiments were premixed prior to launch, and only about half of the proteins were able to be reloaded due to insufficient supply of the proteins. Many of the poor vapor diffusion results came from the proteins that could not be reloaded. Another factor that may have affected the study's success rates is the extended mission. The experiments were originally proposed to stay in orbit for three months (the optimal time for PCG experiments); however, they ended up spending about six and a half months in orbit. Although this extension did not appear to affect the liquid diffusion experiments, it did have a considerable adverse effect on the vapor diffusion experiments—once the crystals were fully grown, they began to degrade.

There were many cases in which the mosaic spread and resolution of the microgravity-grown crystals were only slightly better than the ground controls, and the results were not as good as those from previous PCG experiments in microgravity. This may be due to snap freezing the crystals, which changes the mosaic spread and affects the quality of the resolution. It is important to consider whether snap freezing destroys the advantage of growing protein crystals in microgravity. One way to potentially avoid snap freezing is by using neutron diffraction (if the crystals are large enough).

However, the study did yield many positive results. There were many cases in which the crystals grew to the entire size of the chamber; and the study yielded the highest-resolution data set ever for a TB protein crystal. For the bacterial protein GBS Sortase B, although there was little difference in crystal size or mosaic spread between microgravity-grown crystals and ground controls, there was a considerable difference in the signal-to-background ratio—the microgravity-grown crystals diffracted about 0.35 angstroms, better than the best crystal for this protein ever grown on Earth. This level of resolution improvement allows scientists to see more features on the electron density map and, thus, to better trace the chain and more accurately position the amino acids.

In an upcoming study, the investigators will examine why protein crystals may grow better in microgravity. The investigators have hypothesized that the improved quality of microgravity-grown protein crystals is the result of two macromolecular characteristics that exist in a buoyancy-free, diffusion-dominated solution—slower crystal growth rates (due to slower protein transport to the growing crystal surface) and predilection of growing crystals to incorporate protein monomers versus higher protein aggregates (due to differences in transport rates).

Crystals for Neutron Crystallography—Dr. Joseph Ng

This study sought to produce crystals of inorganic pyrophosphate phosphatase—a very stable protein associated with several health problems—of sufficient size for neutron crystallography. Determination of the enzyme's structure with a high enough resolution to reveal the hydrogen atoms (hydrogen comprises half of the atoms in proteins) could lead to the development of an inhibitor.

The study consisted of two Granada Crystallization Facility (GFC) units, each containing 20 Granada Crystallization Boxes (GCB). Each GCB holds 10 large-diameter (2 mm) capillaries, so each GFC unit contained 200 capillaries. Each capillary was filled with protein solution set to equilibrate with a precipitant that slowly diffuses across a gel buffer to facilitate counter diffusion crystallization. As the protein solution meets the diffusion interface, it produces a gradient of saturation along the length of the capillary. Both GFC units launched on SpaceX-3 on 4/18/14. One unit spent 30 days in orbit (returned on SpaceX-3 on 5/18/14), and the other unit spent six months in orbit (returned on SpaceX-4 on 10/25/14).

The short-term (30-day) experiment yielded microgravity-grown crystals that varied in size along the length of the capillary, with some of the largest crystals growing to almost the full diameter of the capillary. Compared with ground controls, the microgravity-grown crystals were much larger and had a higher I/ò as a function of resolution.

Day 1 Session Summaries

The full-term (six-month) experiment yielded microgravity-grown crystals so large that they could be seen in the capillaries without any magnification. A large number of small-volume crystals grew in one end of the capillary, and crystal size increased along the length of the capillary. Many microgravity-grown crystals grew to the full 2-mm capillary diameter—an ample size for neutron diffraction. The ground-control capillaries did not contain nearly as many crystals, and the crystals were much smaller.

Both X-ray diffraction and neutron diffraction were performed on the crystals. The best microgravity-grown crystals were compared with the best Earth-grown crystals (although it was difficult to find high-quality Earth-grown crystals because the crystal volumes were so much smaller). For neutron diffraction analysis, the crystals were mounted in the center of a spherical array of 30 detectors at Oakridge National Laboratory. The microgravity-grown crystals remained in the capillaries for neutron diffraction analysis, as they had grown to the full diameter of the capillary. They were held securely in place, thus eliminating the need for manipulating the crystals.

Overall, the microgravity-grown crystals had higher resolution and a much higher I/ò compared with ground controls, resulting in electron density maps with greater detail and higher accuracy. The neutron diffraction analysis also revealed the placement of hydrogen atoms in the structures. This allowed researchers to know the orientation of water in the structures, which is important because it helps them better understand the enzyme's mechanisms.

Currently, less than 0.08 percent of solved protein structures have been solved using neutron diffraction. This is because neutron diffraction requires large-volume crystals, which are difficult to grow on Earth. If microgravity can be instrumental in providing conditions to increase the volume of crystals, it could provide a gateway to solving more protein structures using neutron diffraction.

Merck Pharma Program—Paul Reichert

There is a great opportunity to use crystalline suspensions for applications in the pharmaceutical industry. Pharmaceutical companies, such as Merck, are interested in crystalline biologics for drug discovery (structure-based drug design), product development (controlled release, high-concentration formulations, and subcutaneous and pulmonary delivery), and manufacturing (more efficient purification and stable concentrations that make storage easier).

Pharmaceutical companies are mainly interested in protein crystallization research in microgravity on monoclonal antibodies, which must be given in very high doses and are not very soluble. Protein crystallization could be used to make concentrated crystalline suspensions of monoclonal antibodies to use as injectable products. If pharmaceutical companies could produce small, uniform particles to put in an aerosol, the drugs could be sent to the lungs for systemic delivery.

For manufacturing, pharmaceutical companies are in need of a simple technique to replace the current extensive purification process to achieve more cost-effective products. Pharmaceutical companies also seek a solution for storage issues. Monoclonal antibodies are shipped in large bags from the place where the active ingredients are made to formulation sites around the world. If pharmaceutical companies could develop a concentrated crystalline suspension that is stable at room temperature, it would be of tremendous value.

Doing crystalline biologics research in microgravity has several benefits. Microgravity provides reduced sedimentation, minimized convection currents, and reduced molecular diffusion rates. Microgravity-produced crystals are larger, higher order, and purer, and microgravity enables the production of more uniform crystalline suspensions. In particular, pharmaceutical companies can take advantage of the minimized convective currents in microgravity to control nucleation and subsequent crystal growth. To achieve more uniform suspensions with high purity for pharmaceutical applications, there is a need for more nucleation and less crystal growth—the opposite of what most crystallographers are trying to do.

Merck conducted protein crystallization experiments on 11 Space Shuttle missions from 1993 to 2003. These experiments yielded microgravity-grown crystals that are 40 percent to 50 percent larger than ground controls. The experiments also yielded a more uniform crystalline suspension (20-micron crystals) with better dissolution properties. A one-liter bottle of a drug from one experiment generated enough crystals to use in primate studies for about two years, greatly driving the research forward. However, there were also times when the experiments yielded poor quality crystals, mainly due to issues such as multiple launch scrubs, crew activity, hardware issues, differing quality between batches of proteins, etc.; but the overall process of preparing and conducting crystallization research in microgravity led investigators to learn more and improve their process. This highlights the importance of providing investigators with multiple opportunities to fly their experiments.

Recently, Merck conducted two protein crystallization studies on the ISS National Lab that focused on monoclonal antibodies being investigated for multiple diseases. The first experiment, launched on SpaceX-3 in April 2014, sought to produce single crystals for structure determination. The Mab1 sample was stored in flight stowage bags on the ISS for the duration of the experiment. The experiment yielded microgravity-grown crystals that were 40 percent larger than the ground controls. However, although the microgravity-grown crystals diffracted better than ground controls, the structure of the protein could not be solved due to technical issues.

Merck launched another experiment on SpaceX-6 in May 2015 that aimed to produce crystalline suspensions for multiple pharmaceutical applications, including drug delivery, purification, and storage of bulk active ingredients. Samples of Mab2 and Mab3 were put into a high density protein crystallization growth (HD-PCG) unit, and a MERLIN incubator was used to ramp up the temperature from 4°C to 30°C. The Mab2 sample produced a few malformed crystals; the Mab3 sample did not produce any crystals. There were no anomalies with the hardware, incubators, or processing. However, one lesson learned is that temperature control is crucial, and the temperature must reach 30°C to achieve crystal growth. Merck is in the process of planning additional protein crystallization experiments for the ISS National Lab, which are expected to launch on SpaceX-10.

THEORETICAL PREDICTION

Jim Baird presented on the physical chemistry of protein crystallization. Nature has made protein crystallization difficult because of the deleterious effects that would otherwise ensue if crystals were to form in a living organism. Nevertheless, water soluble proteins can be crystallized from isothermal, pH buffered, aqueous solutions of strong electrolytes under sufficiently non-physiological conditions.

Kinetics describes the process by which the growth solution approaches equilibrium. Crystallization experiments are said to be under kinetic control when the protein concentration in the growth solution is greater than the equilibrium solubility. Thermodynamics describes the state of equilibrium. Crystallization experiments are said to be under thermodynamic control when the protein concentration in the growth solution is equal to the equilibrium solubility. Baird concluded that kinetics can affect crystal shape and thermodynamics can affect crystal size.

Day 1 Session Summaries

I PANEL 1: MOLECULES OF INTEREST

Panel Members

Lawrence DeLucas
University of Alabama at Birmingham
www.uab.edu/csb/faculty/article1

Eric Johnson
Pfizer
www.pfizer.com/research/science_and_technology/rd_locations/ca_la_jolla

Joseph Ng
iXpressGenes, Inc.
www.ixpressgenes.com

Paul Reichert
Merck
www.merck.com/about/our-people/paul-reichert.html

Ken Savin
Eli Lilly and Company
www.lilly.com/home.aspx

Diana Tomchick
American Crystallographic Association
www.amercrystalassn.org

Key Points

- Key molecules of interest include 1) full-length, biologically relevant protein constructs for which it is difficult to get crystals or the crystals have poor diffraction quality; 2) membrane proteins; and 3) protein-protein complexes.
- Small molecule crystallization is also of interest.
- There is a need for larger crystals as well as crystals of uniform size.
- There is value in sharing lessons learned from past protein crystal growth (PCG) flight experiments.

Challenges Noted

- Currently, there is a steep learning curve for members of the PCG research community unfamiliar with the process of doing PCG in space.
- Transitioning from ground hardware to flight hardware is daunting.
- The stability of crystals over the duration of the spaceflight and return is of concern due to the risk of crystal degradation.
- There is often not enough purified protein to re-load in the event of a flight delay.

Panel 1 focused on identifying the key molecules or classes of molecules of interest for a protein crystallization research program on the ISS National Lab. Key targets identified during the discussion include 1) full-length, biologically relevant protein constructs for which it is difficult to get crystals or the crystals have poor diffraction quality; 2) membrane proteins; and 3) protein-protein complexes. There is great interest in membrane proteins among structural chemists, and NIH is currently very interested in membrane proteins. Participants agreed that it is important to do a basic experiment to determine if crystallization of membrane proteins in lipidic cubic phase (LCP) is possible in space. Protein-protein complexes are of interest because understanding how proteins interact is important for drug development; and drug design requires high-resolution protein structures. It was noted that there is a need for both larger crystals as well as crystals of uniform size. In addition to proteins, small molecule crystallization is of interest, particularly to pharmaceutical companies.

Subsequent discussion focused on potential hurdles involved with translating PCG research from the ground to the ISS. It was noted that there is currently a steep learning curve for members of the PCG research community who are not familiar with the process of doing PCG in space. Potential users need to understand the timeline for spaceflight experiments to determine if their projects would fit well into the process. Potential users also lack an understanding of flight hardware, and it is daunting for researchers to switch from hardware they are familiar with in their lab on the ground to flight hardware that they have no experience with. If the plates that researchers use in their labs could be frozen and flown, it would make the transition from a ground experiment to a flight experiment much easier; however, it is not known if this is feasible.

A question arose about the need to systematically compare crystallization techniques and determine the optimal technique for crystallizing different types of molecules in space. However, it was noted that each lab has its own technique using specific hardware; and if that hardware could be flown as is, the researcher would not have to spend time and effort adapting it. If the hardware does need to be adapted for spaceflight, one way to make the transition easier would be making sure the hardware is SBS compliant. It was also noted that hardware that allows for in-situ diffraction analysis would be beneficial.

Another potential hurdle to conducting PCG research in space is the stability of crystals over the duration of the spaceflight and return. Flight delays and scrubbed missions add to this problem. In particular, membrane proteins are not stable, and data should be collected as soon as the crystal reaches its full size, otherwise the crystal starts to degrade. In many cases, crystals should not stay on the ISS for more than 30 days, or they risk degradation. Another problem that affects the quality of crystals is that often there is not enough of the purified protein to re-load in the event of a flight delay. One option to address this issue is to preserve the purified protein by freezing it; however, this is not ideal, because freezing and thawing could affect crystal quality. Additionally, freezing would not work for membrane proteins. Another possible way to address this issue is through automated protein loading, which could help reduce the time it takes to load protein samples and allow additional time to purify more protein and re-load in time for launch.

It was agreed that there is value in sharing with the PCG research community lessons learned from past PCG flight experiments. This would help to communicate what has been done and what did and did not work so that potential users would not have to start from scratch.

II PANEL 2: STATE-OF-THE-ART IMAGING AND ANALYSIS

Panel Members

Leighton Coates
Oak Ridge National Laboratory
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Peter Moeck
Microscopy Society, Portland State University
www.web.pdx.edu/~pmoeck/

Joe Reibenspies
Texas A&M University
xray.tamu.edu

Andrey Kovalevsky
Oak Ridge National Laboratory
neutrons.ornl.gov/contacts/kovalevskyay

Key Points

- The two main imaging and analysis techniques for PCG research are X-ray diffraction and neutron diffraction.
- Because neutrons are uniquely sensitive to hydrogen atoms, neutron diffraction allows for macromolecular structure-function studies in unprecedented atomic detail.
- Electron microscopy (EM) provides the advantage of not having to grow 3-D crystals; however, EM is only complementary to other methods.
- The Oak Ridge National Laboratory Spallation Neutron Source is the most powerful pulsed neutron source in operation, and researchers can utilize 19 different instruments.
- Currently, researchers can apply for beam time at the Oak Ridge National Laboratory Spallation Neutron Source through a peer-review process; however, it may be possible to reserve a certain amount of beam time per cycle for a constant flow of PCG researchers.
- Kapton® tubing and PTD tubing work well for X-ray diffraction.
- Metal-organic frameworks (MOFs) could be used to grow more ordered crystals for imaging and analysis.

Panel 2 examined the current ways in which crystal imaging and analysis are done and how this could influence the ways in which PCG research on the ISS National Lab is conducted (e.g., the optimal hardware, protein preparation techniques, and crystal characteristics for imaging and analysis). Currently, there are two main imaging and analysis techniques used for PCG research—X-ray diffraction and neutron diffraction. X-ray diffraction reveals protein structure details; however, neutron diffraction reveals atomic details and allows for macromolecular structure-function studies in unprecedented atomic detail. Because neutrons are uniquely

sensitive to hydrogen, neutron diffraction can be used to precisely locate hydrogen atoms, enabling a more accurate determination of molecular structure. It was noted that electron microscopy (EM) provides the advantage of not having to grow 3-D crystals. However, it was agreed that EM is a complementary technique, and 3-D crystals will always be needed.

The Oak Ridge National Laboratory Spallation Neutron Source produces neutrons in pulses (60 Hz) and is the most powerful pulsed neutron source currently operating. The research facility operates 19 different instruments, including the IMAGINE beamline (a quasi-Laue single crystal neutron diffractometer) and MaNDi (macromolecular neutron diffractometer, a time-of-flight wavelength resolved Laue diffractometer designed for flexibility and high signal-to-noise data collection). Although images take hours to produce using neutron diffraction, typically only four to six images are needed. Data collection can be done at room temperature (the crystals do not need to be frozen) and takes three days to two weeks (the time needed depends on the crystal's size and symmetry). The current procedure for researchers to acquire neutron beam time at Oak Ridge National Laboratory is to apply through a peer-review process. However, instead of researchers approaching the lab on a case-by-case basis, it may be possible for CASIS to submit a programmatic proposal to the lab to reserve a certain amount of beam time per cycle for a constant flow of PCG researchers to utilize.

Subsequent discussion focused on optimal techniques for growing high-quality crystals for imaging and analysis. It was noted that in PCG, there are thousands of conditions that cannot be controlled, and crystallization can be viewed as more of a knack than an art. Kapton® tubing and PTD tubing work well for X-ray diffraction because both have very low background. Sometimes the best analyses come from tubes straight from the factory that are not cleaned—a speck of dust in the tube can help with nucleation. One approach to grow more ordered crystals for imaging and analysis could be to use a metal-organic framework (MOF) or some type of repeating grid that the proteins can organize on. There are many ways to make templates so that molecules are not attaching randomly—in a self-assembling matrix, every time a molecule is caught, it orients in the same way to create the same pattern in each grid.

III PANEL 3: LABORATORY-BASED CRYSTALLOGRAPHY

Panel Members

John Clemente	**Edward Pryor**	**Ben Apker**	**Issa Isaac**
Art Robbins	*Anatrace*	*MiTeGen*	*Molecular Dimensions*
www.artrobbins.com	*www.anatrace.com*	*www.mitegen.com*	*www.moleculardimensions.com*

Key Points

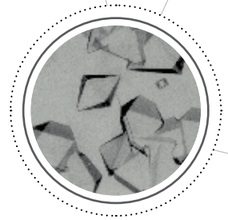
- Biotechnology and laboratory equipment companies would like users to communicate the types of instruments and services they wish were available.
- A common problem with 96-well plates currently available is leakage between wells during spaceflight due to rounded edges.
- A common problem for vapor diffusion experiments is leakage through the air gap between the protein and reservoir.
- Companies could machine a small version of new hardware under development to test for issues such as leakage before proceeding with development.
- Plates need to have a sizing capability, as larger volumes are necessary to grow crystals for neutron diffraction.
- Users have trouble getting crystals out of the hardware for diffraction analysis and do not want to manipulate fragile crystals; some chips are designed to be harvestable and peel out of the hardware.
- Hardware that enables in-situ diffraction analysis is of interest; however, background is a problem with in-situ diffraction, particularly for neutron diffraction.
- Adapting hardware familiar to users would make the transition from ground to spaceflight easier; and keeping plates SBS-compliant is crucial.
- Biotechnology and laboratory equipment companies could help facilitate services such as sample preparation and re-loading in the case of a scrubbed mission.

Panel 3 focused on PCG tools and techniques currently used in ground-based labs and what is needed when transitioning to spaceflight. The discussion began with brief presentations from the panelists (representatives from companies that provide equipment, materials, and other supplies used in labs on the ground), providing an overview of each company and the types of the instruments and services each provides. Panelists noted that it is important for users to communicate the types of products that they wish were available—even if a suggested product is not developed, it could spark conversations about products that could be developed.

The discussion that followed highlighted some issues with current PCG equipment when taken from the ground to spaceflight. A common issue with 96-well plates currently available from several companies is leakage between wells during spaceflight, particularly upon re-entry impact. Some plates have rounded edges instead of sharp 90-degree angles, and the liquid goes up the wall and leaks over the rounded edge. Additionally, for vapor diffusion, there is an air gap between the reservoir and the protein that liquid can leak through. One possible solution to this issue could be putting a tunnel where the vapor diffusion occurs that is long enough so that any liquid that gets in would not go all the way through. It was suggested that when companies are developing a new plate design, they could machine a small version that could be tested for issues such as leakage upon re-entry to gain insight into whether the design will work before time is spent making a mold and continuing development. The need for plates to have a sizing capability was also noted, as larger volumes are needed to do PCG for neutron diffraction.

Additionally, it is often difficult to get crystals out of the hardware to conduct diffraction analysis, and users want to avoid manipulating the crystals, as they are extremely fragile. To address this issue, some chips are designed to be harvestable, with rear foil that can be pulled back and scooped out. The ability to image crystals in the hardware without having to remove the crystals would be beneficial; however, background from in-situ diffraction is a problem, particularly for neutron diffraction. Companies would need to see how many users would take advantage of in-situ diffraction at room temperature, and it was noted that for in-situ neutron diffraction, the hardware materials would need to contain minimal hydrogen and must be nonporous.

The discussion echoed the earlier point that it would be beneficial to take advantage of hardware familiar to users and adapt that hardware for use in spaceflight. It was reiterated that keeping new plates SBS compliant is critical in translating from ground to flight and that automation for protein loading could be beneficial. It was noted that biotechnology and laboratory equipment companies could help facilitate tasks such as getting samples ready or being prepared in the case of a scrubbed mission so users can re-load their protein quickly.



Day 1 Session Summaries

IV PANEL 4: SPACE-BASED CRYSTALLOGRAPHY CAPABILITIES

Panel Members

Dan Connor
University of Alabama at Birmingham
www.uab.edu/engineering/eitd/

Hiroaki Tanaka
Confocal Sciences
www.confsci.co.jp/index_e.html

Marc Pusey/ Sridhar Gorti
Marshall Space Flight Center
www.nasa.gov/centers/marshall/

Greg Jenkins
Moseley Technical Services
www.moseleytechnical.com

Eugene Boland
Techshot, Inc.
www.techshot.com/aerospace/index.php

Mary Murphy
NanoRacks, LLC
nanoracks.com

Tim Allen
Teledyne Brown Engineering
tbe.com

Key Points

- Implementation partners focus on getting experiments to the ISS National Lab so investigators can focus on their science.
- Some implementation partners provide an end-to-end process, from assessing a protein's viability all the way to diffraction analysis of crystals.
- There are potential advantages to providing real-time, in-flight images of crystal growth; however, the benefits must be weighed against potential impacts on the science and cost.
- Containing spaceflight samples is not expensive, but adding video, temperature control, interfaces, software, etc. drives up the cost.
- Any increase in ISS crew intervention would have to fit within the crew's tight schedule restraints.
- It is important for users to submit a hazardous materials list as early as possible. Review usually takes three to six months, but it can take up to nine months.
- ISS National Lab program requirements are currently undergoing review because many date back to the Space Shuttle era.
- Users have a hard time understanding the flight hardware and capabilities available and would like ISS National Lab facilities information provided in a spreadsheet that allows for sorting by characteristics of interest.

Panel 4 focused on current PCG flight hardware and capabilities. Panelists included implementation partners that each gave a brief presentation on the hardware they provide. Implementation partners are seeking to better understand the needs of the PCG research community and identify any gaps that need to be addressed.

The role of implementation partners is to help investigators translate their research from the ground to spaceflight. Implementation partners focus on the process involved with carrying out an experiment onboard the ISS National Lab, allowing investigators to focus on their science. Some implementation partners meet with investigators and provide an end-to-end process—from assessing the protein for viability to optimizing conditions for that protein, purifying the protein, launching the experiment, returning the experiment, and performing X-ray or neutron diffraction. Sometimes support is provided to remove crystals from the hardware for neutron diffraction.

Additionally, some implementation partners have developed a process to rapidly image PCG plates that return from spaceflight to document the results as quickly as possible. A question was raised about the potential benefits of providing real-time, in-flight images (time-lapsed photography or video) of crystal growth. It was noted that there could be advantages to watching crystal growth in real time, and an added benefit would be the applications for student outreach. However, those benefits would need to be weighed against any potential impacts in-flight imaging could have on the science (such as the sample being disturbed by temperature changes, crew handling, etc.). It was also noted that if extra crew time is needed for in-flight imaging, it could drive up the cost. Although the aim is usually for experiments to require minimum crew intervention, ISS crew have voiced an interest in playing a more active role in ISS research; yet, any increased crew intervention would have to fit within the crew's very tight schedule constraints. Implementation partners pointed out that simply containing the samples is not very expensive, but adding video, temperature control, interfaces, software, etc. drives up the cost.

Implementation partners discussed some of the requirements for sending PCG experiments to the ISS National Lab, such as getting all materials approved by toxicology. All investigators must submit a hazardous materials list; and approval depends not only on the specific chemicals, but also on the volume, concentration, etc. Implementation partners stressed the importance of submitting a list of materials to toxicology as soon as possible because the review period can be lengthy. Review and approval usually takes three to six months; however, it can take as long as six to nine months. It was noted that all ISS National Lab requirements are currently being updated because many of the requirements were developed during the Space Shuttle era.

The discussion that followed highlighted that the PCG research community has a hard time trying to understand the flight hardware and capabilities available. The Program Science Office is updating the ISS facilities information with all current implementation partners and existing facilities onboard the ISS, along with the characteristics of the facilities and their availability. Users expressed a desire to have such information made available in a spreadsheet that can be sorted according to characteristics of interest.

Day 2 Session Summaries

Breakout Groups

Overview of Breakout Goal

By dividing attendees into smaller groups with distributed expertise, discuss in detail specific questions surrounding PCG in microgravity with the goal of providing the basis for Workshop Recommendations.

Breakout Group Trigger Questions

MOLECULES OF INTEREST

- *Are membrane proteins and protein-protein complexes the highest priority for spaceflight crystallization?*
- *Are inorganic molecules of interest? And are the conditions/hardware for their crystallization in microgravity different from membrane proteins?*
- *Would you characterize protein-protein complex crystallization as early stage in depth of understanding of how to crystalize? What are the gaps in technology or protocols that need to be solved to make this viable?*
- *Are the requirements for production of crystalline solutions for drug formulation/stability studies different than those for producing crystals for structural determination focused on drug discovery?*
- *Is the mathematical model described yesterday to rank space-grown proteins in order of crystal size/mosaicity for X-ray crystallography and neutron diffraction an appropriate screen for selection/prioritization of PCG experiments?*
- *What set of tests (freeze/thaw, temperature, etc.) should be developed for all ISS National Lab candidate proteins before they launch to increase the likelihood of success? Would a consortium of mentors be appropriate to assist new users through this process?*
- *To reduce the issues with launch delays, how may we optimize and leverage commercial expertise to assist in production of proteins for spaceflight crystallization?*
- *Is it preferred to freeze for launch or to find a reliable method to physically separate the two solutions until you reach microgravity? Or do we need the capability for both to support different protein solutions?*

IMAGING AND ANALYSES

- *What type of inflight monitoring (imaging or other) would be helpful to developing the understanding of how crystallization is occurring and understanding the factors that lead to appropriate crystal results?*
- *Are X-ray and neutron diffraction the primary analytical tools in all cases? Even crystalline solution evaluation?*
- *Are we limited to liquid-liquid diffusion crystallization to grow crystals large enough for neutron diffraction?*
- *Is the recommendation to provide easy access to proteins to be removed from hardware for analysis or is it preferred to have hardware that the crystals can be analyzed directly?*
- *Should there be a program to perform standardized tests on every space-grown crystal? Should those tests be performed with the same analytical facilities? Should the data be published on-line similar to or in collaboration with databases for current crystal structure data?*

LABORATORY COMPATIBILITY

- *Does the mindset of "spaceflight preparation" result in improved laboratory practices that reduce error and increase crystallization success even in ground control over "state of the art" on Earth?*
- *How important is being able to eliminate optimization of crystallization setup for unique spaceflight hardware versus having a unique hardware built to work in concert with microgravity phenomena around mass transport and fluid dynamics?*
- *Do we agree that there are certain geometries that will work with surface tension to prevent undesirable mixing? Do we believe we understand what these are (i.e., ledges and pining points)?*
- *What materials cannot be used for crystallization chambers? For X-ray diffraction chambers? For neutron diffraction chambers?*

FLIGHT HARDWARE

- *What are your top five characteristics of a successful space-based crystallization tray/system?*
- *Has there been loss of samples in transportation between landing site and laboratory? To what degree?*
- *To develop a hardware selection matrix of available hardware, what are the "headers" of the array (i.e., capillary size, volume, and material)?*

Breakout Group Assignments

BREAKOUT	NAME	AFFILIATION/TITLE
1	Tim Allen	*Teledyne Brown Engineering*
1	Stephen Aller	*University of Alabama at Birmingham*
1	Jim Baird	*The University of Alabama in Huntsville*
1	Brad Carpenter	*NASA's SLPSRA*
1	John Clemente	*Art Robbins*
1	Mary Murphy	*NanoRacks*
1	Joseph D. Ng	*The University of Alabama in Huntsville/iXpressGenes, Inc.*
1	Edward Pryor	*Microlytic (acquired by Anatrace)*
1	Joseph Reibenspies	*Texas A&M University*
1	Paul Reichert	*Merck Research Laboratories*
1	Michael Roberts	*CASIS, Senior Project Scientist*
1	Mark Uhran	*Oak Ridge National Laboratory*
2	Benjamin Apker	*MiTeGen*
2	Diego Arias	*JAMSS America, Inc. (JAI)*
2	Gloria Borgstahl	*University of Nebraska Medical Center*
2	Leighton Coates	*Oak Ridge National Laboratory*
2	Dan Connor	*University of Alabama at Birmingham's Center for Biophysical Sciences and Engineering*
2	Kirt Costello	*NASA, ISS Program Scientist*
2	Todd Link	*University of Texas MD Anderson Cancer Center*
2	Donnie McCaghren	*NASA's Marshall Space Flight Center*
2	Timothy Mueser	*University of Toledo*
2	Ken Savin	*Eli Lilly and Company*
2	April Spinale	*CASIS, Operations Team*
2	Hiroaki Tanaka	*Confocal Science, Inc.*
2	Diana Tomchick	*American Crystallographic Association/UT Southwestern*
3	Eugene Boland	*Techshot, Inc.*
3	Larry DeLucas	*University of Alabama at Birmingham*
3	Sridhar Gorti	*NASA's Marshall Space Flight Center*
3	Issa Isaac	*Molecular Dimensions*
3	Greg Jenkins	*Moseley Technical Services*
3	Eric Johnson	*Pfizer, Inc.*
3	Andrii Y. Kovalevskyi	*Oak Ridge National Laboratory*
3	Peter Moeck	*Portland State University*
3	Marc Pusey	*iXpressGenes, Inc.*
3	Debbie Wells	*CASIS, Portfolio Project Manager*
3	Liz Wilson-Kubalek	*The Scripps Research Institute*

Day 2 Session Summaries

Breakout Group Shared Feedback (Basis for Final Recommendations)

The following are the key points collected from the breakout groups for the four focus areas: molecules of interest, imaging and analysis, laboratory compatibility, and flight hardware. These key points were the basis for the final recommendations coming out of the workshop.

MOLECULES OF INTEREST

- Molecules of interest include any protein of high biological interest with an indication of commercial interest or value that 1) shows the need for improved diffraction and/or an improved electron density map or 2) a high-quality diffraction resolution and/or a high-quality electron density map cannot be obtained on Earth. Areas of interest include:
  - *Ligand validation*
  - *Enzymes—proton transfer*
  - *Ion channel proteins*
  - *Configuration of side chains*
  - *Membrane proteins—early wins are needed to draw users to the ISS National Lab*
  - *Inorganic molecules—some are air sensitive and need sealed capillaries*
  - *Nanocrystals (very tiny structures)*
- Specific organic molecules of interest include:
  - *Protein complexes*
  - *Member proteins—not all membrane proteins will benefit from microgravity research; however, investigators need to understand there is potential value*
  - *Small molecules—better outreach is needed to inform researchers interested in small molecules of the availability of the microgravity environment onboard the ISS National Lab*
- Specific inorganic molecules of interest include:
  - *Semiconductor/liquid crystals*
  - *Zeolites*
- There is a need to grow both large crystals as well as small uniform crystals, and hardware and processes need to be available to support both.
- The following checklist could be used to evaluate specific proteins under consideration for microgravity research:

1. *Is it possible to achieve nucleation?*
2. *Can the protein be produced in enough quantity?*
3. *If crystals have been obtained for the protein, do the crystals need to be larger or more uniformly produced? Or do the crystals need a better diffraction resolution?*
4. *Are some characteristics of the protein's stability known (i.e., temperature range)?*

- If the appropriate partnership can be developed to validate the mathematical model, once the model is validated, the community could determine the best role of the model in the process of identifying candidate proteins.
- There is a need to do more fundamental research on how to do crystallization for any protein in a spaceflight environment.
- There are still those in the PCG research community that downplay the utility of doing PCG in microgravity; therefore, there is a need for additional outreach within the community. Such outreach should point to literature that describes the advantages of growing both organic and inorganic crystals in a microgravity environment.
  - *YouTube, Twitter, and Facebook may be good ways to target new audiences. Traditional publications do not hit as broad an audience as these other outlets can.*
- There is a need to explore relationships with international societies to increase awareness among structural biologists and chemists of the available capabilities for PCG research onboard the ISS National Lab. In addition to the ISS hardware capabilities literature that NASA has developed, a sortable spreadsheet would help new-to-space users understand what is available.
  - *Holding a workshop or session at the American Crystallographic Association's annual meeting would require planning to begin right away for the 2017 meeting.*
- Historically, the focus has been on establishing the comparative advantages of doing PCG in microgravity. However, in addition to publication and results demonstrating the advantages, reviews on the current state of the art are needed that target the whole microgravity research field, not just the PCG research community.
- There is a need both to expand the PCG spaceflight research community and also to unequivocally establish the comparative advantages. The community needs to choose one of these to make the top priority and focus resources on it.
- A checklist is needed for investigators who are new to doing PCG in space. Lessons learned, such as having enough protein available in case the mission is scrubbed, should be provided.

IMAGING AND ANALYSIS

- In-flight imaging is not required for the science; however, it would be very useful and nice to have for both the science as well as educational outreach.
  - *Previously, it was thought that providing an imaging capability took space away from being able to fly proteins of interest. Yet, it would be ideal if in-flight imaging could be done in way that still maximizes the available space for crystallography.*
  - *If it is necessary to handle the crystals to obtain in-flight imaging, it must be determined if the imaging is worth it. Additionally, crew time is expensive, so imaging would likely need to be done without crew involvement.*
  - *There are trade-offs associated with handling crystals too much; however, there is tremendous value in the excitement associated with investigators having in-flight imaging, and it could be something to consider with individual investigators to see if it could be done easily.*
  - *In-flight imaging could also be valuable for situations in which investigators want to know when nucleation took place or when the crystal grew and then degraded. Right now, that cannot be seen in studies. There may be some cases in which an investigator may want to trade protein space for in-flight imaging capabilities.*
  - *Imaging does not need to be real-time; it could just be recorded and use something as basic as an iPhone camera.*
- There is a need for vapor diffusion, liquid-liquid diffusion, and batch capabilities for neutron diffraction. The focus cannot only be on one—capabilities are needed for all three.
- For structure determination, X-ray diffraction and neutron diffraction are the primary analysis tools. But for smaller crystals (nanocrystals), there are some additional options.
- Access to the crystals needs to be one of the top characteristics of hardware—the ease of getting crystals out. For analysis, it is better to be able to remove the crystal from the hardware and mount it. It is not always possible to do imaging in the hardware.
- If it is possible to image through hardware, that may be preferable—some investigators have trouble getting high-quality crystals out of the hardware to do imaging without damaging the crystals. This is mostly the case with crystals that have adhered to the hardware. The sticky plastic sometimes requires quite a bit of manipulation to get the crystals out of the hardware.
  - *Investigators could be asked whether their protein of interest may have trouble with adhering to know whether to try to grow the protein in hardware that can be imaged and analyzed without having to remove the crystal.*
  - *It is hard to know which proteins are more likely to adhere to the hardware—it appears to be random. It is also a matter of which part of the crystal is adhered. Using a Teflon surface may reduce sticking problems. A thin Teflon sheet allows investigators to easily push crystals off without cracking them. However, adhesion of the drop to the surface is also needed so it does not slip.*
  - *In an ideal world, it would be best to never touch a crystal. If a crystal is adhered to the hardware, trying to release it will likely damage the crystal. The whole idea of growing crystals in microgravity is so the crystal grows more perfectly. Investigators do not want to grow a perfect crystal and then introduce defects trying to remove the crystal from the hardware. This is why in-situ capillaries are attractive.*
- This question really impacts what types of hardware CASIS considers and how CASIS would make hardware available for the crystallization program. If the hardware is SBS compliant, that is a completely different thing than using capillaries. CASIS is trying to get an idea of prioritization for hardware.
- The reason there are many different types of plates and ways to grow crystals is that the type of plate or method used is sometimes dependent on the protein the investigator is trying to grow and the expertise of that particular investigator. There is a barrier of investigators not wanting to try new methods. As an engineer, you would like everyone to do PCG the same way; however, in the academic world, you would not want to try to force everyone to do PCG the same way because it would eliminate a lot of investigators.
- Currently, users have no way to test hardware to see which is best for their protein, and that is needed. Investigators need to be given a variety of options because some proteins work better in certain types of hardware. It would be beneficial to have hardware available in the lab for testing so investigators can determine what hardware works best for their protein.

LABORATORY COMPATIBILITY

- Optimization for spaceflight improves lab procedures and quality control, which can be translated to standard ground crystallization. When preparing an experiment for spaceflight, investigators tend to focus more on the experiment and multitask less. Lessons learned from optimizing for spaceflight can be applied to ground programs to improve what is being done in the lab. Although this benefit has intangible value, it is important to capture.

Day 2 Session Summaries

- CASIS should not focus on solving problems of the PCG research community, such as how to get crystals out of hardware for analysis. The PCG research community has been working to solve that problem for years. Instead, CASIS should focus on selecting four to five of the best methods known and make sure those are totally enabled. Then, investigators could choose among those options.
- Investigators need to begin with the end in mind and should know the type of analysis that they would like to do before they decide what hardware to use—it cannot wait until after obtaining the crystal.
  - » *Quartz works well for in-situ X-ray and neutron diffraction; however, hardware should be designed for easy crystal removal.*
- Some investigators want an end-to-end solution, someone to go through the process of optimizing and loading the protein (either by hand or with automation) for them.
- Investigators would like hands-on experience to try out different types of hardware and see what works best. CASIS could choose a subset of companies and investigators and send the investigators hardware to test in their ground-based labs.
  - » *When an investigator first connects with CASIS to fly proteins, the investigator could be given time to test different types of hardware to see what proteins work best in which hardware. That way, when investigators make a final proposal for flight, they can narrow it down before they write the proposal.*
  - » *Testing could also be done with a broader audience, including those who are not primarily interested in microgravity research, which could spark an interest.*
- CASIS could start with 10 different pieces of hardware from six or seven companies and down-select to two to five pieces that work best for the greatest number of users in the community. Then, a relationship could be developed with those companies so that the hardware would be ready for investigators to use through a sustainable, predictable model for flight. There could be placeholders for the hardware that just need to be filled with users. Using this model, CASIS could anticipate the cost, and the schedule would be somewhat fixed. Such a model would be much more efficient than a completely user-driven model that has to be turned on for each individual user, which is what currently happens when a project is selected for flight. Instead of being on-demand, it would be a service model for investigators to utilize. This would shift the mindset from enabling one user to do one experiment to that of a laboratory and services that the lab can provide. This model would not preclude a one-off that does not fit into the model—it does not disenfranchise that opportunity.
  - » *Additionally, this model could foster repeat users, so there is the potential for a more experienced user base. Repeat users may learn new things after flying several experiments, expanding their knowledge base. However, some users may not achieve a significant enough effect from microgravity to return.*
- CASIS would like to aim for a shorter timeline to get experiments to the ISS National Lab, along the lines of six months.

FLIGHT HARDWARE

- Capabilities that flight hardware must have include:
  - » *A way to keep the two components separate for controlled release: Keeping the protein and precipitant separated in the hardware would allow investigators to not have to re-load or use freezing in the case of a scrubbed mission.*
  - » *High-fidelity hardware replicates for ground testing: Investigators need to have time to optimize crystallization on the ground before flight, so the hardware should be available in appropriate quantities to do that.*
  - » *Ease of removal to minimize the handling of the crystal: The hardware should be made in such a way that it is easy to get the crystal out.*
  - » *Size that is scalable to crystal size: The hardware should be scalable from 100 nanoliters up to 200 microliters or, in some cases, up to 1 mL.*
  - » *Disposable wells for protein: Cleaning hardware should be avoided because it could damage the wells, which could result in a change in crystal characteristics. Additionally, if the crystal is attached to the surface of a well and needs to be removed, the well may be damaged in the process of trying to remove the crystal.*
  - » *Materials need to be non-permeable and stable.*
- Capabilities that would be nice to have include:
  - » *SBS compliance: It would be nice to have the capability to fly what is already being used in the lab (i.e., a 96-well plate), so that users do not have to adapt their science to different flight hardware.*
  - » *The ability to image through hardware, although the trade-offs would need to be examined.*
  - » *A way to address evaporation issues.*

Program Funding Strategies

Funding to support PCG research on the ISS National Lab is needed in two areas: 1) work done by principal investigators in the lab for experiment preparation and analysis of results and 2) implementation services (translating the way in which an experiment is carried out in the lab on the ground to a way in which it can be carried out in space). CASIS receives the majority of its funding from NASA and directs this funding primarily toward supporting implementation services. Thus, CASIS is looking for innovative ways to leverage its funding with other sources of funding (e.g., through collaborations, consortia, sponsored programs, etc.) to create a robust PCG research program on the ISS National Lab. There is a consensus that it will be crucial for the PCG research community to assemble a compelling story about the importance of funding PCG research in microgravity.

Examples of entities currently funding PCG research include NIH (the NIGMS division funds structural biology, but the research must be disease-related); NSF (the Molecular and Cellular Biology Division/Molecular Biophysical Department); the Howard Hughes Medical Institute; the Welch Foundation (only funds research done in Texas); the Gates Foundation (only funds research aimed at disease treatment); the American Heart Association; and the Cystic Fibrosis Foundation. NSF may fund PCG research aimed at understanding a particular mechanism or something unique about a protein. It was noted that NIH does not often fund research aimed at growing crystals, except, perhaps, for membrane proteins. Instead, NIH usually focuses on funding research after a crystal is obtained. Examples of entities currently funding inorganic crystal growth research include the Department of Defense, the Department of Energy, the Petroleum Research Fund, and NIST (although not often outside of the organization).

NASA's National Space Biomedical Research Institute (NSBRI) was suggested as another potential source of funding. It was noted that although NSBRI is already working with CASIS on the Good Health campaign and is part of the CASIS steering committee, NSBRI has not been specifically approached about supporting PCG research. It was suggested that the PCG research community create a document encouraging NASA to provide additional funding for PCG research; however, this may not be possible due to NASA's budget constraints. For NASA, a potential pitch angle could be PCG research for improved long-term storage of drugs for astronauts on long-duration missions. It was also noted that one area in which NASA may be supportive is in developing a standing capability for PCG research on the ISS National Lab. But CASIS would need to present a compelling argument as to why PCG capability on the ISS National Lab is a valuable enabling technology and would need to demonstrate a long line of potential users.

For other government agencies, such as NIH and NSF, CASIS is aiming to re-establish the connections NASA had with the agencies during the Shuttle era. The idea would be to establish a sponsored program with an agency focused on areas in which the agency has a specific interest (e.g., proteins of interest or disease areas of interest) and run a competitive challenge in which the PCG research community submits proposals for funding within the sponsored program.

It was noted that NIH's Protein Structure Initiative ended July 1, 2015, and there are several large centers (e.g., Argonne National Laboratory, Brookhaven National Laboratory, Lawrence Livermore National Laboratory, etc.) that are trying to determine their next step and convince NIH that more funding is needed. These centers have many proteins for which crystals were obtained that are not of high enough quality to determine the protein's structure. There are hundreds of such proteins that could be crystallized onboard the ISS National Lab to obtain higher quality crystals for structure determination. It was suggested that a partnership could be formed with NIH and the centers to work on these proteins.

Venture capitalists and angel investors do not usually fund basic research; and to obtain this type of funding, commercial viability is important. It was suggested that a commercial center be created to offer crystallography services to work on structure determination for pharmaceutical companies or angel investors. Pharmaceutical companies may be willing to pay for an outside entity to generate structural data on a given protein or set of proteins. Angel investors may also be willing to invest in such an entity and sell the structural data to pharmaceutical companies.

Another suggested approach to obtain funding is to find a common cause tied to PCG research that the public would support, such as education. Many foundations, such as the Lilly Foundation, are strong supporters of science, technology, engineering, and math (STEM) education projects. A PCG research campaign could incorporate a major STEM education component to attract funding from foundations. Several members of the PCG research community have experience leading STEM education programs in the area of PCG and could provide expertise.

Day 2 Session Summaries

STEM Education/Outreach

All CASIS-awarded principal investigators are encouraged to participate in STEM education events and stream their STEM content through education programs associated with their company or institution. To further extend educational outreach in the area of PCG, a large-scale PCG research campaign that integrates a major STEM education component could be initiated.

It was noted that a key to success in obtaining support from foundations for an education program is having a well-developed plan that clearly describes the goals of the program, how the goals will be achieved, what the impact will be, and how the program material ties in with the focus area of the particular foundation. It was also noted that it takes time to get funding from foundations, so it is best to approach a foundation early.

Some of the workshop participants discussed their experience leading STEM education programs in which students helped with protein sample loading. In one program, students in grades five through eight helped load protein samples into capillaries. The program was deeply impactful, and the students did high-quality work. In another program, 80,000 college students in more than 30 states loaded protein samples. It was pointed out that such a program is a good way to introduce PCG to students on college campuses.

Another type of STEM education program involves teaching the basic principles of PCG research using simple protein crystallization kits in the classroom and using the topic of PCG research in space as inspiration to get students excited about what they are learning. Some of the workshop participants discussed their experience in doing basic PCG experiments with elementary students using lysozyme crystallization kits (it was noted that Anatrace sells such kits). This type of experiment is safe and easy and can be accompanied by a video explaining that the same type of experiment can be done in space to obtain better crystals.

A possible outlet for PCG educational outreach is Challenger Center and its network of Challenger Learning Centers. CASIS recently entered into an MOU with one of the Challenger Learning Centers to provide science content for the center's Earth Odyssey Mission simulation. For a PCG mission, a PCG principal investigator could be featured in a scripted video explaining what PCG is and why it is important. The video could show a scientist loading protein samples and wishing the students luck with their mission. Then the students would participate in a mission simulation that includes a video of astronauts doing PCG in space. After the students complete the simulation, their teachers would be given a protein crystallization science kit to take back to the classroom. Using the kits, the students could load their own protein samples and wait for crystals to grow. After 30 days, the students could harvest their crystals and compare them with the crystals grown onboard the ISS National Lab. The program would allow teachers to cover a wide variety of STEM topics, including gravity and microgravity, convection, and mass transport. Curriculum on these topics could be provided along with the kits to support teacher lesson plans. It was noted that such a program could also be repurposed for a variety of STEM education uses, such as programs in school districts, out-of-school informal learning networks, and Boys & Girls Clubs of America.

Other suggested approaches for PCG-related STEM education include creating a PCG video game (much like the protein folding game "Foldit"), using a 3-D printer to make protein crystal models to use in a hands-on display, developing a protein crystallization cookbook with instructions for purifying proteins from foods and crystallizing them, and using lasers to show diffraction patterns to explain X-ray diffraction techniques to students.

One of the challenges in educational outreach is finding effective distribution channels. It was noted that one effective way to find distribution channels is by leveraging connections at teacher workshops, such as those held by NSF and the Albert Einstein Distinguished Educator Fellowship Program. It was also noted that targeted outreach aimed at teachers is important because one teacher can reach hundreds of students, and providing STEM-related professional development for teachers can inspire them and transform the way they approach science in the classroom.

Next Steps

CASIS intends to use the information obtained through this workshop and the ongoing support of this group to outline an ISS National Lab program for repetitive, low-cost crystallization in microgravity. The next steps in the development of such a program include two phases:

- **Phase 1 will aim to develop at least three different types of hardware configurations, a concept of operations, and user guides by July 20, 2016.** The operations should be low-cost, standardized, scheduled, and of consistent quality and will be managed internally by the CASIS operations team. Partners such as iXpressGenes, JAMSS, or others may be asked to contribute as consultants. An implementation partner solicitation is planned for Q4 of FY2016; and a customer solicitation is planned for Q1 of FY2017.
- **Phase 2 will run in parallel with Phase 1.** Its aim is to develop a plan with the CASIS commercial innovation and portfolio management teams on how to generate "customers" to close the business model. The goal is to generate full cost pricing for commercial users with a price per tray. The trays will be standard SBS compliant crystallization trays that require little or no development time. An ISS National Lab crystallization program overview will be provided at the American Society for Gravitational and Space Research conference in October 2016. The results of Phase 2 should be ready to implement in FY2018.

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2015 MICROGRAVITY PROTEIN CRYSTAL GROWTH WORKSHOP

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