Workshop on Advanced Topics in EM Structure Determination: Optimization and Validation.

November 11–16, 2012.

National Resource for Automated Molecular Microscopy
Center for Integrative Molecular Biosciences
The Scripps Research Institute

Sunday November 11 **Theme: Setting the Goals**

4:00 pm ***Registration***

4:45 pm **Welcome** **Bridget Carragher**

Session Chair: Clint Potter

5:00 pm **The Biological Challenges** **Tom Walz**
This introductory talk will describe the big picture: define what we mean by Optimization and Validation in the various subsections of our discipline: 2D crystallography, helical, icosahedral, single particle and tomography. What are the problems in each area; past, present and future of what might be possible. Perhaps compare and contrast with other methodologies (X-ray, NMR, LM, etc.), and why do we need to pay attention especially to validation in some areas. In particular why is single particle work so problematic? Perhaps illustrate these points with great successes and embarrassing failures (without offending people). Do the problems get bigger as we approach atomic resolution? What are the challenges in looking at intermediate states? How do we identify bad images? What is the current state of the art and the future prospects? What should we pay attention to and what should we NOT do? We expect the talk will focus on single particles which is where the likelihood of making mistakes is greatest and where there seems to be the most interest in the field right now.

6:00 pm **The Technical Challenges** **Niko Grigorieff**
Overview of technical challenges and possible solutions (may cover hardware, software, prep methods, processing etc.) What do we need to do to get to atomic resolution with particles other than viruses? What are the latest ideas in the field? How will we deal with small particles, membrane proteins, heterogeneity? How do we identify bad images? A few sentences about the various topics that will be covered later in the meeting would be appropriate. The goal of atomic resolution is laudable, but subnanometer (or even poorer) resolution is sometimes very useful for answering some questions – are we effectively matching the resolution to the question? What can we NOT do by the single particle approach?

7:00 pm ***Opening reception***

Monday November 12 **Theme: Specimen preparation**

8:00 am Breakfast

Session Chair: Ron Milligan

9:00 am Introduction to domain/subunit identification and labeling strategies
David DeRosier

Not all 3D maps – in fact very few of them - are at a resolution sufficient to directly identify subunits or domains. What have been the approaches to identification of domains and subunits in the past: Difference mapping applied to complexes of differing composition or with specific protein domains deleted, antibody labeling, labeling with ligands, etc. Why is this so difficult? What are the specific and general problems associated with these approaches: changing conformation in response to labeling or deletions? How might one distinguish between a large conformation change and mass difference in a localization experiment. Identification of specific sequences in a protein or complex: cysteine identification with covalent labeling using undecagold / nanogold / tetrairidium, etc. The use of GFP, SH3 or biotin domains (small inserted domains). Advantages and disadvantages. Problems of size of label versus size of the complex, linker length, visibility, mobility. Identification of complexes in situ. Applications in cryo-tomography of sectioned cells or in whole mounts. Clearly we'd like a method for EM like the GFP approach that has been so successfully used in LM. What are the requirements? Which approaches have been tried in the past? How successful have they been? Which approaches look like the most promising? How should we go about developing a robust, easily applicable technology? A brainstorming approach here might be very useful in stimulating work in this area.

10:15 am Coffee Break

10:45 am Optimizing specimen preparation **Holger Stark**

It is extremely important to get clean well-characterized specimens. Are there any general conditions for optimizing conformational and compositional stability or are these parameters specimen dependent? Are subunits present in correct stoichiometry? Can we stabilize the complex by adding ligands: substrates/substrate analogues, non-hydrolysable nucleotide analogues, inhibitors (after this the complex may need additional purification steps)? What affinity is necessary for complexes to be stable during handling and grid preparation? Should we use glutaraldehyde (GraFix, or just simple fixation) or other bi- or multi-functional crosslinkers to prevent subunit loss or to stabilize conformations? Next, what can we do to optimize the procedures for getting material on to the grid? Are there ways to improve the adsorbtion, distribution and/or orientation of your specimen of interest on the grid? Grid treatments coupled with specimen modification (e.g. his-tagging plus lipid players, or changing the charge on the grid surface, etc.) Are there general ways to improve the yield of ice layers of suitable thickness (e.g. using detergents or proteinaceous surfactants to improve spreading?) For complexes, fibers, and whole mounts of cells or bacteria, how do we ensure that we get sufficient material on the grid and not have it all blotted away? For sectioning, are there pointers for getting a high yield of good sections and transferring them successfully to a support grid?

12:00 Lunch

1:00 pm Fab labeling as a strategy for small molecules **Yifan Cheng**

How easy is it to get stably attached, non-floppy Fabs. What affinity is required to make a stable complex that will work for EM? Is labeling done in the test tube, on the grid? Is

the Fab-Ag complex stable, how long does the process take, etc. etc.? How feasible is it to do many proteins? Is the processing of the images challenging. How is it accomplished? What is the resolution we might expect? How accurately can the epitope be localized in the final 2D / 3D map? How might we improve this approach?

1:30 pm Spotiton: A new approach to EM specimen preparation Tilak Jain
A novel approach to specimen preparation using picoliter dispensing and engineered substrates will be described. The potential advantages include greater control of the ice thickness and quality, and substantially reduced sample volume. Are there problems associated with spot spreading, evaporation? Is there damage to the specimen during spraying – e.g. do fibers shear or complexes disintegrate? How many spots can one get on the grid? How do you do this for different samples and avoid cross-contamination? Is this easy, convenient, useful, affordable?

2:00 pm DOLORS: Versatile Strategy for Internal Labeling and Domain Localization in Electron Microscopy Ian MacRae
This talk will present the technical details of a new labeling strategy for accurately determining the position of internal sites in EM maps. DOLORS uses monovalent streptavidin, which is readily visualized by EM and can be added to biotin-tagged sites in the target protein post-translationally. Internal labels generally display less conformational flexibility than terminal labels, providing more precise positional information. Automated image acquisition and data processing are used to rapidly generate assemblies of unique 3D models allowing the attachment sites of labeled domains to be accurately identified and thus provide an overall architectural map of the molecule.

2:30 pm New innovations for capturing macromolecules Debbie Kelly
What issues do these new approaches solve and how are they implemented. This talk will discuss new innovations for capturing and viewing dynamic biological machinery in vitreous ice and in liquid environments. A recently optimized "gentle blot" technique to improve the yield of active complexes in frozen-hydrated single particle specimens will be described. A new system that uses functionalized silicon nitride devices to capture assemblies in a nanofluidics chamber will be described. This chamber fits within a TEM specimen holder and while inserted in the column, is completely isolated from the vacuum system. This allows imaging of macromolecules within a "nanoscale biosphere".

3:00 pm Break

3:00 pm Demos

3:00 pm Leginon Users Group Meeting. Anchi Cheng, Jim Pulokas
Room 200
This meeting will focus on a discussion of feedback from users and future directions for development.

3:00 pm Dynamo: software for subtomogram averaging. Daniel Castaño Díez
Room 107
The free software package Dynamo offers a user friendly framework for the management of subtomogram averaging projects in a wide variety of computing environments: from personal desktops to multicore machines and clusters of CPUs or GPUs. This presentation will give a general overview of the operation of the Dynamo user interfaces, focusing on the most basic procedures in the subtomogram averaging pipeline: extraction and alignment of subtomograms, classification, and data management and visualization.

3:00 pm Experience K2 Summit™, the First Counting, Super-Resolution, Direct Detection Camera for TEM. Christopher Booth
Room 143
K2 Summit™ is a second generation Direct Detection Camera for Electron Microscopy. The K2 Summit™ is unique in its ability to count individual image electrons, providing counted and Super-Resolution images in real time. The counting and Super-Resolution imaging modes provide a significant enhancement to the DQE of the detector. When combined with dose-fractionation imaging mode (to break a single exposure into a series of sub-frames), this camera allows an unparalleled capability to collect the best structural biology data possible.

3:00 pm Acquiring and Using Movies with a Direct Electron Camera
Benajmin Bammes
Room 147

3:00 pm Spotiton (v0.75): picoliter dispensing for cryoEM Tilak Jain
Room 149
A demonstration of the Spotiton system (v0.75), an automated platform for cryo-EM specimen vitrification. Spotiton uses three piezo inkjet heads to aspirate, position and dispense picoliter volumes of specimens into targeted micro-scale regions of a single EM grid. Using camera vision and precise timing (millisecond level control), three different specimens can be vitrified onto the same grid.

3:30 pm RELION: a stand-alone program for Bayesian image refinement
Sjors Scheres
Room 107
RELION: a stand-alone program for Bayesian image refinement. The demonstration will cover how to preprocess images, starting from micrographs and picked particle positions; how to calculate reference-free 2D class averages; how to classify structural heterogeneity in 3D; and how to perform high-resolution "gold-standard" FSC-based 3D refinements on homogeneous data sets. Useful tricks for data analysis will also be discussed. Taken together, the subjects covered provide a semi-automated and easy-to-use solution for many single-particle projects.

3:30 pm Experience K2 Summit™. Christopher Booth
Room 143

3:30 pm Movies on a Direct Electron Camera
Room 147 Benajmin Bammes

3:30 pm Spotiton (v0.75): picoliter dispensing for cryoEM Tilak Jain
Room 149

4:00 pm Panel Discussion: David DeRosier (Chair)
For these panel discussions, all participants are encouraged to submit questions during the day that will then be discussed in an open forum. Questions are preferably related to topics on which presentations have already been made and should expand on or clarify the lectures of the day. The Chair is encouraged to plant some seeds (moles) in the audience to get the discussion going.

5:00 pm Poster Session
Poster Set Up Schedule:
Last name beginning with A-L. Setup Sunday night or Monday Morning. Take down on Tuesday evening.
Last name beginning with M-Z. Setup Wednesday Morning. Take down on Friday morning.

6:30 pm Dinner

7:30 pm Research Talk: Opening windows into the cell: Focused ion beam micromachining of eukaryotic cells for cryo-electron tomography. Elizabeth Villa
Cryo-electron tomography (cryo-ET) provides unprecedented insights into the 3-D macromolecular organization of cells in their native state. However, the thickness of most cells makes them inaccessible to cryo-ET. This talk will show how focused ion beam (FIB) milling can be used to prepare 200-500 nm lamellae from intact cells, opening large windows into the cell's interior, exposing their landscapes at molecular resolution. The use of cryo-FIB/ET to study diverse cellular environments at molecular detail, including the structural dynamics of the nuclear pore complex, actin networks, and the distribution of macromolecular complexes within organelles such as mitochondria will be illustrated.

Tuesday November 13 Theme: Image acquisition

8:00 am Breakfast

Session Chair: Bob Glaeser

9:00 am Introduction and new approaches Wah Chiu
What are the pros and cons of using a high-end microscope such as a JEOL or a Krios? Are "regular" 200kV instruments just as good if we use the right imaging conditions? What progress has been made with using phase plates? What are the advantages and disadvantages of phase plates per se? What are the advantages and disadvantages of the various phase plate solutions that are out there? There have been some impressive images presented at recent conferences. However, is the technology limited to moderate resolution investigations, depending on the type of phase plate used? How easy is it to use a phase plate? How long does each "plate" last? Are the plates expensive? Are they easy to exchange? Is the exchange technology expensive? Will this become mainstream technology or only have specialized applications? What other innovations are coming along? DDs, Cs correctors, etc.

10:15 am Coffee Break

10:45 pm Optimizing image acquisition John Rubinstein
When making use of regular (not high-end) microscopes, what are optimal imaging conditions? Settings for kV, C1, C2 settings, exposure time, amount of irradiation, rate of irradiation, beam tilt corrections, etc. etc? Is this different when using high-end scopes and DDs?

11:45 Group Photo

12:00 pm Lunch

1:00 pm Direct Detectors Forum David Agard (Discussion Leader)
Short contributions from people who have real life experience with these instruments. Yifan Cheng (K2), Richard Henderson / Sjors Scheres (Falcon), Hong Zhou (DE12), Niko Grigorieff (DE12, Falcon, K2).
The session will start with a brief overview by the discussion leader describing the background and expectations for the new generation of detectors. This will be followed by talks on experience with the DE, Gatan and FEI cameras, with "open mike" and questions at the end. We hope the talks will highlight the reality of using DDs. Basic questions might include: should we use film, CCDs or DDs to collect the data? Are there circumstances or specimens for which one acquisition approach is better than another? DDs are the new wave in image acquisition. Are they as good as advertized? Do they solve all our problems? Should everyone be using direct detectors to record our images? What's the utility of single electron counting? Should we routinely correct for specimen drift and movement and record images by single electron counting? What are the practical concerns with single electron counting - dose rate, exposure time, movement, drift correction, etc. Does each image take a long time to acquire? Is this a nuisance? What's the rate-limiting step? Will this get us all to high resolution?

2:45 Coffee break

3:15 – 5:00 Additional High-End Instrumentation Forum
Bob Glaeser, Discussion Leader
3:15 – 3:30 Holger Stark
3:35 – 3:50 Henning Stahlberg
3:55 – 4:10 Bob Glaeser
4:15 – 4:30 Q&A on topics of interest that were not covered by the speakers
The session will start with three talks on selected topics about not-yet-conventional, "frontier instrumentation" that currently is in an early stage of development and/or characterization. Topics may be selected from: 80kV vs 300kV scopes, STEM vs CTEM, brighter guns (e.g. X-FEG), wet-specimen holders, dynamic TEM, correctors, condenser zoom (parallel illumination), phase plates, FIB, cryo-sectioning, sample-spotters, what else? These additional features are expensive! Speakers are charged to give a critical review of:
Are they worth it? What do they get us? What does the future hold?

4:30 – 5:00 "Open-mike" discussion at the end, on how to provide universal access in view of the high cost of: purchase, annual service, support infrastructure.
Is the beam-line model a good one for the cryo-EM community? Are there better alternatives?
It seems like each high-end add on costs an additional ~$1,000,000. Will NIH be willing to pay for these or do we need to look elsewhere to fund these additions? Overall, how do we see EM structure determination 5 – 10 years from now? As most of us cannot afford the initial cost – or for that matter, the running costs – of the new high-end instruments, will we all go to a few dedicated image acquisition facilities to collect data? These would have high-end scopes with all the bells and whistles imaginable (or maybe not!), as well as having high end preparative instrumentation available.

How will such facilities be run and supported? Will they run as NIH supported facilities that are freely available for grant holders? Or will they be run as fee for service facilities?

5:00 pm Manufacturers Exhibit

5:00 pm **Experience K2 Summit** **Christopher Booth**
Room 143

5:00 pm **Movies on a Direct Electron Camera**
Room 147 **Benajmin Bammes**

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6:30 pm ***Dinner***

7:30 pm **Research Talk: Furthering our understanding of microtubule dynamic instability by cryoEM** **Gabe Lander**

It is known that the binding and hydrolysis of GTP by tubulin underlies their stochastic growth and shrinkage (dynamic instability), yet a mechanistic explanation for the molecular motions involved in this process remains elusive. Through technological improvements in cryo-electron microscopy sample preparation, instrumentation, and image processing, we have determined the structures of microtubules before and after GTP hydrolysis to better than 5 Angstroms resolution, sufficient to describe a physical model for the interrelationship between nucleotide state, tubulin lattice contacts, and microtubule stability.

Wednesday November 14 **Theme: Processing**

Session Chair: Bridget Carragher

8:00 am ***Breakfast***

9:00 am **Introduction and new approaches** **Pawel Penczek**
3D reconstruction methods and image restoration techniques. Heterogeneous populations. The ISAC algorithm. What are the hot topics in processing? Mathematical approaches and available software. Introduction to the methods of data processing, the success stories and the failures. Where are the greatest challenges right now and how are we approaching these? Do we need completely new algorithms or just incremental improvements on the current ones? How to detect heterogeneity? What are the signs that it is present in a dataset? How to distinguish conformational and compositional variability? What to do about it? Sub-averaging within populations. Mistakes to avoid!

10:00 am ***Coffee Break***

10:30 am **Optimizing image processing** **Sjors Scheres**
Conventional and ML 3D (projection matching), refinement (and the differences between them), Bayesian extension, how to avoid overfitting, how to avoid model bias, multi-reference refinement (classification).

11:30 am **Single-Particle Electron Microscopy Reveals Extensive Conformational Variability of the Ltn1 E3 Ligase**

Dmitry Lyumkis

Ltn1 is a 180 kDa ubiquitin ligase that associates with ribosomes and marks aberrant, translationally arrested nascent polypeptide chains for proteasomal degradation. A variety of single-particle EM strategies were used to show that Ltn1 has an elongated form and presents a continuum of conformational states about two flexible hinge points, while its overall architecture is reminiscent of multi-subunit cullin-RING ubiquitin ligase complexes (CRLs). A model of Ltn1 function, based on its conformational variability and flexibility, describes how these features may play a role in cotranslational protein quality control.

12:00 ***Lunch To Go***

12:00 pm **Experience K2 Summit** **Christopher Booth**
Room 143

12:00 pm **Movies on a Direct Electron Camera**
Room 147 **Benajmin Bammes**

12:30 pm **Experience K2 Summit** **Christopher Booth**
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12:30 pm **Movies on a Direct Electron Camera**
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Afternoon free to explore San Diego!

Thursday November 15 **Theme: Validation**

8:00 am ***Breakfast***

Session Chair: Joachim Frank

9:00 am **Introduction** **Richard Henderson**

The underlying question is, under what circumstances can we believe what we get from single particle processing? (or for that matter, any other of our sub disciplines - 2D, helical etc.?) What are the tests that we can do to determine if our output is meaningful and believable? Does common sense play a role here? Should we be thinking of implementing standard validation procedures for every 2D and 3D investigation that is submitted for publication? If so, can we agree on one, reliable, relatively simple procedure that is acceptable to all? What are the current options? This is perhaps an opportunity to brainstorm about new ideas. Are there new ideas that we should be thinking about?

10:15 am ***Coffee Break***

10:45 am A case study for validation Steve Ludtke

The question of how to assess the overall accuracy and resolution of maps in cryo-EM has become a focus of much attention over the last 1-2 years. Structures are often published with widely varying levels of detail, yet claim the same resolution. Low resolution structures are published with no way for a reviewer to assess whether the structure is reliable or not. New methods, and reinvention of existing methods over the last year has finally begun to produce some solutions for these problems. Rosenthal & Henderson's tilt validation method provides one way to experimentally confirm the accuracy of low resolution structures, but is not without its own unique challenges. The concept of refinement in multiple software packages to assess the similarity of the resulting map can be reassuring, but doesn't really prove that the structure is unique and accurate on its own. In resolution assessment, the concept of a "gold standard" FSC, which is really just going back to the original FSC definition, and doing what we should have been doing all along, is now emerging as a standard for the field. We have applied these methods and more to a number of test specimens, including the contentious IP3 receptor structure as well as some less controversial structures, and show how these tools can be used to make scientists both inside and outside the cryo-EM field more confident of the maps we publish, and, indeed, that using these methods can actually improve the quality of our published maps, not just our confidence in them.

12:00 Lunch

1:00 pm TBD

Rather than a research talk, we especially want a discussion on the technical issues that were faced and overcome.

1:30 pm Panel Discussion: Richard Henderson (Chair)

For the panel discussions, all participants are encouraged to submit questions that will then be discussed in the session. Questions are preferably related to topics on which presentations have already been made – and should expand on or clarify the lectures.
Other possible topics:
With increased output from automation and the new direct detectors we are experiencing a deluge of data. It's filling up our discs and requiring increasing efforts to manage it and back it up. Is CPU, disk, etc. a bottleneck to progress? What are the proposed solutions? Should we look at preprocessing before committing images to storage? Are there ways to identify "bad" images and dump them. Are there other communities that we should be looking to, to help us solve our problems?

2:30 pm Demos

2:30 pm Appion Users Group Meeting Anchi Cheng, Amber Herold
Room 200

This meeting will focus on a discussion of feedback from users and future directions for development.

2:30 pm Maskiton demo Craig Yoshioka

A demonstration of Maskiton, a tool for interactively creating custom masks and using them to perform 2D classification. Maskiton uses HTML5 technologies and a sophisticated server-side caching architecture to provide fast processing and feedback to users for working with their data directly in a web browser.

2:30 pm Experience K2 Summit™. Christopher Booth
Room 143

2:30 pm **Movies on a Direct Electron Camera**
Room 147 **Benajmin Bammes**

3:00 pm **Experience K2 Summit™.** **Christopher Booth**
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3:30 pm **Poster session**
Poster Set Up Schedule:
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5:00 pm ***Buses leave for dinner***

6:00 pm ***Conference dinner***
Whisknladle Restaurant
1044 Wall Street, La Jolla, CA 92037
http://whisknladle.com/
(858) 551-7575

Friday November 16 **Theme: EM as a Hybrid Method**

Session Chair: Jack Johnson

8:00 am ***Breakfast***

9:00 am **Computational approaches** **Andrej Sali**
This talk will review integrative structure determination of macromolecular assemblies, with an emphasis on using data generated by electron microscopy. The first topic will be the use of class average images directly as spatial restraints on the assembly structure. The second topic will be simultaneous flexible fitting of multiple subunit structures into an electron microscopy derived density map. Both methods are implemented in an open source IMP package, which will also be outlined.

10:00 am ***Coffee Break***

10:15 am **Probing molecular architecture and interactions using X-ray and EM**
Ian Wilson
Human antibodies are able to broadly neutralize HIV-1 and influenza virus. Using a combination of X-ray and EM techniques, key antigenic sites on the viral surface proteins can be mapped rapidly (EM) and in detail (X-ray) to determine the antibody mechanism of action. This structural and functional information is then used for structure-based design of an HIV-1 vaccine or for a universal flu vaccine.

11:15 am ***Coffee Break***

11:30 am **Structure of the ATP synthase and respiratory chain complexes in the inner mitochondrial membrane**

Werner Kühlbrandt

Mitochondria produce most of the ATP in animal cells. It is widely assumed that the complexes that carry out oxidative phosphorylation are randomly distributed in the inner mitochondrial membrane. Electron cryo-tomography (cryo-ET) was used to show that in fact the mitochondrial ATP synthase forms extensive rows of dimers along highly curved cristae membrane ridges. A 3D map of the ATP synthase dimer obtained by sub-tomogram averaging clearly resolves the central and peripheral stalks. While details of molecular interactions within the dimer may vary between species, the overall dimer structure and the high local membrane curvature are highly conserved, pointing to a fundamental role in mitochondrial energy conversion.

12:30 **Wrap-up**

1:00 ***Lunches To Go and End of Workshop***