Invited Speaker

604 Prospects of nanofluidic cavities for cryo-EM sample preparation
Dr. Arjen J. Jakobi[1,2], Stefan Huber[2], Edin Sarajilic[3], Roeland Huijink[3], Dr. Wiel H. Evers[2]
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398 Enabling discovery by in-cell structural biology
Dr Julia Mahamid[1]
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Oral Presentation

23 Single particle CryoEM structures using iDPC-STEM from 4D-STEM detectors at near-atomic resolution
Daniel Mann[1], Aikaterini Filopoulou[1], Max Leo Leidl[1,4], Mr Maarten Wirix[2], Mr Ivan Lazic[2], Knut Mueller-Caspary[4], Mr. Carsten Sachse[1,3]
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363 EasyGrid: a new automated cryo-multimodal sample preparation
Dr Caroline Bissardon[1], Victor Armijo[1], Dr Olivier Gemin[1,2], Franck Felisaz[1], Jérémy Sinoir[1], Arthur Felisaz[1], Thibault Deckers[1], Dr Léa Lecomte[1], Raphael Cohen[1], Christopher Rossi[1], Robert Janocha[1], Kevin Lauzier[1], Dr Romain Linares[1], Dr Michael Hons[1], Dr Sarah Schneider[1], Dr Matthew W. Bowler[1], Dr Peter Cloetens[3], Dr Wojciech Galej[1], Dr Julia Mahamid[2], Dr Sebastian Eustermann[2], Dr Simone Mattei[2], Florent Cipriani[1], Gergely Papp[1]
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381 Damage reduction in amorphous ice by a non-conventional scan strategy in cryo-STEM
Abner Velazco Torrejón[1], Mr. William Bowles[1], Dr. Maud Dumoux[1], Dr. Judy Kim[1], Prof. Angus Kirkland[1,2]
[1]Rosalind Franklin Institute, Harwell Science and Innovation Campus, Didcot, United Kingdom, [2]Department of Materials, University of Oxford, Oxford, United Kingdom
567 Towards Atomic Resolution of Cryogenic Ptychography Single-Particle Analysis (Cryo-EPty SPA)
Mr. Yu Lei[1], Mohammed Yusuf[2], Adrián Pedrazo-Tardajos[2], Judy Kim[2], Julie Staunton[1], Angus Kirkland, Dr Peng Wang[1], Chen Huang[2]
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573 Analyzing conformational and compositional heterogeneity of macromolecular complexes by cryo Electron Microscopy: Zernike3Deep and HetSIREN
Dr. David Herreros Calero[1], Carlos Óscar Sánchez Sorzano[1], José María Carazo[1]
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1009 Setting up fringe-free imaging for SPA on Talos Arctica and Titan Krios microscopes
Dr Nadav Elad[1], Dr Roman Kamyshinsky[1]
[1]EM Unit, Department of Chemical Research Support, Weizmann institute of science, Rehovot, Israel
313 Novel combination of integrated FLM with cryo-FIB allows targeted cryo-ET sample preparation from challenging samples
Marit Smeets[2], Samuel Zachej[1], Wessel Teunisse[2], Jakub Javůrek[1], Dominik Pinkas[3], Vlada Filimonenko[3]

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513 Microfabricated Sample Carrier for Cryogenic Electron Microscopy

Dr Vasilis Papadimitriou[1], Dr Evgeniya Pechnikova[1], Mr Merijn Pen[1], Hugo Pérez-Garza[1]
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582 Joint Ptychographic Tomography of Frozen Hydrated Proteins

Georgios Varnavides[1,2], Dr. Yue Yu[3], Berk Küçükoğlu[4], Dr Stephanie Ribet[2], Dr Mary Scott[2,5], Dr Henning Stahlberg[4], Dr Colin Ophus[2]
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638 Beyond Ribosomes: In Situ Structural Biology of Diverse Targets in C. reinhardtii

Dr Ron Kelley[1], Dr Xianjun Zhang[1], Dr. Martin Obr[1], Mr. Sagar Khavnekar[1], Dr Jessica Heebner[1], Dr Saikat Chakraborty[1], Dr Ricardo Righetto[2], Dr Florent Waltz[2], Dr Alicia Michael[2], Dr Wojciech Wietrzynski[2], Dr Grigory Tagiltsev[3], Prof John Briggs[3], Prof Juergen Plitzko[3], Dr Ben Engel[2], Dr. Abhay Kotecha[1]
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674 On how to tame the beast: Towards a high-throughput plasma FIB pipeline

Elisa Lisicki[1,2,3], Dr. Tatjana Taubitz[1,3], Prof. Dr. Stefan Raunser[1], Dr. Sebastian Tacke[1]
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770 An Improved Method for Growing Primary Neurons on Electron Microscopy Grids Co-Cultured with Astrocytes

Mrs Ishika Kumar[1], Mrs Anju Paudyal[2], Mrs Anna Kádková[3], Mrs Michelle Stewart[2], Mr Jakob Balslev Sørensen[3], Mrs. Julika Radecke[1,3]
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Poster Presentation

25 The Electron Bio-Imaging Centre (eBIC) at Diamond Light Source, UK

Lorna Malone[1], Dr Eilis Bragginton[1]
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219 Thermo Scientific Smart EPU Software: Towards autonomous screening

Mr. Fanis Grollios[2], Mr. Holger Kohr, Julio Ortiz[1], Mr. Edward Pryor[3]
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301 Sub 3Å cryoEM structure using a standard LaB6 120kV TEM upgraded with direct electron detector

Hari Venugopal[1], Jesse Mobbs[3], Cyntia Taveneau[2], Daniel Fox[2], Gavin Knott[2], Rhys Grinter[2], David Thal[3], A/prof Georg Ramm[1]

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571 Glacios 2 Cryo-TEM and Smart EPU Software streamline Cryo-EM for drug design with higher throughput

Adrian Koh[1], Michael Adams, Lingbo Yu[1], Fanis Grollios[1], Bart van Knippenberg[1], Abhay Kotecha[1], Basil Greber[2], Oliver Raschdorf[1], Julio Ortiz[3], Edward Pryor[4], Jeffrey Lengyel[4]
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624 Cryo lift-out technique to study host-pathogen interaction on cell monolayer

Dr Gautham Hari Narayana Sankara Narayana[1], Sylvie GOUSSARD[1], Guillaume DUMENIL[1], Daria BONAZZI[1]
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773 Cryo-EM and ED are driving structural studies at the University of Warsaw

Dr Tomasz Góral[1], Dr Szymon Sutuła[1], Prof Krzysztof Woźniak[1,2,3]
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884 Quantitative Super-Resolution Methods for Cryo-CLEM

Laura C. Zanetti-Domingues[1], Michael Hirsch[1], Lin Wang[1], Tara A. Eastwood[2], Karen Baker[2], Daniel P. Mulvihill[2], Sheena Radford[3], Jim Horne[3], Paul White[3], Dr Gea Van De Kerkhof[1], Benji Bateman[1]
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930 VitroJet: ice thickness control and measurement for time-efficient single particle structure determination3052

Rene Henderikx[1,2], Prof. Peter Peters[2], Dr. Bart Beulen[1]
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Late Poster Presentation

1262 Sub-4-Å cryo-EM structure of apoferritin from a basic 120 keV TEM with thermionic electron gun

Dr. Alexander Neuhaus[1], Dr. Anja Blanque[1], Prof. Dr. Christos Gatsogiannis[1]
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1280 Cryo-EM structure of wild-type Orsay virus: preliminary insights into the assembly mechanism

Dr. Antonela Stagnoli[1], Dr. Victoria G. Castiglioni[2], Technician Ander Vidaurrazaga[1], Dr. Santiago F. Elena F. Elena[2], Dr. Nicola G. Abrescia[1,3]
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1294 Cryo-Volume Electron Microscopy characterization of chlorophyll deficient microalgae

Phd Student Malene Olsen[1], PhD Maryna Kobylynska[2,3], Professor Poul Erik Jensen[1], Professor Pippa Hawes[4], Professor Roland Fleck[2,3]

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604

Prospects of nanofluidic cavities for cryo-EM sample preparation

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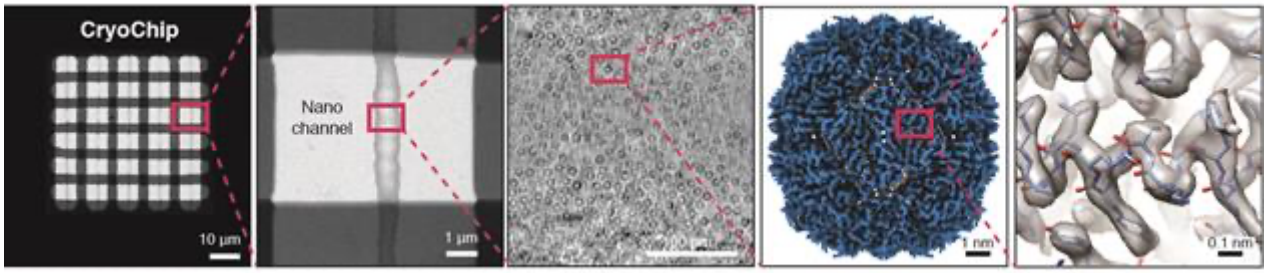
IM-11 (1), Lecture Theater 5, august 29, 2024, 10:30 - 12:30

Cryogenic electron microscopy (cryo-EM) has become an essential tool for 3D structure determination of biological macromolecules. Despite many technical advances, the difficulty to reliably prepare samples with uniform ice thickness and the excessive sample loss during grid preparation still present a barrier for routine high-resolution imaging and limit the current throughput of the technique [1,2]. Nanofabrication techniques employed for Micro-/Nanoelectromechanical Systems (M/NEMS) provide new opportunities to miniaturise and automate cryo-EM sample preparation. We have recently shown that MEMS-based nanofluidic sample supports with well-defined geometry can be used to prepare cryo-EM specimens with uniform ice thickness from picolitre sample volumes and allow for high-resolution structure determination [3]. Despite these promising prospects, several key challenges remain to be addressed to transform this approach into a viable alternative for widely used holey support films, most prominently mitigation of beam-induced specimen movement and putative interaction with the water-support film interface. I report on the present status of our developments, describe recent efforts in addressing some of the outstanding challenges and elaborate on others that require additional work to resolve. I will also showcase recent results demonstrating the potential of new chip generations to provide new capabilities for further automation of the cryo-EM workflow, and to explore new frontiers for cryo-EM applications such as time-resolved imaging and high-throughput screening.



Keywords:

Cryo-EM, sample preparation, MEMS, nanofluidics

Reference:

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398

Enabling discovery by in-cell structural biology

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IM-11 (2), Lecture Theater 5, august 30, 2024, 10:30 - 12:30

Most structural biology focuses on the structure and function of individual macromolecular complexes, but falls short of revealing how they come together to give rise to cellular functions. Here, cryo-electron tomography (cryo-ET) provides a unique opportunity for obtaining structural information across a wide range of spatial scales - from intact cells, 3D cultures, tissues and model organizms frozen in their close-to-native state, to individual macromolecular assemblies embedded in their native functional environments. We develop and employ advanced sample preparation techniques for in-cell cryo-ET, including cryo-focused ion beam thinning guided by 3D correlative fluorescence microscopy. Preparations of such site-specific 'electron-transparent windows' in appropriate cellular model systems visualizes molecular structures directly from three-dimensional stills of intact cells and can reveal their molecular sociology. Using the genome-reduced human pathogen Mycoplasma pneumoniae as a minimal cell model, we further demonstrated the synergistic application of whole-cell crosslinking mass spectrometry and cryo-ET to determine an in-cell integrative structural model of actively transcribing RNA polymerases coupled to translating ribosomes. Recent computational breakthroughs now allow resolving these molecular machines to near-atomic resolution directly inside the cell, reveal small molecule antibiotics bound to their active site in ribosomes within the intact pathogen, and provide snapshots of their structural dynamics along reaction cycles. We will discuss novel technologies that extend these applications and depth of information to mammalian cells and tissues. These cutting-edge methodologies unlock an enormous potential for system-spanning discovery enabled by label-free in-cell structural biology.

Keywords:

cryo-FIB, cryo-CLEM, cryo-ET, integrative modeling

23

Single particle CryoEM structures using iDPC-STEM from 4D-STEM detectors at near-atomic resolution

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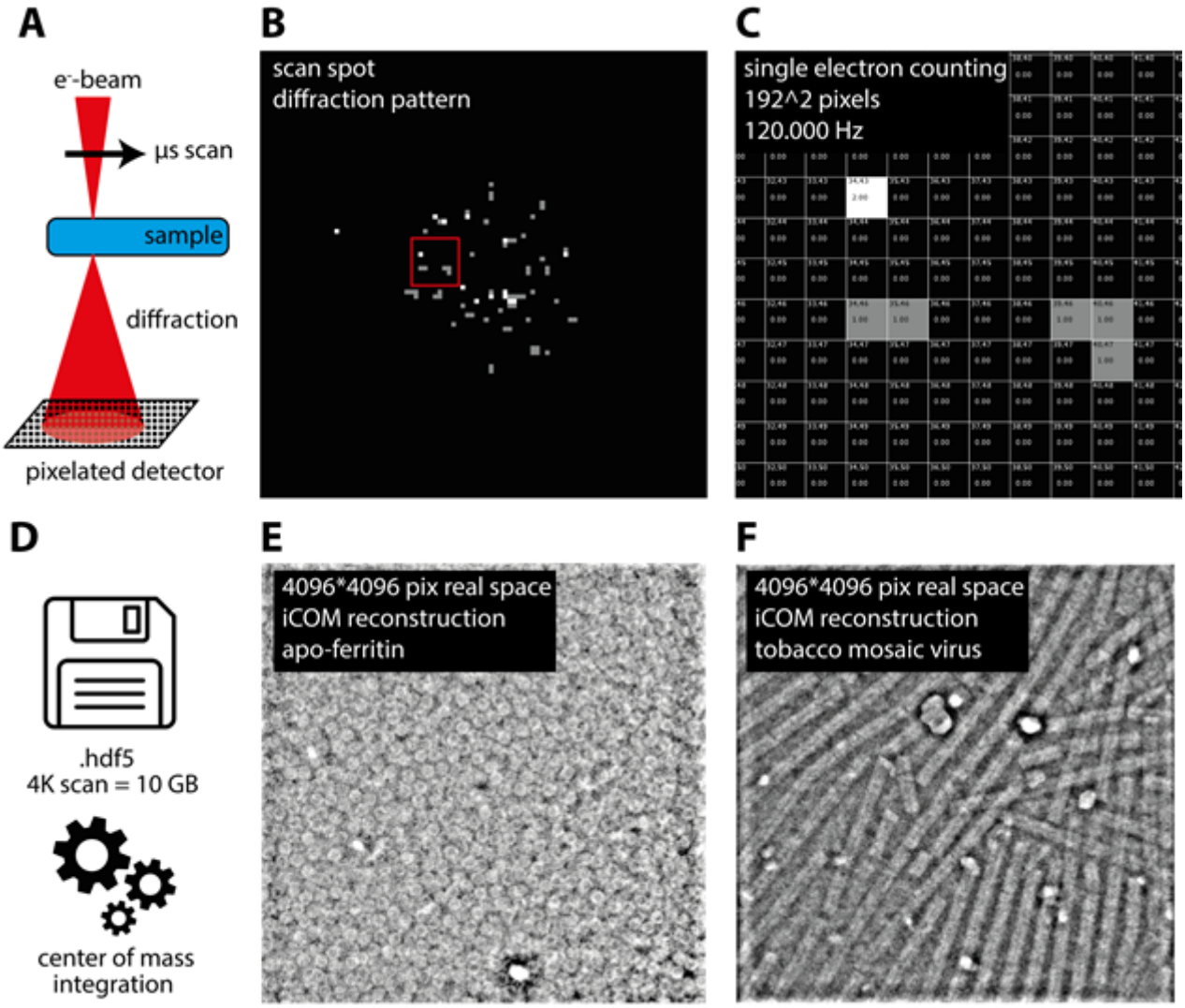
IM-11 (1), Lecture Theater 5, august 29, 2024, 10:30 - 12:30

CryoSTEM can image frozen-embedded biomolecules in focus without an oscillating contrast transfer function, enabling complete information transfer in each image. Using integrated differential phase contrast (iDPC)-STEM, one can obtain three-dimensional reconstructions of proteins using single particle analysis. We have recently demonstrated how iDPC-STEM can be used to reconstruct protein structures to near atomic resolution (Lazic et al. Nat.Meth. 2022), depending on the chosen convergence semi angle (CSA) of the incident beam. During scanning the electron dose rate is orders of magnitude higher compared to parallel illumination as the dose is distributed in a very small area (Ang2) and for a very short time (50 ns – 10 µs). We compare beam damage of conventional TEM and iDPC-STEM on the same frozen-embedded protein sample. We will demonstrate how this technique can be further applied to a variety of test samples and how alternative ptychographic image reconstruction methods such as single side band (SSB) can be used to further enhance the image information.
iDPC-STEM using segmented detectors is an approximation for integrated center of mass (iCOM) reconstructions. Therefore, we recorded pixelated 4D-STEM data of frozen-embedded protein molecules with a 300 kV Titan Krios G4i equipped with a fast pixelated 4D-STEM detector (Dectris Arina), derived the corresponding iCOM images and reconstructed protein structures to near-atomic resolution.



Keywords:

CryoEM, iDPC-STEM, 4D-STEM, SPA

363

EasyGrid: a new automated cryo-multimodal sample preparation

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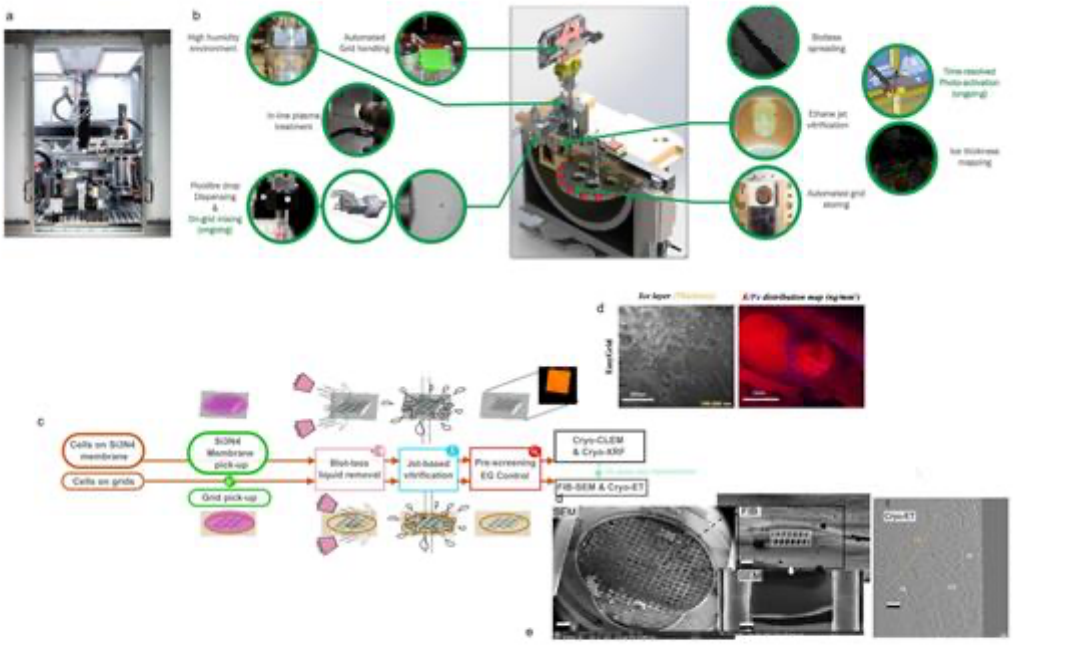
IM-11 (1), Lecture Theater 5, august 29, 2024, 10:30 - 12:30

Background incl. aims
In structural biology, Cryo-EM is one of the major imaging technologies. Even if the limits of the EM towards higher resolutions and faster data-collection have been really improved, the sample preparation step still remains poorly automated and difficult to manage for untrained and trained users (reproducibility issues). Many developments (e.g., Vitrojet1, Chameleon2) exist to make cryo-EM sample grid preparation easier, faster, and more reliable, and some of them also offer time-resolved module than remains a less user-friendly solutions (TrEM3, Joachim Franck's mixing/spraying method4). However, no machine currently offers a universal and easy solution. Several relative issues also exist in the field of X-ray imaging where there is no unique, reproducible sample preparation. This is the main reason why the instrumentation team of EMBL-Grenoble began the development of the EasyGrid5 device in 2017 that aims to provide a versatile tool that addresses most of the current difficulties encountered in the cryogenic preparation of diverse sample supports.

Methods & Results
This fully automated procedure (Fig.1a,b) uses in-line plasma treatment, picolitre drop dispensers, blotless liquid removal, ethane jet vitrification, automated cryo-storage and sample quality control at cryogenic temperature. The in-depth vitrification step is crucial to study native sample conditions for cells. Here we report initial results in two main domains; thin film sample vitrification for SPA experiments (cryo-EM), and whole-cell vitrification for cryo-ET/-XRF deep in large cells (Fig 1c,d,e).

Conclusion
EasyGrid proposes a versatile and robust multimodal sample preparation framework that can be easily adapted to a large variety of sample preparation protocols: cell freezing, classical SPA sample grid preparation, light triggered time resolved freezing, on-grid mixing, and this for diverse imaging modalities requiring different supports and conditions. The adherent cell vitrification protocol has been adapted for XRF using Si3N4 membranes (Silson), however, other supports can also be envisaged. The blotless liquid removal process allows for control of the ice layer thickness, a crucial parameter to optimise, to limit radiation damage on the sample during data-collection and maximise signal and contrast. The fully automated sample preparation procedure, leveraging precise robotics and an axis control system with sub-millisecond time-base, is highly reproducible. Hence, process optimization for different samples becomes accessible, and the screening of each process parameter can be reliably executed.



Keywords:

ElectronMicroscopy, X-rayFluorescence (XRF), Single-particleAnalysis, Cryo-samplePreparation

Reference:

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[3] Kontziampasis et al 2019. IUCrJ 6, 1024-1031.
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381

Damage reduction in amorphous ice by a non-conventional scan strategy in cryo-STEM

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IM-11 (1), Lecture Theater 5, august 29, 2024, 10:30 - 12:30

Background
For many important classes of materials, electron beam damage in transmission electron microscopy (TEM) has a detrimental effect that limits the capability of the instrument to obtain information at different scales, atomic or structural scale, for instance. Organic and biological materials are among the most beam sensitive materials. Biological samples are commonly studied using cryo-TEM, where the samples are preserved in amorphous ice and illuminated by a parallel beam. Unfortunately, the ice matrix is also sensitive to the electron beam. For these reasons, electron doses in the order of only 10's of e-/Å² are usually employed in cryo-TEM. Potentially, scanning transmission electron microscopy (STEM) offers some benefits over TEM such as the compositional contrast, through the high angle annular dark field (HAADF) signal, and the capability to observe thicker specimens [1]. In STEM, it is well known that beam damage can extend over an area larger than the size of the scanning probe so areas that come later in the scanning sequence can be damaged prior to observation with the probe. This effect can have many physical sources, which can depend heavily on the material itself: diffusion of heat, diffusion of radicals, delocalized inelastic scattering, electrostatic charge, etc. [2]. Some of these effects are dynamic in nature and have both a spatial and a temporal scaling parameter. It has been shown that this behavior can be modeled as a diffusion process and that beam damage can be mitigated by changing the temporal distribution of the electron dose [3]. In this work, since evidence of a diffusion type damage behavior was found in vitreous ice, we compare beam damage when using an interleaved scan, see Figure 1, and the conventional raster scan with the aim to mitigate damage effects and increase the applicability of cryo-STEM on biological samples.
Methods
To make the experiments reproducible, vitreous ice served as a test sample. A JEOL ARM300F2 microscope operated at 300 kV and equipped with an external scan generator was employed to scan two adjacent areas using raster and interleaved scan. In an effort to perform the experiments in areas of relatively uniform thickness, the experiments were carried out close to the center of the holes of quantifoil grids filled with ice, see Figure 1. An electron dose budget approximately 100x higher than in conventional cryo-TEM was used in order to make the effect of damage more evident and easier to quantify. The quantification was done from images acquired using the HAADF analog detector where darker areas indicate more damage after mass removal when scanning. The electron dose was fractionated by sequential acquisitions for both scan methods and also compared to single acquisitions, maintaining a constant total dose.
Results
A significant reduction of beam damage of more than 20% is observed when using interleaved over raster scan when scanning with long dwell times (more than 250 µs) and only a few passes (two or less); as shown by the use of line profiles in Figure 1. However similar amounts of damage occurred when using a short dwell time (20 µs) and several passes (around 25). Our results also show that the damage rate increases as the vitreous ice thickness decreases.
Conclusion

Our observations support the diffusion like process, at least for this specific material. It shows that beam damage is not only proportional to the total electron dose, but also the distribution in time and space plays an important role. Further work is required to identify the physical nature of the diffusion process and its link to beam damage.

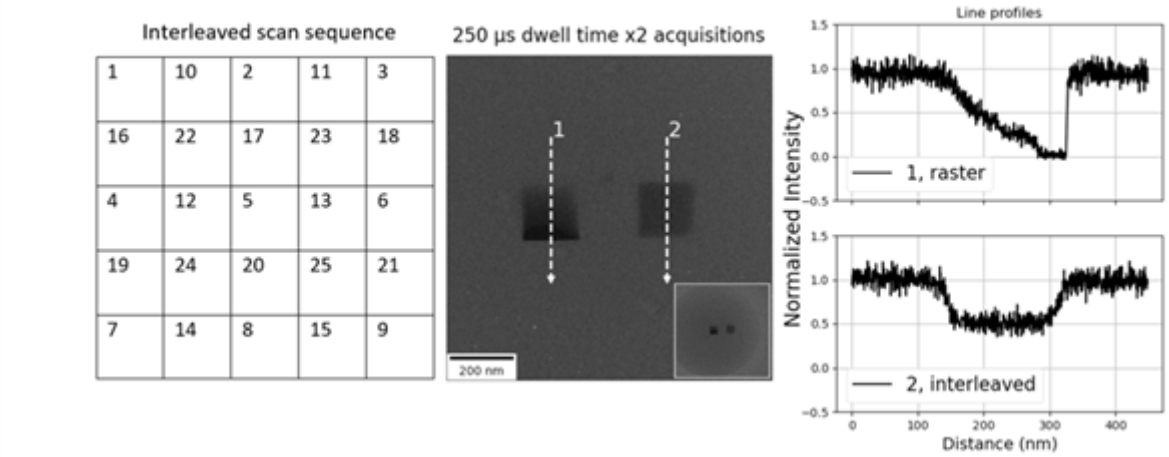

Figure 1. Left, example of an interleaved scan sequence for a 5x5 frame size. The numbers indicate the sequence of the scan positions. The same total number of positions are visited as in a conventional raster scan. Middle, experiment in a hole of a quantifoil grid filled with vitreous ice. Dark areas indicate beam damage after scanning with raster (left) and interleaved (right) patterns using 250 µs dwell time, 2 acquisitions, 512x512 frame size, 0.16 nm pixel size, 1 Å probe size approx. The same total dose was applied to the sample in both cases, 4000 $e^-/Å^2$ approx. An overview image acquired at a lower magnification is shown as an inset in the lower right corner. Right, normalized line profiles for the raster and interleaved scanned areas. The line profiles were normalized with respect to the mean of a similar line profile taken in between the two scanned areas, undamaged region. The acquisitions were vacuum subtracted, and the zero level indicates vacuum.

Keywords:

Cryo-STEM, diffusion process, beam damage.

Reference:

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567

Towards Atomic Resolution of Cryogenic Ptychography Single-Particle Analysis (Cryo-EPty SPA)

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IM-11 (1), Lecture Theater 5, august 29, 2024, 10:30 - 12:30

Background
Rapid advances in cryo-electron microscopy (cryo-EM) have made it possible to visualize three-dimensional structures of a wide range of biological macromolecules at near-atomic resolution in a close-to-native, frozen-hydrated state 1. However, the high electron irradiation sensitivity inherent in biological samples leads to a low signal-to-noise ratio and low contrast in EM images. While cryo-EM single particle analysis (SPA) usually records microscopic movies with high defocus to improve contrast, high-defocus condition attenuates low-frequency information and rapidly reverses high-frequency information in the phase contrast transfer function (PCTF), which causes difficulties in accurate identification, classification and alignment of particles to a common reference. Ptychographic diffractive imaging, a technique capable of reconstructing phase information from diffraction patterns using an iterative algorithm known as ePIE (Fig. 1a), holds great promise for achieving super-resolution, high-contrast, low-dose, and 3D imaging of biological samples in vitreous ice at low doses2. Moreover, ptychography utilizes the entire diffraction pattern , making it particularly dose efficient, especially when using direct electron detector data with a high signal-to-noise ratio at a low electron dose 3. Using cryogenic electron ptychography (cryo-EPty) [4], we have successfully reconstructed the 2D phase images of rotavirus double-layer particles (DLPs) measuring 76.5 nm in diameter at doses of 20 e/Å2, corresponding to a convergence semi-angle (CSA) of 4.83 mrad, employing approximately 300 particles. Additionally, we have demonstrated the visualization of 3D structures by integrating SPA 4. The resolution of the 3D map was estimated to be 1.86 nm at the nanometer level. These advancements underscore the potential for combining cryo-EPty and single-particle analysis to reach atomic-level resolution in imaging . We aim to increase the resolution achieved by not only increasing the number of particles used as is done in traditional SPA but by increasing the optically limited resolution with a larger convergence semi-angle.

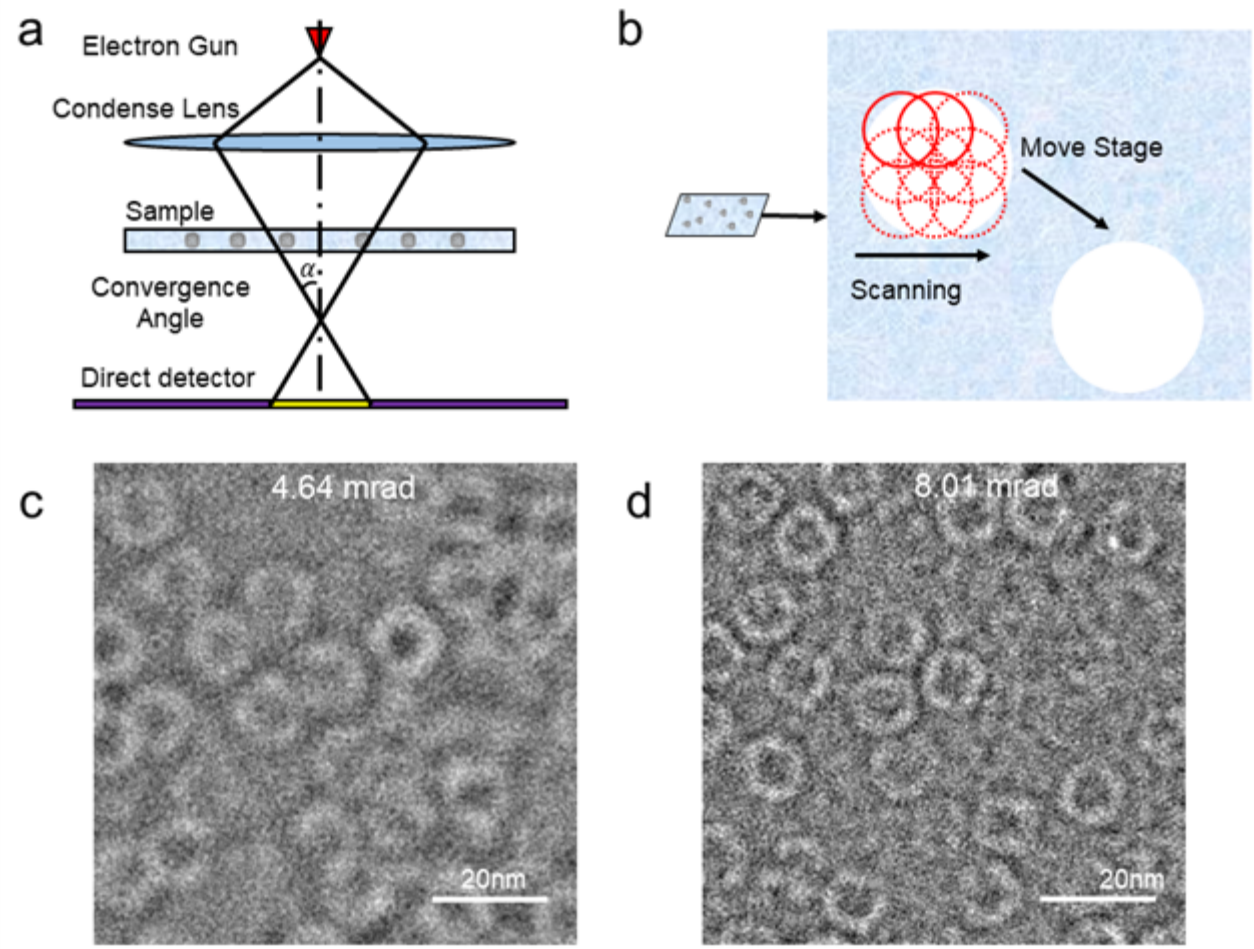
Methods
We used apoferritin embedded in vitreous ice as a benchmark sample. Experimental cryo-EPty SPA datasets (Fig. 1b) were acquired in a scanning diffraction configuration, in which a defocused probe is scanned over a sample at cryogenic temperatures for probe overlap and information redundancy of approximately 85% as is commonly employed for defocused cryo-EPty 5. The use of a defocused probe enables a lower electron fluence than employed in physical sciences while maintaining full coverage of the scanned specimen area.

Results
We reconstructed 2D ptychographic phases of apoferritin particles with varied CSAs (i.e., 4.64 and 8.01 mrad) as shown in Fig. 2a-b to obtain information transfer over a greater range of spatial frequencies. We will demonstrate that using the SPA pipeline with the ptychographic phase, high-resolution 3D density maps of apoferritin can be reconstructed from the stack of particle phases. Using a Fourier Shell Correlation, we anticipate that the 3D density maps will approach a resolution of a few angstroms with a greater number of particles. Our findings show that larger convergence

angle datasets produce higher-resolution information that can be combined with essential lower spatial frequency information for data across a greater range of spatial frequency.

Conclusion

Our findings suggest that the promise of cryo-EPty combination with SPA will pave an alternative method for high-resolution 3D reconstructions of biological samples, potentially reaching atomic resolution .



Keywords:

4D-STEM, Ptychography, SPA, Cryo-EM

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573

Analyzing conformational and compositional heterogeneity of macromolecular complexes by cryo Electron Microscopy: Zernike3Deep and HetSIREN

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IM-11 (1), Lecture Theater 5, august 29, 2024, 10:30 - 12:30

BACKGROUND INCL. AIMS
Biological macromolecules have varying degrees of conformational flexibility and compositional heterogeneity that complicates the image processing of large sets of cryo Electron Microscopy images, especially when flexibility is continuous or there are many compositional options. New approaches like HetSIREN (1) and the Zernike3D family of algorithms (2) are capable to generate conformational landscapes representing complex heterogeneity cases while also producing higher resolution maps of the different states. Additionally, novel meta-approaches, like the ones pioneered by Scipion Flexibility Hub (3,4), start bringing consistency and reliability to this emerging field of flexibility analysis and map quality improvement.

METHODS
HetSIREN and Zernike3Deep are two cutting-edge deep learning algorithms meticulously designed to address the analysis of structural heterogeneity in cryoEM single particle analysis. These new algorithms not only excel at segregating particles into distinct 3D conformational states but also places special focus on the representation of structural features and map quality to enhance the biological interpretability. These new algorithms are distributed as part of the Scipion Flexibility Hub (4), together with new tools designed to analyze and integrate findings across different conformational landscapes.

HetSIREN performs a constrained heterogeneous reconstruction or refinement, relying on a structural prior in real space. This approach decodes high-resolution stable and non-stable structural states, providing a more sensible picture of the structural landscape explored by a biomolecule.

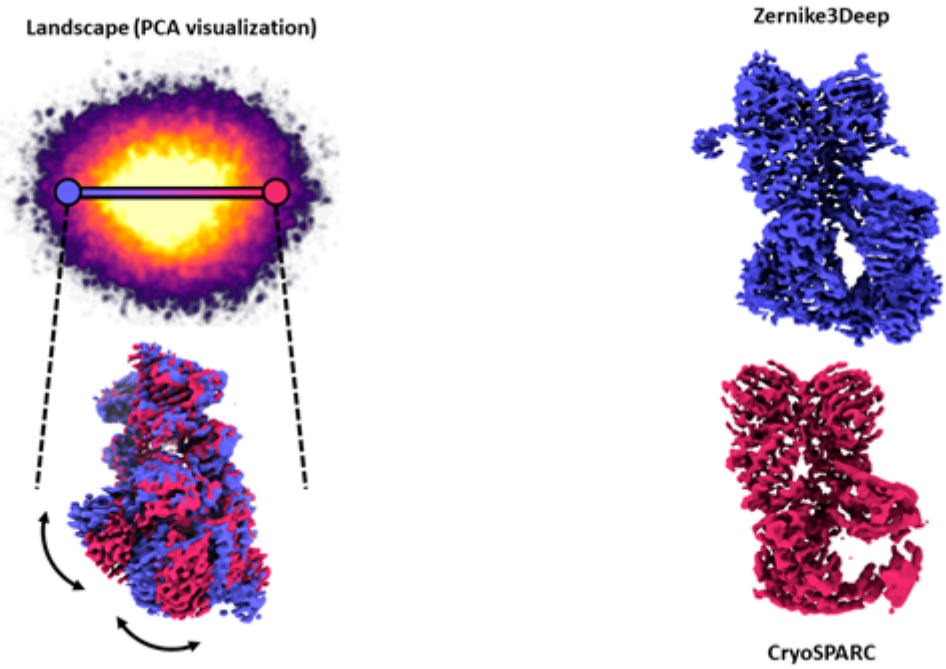
In contrast, Zernike3Deep relies on a semi-classical neural network that is specifically designed to learn mathematically meaningful latent spaces. These spaces are to be used in conjunction with the Zernike3D basis to accurately expand molecular motions. The decoded motions are suitable not only for modeling high-resolution structural states captured in the conformational landscape but also for applying as a correction for motion blur artifacts during the reconstruction process. This application increases the resolution of molecular regions that are moving and difficult to resolve.

RESULTS
HetSIREN and Zernike3Deep have proven themselves as versatile tools for the heterogeneity analysis of cryoEM particle datasets with varying characteristics, emphasizing the importance of accurately representing structural features to enhance biological interpretation. To illustrate the capabilities of these methods, we present an analysis of the Gr-HsP90-FKBP51 complex, a highly dynamic and biologically significant biomolecule. Its understanding is hindered by motion blurring, which obscures both its structural characteristics and dynamic behavior.

The accompanying graphic summarizes the analysis conducted by Zernike3Deep, depicting the PCA representation of the conformational landscape, along with the primary motion described along the first principal component. Additionally, the graphic also compares the motion-corrected map derived from Zernike3Deep heterogeneity analysis with the one obtained without correction by a classical method in the field (CryoSPARC (5)). The results underscore the importance of incorporating advanced heterogeneity analysis into cryoEM workflows, enabling the recovery of structural information that would otherwise be overlooked by the conventional assumptions of more traditional methods regarding reduced and discrete structural heterogeneity.

CONCLUSION

CryoEM structural analysis is undergoing a revolutionary shift towards extracting richer and more accurate conformational landscapes. The advent of new heterogeneity algorithms moves beyond the limiting assumptions of classical methods, significantly reducing the amount of structural information discarded during estimation processes. In this work, we introduce two innovative deep learning methods for advanced heterogeneity analysis, named HetSIREN and Zernike3Deep, as well as a meta-analysis environment named Scipion Flexibility Hub. These methods are designed not only to accurately estimate conformational landscapes and structural states but also to place a strong emphasis on the representation of structural features. These approaches aim to enhance the biological interpretability of the estimated structural states and improve the resolvability of highly dynamic molecular regions.



Keywords:

cryoEM, Image Processing, Conformational Variability

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1009

Setting up fringe-free imaging for SPA on Talos Arctica and Titan Krios microscopes

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IM-11 (1), Lecture Theater 5, august 29, 2024, 10:30 - 12:30

Background:
Single particle cryo-EM requires large image data sets, while instrument time and sample availability are in many cases limiting factors. Increasing the data collection efficiency is therefore highly advantageous. One of the methods for increasing imaging efficiency is by reducing the diameter of the beam, such that more images can be taken from suitable sample areas on the TEM grid. In normal TEM imaging mode, the diameter of the beam is limited by appearance of Fresnel fringes from the C2 aperture. Fringe-free illumination reduces Fresnel fringes at the beam edges by simultaneously imaging the sample and C2 aperture in focus. The beam diameter can therefore be reduced significantly, allowing the collection of a larger number of images per-hole and higher throughput.

Methods and Results:
Installation of fringe-free illumination is commercially available from Thermo Fisher Scientific (TFS) and includes mechanical adjustment of the stage's eucentric height. However, purchasing and implementing the TFS product is not always possible or is simply unnecessary. Instead, the microscope can be aligned for fringe-free imaging by the local facility, and the alignment stored in a separate file. We have implemented this method on our Titan Krios G3 (3-condenser lens system) and Talos Arctica (2-condenser lens system), both resulting in equally high-resolution structures as in the normal alignment mode. On the Talos Arctica, fringe-free alignment using a 20 um condenser aperture results in a parallel illumination beam diameter of about 750 nm. The use of a small condenser aperture and imaging far from the original eucentric focus introduces several challenges in alignment and automated acquisition, however these are solvable.

Conclusions:
Fringe-free illumination increases SPA imaging throughput and can be implemented by experienced microscopists in both 2 and 3-condenser lens EMs. I will present the procedure for aligning both microscope types, as well as a script for quickly switching between the two alignment modes before starting EPU sessions.

Keywords:

single particle cryo-EM, fringe-free illumination

313

Novel combination of integrated FLM with cryo-FIB allows targeted cryo-ET sample preparation from challenging samples

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IM-11 (2), Lecture Theater 5, august 30, 2024, 10:30 - 12:30

Cryo-electron tomography (cryo-ET) has revolutionized structural biology by enabling the observation and characterization of biological samples at a molecular level in their near-native state [1]. This technique provides an unprecedented level of structural detail, but it necessitates thin specimens (typically below 200nm) for effective electron beam penetration. Presently, the prevailing method for achieving such thinning involves Cryogenic Focused Ion Beam (cryo-FIB) milling [2].

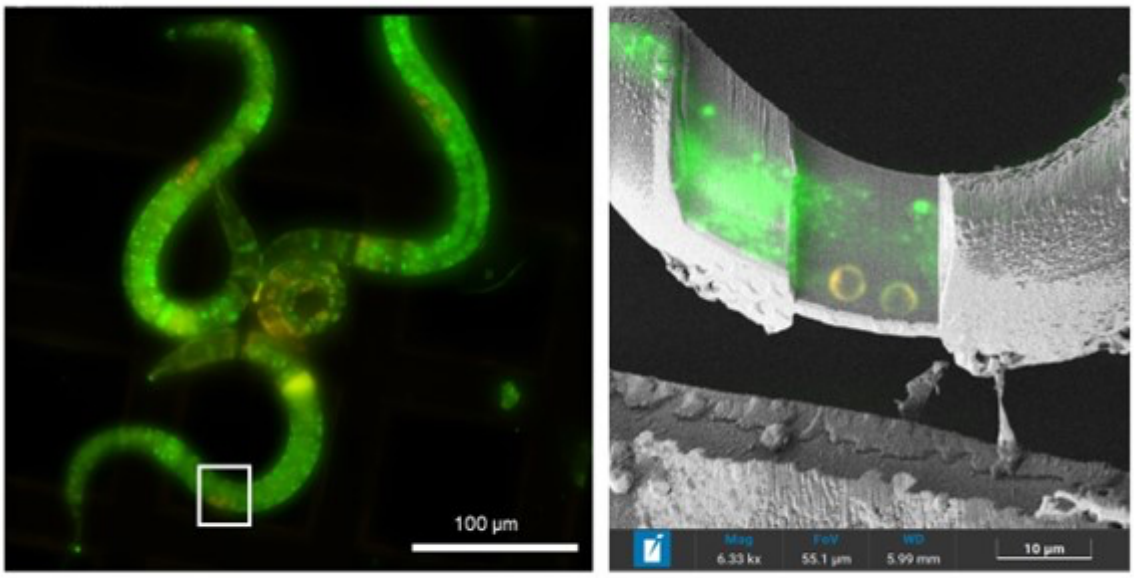
During this milling process, it is easy to miss the regions of interest (ROIs). Fluorescence labeling and fluorescence light microscopy (FLM) can improve targeting [3]. However, the conventional use of a stand-alone cryo-FLM invokes extra transfer steps, increasing the risk of ice contamination, devitrification, and mechanical damage [4]. Moreover, the correlation of the two imaging modalities is cumbersome and often inaccurate, leading to the loss of ROI inside the lamella, rendering it useless, reducing the overall efficiency of the whole workflow.

To address these challenges we present the novel combination of the TESCAN AMBER cryo- FIB/SEM with a fully integrated high quality FLM: Delmic's METEOR system [5]. Thanks to the software integration and position of FLM parallel to the FIB, this novel combination allows smooth and accurate 3D correlation of FLM , FIB and SEM data. Furthermore, the integration of the FLM significantly reduces handling and ice contamination. Additionally, a full collision model allows safe control of the microscope ensuring novel users can adapt the technique quickly. The high-end fluorescent light microscope provides excellent resolution and brings background noise to an absolute minimum allowing researchers to image and mill challenging samples.

In this talk we will delve into the practicalities of this accurate, easy-to-learn and high-throughput correlative cryo-FIB workflow. We will show that the workflow can be used for targeted fabrication of cryo-lamellae from thin specimen (on-grid lamella), larger volumes frozen by waffle method , and cryo lift-out from the most challenging samples. Additionally, we will present the latest results showing EM and FLM data of high-pressure frozen C. elegans and plunge frozen U2OS cells. We will also provide an outlook and highlight the potential of plasma FIB milling, enabling analyses of large biopsies, tissues and small organisms are also discussed.

The novel combination of METEOR with TESCAN AMBER cryo-FIB/SEM allows targeted preparation of cryo-TEM specimen from challenging samples, like high-pressure frozen samples and flat U2OS1 cells.

Acknowledgement
We acknowledge the Electron Microscopy Core Facility, IMG ASCR, Prague, CR, supported by MEYS CR (LM2023050 Czech-BioImaging) and OP RDE (CZ.02.1.01/0.0/0.0/18_046/0016045, CZ.02.1.01/0.0/0.0/16_013/0001775).



Keywords:

CLEM, Cryo-FIB, Cryo-ET

Reference:

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513

Microfabricated Sample Carrier for Cryogenic Electron Microscopy

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IM-11 (2), Lecture Theater 5, august 30, 2024, 10:30 - 12:30

Cryo-electron microscopy (CryoEM) has been proven as invaluable tool for small biomolecule structure determination ranging from proteins to viruses and DNA. Nevertheless. since the introduction of TEM and cryoEM the sample carrier (grid) has remained largely unchanged. Meanwhile microfabrication has made leaps due to the semiconductor industry. In this abstract we present a silicon-based sample carrier made with microfabrication techniques.

Current sample carriers consist of grid made by soft metals typically copper or gold and covered by a holey carbon film or lately higher end carriers are covered by a gold film[1] . Despite their desirable high thermal conductivity, soft metals carriers are very easy to bend, and require gentle handling . Clipping the grid (i.e. to create AutoGrids) is required to be compatible with autoloaders. In addition, due to their low robustness, their surface is typically wavy (not flat), making sample preparation far from ideal, especially for applications such as cryo-tomography. The carbon film transfer typically happens on an individual carrier level, resulting in surface inconsistencies between carriers even from the same fabrication batch. Furthermore, any process in the individual carrier level lengthens the fabrication process and hence the lead times.

Our proposed carriers use nanotechnology materials and, via the introduction of microfabrication techniques, we aim to improve all the aforementioned limitations of the current carriers. Our microfabricated devices consist of a silicon frame, holey silicon nitride membrane and a monolayer Graphene layer (Figure 1). Silicon offers a rigid and robust frame, making the handling and sample preparation much more forgiving. Our carriers can be clipped in the standard AutoGrid following the same process and tools as the current grids, but our microfabrication process enables the possibility to produce carriers with the geometry of an assembled AutoGrid, which would help to skip the tedious process of clipping. By meticulously controlling the deposition of silicon nitride on the silicon substrate, we produce a highly reproducible, atomically flat surface. In addition, microfabrication allows precise control material properties such as internal stress that can improve robustness. A large amount of literature reports the advantages of graphene for cryoEM [2-5], ranging from eliminating one air water interface to enabling affinity grids and controlling ice thickness via graphene hydrophilicity treatment. In order to guarantee the scalability of our production, we have developed the processes to introduce graphene on the wafer level, ensuring consistent quality and surface properties for carriers from the same batch.

Initial characterization of the carriers has been performed, with more extensive experimental results expected in the coming months. The first results are very promising. The Graphene coverage, which depends on the hole size, showed a coverage of 66% for 6µm holes, 85% for 3.5µm holes and 96% for 2µm holes. We have confirmed the compatibility of our chips with different vitrification methods (i.e. plunge freezing and jetting). The acquired ice quality results, obtained with an unoptimized protocol using a Vitrobot, indicate amorphous ice of a uniform thickness of approximately 40nm (Figure 2). Several methods for the hydrophilization of graphene have been investigated depending on the users equipment, ranging from O2 & H2 plasma and glow discharge, to annealing and chemical modification of graphene.

We believe that silicon-based sample carriers are the logical next step to enhance and advance the capabilities of cryoEM. Leveraging microfabrication technologies provides us with unprecedented possibilities to customize the layout/geometry of the chip, allowing us to meet the experiment´s specific requirements precisely.

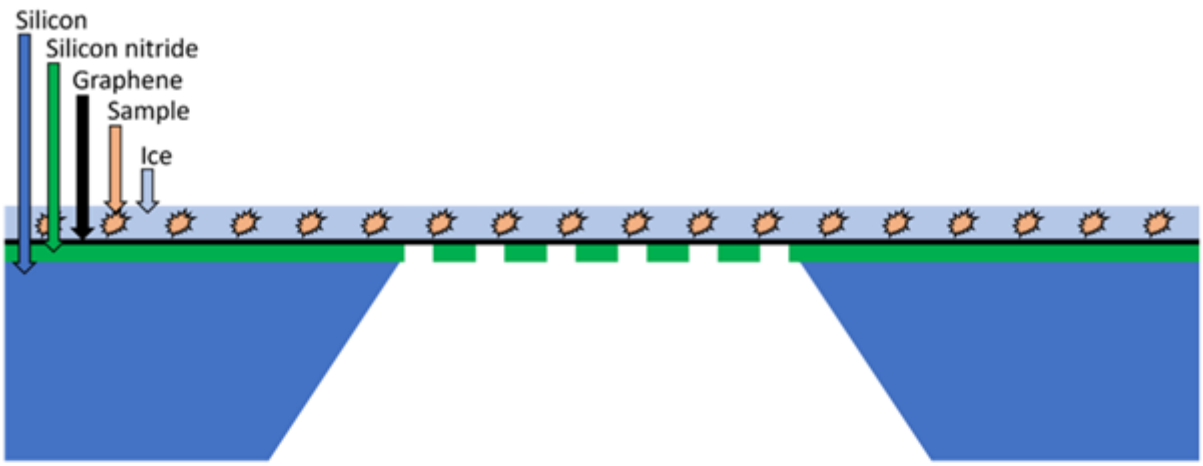

Fig. 1. Schematic of the Sample Carrier. The image shows the resulting architecture of our Silicon-based devices. As observed, a monolayer Graphene lays over a holey Silicon Nitride film.

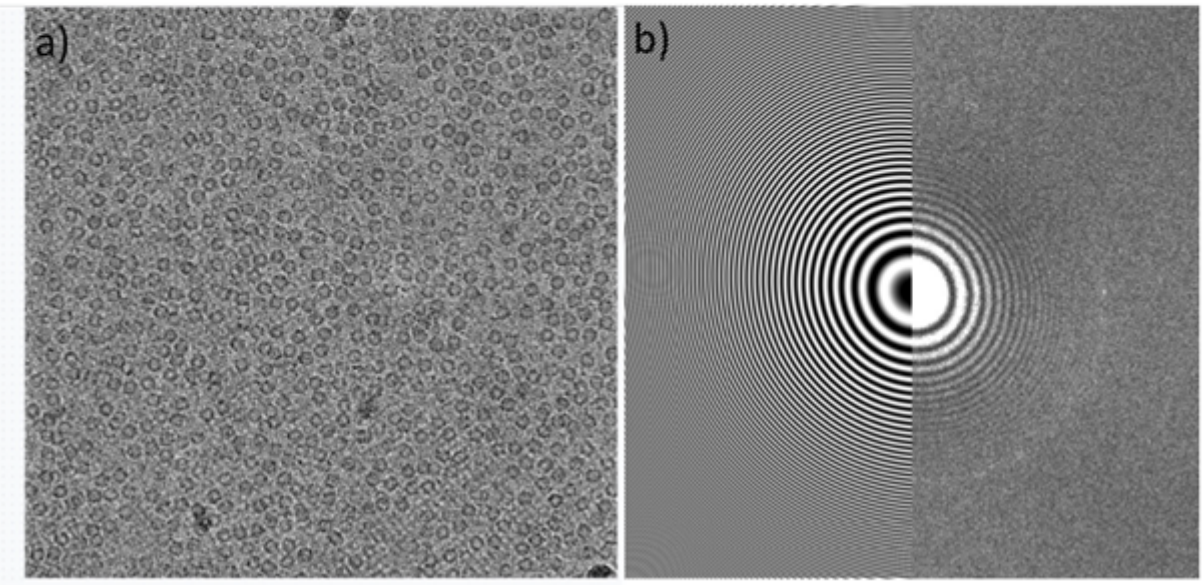

Fig. 2. Ice quality results. a) a monolayer of apoferritin with good distribution can be seen. B) The FFT indicates good quality of amorphous ice

Keywords:

CryoEM, graphene, Silicon based carriers

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582

Joint Ptychographic Tomography of Frozen Hydrated Proteins

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IM-11 (2), Lecture Theater 5, august 30, 2024, 10:30 - 12:30

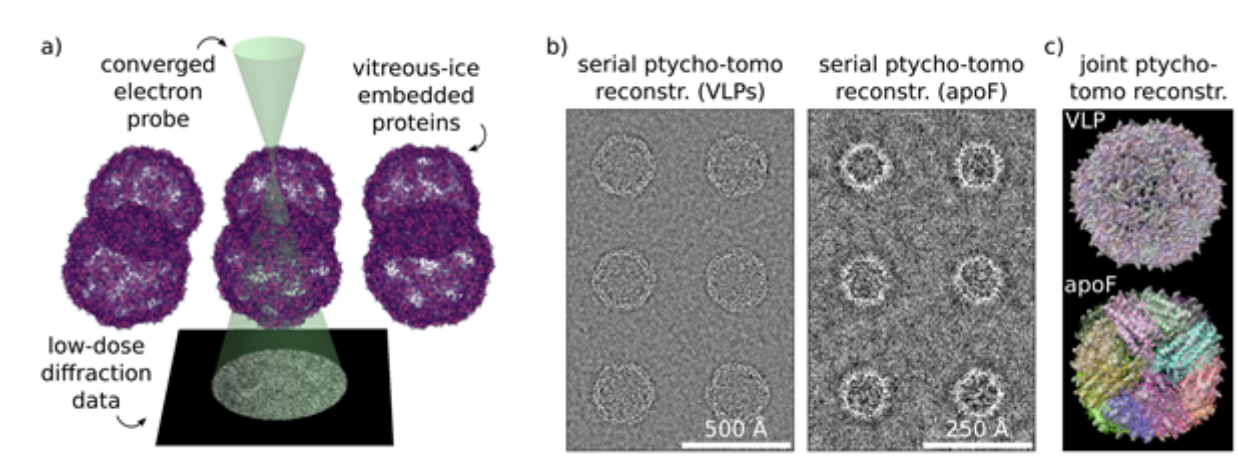
Single particle analysis (SPA) of frozen-hydrated proteins using cryogenic electron microscopy (cryo-EM) enables the three-dimensional structure determination of biomolecules with ångström resolution. Despite the remarkable advances enabled by cryo-EM SPA, the technique requires extensive data acquisition and processing and suffers from size limitations. Cryo-EM techniques are limited for very large biomolecules and very small proteins, due to the presence of multiple-scattering and poor contrast arising from the low electron fluence necessary to prevent sample damage respectively.

Scanning transmission electron microscopy (STEM) techniques have traditionally not been applied to the study of biological samples, due to the high fluence requirements of the most easily interpretable imaging modality using high angle annular detectors (HAADF). However, considerable efforts have recently been employed to apply phase-contrast STEM methods to study biological structures [1,2,3]. Among these techniques electron ptychography, where one iteratively reconstructs the scattering potential using a set of converged beam diffraction patterns (4D-STEM), stands out due to its high dose-efficiency and relaxed sampling requirements [4].

Cryogenic electron ptychography has recently been used to obtain sub-nanometer resolution of apoferritin samples using a relatively small number (~11,000) of high signal-to-noise reconstructions [5]. This “serial” approach, where one uses the 4D diffraction datasets to reconstruct 2D projection images which are then subsequently used to reconstruct a 3D volume using standard cryo-EM methods, is not maximally dose efficient. In this talk, I will propose an alternative technique we term “joint” ptychographic tomography SPA, where the 3D volume is reconstructed directly from the 4D data. This has multiple advantages over 2D projection-based techniques: first, nonlinearities arising from multiple scattering in the sample can be accurately modeled; second, it enables 3D regularization directly which can more effectively fill-in information from missing projection directions; and finally, it can more accurately capture amplitude and phase variations of the scattering potential.

Figure 1 illustrates the technique on small (1728 particles) simulated datasets of virus-like particles (PDB ID:1dwn) and apoferritin (PDB ID: 8rqb). Representative reconstructed micrographs are shown in Fig. 1b for the two proteins using electron fluences of 45e/Å2 and 35 e/Å2 respectively. These are used to reconstruct 3D volumes “serially” using the commonly used SPA software cryosparc with and without imposing symmetry. Alternatively, the volumes can be directly reconstructed using our joint ptychographic-tomography implementation in the open-source software py4DSTEM [4]. The resulting 3D maps, together with the respective docked models are shown in Fig. 1c.

We estimate that, for properly oriented poses, joint ptychographic-tomography offers a 10-20% improvement in resolution, as assessed by gold-standard Fourier shell correlation. Finally, we illustrate how joint ptychographic tomography can be used to estimate the unknown tilt orientations directly from the 4D data and show progress towards experimental results.



Keywords:

single-particle analysis, ptychography, tomography, phase-retrieval

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638

Beyond Ribosomes: In Situ Structural Biology of Diverse Targets in C. reinhardtii

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IM-11 (2), Lecture Theater 5, august 30, 2024, 10:30 - 12:30

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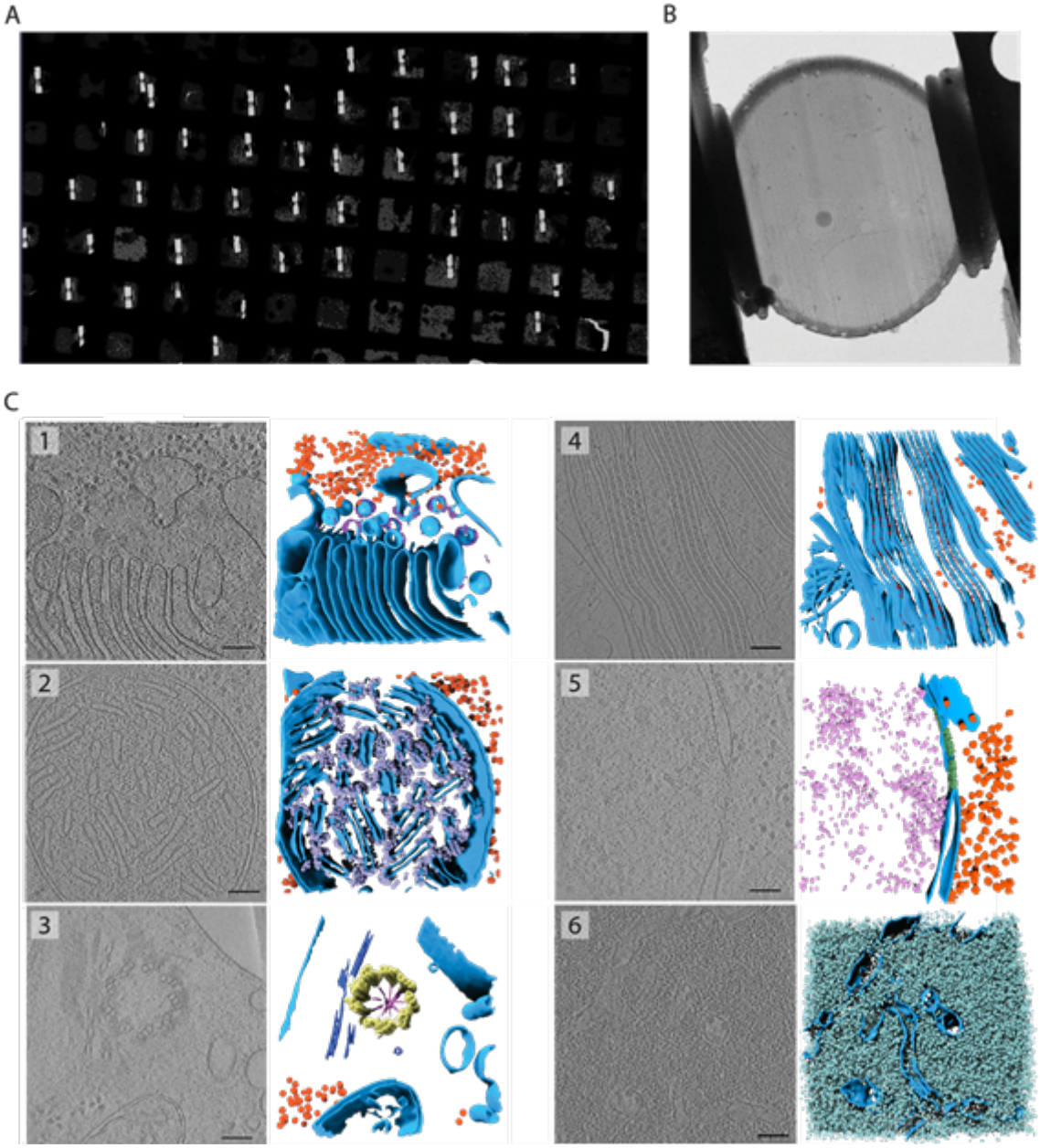
Understanding high resolution protein structure in the context of the whole cell is the vision of visual proteomics. With the advent of high-throughput cryo-electron tomography and cryo-FIB milling, paired with cutting edge computational techniques, achieving such an ambitious goal is no longer a far-reaching dream. The new generation of cryo-FIB from Thermo Fisher Scientific uses plasma to generate focus ion beam, which reduces redeposition and ion beam damage, substantially improving throughput. In preparation for a large-scale effort towards visual proteomics of the model organism Chlamydomonas reinhardtii, we have prepared enough lamellae to allow for acquisition of more than 1800 tomograms of different cellular compartments (Fig 1).

Initial results indicate that the data is of sufficient quality to achieve sub-nanometer resolution for selected complexes using a fraction of the dataset. Six tomograms from the same lamella were used to determine the structure of 80S ribosome at 6Å using template matching1 and subtomogram averaging. Encouraged by this, a number of other targets were selected among the collaborators for further study such as ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), ATP synthase, nucleosomes, photosystem II, and microtubules.

While some proteins were in such high abundance that template matching alone was sufficient to obtain a sub-nanometer structure, as in the case of Rubisco where we were able to determine a 7Å structure from within the pyrenoid compartment, other targets proved more of a challenge and necessitated a combination of computational techniques to achieve results. The denoising neural network cryoCARE was implemented on all datasets to increase template matching accuracy. In the case of microtubules, which are rare in C. reinhardtii, filament tracing in Amira (Thermo Fisher Scientific) was necessary to determine initial centerlines for reconstruction along individual protofilaments.

Photosystem II (PSII), which is present in very high abundance in the chloroplast, proved to be a challenging target as it is a membrane embedded protein with only a small region protruding into the lumen of the thylakoid membrane. Multiple template matching methods failed to identify most of the visible particles and unfortunately noise2noise denoising tended to blur or erase the small densities along the membrane. We therefore employed a novel approach that paired two deep learning networks to generate PSII candidate coordinates which could be further refined for classification and subtomogram averaging. The first network is a regression UNet trained on purely synthetic data2 to denoise cryo-tomographic data. The denoised data was then used to train a 2.5D UNet to segment membrane, ribosomes, and all densities that protrude into the thylakoid lumen as PSII (Dragonfly 2022.2, Object Research Systems). Coordinates (without any angular information) were extracted from the PSII class and used as particle picks, which were then aligned using the surrounding membrane. Work is ongoing, but initial results from 24 datasets are very promising.

The scale of this dataset is exciting, but the huge number of molecular complexes within living cells makes it difficult to identify, confirm the identity of, and determine each structure by just one group. Achieving a full visual proteome of C. reinhardtii will necessitate a large collaborative effort. To that end, we would like to create an open access database for C. reinhardtii to accelerate annotation and curation, enable further cell biology research, and develop new computational tools for in situ cryo-ET. Along with sharing the raw data, reconstructed tomograms, denoised datasets, and structural determinations, we will provide high quality segmentations of selected datasets created using 2.5D (Dragonfly) and 3D (MemBrain Seg3) UNets. This project has the potential to provide invaluable insights into cellular processes and will hopefully lay the foundation for other large-scale studies of other species.



Keywords:

CryoET, FIB-milling, plasmaFIB, visual proteomics

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674

On how to tame the beast: Towards a high-throughput plasma FIB pipeline

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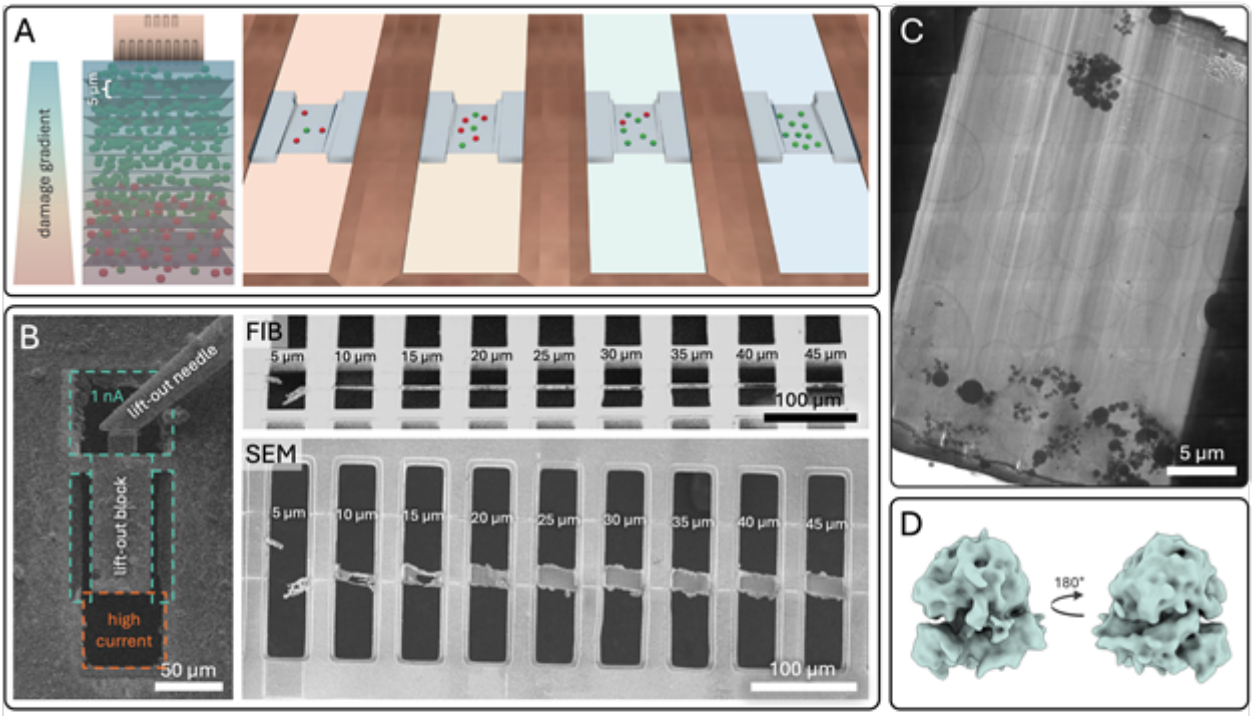
IM-11 (2), Lecture Theater 5, august 30, 2024, 10:30 - 12:30

Background incl. Aims: Recent advances in data acquisition, processing and sample preparation [1,2,3] have made cryo-electron tomography (cryo-ET) a powerful technology for investigating subcellular processes in situ. However, its usage for clinically relevant systems such as tissues, organoids and other 3D cell cultures remains limited due to the increased sample preparation complexity: With increasing sample thickness, direct milling on its substrate is not always possible. Alternatively, region-of-interests can be removed from the bulk, lifted to a new sample substrate and thinned finally to an electron-transparent lamella of less than 300 nm thickness. Both approaches, the so-called waffle [1] as well as the lift-out [2] method, are typically performed using a gallium ion beam for milling. Due to the low sputtering yield of gallium and the low ion beam currents available in conventional focused ion beam (FIB) microscopes, the throughput of both techniques is limited and the resulting slow lamella preparation poses a major bottleneck in clinical cryo-ET applications. Substantially faster sample preparation is expected with the recently introduced plasma FIB microscopes [4] because of the higher sputtering yields of the different ion beam species and the higher available ion beam currents offered. However, it is currently not characterised how the different ion species and higher currents of the ion beam might impact the sample integrity and how such structural damage propagates through the sample material. Here, we systematically investigate the structural damage caused by different ion beam species and currents. For this, we compare the structural preservation of proteins (e.g. ribosomes) with increasing distance to the impact centre for increasing beam currents by serial lift-outs [5] (see figures A, B). Our findings can be directly used to improve the efficiency of both standard as well as advanced sample preparation approaches, thereby enabling cryo-ET investigations of clinically relevant patient tissue.
Methods: To ensure uniform sample vitrification, S. cerevisiae were high-pressure frozen with a thickness of 20 µm in 20% dextran. To quantify the impact of strong ion beam currents, 100 µm long lift-out blocks were exposed to standard 1 nA and only one side was treated with a high current (15, 60, 200, 500, 1000, 2500 nA; figures A, B). The lift-out block was then cut into a series of 5 µm thin slices, therefore increasing distance to the high-current impact side with each slice. Each slice was then thinned to less than 300 nm. For detailed damage evaluation, tomograms were acquired and reconstructed followed by ribosome picking and subtomogram averaging (Thermo Scientific Tomography 5, AreTomo, crYOLO and Relion 3.1.2).
Results: We examine in total three different types of damage induced by high-current milling: 1) specimen level damage that is already evident by FIB/SEM imaging, 2) damage affecting lamella quality, such as devitrification and curtaining, that is visible by low-magnification TEM imaging and 3) structural damage on molecular level that is only apparent after tomogram acquisition and subtomogram averaging. Damage at specimen level (figure B) and lamella level (figure C) affect both the quantity and quality of lamellae that can be milled in a given time frame. The third type of damage will elucidate which currents can be used for sample preparation to still achieve high resolution after subtomogram averaging (figure D). The usage of serial lift-out allows us to trace the expected damage gradients after high-current milling for up to 100 µm from the area of impact and gives unprecedented insights into milling-induced damage in biological samples.

Conclusion: Our research provides the first systematic analysis of plasma FIB currents and their induced damage. Having these profound insights into sample-beam interaction will be of great value for developing high-throughput sample preparation pipelines for bulk specimens.



(A) Scheme of serial lift-out with distance-dependent damage in ribosomes, with the left panel showing the whole lift-out block and the right panel the finished lamellae. The right panel illustrates that high damage might result in low numbers of identified ribosomes per lamella as well as low resolution (red ribosomes). In lamellae with low damage the absolute number and the achievable resolution (green ribosomes) will be higher. (B) Lift-out preparation illustrating the usage of high-current milling on only one site of the prepared lift-out block (left panel). Right panels show the FIB and SEM view of prepared lift-out volumes after high-current milling with 2500 nA Xe. The numbers give the distance of the cut from the high-current trench. (C) Lift-out lamella in low-resolution TEM presenting curtaining, but no signs of devitrification (15 nA Xe, 20 µm slice). (D) Subtomogram average of yeast 80 S ribosome.

Keywords:

Serial-lift-out, plasma-focussed-ion-beam/scanning-electron-microscopy (PFIB/FIB/SEM), cryo-electron-tomography (cryo-ET)

Reference:

[1] Kelley, K., Raczkowski, A.M., Klykov, O. et al. Waffle Method: A general and flexible approach for improving throughput in FIB-milling. Nat Commun 13, 1857 (2022). https://doi.org/10.1038/s41467-022-29501-3

[2] Schaffer, M., Pfeffer, S., Mahamid, J. et al. A cryo-FIB lift-out technique enables molecular-resolution cryo-ET within native Caenorhabditis elegans tissue. Nat Methods 16, 757–762 (2019). https://doi.org/10.1038/s41592-019-0497-5

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[5] Schiøtz, O.H., Kaiser, C.J.O., Klumpe, S. et al. Serial Lift-Out: sampling the molecular anatomy of whole organisms. Nat Methods (2023). https://doi.org/10.1038/s41592-023-02113-5

770

An Improved Method for Growing Primary Neurons on Electron Microscopy Grids Co-Cultured with Astrocytes

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IM-11 (2), Lecture Theater 5, august 30, 2024, 10:30 - 12:30

With the increasing popularity of cryo-electron tomography (cryo-ET) in recent years, the quest to establish a method for growing primary neurons directly on electron microscopy grids (EM grids) has been ongoing. Here we describe a straightforward way to establish a mature neuronal network on EM grids, which includes formation of synaptic contacts. These synapses were thin enough to allow for direct visualization of small filaments such as SNARE proteins tethering the synaptic vesicle (SV) to the active zone plasma membrane on a Titan Krios without prior focused ion-beam milling.

Keywords:

primary neurons; astrocytes; cryo-EM; synapse

Reference:

Radecke, J., Seeger, R., Kádková, A., Laugks, U., Khosrozadeh, A., Goldie, K. N., Lučić, V., Sørensen, J. B., & Zuber, B. (2023). Morphofunctional changes at the active zone during synaptic vesicle exocytosis. EMBO Reports, 24(5). https://doi.org/10.15252/EMBR.202255719

Kumar, I.; Paudyal, A.; Kádková, A.; Stewart, M.; Sørensen, J.B.; Radecke, J. An Improved Method for Growing Primary Neurons on Electron Microscopy Grids Co-Cultured with Astrocytes. Int. J. Mol. Sci. 2023, 24, 15191. https://doi.org/10.3390/ijms242015191

25

The Electron Bio-Imaging Centre (eBIC) at Diamond Light Source, UK

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Poster Group 2

Three-dimensional electron microscopy (3D EM) includes multiple techniques for studying large macromolecular assemblies and cellular machinery. 3D EM provides the ideal tools for discovering the structures of individual proteins and small complexes in vitro, as well as understanding their function in a larger cellular context in situ. State-of-the-art electron microscopy is often too expensive and technically demanding for individual labs to house and operate. For this reason, the Electron Bio-Imaging Centre (eBIC) was established as the UK's national centre for cryo-electron microscopy (cryoEM) following the award of a £15.6 million grant from the Wellcome Trust, the Medical Research Council (MRC) and the Biotechnology and Biological Sciences Research Council (BBSRC).

eBIC's vision and mission:
- Establish state-of-the-art facilities for cryo-electron microscopy (cryoEM) and cryo-electron tomography (cryoET)
- Provide 24/7 free user access to high-end microscopes through peer-review process
- Develop cutting-edge technology with in-house research program
- Grow and train cryo-EM user community
- Foster integrated structural biology within Diamond and beyond

eBIC is a CryoEM center providing scientists with state-of-the-art experimental equipment and expertise in the field of cryo-electron microscopy, for single particle analysis, electron tomography and electron diffraction. The location of eBIC enables scientists to combine their techniques with many of the other cutting-edge approaches that Diamond offers.

Currently eBIC houses five Titan Krios microscopes, a Talos Arctica, two Glacios, a Scios and an Aquilos cryo-FIB/SEM, and a Leica CryoCLEM.

Keywords:

cryo-EM, cryo-ET, micro-ED, community, training

219

Thermo Scientific Smart EPU Software: Towards autonomous screening

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Poster Group 2

Background incl. aims
Over the years, the Cryo-EM community has anticipated the achievement of software automation that would simplify the Single Particle Analysis (SPA) data acquisition workflow without requiring any user setup. This advancement would enable researchers, regardless of their expertise in applications or microscopy, to effectively utilize high throughput cryo-electron microscopes, particularly in industrial settings or entry-level laboratories where efficiency and user-friendliness are crucial barriers to adoption. Notably, one of the primary challenges in SPA is the automation of screening. Thermo ScientificTM Smart EPU Software currently offers a workflow that enables automated screening of multiple samples. Here, we are expanding the capability of Smart EPU to make SPA screening a fully unattended and robust process.

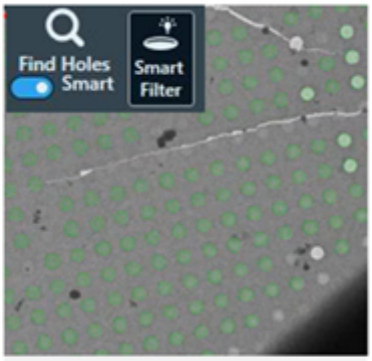
Methods
By utilizing EPU, EPU Multigrid, EPU Quality Monitor (EQM), Embedded CryoSPARC Live, a novel set of AI/DL algorithms, and CryoFlow we can now demonstrate a workflow that allows users to screen an entire cassette of samples on an autoloader system without any manual interventions.
To achieve such a prominent level of automation, various aspects of the workflow have been optimized. User workflows for screening often involve acquiring and analyzing small datasets from multiple grids under different conditions to identify optimal ice thickness and particle behavior. These tasks are laborious, as users must load each grid, select grid squares with varying ice thickness, identify foil holes, select foil holes with varying ice thickness, curate initial selections, and perform data analysis on acquired high-resolution images to determine favorable conditions for further collection of large datasets. Smart EPU presents a set of innovative AI/DL plugins that automate those steps that hitherto needed manual actions. To increase robustness, we also introduced two new set of features: one to access foil hole selection by ice thickness deterministically, using plasmon imaging, which is particularly useful when working with gold grids; secondly, when the image analysis of a screening session reveals a particle preferential orientation issue, Smart EPU fully supports the session setup for automated acquisition on tilted specimens.

Results
Experimental results indicate that the autonomous algorithms perform comparably to an experienced user with a significant gain of time. Furthermore, we combine these innovations with a redesigned user interface that allows users to initiate the setup process with just one click. This screening solution is connected to EQM and Embedded CryoSPARC Live, enabling real-time acquisition adjustments and image analysis.

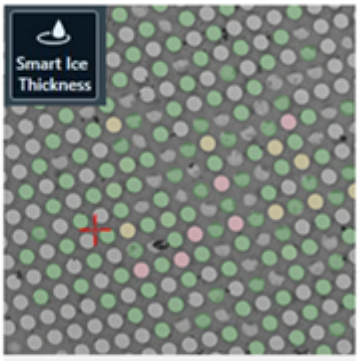
Conclusion
With these advancements, users will be able to load their samples, quickly initiate the screening process on site, and visualize remotely the results on the web portal of our data integration platform, CryoFlow, while still acquiring or after completion of the screening session. This new strategy allows

users an easier and efficient process for a more comprehensive understanding of their samples and better decision making about further data collection.

Smart Selection of Grid Squares



Smart Hole Finder Smart Filter



Smart Ice Thickness Prediction

Keywords:

acquisition software; automation; AI methods

Reference:

1. Wu, Mengyu, and Gabriel C. Lander. Biophysical journal 119.7 (2020): 1281-1289. ttps://doi.org/10.1016/j.bpj.2020.08.027
2. Bepler, Tristan, et al. Journal of Structural Biology 214.4 (2022): 107913. https://doi.org/10.1016/j.jsb.2022.107913
3. Egri, Shawn B., et al. Nature Communications 14.1 (2023): 2527. https://doi.org/10.1038/s41467-023-38251-9
4. Hagen, Wim JH. bioRxiv (2022): 2022-04. https://doi.org/10.1101/2022.04.27.489675

301

Sub 3Å cryoEM structure using a standard LaB6 120kV TEM upgraded with direct electron detector

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[1]Ramaciotti Centre for Cryo-EM, Monash University, Melbourne, Australia, [2]Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Australia, [3]Monash Institute for Pharmaceutical Science, Monash University, Melbourne, Australia

Poster Group 2

Background incl. aims
Cryo electron microscopy (Cryo-EM) single particle analysis (SPA) has become a major structural biology technique in recent years. High-resolution cryo-EM structures typically require higher voltage cryo-TEMs with coherent FEG sources, stable columns, autoloader systems and direct electron detectors. These setups are specialised for Cryo-EM work and expensive to establish and maintain. More recently the concept of using 100keV cryo-TEMs has been introduced as a way to make cryoEM more affordable and hence accessible to a larger group of researchers. However, so far, the implementation of these 100keV have relied on specialised cryo-TEMs with FEG sources and better and more stable optics than usually present in common 120keV TEMs.

Methods
We here explored whether a standard 120keV TEM, commonly available at many places worldwide, can be upgraded with a direct electron detector and used for higher resolution cryo-EM. Our setup consisted of a TFS Tecnai Spirit G2 with lanthanum hexaboride (LaB6) thermionic electron emitter and with a Gatan 626 cryo-transfer holder. The microscope was upgraded with a Gatan Alpine direct detection camera optimised for 100 to 200keV. Automated data collection was set up using Serial EM.

Results
Using this imaging configuration, we were successful in achieving a 2.7Å reconstruction for the cryo-EM standard apoferritin. We were also able to resolve a more challenging small 64kDa protein haemoglobin to 4.5Å. Furthermore, we solved an asymmetric membrane protein, 160kDa GPCR (M4 muscarinic acid receptor) to a resolution of 4.5Å. Importantly, all the results were achieved using limited datasets ranging from 700-1500 movies, making it feasible to collect these datasets with a side entry cryo-holder.

Conclusion
These results showcase a potentially widely accessible microscope configuration to obtaining interpretable cryo-EM structures. However, we suggest that the true value of using standard 120keV TEMs is in the potential for many EM facilities and laboratories to setup high quality cryo-EM SPA sample screening without the need to procure specialised Cryo-TEMs. This could hence help to considerably lower the entry barrier for cryo-EM SPA and contribute to the “democratisation” of Cryo-EM.

Keywords:

democratisation, structure, GPCR, screening, optimisation

571

Glacios 2 Cryo-TEM and Smart EPU Software streamline Cryo-EM for drug design with higher throughput

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[1]Thermo Fisher Scientific, Materials & Structural Analysis, Eindhoven, The Netherlands, [2]The Institute of Cancer Research, Chester Beatty Laboratories, London, UK, [3]Thermo Fisher Scientific, Materials & Structural Analysis, Dreieich, Germany, [4]Thermo Fisher Scientific, Materials & Structural Analysis, Hillsboro, USA

Poster Group 2

Background incl. aims

Cryo-EM has significantly impacted the field of structural biology due to its capabilities to resolve the three-dimensional structure of proteins, protein complexes and other biological macromolecules at high or even atomic resolution. However, acquiring high-quality data still largely depends on the expertise of the microscope operator. This limits the speed of adoption as researchers must invest considerable time into understanding microscopy and the technicalities of the workflow. Here, we show how the Thermo Scientific™ Glacios 2™ cryo transmission electron microscope (Cryo-TEM) with low-energy-spread Cold Field Emission Gun (E-CFEG) and combined with Thermo Scientific™ Smart EPU Software enables users of all expertise levels to acquire high-quality cryo-EM data.

Methods
In collaboration with the Greber lab (Institute for Cancer Research), several high-resolution structures of the 85 kDa Human CDK-activating kinase (CAK) were determined. CAK is a master regulator of cell growth and division and is a promising target for cancer therapeutics.

Results
Structures of CAK were rapidly determine in free and nucleotide-bound states as well as in complex with 14 inhibitors(1). In addition to achieving high-resolution structures from large datasets, ~4 Å and ~3 Å-resolution structures of ligand-bound complexes were determined using from only 1 hour and 4 hours of data collection respectively. Furthermore, in combination with an E-CFEG we have been able to generate a 1.5 A reconstruction of Apoferritin; the highest resolution 200 kV structure to date.

Conclusion
These results show the use of cryo-EM to enable structure-based drug design.

Keywords:

Cryo-EM; Cold FEG; automation; throughput

Reference:

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624

Cryo lift-out technique to study host-pathogen interaction on cell monolayer

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[1]Institiut Pasteur, Paris, France

Poster Group 2

Cryo Lift-out Technique to Study Host-Pathogen Interaction on Cell Monolayer
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1Pathogenesis of Vascular Infections Unit, Inserm U1225, Institut Pasteur, Paris, France.

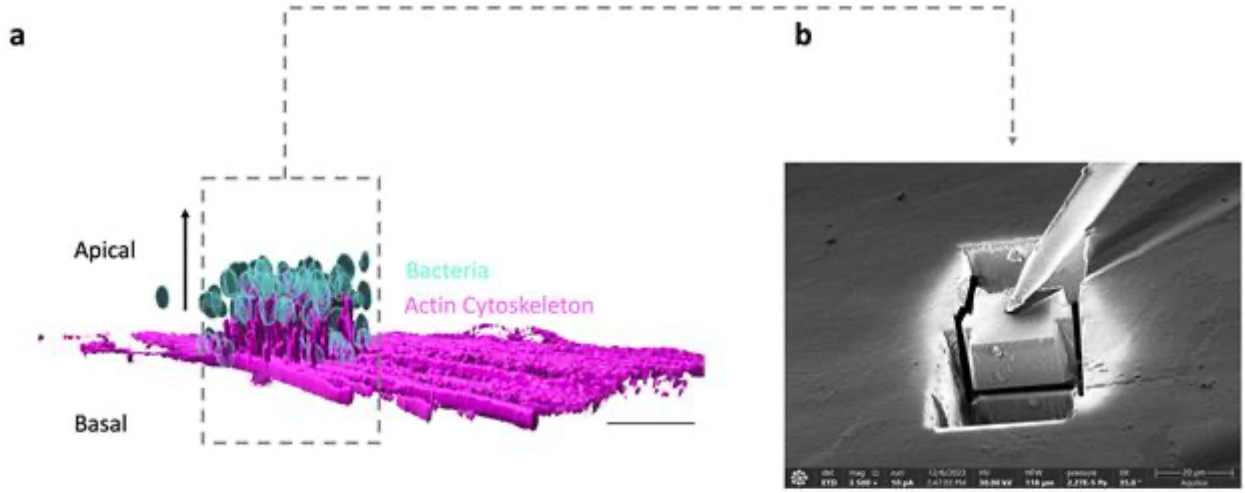
Background:
Cryo lift-out technique is an advanced sample preparation technique for cryo-TEM microscopy. Here, a bulk from the sample surface is lifted out in a vertical orientation using a cryo-needle and thinned using FIB milling, allowing the study of tissue or thicker samples in TEM. In addition to that, the recent development of serial lift-out technique would allow us to understand the information along the volume of the sample. However, in cell biology researc, traditional cryo-FIB that yields horizontal lamella is used, which poses a bottleneck to study the vertical structures in the cells (missing wedge issue). Lift-out is not possible on cell monolayers because of the smaller thickness of the samples. Therefore, we developed a sample preparation technique using hydrogels to grow cell monolayers, followed by high-pressure freezing (HPF) and then lift-out to study the vertical (apico-basal oriented) structures in cells.

Methods:
Optimized hydrogel preparation is vital for performing lift-out on cell monolayer. Here, we used acrylamide gels that have better water content which suits cryo-microscopy. HUVEC cells are used as a model because of our interest in studying a novel (unpublished) actin cytoskeleton structure that is formed from the apical side of the cell to the basal (through the cell volume), due to an extracellular bacterial infection. The hydrogel-Cell-Bacteria sample is frozen using HPF technique, and the lift-out and FIB thinning were performed on Aquilos-2 FIB SEM. Tomograms were acquired using Glacious or Titan TEM.

Results:
Hydrogel preparation, including parameters of polymer concentration, height, and width of the gels, is optimized. The lift-out, which is a completely manual process, has been semi-automated with the help of the IFLM module to perform correlation microscopy using the Maps software. At present, we are replacing the half-moon grids with a rectangular track to perform serial lift-out with the cell samples. The ongoing efforts are to obtain the region of interest with the help of Cryo-CLEM and IFLM techniques to build tomograms.

Conclusion:
Cryo-EM's contribution to cell biology research provides a huge potential to understand the proteins' structures, arrangements, and their microenvironment in their native state. On the contrary, apico-basal polarity and corresponding structures are common in cell biology. For instance, structures such as cell-cell junctions, intestine villus, pedestals, podosomes have specific functions and unique orientation. To study these structures at their native orientation at TEM is physically not possible, due to its alignment along the beam path and missing wedge issue. Therefore, we have developed a

sample preparation technique that would allow the researchers to use cell monolayers to perform lift-out techniques to study the vertically aligned structure in a horizontal lamella.



a. Imaris reconstriction of bacterial infection on HUVEC cell

b. Cryo needle attached to the apical side of the infected cell and ready to lift-out

Keywords:

Cell monolayer, Hydrogel, HPF, Lift-out

Reference:

Reference:

- Schiøtz, Oda Helene, et al. "Serial Lift-Out: sampling the molecular anatomy of whole organisms." Nature Methods (2023): 1-9.
- Schaffer, Miroslava, et al. "A cryo-FIB lift-out technique enables molecular-resolution cryo-ET within native Caenorhabditis elegans tissue." Nature methods 16.8 (2019): 757-762.
- Parmenter, Christopher David, and Zubair Ahmed Nizamudeen. "Cryo-FIB-lift-out: practically impossible to practical reality." Journal of Microscopy 281.2 (2021): 157-174.
- Wagner, Felix R., et al. "Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomography." Nature protocols 15.6 (2020): 2041-2070.

773

Cryo-EM and ED are driving structural studies at the University of Warsaw

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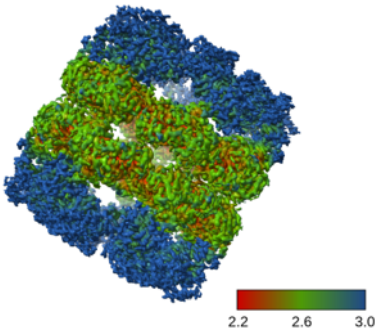
[1]Cryomicroscopy and Electron Diffraction Core Facility,Centre of New Technologies, University of Warsaw, Warsaw, Poland, [2]Biological and Chemical Sciences Research Centre, University of Warsaw, Warsaw, Poland, [3]Department of Chemistry, University of Warsaw, Warsaw, Poland

Poster Group 2

Background incl. aims
In 2019 the University of Warsaw purchased and installed one of the first cryo-EM microscopes in the country - the 200kV Glacios equipped with a Falcon3EC camera and a phase plate solution. In the next few years the Cryomicroscopy and Electron Diffraction Core Facility has been established and started providing many local structural biologists and chemists with a direct access to this groundbreaking and Noble-winning cryo-EM technology. To date, there have been only two cryo-EM Core Facilities operating in Poland which provide services in all cryo-EM modalities.
Methods
This poster shows the current possibilities of our Core Facility and a range of services which are offered to our users. We follow the open-access policy and welcome users from both national and international academic institutions as well as industry.
Results
Our recent developments include benchmarking the Single Particle Analysis (SPA) reconstruction of GroEL with GroTAC peptide at the 2.45Å resolution level with local resolution reaching 2.2Å (PDB: 8S32) and the 2.27Å reconstruction of the AbiK bacterial polymerase (PDB:7R06). The results are further enhanced with the upgrade of the microscope to the micro-ED functionality allowing for a rapid structure determination of small molecules and/or proteins based on electron diffraction data. On top of that, we have recently completed a process of building up a €0.5M-worth IT infrastructure support for the cryo-EM data storage (up to 1.6PB dedicated storage space) and expanding on the data processing capabilities (utilising our 200 GPU-based computer cluster) which will be a unique set-up of its kind not only in Poland but also in Central Europe.
Conclusion
We highlight the importance of smaller cryo-EM Core Facilities such as ours to serve as a first point of contact for users, in particular for those who are new to the cryo-EM field and would like to explore different possibilities of getting high quality data prior to applying for the measurement time on high-end 300kV microscopes. We also show that being able to offer all cryo-EM modalities in one instrument (SPA, cryo-ET and micro-ED) significantly boosts a research potential and opens up new possibilities across many Life Science applications.



Keywords:

cryo-EM, SPA, micro-ED, cryo-ET

Reference:

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884

Quantitative Super-Resolution Methods for Cryo-CLEM

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[1]Central Laser Facility, Didcot, United Kingdom, [2]School of Biosciences, University of Kent, Canterbury, United Kingdom, [3]Astbury Centre for Structural Molecular Biology, School of Molecular and Cellular Biology, Faculty of Biological Science, University of Leeds, Leeds, United Kingdom

Poster Group 2

Background
Cryogenic ultrastructural imaging techniques such as cryo-electron tomography have produced a revolution in how the structures of biological systems are investigated. This has been done by enabling the determination of structures of protein complexes immersed in a complex biological matrix within vitrified cells and model organisms. However, so far successes have mostly been limited to highly abundant complexes or to structures that are relatively unambiguous and easy to identify through electron microscopy.

To realize the full potential of this revolution, we need to be able to pinpoint lower abundance species and obtain functional annotations on the state of objects of interest. This would then be correlated to ultrastructural information to build a complete picture of the structure-function relationships underpinning biological processes. Fluorescence imaging at cryogenic conditions has the potential to meet these demands. However, wide-field images acquired at low numeric aperture (NA) using an air immersion objective have a low resolving power and cannot provide accurate enough three-dimensional (3D) localization to enable the assignment of functional annotations to individual objects of interest or target sample debulking to ensure the preservation of the structures of interest. It is therefore necessary to develop super-resolved cryo-fluorescence workflows capable of fulfilling this role and enabling new biological discoveries.

Here, we will present the current state of our development of super-resolution solid immersion lens stochastic optical reconstruction microscopy (superSIL-STORM) [1]. The superSIL lens can go to larger magnifications than regular air objective lenses, with the advantage over traditional oil immersion lenses that they are cryo compatible. We will show the application of superSIL-STORM to various protein clusters in E. coli cells and discuss the advantages and limitations of the technique. We will then further discuss its future applicability to cryogenic correlative light and electron microscopy (cryo-CLEM) workflows.

Methods
Our SIL assemblies were made in-house using nanolenses (Hyper Hemispheric Ball lens, Cubic Zirconia, 1.0 mm diameter x 0.73 mm thickness, λ\4 flatness - Knight Optical, LBB2018-C), stainless steel foil (FE22-FL-000120) and cryo-compatible adhesive (Loctite Stycast, 2850 FT) [1,2].

We used 100 nm TetraSpeckTM microspheres as fiducial markers. For plunge-freezing of the samples we used an FEI Vitrobot Mark V, using manual blotting. SuperSIL assemblies were glow discharged before use. A 1% PEI solution was applied to the substrates before adding the cells and then the fiducial markers.

For fluorescence imaging an in-house built super-resolution cryo-STORM setup was used, as described in [1]. STORM analysis was conducted using standard STORM software: the preinstalled

ImageJ plugin for ThunderSTORM, the source code from https://github.com/ZhuangLab/storm-analysis for DAOSTORM. For ThunderSTORM, an initial Lowered Gaussian filter was applied before a maximum likelihood Gaussian fit searched for the single molecule PSFs. For DAOSTORM the value for "model" was set to "2D". Drift correction of the sample was done through in-house constructed algorithms [1].

Results

To demonstrate the capabilities and limitations of the superSIL-STORM technique, we study two different protein clusters in E. coli cells.

We assess the ability of single-colour superSIL-STORM to analyse protein clustering in VNp-LZ-mNeonGreen expressing E. coli. VNp-LZ is a short recombinant peptide which is introduced in bacteria to favour the formation of internal vesicles and has been shown by EM to cluster around them. Our superSIL-STORM analysis reveals clustering of the protein around empty spaces within the cells, which are likely to correspond to the internal vesicles shown previously [3]. Through further data analysis, we estimate most of the protein clusters in 100 nm large patches, with an overall distribution of nearest neighbours ranging between 25 nm - 200 nm. Future studies combining these results with EM work for cryo-CLEM will help to reveal the nature of these clusters.

Next, we assess proteins in E. coli cells that are involved in the outer membrane protein (OMP) secretion pathway: the BAM complex. Various mechanisms for how the BAM complex translocates proteins across the outer membrane have been suggested before, but more information is needed for a complete understanding of how this is done. We look at clustering of the major BAM complex subunit BamA with either the periplasmic chaperone SurA or the client OmpA. Overall, we find strong co-clustering of BamA with OmpA, but not of BamA with SurA. The first indicates an agreement with previous findings that OMPs are turned over in patches, away from the original OMP insertion sites. The latter indicates that the co-location of BamA with the chaperone is transient in nature and may depend on a variety of factors, including the metabolic state of the imaged cell.

Conclusions

We have developed workflows for the use of superSIL-STORM. We present here the basic principles of this technique and we show its application to study membrane protein complexes in E. coli. Further work is taking place to integrate this technique in cryo-CLEM workflows.

Keywords:

STORM, superSIL, membrane proteins, cryo-CLEM

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930

VitroJet: ice thickness control and measurement for time-efficient single particle structure determination3052

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Poster Group 2

Background incl. aims
Single particle cryo-electron microscopy (cryo-EM has emerged as a prevalent technique for structural biology due to its capacity to visualize of individual macromolecules, which is applied across a variety of diverse projects. However, the current practice of thickness screening and hole selection for data acquisition within the electron microscope consumes significant beam time. We aim to control and measure ice thickness during sample preparation, to predictably achieve high resolution.
Methods
The VitroJet is utilized for cryo-EM sample preparation, leveraging its automation to enhance productivity and increase accessibility for novice users. Pin-print sample deposition and jet vitrification combined provide a reproducible framework for generating high-quality grids.
Results
Multiple case studies employing the VitroJet showcase its benefits across various sample categories: membrane proteins, nucleosomes, fatty acid synthase, tick-borne virus, lipid nanoparticles, tobacco mosaic virus, and bacteriophages. By adjusting pin-printing velocity and standoff distance from pin to grid, we effectively regulated the median ice thickness, achieving a standard deviation below ± 11 nm for thicknesses up to 75 nm. The integrated optical camera enables precise measurement of ice thickness in individual holes with an accuracy below ± 20 nm within the 0 - 70 nm range. Data collection at 30 nm to 70 nm ice confirms the importance of ice thickness for time-efficient structure determination, with 3.7 times fewer particles needed to attain a resolution 3 Å.
Conclusion
Users in several labs worldwide have validated the versatility of the VitroJet across various sample types and workflows. The capability to control and measure ice thickness empowers users to optimize layer thickness on-the-fly during grid preparation, without the need of cryo-EM. Furthermore, the estimation of ice thickness facilitates targeted selection of holes for data collection based on optical imaging. This underscores how the VitroJet accelerates the entire workflow and enhances the efficiency of utilizing microscope infrastructure for data collection.

Keywords:

Sample preparation, reproducibility, productivity, versatility

Reference:

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1262

Sub-4-Å cryo-EM structure of apoferritin from a basic 120 keV TEM with thermionic electron gun

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Poster Group 1

Background incl. aims: Cryogenic electron microscopy (cryo-EM) has developed into one of the most powerful techniques in structural biology since direct electron detectors (DEDs) became available some 10 years ago. High resolution cryo-EM structures are usually solved with high-end microscopes (e.g. the Thermo Fisher Titan Krios) or well-equipped medium range microscopes (e.g. Thermo Fisher Galcios). These microscopes operate at 200 kV or 300 kV and are equipped with field emission guns (FEGs), DEDs and often with energy filters. Here we analyzed the performance of a current entry-level transmission electron microscope (TEM) with an up-to-date scintillator camera.
Methods: We used a Thermo Fisher Talos L120C with a Gatan 698 Elsa cryo-transfer holder to solve the structure of mouse heavy chain apoferritin (mFTH1) by single particle cryo-EM. This microscope is equipped with a thermionic LaB6 electron source and a Thermo Fisher CETA-F scintillator camera. It does not have an energy filter and is usually used for conventional EM, negative staining EM and only for very basic screening of cryogenic samples.
Results: Here we present a 3.8 Å structure (FSC = 0.143) of apoferritin solved by single particle cryo-EM using a Thermo Fisher Talos L120C. At this resolution the protein backbone is clearly visible and most amino acid side chains produce recognizable densities. To our knowledge this is by far the highest resolution for a single particle cryo-EM structure solved with an entry-level 120 kV TEM. We claim that the decent detector quantum efficiency (DQE) and fast readout speed of the CETA-F camera contributed to achieving high resolution.
Conclusions: A resolution below 4 Å for single particle reconstructions was first achieved about 15 years ago and this resolution range was reserved for high-end and medium range microscopes until now. Our results show that a modern compact and affordable electron microscope can reach near-atomic resolutions in single particle cryo-EM and can be used for much more than analyzing particle distribution and ice quality.

Keywords:

SPA, cryo-EM, structural biology

1280

Cryo-EM structure of wild-type Orsay virus: preliminary insights into the assembly mechanism

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Poster Group 1

Background
The Orsay virus (OrV) is a non-enveloped, icosahedral virus with a bipartite, positive-sense, single-stranded RNA genome (RNA1 and RNA2). It naturally infects the nematode Caenorhabditis elegans. Previous crystallographic studies have resolved the structure of an OrV-like particle (360 Å in diameter) at a resolution of 3.2 Å [1]. One hundred-eighty copies of the capsid protein (CP) assemble the icosahedral proteinaceous shell in a T = 3 lattice. Additionally, other studies have demonstrated the presence of a pentameric fiber, which may facilitate viral attachment and infection of the host cell [2-3]. Here we aim to elucidate the structure of the wild-type virus and to provide an understanding of its assembly mechanism and role played by the genome in this process.
Methods
Wild-type viruses obtained from the infection of C. elegans were purified, vitrified, and subsequently analyzed by cryo-EM using high-resolution microscopes. RELION, Chimerax, Phenix, and Coot software were employed for map analysis and localized reconstruction technique to study the structure of the fiber and the CP-genome interactions.
Results and Conclusion
We have determined the structure of the wild-type OrV virion by cryogenic electron microscopy to 2.5 Å resolution. The reconstructed 3D electron density, in addition to showing an ordered icosahedral capsid, unravels a previously unreported defined density network beneath the viral capsid that may be related to the interaction between the CP and the bi-partite RNA genome. Our efforts are currently focused on improving the resolution and implementing symmetry relaxation techniques, as well as biochemical techniques that will allow us to structurally elucidate possible sites of interactions between the bipartite genome and the CP and its evolutionary relationship with other RNA viruses. Additionally, we are studying the structure of a pentameric virion-associated fiber and its potential role in host cells infection.

Keywords:

Virus, RNA, Cryo-EM, Capsid Protein

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1294

Cryo-Volume Electron Microscopy characterization of chlorophyll deficient microalgae

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Poster Group 1

Microalgae are a novel, valuable food source with multiple benefits including high nutritional value, and potentially with a more sustainable production compared to conventional food protein sources such as soybean, corn and meat. Nevertheless, consumer studies reveal that when applied in food the inclusion levels must be kept low, mainly due to the intense green colour of the algae and taste. By using UV-radiation combined with a visual screening process, we have obtained chlorophyll deficient mutants of the two microalgae species: Chlorella vulgaris and Nannochloropsis oceanica.

Characterization and comparison of the algal mutants and wild types with respect to growth kinetics and biomass composition is ongoing. However, light microscopy indicates that the morphology of the pale mutants is very different from the wild types. Therefore, Focused Ion-Beam Scanning Electron Microscopy (FIBSEM) has been applied to describe these morphological differences and to characterize the algal mutants at the ultrastructural level. The FIBSEM is well suited for site-specific sectioning where the FIB milling exposes a surface that can be visualized either with FIB or SEM to examine the microstructure within each layer and the interfacial structure between layers. By collecting ultrastructural data from a large sample volume and in turn interrogated in silico; novel understandings of the interconnectivity between organelle through an entire cell can be deduced. To date, this has only been successfully applied to chemically processed tissue, with their associated artefacts e.g., significant shrinkage, loss of intracellular soluble content and disruption to membrane structure. Non-chemical fixation using low temperature allows cells and tissues to be studied close to their native biological state. This is a major advantage for the characterization of poorly understood ultrastructural changes such as in the novel microalgae mutants generated in our study. To elucidate and compare the cell morphology of chlorophyll deficient mutants and wild type strains of the single celled microalgae Chlorella vulgaris and Nannochloropsis oceanica we have applied cryo electron tomography by controlled milling of vitrified cells using focussed ion beam milling FIBSEM microscopy at cryogenic temperatures (cryoFIBSEM).

By using a novel cryo vEM (cvEM) approach to generate larger 3D volumes than possible with cryoET through high-resolution sectioning in a cryoFIBSEM, we have been able to observe the microalgae strains close to their native biological state. By successfully overcoming the beam sensitivity of the vitrified samples and avoiding heavy metal contrast, as well as addressing significant challenges in generating sufficient signal-to-noise ratio, we have been able to define the ultrastructural details of the algae. This new approach has revealed significant differences in the ultrastructure between the mutants and the wild types including deformation of the thylakoids and reduced volume of the chloroplast.

Keywords:

Microalgae, volume electron microscopy, FIBSEM-cryo