Invited Speaker

1181 Integrated structural cell biology of pathogen-host interactions
Prof Kay Grünewald

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[1]Monash Ramaciotti Centre for Cryo EM, Clayton, Austalia

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[1]Thermo Fisher Scientific, Brno, Czech Republic
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1155 Structural investigation of the 40S hnRNP particles

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1157 Xenon PFIB-milling and cryo-electron tomography of bacterial anti-phage effectors

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1160 Visualization of the in situ molecular architecture of tau pathology in the murine brain

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Mr Hans Radhoe[1], Dr Sina Sadighikia[1]
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1312 In situ cryo electron tomography enables localization-dependent structural studies: Finding symmetric nuclear pore complexes

Jenny Sachweh[1,2], Bernhard Hampoelz[1], Sven Klumpe[3], Florian Wilfling[1], Martin Beck[1,2]
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1323 Structural biology analyzes of photoreceptor outer segment: a closer look at PDE6

Dr Vineeta Kaushik[1], MSc Luca Gessa[1], Dr Sławomir Tomczewski[1,2], MSc Łukasz Olejnik[1], MSc Nelam Kumar[1], Dr Humberto Fernandes[1,2]
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1326 Charting the molecular landscape across layers of vitrified mammalian hippocampus using electron cryotomographyaa

Mr Jake Smith[1], Dr Calina Glynn[2], Dr Matthew Case[2], Miss Rebecca Csöndör[2], Miss Maria Sanita[2], Dr Thomas Glen[2], Dr Michael Grange[1,2]
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Integrated structural cell biology of pathogen-host interactions

Prof Kay Grünewald

LS-08, Lecture Theater 4, august 28, 2024, 10:30 - 23:30

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Enabling discovery with in-cell Cryo-ET & AlphaFold: Identification of a non-canonical translocation complex

Rasmus Kjeldsen Jensen[1], Liang Xue[1], Federico Marotta[1], Joseph Chris Somody[1], Joel Selkrig[2], Swantje Lenz[3], Nassos Typas[2], Maria Zimmermann-Kogadeeva[2], Juri Rappsilber[3], Peer Bork[1], Julia Mahamid[1]
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LS-08, Lecture Theater 4, august 28, 2024, 10:30 - 23:30

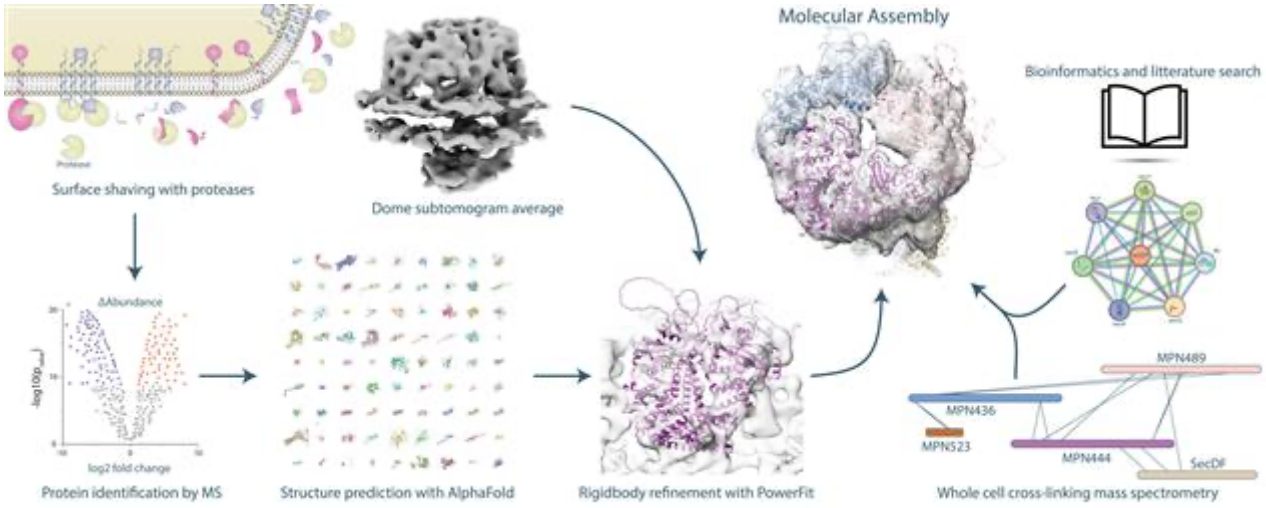
Background incl. aims.
Mycoplasma pneumoniae is a genome-reduced pathogenic bacterium that is the causative agent of a number of respiratory diseases and an established prokaryotic minimal cell model. While the drastic gnome reduction that Mycoplamsa pneumoniae underwent results in only about 500 proteins being expressed, approximately 20% of its genes remain completely uncharacterized. Here, I present our developed approaches aimed at advancing functional identification of previously uncharacterized proteins by in-cell structural biology.

Methods.
Mycoplasma pneumoniae was grown on cryo electron microscopy grids, frozen in vitrious ice, and tilt series was collected on the electron microscope. Particles were first segmented manually in the tomograms, where after deep neural network models were trained to further pick particles. The final cryo electron tomography map was generated by subtomogram averaging. Structures of potential candidates for the proteins were predicted using AlphaFold and rigid body refined against the map. Further proteins were identified using proteomics including, surface shaving mass spectrometry and in-cell cross-linking mass spectrometry. Structural and sequence homology searches were performed to identify functional domains in the uncharacterized proteins.

Results.
The small size of Mycoplasma pneumoniae allowed us to collect over 600, high-resolution cryo-electron tomography datasets without thinning the sample, thereby setting the stage for visual proteomics of an entire cell. In the resultant tomograms, we observed an abundant integral membrane protein complex with a large extracellular dome-like structure. We resolved the protein complex to sub-nanometer resolution by subtomogram averaging. To identify the components constituting the complex, we generated a candidate list of cell surface proteins by enzymatic surface shaving coupled to mass spectrometry, and predicted their structures using AlphaFold. The predicted structures were systematically fitted into the subtomogram averaged map, enabling us to identify three homologous lipoproteins constituting the major part of the extracellular dome. Whole-cell cross-linking mass spectrometry revealed that the three proteins form a hetero-trimeric complex. The cross-linking data also showed that an additional lipoprotein, as well as the integral membrane protein SecDF, are also present in the complex. SecDF is required for efficient translocation by the Sec translocation machinery in other bacteria. Fitting the AlphaFold models of the Sec translocon proteins secA and secYEG into the STA showed that the remaining Sec components are also present in the dome-like structure, and provide a first structural model of the conserved bacterial translocation machinery. The three homologous extracellular lipoproteins are uncharacterized, with yet unknown functions, but are all essential for Mycoplasma pneumoniae. Structural homology searches revealed that the extracellular proteins contain a prolyl isomerase domain found in

periplasmic and extracellular chaperones in other bacteria. Furthermore, only one of the three proteins retains the conserved residues important for substrate binding and catalysis. This is also the component that exhibits a prominent contact with a heterogenous substrate density observed inside the extracellular dome. Classification of the particles showed that a sub-population of the complex interacts with the membrane-associated ribosomes. The fraction of ribosome associated complexes increased when Mp was exposed to antibiotics which halt protein production, indicating stabilization of an otherwise transient interaction. In the antibiotics-treated cells, we also observed a more open conformation of the complex corresponding to the resting state where no translocation occurs. This allowed us to describe the conformational changes that take place upon translocation, especially in secDF.

Conclusions.
Our data suggest that we have discovered a non-canonical sec-translocation complex involved in protein translocation and folding in Mycoplasma pneumoniae, and demonstrates how in cell cryo ET can contribute to de novo structure determination and functional assignment of previously uncharacterized proteins.



Keywords:

In-situ Structural-Biology, cryoEM, Microbiology, Proteomics

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Cryo-FIB milling and Cryo-electron tomography of tissue biopsies: BBQ method

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LS-08, Lecture Theater 4, august 28, 2024, 10:30 - 23:30

Cryo-electron tomography (CET) allows for unique insights into molecular structures in situ down to near atomic resolution. To access thicker cellular areas, thin samples of less than 250nm thickness are required and cryo-lamellae can be obtained by cryo-focused ion beam (cryo-FIB) milling. Specialized cells in tissue display unique molecular architectures that are not easily reproduced in isolated cell culture. While cryo-lamellae can be routinely prepared for cells in culture, there are only a few published examples of tissue CET due to the technical challenges associated with obtaining cryo-lamellae of tissue samples. To explore the full potential of CET for cell and developmental biology, better methods for cryo-lamellae preparation of tissues are required. Here we show a new workflow for cryo-FIB milling specifically for tissue samples. The workflow uses standard sample preparation equipment and on grid milling on a Gallium FIB and allows to routinely obtain cryo-lamellae on high-pressure frozen samples. The technique can be combined with automation and allows for the generation of tissue cryo-lamellae in a timely manner. Examples for several tissues obtained from mice and zebrafish are presented.

Keywords:

tomography, biopsy, cryo-ET, cryo-FIB, HPF

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Synthesis, Characterization, and Biological Evolution Indole Molecules

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Poster Group 2

Aims: Lung cancer is the most prevalent and mortal cancer in the world. The percentage of 85 of lung cancer incidence belongs to non-small cell lung cancer type (NSCLC). Therefore, it is crucial to develop reliable and efficient curing approach and therapeutics to treat this cancer type in order to suggest life prolonging and pain ameliorative option to the sufferers [1]. Heterocyclic compounds are within the carbon ring structure; contain hetero atoms such as Sulfur, Nitrogen, Oxygen, Selenium and Silicon into the ring. Hetero aromatic compounds form the basic structure of many drug molecules. Nitrogen-based heterocyclic compounds constitute an important and unique class among the application areas of chemistry and organic chemistry. Nitrogen-containing hetero aromatic molecules have contributed to the development of many organic synthesis protocols and different application areas in the last two decades. N-heterocyclic compounds found in nature, in addition to having physiological and pharmacological properties, constitute the basic building blocks of many biologically important molecules, including many vitamins, nucleic acids, pharmaceutical products, antibiotics, dyes and pesticides. In addition, N-heterocyclic compounds form an integral part of many pharmacologically active molecules, base pairs of DNA and RNA (guanine, cytosine, adenine and thymine) as well as purines, pyrimidines, etc. It consists of N-heterocyclic compounds such as. These nitrogen-containing heterocycle molecules with diverse properties and applications have gained importance in the field. Furthermore, electron-rich Nitrogen-containing heterocycle-containing organic molecules not only readily accept or donate a proton, but also have a variety of weak bond interactions; It can also easily establish some of the intermolecular forces such as hydrogen bond formation, dipole-dipole interactions, hydrophobic effects, van der Waals forces and π-stacking interactions of Nitrogen organic compounds [2-3].
Methods: In this work, New indole compounds were designed, which is crucial for synthetic organic chemistry and medicinal chemistry. Novel indole derivatives with NO2 have a great deal of potential for biological activity. Many pharmaceutical compounds with indole in them are on the market today. Thus, the synthesis and characterization of indole derivatives are critical to the identification of novel therapeutic compounds. The biological activity of the new synthesis molecules obtained in this study was obtained on lung cancer cells (A549) by MTT cytotoxicity test.
Results and Conclusions: The IC50 values obtained as a result of the Mtt test were found to be 4.62µm in the molecule coded EH-303 for 24 hours at the lowest concentration, while the lowest value for 48 hours was 8.17µm in the molecule coded EH-300.

Keywords:

Lung cancer, compounds, organic synthesis,

Reference:

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Arctis WebUI redefines lamella preparation for cryo-electron tomography workflow

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Poster Group 2

Background
Cryo-electron tomography has emerged as a valuable technique for visualizing proteins and protein complexes in their natural cellular environment. To achieve high-resolution imaging, the preparation of thin cryo-lamellae containing the region of interest is a critical step. However, the usual methods for lamella preparation are often time-consuming and require the use of several applications, presenting challenges for inexperienced users in the cryo-tomo workflow.

Methods
The Arctis cryo-Plasma Focused Ion Beam system plays a pivotal role in the automated production of cryo-lamellae from cells. The system is supported by the Arctis WebUI, with a web-based user interface that streamlines the creation of cryo-lamellae with minimal user inputs. WebUI is a unified platform where users can map and navigate through the sample using electron, ion, or optical tilesets. Additionally, the integration of a fluorescent light microscope enables in-situ targeting of specific cellular structures. Prepared lamellae are transferred to a Transmission Electron Microscope for cryo-electron tomography. The Thermo Fisher Scientific Selectris Imaging Filter is utilized to significantly enhance contrast during data acquisition through zero-loss filtering. Data collection is facilitated by Tomography 5 software, enabling unattended data acquisition, seamlessly connected with Tomo Live software, allowing automatic on-the-fly data reconstruction.

Results
We will provide a comprehensive overview of the application workflows for lamella preparation facilitated by the Arctis WebUI. The key advancement is the use of the fully automated optical tileset, which allows the selection of cells directly based on their fluorescently labelled features that are not visible through standard electron or ion imaging. With the combination of the 3D targeting solution, users gain valuable insights into the cellular content and can precisely identify target areas for lamella preparation. The automatic determination of ROI depth in the optical stack significantly accelerates and simplifies the 3D targeting process, eliminating the requirement for manual browsing through the entire optical stack. Once the lamellae are milled, they are transferred to a cryo-Transmission Electron Microscope through the common sample loading interface and utilized for cryo-electron tomography data collection.

Conclusion
With the capability to seamlessly process multiple (up to 12) grids with cells, Arctis WebUI streamlines the preparation of cryo-lamellae for tomography, enhancing efficiency and productivity in the field of cryo-electron microscopy. The integration of optical tileset and 3D targeting enables more precise lamella placement, guarantees data collection from specific sites in cryo-TEM, and ensures the presence of accurate structures in the resulting tomography data. These workflows significantly reduce the need for extensive user input, allowing researchers to concentrate on solving biological problems while the WebUI automates the sample preparation process for cryo-tomography.

Keywords:

Arctis WebUI, cryo-electron tomography, cryo-lamellae

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Beyond Ribosomes: In Situ Structural Biology of a Challenging Target in C. Reinhardtii

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Poster Group 2

Background
Understanding high-resolution protein structure in the context of the whole cellular environment is the vision of visual proteomics. With the advent of high-throughput cryo-FIB and cryo-electron tomography (CryoET), 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 ions, which reduce 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. Initial results demonstrate that the data is of sufficient quality to achieve sub-nanometer resolution (6Å) for the 80S ribosome using a fraction of the dataset (six tomograms). Together with collaborators, multiple proteins were selected as targets for sub-tomogram averaging (STA). One of these proteins required the development of a novel workflow. 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. The denoising neural network cryoCARE was implemented on all tomograms in the dataset to increase template matching accuracy. Unfortunately, in the case of PSII, denoising tended to blur or erase the small density that projects from the membrane which led to significant under picking when template matching was performed.
Methods
An alternative strategy that paired a regression denoising UNet with a semantic segmentation UNet was employed to generate candidate coordinates for further averaging and classification. Regression denoising: Synthetic training data for regression UNet was generated using CryoTomoSim. Using a box of 400x400x50 pixels, a mixture of medium and small proteins were modeled in four layers at 7.84 Å /pixel, exact protein identity is not important to training. 10 membrane vesicles were modeled. Iterations for each layer were 500, 500, 4000, 8000, and particle density was 0.8. Finally, the vitreous ice option was used. The output or ideal tomogram was simulated at -1 micron defocus, -89 to 89 tilt, 0.5 degree tilt increment and total dose = 0. The input or noisy tomogram was simulated at -3 micron defocus, -60 to 60 tilt, 3 degree tilt increment, total dose 80. Both datasets are loaded into Dragonfly 2022.2 (Object Research Systems) and used as training input and output for a regression 2.5D UNet with architecture: depth level 5, initial filter count 64, slice count 5, patch size 128, Loss function ORSMixedGradientLoss. Training proceeded for 46 epochs using a total of 15,840 patches. Segmentation: 2.5D Segmentation UNet for PSII was trained using Dragonfly 2022.2 according to protocol. In brief, Segmentation Wizard was used for manual annotation of 5-6 training slices and a generic UNet of the following architecture was trained: (2.5D: 3 slice, depth level: 5, initial filter count: 64, patch size: 128). Training data included slices from tomogram numbers: 24, 373, 473, and 900. Aside from patch size, all hyperparameters are left as default. Training labels were Membrane, ATP Synthase, Ribosomes, PSII and Background. Post segmentation, PSII class was

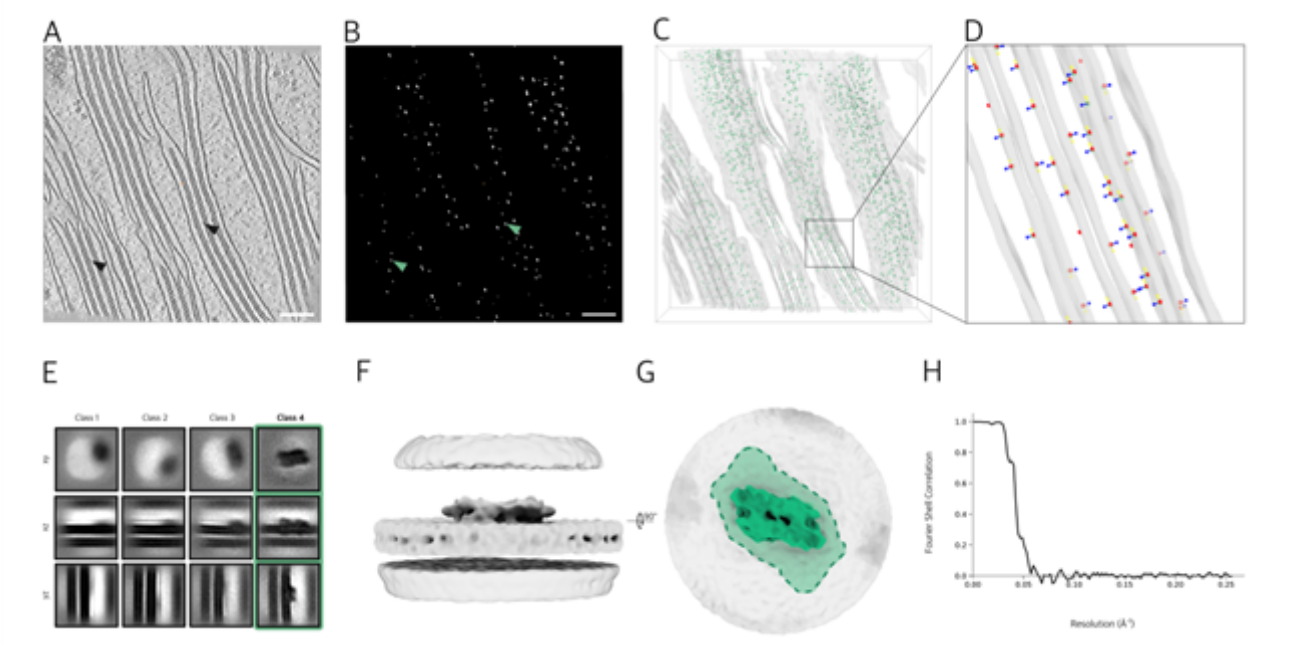
extracted for each tomogram, split into connected components, and center of mass X, Y, Z coordinates were calculated for each label and exported to CSV for subtomogram extraction. Once trained, both UNets are applied to 41 tomograms to generate 52,000 PSII candidates which can be extracted for the STA workflow.

Results

With the initial set of 52,000 candidate coordinates, one high-quality class emerged during classification and as of the writing of this abstract an 18Å structure has been achieved (Fig 1). Ongoing work is in progress to apply this workflow to an additional 200 tomograms. With more candidate coordinates, optimized alignment of sub-tomograms, and further post-processing, we are confident we can improve the resolution of the in situ structure of PSII.

Conclusion

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. Challenging targets such as PSII demonstrate the need for new, creative methods or combinations of techniques to facilitate in situ structural determination. 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 Seg) UNets. This project has the potential to provide invaluable insights into cellular processes and will hopefully lay the foundation for future large-scale studies of other species.



Keywords:

CryoET, Deep Learning, Visual Proteomics

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Benzofuran-Tetrahydrodipyrazolopyridine Hybrids: Novel Compounds for Potential Lung Cancer Treatment

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Poster Group 2

Aims: Benzofuran and tetrahydrodipyrazolopyridine are heterocyclic compounds with diverse biological activities. Benzofuran derivatives have potent anticancer, antiviral, and antimicrobial properties [1]. Tetrahydrodipyrazolopyridines are used in various pharmaceuticals and exhibit anticancer, antiallergic, and antiherpetic properties [2, 3]. The hybridization of these two molecular frameworks presents an innovative approach to crafting novel compounds with significant biological activity. Lung cancer is one of the most common types of cancer worldwide and is a serious health problem affecting both men and women. Although current lung cancer treatment methods are effective against certain types of the disease, many patients may develop resistance or develop side effects. Therefore, the discovery and development of new molecules that are more effective, have fewer side effects and can prevent the development of resistance is an important need in lung cancer treatment [4].
Methods: In this study, benzofuran-tetrahydrodipyrazolopyridine hybrids were successfully synthesized with high efficiency via a room-temperature condensation reaction. The synthesis involved the benzofuran derivatives with an aldehyde functional group, ethyl acetoacetate, hydrazine, and ammonium acetate. The biological activity of the new molecules synthesized within the scope of the study was obtained by MTT test on A549 lung adenocarcinoma cells.
Results and Conclusions: The resulting hybrids were characterized using proton (1H) and carbon (13C) nuclear magnetic resonance (NMR) spectroscopy, which confirmed their structure and purity. As a result of the Mtt test, the IC50 values for the molecule coded DB-1 were determined as 5.26µm for 24 hours at the lowest concentration, while the 24 hour value for the other molecule coded DB-2 was determined as 15.58µm.

Keywords:

Benzofuran, tetrahydrodipyrazolopyridine, lung cancer, hybrid

Reference:

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Structural Analysis of COPI Pathway in Chlamydomonas reinhardtii

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Poster Group 2

Recent advancements in FIB-milling throughput allow production of large tomography datasets from lamellae. Combined with improved processing of tomography data this has made it possible to produce sub-nanometer resolution for large protein complexes abundant in the cell. Despite these advances, in-lamella studies still mostly determine the consensus protein structure. In this study we aimed to correlate structural variability of the target protein with its in situ biological context to structurally describe a biological pathway. In particular we focused on the COPI pathway. Using a large tomography dataset from lamellae prepared by high-throughput plasma-FIB-milling we applied subtomogram averaging to determine the structure of the COPI complex. Next, we used image classification to analyze the biological cues involved in the pathway (e.g. binding of small proteins, cargo sorting, etc.). Further we correlated the results of image classification with different levels of biological context: from local lattice architecture to interactions at the organelle level, to describe different stages of COPI pathway. All in all, we showcase the potential of structural studies of biological pathways in situ.

Keywords:

cryoET, cryo-FIB-milling, COPI, membrane trafficking

1155

Structural investigation of the 40S hnRNP particles

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Poster Group 2

Heterogeneous nuclear ribonucleoproteins (hnRNPs) constitute a prominent family of RNA-binding proteins abundant within the nucleus. They play pivotal roles in various aspects of nucleic acid metabolism, encompassing mRNA stabilization, alternative splicing, transcriptional and translational regulation, RNA export, and degradation [1]. Early investigations in the 1970s revealed that upon lysing nuclei without RNase inhibitors, a significant portion of pre-mRNA formed a distinct protein-RNA complex, sedimenting at 40S [2]. Notably, the core constituents of this complex were identified as hnRNP C1/C2, hnRNP A1/B2, and hnRNP A2/B1. This observation brought up the intriguing proposition that the 40S hnRNP particle might serve as an analogue to the DNA nucleosome [3]. Our objective is to describe the biogenesis of the 40S particle, provide a structural description of the 40S particle using cryo-electron microscopy (cryo-EM) and visualize it in its native context. We have generated TRex-293 cell lines expressing FLAG-tagged hnRNPC1/C2 proteins, isolated the 40S particles and analyzed them by negative staining EM. Furthermore, to investigate the ribonucleosome within intact cells, we have immunostained the key ribonucleosome components and generated thin lamellae using cryo-focused ion beam scanning electron microscopy (cryo-FIB/SEM). Using cryo-electron tomography we have acquired data from FIB-milled lamellae.

Keywords:

40S particles, immunostaining, cryo-FIB, cryo-ET

Reference:

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1157

Xenon PFIB-milling and cryo-electron tomography of bacterial anti-phage effectors

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Poster Group 2

Introduction

Bacterial abortive infection responses to bacteriophage infection play critical roles in bacteria population survival by limiting viral propagation, through activation of programmed cell death or metabolic arrest of the infected bacteria. Cyclic oligonucleotides produced by the bacteria in response to phage infection are responsible for activating certain abortive infection effector proteins. One such example is TIR-SAVED, which has been shown to degrade NAD+ when activated by cyclic tri-adenylate. Previous analysis by cryo-electron microscopy (cryo-EM) demonstrated that TIR-SAVED oligomerises into extended helical filaments in vitro when activated by cyclic oligonucleotide binding [1].
Here, we develop a high-throughput approach based on the Waffle method [2] to imaging filaments in situ in high numbers of high-pressure frozen bacteria using xenon plasma ion sources for lamella preparation. We use cryo-electron tomography (cryo-ET) and subtomogram averaging (STA) to characterise the structure and higher-order organisation of these filaments in a cellular context, as well as the morphological changes to the bacterial cells upon activation of the TIR-SAVED system. This research will provide insights into ancestral mechanisms of antiviral defence, and further our understanding of the innate immune systems of prokaryotes.

Materials and Methods

E. coli C43 (DE3) transformed with plasmids as for the plasmid immunity assay specified by Hogrel et al. [1] were incubated overnight at 37 °C in LB before the addition of D-lactose and L-arabinose to induce activation of the TIR-SAVED system.
Bacteria were vitrified by high pressure freezing and lamellae milled using xenon and argon plasma with an Arctis dual-beam FIB/SEM microscope following an adapted version of the Waffle Method [2].
Dose-symmetric tilt series were collected with a Titan Krios equipped with a Falcon 4i camera and Selectris energy filter. Warp was used for gain correction, CTF estimation, motion correction and tilt series stack generation. AreTomo was used for tomogram reconstruction. Particle picking for subtomogram averaging used a combination of IMOD, EMAN2, STOPGAP and custom scripts, and RELION was used for subtomogram averaging.

Results

Full induction of the TIR-SAVED system resulted in cell death of the bacteria. When partially induced to capture an intermediate state before total cell death, electron-dense regions were observed at the poles of cells. These regions of uncharacterised composition displayed increased sensitivity to radiation damage caused by electron beam exposure during tilt-series acquisition. Bacteria cells containing a TIR-SAVED mutation (E84Q) that inhibits its NAD+-degrading activity did not typically contain these inclusions.
Filaments were observed in reconstructed tomograms of bacteria containing TIR-SAVED-E84Q in both single filaments and as tight bundles of filaments. Subtomogram averaging of the bundles has

revealed the in-situ structure of a TIR-SAVED-E84Q helical filament at a resolution of 12 Å. This analysis shows large variations in helical parameters such as pitch along the length of each filament, indicating significant flexibility in the oligomeric structure. Mapping these averaged structures back into the cellular volumes reveals that the bundled filaments are arranged into hexameric assemblies.

Conclusion
Here, we present the first in-situ investigation of bacterial abortive infection systems by cryo-electron tomography. Our study gives insights into the structure and higher-order organisation of TIR-SAVED in cells, in addition to the morphological effects of the activation of TIR-SAVED on the whole bacteria.

Keywords:

Cryo-ET, FIB-milling, STA, bacteriophage infection

Reference:

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1160

Visualization of the in situ molecular architecture of tau pathology in the murine brain

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Poster Group 2

Background incl. aims
The accumulation of pathological tau protein aggregates is a hallmark of numerous neurodegenerative diseases, including Alzheimer's disease. The accumulation of misfolded tau in neurons is toxic, it disrupts cellular physiology, leading to neuronal death and the propagation of tau misfolding throughout the brain. Effects of tau pathology include disrupted axonal transport, mitochondrial and lysosomal dysfunction, and synapse degeneration. Despite advancements in understanding tau pathology, the relationships between initial tau misfolding, fibril formation, pathology propagation across connected neurons, and subsequent cytotoxicity on the level of individual neurons remain unclear. We aim to visualize the pathological changes in molecular architecture directly in the vitrified brain tissue of the murine model for tauopathy.

Methods
To visualize the native ultrastructure we use vitrified fresh brain without staining or fixation. We combine cryo-plasma-focused ion beam milling (FIB) and bio-contrast scanning electron microscopy (SEM) imaging with cryo-electron tomography (cryo-ET) on lamella. The cryo-plasma-FIB/SEM setup of the Helios Hydra V microscope allows imaging of non-stained vitrified hydrated biological samples with high biological contrast in nanometer resolution permitting volume imaging covering a much wider area than typical lamella used in cryo-ET.

Results
In this poster, we present our in situ visualization workflow and showcase preliminary bio-contrast cryo-plasma-FIB/SEM images and tomographs of murine brain tissue affected by tauopathy.

Conclusions
We showed that the novel bio-contrast cryo-plasma-FIB/SEM imaging workflow can be used for ultrastructural characterization of pathological tissues without chemical fixation and that the combination with lamella lift-out and in situ cryo-ET provides an excellent tool for uncovering the details of cellular mechanisms of neurodegeneration.

Acknowledgment
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Keywords:

in-situ cryo-ET, cryo-plasma-FIB/SEM, bio-contrast, tauopathy

1206

Streamlining Graphene Liquid Cell Preparation: VitroTEM's Naiad

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Poster Group 1

Graphene Liquid Cells (GLCs) offer unprecedented capabilities for imaging samples in their native hydrated state, providing insights at atomic resolution [1]. However, manual assembly of GLCs presents significant challenges, limiting their widespread adoption. This poster presents VitroTEM's Naiad system, a revolutionary approach to GLC preparation. By employing a layered structure consisting of monolayer graphene sheets on standard TEM grids, the Naiad system rapidly constructs GLCs, encapsulating liquid samples for imaging [2]. Our poster showcases images of ferritin particles in their native environment, demonstrating the system's efficacy in biological materials research. Additionally, we present atomic resolution images of Au nanoparticles, highlighting its utility in nanomaterial science. The Naiad system simplifies GLC assembly, enabling researchers to focus on sample imaging rather than grappling with graphene preparation. This poster emphasizes the Naiad system's potential to accelerate discoveries in diverse fields reliant on high-resolution imaging of liquid-phase samples.

Fig. 1: Ferritin particles encapsulated in GLC pockets.
Fig. 2: High resolution TEM image of Au nanoparticles encapsulated in a very thin GLC pocket.

References:
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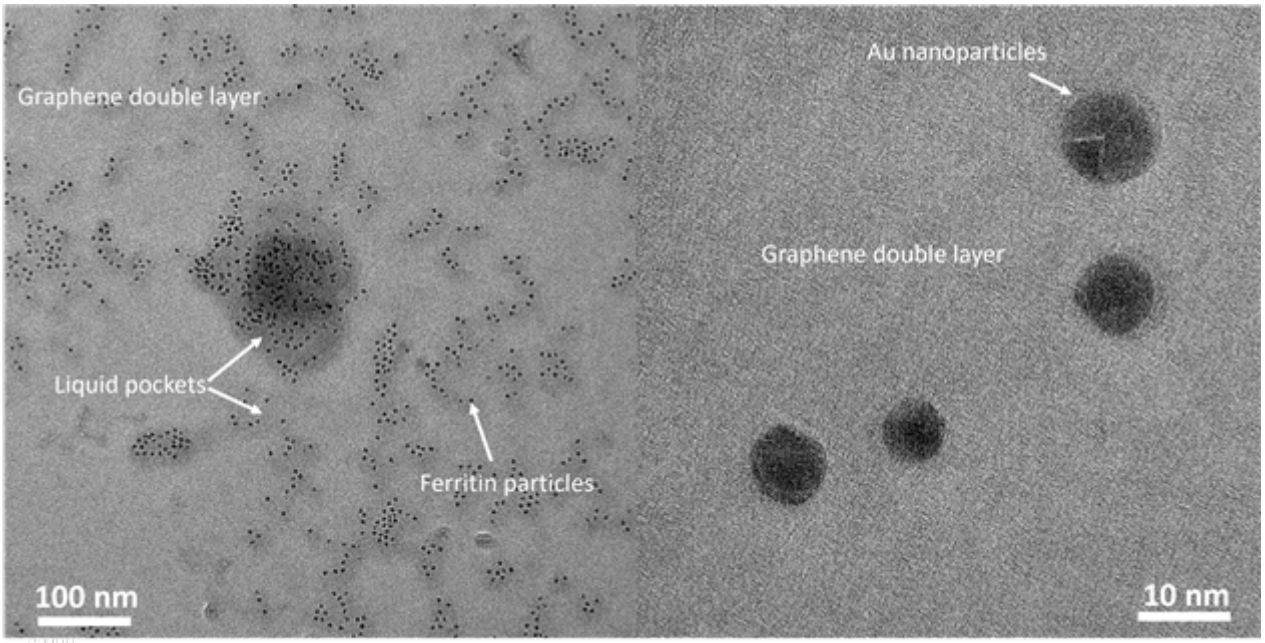
[2] van Deursen, Pauline MG, et al. Advanced Functional Materials 30.11 (2020): 1904468.



Keywords:

Graphene Liquid Cells, CryoEM, Insitu

Reference:

[1] Park, Jungjae, et al. ACS nano 15.1 (2021): 288-308.
[2] van Deursen, Pauline MG, et al. Advanced Functional Materials 30.11 (2020): 1904468.

1306

The apoptosome assembly in situ

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Poster Group 1

Background incl. aims
The apoptosome is a large protein complex crucial in initiating programmed cell death (apoptosis). It forms upon initiating apoptosis and recruits procaspase-9 to activate the caspase cascade, eventually leading to the dismantling of the cell. Although experimental structures of the apoptosome are available, the occurrence of the complex has yet to be shown in situ. In vitro, it is a heptameric assembly of the apoptotic protease-activating factor 1 (Apaf1) and bound cytochrome c [1,2]. Using Cryo-Correlative Light Electron Microscopy (Cryo-CLEM), our lab has found that dense meshworks in the cytosol of apoptotic cells correlate with foci of Apaf1-GFP. Our cell biological data indicate that these foci are functional apoptosome equivalents [3]. Interestingly, these foci disassemble when cells survive apoptotic signals, indicating a potential regulatory function. Based on these findings, we aim to uncover the underlying structure of these dense Apaf1 meshworks within cells.

Methods
To further investigate the Apaf1 dense meshwork, we increased our dataset of electron cryo-tomograms of cryo-FIB milled cells. Additionally, we used a modified sample preparation protocol to unroof cells on cryo-EM grids to obtain tomograms of the dense meshwork in a close-to-native environment without needing prior FIB-milling [4]. We are now implementing a computational pipeline to analyse the amorphous meshwork using Rasterized Subtomogram Extraction (RSE), in which subtomograms from a region of interest are sampled in regular intervals, combined with the latest Relion 5 tomography pipeline for subtomogram classification and averaging.

Results
At present, we acquired a dataset of 12 tomograms of cryo-FIB milled cells and 52 tomograms of unroofed cells showing the Apaf1 dense meshwork. In these tomograms, we can outline the dense meshwork corresponding to the Apaf1-GFP signal of Apaf1 foci. Our cell biological experiments show that the assembly is based on defined molecular interactions, indicating a higher-order structure of the meshwork. However, the dense meshwork has a continuous, cloud-like appearance, in which it is challenging to discern the repeating subunits for subtomogram averaging. We, therefore, implement a systematic approach to extract subvolumes, which we will extensively classify and align in many iterations. Furthermore, our cell biology data indicates that caspase-9 plays a role in the assembly of Apaf1 foci. We therefore consider that caspase-9 might be a structural component of the foci.

Conclusion
By Cryo-CLEM we demonstrated that Apaf1 is associated with high-density amorphous meshworks in cryo-electron tomograms of apoptotic cells. These meshworks are notably different from the currently documented, discrete heptameric apoptosome wheel and thus likely represent an alternative in situ apoptosome assembly structure. Moving forward, we aim to reveal the structure of the meshwork at high resolution. We then want to identify the potential meshwork subunits, construct structural models and understand the biological function of the assembly. In particular, we want to understand the molecular basis of the transientness of Apaf1 foci and whether it serves to regulate apoptosome function.

Keywords:

Apoptosome, Cryo-ET, Cryo-CLEM, Subtomogram Averaging

Reference:

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1312

In situ cryo electron tomography enables localization-dependent structural studies: Finding symmetric nuclear pore complexes

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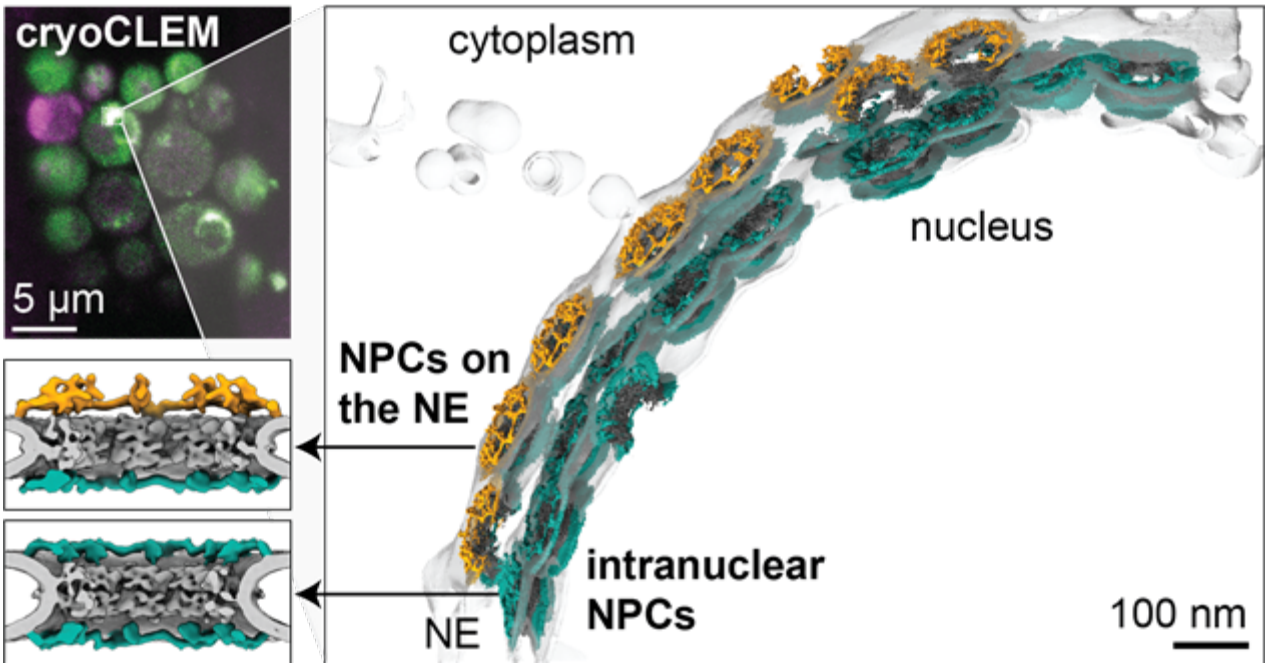
Poster Group 1

Nuclear pore complexes (NPCs) bridge across the nuclear envelope and mediate nucleocytoplasmic exchange. Numerous copies of over 30 distinct protein species assemble to form each nuclear pore complex. The NPC's core scaffold is symmetric across the nuclear envelope. Distinct peripheral nucleoporin complexes are attached to the cytoplasmic and nuclear face, where they ensure transport directionality and fulfill compartment-specific roles, such as genome organization. It remains enigmatic how such compositional asymmetry is established on a scaffold architecture that exposes the same interaction surfaces to either side. Here, we combine in situ cryo-electron tomography (cryoET), subtomogram averaging, and template matching with live cell imaging to address this question in both budding yeast and isolated cells from Drosophila melanogaster ovaries. We propose that the cell uses the surrounding cellular milieu as a cue for the correct allocation of components specific to the two faces of the NPC. This would predict the existence of symmetric NPCs when exposed to the same environment on both sides. To test this prediction, we study pores outside the nuclear envelope. Such ectopic NPCs naturally occur, but are either rare, transient, or prevalent in cell types that are not amenable to standard cryoET sample preparation. We use genetically engineered systems to enrich pores in either intranuclear or cytoplasmic double membranes. We show that in contrast to pores at the nuclear envelope, such NPCs are symmetric across the membrane. We furthermore demonstrate that the peripheral nucleoporin configuration depends on the nucleotide state of the small GTPase Ran. Our data indicate that the nuclear transport system is self-regulatory, namely the same molecular mechanism controls both transport and transport channel composition.
Our findings exemplify that not only structural determinants but also the local cellular environment shape the composition and architecture of macromolecular assemblies, highlighting the importance of in situ structural biology.



Keywords:

nucleus, NPC, cryoET, FIB-SEM, correlative

1323

Structural biology analyzes of photoreceptor outer segment: a closer look at PDE6

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Poster Group 1

Background incl. aims
Sight is perhaps the most spectacular human sense. The front of the eye allows light to pass through and channels it to the photoreceptor layer on the retina. There it triggers biochemical reactions that ultimately are converted to electrical signals that are sent and processed in the brain to generate images. Many components are required to create a vision, and it took millions of years of evolution to arrive at such a phenomenal organ. The conversion of photons to electrical pulse relies on the phototransduction cascade that involves many proteins at the outer segments of the cells and on its stacking of discs. There is a long-standing observation that in addition to the phototransduction activation, light stimulus also evokes morphological changes in the photoreceptors, still, the molecular determinates of this phenomenon have remained evasive. Recent developments in the Optical Coherence Tomography (OCT) field (including Optoretinography (ORG)) allow direct imaging of photoreceptors, and offer a new biomarker with the measurement of morphological changes detected upon light stimulus. At the same time, there is now evidence that a phosphodiesterase enzyme, namely Phosphodiesterase 6 (PDE6), that is at the core of the phototransduction signal pathway and is attached to the disc in the photoreceptor's outer segments, may bridge consecutive discs. Its structure has been determined in slightly different conformations that render the different protein lengths, thus opening the possibility that PDE6 is the molecular driver of the morphological changes observed. Our goal is to test if PDE6 is the molecular driver behind the observed morphological changes and a ORG biomarker.
Methods
To test our hypothesis, we isolated rod PDE6 and full outer segments (ROS) and employed Cryo-Electron Tomography (cryoET) and Cryo-Soft X-ray Tomography (cryoSXT) to test our theory in the cellular environment. The resolutions archived are modest compared to the Cryo-Electron Microscopy (cryoEM) structures already determined for PDE6, but still adequate to measure ROS disc spacing under different conditions and add to the “big picture” of the observed morphological changes. Importantly, the measurements are done with PDE6 in its natural environment and in the presence of relevant membranes. Additionally, we are also imaging fixed ROS, isolated under different conditions with Transmission Electron Microscopy (TEM).
Results
We have established the conditions to isolate and deposit ROS in cryo-grids, freeze the samples, prepare lamellas and collect tomograms. The tomograms are then processed so that information on the ROS disc spacing, under different conditions, is collected and analyzed. Such knowledge may have implications for therapeutics diagnostics and functional imaging of photoreceptor physiology.
Initial TEM images were obtained, and larger sample sets are being prepared.
Conclusion
We are at the initial sets of this project, but we have overcome already some technical difficulties, and expect now to collect larger data sets to test our hypothesis. At the same time, we are looking for other possible microscopic techniques that may add information to the observed morphological phenomenon.

Keywords:

Photoreceptor outer-segment, cryoET, cryoSXT, TEM

1326

Charting the molecular landscape across layers of vitrified mammalian hippocampus using electron cryotomographyaa

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Poster Group 1

Background incl. aims
Our understanding of neurological function and disease relies on knowledge of both the cellular architecture of brain tissue and the molecular organisation within neurons and glia. Electron cryotomography (cryo-ET) is a powerful technique for investigating the structure and distribution of biological macromolecules in their native, cellular environment. Traditional sample preparation for cryo-ET of tissues involving chemical fixation and microtomy leads to artefacts that can obscure high-resolution detail, impeding biological interpretation on the molecular scale. Alternatively, tissues can be studied in their native, hydrated state by preparing vitreous samples with high-pressure freezing (HPF), combined with nanofabrication approaches such as focused ion beam (FIB) milling.
The advent of plasma sourced focused ion beam (PFIB) instruments offers enhanced milling speeds afforded by the greater beam coherence at high currents that plasma sources provide [1]. This has the potential to greatly improve the throughput of sample preparation for thick tissue samples. Similarly, recent advancements in cryogenic lift-out of high-pressure frozen samples on grids have shown the ability to excise large sections of material from HPF samples, and has enabled the analysis of more complex biological models such as C.Elegans embryos and organoids by cryo-ET [2,3]. Applied to mammalian tissues, the approach promises to unlock the molecular characterisation of brain organisation. Here we outline a robust technical workflow for fabricating brain tissue lamellae using PFIB. We leverage serial lift-outs to access new sampling geometries from HPF samples spanning the mouse hippocampus CA1 stratum radiatum.
Methods
The brain of a 184 day old female C57BL6 mouse was divided into hemispheres and sectioned to 100 µm using a vibratome on ice where 2 mm biopsies of hippocampus were taken for incubation in cryoprotectant. Hoescht 33342 nuclear stain was added prior to vitrification in copper-coated gold carriers using the EM ICE HPF (Leica). The sample was loaded into the Helios Hydra G5 PFIB (TFS), where fluorescence mapping was performed using an integrated light microscope (Delmic Meteor) within the microscope chamber. A 350 µm region of CA1sr was identified and adjacent trenches were milled with 200 nA Xe plasma. Undercuts were performed at 4 nA to relieve the sample from the bulk of the material. A cryogenically cooled tungsten manipulator (TFS) adapted with a copper block attachment was then used to lift out the sample, and serial sections were deposited on a 400 x 100 mesh copper support grid (Agar Scientific). Automated lamellae thinning was performed using Xe for Rough (4nA), Medium (1nA) and Fine (0.1nA) milling followed by manual polishing with argon (60-20pA). Tilt series were acquired using a Titan Krios G4 with an energy filtered Falcon 4i detector (TFS). Tilt series were preprocessed in Warp and reconstructed with WBP in AreTomo before visualisation in IMOD.
Results
A novel milling approach was developed to isolate sections spanning mouse hippocampal layers for serial lift-out from HPF carriers. Firstly, the location of the pyramidal cell layer was identified using a correlated light and electron microscopy (CLEM) approach. The distribution of fluorescently labelled nuclei allowed for the cell body layer of the CA1 to be identified and guided the lift-out of targets spanning from the CA1 stratum oriens to the CA1 stratum lacunosum. The use of the xenon plasma

to shape the sample allowed deep trenches adjacent and above the region to be milled in less than 30 minutes. This utilised beam currents ranging from 60-200 nA, with resulting lamellae showing no visible damage artefacts. The orientation of the milling enabled undercuts to be made using a 90º relative stage rotation so that the tissue could be excised. Subsequently, we could deposit up to 42 serial sections that sample across a 350-400 µm region containing multiple layers of the CA1. Sections were thinned to TEM transparency with >90% success, where 246 tilt series were collected in the CA1 stratum radiatum (CA1sr).

From these data, the cellular organisation and content across the mammalian CA1sr could be characterised. These include multiple different cell types, such as glia with patches of ribosomes in cell bodies (n=12/251) and neuronal projections (n=79/246). Cellular features observed include microtubules (n=221/246), actin (n=75/246) and endoplasmic reticulum (ER) (n=38/246). Manipulation of the geometry of the lift out by isolating a section of tissue within the plane of the sample provided access to a previously unobserved view of the CA1 apical dendrite cytoskeletal network in 14% (n=35/246) of tomograms. This perspective revealed networks of cytoskeletal elements, ER, and mitochondria. Furthermore, 9% of tomograms in this dataset contained a confirmed synapse (n=22/246), with a further 11% (n=27/246) showing putative synapses. The quality of the tomograms reconstructed from this tissue sample is sufficient to identify canonical synaptic features including cleft proteins, post-synaptic density, active zone organisation and synaptic vesicle endocytosis.

Conclusion

This work presents a routine approach for generating lamellae for cryo-ET of multiple layers of mammalian hippocampus from a single lift out using plasma FIB. The developed pipeline incorporates a cryo-CLEM aspect to guide the lift-out of targets spanning 350-400 µm across the CA1 region, generating over 40 deposited sections of high-pressure frozen brain biopsy in a single 24-hour microscope session. Semi-automation of lamellae thinning using a hybrid Xe/Ar plasma approach produces sufficiently thin lamellae to characterise cellular organisation and content across the mammalian CA1sr. By lifting out a section of tissue in the plane of the sample, previously inaccessible views of the CA1pyr apical dendrite cytoskeletal network could be imaged in vitrified samples. The spatial relationship between sequential lamellae, which sample every 5-10 µm of the CA1sr, demonstrates the feasibility of acquiring cellular cryo-ET data with enhanced biological context using our approach. The workflow we present here has the potential to be adapted for different cryogenically preserved model systems and tissues, opening up possibilities for observing biomolecules within their most native environment. Our approach paves the way for more comprehensive, contextually rich structural studies of brain tissue at the molecular level.

Keywords:

Plasma-FIB, Cryo-ET, Lift-Out, Structural Neurobiology

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