公開

研 究 終 了 報 告 書

「高速量子波面モジュレーション・クライオ電顕」

研究期間： 2018 年 10 月～2022 年 3 月
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1． 研究のねらい (Research aim)

The goal of this project was to improve the overall performance of cryogenic electron microscopy (cryo-EM) for its use in high-resolution studies of biological samples. The transmission electron microscope is a quantum beam device. It uses a highly coherent beam of electrons accelerated to relativistic energies (100 – 300 keV) to image thin (<100 nm) samples with atomic resolution. Unfortunately, the full optical performance of the electron microscope is directly applicable only to inorganic material samples that do not deteriorate under electron beam bombardment. Biological samples and organic soft material specimens are quickly destroyed by the electron beam and therefore require careful observation with limited radiation exposure. Here come into play two of the fundamental quantum properties of electrons, which regrettably have opposite contributions. Firstly, the matter wave nature of electrons is highly beneficial and is used to generate phase contrast and thereby extract more information from the sample. Secondly, because of the limited beam exposure, the quantum counting ("shot") noise dominates the images, especially for the most valuable high-resolution signal components. Consequently, individual images are very noisy and many thousands of them must be averaged to produce a high-resolution reconstruction. There are very few remaining opportunities to improve the signal-to-noise ratio. Direct electron detectors have been the main drivers of the cryo-EM "resolution revolution" in the past decade. They offer much better detective quantum efficiency, i.e., better signal-to-noise ratio at the output, and high framerate that enables data collection in the form of multiframe movies. However, at present the time domain in the movies is not fully utilized and is used only to correct beam-induced sample motion and to weight the effect of radiation damage. The optical parameters of the microscope are kept constant during movie recording, which makes the data unilateral and monotonous.

The aim of this project was to introduce a time-varying optical modulation of the electron wave that will add additional dimensions to the data and offer opportunities to improve data quality; and ultimately the resolution and contrast in cryo-EM studies of biological systems. The first novel idea was to design and implement a real-time synchronous defocus modulator, which can be used to apply custom time-varying defocus profiles to the acquired movies. This will enable tuning of the balance between contrast and high-resolution signal preservation as the sample is gradually damaged during an exposure. The second idea to explore was to design and incorporate a high-speed astigmatism modulator, which can be used to modulate the image astigmatism in real time. In general, the new modulators are targeted at transforming the electron microscope into a flexible and adaptable optical platform that can be tuned to extract the most information from a broad range of biological samples.

2. 研究成果（Research results）

（1）概要（Overview）

The project achieved its main goal of real-time synchronous modulation and detection of the electron wave. Theoretical and numerical investigations of the proposed defocus modulator designs revealed practical challenges related to the magnitude of the required electromagnetic field, which were overcome through a new approach based on applying an electrostatic potential on the objective lens aperture. After assessing the simulation results for the defocus modulators, the plan for an astigmatism modulator was discontinued because it would have required even higher fields that would not have been possible in practice. The aperture-based defocus modulator was tested both numerically and experimentally and the results confirmed its performance and suitability for fast and accurate defocus modulation (FADE). Consequently, redesigned objective lens aperture hardware was installed on the development microscope system at the University of Tokyo, thus enabling the start of FADE experiments. In the same period, the design and construction of the FADE modulation control hardware was completed, and it was also installed on the microscope.

Cryo-EM experiments on the FADE system begun around the middle of the third year (2020). They revealed numerous practical problems with the microscope, the specimen cryo-holder, and the camera, that had to be overcome to reach satisfactory experimental performance. Through systematic investigation and mitigation of these issues, the first successful FADE datasets were acquired in the beginning of the fourth year of the project (2021). After overcoming further challenges related to the processing of the non-standard FADE data, the first high-resolution 3D cryo-EM reconstructions were produced and a new resolution record for this type of electron microscope was achieved.

In the beginning of the fourth year (2021), one of the topics planned for future expansion of this project, namely modulation of the incident wave, was adopted in place of the originally planned astigmatism modulator. An electrostatic dese modulator (EDM), which allows high-speed beam intensity modulation, was installed on the development system. Because the main experimental focus was on FADE, there was a chance to collect only a couple of test datasets with EDM. They confirmed the proper operation of the hardware and its added capability for beam intensity modulation. Further EDM-based investigations will be carried out on a new microscope platform in the future.

The successful realization of FADE and the performance limitations of the test microscope motivated a funding search that led to the procurement of a new state-of-the-art microscope (JEOL CryoARM 200). It will become the next research and development platform at University of Tokyo for FADE, EDM, and other novel cryo-EM technologies. The microscope was delivered at the end of this project's period (March 2022) and will enable the continuation of our research on pushing the performance boundaries of cryo-EM and expanding its reach towards more challenging samples and higher resolutions.

（2）詳細（Result details）

Research topic A “Simulation, design, and testing of a high-speed defocus modulator”

In the first stage of the project, I performed numerical simulations of the originally proposed designs for a defocus micro-modulator. They were based on an electrostatic or magnetic micro-lens that will be positioned at the back-focal plane of the objective lens. The simulations revealed that because of the space restraints at the installation location, the electrostatic potential or the electric current required to achieve the desired modulation will be on the limits of what is practically possible. After further deliberation of this problem, I came up with an idea for a much simpler modulator design that does not rely solely on the focusing power of a micro-lens to change the defocus. The new design was based on voltage biasing of the objective lens aperture which modifies locally the primary beam energy and then utilizes the chromatic aberration of the objective lens to produce defocus offset (Figure 1). This approach is much simpler to realize in practice because it does not require fabrication of micro-lenses. Curiously, it also exploits an undesirable property of the objective lens, namely its chromatic aberration, to achieve a positive outcome. Simulations of the new design showed that it would indeed work and will be able to achieve defocus modulation magnitudes much larger than those possible with micro-lenses. Following the simulations, in collaboration with the engineers of the Japanese electron microscope manufacturer JEOL, we performed initial modulation magnitude tests on an R&D microscope at their factory. The experimental results were in very good agreement with the theoretical predictions. The measured modulation strength differed by less than 10 % from the numerical prediction. Furthermore, the linearity of modulation was better than 1 %. These results were excellent, considering the complex ground plane geometry that surrounds the aperture area. At this stage, we published our theoretical and numerical simulations, and the initial experimental results in a general methods paper that outlined the principles, modulation strategy ideas, and expected benefits from the new method. We called the technique FADE (Fast and Accurate DEfocus).

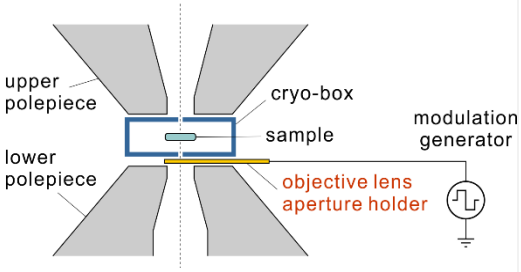

Figure 1. The new design for defocus modulator based on applying electrostatic potential on the objective aperture.

Following the very successful preliminary tests, we designed a modified objective lens aperture holder that was optimized for FADE. After some delays due to COVID, the new holder was installed on a general-purpose JEM-F200 electron microscope at the University of Tokyo in the middle of the third year of the project (2020).

Research topic B “Design and construction of a high-voltage modulation control unit”

I began design and construction of the modulation control unit (MCU) in the second half of the second year of the project (2019). My original plan was to use a high precision 16-bit

digital-to-analog converter (DAC) followed by a high-voltage linear amplifier to generate the necessary modulation signal. After doing some research and comparing various electronic parts, I came up with a simpler solution that used a high-voltage DAC (HVDAC) designed for control of digital light processing (DLP) chips. It can output up to 200 V signals with 14-bit precision. Such voltage range can generate defocus modulation of up to ~900 nm, which was more than sufficient for performing initial experiments. For controlling the HVDAC and interfacing it with the rest of the microscope system, I selected the Raspberry Pi 4 single board computer that uses a Linux-based operating system and has multiple digital and analog inputs and outputs, a standard LAN port, and supports a broad range of programming languages. The construction of the MCU was completed before the middle of the third year (2020). Initial testing revealed some issues related to noise and jitter in the modulation signal. These issues were resolved through testing on the microscope system and modifications of the grounding and power supply configurations, and low-level precise timing in the Python scripts that control the generation of the modulation pulses.

For the new state-of-the-art electron microscope that will become the next FADE platform (see section 3 below), we opted to purchase an off-the-shelf high-precision high-voltage source measure unit (SMU) to act as an MCU. It has a very accurate bipolar output with a range of ±1 kV, a network interface, and a scripting language support. This instrument will provide a much broader defocus modulation range and even higher modulation accuracy.

Research topic C "FADE experiments with cryo-EM test samples"

After the installation of the modified aperture holder and the MCU on the development microscope, I begun cryo-EM experiments. The initial impression of the system was quite discouraging because there were numerous practical issues that caused severe data quality degradation. Most of the problems were related to the old age of the specimen cryo-holder and the K2 direct-detector camera. Because of the prohibitively high costs of service and repair for these items, I resorted to DIY and systematically investigated and eliminated the problems, including recalibration of the microscope control software, cleaning and self-refurbishment of the specimen cryo-holder, numerous steps to improve the stability of the camera and reduce artifacts, installation of remote operation software for overnight monitoring of the data collection from home, etc. As a result of these improvements, the success rate of the experiments and the quality of the data improved dramatically. By the middle of the fourth year of the project (2021), apoferritin test sample datasets were routinely reaching sub-2 Å resolutions. In the last few months, I focused on reverse-engineering and modifying subroutines in the Relion reconstruction software to

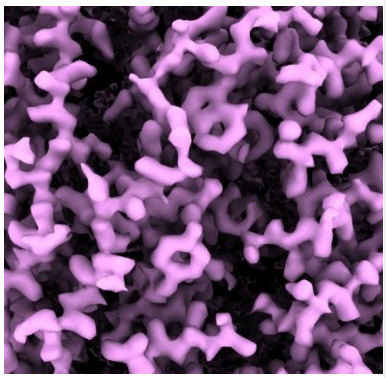

Figure 2. A new resolution record of 1.77 Å was achieved with nano-FADE.

enable FADE data processing. Consequently, I am currently able to process FADE datasets with small-amplitude nanometer-level defocus modulation, which I call "nano-FADE". The processing is still quite cumbersome and involves multiple manual steps, but the results are very encouraging. With the nano-FADE approach, I was able to set a new cryo-EM resolution record of 1.77 Å for a general-purpose electron microscope (Figure 2).

Research topic D "Fast electrostatic beam intensity modulation"

The electrostatic dose modulator (EDM) is based on a commercial high-speed electrostatic shutter capable of chopping the beam at up to megahertz rates. Due to my occupation with FADE experiments, I was only able to perform a couple of experimental tests of EDM and confirmed its proper operation. Further experimental investigations of the possible benefits of EDM will be performed on the new JEOL CryoARM 200 microscope.

Research topic E "Cryo-EM studies of G-protein coupled receptors"

My goal with the FADE project was to ultimately improve the performance of cryo-EM for challenging samples, such as membrane proteins, that are of great importance for understanding the causes and mechanisms of disease. Furthermore, with the expanding role of cryo-EM in drug discovery, I hope to contribute to the development of treatments that will give back to society and improve people's lives. In this respect, G-protein coupled receptors (GPCRs) are of great significance because they are implicated in numerous chronic diseases, such as diabetes, asthma, migraine, allergies, cardiovascular and nervous system disorders, cancer, and others. Consequently, they represent the largest group of drug targets and are the subject of more than 30 % of approved drugs. During the period of the PRESTO project, and in parallel with the development of the FADE method, I was collaborating with a GPCR pharmacology team from Monash University, Australia, on cryo-EM structural studies of numerous GPCR complexes with endogenous and synthetic ligands. In the last three years, we determined high-resolution structures and elucidated the activation mechanics of more than 50 receptor complexes. This is an unprecedented achievement that is further emphasized by the fact that approximately half of the structures are at resolutions better than 2.5 Å. To achieve this level of performance, I meticulously tested and carefully optimized several cryo-EM experimental factors which led us to the culmination of this collaboration with the recent publication of the first sub-2 Å and the first receptor-only GPCR structures. To put this in perspective, in the beginning of 2019 virtually all cryo-EM GPCR structures were at worse than 3 Å resolution. Although the new FADE approach was not yet ready for use in these studies, they made significant contributions to the structural and pharmacological knowledge of GPCRs. They also set a solid foundation and primed our experience for the future applications of FADE in such challenging projects.

In the last few months, I did collect a couple of GPCR cryo-EM datasets on the FADE development microscope. Unfortunately, its basic performance is limited by numerous

practical issues, as described above, that preclude the collection of large high-quality datasets that are imperative for high-resolution GPCR reconstructions. However, this is just a temporary setback that will hopefully be resolved soon with the installation of the new microscope equipped with FADE and EDM (see section 3 below).

3. 今後の展開 (Future deployment/expansion/directions)

The FADE and EDM technologies will continue to be in the center of our research. With the installation of the new FADE- and EDM-equipped JEOL CryoARM 200 electron microscope at the University of Tokyo in the spring of 2022, we hope to not only push the performance limits with test samples, but also to gradually transition to investigations of pharmacologically important samples, such as GPCRs. We will also be able to research cryo-tomography applications of FADE and EDM, which unfortunately could not be explored thus far because of the technical limitations of the development microscope system. Our presentation of the FADE method at the recent 3DEM Gordon Conference generated much interest from the cryo-EM community. Depending on the progression in quality of experimental results and the software support for data processing, it may take a few years for FADE to start generating results from actual samples. If the method gains a broader adoption, it could help to expand the already significant impact of cryo-EM in other areas of research, such as medicine and drug development.

4. 自己評価 (Self assessment)

Overall, I am very satisfied with the scientific advances that were made in this project. I was able to overcome numerous challenges in terms of theoretical design and experimental performance and achieved the first real-time defocus modulation in cryo-EM, setting a new resolution record in the process. Nevertheless, the real impact of the new FADE and EDM technologies is still to come. The state-of-the-art microscope equipped with FADE and EDM that was recently installed at the University of Tokyo will allow us to expand our experimental investigations to pharmacologically relevant targets and other imaging modalities, like cryo-tomography. With such applications, we hope to contribute to studies in important areas, such as chronic and infectious disease research and drug/vaccine development. We hope that the technologies invented in this project will proliferate positive scientific impact and thereby help to give back to society and to improve people's well-being in the future.

5. 主な研究成果リスト (List of major research results)

(1)代表的な論文(原著論文)発表 (Summary of representative original papers)

研究期間累積件数:20件

1. R Danev*, H Iijima, M Matsuzaki, S Motoki. "Fast and accurate defocus modulation for improved tunability of cryo-EM experiments". *IUCrJ* (2020) **7**.

This is the original publication of the FADE method and the defocus modulator design. In the paper we described our theoretical investigations of the electrostatic and magnetic micro-

lens modulators and their practical disadvantages. Later, we presented the new design based on voltage biasing of the objective lens aperture and experimental results showing its linear behavior in very good agreement with the numerical simulations. Finally, we proposed several defocus modulation recipes tailored for various applications.
2. TM Josephs, MJ Belousoff, YL Liang, SJ Piper, J Cao, DJ Garama, K Leach, KJ Gregory, A Christopoulos, DL Hay, R Danev*, D Wootten*, PM Sexton*. "Structure and dynamics of the CGRP receptor in apo and peptide-bound forms". *Science* (2021) **372.**
In this paper we published the first receptor-only cryo-EM structures of GPCRs. This is a groundbreaking achievement that opens the doors to cryo-EM investigations of inactive and pre-activation states of receptors, which until now have been accessible only by X-ray crystallography. The presented data also includes an innovative integrative structural approach that combined cryo-EM dynamics with hydrogen-deuterium exchange mass spectrometry.
3. R Danev*, M Belousoff, Y-L Liang, X Zhang, F Eisenstein, D Wootten, PM Sexton. "Routine sub-2.5 Å cryo-EM structure determination of GPCRs". *Nature Communications* (2021) **12**.
This work summarizes our continuing methods development efforts in pushing the performance limits of cryo-EM for challenging membrane protein samples, such as GPCRs. In the past three years we determined more than 50 high-resolution GPCR structures and in parallel systematically investigated and carefully optimized several experimental parameters. This led us to an experience and reproducibility level where nowadays we routinely determine GPCR structures at resolutions better than 2.5 Å.

（2）特許出願 (Patent application)

研究期間全出願件数：0 件

My original plan was to submit patent applications covering the new technologies that will be developed in this project. However, after further consideration I decided that the effort and time required to prepare such applications would be better spent on actual scientific research and further technology development. It is my belief that patent applications, especially in public-funded research, create hurdles, slow down the dissemination, and increase the costs of products that would otherwise, through open competition, reach the society much faster and at a lower cost.

（3）その他の成果（主要な学会発表、受賞、著作物、プレスリリース等）

I presented the FADE method at the first 3DEM Gordon Conference after a two-year hiatus due to the COVID pandemic. It took place in the US in the beginning of November 2021. The method and the results were met with great enthusiasm by the cryo-EM community. This in-person meeting also helped me to establish new connections discuss potential collaborations.