（別紙様式５）

変革を駆動する先端物理・数学プログラム（FoPM）

国外連携機関長期研修　報告書

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Due to the current situation, I decided to cooperate with a researcher especially familiar with image analysis of microtubule associated proteins. We aimed at solving the microtubule structural transition triggered by a microtubule minus-end binding protein, CAMSAP3, to clarify how its minus-end stabilization function works. Our goal is to analyze the local microtubule lattice structure using the algorithm called local-CFT that I developed recently.

In my plan, I would purify a part of CAMSAP3 protein, D2-CKK, from genetically engineered bacterial cells, and profile the purified protein sample to confirm that it retains the inherent property: microtubule minus-end capping. After cryogenic electron tomography (cryo-ET) image acquisition, the cooperator would process the data with deep learning for further analysis. Although the cooperator is more used to take tomographies, images were taken on my side because sending biological samples are not recommended during the pandemic (it may take a few months to arrive, which is too long to keep the samples frozen).

1. DNA construction and purification of D2-CKK

DNA coding D2-CKK region from human CAMSAP3 was conjugated with N-terminal mCherry fluorescent protein gene (for the future fluorescence microscopy) and inserted into a plasmid vector. This plasmid was transformed into bacteria (*E. coli*) for protein expression and purification.

After several trial, I found a protein expression condition and purification workflow that yield high-purity D2-CKK protein, which was confirmed by electrophoresis (Fig. 1A).

2. Microtubule nucleation assay with D2-CKK

D2-CKK is reported to promote microtubule nucleation and block the minus-end from depolymerization by coating the end. To check the D2-CKK I purified is functional, I mixed the purified D2-CKK with fluorescently labeled tubulin and observed under total internal reflection fluorescence microscope (TIRFM). If the protein is functional, polymerized microtubules should be capped by the fluorescence puncta emitted from the mCherry protein conjugated to D2-CKK.

As shown in Fig. 1B, after 15-min incubation, tubulin molecules polymerized into microtubules (magenta channel) and D2-CKK (green channel) capped their tips. This result indicates that, under this condition, I successfully reconstituted the state in which D2-CKK exclusively coated the microtubule minus-end.

3. Cryo-ET image acquisition

Based on the nucleation protocol in 2., I prepared cryo-ET grid sample. D2-CKK-capped microtubule were prepared in tubes and dropped on cupper grids for cryo-ET. After 15-minute nucleation in total, grids were snap frozen with liquid ethane for cryo-ET image acquisition. Unfortunately, I tried to find D2-CKK-capped microtubule tips but was not able to find any microtubules.

There are two reasons that possibly caused this failure. First, D2-CKK promoted microtubule nucleation less in tube and on the grid than in the glass chamber, a highly spatially restricted space. The other possible reason is that microtubule did not attach on the cryo-ET grids as well as in glass chamber. Cryo-ET grids are usually charged and were pre-processed by glow discharging just before the sample preparation. Microtubules are also known to negatively charged at their surface, so that the electrostatic repulsion might have caused the low efficiency of grid loading.

4. Re-analysis of existing tomographic data

I planned to send tomographic data to the cooperator for further analysis. Since I failed to acquire images of

D2-CKK-bound microtubules, I asked the cooperator for the similarly useful images. Previously, he had tried to analyze microtubule-bound full-length CAMSAP3 proteins but quality of the tomograms he got did not satisfy the quality of conventional structural analysis of CAMSAP3. The reason probably is that full-length CAMSAP3 is a highly disordered peptide so that it is not easily applicable to *in vitro* study. I thought images of full-length CAMSAP3/microtubule mixture could be useful for the analysis of microtubule lattice structure for my purpose.

The cooperator had reconstructed tomographic images and these images were also processed by cryo-CARE, a denoising method powered by deep leaning. Since the coverage of CAMSAP3 on microtubules were very low, this denoising procedure makes it possible to distinguish microtubule tips bound by CAMSAP3 and those not bound. He collected subtomograms where a CAMSAP3-bound microtubule tip exists in each image, and sent me the raw (non-denoised) subtomograms online. I analyzed the local structural profiles of the microtubules, but they did not differ from the middle region where CAMSAP3 were not bound. I assume that the number of CAMSAP3 bound to microtubule minus-end was not sufficient to trigger local lattice structure transformation.

5. Future plans

Since I could not acquire cryo-ET images this time, I'm planning another date for the same experiments with an improved protocol. Considering the low efficiency of microtubule loading onto the cryo-ET grids, I'm searching for the condition where tubulin nucleates more efficiently and generates more microtubules. In the previous trial I nucleated microtubule under room temperature (25℃) but nucleation and polymerization are much more efficient under physiological temperature (37℃). Now I'm trying nucleation pilot experiments under 37℃, higher concentration of D2-CKK and tubulin.

In parallel with this project, I have tested local-CFT algorithm in many ways and find that it is actually highly sensitive to even a subtle difference in lattice structure. I expect that this will work very nicely with microtubule binder profiling using the cryo-CARE denoising to figure out the correlation between protein occupancy and lattice structural deviation.

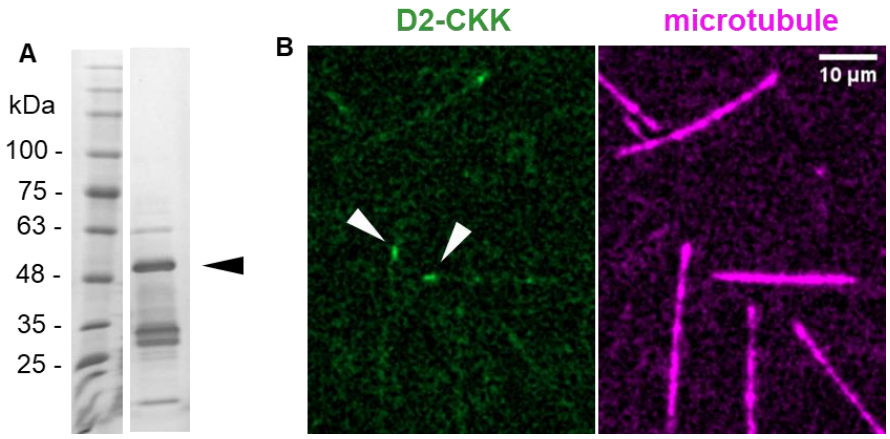

Fig. 1: Analysis of purified D2-CKK protein.