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7. PI 論文、著作

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英文研究介紹

1. 冷凍生物電子顯微技術 (Cryo-Electron Microscopy & Tomography)
2. 生物環境電子顯微技術 (Bio-Environmental 4D Electron Microscopy)

Important cellular mechanisms are carried out through the formation of large macromolecular complexes. These biological macromolecules are responsible for key processes such as cell signaling and reproduction, as well as being crtical in diseases like cancer and viral infections. Understanding of the molecular structure of these macromolecules is not only essential for the comprehension of their function and mechanism, but can also provide clues for the developing therapeutics related to health and disease. Nevertheless, the structures of only a small number of macromolecular complexes have successfully been determined at atomic resolution using x-ray diffraction (XRD), because crystallization of proteins and these macromolecular complexes remains a major hurdle to structural analysis with XRD.

Cryo-electron microscopy (Cryo-EM) is a technique to freeze a hydrated sample and derive the 3D structures of the biological macromolecules using an electron microscope. In Cryo-EM, the samples do not need to be crystallized as in x-ray crystallography. However, Cryo-EM technique still lacks the resolution to determine the atomic structure of biological macromolecules—due to the radiation damage which limits the resolution in most biological materials. In our research, we develop a new technique, the cryo-specimen electrification (CSE) technique, which reduces radiation damage to biological samples during electron beam irradiation. A unique cryo-charging specimen holder have been developed for CSE experiments. The ion doped cryo-specimen after charging can not only trap and store charge in equilibrium with the free and mobile charges, but also has a conductivity level close to that of metals. Hence it can promptly return electrons to ionized atoms and fragments in the frozen sample to efficiently repair the radiation damage, and thus greatly increase the electron dosage tolerance of the sample under electron beam exposure. Therefore, the CSE technique accompanied with Cryo-EM has opened up the possibility of studying the frozen biological sample at higher resolution. In addition, we will develop the cryo-scanning electron microscopy to observe the nano-scale cell organelles and further understand their various structures.

The cryo-electron microscope is shown in Figure 1, and the Vitrobot system used for prepapring the vritrified icy specimen is shown in Figure 2. Using cryo-EM, we have been able to solve less than 1nm resolution protein structure. Our lab has worked on many proteins and are able to solve close to or less than 1nm resolution protein structure. Shown in Figure 3 and 4 are two proteins solved by my students in my laboratory for the past few years. Figure 3 is the 3D structural reconstruction of hemoglobin protein, the resolution obtained is 15 Å (pixel size of 1.3 Å). Figure 4 is the GroEL protein which is a protein folding structure of protein molecules, the resolution obtained is 11.6 Å (pixel size of 2.0Å). The Cryo-EM image of GroEL protein is shown in figure 5.

We have also attempted to solve the 3D structure of Herpes simplex virus (HSV) which causes a variety of diseases such as chicken pox and cold sores. The structural classification of this HSV is illustrated in Figure 6 and its initial 3D reconstructed model has been obtained and shown in Figure 7. The following refinement procedure is in progress. *以上各圖形請參見以下中文研究介紹.

Employing the Cryo-EM technique, we are going to conduct and develop following research topics, including: (1) high-resolution 3D reconstruction of FtsK division protein and its function analysis; (2) complete structure expression and reconstruction of XerC and XerD proteins and the study of their biological functions; (3) structure reconstruction of FtsK, XerC/D, and DNA complex and the research of this complex mechanism; (4) high-resolution structure reconstruction of SpoIIIE division protein and its function analysis; and (5) drug development based on the structural and functional study of above-mentioned cell division proteins. These research topics related to the cell division in our research work should help in diseases and cancer control. In the future, we will also collaborate with biologists, biochemists, and medical doctors to conduct many other interesting research topics using Cryo-EM. We believe that the future application potential of the Cryo-EM plus the merit of the CSE technique should be beyond our expectation.

生物環境電子顯微技術 (Bio-Environmental 4D Electron Microscopy)

In the research of bio-environmental 4D electron microscope, Prof. Chih-Yu Chao uses differential pumping and microfluidic techniques to circulate water in the specimen chamber of electron microscope, and have established the first biological transmission electron microscope (Bio-TEM) for controlling a thin water environment surrounding the biological samples with temperature and pressure control [1,2]. Prof. Chao have applied his technique to study the phase transitions of various liquid crystal films and obtained a lot of results, and he also received more than ten U.S. patents regarding this technique. The ultimate goal of the Bio-TEM (or Bio-STEM) is to observe the live cells in aqueous environment up to subnanometer resolution in TEM. Although the invention of Bio-(S)TEM by Prof. Chao was not generally recognized in our country since 2004, its feasibility has been proved to work using a STEM by other research team from Oak Ridge National Lab and NIH in U.S. in 2009 and 2011 [3,4]. Our research team will also actively develop Bio-STEM related technologies, combined with nano-targeting technique to enhance the Bio-TEM resolution to the atomic level, since this technique is quite important for the observation of protein interactions in live cells.

In the near future, we will actively improve our Bio-STEM’s capability by developing new nanoparticle labeling methods and advanced techniques that stabilize live cells in liquid environment under normal pressure to prevent the bursting of cells in low-pressure environment. We will apply this live imaging technique to some cancer- and neuron-related research topics. In the aspect of cancer research, we will focus on how to inhibit the cancer cell division by blocking the functions of some cell division proteins. Cancer drug development based on the structural and functional study of target cell division proteins will be conducted. The research related to the cell division should have great help in cancer control.

Observing synaptic connection structure using Bio-STEM would provide a basic model to characterize the effects of neuron-related diseases in changing the neurotransmission. We will

visualize the movement of secretary vesicles at the synapse, and understand how the vesicle recycling are modulated to evaluate how neurons response to *degeneration*. We will also apply Bio-STEM to observe the changes in the architecture of the synapses and the synaptic connections at different stages in order to characterize the synapse structure change to understand the effects of *neuron degeneration* which leads to many neuron-related diseases. The other topics like how the neurotoxin molecule leads to the malfunction of the proteasome in Parkinson's disease and what's the mechanism of abnormal protein production by protease in Huntington patient will also be conducted in our research. On the other hand, neuronal differentiation and migration is the fundamental cell behavior by which neurons travel from their origin to their final position in the brain. To visualize the neuron stem cell differentiation and neuronal migration will provide insight into the cellular and molecular mechanisms by which complex nervous systems grow and develop. Therefore, observing these events in real-time mode and at nm resolution will promise the understanding of their functional mechanism. Besides, we will also design and construct a gradient-pressure electron column for environmental SEM imaging on above-mentioned research topics. The obtained SEM images can be complementary to the results of the high-resolution Bio-STEM.

Moreover, our team will also establish the Cryo-SEM technique and develop the 3D imaging and reconstruction technology for cryo-processed biological samples. Using above techniques, we can construct a portion of the brain neural structure of insect or animal by reverse engineering the Cryo-TEM and Cryo-SEM brain cell images, and develop microscopic image to neural network transformation algorithm to construct brain neural network.

Objective of the our research

1. Conventional TEM used by biologists and medical researchers can only observe dead sliced cells that have been frozen or dried and stained, but our Bio-TEM(STEM) can achieve the very challenging task of observing the dynamic reactions of living cells.
2. Our Bio-STEM technique combined with nanoparticle-labeling and several unique sample preparation methods have opened up the possibility of studying the living biological samples in liquid environment *at normal pressure*, which is larger than the sample pressure (~100 torrs) of the other Bio-STEM designed by the research team from Oak Ridge National Lab and NIH in US in 2009. This unique technique can provide enough pressure to prevent the *bursting of cells* in low-pressure environment.
3. In the aspect of cancer research, we will focus on how to inhibit the cancer cell division by blocking the functions of many cell division proteins, such as FtsK, FtsZ, FtsA, XerC, XerD, and SpoIIIE etc. Drug development based on the structural and functional study of these cell division proteins will be also conducted. The research related to the cell division should have great help in diseases and cancer control.
4. Synapses are the basic functional units in the nervous system. When neuron degenerates, the synaptic connection would be modulated but the detailed changes in the structure could not be verified by traditional approaches so far. To relay the neurotransmission, the neurotransmitter diffuses out of the fused synaptic vesicle and then binds to the postsynaptic membrane. Observing these events at nm resolution and in real-time mode promises the understanding of

the functional change in single synapses, which may provide a basic model to characterize the effects of neuron-related diseases in changing the neurotransmission.

5. Vesicle recycling is known to determine the synaptic efficacy. The number of vesicles in an axon terminal is limited and the vesicles need to be reused to maintain efficient synaptic transmission during repetitive stimulations. Many proteins are involved in these exo-endocytosis pathways and live imaging technique with nm resolution can provide the information about how the vesicle movement is modulated. The obtained results can help us to evaluate how neurons response to *degeneration* which leads to many neuron-related diseases. The other topics like how the neurotoxin 6-hydroxydopamine (6-OHDA) molecule leads to the malfunction of the proteasome in Parkinson's disease and what's the mechanism of abnormal production of CpA and CpB proteins by protease in Huntington patient will also be conducted in our reseach.
6. Neuronal differentiation and migration is the fundamental cell behavior by which neurons travel from their origin to their final position in the brain. Neurogenesis initiated by neural stem/progenitor cells is responsible for populating the growing brain with neurons. Migration during development brings different classes of neurons together so that they can interact appropriately, and the neural function depends on precise connections made by neurons and their targets. To visualize the neuron cell morphological changes during differentiation and neuronal migration will provide insight into the cellular and molecular mechanisms by which complex nervous systems grow and develop. We will also research the connection changes between cell-cell interaction in normal and degeneration induction to understand the process of neuronal migration.
7. The construction of brain neural network is an important scientific topic in this century. In our research, we will construct a portion of the brain neural structure of insect or animal by reverse engineering the Cryo-TEM and Cryo-SEM brain cell images, and develop microscopic image to neural network transformation algorithm. We will further conduct the neural network to neural simulations.

The **significant aims** of the our research in the near future are:

1. To develop Bio-STEM related technologies such as nanoparticle-targeting and labeling techniques to enhance the Bio-STEM resolution to near-atomic level. The goal of Bio-STEM is to observe the live cells in *aqueous environment* and *at normal pressure* with subnanometer resolution in STEM.
2. To understand how to inhibit the cancer cell division by blocking the functions of some cell division proteins such as FtsK, FtsZ, FtsA, XerC, XerD, and SpoIIIE etc.
3. To visualize and understand the synaptic connection structure to provide a basic model to characterize the effects of neuron-related diseases in changing the neurotransmission.
4. To understand how the vesicle recycling in synapse are modulated to evaluate how neurons response to *degeneration* which leads to many neuron-related diseases.
5. To find the mechanism of abnormal production of CpA and CpB proteins by protease in Huntington patient.
6. To realize how the neurotoxin 6-hydroxydopamine (6-OHDA) molecule leads to the

malfunction of the proteasome protein in Parkinson's disease.

7. To study the neuron stem cell and find the cellular and molecular mechanisms for neuronal differentiation and migration to understand how the complex nervous systems grow and develop in the brain.
8. To find the key proteins involving in the cell-cell interaction and characterize the connection changes between cell-cell interaction in normal and degeneration induction to understand the process of neuronal migration.
9. To build up the high-resolution bio-tissue Cryo-TEM and Cryo-SEM imaging procedure, and develop the microscopic image to neural network transformation algorithm and conduct the neural network to neural simulations.

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中文研究介紹

1. 目標

目前人類基因體已完全解碼，人類已進入後基因時代，但對於這些基因所表現出的蛋白質卻仍然未能有充分的瞭解，蛋白質在細胞內扮演非常重要的角色，舉凡細胞分裂、代謝反應、訊息傳遞、基因的複製與修復、養分的運送等，都需要蛋白質分子參與反應，蛋白質分子之間透過彼此交互作，用形成複雜的蛋白質網絡，而只要其中某一蛋白質表現失衡，即有可能使蛋白質網絡受到破壞，連帶造成細胞的病變，為了研究蛋白質與疾病之關係，現已經有所謂的蛋白質體學（proteomics）之誕生，其重點偏向系統生物學，研究多種蛋白質組成的系統，往往藉由一段序列與資料庫比對來推測蛋白質的特性，並建構出蛋白質間互動之關係，此方法雖能有效的將蛋白質分類建檔，但若想從分子尺度來解釋蛋白質的功能與交互作用，勢必要從最基本的蛋白質三維結構出發，結構生物學不僅可以驗證我們假設的正確性，另外亦讓我們能從實際的影像歸納出合理的推測與解釋，此將加速我們對功能性蛋白質之瞭解，也將會在製藥、疾病防治、基因複製與修復上提供重要的資訊。

2. 成果介紹

傳統結構生物學賴以使用的技術主要有 X 光結晶繞射與核磁共振(NMR)光譜法技術，X 光結晶繞射法是使純化後之高純度蛋白質在特定環境下形成具有規律堆疊的蛋白質晶體，然後收集 X 光的繞射圖紋後，藉由分析繞射圖紋可以計算出蛋白質的立體空間結構。而核磁共振(NMR)技術，主要是以收集蛋白質中各氫、碳及氮原子間的相對距離，再利用此相對距離資料推算出蛋白質的立體結構。以上兩種方法皆有其限制，首先若想要得到蛋白質之結晶，蛋白質之純度至少要達到 99%以上的程度，並且通常需要毫克（mg）的量，這考驗生物學家在純化表現上之功力，而就算達到此基本門檻，蛋白質結晶之參數條件也需要有化學背景知識豐富之技術人員不斷的嘗試才有可能取得，對於不溶於水的膜蛋白而言，那更是不可能的任務，另外蛋白質結晶學被人詬病的一點，就是他所解出的結構並非蛋白質分子在生理環境下之原型（native form）。另外針對核磁共振(NMR)技術而言，因為電子自旋之交互作用必須在一定的距離以內才有足夠之訊號，因此若蛋白質分子過大，則原子之間的相對距離將難以解析，所以並不適合用於較大的蛋白質分子。大約在 20 年前，科學家們已經有辦法製備能在電子顯微鏡高真空環境下，能維持完全水合之原型蛋白質試片，並且其所能夠承受之電子劑量高出平常之蛋白質分子 5～7 倍，此一技術稱為冷凍電子顯微鏡（Cryo-EM）[1]，即利用急速冷凍之處理，將蛋白質分子包埋在非結晶態之冰當中，此技術克服了以上所提蛋白質結晶學以及磁核共振之限制，因它在試片處理之參數調控非常容易，且蛋白質的純度只需 95%，而量也只需數微克（μg），這對於一般具有生物背景之技術人員來說是相當容易達到的，另外相較於核磁共振之技術，它所能解的蛋白質分子大小，從幾萬到幾百萬道耳吞（Dalton）皆可。

我們的 200kV 冷凍電顯如圖一照片顯示, 而冷凍製冰系統則如圖二所示。本團隊使用冷凍電顯技術，我們目前已能解出小於 1nm 解析度的蛋白質結構，本實驗室曾先後對數個蛋白質進行電顯取像工作，所得蛋白質結構皆接近或小於 1nm 之解析度，以下這兩個蛋白質結構即是本實驗室同學們近期所得 3D 蛋白質結構重建的成果，圖三所示為協助運送氧氣的血紅蛋白(Hemoglobin)重建後的結果，其解析度為15 Å (pixel size 為 1.3Å); 圖四則是輔助蛋白質折疊的 GroEL 蛋白質分子的結構，其解析度為 11.6 Å (pixel size 為 2.0 Å)，圖五為其冷凍電子顯微鏡之原始照片。此外，我們也嘗試解單純皰疹病毒 Herpes simplex virus (HSV) 之三維立體結構, 此病毒透過接觸傳染，會使人類產生皰疹或水痘，此病毒的三維重建分群結果可參考圖六所示, 而初步 HSV 之 3D 結構重建結果則如圖七所示，接著往後之結構細緻化還需不斷持續進行中。

傳統結構生物學現今賴以使用解生物大分子的技術仍是以 X 光結晶繞射為主，但其困難性在於使高純度蛋白質大分子在特定環境下形成具有規律堆疊的蛋白質晶體，且此技術被人詬病的一點，就是所解出的結晶結構其實並非蛋白質分子在生理環境下之原型。所幸科學家們目前已經有辦法製備能在電子顯微鏡高真空環境下維持完全水合之原型蛋白質試片，此一新穎之技術稱為冷凍電子顯微術（Cryo-EM），此技術克服了蛋白質 X 光結晶繞射技術之限制，但 Cryo-EM 的缺點是尚未具有原子級的解析能力，而限制 Cryo-EM 解析度最根本的問題是在於輻射傷害[2]，因為在非結晶態冰生物試片中並無自由電子，故不具導電性，在電子束照射下會產生永久性無法修復的輻射傷害。為了解決冷凍生物試片的輻射傷害問題，本團

隊於 2007 年成功發展出一種使冷凍試片帶電的技術，該發明乃是藉由對非結晶態冰生物試片施以適量電解質摻雜並予以充電，使得摻雜充電過後的非結晶態冰，變成有接近導體的行為，故當有輻射傷害產生時，試片當中的自由電子能迅速返還修復因輻射傷害而失去電子的蛋白質帶電分子片段與水分子的自由基，藉此可增加入射電子的劑量而能夠清楚觀察生物材料的原型，並同時使得電顯照片之訊噪比提高進而能達到更高的解析能力。在將近三年來不斷地嚐試與進行蛋白質結構的重建，目前本團隊已能在短時間內解出小於 1nm 解析度的蛋白質結構，所得最佳解析度為環節動物中普通蚯蚓(*Lumbricus terrestris*)之血紅蛋白分子的結構(見圖三)，目前這些結果在國內 Cryo-EM 的領域中都是居於領先地位。

目前世界上 Cryo-EM 技術對非病毒類的蛋白其最佳解析度約可達 4.0-4.5 Å 左右，與已知氨基酸序列的原子級解析 3.6 Å 已相距不遠矣，若是再配合本團隊所開發的冷凍試片充電(CSE)之實驗技術應有機會能將 Cryo-EM 技術在解一般蛋白結構的解析度推進到原子級(<3.6Å)的解析能力。另外，我們相信 Cryo-EM 技術可以整合跨領域的研究資源的潛力無限，也將開啟整合物理、生化與醫學應用研究新的里程碑。

此外，我們的團隊也將著手進行發展低溫掃描式電子顯微鏡(Cryo Scanning Electron Microscope, Cryo SEM)，尤其 Cryo SEM 已逐漸成為生物顯微領域不可或缺的技術之一，而一般生物樣品由於含有水份，因此多呈現液態或半液態，Cryo SEM 技術係以急速冷凍的方式來固定樣品，以保留樣品內的水份與組成，並使其在電子束的照射下維持穩定。再者，一般製作奈米結構所用的聚焦離子束系統(Focused Ion Beam; FIB)，由於具有移除材料(切削、蝕刻)的功能，因此可與電子顯微鏡及低溫傳輸系統結合，成為低溫雙束型聚焦離子束/電子束(Cryo Dual -Beam FIB; DB-FIB)系統，具有定點上二維及三維細胞內部的解析能力。

FIG.1 200kV 穿透式電子顯微鏡。

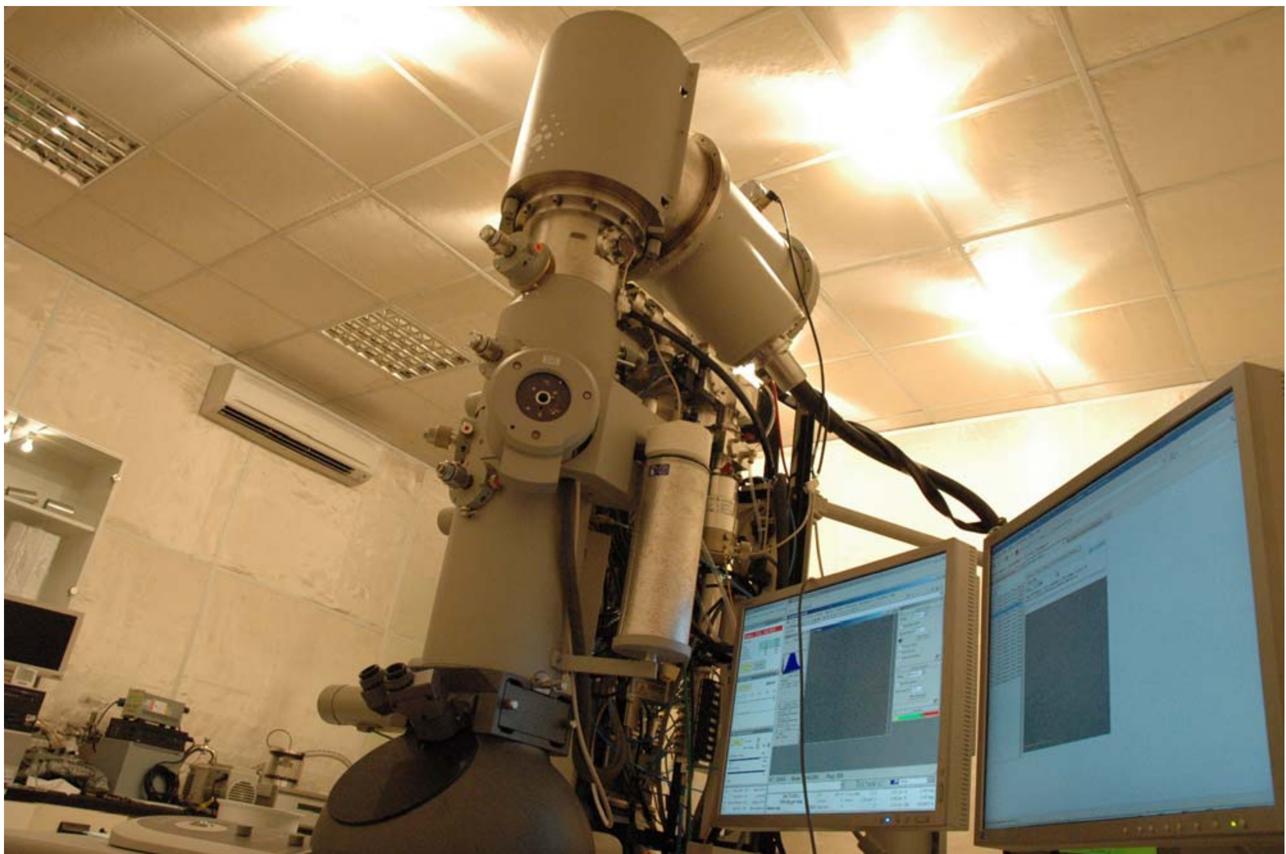


FIG.2 冷凍試片製造機。

FIG. 3 **Hemoglobin** 正視圖

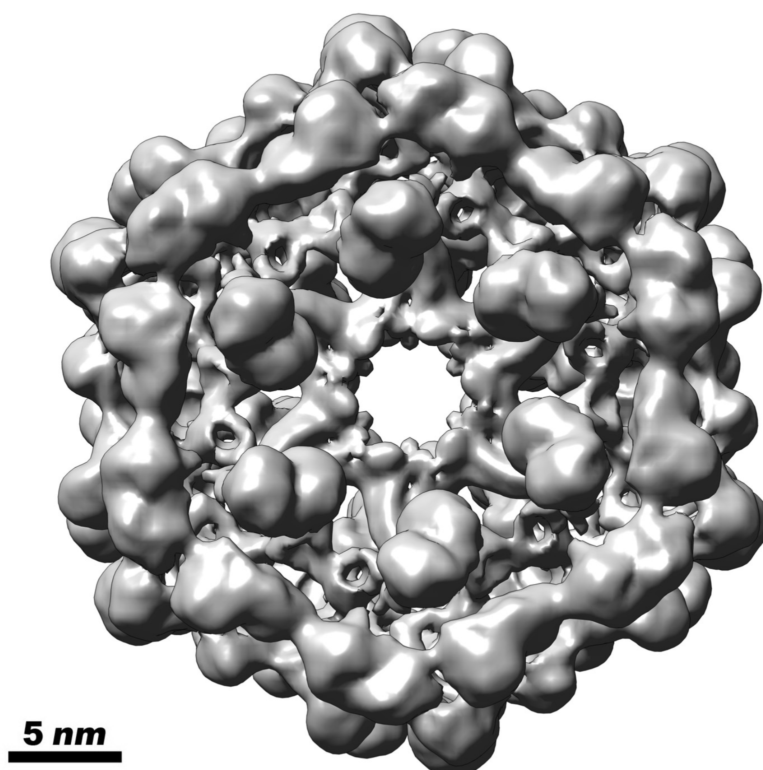


FIG. 4 **GroEL** 蛋白酶側視圖。

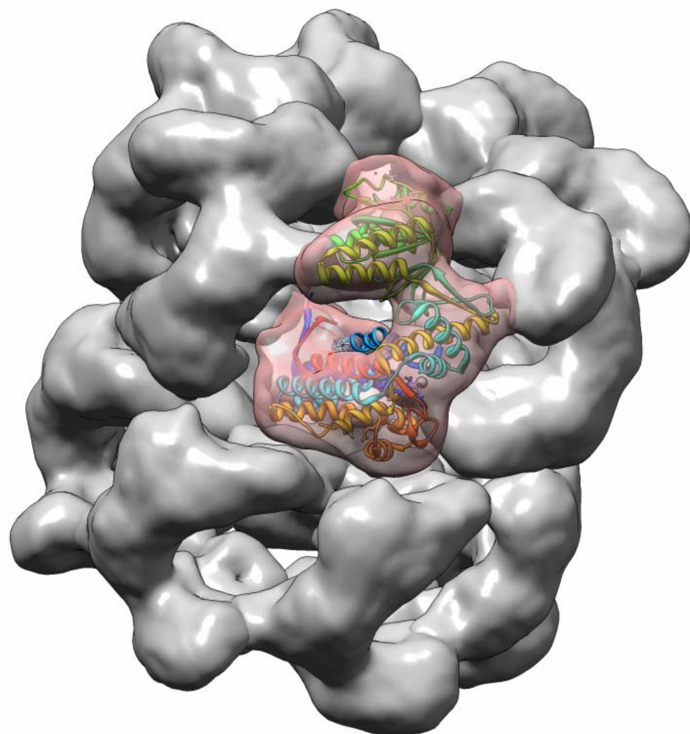


FIG. 5 **GroEL** 蛋白酶之冷凍電子顯微鏡照片。

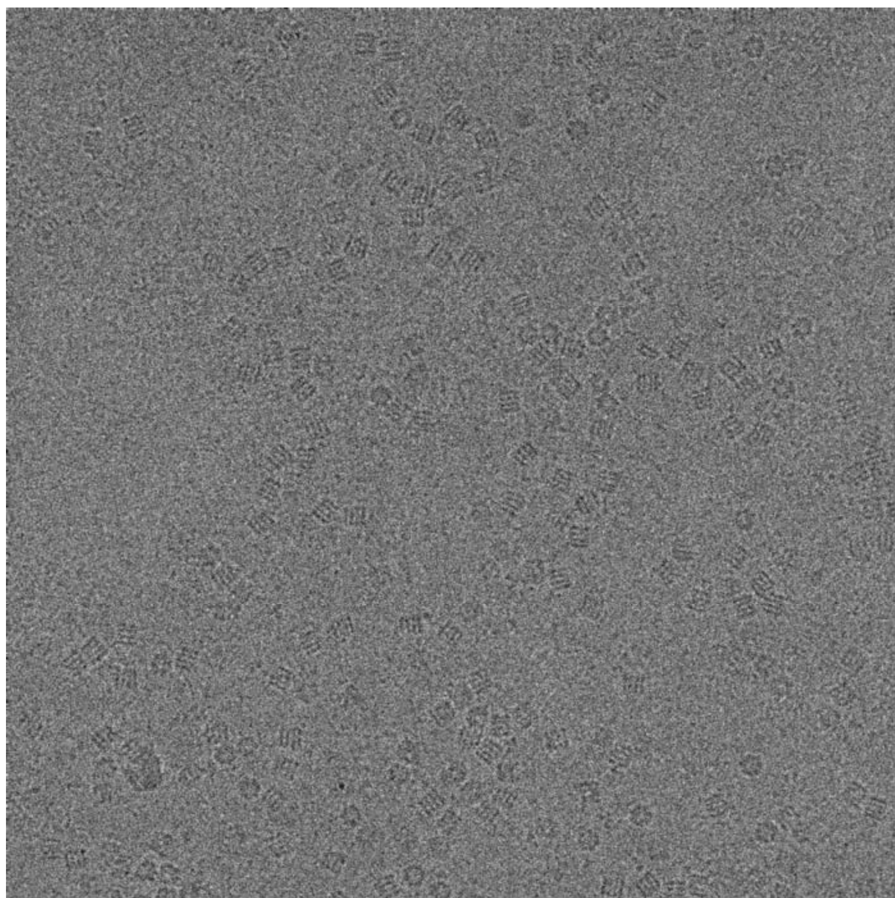


FIG. 6 病毒結構重建分群步驟。

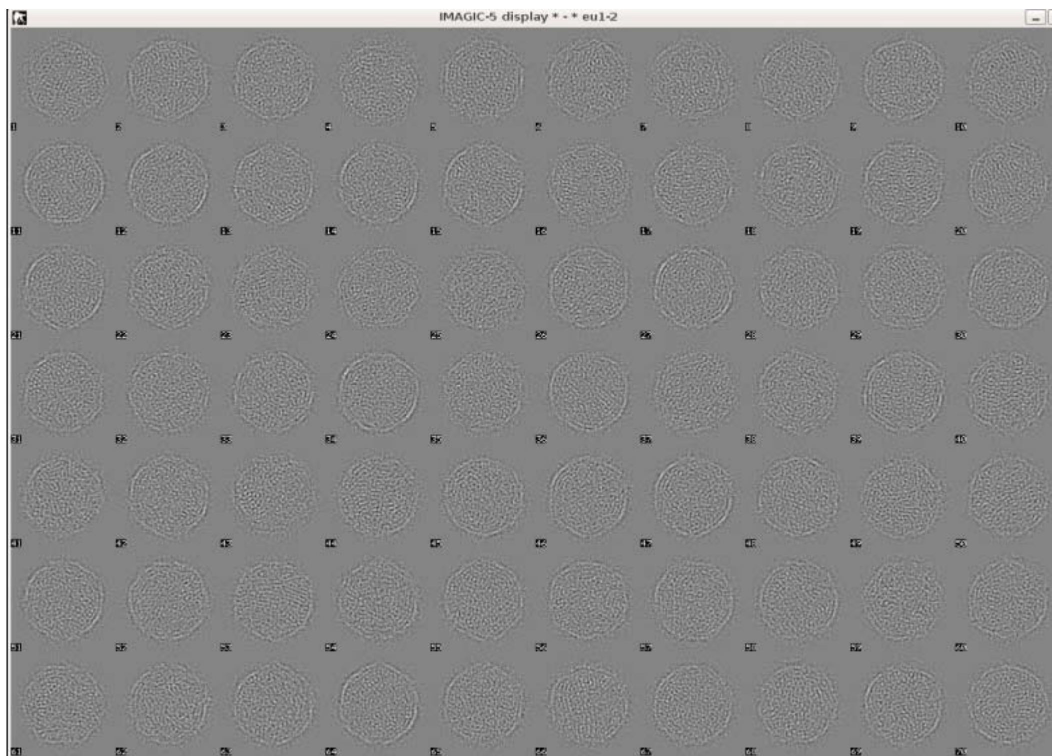
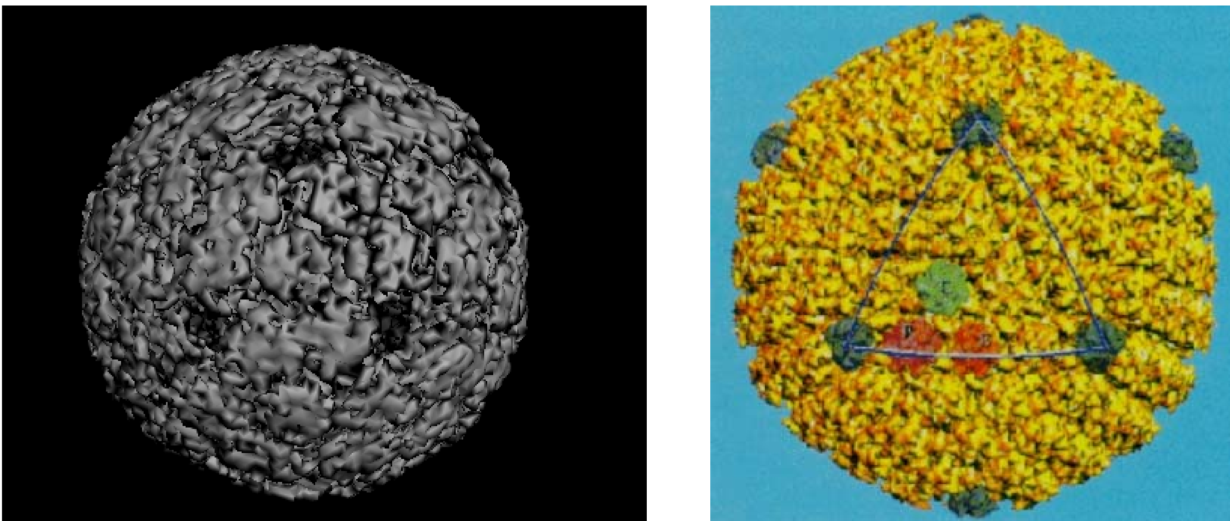


FIG. 7 HSV 病毒三維立體結構。



http://pharmadata.starconet.com/Datas/FR/conseils/datas/homeo/herpes.htm

一般 Cryo DB-FIB 分析的流程可分為兩個步驟，首先需對觀測樣品進行冷凍處理，主要目的在於固定樣品狀態、強化樣品對高能量粒子入射之抵抗力、以及避免樣品在真空中產生揮發。冷凍處理過程的關鍵在於必須具有夠快的降溫速率，以生物細胞的冷凍為例，若降溫速率不足，細胞內的水份將會結晶析出，導致細胞內含水區域的組成分離，當結晶尺度過大，亦會對細胞內的微結構產生破壞，因此必須以極快的速率降溫，才能達到細胞組成結構保存之目的。

冷凍樣品經冷凍處理後，即可置入 Cryo DB-FIB 系統進行進一步觀測。對一般 DB-FIB

系統而言，在對材料進行切削前，會以離子束輔助沉積在分析區域上鍍覆一層金屬，以保護樣品表面結構。然而在 Cryo DB-FIB 系統中，一般的輔助沉積方法將會因蒸汽量超出飽和蒸汽壓而產生過厚且導電性不佳的金屬沉積物，不適合作為截面研磨的保護層。解決方發之一為先將金屬源溫度降低，同時將金屬源噴嘴與樣品間距離拉長，在不使用離子束輔助的情況下短暫通入金屬氣體，即可獲得一層較薄的金屬層，最後以離子束適度掃描使其平坦。對於觀測截面的溝槽加工，可依設定尺寸及時效選取合適的離子束電流來研磨，再依序遞減離子束電流來拋光截面。對大多數的冷凍樣品而言其導電性並不佳，使得離子束易累積於失去導電層保護的分析區域上，導致加工位置飄移，欲克服此問題，可在離子束加工的同時，入射適當的電子束進行中和。此外，確定樣品與低溫載台間的熱平衡亦是重點，否則溫度變動所產生的樣品飄移亦會干擾加工位置的準確度。在完成樣品截面溝槽的製備後，即可以電子束進行截面觀測。圖八所示即為本團隊利用 Cryo DB-FIB 系統所拍攝之 SEM 影像。未來，我們亦會使用冷凍掃瞄式電顯技術來觀察細胞內奈米尺度的胞器與其組織型態做進一步瞭解。

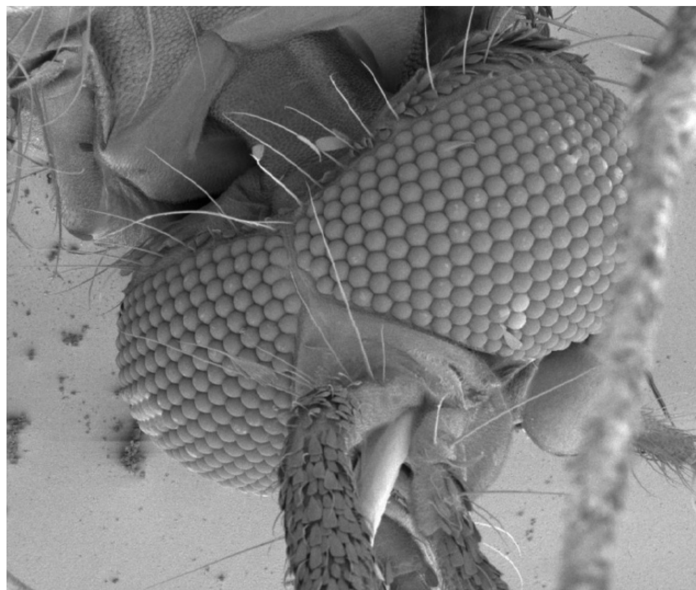


FIG. 8 以 Cryo DB-FIB SEM 系統所拍攝之影像(蚊子的眼睛)。

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