

DOI: 10.13376/j.cbbs/2025085

文章编号: 1004-0374(2025)07-0854-14



孔亮亮, 高级工程师, 国家蛋白质科学研究(上海)设施电镜系统负责人。长期从事生物电镜技术工作, 致力于生物大分子复合物的高分辨率三维结构及其方法学研究, 具有多种型号电镜建安、运维和电镜平台管理经验。作为项目负责人承担国家重点实验室开放课题项目和“联合举办‘整合结构生物学’技术培训及研讨会”项目及上海市科学技术委员会科研计划项目电镜部分各一项, 作为关键技术骨干主导完成中国科学院重大科技基础设施维修改造项目和中国科学院修购专项设备项目各一项。以第一作者或通讯作者身份在 *Cell*、*Nature* 和 *Nat Commun* 各发表 1 篇研究论文, 以协同作者身份在 *Nature*、*Nat Commun*、*Cell Res*、*Sci Adv* 等杂志发表 8 篇研究论文。申请专利 2 项, 涉及冷冻制样技术。撰写一部科学专著和一部科普专著中的电镜篇章。任上海市显微学学会冷冻电镜专业委员会委员。



丛尧, 中国科学院分子细胞科学卓越创新中心(上海生物化学与细胞生物学研究所)研究员、博士生导师, 曾兼任国家蛋白质科学研究(上海)设施冷冻电镜系统副总设计师。长期从事基于冷冻电镜的蛋白质质量控制大分子机器和纤毛运动调控大分子机器的动态与原位结构研究, 新冠病毒及肠道病毒的结构研究及抗体和抑制药物发展, 并致力于冷冻电镜重构算法及亲和载网等新方法的发展, 取得了一系列有国际影响力的研究成果。近五年来, 在 *Nature*、*Mol Cell*、*Sci Adv*、*Nat Commun*、*Cell Res*、*PNAS* 等国际知名学术期刊发表通讯及共同通讯作者论文 20 余篇。中国科学院“百人计划”、国家自然科学基金委员会首届“优秀青年”及上海市“优秀学术带头人”获得者, 并荣获中国生物物理学会“女科学家优秀科研成果奖”等。承担多项科技部、基金委、中国科学院的科研项目, 担任 *Sci Rep*、*QRB Discov*、*ABBS* 等期刊编委。任中国生物物理学会第十二届理事会理事、上海市生物物理学会第十二届理事会理事和中国生物物理学会冷冻电子显微学分会委员等。

冷冻电镜前沿技术与发展应用

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摘 要: 随着冷冻电镜 (Cryo-EM) 分辨率革命的到来, 冷冻电镜技术已成为生物大分子结构解析的核心手段。这场技术革命的背后, 是多维度技术的协同发展。新兴的冷冻制样技术, 尤其是亲和载网及时间分辨率的

收稿日期: 2025-06-05

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冷冻制样技术, 不仅具有整合生物复合体提纯与冷冻制样、降低气-液界面影响的潜能, 还有望捕捉生物过程的瞬时构象。冷冻聚焦离子束减薄结合荧光定位与高压冷冻技术, 可实现对细胞或组织样品的精准减薄, 助力揭示细胞原位中的“分子社会”及生物过程的时空调控。冷冻透射电镜硬件、控制软件及探测器的不断发展, 极大推进了高通量、高分辨率、自动化的电镜数据采集。近年来, 人工智能(AI)与冷冻电镜技术的深度融合, 正在推进冷冻电镜技术向着捕获生物大分子动态生物过程, 揭示其原位时空调控和“分子社会关系”方向飞速发展, 为揭示生命本质提供了强大的技术支撑。冷冻电镜技术不仅在基础研究中具有重要意义, 在药物开发等应用领域也展现出巨大潜力。本文将围绕冷冻样品制备、冷冻透射电镜及探测器硬件、数据收集及AI应用等方向展开阐述。

关键词: 冷冻制样技术; 冷冻透射电镜及探测器; 电镜数据采集; 人工智能

中图分类号: Q6-33; TN16 **文献标志码:** A

Cryo-electron microscopy: frontiers, advances and applications

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Abstract: Over the past decade or so, cryo-electron microscopy (Cryo-EM) has emerged as a primary approach in the structural determination of biological macromolecules. Behind this revolution lies the concurrent development of all steps of the Cryo-EM workflow. Advances in Cryo-EM sample preparation, particularly affinity grids and time-resolved sample preparation method, not only being able to integrate the purification with vitrification, minimizing artifacts from water-air interface, but also show promise in capturing transient conformational intermediates during biological processes. The combination of cryo-focused ion beam (Cryo-FIB) milling with fluorescence-guided localization and high-pressure freezing allows for precise thinning of cellular or tissue samples, facilitating the revelation of *in situ* “molecular societies” and the spatiotemporal regulation of biological events. Developments in microscope hardware, control software, and detectors have enabled high-throughput, high-resolution, and automated data acquisition in Cryo-EM. Meanwhile, the deep integration of artificial intelligence (AI) and Cryo-EM has propelled the field to new frontiers, enabling the visualisation of time-resolved macromolecular dynamics and the decoding of *in situ* spatiotemporal regulation, providing a powerful technique for revealing the essence of life. Cryo-EM also demonstrates enormous potential in applications such as drug development. Here, we focus our review on Cryo-EM sample preparation, instruments (both microscopes and detectors), data acquisition strategies, and AI applications in the field of Cryo-EM.

Key words: Cryo-EM sample preparation; cryo-electron microscopes and detectors; data acquisition; artificial intelligent

冷冻电子显微镜(cryo-electron microscopy, Cryo-EM)技术将生物样品在毫秒时间尺度快速冷冻在玻璃态的冰中, 应用冷冻透射电镜记录样品在各个角度的二维投影图像, 经计算机图像处理与重构生成生物大分子的三维结构^[1,2]。电子显微镜理念诞生于1928至1933年间, 距今近百年。Ernst Ruska于1933年设计建造了首台电子显微镜, 并获1986年

诺贝尔物理学奖, 以表彰他发明了透射电子显微镜。Aaron Klug开创电子显微晶体学方法, 并建立了从二维电子显微图像重建三维生物结构的方法^[3], 对现代冷冻电镜的发展产生深远影响, 获1982年诺贝尔化学奖。Jacques Dubochet发明了快速冷冻方法, 使水形成非晶态的“玻璃态”冰, 保护生物分子天然结构, 是推动冷冻电镜成为结构解析主流手

段的关键一步^[4]。Richard Henderson 结合冷冻电镜与电子晶体学,解析细菌视紫红质的结构^[5],首次证明电子显微镜可用于解析蛋白质的原子级结构,为冷冻电镜高分辨率结构解析奠定理论基础。Joachim Frank 提出单颗粒重构方法(single particle analysis),高精度解析生物大分子空间结构^[6,7]。2017年,诺贝尔化学奖被授予上述三位科学家,以表彰他们在开发用于溶液中生物分子高分辨率结构测定的冷冻电镜技术方面的贡献。

2013年前后,随着冷冻电镜软硬件技术的发展,尤其是直接电子探测器(DED)的问世,解决了图像漂移问题并提高了冷冻电镜图像信噪比^[8-10],掀起冷冻电镜分辨率革命。目前,Cryo-EM分辨率突破至1.09 Å^[11](图1a),可解析低至50 kDa的生物分子^[12],已成为结构生物学核心技术,推动生命科学进入一个新时代,且其在生物医药领域的应用潜力逐渐凸显。蛋白质数据库(PDB)和电子显微镜数据库(EMDB)中Cryo-EM结构条目呈指数级增长(图2),反映了其广泛应用。

作为Cryo-EM的衍生技术,冷冻电子断层扫描(cryo-electron tomography, Cryo-ET)可在接近天

然状态下,实现对细胞或亚细胞结构中大分子机器的空间分布与结构的原位解析(图1b)。Cryo-ET正发展成为连接结构生物学与细胞生物学的桥梁,使研究者得以从分子级分辨率观察细胞级生命过程,展现出广阔的应用前景与科研价值。

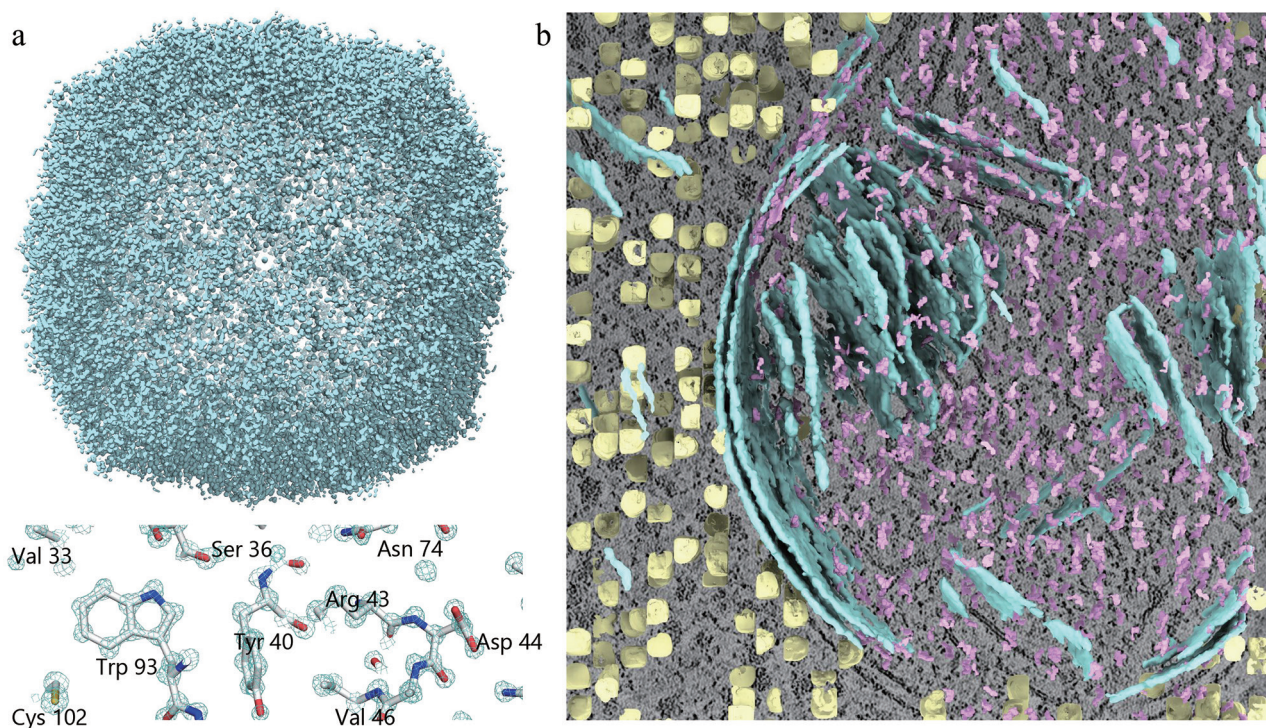
本文将重点围绕冷冻电镜样品制备、冷冻电镜系统及探测器、数据采集等核心技术,从实践应用角度出发,结合前沿技术与发展趋势,尤其是AI在冷冻电镜领域的应用,为读者呈现一幅冷冻电镜技术演进图景并对未来进行展望。

1 冷冻电镜制样前沿技术

冷冻电镜单颗粒分析已成为生物学核心技术之一^[13],但样品制备已成为限制其广泛应用的主要技术瓶颈。超大分子复合体难获得、气-液界面(air-water interface, AWI)造成的样品损伤、分子取向倾向性^[14-18]及瞬时状态难以捕捉等问题,导致样品优化需要投入大量的时间和精力,成为制约结构解析效率与质量的技术瓶颈。

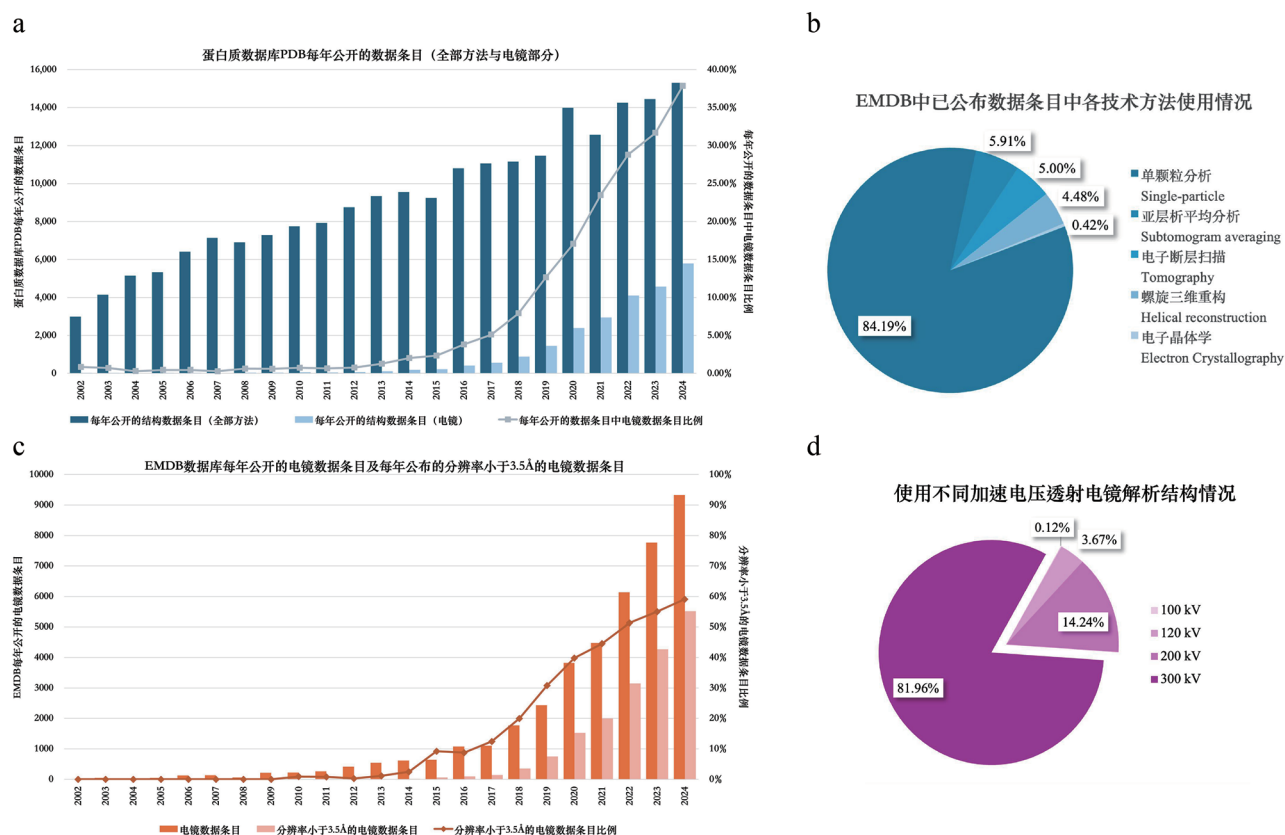
1.1 亲和载网(affinity grid)制样方法

近年来,亲和载网技术通过对载网表面进行功



a: EMDB数据库中记录的使用冷冻电镜单颗粒技术解析的最高分辨率数据条目(EMD-19436)。样品为铁蛋白,报告分辨率为1.09 Å。(上)电镜密度图;(下)局部电镜密度图与拟合的PDB结构模型(PDB: 8RQP)叠加图。b: 运用cryo-FIB和cryo-ET解析的衣藻原位结构渲染图(EMPIAR-11830)。黄色为核糖体,天蓝色为线粒体膜,浅紫色为线粒体内部蛋白质。

图1 冷冻电镜解析生物大分子及原位结构的高分辨率研究成果



a: 蛋白质数据库PDB每年公布的使用全部方法学解析的结构数据条目和使用电镜方法解析的结构数据条目的比较(自2002年EMDB建库至2024年)。b: EMDB中目前公布的数据条目按技术方法分类的情况统计。c: EMDB自2002年以来每年公布的数据条目和公布条目中分辨率小于3.5 Å的数据条目统计。d: EMDB目前公布的数据条目按照使用电镜的加速电压分类的统计。

图2 EMDB和PDB公布的数据条目统计

能化修饰(如亲和配体修饰的脂质单分子层^[18-22]、生物素-链霉亲和素^[23-26]、抗原-抗体^[27-29]等),直接捕获目标蛋白,减少AWI暴露引起的样品损伤及分子取向倾向性,并富集目标蛋白,为高效冷冻制样提供了革新性方案^[30,31]。

亲和载网根据界面修饰策略主要分为四类:(1)功能化脂质单分子层(functionalized lipids monolayers)载网,通过亲和配体(如Ni-NTA、小分子探针)修饰的脂质层捕获目标蛋白。如通过Ni-NTA脂质层捕获带有His标签的蛋白^[19,20],近年利用小分子配体功能化的脂质层捕获p97双六聚体等^[21]。(2)链霉亲和素二维晶体(streptavidin 2D crystals)载网,利用链霉亲和素与生物素化复合体间的超高亲和力实现靶标捕获^[23-25]。近年应用该技术解析了难以获得的PRC2-RNA复合体的高分辨率结构,揭示了RNA调控表观遗传修饰的分子机制^[26]。(3)功能化碳基^[32,33]、石墨烯^[34,35]和氧化石墨烯(GO)^[36-39]等为基底的载网。如经Ni-NTA功能化石墨烯特异性

捕获带His标签的20S蛋白酶体^[34],或开发GO共价偶联SpyTag/SpyCatcher的通用策略,实现TRAP1等复合体的高分辨率重构^[38]。(4)锚定抗体载网,如在碳膜覆盖的载网表面物理吸附抗体从而亲和病毒颗粒^[29],或通过化学偶联(如Pyr-NHS)锚定抗体于含sp²杂化的碳膜、石墨烯或GO表面,直接捕获抗原^[40]。如近期发展的锚定抗体的亲和载网(IAAG)策略^[40]使用Pyr-NHS作为连接器结合高亲和力的PA标签/NZ-1抗体系统,直接从细胞裂解液中亲和纯化低丰度目标蛋白,成功实现原子分辨率结构解析。该方法极大简化了样品纯化流程,可富集低丰度蛋白,克服样品取向倾向性问题,并可拓展到锚定诱饵蛋白或其他亲和体系,简便易行,并有望推动冷冻制样技术的革新。

亲和载网技术通过特异性识别,实现对目标复合体的高效富集与特异性锚定^[40]。然而,该技术仍面临非特异性吸附、亲和对(配体)种类有限、碳基材料功能化难度高等挑战。未来,AI驱动的适配

体或纳米抗体等新型探针设计与拓展,微流控自动化制备平台发展等,将有望推动亲和载网技术成为高分辨率冷冻电镜不可或缺的核心技术。

1.2 时间分辨率的冷冻制样技术

目前通用的吸滤 (blotting) 冷冻制样方法能够捕捉秒级及以上的生物过程^[41, 42],但难以捕捉毫秒时间尺度的动态过程,如酶催化、蛋白质折叠与降解、复合体组装、蛋白质-RNA 识别等^[43]。时间分辨率的冷冻电镜 (time-resolved cryo-EM, trEM) 通过精确控制反应时间,冻结瞬时中间态,揭示快速生物过程及动态构象变化的过程与机制^[44]。

trEM 依赖先进的反应控制和制样技术^[43],其反应引发与时间控制方式包括手动混合^[41, 42]、载网上混合^[44, 45]、微流控芯片混合^[46-49]及光触发技术^[50-52]。Nigel Unwin 实验室通过底物喷涂法捕获了乙酰胆碱受体通道开放态^[45];改进的压电分配器 (如 Spotiton) 通过双头设计实现了约 100 ms 的精准时间分辨率控制^[44];微流控芯片混合通过管道长度与流速控制反应时间 (10~1 000 ms),在流道内混合反应物后快速喷至载网冷冻制样,成功捕获了在 GTP 存在时 HflX 介导的核糖体解离过程中的亚稳态构象^[49]。光触发利用天然光敏蛋白 (如细菌视紫红质^[50]) 或光笼化合物^[51]实现精准控制,光耦合冷冻装置在毫秒内完成光激活与冷冻,为膜蛋白动态研究提供新途径^[52]。而制样技术主要采用微流控喷嘴^[46, 53]、超声雾化器^[54]或压电分配^[44, 55]生成液滴,结合自吸式载网^[56]和改良吸滤法^[57]实现毫秒级冷冻制样。另外,新兴技术,如 VitroJet^[58]、cryoWriter 毛细管系统^[59]及纳流控芯片^[60](从皮升级超微量样品解析高分辨率结构),拓展了时间分辨的应用。

trEM 需要精确控制混合与冷冻时间,但样品消耗、混合程度、反应时间精确测量及可重复性等仍具挑战,复杂的设备和专业操作也限制其普及应用。此外,数据处理需要从海量图像数据中识别出极其稀少的瞬时中间态,计算密集。未来,精确高效的自动化微流控技术,以及 AI 驱动的机器学习和结构解析方法,有望挖掘复杂反应路径中的隐藏中间态,显著提升数据处理通量与解析精度,必将推动时间分辨的冷冻电镜技术在探索动态生物过程中展现巨大潜力。

1.3 冷冻聚焦离子束(cryo-focused ion beam, Cryo-FIB)减薄技术

冷冻聚焦离子束 (Cryo-FIB) 结合传统聚焦离子束 (FIB) 的精准加工与低温样品冷冻技术,提供高

质量薄片样品。传统 FIB 使用镓离子 (Ga^+) 束进行微纳加工,常因室温加工导致样品损伤、水挥发或结构塌陷。Cryo-FIB 通过在低温 (通常 $<-170^\circ\text{C}$) 环境下操作,解决了上述问题^[61, 62],为细胞和组织等较厚样品的 Cryo-ET 研究提供了革命性的样品制备手段^[61, 63-65]。

Cryo-FIB 的核心是在低温环境下用离子束对样品进行减薄,降低离子束引起的热效应和辐照损伤,抑制样品升华,保持样品天然状态。冷冻生物样品在 SEM 观察时易产生电荷积累,故需溅射导电层 (如铂或碳) 增加样品导电性。因导电层薄易受离子束损伤,需用 GIS 系统沉积铂保护层减少表面损伤。为保证样品具有良好的导电性,常在保护层上再溅射导电层,便于在 Cryo-FIB 过程中观察。此方法可以保护冷冻含水生物样品 (如细胞、病毒) 免受离子束损伤。

样品经离子束减薄成 100~300 nm 的薄片,适合透射电镜 (TEM) 观察。过薄 (如 $<100\text{ nm}$) 可能因离子束损伤样品超微结构或因机械应力而断裂。因镓离子 (Ga^+) 嵌入样品表层,两面各形成约 30 nm 损伤层。若样品过薄,损伤层占比过高,有效结构信息大幅减少。典型减薄厚度为 100~200 nm (病毒或蛋白复合体),或稍厚的样品适合细胞内细胞器等形态学研究,平衡结构完整性与电子穿透性^[65]。Cryo-FIB 系统通常包括:(1) 离子光学系统 (Ga^+ 或 Xe^+ 等离子体离子源);(2) 扫描电子显微镜 (SEM)——实时成像;(3) 液氮冷却样品台 (约 -170°C);(4) 气体注入系统 (GIS)——沉积保护层 (如铂) 或增强刻蚀;(5) 冷冻传输系统——确保低温转移;(6) 表面溅射系统——溅射导电层以减少荷电效应。

Cryo-FIB 通过精准切割制备近天然状态的薄片样品,显著提升 Cryo-ET 原位结构解析能力。代表性应用包括:解析哺乳动物细胞核孔复合体^[66]、脑细胞超微结构^[67]、肺支原体细胞内 13 种核糖体功能状态^[68];解析内质网膜上翻译-转运复合体精细结构^[69]、核糖体到光系统 II 等多种蛋白复合体^[70]。近期, Dietrich 等^[71]以 4.2 Å 分辨率揭示了线粒体 ATP 合酶在天然膜电位下的动态旋转机制; Xia 等^[72]在宿主细胞中捕获了蓝舌病毒衣壳组装中间态,提出了 RNA 包装与衣壳扩张协同的“双路径”模型; Xing 等^[73]解析了分子伴侣素 TRiC 的原位结构,发现 PDCD5 与 TRiC 的开环构象结合。

Cryo-FIB 面临离子束损伤、设备复杂及成本高昂等挑战。当下开始应用的 Xe^+ 等离子源可减小

Ga⁺ 的注入损伤; 未来, 切削制备薄片的自动化程度将进一步提升, 降低 Cryo-FIB 的学习成本, 提高薄片制备的效率, 降低应用成本。Cryo-FIB 的靶向切削与 Cryo-ET 的整合正推动结构生物学向原位高分辨率时代迈进, 为揭示组织或细胞中的“分子社会”及其时空调控提供了强有力工具。

1.4 高压冷冻(high pressure freezing, HPF)制样技术

高压冷冻 (HPF) 是适用于细胞和组织等厚样品的关键冷冻样品制备技术, 能够有效保存细胞超微结构^[74, 75]。HPF 在约 2 100 bar 高压下以液氮快速冷冻样品 (速率 12 000~25 000 K/s), 在毫秒内将厚度达 200 μm 的样品玻璃化, 避免形成冰晶损伤胞内生物分子超微结构, 保留样品近生理状态。厚样品 (>200 μm , 如大型细胞、细胞团块、组织等^[75]) 因冷冻速度衰减易形成冰晶, 需预先薄片化处理再进行 HPF^[76, 77]。

高压冷冻设备核心部件包括高压腔室、制冷系统、压力控制系统等。主流设备包括徕卡 EM ICE、Boeckeler 的 HPM 010 和 M. Wohlwend 的 HPF Compact 等。一类是在高压下使用液氮对压力室中样品加压和冷却, 采用酒精协调压力积累和冷却; 另一类采用无酒精冷冻原理, 无须液体加压^[78]。目前, 借助新型高压冷冻仪, 全过程只需约 1 s。

相较于投入式快速冷冻 (<20 μm)^[79, 80], HPF 可均匀玻璃化厚度 <200 μm 的样品^[81]。但电镜成像样品厚度需小于 0.3 μm (理想情况 <0.15 μm)^[13], 以确保信噪比。Cryo-FIB 解决了因样品过厚电子束无法穿透样品的难题^[63-65]。Cryo-FIB 结合高压冷冻、冷冻光电联用 (Cryo-CLEM) 和 Cryo-ET, 在原位结构研究中潜力巨大^[76, 82]。Cryo-CLEM 通过荧光定位^[83, 84] 指导 Cryo-FIB 精准减薄; Cryo-FIB 样品提取“Lift-out”技术利用微操作器提取目标区域薄片, 转移至专用冷冻载网^[85-87]; Cryo-ET 实现高分辨率三维重构^[88, 89]。近年来, Cryo-FIB 在硬件上融合 CLEM 技术, 将电子、光、离子三束整合至同一界面, 借助实时荧光成像指导 Cryo-FIB 减薄^[90]; 冷冻光电融合聚焦离子束 (Cryo-CLIM) 技术, 在 Cryo-FIB 中集成 3D 荧光共聚焦模块, 提升了荧光定位的分辨率, 进一步增加了荧光指导 Cryo-FIB 减薄的准确性^[91]。近年来, Cryo-FIB 硬件引入了体电子显微镜 (volume electron microscopy, vEM) 模块, 支持大体积连续成像^[92-94]。上述技术协同可用于解析组织切片、多细胞聚集体, 以及小型模式生物样本的超微结构^[76, 82, 85-87, 95], 极大地拓展冷冻电镜技

术在细胞生物学、病理学等领域的应用边界, 便于更加深入地揭示从分子、细胞到组织层面生命活动的本质, 成为跨尺度生物复杂体系研究的关键工具。

2 高端冷冻透射电镜的配置及特色

2.1 Titan Krios 系列冷冻电镜

Titan Krios 是赛默飞世尔科技公司的高端 300 kV 冷冻透射电镜系列, 从第一代 G1 (原 FEI 公司) 升级至第四代 G4, 近期推出第五代 Krios5。G4 配备 X-FEG 场发射电子枪 (extreme high-brightness field emission gun) 或 C-FEG 冷场发射电子枪 (low-energy-spread cold field emission gun) (图 3)。C-FEG 能量分散低 (≤ 0.3 eV), 支持于最高分辨率 (≤ 2.0 Å) 下实现更高对比度, 适于超高分辨率成像和电子能量损失谱 (EELS) 分析, 但需定期施加电流“flash”枪尖, 以维持发射强度。三级聚光镜系统提供宽范围连续可调平行光照明。无像差成像区域位移 (aberration-free image shift, AFIS)^[96] 技术在不产生离轴彗差和像散的情况下实现 12 μm 电子束位移, 替代机械样品台移动, 显著提升自动数据采集速度。无条纹成像 (fringe-free imaging, FFI) 技术消除电子束边缘波纹, 避免电子束对成像区域以外的损伤, 配合 Nanoprobe 模式拍照, 增加单孔可用图像, 提高通量。Autoloader 自动进样装置可储存 12 个冷冻样品, 提高上样效率, 降低冰晶污染, 支持样品无污染转移至其他配备自动进样装置的显微镜。可选镜筒后置 (post-column) 能量过滤器 (如 Selectris、Selectris X^[97] 或 Gatan Bio-Quantum), 通过零损失滤波减少非弹性散射电子干扰, 提高图像对比度^[98]。

Krios5 进一步扩展 AFIS 范围至 20 μm , 提高成像通量。新引入专为 Cryo-ET 设计的真空样品盒 (vacuum capsule), 适配赛默飞 Arctis Cryo Plasma-FIB 切割, 确保冷冻薄片无污染转移至 Krios5。Cassette 装置固定载网位置, 支持多载网批量设置数据采集任务, 实现长时间连续数据采集, 显著提高效率。

2.2 JEOL Cryo-ARM 300系列电镜

JEOL Cryo-ARM 300 (简称 ARM 300)^[99, 100] 是日本电子株式会社生产的 300 kV 冷冻透射电镜, 配备冷场发射电子枪和四级聚光镜平行照明系统, 通过联动调节光斑尺寸 (spot size)、光束角度 (angle) 和聚光镜光阑, 实现一定宽度的平行光照明 (图 3)。从第二代起具有独特的“科勒模式 (Koehler)”照明^[101], 可消除光斑边缘波纹, 支持单孔多张数据

采集, 结合光束图像偏移的彗差校正实现在光束偏移(约 $7\ \mu\text{m}$) 时的高质量高通量数据采集^[102, 103]。ARM 300 配有直接嵌入镜筒电子光路的 Ω 型能量过滤器, 由四个对称的磁棱镜构成, 置于中间镜与投影镜之间, 出厂高精度合轴校准, 无须因放大倍率改变而调整色差和畸变, 结构简单, 稳定性高^[104, 105]。但受限于光学设计, 不支持低放大倍率下的高衬度图像采集。自动进样装置包括传输盒(4 个样品位)和存储仓(可存放 12 个样品), 可同时完成新旧样品的替换, 减少转移步骤降低冰污染。存储仓样品可保存约 2 周, 冰层增长速率低, 但传输盒需在预储仓抽干液氮, 可能导致样品回收时出现短暂升温 and 冰污染^[106]。ARM 300 已被应用于多个复合体的高分辨率结构解析, 如紫硫细菌光系统捕捉 1- 反应中心(LH1-RC)复合物($2.24\ \text{\AA}$)^[107]、未结合 Zn^{2+} 的人源锌转运蛋白 ZnT7 ($2.2\ \text{\AA}$)^[108] 等。

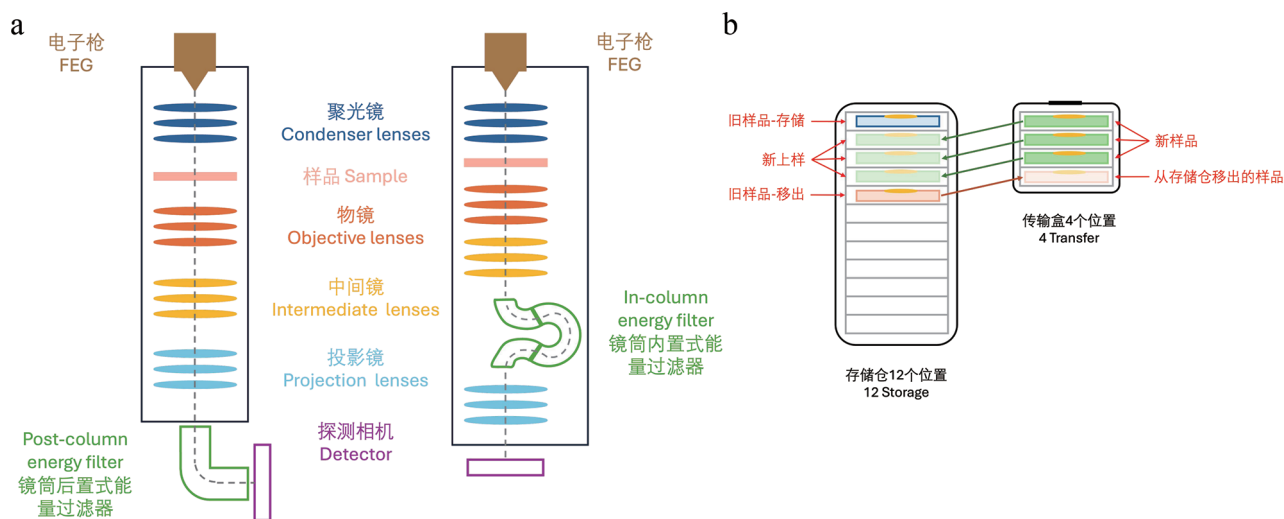
截至 2025 年 5 月 11 日, 应用 300 kV 高端冷冻电镜已达到 $\sim 1.1\ \text{\AA}$ 分辨率水平($1.09\ \text{\AA}$, EMD-19436^[11]; $1.19\ \text{\AA}$, EMD-35984^[109])。此外, 应用 200 kV 及以下冷冻电镜已解析得到较好分辨率的结构。EMDB 数据库记录显示, 200 kV 冷冻电镜解析的结构最高分辨率为 $1.635\ \text{\AA}$ (EMD-14173)^[110], 120 kV 达 $1.92\ \text{\AA}$ (EMD-43576)^[111], 100 kV 达 $2.1\ \text{\AA}$ (EMD-51481), 显示上述中端冷冻电镜亦有广阔的应用潜力和前景。

3 直接电子探测器(direct electron detectors, DED)

直接电子探测器 (DED) 基于互补金属氧化物

半导体 (CMOS) 技术, 取代照相胶片和电荷耦合器件 (CCD) 探测器, 引发冷冻电镜分辨率革命^[8, 112, 113], 常规实现近原子分辨率成像^[114, 115]。生物样品易受电子束辐照损伤, 图像衬度低, 探测器的高量子探测效率 (detective quantum efficiency, DQE) 至关重要。DQE 反映探测器的电子探测效率、空间分辨率和噪声特性, 定义为输出与输入信噪比平方的比值^[113]。在离散采样中可准确分辨的最高频率为采样频率的一半, 即奈奎斯特截止频率 (Nq)。像素化探测器可记录的最大空间分辨率由像素尺寸决定, 故其奈奎斯特截止频率为 $1/(2 \times \text{pixel spacing})$ 。

目前, 应用于冷冻电镜的主流 DED 包括 Gatan 的 K2/K3 相机、Thermo Fisher 的 Falcon 系列以及 Direct Electron 的 DE 系列。K2/K3 相机支持电荷累计 (linear) 和计数 (counting) 读出模式。Linear 模式可实现数据快速积分, 但能量波动和读出噪声降低 DQE。Counting 模式识别单个电子事件, 设定阈值消除能量波动带来的噪声, 提升 DQE。在 Counting 基础上还可采用超分辨率 (super resolution) 模式, 通过对电子事件的亚像素定位突破奈奎斯特极限^[10]。目前 K3 内部帧率 1 500 fps 降低重合损失, 提高 DQE, 相机尺寸 $5\ 760 \times 4\ 096$ 支持大区域成像, Counting 模式可在 $10 \sim 20\ \text{e}^- \cdot \text{\AA}^{-2} \cdot \text{s}^{-1}$ 下运行, 成像速度快。K3 还具备相关双采样 (CDS) 模式, 通过采集前后两次读取所有像素的电压来抑制噪声, 提高衬度^[116]。Falcon3 相机也开始有电荷累计 (integrated) 和计数 (counted) 两种读出模式^[117]。Falcon 4i 新增电子事件表示 (electron event representation, EER) 模式, 记录



a: 透射电子显微镜两种不同能量过滤器示意图; b: JEOL ARM300自动进样装置示意图。

图3 冷冻透射电镜关键部件与技术装置示意图

每个电子事件的坐标和时间戳, 通过亚像素定位突破奈斯特极限, 且数据体积较之前 MRC 格式大幅度压缩^[118]。EER 模式支持灵活时间窗口划分, 优化运动校正与剂量加权。Falcon 4i 搭配 Krios G4-Selectris 系列能量过滤器, 协同提升数据采集体验和效果^[119]。此外, Direct Electron 目前应用在生物领域的 DE-64, 亦可助力高分辨率结构研究^[120, 121]。

4 冷冻电镜数据收集

4.1 冷冻电镜自动化数据收集软件

冷冻电镜自动化数据收集软件通过自动拍摄载网图像、定位和识别数据采集区域及高通量连续采集, 减少人工操作, 极大提高效率。主流软件包括 Thermo Fisher Scientific (下文简称“TFS”) 的 EPU/TOMO^[122]、科罗拉多大学 Boulder 分校开发的 SerialEM^[123, 124] 及纽约结构生物学中心的 Leginon^[125]。

TFS 为旗下电镜开发了自动采集单颗粒数据的 EPU 软件, 支持多种探测器及能量过滤器, 允许参数定制和连续高通量采集; 还为 Cryo-ET 数据自动采集开发了 Tomography (TOMO) 软件, 精确控制样品台移动和倾转, 可以采用剂量对称倾转方案确保电子剂量最优化, 实现 Cryo-ET 数据的自动化批量采集。TFS 软件界面设计和操控逻辑直观, 易于初学者上手, 但其商业化框架限制灵活性, 难以定制复杂实验。

SerialEM 是免费开源软件, 适配所有主流透射电镜和探测器, 覆盖控制、采集及处理等各方面。同时, 其脚本化平台支持定制化功能脚本, 最初聚焦 Cryo-ET^[123], 现已广泛用于 SPA^[124, 126] 和电子晶体衍射数据^[127, 128] 收集。SerialEM 功能强大, 是当前应用较为广泛的电镜数据收集软件, 也是数据收集方法开发创新的重要软件平台。但其灵活性和丰富的功能也带来一定使用门槛, 复杂参数体系与多样化的脚本调用对初学者构成挑战。

Leginon 为免费开源软件, 融合电镜控制、图像采集和机器视觉, 并整合多种数据处理软件包便于实时数据评估和处理, 支持 SPA、Cryo-ET 及电子晶体衍射, 兼容性强^[125, 129-132], 但对初学者亦存在挑战。Gatan 发展的 Latitude®S 适配旗下的 K2/K3、OneView 相机及能量过滤器, 专注 SPA。JEOL 的 JADAS^[133] 软件针对 SPA 数据自动收集, 适配 JEOL 旗下电镜。

4.2 冷冻电镜数据收集的提速

冷冻电镜数据的数量与质量决定三维重构的分辨率。早期需要依赖人工定位样品采集区域、调试电镜 (如零慧差合轴、消除透镜相差、能量过滤器零峰对中等) 及设置收集参数 (如样品台漂移倾斜角度和稳定等待时间、对焦等), 耗时长、效率低, 需丰富经验^[134]。自动化数据收集软件显著提升效率, 初期实现机械操控稳定性和光学系统精准对齐^[129, 135], 后升级为可自动完成从载网→栅格→孔洞→目标的多尺度成像, 整合光学校正并加入模式选择和参数预设, 减少调试和设置时间, 实现高质量图像的自动化采集^[134, 136]。

传统样品台机械移动定位极大限制了数据采集速度。SPA 数据采集中采用光-图像偏移 (beam-image shift), 通过控制电子束倾斜引起的图像偏移进行导航, 增加单次机械移动所获取的目标数, 极大提升了数据采集速度^[96, 102, 137, 138]。随后, Cryo-ET 数据采集利用几何模型和每个倾斜角度像差校准实现倾斜后精准多点成像^[139-142], 减少倾斜后稳定和重新定位时间^[141], 速度提升至少 3 倍^[143]。此外, 机器学习算法在目标识别和选择上的应用, 也将极大提升区域选择的效率^[122, 132, 142]。

5 AI在冷冻电镜领域的应用

近年来, 人工智能 (AI) 技术飞速发展, 也为冷冻电镜领域带来巨大突破, 尤其针对极具组成与构象异质性 (compositional and conformational heterogeneity) 的动态体系。cryoDRGN 软件^[144] 利用深度神经网络直接重建三维密度图的连续分布, 描绘了单颗粒数据集中构象异质性的完整图谱, 显著提升了对分子动态性的解析能力。CryoTRANS 软件^[145] 通过构建密度图间的自监督伪轨迹, 并以神经网络参数化的常微分方程进行建模, 在保留高分辨率结构细节的同时, 实现了对稀有构象的高效重建。3DFlex 软件^[146] 则基于局部几何结构保持不变的原理, 从二维图像中恢复高分辨率三维密度, 构建蛋白质在连续构象空间中的运动模型。其他软件如 CryoSTAR^[147] 和 Zernike3D^[148] 等工具, 进一步扩展了动态结构分析的技术边界。在数据预处理方面, 深度学习同样展现出强大优势。Topaz^[149] 和 Warp^[150] 等软件实现了电镜图像的高效降噪, crYOLO^[151] 与 EPicker^[152] 等显著提升了颗粒挑选的速度与精度。

在冷冻电子断层扫描 (cryo-ET) 方向, AI 也正推动其流程全面自动化与分析精度的提升。

SPACEtomo 软件^[142]借助机器学习实现了薄片识别、生物特征分割、目标选择与倾斜系列采集的全流程自动控制,显著提高了数据采集效率。针对 cryo-ET 数据中普遍存在的低信噪比与缺失锥 (missing wedge) 问题, IsoNet 软件^[153]通过深度学习迭代恢复缺失信息,同时增强图像质量; DeepDeWedge^[154]则利用自监督机制实现降噪与缺失补全的统一建模。在数据分析方面, AI 对自动图像分割与颗粒识别的推动尤为显著。与传统模板匹配方法相比,基于深度学习的 DeepFinder^[155]、DeepETPicker^[156]、TomoTwin^[157]、MiLoPYP^[158]及 EMAN2 软件包中的 e2gmm^[159]等显著提高了识别效率与准确性; Ais^[160]等图像分割工具提升了亚细胞结构的分辨能力; AI 驱动的子断层平均方法 cryoDRGN-ET^[161]则为原位构象动态分析提供了全新视角。

6 展望

冷冻电镜软硬件技术的持续发展,尤其是人工智能与冷冻电镜的深度融合,正在推进冷冻电镜技术向着捕获生物大分子时间分辨率动态生物过程,揭示其原位时空调控和“分子社会关系”方向飞速发展。同时,电镜技术的快速发展使数据采集速度大幅提升,对数据存储的容量、速度与可靠性以及处理的算法算力形成了前所未有的挑战。然而,挑战亦孕育机遇。尤其在 Cryo-ET 领域,伴随海量数据的持续积累,识别原位未知蛋白、解析其相互作用网络并揭示其参与生命活动的机制,正成为未来研究的重要方向。未来,时间分辨冷冻电镜结合高通量自动化数据采集,必将产生大量蕴含瞬时中间态的图像数据,发展建立利用 AI 工具挖掘分辨瞬时中间态的结构及其可能发生的过程,有望极大提升对生物大分子体系的动态瞬时过程的解析能力。同时, AI 有望推动 Cryo-ET 原位结构解析的全流程自动化,包括数据采集、三维重构、图像降噪、缺失信息补全、目标体系识别与子断层平均,从而显著提升数据采集、分析和重构的吞吐量、准确性和分辨率,有望系统阐释生物大分子复合体的原位时空动态调控机制、网络及其参与的生命过程。另一方面, AI 目标探测结合结构预测和细胞内交联质谱 (in-cell XL-MS) 等新技术,也将为在细胞原位采集的大量断层扫描 (tomogram) 数据中大量未知结构的识别与功能注释提供全新路径,有助于构建细胞尺度的精细结构图谱,拓展 Cryo-ET 在结构细胞生物学中的应用边界,更全面地描绘细胞内“分子社会”

成员及其时空调控网络。

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