Super-resolution cryogenic correlative light and electron microscopy reveals protein organization in the context of intact cellular ultrastructure

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Introduction

To understand how cells work, we need elucidate how proteins interact inside cellular ultrastructure. Super-resolution microscopy, e.g. stochastic optical reconstruction microscopy (STORM) [1], underpins our understanding of interacting molecular networks in cells at the resolution of dozens of nanometres. However, to ascertain protein structure and function relationship, cryogenic correlative light and electron microscopy (cryo-CLEM) [2] is highly sought after because it combines the functional information from molecular tagging in light microscopy with the intact ultrastructure information in electron microscopy. The challenge is the discrepancy in resolving power and imaging volume between cryo-EM and conventional cryo-FM. To address this challenge, we developed cryogenic STORM (cryo-STORM) to achieve sub-10 nm localization precision [3], and 3D Double Helix STORM with extended imaging volume to a few microns in a single shot. We are developing super-resolution cryo-CLEM workflow, aiming at unravelling the structure-function relationship of proteins and their partners throughout the cells with unprecedented precision.

Cryo-STORM incorporating solid immersion lenses images cells with 12 nm resolution

In STORM, light induced stochastic fluorescent switching from certain fluorophores enables time-resolved sequential localizations of single fluorescent molecules at nanometre scale precision, surpassing the classic diffraction limit (~ 250 nm). The resolution in this case is governed by the precision with which individual molecules can be localized. This depends on the numerical aperture (NA) of the optics and the number of collectable photons emitted by the samples. All fluorophores emit many more photons under cryogenic conditions [4], therefore the resolution can be improved substantially by deploying STORM technique under cryogenic conditions. However, the challenge came from that, high NA liquid immersion objectives are incompatible with the ultra-low temperatures, e.g. -196 °C in liquid Nitrogen, under cryogenic conditions. Our approach to address this challenge was to deploy super-hemispherical solid immersion lenses (superSILs) in cryoSTORM. SuperSILs are truncated balls made of solid materials of high refractive index, e.g. 2.17 in cubic zirconia, that fill the gap between the objective and the sample. By using a superSIL to couple the sample to a dry objective, the effective NA of the latter is enhanced up to the value of the refractive index of the superSIL. The breakthrough was the realization that the high NA delivered by superSILs is eminently compatible with cryogenic conditions and particularly beneficial for STORM resolution enhancement. In addition, cryo-fixation preserves biological contents in amorphous ice, which is the optimal method to maintain their native state [5].

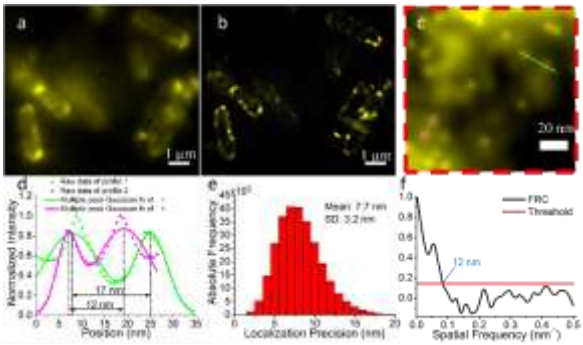

Figure 1. Cryo-STORM of *Escherichia coli* cells. (a) Wide-field and (b) STORM image of a field of cells. (c) The enlarged image of the region of the cell indicated by the read dashed boarder box in (b). (d) Line profiles of the cross-section of two adjacent single molecules. (e) Localization precision histogram. (f) FRC curve.

We developed a superSIL-based cryo-STORM system, and imaged plunge-frozen *Escherichia coli* cells expressing the ATP binding cassette (ABC) transporter protein PH1735, a putative multidrug transporter, fused to enhanced Green Fluorescence Protein (EGFP). Representative conventional wide-field and STORM images are shown in Fig. 1a,b. From the STORM image, we can observe single PH1735-EGFP molecules embedded in the membrane together with larger features. Line profiles of the cross-section of two adjacent single molecules marked by dashed lines in Fig. 1c, an zoom-in view of the area marked by red dashed border box in Fig. 1b, reveal two pairs of molecules separated by ~12 and ~17 nm (Fig. 1d). This resolution is consistent with the observed localization precision of 7.7 nm (Fig. 1e), and the resolution evaluated by Fourier Ring Correlation (FRC) method (Fig. 1f). The measured separation of 12–17 nm probably represents a nucleotide-free inward-open transporter, which is consistent with distances measured on other ABC transporters such as McjD [6]. Our ability to resolve individual proteins in a dimer shows that the resolution of cryoSTORM incorporating SILs is adequate to probe macro-molecular organization in cells.

Double Helix optics enables 3D deep imaging in cryo-STORM

Biological systems exist in a 3D environment, however, accessing axial information has long been a challenge in light microscopy. The challenge is deeply rooted in the restricted imaging depth, typically below 1 µm, in light microscopy. Moreover, the light emitted from out-of-focus fluorophores causes high background and reduces signal-to-noise ratios in the images of in-focus object. An approach to localize individual

molecules in 3D is to alter the intrinsically symmetric shape of their point-like images, i.e. Point Spread Functions (PSFs, Fig. 2a), and then the axial information of a molecule can be encoded in the characteristic PSF shapes across imaging depth. By introducing a double-helix phase mask in a STORM microscope [7], the lateral cross-section of a PSF decomposes into two side lobes that rotate around each other as the axial position is changed (Fig. 2b). In lateral directions, the angle between the two side lobes provides critical information of the axial position of the molecule in an extended imaging depth (a few microns).

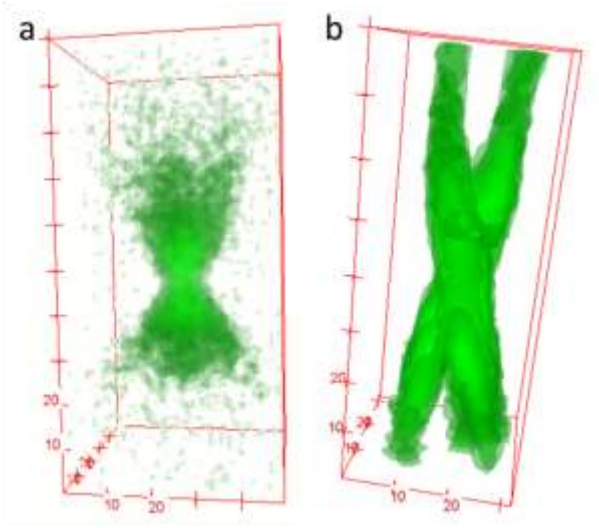

Figure 2. Point Spread Function in (a) Conventional wide-field fluorescence microscopy and (b) Double Helix optics-based microscopy.

Cryo-focused ion beam /scanning electron microscopy offers nanometre scale volume imaging of intact cellular ultrastructure

Beyond optical microscopy, cryo-EM has the power to image biological samples at atomic level resolution [8]. Cryo-focused ion beam /scanning electron microscopy (Cryo- FIBSEM) is a powerful tool for volumetric structural imaging of whole cells by serial milling and imaging, achieving a few nanometre resolution [9]. In SEM, electrons interact with a sample and generate secondary electrons from which EM images can be obtained. FIB shares the same physical principle as SEM, but uses a beam of ions to interact with samples. FIB can directly mill the specimen surface through the sputtering process, which can be controlled at nanometre scale precision. Cryo-FIBSEM integrates a cryo-SEM system with a FIB instrument, allowing SEM imaging of FIB-milled surfaces of frozen vitrified biological samples kept in their near-native state. The capability of precise sample milling under cryogenic conditions is also crucial for the production of ultrathin lamellas required for ultra-resolution electron tomography (ET) in cryogenic transmission electron microscopy (cryo-TEM) [10].

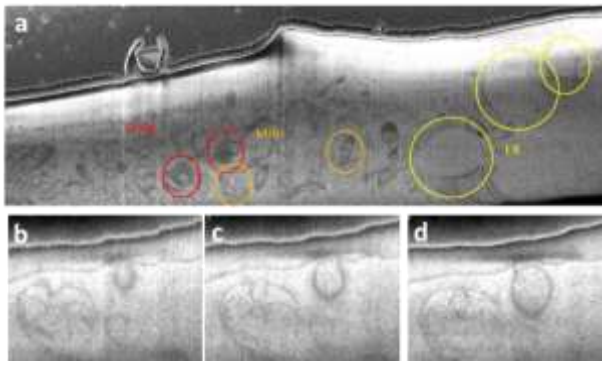

Figure 3. Cryo-FIBSEM of a Chinese hamster ovary cell. (a) Membranes from cellular organelles as marked in the image. (b-d) Consecutive slices of endosomes and a Multivesicular body.

Central Laser Facility's Octopus group is commissioning a ZEISS Crossbeam cryo-FIBSEM system, funded by a BBSRC Alert 18 grant with a substantial contribution from the CLF. The cryo-FIBSEM image of a Chinese hamster ovary (CHO) cell is shown in Fig. 3. In Fig.3a, multiple cellular organelles, such as Golgi, Mitochondria and Endoplasmic Reticulum (ER), are clearly visible. Volumetric information of endosomes and a possible Multivesicular body can be obtained from the consecutive slices of the image series (Fig. 3b-d).

Super-resolution cryo-CLEM discovers protein structure and function relationship in cells' native state

To answer key questions concerning the *in situ* structural biology, super-resolution cryo-CLEM workflows are under development to combine super-resolution cryo-STORM with cryo-FIBSEM and/or cryo-ET. Some initial result is shown in Fig.4. Here, ABC transporter protein PH1735 fused with EGFP in a plunge-frozen *Escherichia coli* cell was firstly imaged in cryo-Airyscan microscopy incorporating a superSIL, and then imaged in cryo-FIBSEM. The super-resolution microscopy (yellow color rendered) and EM (grey scale rendered) images were aligned and superimposed to show correlative information. Protein organization from specific labelling is observed in the super-resolution microscopy image, and cellular ultrastructure is observed in the FIBSEM image.

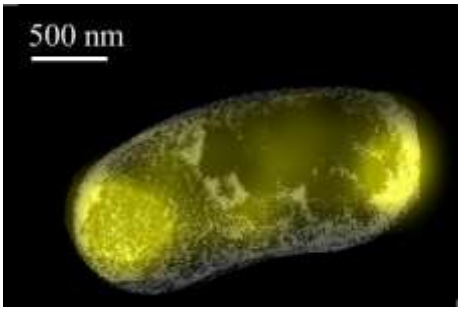

Figure 4. Super-resolution cryo-CLEM image of an *Escherichia coli* cell. Cryo-superSIL-Airyscan microscopy image of PH1735-EGFP in rendered in yellow color, while cryo-FIBSEM image of cellular ultrastructure is rendered in grey scale.

In the workflows, frozen vitrified samples are imaged in cryo-STORM to locate regions of interest in 3D with around 10 nm resolution, and then these images are used to guide FIBSEM preparation of lamellae suitable for cryo-ET. A new type of cryo-STORM, namely Minimal Photon Fluxes (MINFLUX) [11], is also used to identify individual molecular species at ~1-3 nm resolution in 3D. The information is used to annotate molecular species in tomograms and to determine which molecules imaged in the tomograms should be averaged for structure determination. In this way, we will achieve *in situ* structure determination of molecules and molecular complexes at specific times and in specific compartments, allowing us to unravel the structure-function relationship of proteins and their partners throughout the cells.

Conclusions

Super-resolution cryo-CLEM, with minimal discrepancy in resolving power and imaging volume, gathers the strengths of fluorescence microscopy for high specificity imaging with ultrastructure imaging provided by electron microscopy, which promises new insights into biological processes and molecular anatomy. Super-resolution cryo-STORM using Double Helix optics enhances the resolution performance of conventional cryogenic light microscopy by an order of magnitude in 3D. Furthermore, the lateral resolution can be improved to around 10 nanometres by incorporating superSILs in cryo-STORM. This

paves the way of imaging single macro-molecules using optical means. Super-resolution cryo-CLEM workflow we are developing will represent a breakthrough for *in situ* structure determination and structure-function relationship elucidation of proteins and their partners. We expect the method will be applied in a wide range of biological research areas.

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