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Blotting-free and lossless cryo-electron microscopy grid preparation from nanoliter-sized protein samples and single-cell extracts

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ABSTRACT

We present a sample preparation method for cryo-electron microscopy (cryo-EM) that requires only 3–20 nL of sample to prepare a cryo-EM grid, depending on the protocol used. The sample is applied and spread on the grid by a microcapillary. The procedure does not involve any blotting steps, and real-time monitoring allows the water film thickness to be assessed and decreased to an optimum value prior to vitrification. We demonstrate that the method is suitable for high-resolution cryo-EM and will enable alternative electron microscopy approaches, such as single-cell visual proteomics.

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1. Introduction

In recent years, transmission electron microscopy (TEM) of vitrified specimens (cryo-EM; Dubochet et al., 1988) has become a powerful technique for the high-resolution structural analysis of biological matter (Liao et al., 2013), and is now increasingly recognized as a mainstream tool in biology (Callaway, 2015; Kuhlbrandt, 2014; Nogales, 2015). Several technical achievements have made this development possible, the most prominent being the recent introduction of direct electron detection (DED) cameras (Milazzo et al., 2011; Ruskin et al., 2013; Veesler et al., 2013), and the availability of improved data processing algorithms (Grigorieff, 2007; Lyumkis et al., 2013; Scheres, 2012).

Cryogenic sample-grid (cryo-EM grid) preparation and imaging methods ensure that biological specimens withstand the ultra-high vacuum inside electron microscopes, allow their investigation while trapped at physiological conditions that conserve the structural arrangement of the biomolecules and reduce the effect of radiation damage (Baker and Rubinstein, 2010; Dubochet et al., 1988; Lepault et al., 1983). However, these preparation methods have not improved significantly over the last 20 years and have some major drawbacks: a 2–4 μL sample volume is required, and 99.9% of the sample volume is lost during grid preparation, due to an extensive blotting step made with filter paper (Kemmerling et al., 2012). Furthermore, blotting with filter paper can lead to protein aggregation or denaturation. The current state of the art sample preparation methods are recognized as one of the most significant limitations in cryo-EM (Glaeser, 2016).

Improved cryo-EM grid preparation strategies that reduce sample consumption are now an essential requirement. A device that combines ink-jet picoliter dispensing with a plunge-freezing apparatus was presented in 2012 (Jain et al., 2012), and was recently refined by the use of self-blotting grids to allow cryo-grid preparation in combination with ink-jet spotting (Razinkov et al., 2016). Here, we present a paper blotting- and spotting-free method that requires total sample volumes of just a few nanoliters (e.g., 3–20 nL). It uses a microcapillary to directly 'write' the sample on holey carbon EM grids and subsequently vitrifies the deposited liquid, producing thin layers of vitrified specimen in the holes of the carbon film.

2. Material and methods

The instrument presented here is described in the Section 3. Additional aspects were published previously, such as the conditioning and preparation of nanoliter volumes for negative stain EM, or the single-cell lysis and aspiration device using a microcapillary electrode (Arnold et al., 2016; Kemmerling et al., 2013). Supporting Information S1 provides a detailed description of the

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setup, including a parts lists and details of the method. Supporting Information S2 describes the control software.

2.1. Chemicals and buffers

All chemicals were ordered from Sigma-Aldrich, Switzerland if not otherwise indicated. The following buffers were used: *PBS*, Dulbecco's Phosphate Buffered Saline, 2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.9 mM $Na_2HPO_4{\cdot}7H_2O$, pH 7.4; *Tris-HCl buffer*, 20 mM Tris-HCl, 50 mM NaCl (pH 7.4); *HEPES buffer*, 10 mM HEPES pH 8.0, 50 mM KCl.

2.2. Test samples

The test samples were (i) tobacco mosaic virus (TMV) in PBS containing 0.1% decyl-β-D-maltopyranoside (DM, Anatrace), (ii) a mixture containing apoferritin and phages against *Escherichia coli* (vB_EcoM_CBA120) in Tris-HCl buffer, and (iii) a purified 200 kDa membrane protein (name not disclosed) reconstituted in amphipols. In this case, the bulk membrane protein sample was applied to a S200 10/300GL size exclusion chromatography column equilibrated with HEPES buffer complemented with 5 μM sodium azide. A single 300 μl fraction corresponding to the center of the major A280 peak was used to prepare cryo-EM grids (a) by using the cryoWriter and (b) by using a Vitrobot IV plunge-freezer (FEI, USA). The protein concentration of this fraction was 0.35 mg/mL as determined by absorbance measurement using an UV–vis spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific Inc.) and assuming Abs (0.1%) = 1. (iv) Urease from *Yersinia enterocolitica* bacteria at a concentration of 0.35 mg/mL in PBS buffer.

2.3. EM grids

Various EM grids were used with the cryoWriter, such as 200-mesh copper grids with holey carbon film (R2/2, R2/1, and R1.2/1.3, Quantifoil, Germany), as well as holey carbon grids with an additional layer of continuous carbon, and lacey carbon grids. EM grids were glow discharged for 30 s in air plasma immediately before use. Note that the flatness of the grid is an important aspect, both for the mechanical writing step and for efficient heat-transfer when the grid is on the temperature controlled dew point stage (DP-stage) of the cryoWriter.

2.4. Cryo-grid preparation

The two deposition protocols used with the cryoWriter setup are detailed in the sections below and in Supporting Information S1.

2.5. Electron microscopy and single particle analysis

Unless otherwise specified, the EM grids with test samples were placed in a Gatan-626 cryo-holder and imaged in a Philips CM200 (FEG) TEM operated at 200 kV and equipped with a TVIPS F416 4k CMOS camera (TVIPS, Germany).

Images of urease for single particle analysis were acquired using a FEI Titan Krios, operated at 300 kV and equipped with Gatan Quantum-LS (zero-loss slit width of 20 eV) and Gatan K2 Summit DED camera. Low dose conditions were applied with a total electron dose of ~50 e^-/A^2 at a dose rate of 5–6 e^-/physical-pixel/s for a stack of 40 images obtained in movie mode. Drift- and contrast transfer function (CTF) correction was applied to the urease data with the Zorro software (McLeod et al., submitted) using CTFFIND4 (Rohou and Grigorieff, 2015). Approximately 10,000 particles were manually selected from the 51 micrographs recorded using EMAN2 (Tang et al., 2007) and subsequently processed in RELION-1.4 (Scheres, 2012). After particle alignment, around 8700 particles yielded good 2D class averages that were subsequently used for the 3D classification. The two best 3D classes were selected (containing ~7800 particles) and iteratively refined. A final sharpening of the map was then performed, yielding the final 3D reconstruction of the urease complex. The atomic model (PDB: 10.2210/pdb4z42/pdb) was fitted into the 3D map using the UCSF chimera software (Pettersen et al., 2004).

3. Results

3.1. Principles and setup

The cryoWriter setup and the principles of the method are depicted in Fig. 1; further details can be found in Supporting Information S1 and S2. The cryoWriter is integrated into a liquid handling and transfer system developed previously for nanoliter-volume sample conditioning and EM grid preparation for negative stain and trehalose embedding (Arnold et al., 2016) and is not enclosed in a humidity-controlled environment. The method uses a high-precision pipetting system to control liquid uptake and dispensing by a microcapillary, a temperature-controlled DP-stage to control the environment of the EM grid, a real-time monitoring system to control the thickness of the sample layer, and a pick-and-plunge-mechanism for sample vitrification. The entire process is controlled by an openBEB (Ramakrishnan et al., 2014) plug-in developed in LabVIEW (Elliott et al., 2007) and can be automated using macros (see Supporting Information S2).

The setup for cryo-grid preparation and the basic procedure (Fig. 1) are as follows: (i) *Grid cooling*: a glow-discharged standard holey carbon film EM grid held by tweezers is positioned on the cold DP-stage. (ii) *Sample up-take*; a microcapillary and high-precision pump system are used to aspirate a liquid plug from a sample stock or the lysate of a single cell immediately after electrolysis, as described previously (Arnold et al., 2016; Kemmerling et al., 2013). (iii) *Sample deposition* (Fig. 1a), the microcapillary tip is brought into close proximity of the grid surface. The sample is then dispensed from the microcapillary to form a small liquid droplet that spans the gap between the microcapillary tip and the hydrophilic (glow-discharged) holey-carbon surface of the EM grid. Simultaneously, the grid is moved relative to the nozzle in a sinusoidal or circular pattern; the liquid sample is spread-out over the surface filling the holes of the carbon film. (iv) *Sample stabilization and thinning* by controlled water evaporation (Fig. 1b and dedicated section below); the temperature of the DP-stage is set to keep both the EM grid and the suspended sample at a specified temperature close to the dew-point temperature of the room, throughout. Note that the grid is not immediately vitrified, but can be kept on the stage for a short time (between <1 and 10 s, depending on the chosen offset temperature), so that a certain amount of sample water can evaporate. Interference/scattering effects from interaction of the grid and applied aqueous sample with a 780 nm laser beam can be used to monitor the evaporation (Fig. 2). The controlled loss of some water is important to ensure that a thin water film spans the holes of the carbon layer. (v) *Cryo-grid pick-and-plunge freezing* (Fig. 1c); the mechanism is triggered by the monitoring system or after a pre-set time, rapidly plunging the grid (and tweezer tips) into a liquid ethane/propane (40:60) bath (Tivol et al., 2008) for vitrification (see also Supporting Information S1 and Supplementary Movie 1).

Two deposition protocols can be used with this basic procedure, depending on the experiment and sample. In Protocol 1, a relatively large volume (20 nL) is deposited on the grid, excess sample is removed and recovered by re-aspiration with the dispensing microcapillary; this protocol is ideal for the preparation of large

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