White Rose Research Online

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk
Universities of Leeds, Sheffield and York

Deposited via The University of Leeds.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/177615/

Version: Accepted Version

Article:
Klebl, DP, Sobott, F, White, HD et al. (2021) Fast Grid Preparation for Time-Resolved Cryo-Electron Microscopy. Journal of Visualized Experiments, 177. e62199. ISSN: 1940-087X

https://doi.org/10.3791/62199

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

UNIVERSITY OF LEEDS
University of Sheffield
UNIVERSITY of York

Fast Grid Preparation for Time-Resolved Cryo-Electron Microscopy

David P. Klebl[1], Frank Sobott[2], Howard D. White[3] & Stephen P. Muench[1*]

[1] *School of Biomedical Sciences, Faculty of Biological Sciences & Astbury Centre for Structural and Molecular Biology, University of Leeds, Leeds, LS2 9JT, UK*.

[2] *School of Molecular and Cellular Biology, Faculty of Biological Sciences & Astbury Centre for Structural and Molecular Biology, University of Leeds, Leeds, LS2 9JT, UK*.

[3] *Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, Virginia, USA.*

*corresponding author s.p.muench@leeds.ac.uk

Keywords: cryo-EM; sample preparation; time-resolved

Summary Here we provide a detailed protocol for the use of our rapid grid making device for both fast grid-making and for rapid mixing and freezing to conduct time-resolved experiments.

Abstract

The field of cryo-electron microscopy (cryo-EM) is rapidly developing with new hardware and processing algorithms producing higher resolution structures and information on more challenging systems. Sample preparation for cryo-EM is undergoing a similar revolution with new approaches being developed to supersede the traditional blotting systems. These include the use of piezo-electric dispensers, pin printing and direct spraying. As a result of these developments the speed of grid preparation is going from seconds to milliseconds providing new opportunities, especially in the field of time-resolved cryo-EM where proteins

and substrates can be rapidly mixed before plunge freezing, trapping short lived intermediate states. Here we describe, in detail, our standard protocol for making grids on our in-house time-resolved EM device both for standard fast grid preparation and also for time-resolved experiments. The protocol requires a minimum of about 50 µL sample at concentrations of ≥ 2 mg/mL for the preparation of 4 grids. The delay between sample application and freezing can be as low as 10 ms, one limitation is increased ice thickness at faster speeds and compared to the blotting method. We hope this protocol will aid others in designing their own grid making devices and those interested in designing time-resolved experiments.

Video Link

-

Introduction

Background

Recent developments in cryo-electron microscopy (cryo-EM) have enabled structural studies of increasingly complex systems at high resolution. With few exceptions, such studies have been limited to biological macromolecules at equilibrium [1] or relatively slow reactions [2]. Many processes *in vivo* occur on a faster timescale (milliseconds) and there is increasing interest in time-resolved cryo-EM (TrEM) on these timescales [3]. However, conventional cryo-EM sample preparation by the blotting method is too slow for millisecond TrEM.

The blotting method has other limitations besides poor time resolution. Proteins and protein complexes can suffer from denaturation or preferred orientation on grids [4]. Reducing the exposure time to the air-water interface during sample preparation has been shown to

mitigate preferred orientation and protein denaturation [5,6]. Thus, fast grid preparation not only enables millisecond TrEM but can also improve grid quality.

Currently, there are three different approaches to automated grid preparation. The first approach uses a pin or capillary which holds a small amount of sample. After establishing contact between the liquid and the grid surface, the sample is 'written' onto the grid [7,8]. The sample application process is relatively slow and takes a few seconds. An alternative approach uses controlled droplet generation by a piezo dispenser and self-wicking grids [9]. This allows faster dispense to freeze times, but is still limited by droplet and wicking speed (currently reaching 54 ms). The fastest approach so far is the direct spray approach, in which the sample is atomized in a spray nozzle and the small (~ 10 – 20 µm) and fast (> 5 m/s) droplets spread upon contact with the cryo-EM grid. The sample spray can be generated through different ways such as airblast atomizers, surface acoustic waves or ultrasonic humidifiers [10-13]. In our experience, the ice thickness with the direct spraying approach is greater but direct spraying enables dispense to freeze times < 10 ms.

This protocol describes step-by-step how a time-resolved EM device (TED) equipped with a microfluidic spray nozzle can be used to prepare grids on a fast timescale [14,15]. The device has been used to prepare grids with a minimum delay time of 6 ms between sample application and freezing and to rapidly mix and freeze two samples. The design of the TED is based on a previous version [16] and is similar to other spray-based time-resolved cryo-EM devices [17].

First, the four main parts of the TED setup are described: The core of the TED is the liquid handling unit, which is responsible for sample aspiration and dispensing. A pneumatic plunger

moves the grid through the spray into the liquid ethane. Generation of the spray is achieved with microfluidic spray nozzles and freezing is done in a liquid ethane container, which are described briefly. Lastly, the additional features to control the grid environment, especially humidity, are highlighted. This is followed by detailed protocols for the operation of the device and for conducting TrEM experiments. Representative results are given for fast grid preparation and a simple TrEM experiment.

Experimental Setup

The liquid handling unit

The liquid handling system of the TED is formed by three syringe drive pumps ('pumps 1 – 3'), each equipped with a rotary valve (Figure 1). A power supply provides pumps 1 – 3 with 24 V DC. Communication with the control software (written in Visual Basic and C++) is via a RS232 interface to pump 1. Commands are distributed through the serial I/O expansion ports from pump 1 to pumps 2-3. Pumps 1-3 are equipped with glass syringes ('syringes 1-3', we use 250 µL/zero dead volume syringes here). Each valve has two positions, 'load' and 'dispense'. The 'load' position is used to aspirate sample into the syringe. A short piece (~ 3 - 4 cm) of 1/16" O.D., 0.01'' I.D. FEP tubing is connected via ETFE/ETFE flangeless fittings to the 'load' position of valves 1-3. This short piece of tubing reaches into the sample reservoir (typically a 1.5 mL or 0.5 mL plastic tube). The 'dispense' position leads to the spray nozzle. Connection between the 'dispense' outlet and the spray nozzle is made by PE tubing (~ 20-30 cm length, 0.043" O.D., 0.015" I.D.), with a short piece of sleeve tubing (~ 0.5 cm) and ETFE/ETFE flangeless fittings.

The pneumatic plunger

The TED uses a pneumatic plunger to accelerate the grid and move it through the sample spray into the liquid ethane container. Negative pressure tweezers hold the grid, screwed into a home-built holder which is mounted to a dual rod pneumatic cylinder (Figure 2 A).

Pressure is supplied from a large nitrogen gas cylinder (size W), equipped with a multistage regulator (0 – 10 bar, 'main pressure'). Flexible reinforced PVC tubing (12 mm OD.) connects the regulator to a 12-port manifold where pressurized nitrogen is delivered to the nozzle and the pneumatic plunger. Gas flow through the nozzle is constant, regulated directly at the nitrogen cylinder (main pressure). The connection to the nozzle is made with PU tubing (4 mm O.D., 2.5 mm I.D.), a short piece of PE tubing (~ 8 cm length, 0.043" O.D., 0.015" I.D.) and appropriate connectors. Pressure on the pneumatic plunger is controlled through an solenoid valve. PU tubing (4 mm O.D., 2.5 mm I.D.) connects the solenoid valve with a regulator and the pneumatic plunger, to allow a reduced plunge pressure (≤ main pressure). The solenoid valve is computer controlled. A schematic overview of the setup is given in Figure 2 B.

Note that with this setup the plunge pressure is always equal or smaller than the spray gas pressure (main pressure). However, the setup can easily be changed by incorporating a second regulator upstream of the spray nozzle to allow higher plunge speeds at low spray gas pressure. High pressures (>> 2 bar) can damage the PDMS spray nozzle.

Caution:
This is a pressurised system and the 'main pressure' should always be < 7 bar.

Pressures between 0.5 and 2 bar are typically used for the pneumatic plunger and show an approximately linear relation between pressure and speed (at the vertical position of the spray). Plunge speeds are measured with an oscilloscope, connected in line with a slide potentiometer (10 kΩ) and in parallel with a 2 kΩ resistor (Figure 2 C). A power supply provides the potentiometer with 9 V DC. While the approximate plunge speed is set prior to the experiment by setting the plunge pressure, the potentiometer gives a precise readout of the speed after the experiment.

Spray nozzles and liquid ethane container

The fabrication and operation of gas-dynamic virtual nozzles for spray-based sample delivery has been described elsewhere in detail [15]. As described above, the 'dispense' outlets of valves 1-3 are connected to the liquid inlets of the nozzle (Figure 3 A). The pressurized spray gas is connected to the gas inlet of the nozzle. The inlets in the PDMS spray nozzles are such that 0.043" O.D. PE tubing can be used directly without the need for fittings. Our nozzle design contains a 'jet-in-jet' geometry for mixing of two samples, similar to the device described in ref. [18]. A schematic of the design is shown in Figure 3 B, a microscopic image of a nozzle is shown in Figure 3 C. The layout of the microfluidic device requires the use of three syringes to mix two samples. The spray nozzle is typically positioned at 1-1.5 cm distance from the grid (during sample application).

We use liquid ethane as a cryogen, in a liquid ethane/nitrogen container as used for the standard blotting method. Vertical positioning of the liquid ethane cup is achieved with a laboratory lifting platform.

Control of the spray and grid environment

The plunger and spray nozzle are contained within a custom built PMMA (acrylic glass) box with a double door (Figure 4 A). High relative humidity inside the box is achieved by an air-humidification system at the back of the TED (Figure 4 B). Air is supplied by a pump and fed into a first 10" canister (typically used for under sink water purification). The canister is filled with a low (~ 5-10 cm) level of water and also houses a humidifier unit. Mains power to the humidifier is controlled by a digital humidity/temperature controller and a humidity/temperature sensor located inside the acrylic glass box. The controller is set to turn off the pump when the relative humidity reaches ≥ 90 %. Humidified air from the first canister is pumped through a diffuser, immersed in water in a second 10" canister and then enters the acrylic glass box.

Caution:
Because the sample is aerosolized in the spray nozzle, hazardous biological or chemical specimen are not suitable as samples.

The run sequence

The 'run script' button in the control software initiates the run sequence. This sequence of commands can be pre-defined in a script file and altered through the software. The most important variables are explained here:

Spray speed: The spray speed determines the liquid flowrate used by the syringe pump. The flowrate can be calculated as follows:

The syringe pump motors used here have a fixed step size. The full range of the pump is divided into 48,000 steps.

The second important factor is the syringe volume. We typically use 250 µL syringes.

The spray speed in the control software is set as number of steps/second. A spray speed of 1000 steps/second corresponds to:

$$1000\frac{steps}{s} * \frac{250\ \mu L}{48000\ steps} = 5.2\frac{\mu L}{s}$$

Spray volume: The spray volume determines the total volume to be sprayed. Thus, it also determines the duration of the spray. The spray volume in the control software is set as a number of steps. A spray volume of 2000 steps, at a spray speed of 1000 steps/second, leads to a spray duration of 2 s and a total volume of 10.4 µL.

Pre-spray time: This variable defines the time between initiation of the spray and plunge. It is important to choose the delay time such that:

The spray has sufficient time to stabilize before plunging the grid. Usually, the spray is given 1.5 – 4 s to stabilize before the grid is plunged.

The spray is maintained until the grid has moved through. Usually, the liquid flow (and therefore the spray) is stopped 0.5 to 1 s after the grid has been plunged.

Using a spray speed of 1000 steps/s and a spray volume of 2000 steps, a typical pre-spray time is 1.5 s, for example.

An exemplary sequence of commands is shown in Figure 5 A, the grid position over time is illustrated in Figure 5 B.

Protocol

1. Preparing the System

The following protocol describes how to prepare grids of a single sample. Usually, a minimum of 2 replicate grids are prepared for each sample or condition. For faster plunge speeds (less than ~ 20 ms time delay), 3 or 4 replicate grids are typically prepared to account for a reduced number of thin ice areas.

1.1 The protein sample is diluted to the target concentration in the desired buffer. Typically, final concentrations ≥ 2 mg/mL work well for grid preparation with the TED. Note that the sample can be kept on ice until step 10, from step 10 onwards the sample will spend considerable time at room temperature as it takes approximately 20 min to prepare 3-4 grids.

1.2 Turn on the TED. Then turn on the control PC and start the control software.

1.3 Initialize all syringe pumps by pressing the 'initialize' button (lower black button) on each syringe pump.

1.4 Turn on the potentiometer power supply, set it to 9 V and start the oscilloscope control software.

1.5 Ensure that the regulator valve of the N_2-cylinder is closed. Open the cylinder valve. Then, slowly open the regulator valve setting the outlet pressure to the desired value for the spray gas pressure (typically 1-2 bar). For the PDMS nozzles used here, do not use pressures higher than ~ 2.5 bar to avoid damage to the nozzle. The continuously flowing gas prevents liquid from dripping and accumulating at the nozzle tip which can lead to irreproducible spraying.

1.6 Ensure all syringe pump valves are in the 'load' position. This can be done by switching all valves to the 'dispense' position in the control software and then back to 'load'. Leave all valves switched to 'load'. Set all syringes to zero.

1.7 Any air-bubbles present in the system are removed at this stage. To do this, the syringes may have to be unscrewed, bubbles removed manually and the syringes mounted again when containing no more bubbles.

1.8 The liquid handling components are usually stored in H_2O. Before loading the sample solution, the liquid system is equilibrated with buffer. This is done by washing the tubing with an excess of buffer. An appropriate maximum liquid flowrate is chosen to avoid overpressure on the spray nozzle. Liquid flowrates > 10 µL/s may cause damage to the PDMS nozzles described here.

1.9 Place a 1.5 mL tube containing ≥ 200 µL buffer onto syringe 1 (the top must be pierced to attach to tubing).

1.9.1 Make sure valve 1 is in 'load' position. Switch in the control software, if necessary.

1.9.2 Aspirate the desired amount (typically 50-100 µL) of buffer with syringe 1, through the control software.

1.9.3 Switch valve 1 to the 'dispense' position, through the control software.

1.9.4 Dispense all of the liquid in syringe 1, through the control software.

1.9.5 Return valve to 'load', reset the syringe position to '0' in the program and press initialize on syringe 1, to prepare the system for the next cycle.

Steps 1.9.1 - 1.9.5 are usually repeated three times to ensure thorough washing of the tubing.

1.10 Tweezers with an EM grid are loaded by loosening the plunging arm clamp, placing the tweezers in the clamp and then tightening so that the tweezers will not fall out during high-speed plunging. The top side of the grid faces the spray nozzle.

1.11 Position the spray nozzle. An EM grid (no requirement for any specific grid type) is mounted and manually moved to the vertical position of the nozzle (the 'sample application' position). If nozzle and grid are aligned, liquid will accumulate on the grid after spraying for an extended period. If necessary, adjust the nozzle position.

1.12 Adjust the position of the liquid ethane cup by manually moving the plunging arm with mounted tweezers to its end position (reaching into the ethane cup). When set up correctly, a grid held by the tweezers will reach approximately the centre of the liquid ethane cup.

1.13 Check that the 'run script' will use the desired flowrates, volumes and timings. See section 'The run sequence' above for details.

1.14 Make sure nothing is obstructing the path of the plunger. Aspirate the volume of buffer required for a single run into syringe 1. This is done in the control software, see section 'The run sequence' for typical setting and volumes. Then make sure valve 1 is switched to the 'dispense' position. Perform a test run by pressing 'run script' in the control software.

Caution:

Stay clear of the TED until the run sequence is finished. Moving parts could cause injury!

1.15 When the 'run sequence' is finished, set the pressure on the plunging arm to the desired value (typically 1-2 bar). Only then press 'ok' in the control software to release pressure from the plunging arm. If the 'run sequence' or pressure on the plunging arm need to be adjusted, these settings may be changed at this stage and steps 1.13 - 1.15 repeated.

2. Fast Grid Preparation

2.1 The liquid ethane/nitrogen container is filled, first with liquid nitrogen. When sufficiently cold and free of liquid nitrogen, the cup is filled with liquid ethane. Avoid solidification of the liquid ethane. This step is the same as for conventional grid preparation.

Note: To minimize ethane contamination, ensure that steps 2.2 – 2.13 are performed as quickly as possible

Caution:

Liquid ethane is a cryogen and flammable. Care should be taken when handling.

2.2 Cryo-EM grids are prepared for glow-discharge. We typically use holey carbon grids and glow-discharge for 90 s, at 0.1 mbar air pressure and 10 mA. Usually, no more than 4 grids are glow discharged at a time. The grids are used within 30 min of glow-discharging.

2.3 The tubing is then equilibrated with sample (following steps 1.9.1 - 1.9.5, using sample instead of buffer). If the available sample volume is low, the tubing may be equilibrated with only 1 dead volume.

2.4 Aspirate the amount of sample that is needed for a single run into syringe 1 in the control software (see section 'The run sequence' for details). Then switch valve 1 to the 'dispense' position.

2.5 Check relative humidity has reached the desired value (we typically prepare grids at 60 % relative humidity or higher). Once ≥ 60 % humidity is reached, open the humidity chamber only for a minimal amount of time, to maintain high humidity.

2.6 Place the tweezers, holding a glow-discharged grid, in the pneumatic plunger and fix them. Move the plunger to its start position (at the top).

2.7 Make sure the slider of the potentiometer is in the start position, ready for the measurement, by moving it manually to contact the plunger. Set the trigger on the oscilloscope in the oscilloscope software.

2.8 Place the liquid ethane/nitrogen container.

2.9 Press 'run script' in the control software. When prompted, click 'ok' in the control software to start the run.

Caution:

Stay clear of the TED until the run sequence is finished. Moving parts could cause injury!

2.10 Once the 'run sequence' is completed, release the pressure on the pneumatic plunger by clicking 'ok' in the control software.

2.11 Open humidity chamber, loosen the connection between plunging arm and tweezers with one hand while securing the tweezers with the other. When the tweezers are free, move the plunging arm up while keeping the grid in the liquid ethane. Then transfer the grid to its storage space in the surrounding liquid N_2. After freezing, the grid needs to be kept at liquid N_2 temperature at all times.

2.12 Save the oscilloscope measurement. Manually reset the position of the potentiometer slider and plunger afterwards.

2.13 Repeat steps 2.4-2.12 to prepare replicate grids

2.14 Transfer the grids into long-term storage until grid clipping and data collection.

2.15 Wash the system with buffer, according to steps 1.9.1 – 1.9.5. Then wash the system with H_2O, according to steps 1.9.1 – 1.9.5.

2.16 Turn off the main nitrogen gas regulator and turn off the power.

2.17 Place the ethane/nitrogen container in a fume hood to let it warm up and let the liquid ethane and N_2 evaporate.

3. Time-resolved cryo-EM

When time-resolved experiments are conducted with the TED, there are additional aspects to be considered, although the basic setup and variables remain the same. It is assumed here that two solutions are mixed in a 1:1 (v/v) ratio to produce the final mixture which is deposited on the grid.

Follow the protocol described in '1. Fast grid preparation with the TED', with the following changes:

3.1 Higher stock concentrations are required for mixing experiments than for simple spray experiments. Mixing in a 1:1 (v/v) ratio will result in a 2x dilution of each component.

3.2 For a rapid mixing experiment, use all three syringes rather than just a single one.

3.2.1 Attach tubing to syringe pumps 2-3.

3.2.2 Attach tubing from syringe pumps 2-3 to the spray nozzle.

3.1.3 Equilibrate all three syringes in buffer and sample separately. Typically, syringe 1 is filled with sample A and syringes 2-3 are filled with sample B (see Figure 3).

3.3 The run sequence needs to be changed. An example for a run sequence using all 3 syringes for a rapid mixing experiment is given in Figure 6. See also section 'The run sequence' for details.

3.4 Different time delays can be achieved in two ways:

3.4.1 Changing the plunger speed. By adjusting the plunger speed, the time delay can be changed in a relatively narrow range. For example, with a spray/ethane distance of 2 cm, the plunger can be moved at 1 m/s or 2 m/s to give a time delay of 20 ms or 10 ms, respectively. This is done as described in step 1.15.

3.4.2 The spray/ethane position is changed by adjusting the (vertical) position of the spray nozzle. If the nozzle is positioned at a 5 cm distance from the ethane surface, for example, a plunge speed of 1 m/s gives a time delay of 50 ms.

Achieving significantly longer time delays requires further modifications to the setup.

Note: Due to laminar flow in the flow focussing region of the nozzle, we do not expect significant mixing in this part of the nozzle. Instead, we expect that mixing occurs during spray generation, in droplets en route to the grid and during droplet spreading on the grid. The time of flight for spray droplets to reach the grid is estimated ≤ 1 ms (for a droplet speed of ≥ 10 m/s and a nozzle-grid distance of 1 cm). Thus, only the time between droplet landing and vitrification is considered the 'time delay'.

Representative Results

1. Fast grid preparation with the TED

As a test specimen for fast grid preparation, we have used apoferritin from equine spleen at 20 µM in 30 mM HEPES, 150 mM NaCl, pH 7.5. A reconstruction at 3.5 Å resolution was obtained from 690 micrographs as described in ref. [15] (Figure 7 A). The defocus range was chosen so that particles can easily be identified in the raw images (Figure 7 B). A typical grid prepared with a time delay of 10-40 ms shows sufficient areas of thin ice (Figure 7 C) to allow

for collection of > 1000 micrographs. The resulting resolution is likely limited by ice thickness, tomographic analysis of a number of different grids showed 96 ± 33 nm ice thickness [6].

2. Time-resolved cryo-EM

In order to demonstrate rapid mixing and time-resolution using the TED, we have used dissociation of the actomyosin complex by mixing with ATP as a model reaction [19]. This choice was based on the easy distinction between actomyosin and free F-actin from raw micrographs and the well understood kinetics of the reaction.

In the following, the representative results for TrEM of dissociation of the actomyosin complex by ATP are shown. Detailed experimental procedures and results are provided in ref. [19]. In brief, F-actin and rabbit skeletal myosin S1 were mixed in 10 mM MOPS, 50 mM KAc, 2 mM $MgCl_2$, 0.1 mM EGTA, pH 7 at final monomer concentrations of 40 µM to prepare the actomyosin (AM) complex. AM complex was loaded into syringe 1 and 200 µM MgATP in the same buffer was loaded into syringes 2 and 3. The key experimental parameters for the two different timepoints prepared are listed in Table 1.

In both cases, the result was a 1:1 v/v mixture of AM complex and MgATP, giving final concentrations of 20 µM AM complex and 100 µM MgATP. Calculated mixing to freezing times were 7 and 13 ms as shown in Table 1. The estimated time of flight for the spray droplets between nozzle and grid was < 1 ms.

A small cryo-EM dataset (306 and 123 micrographs) was acquired for each timepoint and the combined data processed using standard helical processing procedures [20]. The resulting

consensus reconstruction (both timepoints combined) is shown in Figure 8 A. The combined data was then subjected to focussed classification and split into an AM-complex and an F-actin class (Figure 8 B, C). Then, the fraction of AM-complex or F-actin particles for each timepoint was calculated and is shown in Figure 8 D.

The second order rate constant for AM-complex dissociation is $1.5 \cdot 10^6$ M^{-1} s^{-1} [21]. Under the assumption that

$$[ATP] \gg [AM-complex]$$

the integrated rate law can be approximated to:

$$[AM-complex] \approx [AM-complex]_0 * e^{-k[ATP]_0 t}$$

Where [AM-complex] is the time-dependent concentration of AM-complex, $[AM\text{-}complex]_0$ is the initial concentration, k is the second order rate constant and $[ATP]_0$ is the (initial) ATP concentration.

Using this equation, the kinetics of AM-complex dissociation were modelled. The model agrees reasonably well with the experimental TrEM data. There is significant per-micrograph variation as shown in Figure 8 D.

Discussion

The protocols in this work can be used for fast grid preparation by direct spraying and TrEM experiments. Fast grid preparation can be used to reduce particle interactions with the air water interface [5]. The main limitations are the available sample concentration and ice

thickness on the grid. Within these limits and provided that the sample quality is good, the protocol produces grids suitable for high resolution cryo-EM.

Troubleshooting

Liquid flowrate and grid speed determine the amount of liquid deposited on the grid. With the example settings given above (liquid flowrate 5 µL/s and plunge speed 1-2 m/s), a good coverage of the grid with droplets is expected. If the nozzle was not aligned well (so spray droplets miss the grid), frozen grids show very few or no droplets.

Contamination of grids cannot be entirely avoided (see Fig. 7 C), but can be reduced by minimizing exposure time of liquid ethane to a humid environment. This is achieved by preparing replicate grids as quickly as possible (≤ 20 min for 4 grids). We typically prepare 4 grids before replacing the liquid ethane.

Vitreous ice on grids may (partially) crystallize if the initial cooling in liquid ethane is too slow. In agreement with a previous study [22], we have found low plunge speeds (< 0.5 m/s) lead to an increase of crystalline ice. Areas of very thick ice (≥ 200 nm) typically show crystalline ice due to inherently slower cooling. If the grid warms up during any of the steps following grid preparation (transfer from liquid ethane to liquid nitrogen, transfer to storage, *etc.*) crystalline ice may occur, too.

Data acquisition for TED prepared grids

The average ice thickness produced by the TED is thicker than the “ideal” cryoEM grid prepared on a standard blotting system. Ice thickness can also vary between acquisition areas.

This means that acquisition areas need to be selected carefully to give the thinnest possible ice. The defocus range may need to be adjusted to obtain high contrast images. When the ice is thick and particles have low contrast, we suggest an initial pilot data acquisition using very high defocus values (3 – 5 µm). Recent work suggests that high resolution can still be achieved using such high defocus values [23].

For conventional cryo-EM data collection, an entire dataset is often collected from a single grid. However, we have found the collection of multiple small datasets and merging of these datasets useful. This way, multiple timepoints can be recorded within limited microscope time and timepoints of interest can be identified.

Processing and analysis of TrEM data

Merging of datasets can routinely be done in most common cryo-EM data processing software. As described above and in the work of others, merging of datasets from separate timepoints can be useful [17]. It is important that particles can be traced back to their original subset (timepoint) throughout the processing.

Conventional kinetic measurements (for example using light scattering or fluorescence) can be a valuable addition to TrEM experiments. Rate constants from biochemical measurements can be used to predict lifetimes of the intermediate state of interest, or in confirming the kinetics observed by TrEM. For a basic introduction to reaction kinetics and their relation to time-resolved structural studies, see ref. [24].

There is a number of possible reasons for differences between TrEM and conventional kinetic experiments. As shown in Fig. 8 D, TrEM data can show significant variations per-micrograph. We also note that preliminary data suggests at least 5 % variability in relative particle numbers between replicate grids. Particle classification (class assignment) could be imperfect, particularly if states are structurally similar or if a structure is highly flexible. Particles, and thus TrEM derived kinetics, might also be influenced by particle interactions with the air-water interface because such interactions are reduced but not eliminated at fast speeds.

This work provides some guidelines for TrEM but we note that this is an active area of research, and we expect further advances in the future. While TrEM experiments require significant experimental effort, they can offer high resolution insight into the dynamics of macromolecular complexes.

Acknowledgements

We would like to thank Molly S.C. Gravett for helpful discussions and the ABSL facility staff for help with cryo-EM data collection. D.P.K. is a PhD student on the Wellcome Trust 4-year PhD program in The Astbury Center funded by The University of Leeds. The FEI Titan Krios microscopes were funded by the University of Leeds (UoL ABSL award) and Wellcome Trust (108466/Z/15/Z). This work was funded by a BBSRC grant to SPM (BB/P026397/1) and supported by research grants to Howard White from the American Heart Association (AMR21-236078) and Howard White and Vitold Galkin from the U.S. National Institutes of Health (171261).

References

1 Murphy, B. J. *et al.* Rotary substates of mitochondrial ATP synthase reveal the basis of flexible F1-Fo coupling. *Science* **364** (2019).

2 Benton, D. J., Gamblin, S. J., Rosenthal, P. B. & Skehel, J. J. Structural transitions in influenza haemagglutinin at membrane fusion pH. *Nature*, 1-4 (2020).

3 Dance, A. Molecular motion on ice. *Nature Methods*, 1-5 (2020).

4 D'Imprima, E. *et al.* Protein denaturation at the air-water interface and how to prevent it. *Elife* **8**, e42747 (2019).

5 Noble, A. J. *et al.* Reducing effects of particle adsorption to the air–water interface in cryo-EM. *Nature methods* **15**, 793-795 (2018).

6 Klebl, D. P. *et al.* Need for speed: Examining protein behaviour during cryoEM grid preparation at different timescales. *BioRxiv* (2020).

7 Ravelli, R. B. *et al.* Cryo-EM structures from sub-nl volumes using pin-printing and jet vitrification. *Nature communications* **11**, 1-9 (2020).

8 Arnold, S. A. *et al.* Blotting-free and lossless cryo-electron microscopy grid preparation from nanoliter-sized protein samples and single-cell extracts. *Journal of structural biology* **197**, 220-226 (2017).

9 Razinkov, I. *et al.* A new method for vitrifying samples for cryoEM. *Journal of structural biology* **195**, 190-198 (2016).

10 Feng, X. *et al.* A fast and effective microfluidic spraying-plunging method for high-resolution single-particle cryo-EM. *Structure* **25**, 663-670. e663 (2017).

11 Ashtiani, D. *et al.* Delivery of femtolitre droplets using surface acoustic wave based atomisation for cryo-EM grid preparation. *Journal of structural biology* **203**, 94-101 (2018).

12 Rubinstein, J. L. *et al.* Shake-it-off: a simple ultrasonic cryo-EM specimen-preparation device. *Acta Crystallographica Section D: Structural Biology* **75** (2019).

13 Mäeots, M.-E. *et al.* Modular microfluidics enables kinetic insight from time-resolved cryo-EM. *Nature Communications* **11**, 1-14 (2020).

14 Kontziampasis, D. *et al.* A cryo-EM grid preparation device for time-resolved structural studies. *IUCrJ* **6**, doi:doi:10.1107/S2052252519011345 (2019).

15 Klebl, D. P. *et al.* Sample deposition onto cryo-EM grids: from sprays to jets and back. *Acta Crystallographica Section D: Structural Biology* **76** (2020).

16 White, H., Thirumurugan, K., Walker, M. & Trinick, J. A second generation apparatus for time-resolved electron cryo-microscopy using stepper motors and electrospray. *Journal of structural biology* **144**, 246-252 (2003).

17 Kaledhonkar, S., Fu, Z., White, H. & Frank, J. in *Protein Complex Assembly* 59-71 (Springer, 2018).

18 Trebbin, M. *et al.* Microfluidic liquid jet system with compatibility for atmospheric and high-vacuum conditions. *Lab on a Chip* **14**, 1733-1745 (2014).

19 Klebl, D. P., Sobott, F., White, H. D. & Muench, S. P. On-grid and in-flow mixing for time-resolved Cryo-EM. *In press* (2021).

20 He, S. & Scheres, S. H. Helical reconstruction in RELION. *Journal of structural biology* **198**, 163-176 (2017).

21 Millar, N. C. & Geeves, M. A. The limiting rate of the ATP-mediated dissociation of actin from rabbit skeletal muscle myosin subfragment 1. *FEBS letters* **160**, 141-148 (1983).

22 Kasas, S., Dumas, G., Dietler, G., Catsicas, S. & Adrian, M. Vitrification of cryoelectron microscopy specimens revealed by high-speed photographic imaging. *Journal of microscopy* **211**, 48-53 (2003).

23 Glaeser, R. M. *et al.* Defocus-dependent Thon-ring fading. *bioRxiv* (2020).

24 Bagshaw, C. A beginner's guide to flow kinetics. *The Biochemist* **42** (2020).

Figure Legends

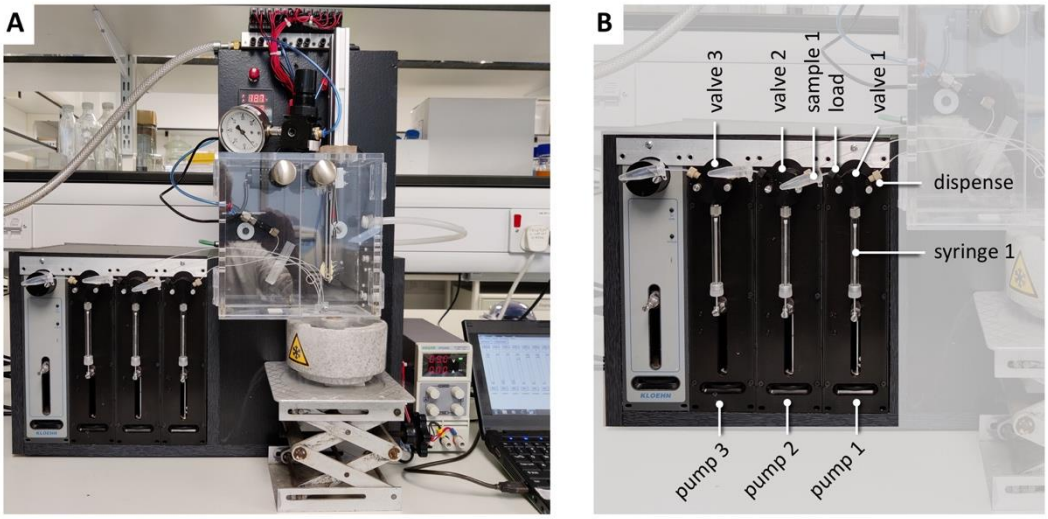

Figure 1: The time-resolved EM device (TED). (A) Overview of the TED. (B) The liquid handling unit with pumps 1-3. The fourth pump on the left is not used in this work. Load and dispense positions are indicated for valve 1, as is the sample reservoir ('sample 1').

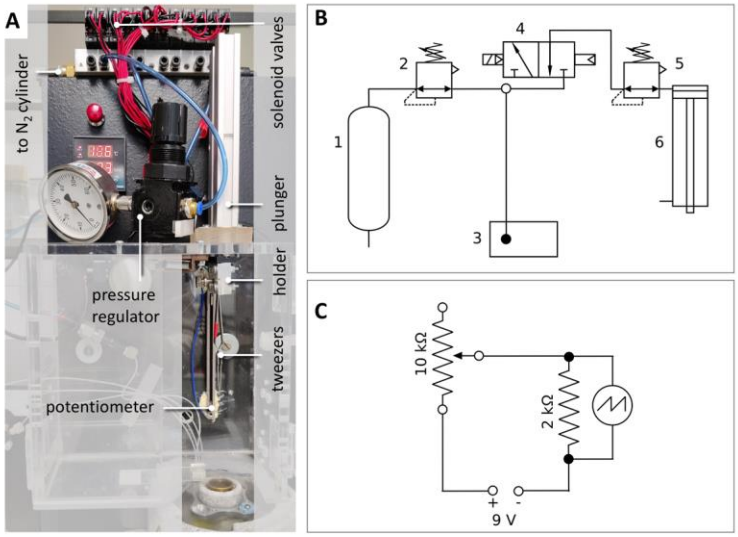

Figure 2: Pneumatic plunger and linear potentiometer. (A) Details of the pneumatic plunger of the TED with important components labelled. (B) Schematic of the pneumatic system of the TED. Numbers correspond to the N_2 cylinder (1), the main pressure regulator (2), the spray nozzle (3), the solenoid valve (4), the plunge speed regulator (5) and the dual rod pneumatic cylinder (6). (C) Schematic of the circuit for the potentiometer.

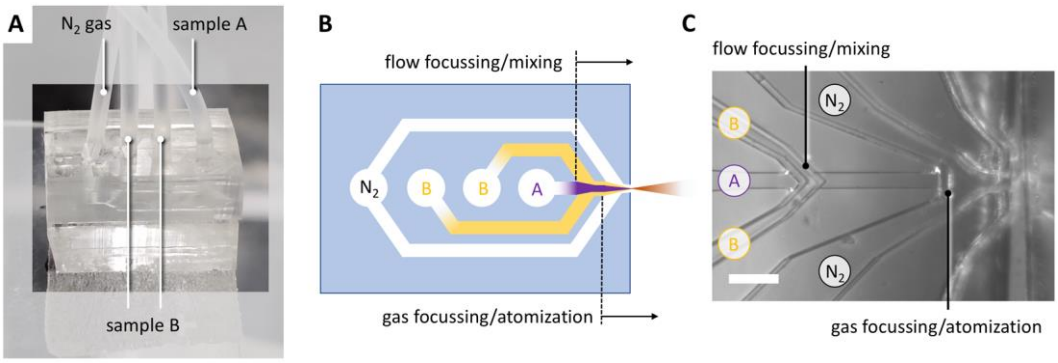

Figure 3: Gas-dynamic virtual nozzles for mixing and spraying. (A) A nozzle with 4 inlet tubes connected, to supply to samples (A and B) and N_2 gas. (B) Schematic of the central section of the PDMS spray nozzles with double flow focussing geometry to achieve flow focussing (for downstream mixing) and gas focussing or atomization. Sample B is shown in yellow and sample A in purple. (C) Microscopic image of the mixing and atomization sections of the microfluidic nozzle. Image from ref. [15]. Scale bar 100 nm.

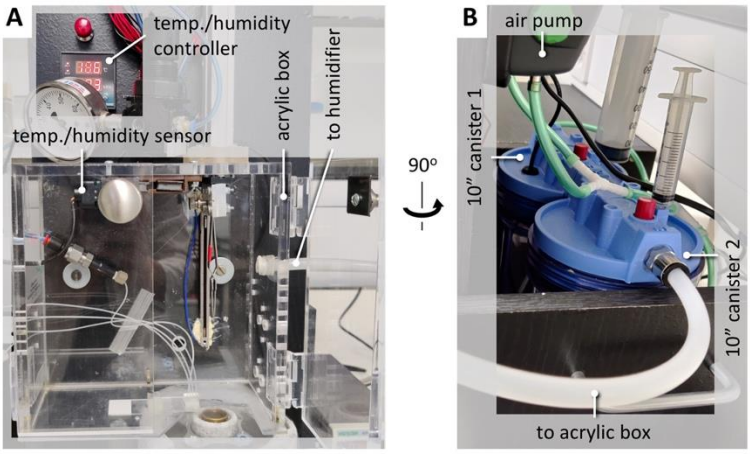

Figure 4: Environmental control of grid and spray in the acrylic glass box. (A) View from the front and (B) from the side of the TED.

A

Variables:	PLUNGE1$ = /1U#2R SPRAY-VOL1$ = /1D2000 S-SPEED1$ = /1V1000 PRE-SPRAY-TIME = 1.5

```
VARIABLE PLUNGE1$                       #variables
VARIABLE SPRAY-VOL1$                    #are defined
VARIABLE S-SPEED1$                      #here
VARIABLE PRE-SPRAY-TIME
MSGBOX PRESS OK TO START RUN            #message
SELPORT 3
WAITFOR 1.0
COMPORT S-SPEED1$                       #set spray-speed
WAITFOR 1.0
COMPORT /_OP1
WAITFOR 0.1
COMPORT /_u1u2u3
WAITFOR 1.0
TIMER0
WAITFOR 0.1
COMPORT SPRAY-VOL1$                     #spray starts
WAITFOR PRE-SPRAY-TIME                  #pre-spray time
COMPORT PLUNGE1$                        #plunge
WAITFOR 1.0
MSGBOX RUN COMPLETE
WAITFOR 1.0
COMPORT /_u1u2u3
SELPORT 1
```

B

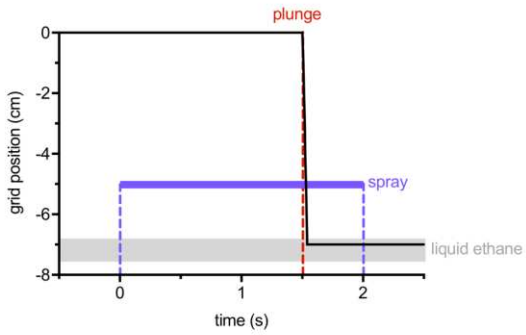

Figure 5: The run sequence. (A) An example run sequence showing variables at top and the sequence of commands at the bottom. Some of the key commands are annotated in red. (B) Grid position over the time course of a 'run sequence' shown as a black solid line. The start position of the grid is at 0 cm. Spray is initiated at t = 0 s for 2 s, the distance between spray and liquid ethane is ~ 2 cm. At 1.5 s, the grid starts moving with 2 m/s, through the spray and into the liquid ethane. The sequence ends as the spray stops after 2 s.

Variables:	PLUNGE1$ = /1U#2R SPRAY-VOL1$ = /1D800 SPRAY-VOL2$ = /2D400 SPRAY-VOL3$ = /3D400 S-SPEED1$ = /1V400 S-SPEED2$ = /2V200 S-SPEED3$ = /3V200 PRE-SPRAY-TIME = 1.5

```
VARIABLE PLUNGE1$
VARIABLE SPRAY-VOL1$                #variables for spray volume and
VARIABLE SPRAY-VOL2$                #spray speed for all 3 syringes
VARIABLE SPRAY-VOL3$
VARIABLE S-SPEED1$
VARIABLE S-SPEED2$
VARIABLE S-SPEED3$
VARIABLE PRE-SPRAY-TIME
MSGBOX PRESS OK TO START RUN
SELPORT 3
WAITFOR 1.0
COMPORT S-SPEED1$
WAITFOR 0.1
COMPORT S-SPEED2$
WAITFOR 0.1
COMPORT S-SPEED3$
WAITFOR 1.0
COMPORT /_OP1
WAITFOR 0.1
COMPORT /_u1u2u3
WAITFOR 1.0
TIMER0
WAITFOR 0.1
COMPORT SPRAY-VOL1$
WAITFOR 0.04                        #liquid flow is started for each
COMPORT SPRAY-VOL2$                 #syringe separately, with 40 ms
WAITFOR 0.04                        #delay between commands
COMPORT SPRAY-VOL3$
WAITFOR PRE-SPRAY-TIME
COMPORT PLUNGE1$
WAITFOR 1.0
MSGBOX RUN COMPLETE
WAITFOR 1.0
COMPORT /_u1u2u3
SELPORT 1
```

Figure 6: Example run sequence for a rapid mixing experiment. Important differences to Figure 5 A are annotated in red.

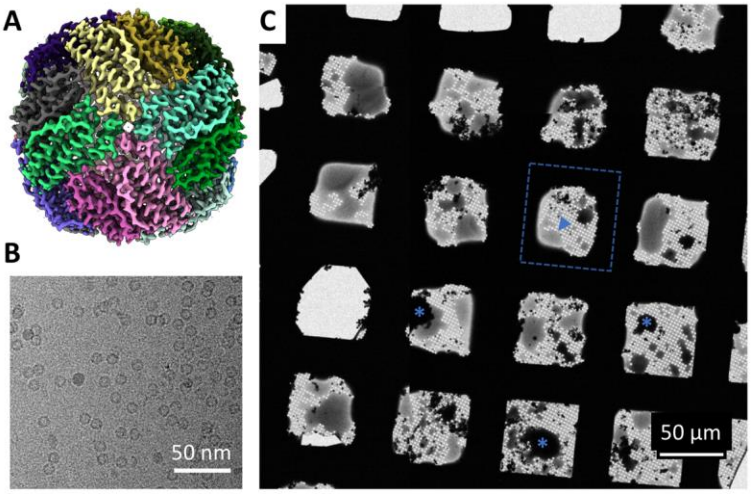

Figure 7: Representative results from a simple spray experiment. (A) 3.5 Å reconstruction of equine apoferritin from a grid prepared in 36 ms between spraying and vitrification (EMD-10533). (B) Raw image of the same apoferritin sample. (C) Low magnification view of the grid,

one exemplary grid-square containing thin ice is highlighted by the dashed blue rectangle, the area of thin ice within this grid-square is indicated by the blue arrow. Areas of thick ice or contamination are indicated with asterisks.

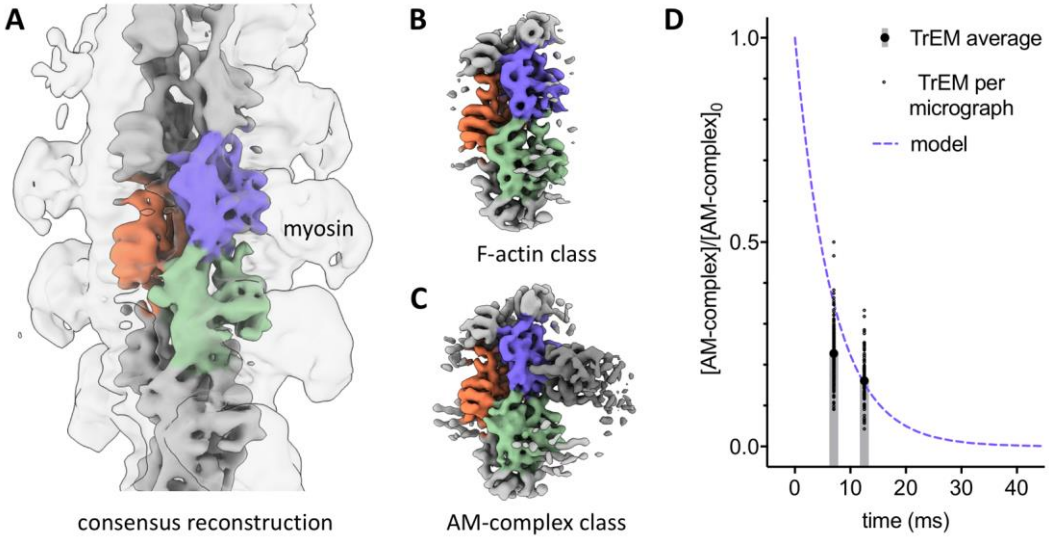

Figure 8: Representative results from a mixing experiment. (A) Consensus reconstruction of the AM-complex shown at a high threshold (coloured) and low threshold (transparent). One myosin-density is labelled and highlighted with a dashed line. The reconstruction was generated from data from both timepoints combined. (B) F-actin class showing no myosin bound. (C) AM-complex class showing myosin bound (myosin in grey, bound primarily to the light red actin subunit). (D) Comparison between kinetic model and experimental TrEM data. Shown is the fraction of AM-complex relative to the initial concentration. Large solid black dots indicate averaged TrEM data, small dots show per-micrograph TrEM data. The purple dashed line represents the kinetic model.

Table 1: Experimental settings for TrEM of AM complex dissociation by ATP

	flowrate (µL/s)			spray/ethane distance (cm)	plunge speed (m/s)	time-delay (ms)
	syringe 1 (AM)	syringe 2 (ATP)	syringe 3 (ATP)			
7 ms	2.08	1.04	1.04	1.4	2.0	7
13 ms	1.04	0.52	0.52	2.0	1.6	13