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Recent advances and current trends in cryo-electron microscopy

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Abstract

All steps of cryogenic electron-microscopy (cryo-EM) workflows have rapidly evolved over the last decade. Advances in both single-particle analysis (SPA) cryo-EM and cryo-electron tomography (cryo-ET) have facilitated the determination of high-resolution biomolecular structures that are not tractable with other methods. However, challenges remain. For SPA, these include improved resolution in an additional dimension: time. For cryo-ET, these include accessing difficult-to-image areas of a cell and finding rare molecules. Finally, there is a need for automated and faster workflows, as many projects are limited by throughput. Here, we review current developments in SPA cryo-EM and cryo-ET that push these boundaries. Collectively, these advances are poised to propel our spatial and temporal understanding of macromolecular processes.

Keywords

cryo-EM; cryo-ET; time-resolved cryo-EM; cryo-FIB; cryo-CLEM

Introduction

Advances in direct electron detectors (DEDs) and software innovations have prompted exponential growth in the number of biomolecular structures solved with cryogenic electron microscopy (cryo-EM). Scientists leverage single-particle analysis (SPA) cryo-EM [1] to determine the structures of purified macromolecules and cryogenic electron-tomography (cryo-ET) [2] to study large assemblies in their native cellular environment. However, for a detailed understanding of molecular function, a mere snapshot of a molecule is not enough. Rather, its interaction with substrates, reaction intermediates, binding partners, and the cellular environment is required to determine its mechanism and function. The last few years have seen constant improvements in sample preparation that facilitate the study of challenging specimens. Meanwhile, accelerated data acquisition schemes and automation have increased throughput [3–6].

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Declaration of Interest

The authors declare no conflict of interest.

SPA largely depends on highly purified and often stabilized molecules that lack their native environment. Often these molecules are reconstituted in complexes that may miss unknown, but physiologically relevant, cofactors. In contrast, cryo-ET can be used to determine the structures of large molecules and complexes in naturally thin or thinned areas of a cell, but it is limited to relatively low-resolution (~ 20 Å) for single-instance detection [7]. However, with large datasets and elaborate data-processing workflows, subtomogram averaging can integrate the signals of several thousands of individual particles [8, 9], yielding a much higher resolution (currently to ~ 3.5 Å in the absence of symmetry [10]).

Acquisition of sufficient data is often challenging. Macromolecules are extremely small relative to the size of a cell and, thus, can be hard to locate. For example, a typical tomogram only spans $\sim 0.01\%$ of the volume of a mammalian cell (estimated based on a cell with a $10\text{ }\mu\text{m}$ radius and a $200\text{-}\mu\text{m}$ thick section). The physical nature of electrons also poses a key challenge for cryo-ET: the apparent mean free path of electrons is only ~ 280 nm in a microscope suitable for cryo-ET [11]. Thus, most areas of a cell are too thick to image directly. Cryo-correlative light and EM (cryo-CLEM) [12–15] and cryo-focused ion beam (FIB) milling [16, 17] attempt to address these challenges and can be combined [18–27]. Cryo-CLEM integrates light microscopy and cryo-ET to locate, for example, cellular structures or fluorescently labelled molecules of interest [12–15, 25]. Cryo-FIB milling leverages a high-current gallium ion beam to create thin sections through cells, termed lamellae (typically $\sim 80\text{--}300$ nm thick) [16, 17, 22–24, 26].

Nevertheless, challenges persist. Here, we review the latest breakthroughs and current developments of cryo-EM techniques with an emphasis on large molecules using time-resolved methods, natively purified samples, and *in situ* imaging methods.

Time waits for no one — The race against the clock to image short-lived intermediates

Many biological processes occur on a timescale of milliseconds or shorter. However, during a classical SPA specimen preparation procedure, the sample is applied to a cryo-EM grid, excess buffer is removed by blotting with filter paper, and the sample is plunged into liquid ethane to rapidly vitrify the biomacromolecule in a native state (Fig. 1A) [28]. This procedure takes at least several seconds from sample application to vitrification – too long to capture short-lived states in many reactions. Therefore, faster vitrification methods to visualize such intermediates are needed.

Time-resolved cryo-EM attempts to capture short-lived molecular states by rapid mixing of reactants and sample vitrification on ms-timescales (Fig. 1B–D) [29–31]. Time-resolved cryo-EM faces two main challenges: achieving efficient mixing of the reactants and obtaining ideal ice-thickness for imaging. Automated devices developed to overcome such obstacles are often built in-house, expensive, and difficult to operate, resulting in low reproducibility. Here, we review recent approaches that enable new insights into physiological molecular dynamics, assembly, and reaction pathways (Table 1) [32–34] and provide an outlook for future applications and developments.

One of the earliest developed methods, ‘spraying-mixing’, leverages passive diffusion after the sequential application of two reactants onto the same grid before flash-freezing within <100 ms [29] (Fig. 1B). Updated versions achieve time resolutions as small as 2 ms but poor sample mixing, especially of large molecules, and uneven ice thickness limits its use [35]. The commercially available Spotiton deposits ~50 pL sample droplets on self-wicking grids with a piezoelectric dispenser. Dandey *et al.* [32] modified a Spotiton by adding a second piezoelectric dispenser to deposit the second reactant. Self-wicking grids contain nanowires to leverage capillary forces that generate a thin buffer film and effectively mix the sample within ~90 ms. A limitation of this technology lies in the requirement of self-wicking grids, which are mainly manufactured in-house through laborious processes, also posing the problem of reproducibility. This issue is partially addressed within a new implementation of the Chameleon, a blot-free plunger based on the Spotiton. The Chameleon can now automatically calculate the ideal wicking time and plunge-freezing speed and enables the user to assess the film thickness prior to imaging [36, 37]. The tight control of these parameters is expected to increase reproducibility.

In contrast, ‘mixing-spraying’ approaches optimize sample mixing using microfluidic devices prior to grid application [30, 38, 39]. So far, this approach has provided insights into the dynamics of biological systems like ribosomal assembly [38, 40, 41]. The primary drawback of mixing-spraying is the complex and expensive manufacturing of microfluidic devices. The poorly understood physical parameters of mixing efficiency and incubation error in microfluidic devices pose additional experimental challenges. Mäeots *et al.* [33] developed a bimodular instrument that consists of a microfluidic module that allows for tuning of flow rate, length of mixing segments, and spraying pressure to deposit the sample on a conventional cryo-EM grid (Fig. 1C). Their detailed characterization of the mixing properties of this device allows for accurate control of the sample preparation in as short as ~30 ms. The use of conventional cryo-EM grids rather than specifically manufactured grids further facilitates the procedure. Finally, the system proposed by Mäeots *et al.* might provide an affordable alternative that is relatively easy to assemble compared to spraying-mixing approaches.

Finally, a third time-resolved cryo-EM approach uses light of specific wavelengths to trigger photoreactions. Light-induced overheating and melting of the vitrified specimens are key challenges. This has led to relatively little progress since the development of the first flash-plunge setups to activate light-sensitive proteins like rhodopsin over 20 years ago [31]. Recently, a UV LED approach was used to induce photochemical reactions directly on a cryo-EM grid (Fig. 1D) [34]. Yoder *et al.* used the chicken proton-gated acid-sensing ion channel 1a (cASIC1a) as a case study. cASIC1a has a well-characterized “closed/resting” state at high pH, and transient “open/conducting” and “open/desensitized” states at low pH. Leveraging their light-coupled cryo-plunger combined with caged protons, the authors vitrified the sample within ~70 ms of exposure at different intensities. No UV exposure and low intensity conditions yielded cASIC1a structures in a “closed/resting” conformation, whereas high-intensity exposure induced an “open/desensitized” state. This example foreshadows the use of light-based time-resolved cryo-EM for both SPA and cryo-ET in structural neuroscience, where caged compounds (e.g. ions, neurotransmitters, ATP) are already routinely used [42]. Despite the advances, the temporal resolution could not

capture the “open/conducting” state of cASIC1a, expected to last only tens of milliseconds, highlighting the need for even faster sample preparation and vitrification techniques.

These blot-free vitrification techniques also overcome additional challenges that plague many specimens, such as preferential orientation and sample aggregation at the air-water interface [43]. Mäeots *et al.* demonstrate reduced sample aggregation on the example of the Cop9 signalosome in complex with its effectors, which is known to aggregate at the air-water interface. Similarly, the new Chameleon was used to determine the structure of deoxyadenosine triphosphate (dATP)-bound inactive class Ia ribonuclease reductase (RNR) from *Neisseria gonorrhoeae* complexes [36]. Ring-shaped RNRs orient perpendicularly to the air-water interface leading to preferential orientation and rapid denaturation, when vitrified using conventional methods. These issues were partially overcome by decreasing the specimen dispense-to-plunge time to 54 ms, which yielded a better angular distribution and a higher fraction of intact RNR rings. Consequently, the development of instrumentation for time-resolved cryo-EM may have wider ramifications for vitrification and sample quality at large [44, 45].

In summary, time-resolved cryo-EM is a fast-developing area of SPA that allows for high-resolution snapshots with millisecond resolution. Recent developments improved the reproducibility of time-resolved cryo-EM. Despite the increase in new technologies and the marketing of commercial instruments, access to time-resolved cryo-EM equipment is still limited, and the use of this instrumentation is challenging. Future improvements will likely focus on reproducibility and ease of use.

You don't know me — We only find what we include

Most structural biology approaches rely on highly homogeneous samples obtained by overexpression and isolation of recombinant proteins. Many macromolecular assemblies are too complex for *in vitro* reconstitution or require extensive prior knowledge. Cryo-EM overcomes some of these limitations by leveraging elaborate computational schemes to classify different molecular states and conformations [46]. Thus, increasingly, researchers have taken to native samples to study complex and dynamic cellular machinery, e.g. the spliceosome in its many functional states and assemblies [47–60] or patient-derived samples such as amyloid fibrils (reviewed in [61]).

SPA has also emerged as a discovery tool for identifying binding partners and regulatory factors, such as translation hibernation factors in oocytes [62] and a translation elongation factor repurposed as an inhibitor in sensory neurons [63]. Two consecutive studies from Ho *et al.* [64] and Terwilliger *et al.* [65] use enriched cell lysate from *Plasmodium falciparum* to determine the structures of large complexes in the mixture. Subsequent *de novo* model building is aided by mass spectrometry and automated. The authors determined multiple structures of previously unidentified proteins, as well as unseen conformations of known complexes such as the 20S proteasome. However, all determined structures stem from relatively large and rigid macromolecules. Despite its great appeal, such an approach requires high-quality electron density maps (<4 Å) to automatically and unambiguously fit

amino acid side chains. All studies so far highlight that only large and abundant complexes with limited conformational heterogeneity are currently tractable.

Come together — The relevance of the molecular context

Cryo-ET is the method of choice for high-resolution 3D studies of complexes in cells (reviewed in [66]) (Fig. 1, bottom panel). During cryo-ET data collection, vitrified cryo-ET specimens are incrementally tilted, and the imaged 3D volume is reconstructed from the acquired 2D projections. Preservation of the cellular context has expanded our understanding of molecular mechanisms and processes. For example, the nuclear pore complex (NPC) illustrates the importance of studying complexes in their cellular environment: *in cellulo* cryo-ET structures of NPCs from *S. pombe* [67], *S. cerevisiae* [68], *C. reinhardtii* [69], and *H. sapiens* [70, 71] reveal that the central channel of NPCs is much bigger than in purified NPCs [72–74]. This difference is likely due to tensile forces that the nuclear membrane exerts on NPCs in intact cells [67]. This result also has implications for cargo transport through the NPC. For instance, HIV-1 capsids can pass through the NPC into the nucleus without disassembly or remodelling of the NPC as had been previously hypothesized [71]. The key to these findings is the cellular context - the complex interplay of NPCs with the nuclear membrane, cytoskeleton, autophagosome, and other factors.

I still haven't found what I'm looking for — The challenge of locating rare or localized molecules of interest

Molecules or states of interest are often transient and localized in specific subcellular areas. Therefore, it is essential to freeze the specimen at the right time and identify areas of interest before cryo-ET imaging or further processing of the specimen. Initially, live-cell imaging can provide valuable information on suitable time points to vitrify the specimen, for example, to capture transient structures such as the contractile actomyosin ring during eukaryotic cell division (Fig. 1, right bottom panel) [75]. After vitrification, cryo-fluorescence imaging of the sample in a light microscope (confocal or widefield) equipped with a cryo-stage facilitates target identification [12, 13, 15]. Open-source solutions facilitate the alignment and cross-correlation of both montages [27, 76], reducing the need for homemade software solutions.

While the resolution of conventional cryo-fLM is acceptable, it is insufficient to determine the precise depth of a target necessary to inform cryo-FIB milling [25, 77–79]. Resolution is further impacted by sample thickness. The low temperatures used in cryo-fLM require objectives with long working distances and cryo-immersion approaches that are compatible with subsequent cryo-ET are currently not available. Super-resolution (reviewed in [80]) is still in early development phases but can deliver sufficiently precise target coordinates for cryo-FIB milling. For instance, Sexton *et al.* combined super-resolution confocal cryo-fLM with cryo-FIB milling and cryo-ET to study *Deinococcus radiodurans* during cell division, uncovering the atypical morphology of its cell envelope and the ultrastructure of division sites [81].

The ice is getting thinner — Thinning of specimens using cryo-FIB milling

Cryo-FIB milling is currently the most advanced method to ablate cell material to generate thin preparations for cryo-ET imaging (Fig. 1, right bottom panel) [16, 17, 26]. Initially, an organo-platinum layer is applied to disperse charge build-up during milling. This is followed by consecutive rounds of cryo-FIB milling with decreasing ion beam currents until the desired thickness is obtained. The milling process is monitored by scanning electron microscopy (SEM). Importantly, correlation of cryo-fLM with the SEM image can be used to guide milling [18–21, 82].

Cryo-FIB milling still suffers from many challenges: the commonly used gallium ion beam becomes divergent at high beam currents, leading to sample damage and gallium implantation into the lamella. Moreover, the procedure is susceptible to ice contamination during milling and transfer between instruments, and the milling process is slow and highly user-involved. Strategies have emerged and continue to evolve to overcome these challenges. For example, plasma cryo-focused ion beams (cryo-PFIB) that use gases, such as oxygen or argon, to generate the plasma, are emerging as an alternative for biological specimens [83, 84]. Cryo-PFIBs remain more focused than gallium ion beams at high beam currents [85], thus reducing implantation effects and sample damage. Early studies confirm that high-resolution features are preserved in cryo-ET of lamellae prepared with a cryo-PFIB [84]. Cryo-PFIBs can also ablate more material than a gallium cryo-FIB, which could pave the way for the preparation of specimen from larger tissue sections.

The main sources of ice build-up on lamellae are water molecules in the imperfect vacuum of the cryo-FIB and re-disposition of ablated material. Additionally, exposure to atmospheric water during transfer of the sample between instruments increases the risk of contamination. These issues are addressed by a combination of milling strategy adaptations and engineering solutions. Initial crude milling and final ‘polishing’ to the desired thickness minimize the time of the final lamella in the cryo-FIB [86]. Tacke *et al.* used cryo-FIB/SEM hardware modifications such as a stage heater, a cryo-shield, and a cryo-shutter to drastically reduce ice build-up [87]. Additionally, a glove box equipped with an adaptor for a previously designed high vacuum cryo-transfer system that is purged with dry N₂ eliminates water exposure during transfer [87, 88]. Collectively, these advances reduce the chance of ice contamination and allow for longer cryo-FIB milling times and automation methods reduce user intervention and speed up the thinning process [86, 89, 90].

Classical cryo-FIB approaches largely rely on conventional plunge-vitrification. However, the achievable depth of vitrification, at which no crystalline ice forms, only allows for vitrification of specimens that are a few microns thick (10–20 µm [91]). New approaches, termed cryo-FIB-lift-out [92–96] and the Waffle method [97] both leverage high-pressure freezing to achieve homogenous vitrification. *In situ* lift-out, the biological material is first exposed using a cryo-ultramicrotome [92, 95]. This facilitates the use of cryo-fLM to identify areas of interest and enables the use of a gallium cryo-FIB to generate a lamella. Finally, the resulting lamella is mounted on a support grid using a micromanipulator where it can be further thinned before imaging.

The Waffle method (named so due to the resemblance of the high-pressure frozen grid to a waffle) leverages high-pressure freezing followed by cryo-FIB milling to solve the common preferred orientation problem that the air-water interface imposes on many samples [97]. In comparison to lift-out, the thickness of the starting material is limited to ~50 μm as opposed to ~200 μm due to the limitation of gallium cryo-FIBs. Cryo-PFIBs will likely push this limit. The Waffle method provides the advantage that densely packed cells or tissue allow for the milling of larger lamellae. Ultimately, this allows for higher throughput on the cryo-electron microscope.

In summary, cryo-CLEM and cryo-FIB workflows are time-consuming and susceptible to contamination of the specimen with ice due to several transfers between instruments. Integrated designs that combine cryo-fLM, cryo-FIB, and SEM in the same instrument, such as the “Photon Ion Electron” (PIE)-scope [82] the Aquilos 2 (ThermoFisher), or the recently released Arctis cryo-PFIB (Thermo Fisher) eliminate the risk of ice build-up during transfers and reduce user intervention. Thus, they are expected to become increasingly more popular. While many projects largely still rely on progressive automation to increase the throughput and accessibility of cryo-ET, the next frontier will be the preparation of samples from more complex multicellular sources.

Where have I seen you before? — 2D template matching to identify molecules in crowded environments

The identification of molecules of interest for structure determination in cryo-ET largely relies on the human eye or semi-automated detection using a 3D template [98]. 3D template matching (3DTM) uses low-resolution information from a template that is either generated from a manual selection of averaged molecules in subtomograms or from known structures (Fig. 1, left bottom panel). This has limited cryo-ET to studies of relatively large and distinctly-shaped complexes such as the ribosome and the proteasome.

The recently developed 2D template matching (2DTM) approach leverages the known 3D high-resolution structures obtained from SPA or X-ray crystallography as search models (Fig. 1, left bottom panel) [99, 100]. In contrast to 3DTM, 2DTM leverages high-resolution information, which is unique to every molecule and assembly, to determine the locations within an image. The method requires only one untilted exposure that retains high-resolution information. The recent integration of 2DTM into the graphical user interface of cisTEM [99, 101] makes 2DTM a user-friendly and relatively fast alternative to manual identification or 3DTM in a thin specimen. In a direct comparison on a bacterial ribosome, 2DTM has higher precision than 3DTM but relies more strongly on a thin sample to retain high-resolution information in the image [99]. Indeed, the method currently has lower sensitivity in samples >100nm [99]. 2DTM is compatible with cryo-FIB milling and multiple templates could be used to coarsely pre-classify particles [102] prior to reconstructing 3D volumes. However, the reliance on high-resolution signal requires higher magnifications than commonly used for cryo-ET. This limits the volume that can be examined at any given time. If the untilted 2DTM image cannot be integrated into the tilt-series acquisition, the additional irradiation of the sample might be undesirable. Finally, despite an estimated

theoretical limitation of ~300 kDa in a 100 nm thick biological sample [100], the use of 2DTM beyond detecting ribosomes has yet to be proven.

Concluding remarks

As new methods and technologies address the challenges associated with specimen preparation and target molecule identification, the scope of SPA and cryo-ET keeps broadening. Once considered separate disciplines, the gap between these two methods is narrowing due to the need for studying molecules in action, in native environments, and at high resolution. Time-resolved cryo-EM has added another dimension, time, whereas cryo-CLEM and cryo-FIB milling have allowed the visualization of macromolecular complexes previously inaccessible cellular areas. While the latest approaches achieve unprecedented resolution in time and space, their increasing cost limits broad access to constantly improving instrumentation. Therefore, there is a critical need for affordable access to instrumentation that will democratize cryo-EM.

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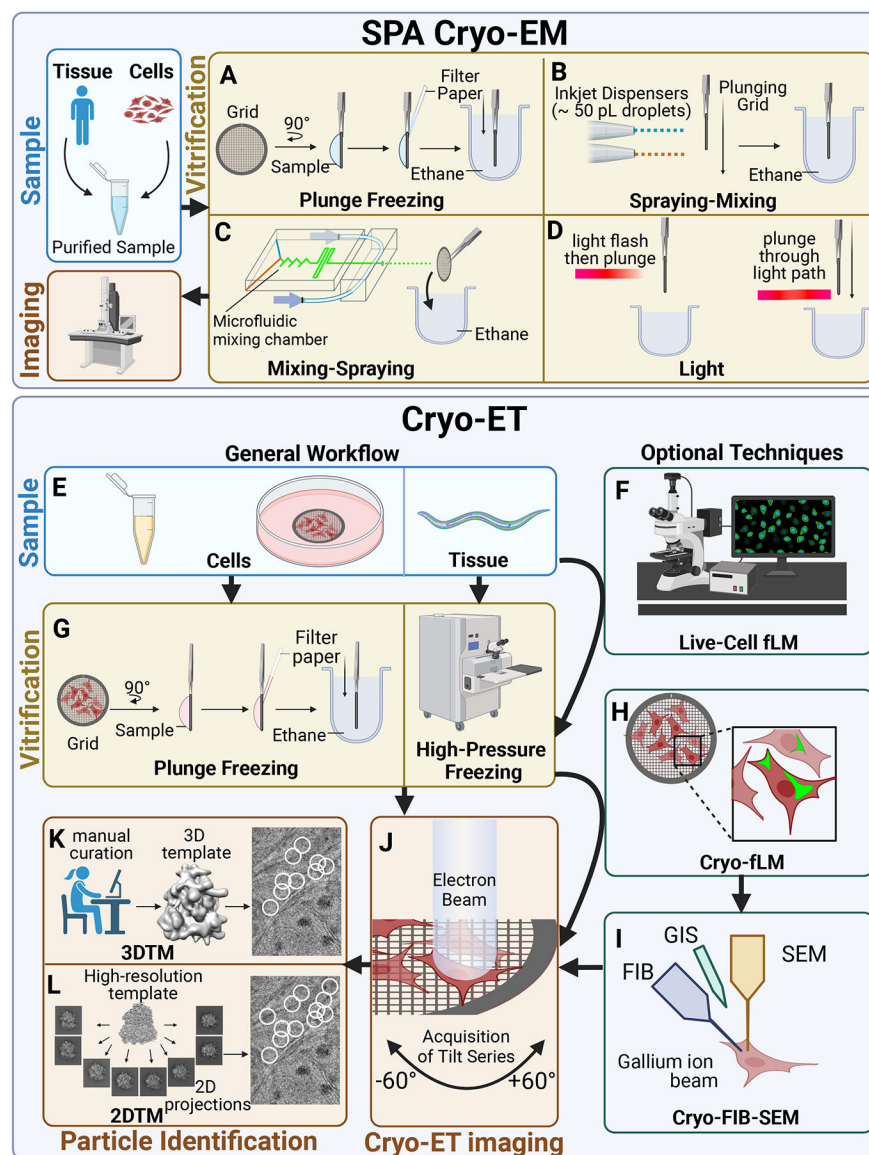


Figure 1. Overview of advances in SPA cryo-EM and cryo-ET.

SPA cryo-EM panel, A typical workflow includes sample extraction and purification from a recombinant or native source such as tissue followed by vitrification by plunge freezing. Vitrification sub-panel (A). Alternative vitrification methods such as spraying-mixing (B), mixing-spraying (C), and light time-resolved cryo-EM (D) facilitate the capture of short-lived states. After vitrification, the specimen can be imaged. **Cryo-ET panel**, Panels on the left outline a general workflow. Cells can either be vitrified by plunge freezing or, like small amounts of tissue, by high-pressure freezing. If the specimen is thin enough and the location of the molecule of interest is known, it can be imaged without further processing. Finally, particles are either identified manually and, potentially, with 3DTM or using 2DTM. Panels on the right outline optional techniques. Live-cell fLM is used to monitor the expression and stage of the sample to determine the optimal timepoint for vitrification. After vitrification,

cryo-fLM can be leveraged to identify the locations of fluorescently-tagged molecules of interest to guide subsequent milling using a cryo-FIB for immediate image acquisition.

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Table 1.

Overview of the challenges and new breakthroughs in the available time-resolved cryo-EM approaches.

	Spraying-mixing	Mixing-spraying	Light-based time-resolved cryo-EM
Sample types	Small molecules	Small and large molecules	Photo-reactive and caged ligand-binding molecules
Challenges	Mixing by diffusion, distribution across the grid, ice thickness, speed	Reproducibility, affordability	Versatility, beam-induced heating of the sample
Technological advances	Devices coupling a dual sample sprayer, self-wicking grids and plunge-freezer	Easy-to-build and affordable devices coupling a microfluidic chip, sample spraying and plunge-freezer	Devices coupling sample irradiation and plunge-freezing

Different time-resolved cryo-EM methods are better suited to resolve time-sensitive reactions of different specimens (row 1). Only small molecules efficiently mix by diffusion on grids with the spraying-mixing technique, while mixing-spraying is applicable to reactants of small and large size. Light-based time-resolved cryo-EM is limited to light-sensitive specimens or light-sensitive caged molecules. The long-lasting difficulties imposed by the sample preparation as well as the access to and reliability of the time-resolved cryo-EM devices (row 2) are addressed by recent technological innovations (row 3) that largely automate this process.