Understanding the invisible hands of sample preparation for cryo-EM

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Abstract

Cryo–electron microscopy (cryo-EM) is rapidly attracting researchers in the field of structural biology. With the exploding popularity of cryo-EM, sample preparation must evolve to prevent congestion in the workflow. The dire need for improved microscopy samples has led to a diversification of methods. This review aims to categorize and explicate the principles behind various techniques in preparation of vitrified samples for the electron microscope. Various aspects and challenges in the workflow are discussed, from sample optimization and carriers to deposition and vitrification. Reliable and versatile specimen preparation remains a challenge and we hope to give guidelines and posit future directions.

Introduction

Cryo–electron microscopy (cryo-EM) is providing macromolecular structures up to atomic resolution at an unprecedented rate. Images of biomolecules embedded in vitreous, glass-like, ice are combined into

a 3D reconstruction. The detailed structural models obtained from these reconstructions grants insight on the function of macromolecules and their role in biological processes. For example, resolving the structure of Tau filaments [1] and amyloid fibrils [2] has revealed insights on the mechanism of Alzheimer's, the most common neurodegenerative disease. Furthermore, in the COVID-19 pandemic, the spike protein that gains entry to human cells was resolved to 3.5 Å by cryo-EM [3]. Understanding the process of virus binding to specific receptors of human cells will aid the fabrication of targeted vaccines, drugs and diagnostics [4,5]. Pharmaceutical organizations have already shown a growing interest in the technique to analyze ligand binding, perform conformational studies and drug testing [6]. More detailed and reliable maps will be generated as the resolution continues to improve through technical developments.

The rise in popularity of cryo-EM as a breakthrough structural biology technique originated from a number of technical advancements, which is often referred to as the "Resolution Revolution" [7]. One of the driving forces was the introduction of a new generation of cameras with direct electron detection and increased frame rate [8]. The new cameras are able to operate in single electron counting mode and acquire movies to compensate for movement during imaging [9]. The cryo–electron microscopes became more amenable for automation through features like the autoloader, constant-power lenses, mechanical stages with decreased drift and aberration-free image shifting. Software packages allow unsupervised data collection of high-quality data for days in a row. Furthermore, data processing became more robust and user friendly [10]. Automated pipelines are being developed to streamline data acquisition and processing [11]. Hardware improvements, primarily focusing on narrowing the energy spread of incident electrons, brought about the resolution record below 1.3 Å [12,13]. These technical upgrades made cryo-EM more accessible to structural biologists. The main impediment in the workflow has thus emerged at sample preparation, leading scientists and engineers to target the hurdle [14].

Sample preparation in single particle analysis involves two main components: sample optimization and grid preparation. Sample optimization involves specimen purification and reaching its optimum biochemical state. Grid preparation describes the steps needed to obtain a sample that is suitable for analysis in the microscope. These steps involve chemical or plasma treatment of the grid, deposition

and vitrification. The first breakthroughs came about from a manual blot-and-plunge method developed in the 1980's [15] and is still being applied to achieve formidable results [16–18].

As more heterogenous and complex samples are being studied, special care is required for their stability [19]. Preparing the sample from a bulk solution onto a grid can have an impact on the state of the molecule. When analyzed in the microscope, vitrified samples are often subject to aggregation, preferential orientation or denaturation [14]. These phenomena can be targeted through sample optimization at the biochemical level or through various parameters in grid preparation. Numerous techniques have been proposed to overcome macromolecular instability, however, the effectiveness of one method over another varies by sample [14,20]. Currently, the results from grid preparation rely mostly on user expertise and experience, which becomes increasingly time consuming and challenging [21].

The many variables encountered in sample and grid preparation make it difficult to tie origin and consequence. The researcher only evaluates the sample at the molecular level at the microscope. While the procedure of preparing grids does not require much time, the iterations needed to obtain the right conditions can cost copious amounts of time and are labor intensive [21,22]. Pragmatically, this makes it difficult to standardize protocols. Although databases have aided in better monitoring of condition and outcomes, [23,24] quantitative comparisons and statistics between different protocols in grid preparation are still lacking. A structured approach is essential in order to investigate trends and understand the underlying mechanisms of sample behavior.

If, among others, these problems are solved, cryo-EM could become a versatile and even more dominant technology in structural biology [25]. New research questions will emerge, and different methodologies will develop as the field grows. Many steps of the workflow can be manipulated, starting with the sample in solution, the EM grid, and the way the sample is deposited and vitrified. In this review, we explicate the fundamentals of the different stages involved in sample preparation (Fig. 1). Based on the requisites of sample preparation, we adumbrate the benefits and drawbacks of the available methodologies.

Sample Optimization

The ideal microscope session results in a dataset with a good concentration of intact particles embedded as a monolayer in thin ice; the particles are randomly oriented, evenly distributed, and the vitrified specimen is free of contamination. The quest begins in the wet lab where the candidate molecule is identified, and the construct is designed. Numerous biochemical assays are performed in isolating and purifying the macromolecule from its cellular environment while attempting to emulate its native-like conditions.

The molecule's native environment is mimicked in a bulk solution by tuning the pH and adding salt, glycerol, or other additives. In creating a film of sample solution intended for the microscope, the high surface-to-volume ratio can give rise to challenges in stability. Stability issues can range from preferential orientation, to denaturation or aggregation of samples [14]. Samples are often divided in different classes, which include cytosolic, membrane proteins and larger macromolecular protein complexes. Cytosolic, or soluble proteins, have a tendency to remain stable in thin films due to the hydrophilic amino acids on their external structure. Membrane proteins, on the other hand, often needing further stabilization. The exposed hydrophobic amino acids that are naturally anchored within the lipid bilayer make them especially susceptible to the air–water interface. Membrane proteins are stabilized by mimicking the lipid bilayer using detergents, amphipols, nanodiscs, and styrene–maleic acid copolymers [26]. These additives form a protective ring around the hydrophobic regions of the sample, with amphipols having a higher affinity than detergents. Lipid nanodiscs and styrene-maleic acids copolymers are more recent developments that have a closer resemblance to the lipid bilayer [26]. Through nanodiscs, different conformations of receptors in pentameric ligand-gated ion channels were resolved, shedding light on its gating mechanism [27]. Another approach to handle fragile proteins is to block the air-water interface. While detergents are often used during the purification process, others are added prior to the sample's deposition on a grid, shielding it from the interface. The addition of the fluorinated detergent CHAPSO has shown promising effects on particle orientation in the case of RNA bacterial polymerase [28]. To preserve larger structures, chemical crosslinking can be applied to maintain

their structural integrity. Crosslinking can be done either in solution or using a sucrose gradient [29,30]. Caution should be taken in rigidifying structures as flexible domains can be locked in one conformation.

Prior to introducing the sample to the electron microscope, the sample in solution can be visually assessed by negative staining. This is used for rapid screening prior to cryo-EM and helps to evaluate the homogeneity of the sample along with its buffer components [31]. While negative staining provides general insight about the molecule, high-resolution details cannot be resolved. Despite the screening benefits, dissociation at the air-water interface [32] or concentration-dependent aggregation [33] of molecules can still arise when preparing the same specimen in the vitrified state [33].

Sample carriers and treatments

Sample carriers are historically circular EM grids of 3 mm diameter that consist of at least two components: a mesh base and foil. The mesh base is made of metal to attain mechanical stability, conduction of the electron beam, and heat dissipation [34]. Commercially available meshes range from 200 - 400 grid bars per inch and are made out of copper, gold or nickel. On top of this mesh, a perforated foil with micrometer-sized holes is placed. Lacey foils provide heterogeneously spaced holes while holey foils offer perforated foils with predefined hole size, space and arrangement. Copper meshes with carbon foils are commonly used, which provide Thon rings for microscope alignment [35]. UltrAuFoil grids are gold meshes with gold foil that increase electric conduction and heat dissipation, improving their stability [36]. Upon vitrification, the difference in thermal expansion between the foil and sample will introduce stress [37]. Stress can result in buckling of the ice layer, leading to beam-induced motion in the microscope, the primary source of resolution loss [37]. The grid geometry is often chosen based on the imaging scheme. The degree of ice buckling can be reduced by decreasing hole sizes to hundreds of nanometers [37]. On the contrary, hole sizes in the micrometer range can be used to acquire multiple images per hole.

Some particles have a tendency to stick to the substrate and are unseen in the holes of the foil. Several additions and alterations to the sample carriers have been presented to circumvent this, roughly classified into two groups: adhering particles to a continuous film or driving particles into the holes. The first group comprises adding a continuous film to which the samples can adhere. Often, these films are made of carbon or graphene (oxide), which can notably increase the number of particles per hole [38–40]. The thickness of these films is generally minimized to reduce extra background noise in microscopy images. It is not trivial to maintain single layers of graphene or graphene oxide sheets [41], however, success using these films was recently shown on the 52 kDa streptavidin particle resolved to high resolution [42,43]. Moreover, the thin films can be chemically treated to increase binding affinity. Functionalized films, for example with Ni-NTA, have shown to bind histidine-tagged particles to increase the number of particles per hole [44]. Antibody binding has also been explored to capture macromolecules in their native state from a cell culture [45]. In addition to increasing the number of particles per hole, these methods have proven advantageous in shielding macromolecules from the air–water interface [46]. The second approach deploys chemical treatment to prevent particles from sticking to the foil and push them into the holes. Treatments through the use of PEGylation have shown potential in distributing particles within the holes of the grid [47]. As no additional film is required, extra background noise in the micrographs is avoided.

To deposit a liquid solution onto a sample carrier, the carrier must be wettable. Plasma treatment is often used in cleaning grids to increase their temporary wettability. In plasma generation, different states of the gas molecules are generated, referred to as a species. The ratio of the species formed depends on the chamber configuration, its operating power, as well as the pressure and flow. The plasma that is generated consists of molecules, ions, electrons and radicals [48]. Radicals of these species have an unpaired valence electron, which makes them extremely reactive. Ions, electrons and charged radicals will be accelerated towards the electrodes. The molecules and uncharged radicals move in the direction of the gas flow. Therefore, the position of the plasma with respect to the grid will affect the amount and type of species that end up at the grid surface. The species etch or bind to the foil, which modifies the surface characteristics of the grid. When ambient air is used in plasma treatment, oxygen and water (dependent on the humidity) are the main reactive species, giving the grid an overall negative charge.

To increase reproducibility of the plasma treatment, a controlled mixture of gases can be used [49]. Hydrogen plasma has been used to make the plasma treatment gentler. Other compounds, such as amylamine, are used to give a positive charge to the grid surface temporarily [50].

Deposition

The ability to deposit a suitable monolayer of macromolecules onto an EM grid still presents an enormous challenge. While frequent successes are observed for the deposition of well-behaved samples, many iterations are often needed for delicate specimens. To attain fruitful depositions, understanding the factors of influence is key. These factors include shear forces in thin film formation, evaporation, the final layer thickness, and molecular dynamics at the various interfaces.

The holes in the foil of the grid have to be filled with a thin layer of liquid. A suitable layer for microscopy is remarkably thin compared to the diameter of the hole itself, for example, a 50 nm layer across a 2 µm diameter hole [51,52]. Thus, it is important to understand how a thin layer can be formed on an EM grid. Two approaches can be used in order to fill the holes. In the first case, the holes must be completely saturated by a large droplet of liquid in order to be filled. After saturating the holes through application of a thick liquid layer, wicking is a necessary step to obtain an appropriate layer thickness. In a second approach, liquid is dragged over the perforated foil and the movement fills the holes. With this technique, a thin layer can be formed without the need of additional wicking. During all thin film creation, the sample encounters shear forces, which originate from a difference in velocity within the liquid. These forces get larger as the length scale and/or time scale gets smaller. It has been hypothesized that shear forces are able to damage fragile proteins [53,54].

After the thin layer is deposited on a grid, it is especially susceptible to evaporation due to the high surface-to-volume ratio. This alters the temperature as well as the pH, salt and particle concentration carrying the risk of conformational changes to the macromolecule [49]. The amount of evaporation is dependent on the deposition environment and the sample temperature, as well as the duration of

exposure. Increasing environmental humidity and/or working at a low temperature, such as 4 °C, evades evaporation [55]. This can be done in combination with temperature control of the sample to dewpoint. The dewpoint temperature is the temperature at which the evaporation and condensation of water are in equilibrium. Alternatively, decreasing the elapsed time between deposition and vitrification will reduce sample evaporation from the grid.

Precise control over layer thickness at the nanometer scale is key, as thinner ice decreases background noise and may lead to higher resolution reconstructions [56]. The dimensions of the particle will determine a minimum layer thickness. Thinner water layers will exclude or denature the particles of interest, while thicker water layers may induce particle overlap. Furthermore, the components in solution will influence the deposition and wicking behavior. There can be variations in viscosity and density between solutions; furthermore, detergents can significantly decrease the surface tension [56]. Thus, each combination of desired thickness and fluid properties will require their tailored deposition settings.

The dimensions of a thin layer (10-100 nm thick) imply that macromolecules have many more interactions with interfaces than in a bulk solution. Understanding interface reactions is critical to control some of the variability in sample deposition. The air–water interface poses a particularly hostile environment for the particle. When the hydrophobic region of a macromolecule encounters the air–water interface, it can lead to its partial to complete unfolding [57]. The number of particles that denature relies on the total amount of interactions as well as their probability to denature. The number of interactions has been estimated based on molecular dynamics, using the layer thickness and molecule diameter: A molecule of 100 kDa in a layer of 100 nm can have 1000 collisions with the interfaces within a second, which indicates that multiple interactions will always take place [57,58]. Minimizing time between deposition and vitrification down to tens of milliseconds has shown be helpful in preserving protein integrity [32].

Different methodologies have been introduced to prepare adequate films for single particle analysis (Table 1). The methods can be classified into three groups: blotting, droplet-based, and scribing.

Blotting

Blotting is currently the standard and most used technique to create a thin film. It has a long history [59–61] and has been the only commercial option until recently, with devices such as the Vitrobot (Thermo Fisher Scientifiic), EM GP (Leica) and the CP3 (Gatan). Several protocols for sample preparation involving blotting are available [62,63] and provide flexibility to the user [64]. In this technique, a droplet of 3–5 µL is pipetted onto a hydrophilized grid that is held by sharp tweezers. A millimeter(s)-thick sample layer fully fills the holes. Then, filter paper is used to wick away the excess fluid on the grid to leave a thin sample layer. Upon retraction of the filter papers, the aqueous bridges between grid and filter paper break. Blotting typically is performed for a number of seconds in an environment with elevated humidity (Fig. 2a). During this exposure, evaporation as well as the air–water interface may influence sample behavior.

Layer thickness by blotting is controlled using blot time, the properties of the filter papers, and the blot force. Blot time controls the number of seconds the liquid is drawn into the filter paper. The properties of the filter papers determine the capillary suction, which together with the contact area dictates the rate at which the sample is taken up by the papers. As the length scale of the filter paper pores is orders of magnitude larger compared to the desired layer thickness, it is considered as one factor that could lead to irreproducibility in layer deposition [65]. A recent study was performed revealing only a few unpredictable initial contact points between sample and filter paper rather than a homogeneous interaction of the blotting paper with the sample. The distance between the fibers of the filter paper and the grid is typically multiple micrometers, leaving space for air to enter. The entrapment of these “air fingers” is deemed a locally violent event where shear stresses might reach the order of kilopascals [65]. Finally, tweezer and location of the grid within the tweezers add variability to the results.

Because blotting instruments can be made on a low budget, modifications have and are being explored to provide a flexible instrument. Blotting devices can provide one-sided/back-sided blotting for delicate or filamentous structures [66,67]. Applying sample and blotting multiple times in order to concentrate particles on the grid has been reported [68]. In combination with binding agents, multiple sample application can be used to bind samples and wash away the residual liquid, creating a purification step

on the grid [45]. Time-resolved studies have also been tested through flashlight additions for cage compounds. Light sources placed in the trajectory of the tweezer to the cryogen can capture different conformations in activation of photolabile compounds in a preset timeframe [69,70].

Droplet-based

While deposition through blotting involves pipetting millimeter-sized droplets, more agile micron-sized droplet formation techniques have been employed in parallel for almost as long [71]. The main motive toward droplet-based techniques has been to capture fast biological processes. Multiple small droplets are generated, which travel and land onto a grid. Deposition may take place on a grid that is in trajectory towards the cryogen, resulting in a fast process. Because of the limited processing time, evaporation of the deposited layer is reduced (Fig. 2b).

Generally 2–40 µl of liquid for deposition is required, due to the dead volume of the device and the number of droplets that are generated [72–74]. In droplet formation, large shear forces can be present. The higher the velocity and the smaller the droplet, the higher the shear force required to form it [75,76]. Droplet formation can occur through various methods.

1. **Ultrasonic spray**: An ultrasonic spray can be used through vibration of an element at very high frequency. Frequencies of over 100 kHz can be used to break up the fluid and generate a spray of droplets sized from 1–10 µm [73,77].

2. **Gas pressure**: The sample solution originates from a nozzle and is surrounded by a high-speed gas sheath. When activated, shear force between the sample and the gas will create a spray of droplets [72,78]. The distribution of droplet diameters can be influenced when changing the gas pressure, resulting in a varying velocity difference between gas and liquid [78]. In order to create a more controlled spray, additional charging of the nozzle is possible. By charging nozzle and sample, the droplet size is more controlled and dispersed, preventing coalescence [74]. A comparison has been made between different sprays with respect to droplet size and thinning on an EM grid [79].

3. **Electrostatic spray**: An electrostatic spray can be generated by applying a high potential (3–10 kV) to the sample and grounding the grid. The repulsive forces between the nozzle and the sample will generate a spray of droplets. This technique was used to make droplets of 0.25–0.5 µm that were deposited in combination with blotting [80].

4. **Inkjet**: Inkjet printing has been presented to generate droplets-on-demand. A pulse train in the piezo-electric dispenser creates a series of single droplets of well-defined diameter (25 µm) and direction [81].

The greatest hurdle in droplet-based methods is the spreading of the liquid onto the grid [79]. As the droplet diameter is typically multiple micrometers, the holes will be filled by a relatively thick layer. Upon contact, the deposited droplets spread over the foil and form spherical caps. The peripheries of these caps are sufficiently thin for imaging. The impact of the droplet as well as the hydrophilicity of the substrate determine the shape of the cap and thereby the useful area for imaging [72,74,78]. This could provide sufficient imaging area for some applications but is often increased by additional thinning of the liquid through wicking. Self-wicking grids provide a method to thin the sample at grid square scale. They enhance wicking through nanowires on the grid bars that create a sponge-like effect [82]. Nanowires are made by treating copper-rhodium EM grids with a basic solution of ammonium persulfate, which leads to the growth of $Cu(OH)_2$ nanowires [82]. Self-wicking grids are commercially available, though in-house fabrication requires a degree of expertise. The total amount of thinning can be influenced by the volume applied, capillary suction of the wires as well as the time. Through these measures, thickness can be tuned using wicking time in the millisecond range, where longer exposures will result in thinner layers. More recently, a hybrid methodology of droplet-based deposition in combination with back-side blotting has been demonstrated [83]. The sample is deposited using ultrasonic spray and wicked through a grid into a glass fiber filter rather than nanowires.

The fastest device holds a record of deposition to vitrification down to 6 milliseconds [84]. Such fast processes will significantly decrease the amount of interactions with the air–water interface [28,51,85]. This

proves beneficial for some proteins, however, instances have been shown where minimizing time negatively impacted the sample [84]. Besides, a short time between deposition to vitrification offers vast potential in capturing molecular dynamics. Mixing samples in transit to vitrification paves the way for analysis of different conformational states in molecular reactions [86,87].

Scribing

In so-called "scribing" methods, an element is moved over the grid to deposit a thin layer. The scribing element is not touching the grid but maintained at a distance of some tens of microns, defined as stand-off distance. The sample forms a liquid bridge between the element and the grid. By hovering the element over the grid, the holes are filled (Fig. 2c). The exerted shear forces depend on the scribing velocity and stand-off distance, comparable to the shear between two parallel plates, estimated to be less than one Pascal [88]. Two different scribing-based methods will be described in more detail:

1. **Pin printing**: In pin printing, a solid metal pin deposits samples using a dip pen method. The solid pin is dipped into a stock solution to collect a sub-nanoliter droplet on the tip of the pin. The pin is moved to the grid to form a liquid bridge, and while moving over the grid surface a thin layer is deposited. During processing the grid is maintained at the dewpoint temperature to minimize evaporation or condensation of water [89].
2. **Capillary writing:** In capillary writing, a sample solution is sucked into a capillary, and forced out by gentle pressure to deliver the sample at the grid surface in a scribing motion [90]. Approximately 3–20 nanoliters of sample is deposited onto the grid as a layer, which has to be thinned to obtain a usable layer thickness. Thinning is achieved by steering the grid temperature to control evaporation, or by reabsorption of excess fluid through the capillary device.

The layer thickness is controlled by the volume that is introduced onto the grid, as well as the scribing velocity and distance between element and grid. The liquid bridge between scribing element and grid will follow the scribing movement, leaving a trace of sample behind [89]. The width of this trace relates to the diameter of the scribing element, where a larger element allows more grid surface coverage per time unit. The Achilles heel of scribing is the deposition time, which is one to several seconds. This

leads to abundant interactions of the sample with interfaces and requires preservation of the layer during processing [89,90]. By manipulating the grid temperature with respect to the chamber temperature and humidity, evaporation or condensation can be modulated to extend the lifetime of a layer [89,90]. Controlled evaporation can be used to thin and concentrate the sample [90].

The scribing motion can be used to deposit sample in a specific pattern and place and onto the grid. This opens up the possibility for deposition of multiple samples or allow samples to interact. In addition, the technique of scribing has been combined with miniaturized protein purification within the scribing element. This drastically decreases the amount of cell lysate and time in purification required, directly linking proteomics with grid preparation for cryo-EM [91].

Vitrification

Following sample deposition onto the carrier, the sample must be vitrified and remain in a glass-like state to preserve its interior features, opening the doors to reconstructions at high resolution [92]. Rapid cooling is essential to produce vitreous ice and prevent ice crystal formation. The required cooling rates to vitrify water have been estimated in a range from 10^5 up to 10^8 K/s, implying there is no clear consensus on the critical cooling rate [93–95]. Addition of cryoprotectants to the sample can decrease the critical cooling rate. The rate that can be achieved is dependent on the type of cryogen, its temperature and the sample. Thermal and physical properties of the cryogen dictate its cooling efficiency. Cryogens tested for cryo-EM include ethane, ethane/propane and freon as well as slush nitrogen. A comparison has been done showing that liquid ethane has the best relative cooling efficiency in single particle studies [96]. Ethane/propane mixtures have been used as they do not solidify when they are at liquid nitrogen temperature and have been shown to have a larger cooling capacity for thicker samples [97].

Currently, plunging a grid into a bath of liquid cryogen, known as plunge freezing, is widely used to vitrify samples of 1 µm or thinner (Fig. 3a). This technique has been the primary method of vitrification since its proof of concept with water [15]. When a grid is plunged into a bath, the grid circumference

makes the first contact with the cryogen. Heat conduction through the grid bars and foil leads to vitrification of the sample [98]. After vitrification, grids have to be handled below -137 °C to prevent devitrification of the ice layer [15,59]. Then the grids are loaded into a side entry holder or they are mounted in a cartridge for further batch processing. This includes automated specimen handling at low temperature inside the microscope. The mounting procedure can be considered a hassle for many novice users as the clipping must be performed at liquid nitrogen temperatures [62,63]. Any handling of grids during or after vitrification runs the risk of contaminating the precious frozen samples with ice crystals.

Recently, jet vitrification has been described to vitrify samples deposited on pre-clipped autogrids (Fig. 3b). Autogrids are the ensemble of the EM grid held within a sturdy copper ring by means of a clip. The grids can then be automatically handled within high-end microscopes containing autoloaders. Jet freezing was originally designed for tissue fixation and subsequent freeze-substitution [99]. In jet freezing for cryo-EM, two cryogen jets are directed towards the center of the grid, aiming at the deposited sample. With continued cooling by the jets the cryogen spreads out, thereby completely cooling down the grid together with the bulky autogrid ring. Measurements have shown that higher cooling rates are achieved by jet freezing compared to plunge freezing [89].

Discussion

Cryo-EM is evolving to become a go-to structural biology technique, provided that sample preparation will keep up with demands. Structural biologists with different backgrounds have already converted to the field as it offers promise for new structures at high resolution. Understanding the role of different macromolecular structures will push for workflow maturation, especially in light of the recent viral pandemic [100]. Cryo-EM is moving at a fast pace, where results can quickly be obtained once the right settings are determined [24]. Faster cameras have been developed to collect more images over a shorter time. Screening microscopes are in high demand so more grids can be prepared and checked. Software is being developed to increase throughput in different steps of data processing [24]. Collecting excess

data and only later filtering out usable particles is the current strategy [101]. However, this strategy does not address the problems of robust and reliable specimen preparation. The speed of developments has put the spotlight on the meticulous and time-consuming number of iterations needed to adjust sample properties. Many rounds of optimization are often needed before having a suitable molecule for data collection [14].

Sample preparation must increase in yield and reach a high standard to keep pace. Easier, soluble proteins have already been resolved or are being resolved now, leaving many complex, heterogeneous samples to be the candidates of future studies [19,102]. At this stage, grid preparation is approached from different angles to learn which of the numerous options is most suitable for a particular sample. Several divergent methods are bundled in devices entering the market [85,89] or are introduced experimentally in labs [72,77,80,83,84,87,90,103]. Miniaturization has pushed for greater efficiency in the overall workflow. New technologies focusing on control and throughput will undoubtedly prove valuable to users trying to optimize their microscope usage. Speeding up the movement of deposition to vitrification has already shown value in the battle against sample denaturation. At the same time, there is a draw toward more flexible, creative, low-cost techniques without the ease of use of commercial frameworks [77]. Since it is still difficult to pinpoint the exact issue that samples encounter on an EM grid, more platforms will surely provide more answers.

The field has the momentum to learn from the upcoming developments and alleviate the bottleneck in grid preparation. Since the cryo-EM infrastructure is already at a high level, a big leap can be taken once sample preparation yield increases and microscopes are used more efficiently. In this phase, streamlining and throughput will play a crucial role in the process, as it does in x-ray crystallography [104]. Foundations of cryo-EM data management are in the works and will be imperative as more scientists contribute to the pursuit of structures [105].

While boundaries are continuously being pushed, there are still limitations in the technique. Size does matter in cryo-EM. The majority of small structures that are often targets in drug discovery are not yet solvable by cryo-EM and are still being resolved through crystallography [106]. At the moment, structures

as small as the 52 kDa streptavidin and the 40kDa SAM-IV riboswitch RNA [107] are the practical limits[108]. With the expansion of the field and new developments, it is foreseeable that closely related techniques will evolve as well. Time-resolved microscopy will likely be used more frequently to observe different macromolecular states [87,102,109–111]. In addition, the behavior of macromolecules in their native environment is of tremendous interest for many. Cell tomography, known for its intricate workflow, is already benefiting from the groundwork in single particle analysis [112]. Based on the rate of advancements in the field, we are optimistic that sample preparation will accelerate in line with the demands. Demystification of the invisible hands in sample preparation will propel cryo-EM as a standard technique in structural biology.

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Competing interests

The University of Maastricht filed patents with Rene Henderikx and Peter Peters as inventors regarding sample preparation for cryo-EM. Giulia Weissenberger and Rene Henderikx are employed by and Peter Peters is shareholder of CryoSol-World that holds licenses for these submitted patents.

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