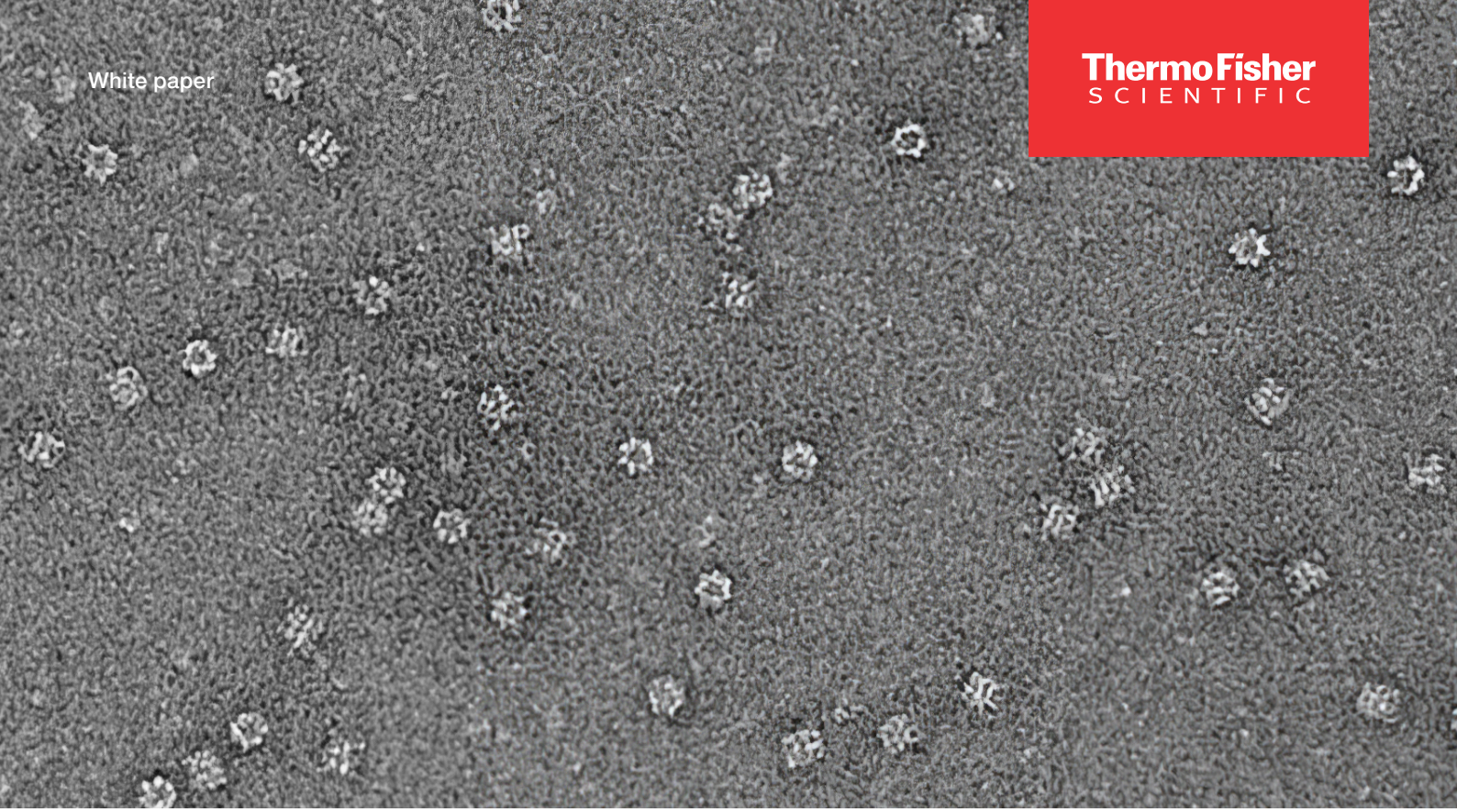
White paper



# Maximizing sample quality for cryo-EM single particle analysis

Sample quality is essential for optimized, high-quality cryo-electron microscopy (cryo-EM) data for single particle analysis (SPA) and reconstruction. The sample preparation process consists of multiple variables that can be adjusted to improve specimen quality. This white paper highlights a number of available options for altering protein behavior during vitrification, which can greatly improve the outcomes of cryo-EM experiments.

Over the past 10 years, cryo-EM imaging has progressively enabled ever higher resolution structures, leading to a number of revolutionary insights. The necessary high-quality images for these results, however, rely heavily on the quality of the samples. For single particle analysis, this consists of a biological macromolecule (normally a protein) that is well-folded, homogeneous, non-aggregated, monodisperse, and preserved in vitreous ice at good density. The resulting sample should have a high number of particles in each image that are not overlapping or otherwise aggregated. If biochemical information is available about native protein structure and oligomeric state, the sample should also conform to this data.

Sample preparation is a critical part of the cryo-EM workflow, and it is therefore important to have a good understanding of each step in the process, along with selection of appropriate reagents, tools, and instruments. A typical sample preparation workflow contains the following steps:

**Table 1. Vitrification workflow**

| | Stage | Summary | Techniques |
|---|---|---|---|
| | **1. Protein expression** | The protein expression system can change protein abundance, as well as solubility, aggregation state, and other factors. | Use the Thermo Scientific™ Gibco™ **Protein Expression System Tool** |
| | **2. Protein purification** | Biochemical purification and buffer condition screening (as well as options such as cross-linking) produce homogeneous, well-folded, and monodisperse protein samples. | A range of chromatography and other tools, as seen on the Thermo Fisher Scientific protein sample preparation page including Thermo Scientific™ **VitroEase™ Buffer Screening Kit** |
| | **3. Quality assessment** | Assessment of purification quality, through biochemical assays and/or negative-stain TEM, helps ensure the sample is suitable for vitrification. | Stability and size assays, as well as room-temperature TEM |
| | **4. Vitrification** | The vitrification process includes many variables that can affect the density, state, and dispersion of the protein on the grid. | Vitrification device configuration along with grid selection and modification |
| | **5. Grid screening** | Identifying the highest quality grids for high-resolution data collection requires careful inspection of the grids. | Cryo-TEM |

## Protein purification and biochemistry

Most successful imaging protocols utilize a protein solution that is approximately 1 mg/mL of homogeneous, well-folded, monodisperse protein. If a sample does not conform naturally to this challenging set of requirements, changes to the protein purification conditions and protocol should be explored.

Adjusting the conditions in which proteins are purified, stored, and utilized is one of the fastest and simplest ways to affect the behavior and condition of the sample.[10,21,32] Variables include (but are not limited to) pH and buffer identity, as well as salt, ligands, detergents, and small molecule additives. Buffer has been shown to improve cryo-EM samples in a number of experiments, including in the analysis of amyloid fibrils[29] and TMEM16.[8] There are many commercially available screens to identify the conditions that are optimal for cryo-EM experiments, including the Thermo Scientific **VitroEase Buffer Screening Kit.**

**Table 2. Common changes to buffer components that can improve protein quality**

| Issue | Buffer components to vary | Potential results |
|---|---|---|
| Aggregated protein and/or compositional heterogeneity | Salt concentration and pH. For membrane proteins, detergents, amphipols or nanodiscs can be used | Cleaner micrographs, fewer clumps, and more usable particles per hole, as well as better 2D class averages[12] |
| Air-water interface damage and/or preferred orientation | Detergent variation, particularly CHAPSO | More orientations, as well as improved 3D reconstruction isotropy[5,10] |
| Need for stabilizing additives | Small molecule additives | Improved complex stability/monodispersity, as well as higher achievable resolution[3] |
| Unexpected interactions between the buffer and sample support | Any, but with a particular focus on detergents (when present) and small molecule additives | More consistent ice along with more even particle distribution[29] |

Simple modifications to the protein surface, such as PEGylation,[33] will change surface charge, and can therefore change vitrification behavior. As a result, this should also be considered as an option with samples that show a tendency to aggregate.

### Cross-linking can stabilize complexes for cryo-EM

Cross-linking is often used in single particle analysis when a macromolecular assembly is fragile, dynamic, or held together by weak interfaces that tend to fall apart (or rearrange) during purification, blotting, and vitrification. By introducing low, carefully titrated covalent stabilization, subunit dissociation and “breathing” motions can be reduced enough to improve particle integrity, 2D class quality, and the fraction of usable particles, especially for multi-protein/RNP complexes that otherwise show compositional heterogeneity. A key tradeoff is that cross-linking can also lock in non-native states, reduce the conformational diversity you actually want to resolve, and/or introduce subtle structural distortion, so conditions are typically screened gently and validated with activity/binding assays and negative-stain EM.

Table 3. Common cross-linking stabilization methods

| Method (common name) | Summary | Typical chemistry and reagents | When to use | Common problems |
|---|---|---|---|---|
| GraFix (gradient fixation)[14] | Rate-zonal centrifugation through a density gradient that contains a fixative | Glutaraldehyde (or other mild fixatives) | For very fragile, multi-subunit complexes; reduces dissociation while purifying | Over-fixation can blur high-resolution features; gradient components must be removed before freezing |
| Gentle solution cross-linking (batch crosslinking)[13] | Adds low-dose crosslinker directly in solution, then quenches | Glutaraldehyde (or formaldehyde-like fixatives) | For quick stabilization when gradients aren't needed | Can induce aggregation when higher sample concentrations are used during reaction |
| AgarFix (agarose fixation)[1] | Embeds sample in an agarose drop; the crosslinker is then diffused and the sample recovered | Mild chemical crosslinkers, which are diffused into the matrix | When batch crosslinking causes aggregation; for "sticky" complexes | The recovery step adds handling time, and diffusion time/conditions need tuning |
| Amine-reactive NHS-ester crosslinking[24] | Crosslinks via lysines/N-termini using NHS esters, then quenches with Tris/glycine | BS3, DSS, or DSG (families widely used in structural biology) | For capturing weak interfaces and stabilizing assemblies; also common in integrative workflows | Can preferentially modify functional lysines, and spacer length/solubility can have an impact; additionally, hydrolysis competes with the crosslinking |
| Zero-length crosslinking[24] | Creates "no spacer" links between proximate functional groups | EDC (carbodiimide, which couples carboxyls to amines) | For mapping very close contacts; sometimes used to rigidify interfaces | Highly condition-dependent due to pH/side reactions; can also be harsh for delicate complexes |

If these methods are not enough to keep the protein in a suitable state, changes to expression construct, vector, and protocol may be required in order to obtain a more soluble, well-folded macromolecule. Thermo Fisher Scientific provides extensive support for relevant expression and purification workflows.

## Protein quality assessment

Before vitrification and high-resolution imaging in a high-end cryo-transmission electron microscope (cryo-TEM) is attempted, it is important to establish that the expression and purification protocol has produced a sample of appropriate stoichiometry and complex stability. Ideally, this means that it consists of well-folded macromolecules in a suitably concentrated, monodisperse, and homogeneous solution. There are a wide variety of biophysical assays that can be used to assess protein state, including:

- **Differential scanning fluorometry**,[18, 19] also known as ThermoFluor, which measures thermal unfolding (melting temperature) through the binding of an extrinsic hydrophobic dye (e.g., SYPRO Orange). These dyes often interact with detergents, so this method is not recommended for membrane proteins.
- **ProteoPlex**,[4] which measures thermal unfolding and aggregation behavior for macromolecular complexes, utilizing a sparse chemical matrix.
- **CPM thermal shift assays**,[2] which uses a thiol-reactive reporter dye to measure thermal unfolding via the exposure of cysteines. This method works well with membrane proteins.
- **SEC-MALS**,[26] which measures absolute mass and polydispersity across SEC peaks, thereby reporting oligomeric states, as well as the presence of aggregates or polydispersity.
- **Mass photometry**,[27] which reports on single-molecule mass distribution via light scattering during transient surface binding. It works at low concentrations and is label-free, aiding in the detection of weak/transient assemblies, or minor populations.
- **Negative-stain electron microscopy**,[28] which provides direct visualization of particle integrity, morphology, and flexibility, making it one of the preferred final screening tools prior to cryo-EM. However, its low resolution, as well as its occasional staining and surface artifacts, make it unreliable for stoichiometry. Thermo Scientific™ Talos™ 12/F200C TEMs are an ideal choice for negative stain pre-screening prior to vitrification, though desktop S(T)EM devices, such as the Thermo Scientific™ Phenom Pharos™ Desktop SEM, can provide a useful alternative. Negative stained samples can be prepared with the ready-to-use Thermo Scientific™ VitroEase Methylamine Vanadate Negative Stain or the VitroEase Methylamine Tungstate Negative Stain.
- **Native mass spectrometry (MS)** [34] measures the mass of proteins and complexes in native-like conditions, enabling the determination of subunit composition, stoichiometry, ligand binding, and heterogeneity. It is best used for soluble complexes, protein-ligand binding, and samples where high-accuracy stoichiometry/composition is important.

The choice of assay is dependent on protein type and the characteristic being investigated (Table 4).

**Table 4. Suggested assays by protein type**

| Protein type | Stability | Size/monodispersity | Pre-cryo-EM integrity check |
|---|---|---|---|
| Soluble globular proteins | ThermoFluor (DSF) | SEC-MALS, mass photometry | Negative stain |
| Multi-subunit soluble complexes | ProteoPlex | SEC-MALS, mass photometry, negative stain, native MS for stoichiometry | Negative stain |
| Membrane proteins | CPM | SEC-MALS | Negative stain |
| Weak/transient assemblies | DSF or CPM (with ligands) | Mass photometry (primary), native MS for stoichiometry/ligand-binding | Negative stain |

# Vitrification

In the vitrification step, the protein solution is dispensed onto a sample support, and then rapidly frozen with the aid of a plunge-freezing device. For high-resolution studies, the resulting vitrified specimen should have well-dispersed protein particles with as high a density as possible without crowding and aggregation. It should be frozen in a flat, continuous ice layer, no thicker than ~100 nm (in order to allow transmission of the electron beam). Note that variations in both the sample support and vitrification settings can have a significant impact on ice thickness, sample density, and dispersion.

## Plunge freezing device

The Thermo Scientific™ **Vitrobot™ Mark IV System** is an established tool that offers semi-automated vitrification for simplified and routine cryo-EM sample preparation. It performs the cryo-fixation process at constant physical and mechanical conditions (i.e., temperature, relative humidity, blotting conditions, and freezing velocity). Improved stability for the cryo-fixation environment can help improve reproducibility and quality of sample preparation prior to cryo-TEM observation. A range of variables can be adjusted in the Vitrobot System in order to alter sample particle dispersion and density. The Thermo Scientific™ **VitroEase Cryo-EM Training Kit**, which offers a detailed visual manual, is also available to help new users learn the intricacies of the vitrification process. Some default settings that work well for many proteins are given below, together with potential adjustments that can be made for specific issues (Table 5).

**Table 5. Default Vitrobot settings and potential adjustments**

| Variable | Suggested setting |
|---|---|
| Temperature | 4°C |
| Humidity | 100% |
| Wait time | 5–10 s |
| Blot force | 0 |
| Blot time | 3–4 s |
| Blot count | 1 |
| Blot offset | 0 mm |
| Drain time | 0 s |
| Plunge speed | Default |
| Cryogen | Fresh liquid ethane |

| Issue | Variable to adjust |
|---|---|
| Ice too thick | Increase blot time or force, increase temperature, reduce sample concentration |
| Ice too thin/empty holes | Decrease blot time or force, increase humidity |
| Particles denatured/ at air-water interface | Reduce wait or blot time, lower temperature |
| Preferred orientation | Reduce blot time, lower temperature, Increase ice thickness, adjust blot force asymmetrically (offset) |
| Aggregation | Lower temperature, reduce wait time or blot force |
| Ice gradient across grid | Adjust blot offset, check pad condition, avoid excessive blot force |

## Specimen support selection

For most cryo-EM experiments, the protein sample is dispensed onto a 3-mm-diameter grid, which is coated with a sample support film (Figure 1).

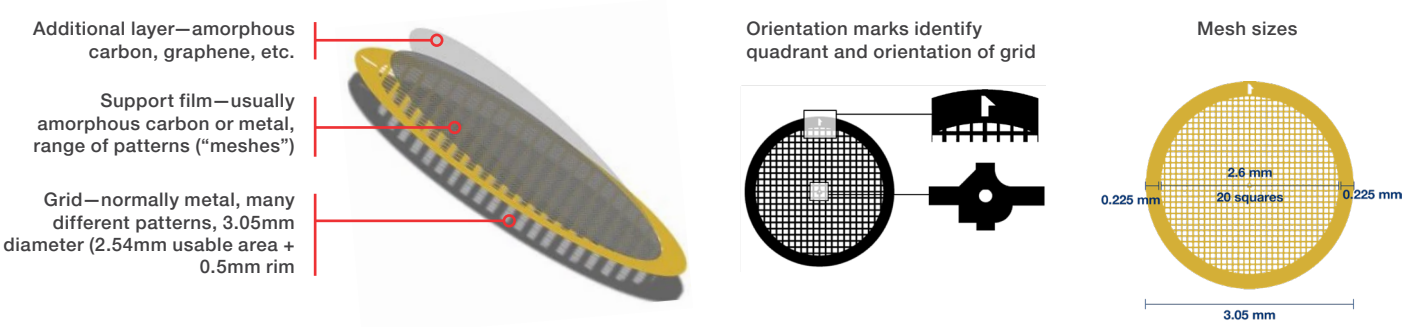
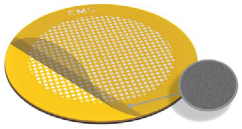


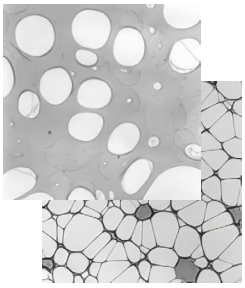

**Figure 1. Diagram of a cryo-EM grid, highlighting the mesh, support film, and other layers. Images courtesy of Quantifoil Micro Tools GmbH.**

Both the grid and the support film can be made from a variety of materials and with a range of different patterns. Grid selection is dependent on both the nature of the protein sample and the type of experiment. For cryo-EM, copper and gold grids are normally used. They are both conductive and radiation hard, which helps to ensure that electrons are rapidly conducted away from the sample, reducing radiation damage. 200–400 size square meshes are usually chosen, which allows for adequate sample support while still maintaining large open imaging areas. There is a broad range of options available for the choice of sample support film (Figure 2). For the highest resolution data, gold foils on gold grids are generally preferred, as these produce flatter, thinner, more consistent ice and reduce specimen movement on exposure to the beam, enabling higher resolution reconstructions.[17, 23]



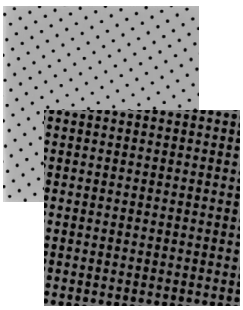
### Continuous films

- Formvar/polymer-resilient, not radiation hard (<60kV scopes only)
- Carbon—more radiation hard but much more fragile



### Holey/lacey carbon

- Random arrangement of random hole sizes
- Can be made in-house or bought from suppliers
- Don't work well with automation
- Good with variable-size samples



### Defined hole films[7]

- Carbon (*e.g.* Quantfoil, C-Flat), $SiO_2$, SiN, polymer
- Holes of pre-defined size and arrangement
- Good with automation
- Radiation stable, but mechanically fragile
- Made with lithography
- Cryo-EM standard choice

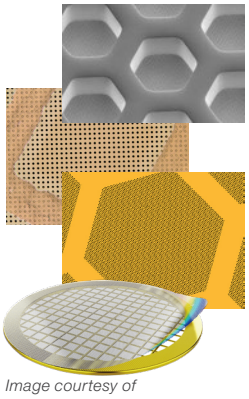
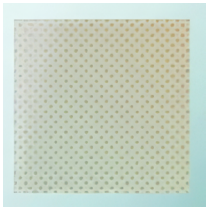


*Image courtesy of Calibre Scientific Ltd*

### Metal foils[11,17,23]

Foils made by lithography from gold, palladium, amorphous nickel-titanium alloy (ANTA) and others *e.g.* UltrAuFoil, HexAuFoil, Au-Flat, ANTCryo

- Overcome issues with foil stability
- Produce flatter, thinner ice
- Reduce specimen movement



### Silicon Nitride/$SiO_2$

- Example: CryoSilico
- Resiliant films, very biocompatible
- Stable at higher temperatures
- Can provide modifiable surface for chemistry

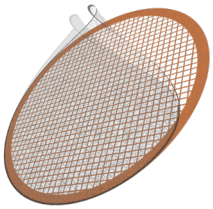


*Image courtesy of EM Resolutions Ltd.*

### Additional layers

- Graphene, GO, amorphous carbon
- Improve distribution and density by providing a surface across holes to absorb particles/provide additional support
- Can be modified to introduce additional grid functionality

**Figure 2. Common options for grid support films. Unless otherwise stated, images courtesy of Quantifoil Micro Tools GmbH.**

Support film hole size can also change the behavior of the grid; as a result, support films are available with a large range of hole sizes, shapes, and geometries. The universal naming convention for grids is R x/y, where R/S is the hole shape ("R" is round and "S" is square), x is the hole diameter, and y is the space between the holes. Some examples of common film geometries for single particle analysis data collection are shown in Figure 3.

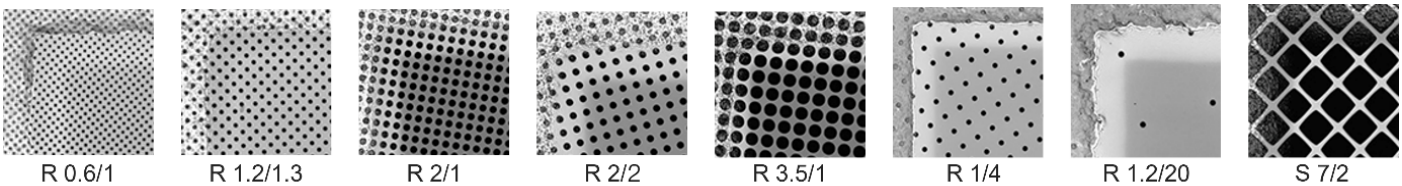



**Figure 3. Common support film geometries used for cryo-EM single particle analysis. Images courtesy of Quantifoil Micro Tools GmbH.**

While carbon and gold supports can have holes as large as 17 µm, these do not generally support thin layers of vitreous ice. Instead, for most cryo-EM imaging, films with holes ≤2 µm are used. In general, smaller holes produce thinner, flatter ice, as they provide greater support. There is evidence that holes as small as 0.3 µm can be beneficial for high resolution data collection.[17] However, considerations such as the number of particles per hole, and the use of multi-shot data collection protocols, mean that the most common foil geometry is R 1.2/1.3. This provides a balance between thin ice and strong support for high-resolution imaging, together with reasonable particle numbers for processing. Larger proteins and viruses may benefit from the use of R 2/2 or R 2/1 (i.e., with 2 µm holes), which improves particle numbers per hole.

The latest innovation in specimen supports for cryo-EM optimize grid and support film combinations to maximize performance. For example, HexAuFoil sample supports utilize gold foils on a gold grid; these minimize beam-induced motion in the plane of the grid, with small, 0.3 µm hole sizes that reduce strain on vitrification and minimize ice thickness, along with a hexagonal mesh grid that provides maximal sample support without compromising the open viewing area.[17]

## Grid preparation

Both carbon and metal foils are hydrophobic, and protein solutions do not naturally spread across the grid (a process called "grid wetting"). As a result, the grids must be placed in a glow-discharger or plasma-cleaner for a few seconds (usually 10–60 seconds, depending on the model and settings) before they can be used. Longer exposure will increase hydrophilicity and grid wetting, but prolonged exposure can damage the support foil and can also lead to dry holes as the protein solution spreads too quickly and becomes too thin. Plasma cleaners can use different gases, such as argon, for this process; this can change wetting behavior and increase reproducibility compared to glow discharging in air.[21] In addition, volatile additives, such as poly-lysine, can be added to the chamber; this modifies the film surface and the behavior of the protein, both on the grid and under the beam.


**Figure 4. Illustration showing the effect of glow discharging on the behavior of a sample solution on a cryo-EM grid. The increase in surface hydrophilicity facilitates the distribution of the aqueous sample across the grid.**

## Grid modifications

In addition to glow discharging of the grid to improve wettability, there are a range of chemical and physical modifications that can be made to the grid surface to alter its interaction with the specimen solution, potentially improving sample stability, density, or dispersion. These include:

- **Additional ultrathin layers of continuous amorphous carbon**, which can be purchased already applied to the grids or added in-house.[21] Many proteins are adsorbed by carbon, which prevents them from appearing within the grid holes. A continuous carbon layer allows proteins to adsorb across the holes, and simultaneously sequesters the protein away from the air-water interface, preventing denaturation. This does increase background noise, so it is only suitable for larger proteins and complexes. Additionally, this modification can have variable effects on preferred orientation, either increasing or decreasing it, depending on the sample.
- **Monolayer graphene**,[9] usually CVD graphene, works similarly to an amorphous carbon layer, but has the advantage of improved electron transparency, so it can be used with smaller proteins. However, graphene can hold significant charge in the beam which can cause damage to the protein being imaged.
- **Graphene oxide (GO) support layers**[22] are similar to graphene monolayers; however, GO flakes are stable and readily purchased from many suppliers, making preparation more straightforward. Conversely, GO flakes are rarely monolayers, or even consistent thickness, and therefore, coverage can vary.
- **Functionalized graphene/GO layers**[15] have been chemically modified to introduce a defined surface charge or capture a specific protein tag. They can alter electrostatics to reduce preferred orientation, sequester particles away from the air-water interface, or reduce non-specific protein binding.
- **PEGylation/passivation of grids**[16] involves the addition of PEG or PEG derivatives to the grid surface, making it more hydrophilic and "non-fouling." It reduces non-specific adsorption onto the grids, thereby increasing partitioning of particles into the holes. Combined with PEG spacers, the technique can also offer control of sample particle orientation and/or distance from grid.
- **Affinity grids** have been chemically modified to include functional layers that bind tagged samples, sequestering protein away from the air-water interface and increasing the concentration of scarce samples. Many different varieties exist, including those with Ni-NTA[25] and other common tags, such as SpyCatcher.[30] An alternative to chemical modification is the growth of a strepavidin monolayer crystal on the surface of the grid.[6] This is useful for biotinylated proteins, and the streptavidin background can also be easily subtracted from images in Fourier space.[31]

Table 6 provides a guide to which of the wide range of possibilities for grid modifications are likely to be the most use for a given imaging issue.

**Table 6. Common sample issues and possible grid modifications to address them**

| Issue | Relevant grid modification | Outcome |
|---|---|---|
| Low particle density in holes | Ultrathin carbon, graphene/GO | Provides a protein adsorption surface across holes, increasing partitioning to holes |
| Air-water interface denaturation/particles are all at surface | Graphene/GO, affinity grids (Ni-NTA, streptavidin), PEG passivation | Moves particles to grid surface, away from air-water interface |
| Strong preferred orientation | Graphene/GO (including functionalized/charged graphene), ultrathin carbon, PEG-SAMs, affinity capture with altered tether geometry | Changes the dominant adsorption interface and/or electrostatics |
| Beam-induced motion is limiting resolution | Graphene/GO or change to gold film and reduce hole size | Stabilizes ice and reduces charging/motion |
| Scarce sample that needs concentrating | Ni-NTA lipid monolayer, streptavidin grids, GO (which often allows for lower concentrations) | Increases on-grid concentration and/or capture efficiency |

## Screening

With so many possible variables, a robust screening protocol is essential for optimizing the vitrification protocol. This can be done by performing diagnostic cryo-EM imaging before high-resolution data acquisition begins. This step qualitatively assesses if the sample is a promising target for 2D class average analysis while also, simultaneously, obtaining an initial low-resolution reconstruction. During this step, the sample is pre-screened to evaluate the following properties:

- Protein concentration, stability, and distribution
- Ice quality, thickness, and uniformity across the grid

Grid screening is often repetitive, testing multiple sample and vitrification options in order to identify optimal conditions. **Thermo Scientific Smart EPU Software** offers fully automated grid screening to simplify this process and increase throughput.[35] During the screening phase, throughput can be further increased with EPU Multigrid Software, which enables the imaging of multiple grids in a single, unattended session.

If the sample is promising, a larger set of images may be acquired to facilitate further 2D and 3D analyses. At this stage, only a moderate resolution 3D map (>3 Å) is required. Thermo Scientific™ **Glacios™ Cryo-TEMs** and Thermo Scientific™ **Tundra™ Cryo-TEMs** are well suited for this screening, and offer robust, contamination-free connectivity with the higher resolution Thermo Scientific™ **Krios™ Cryo-TEM**.

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