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Grid preparation for detergent-solubilized GPCR samples

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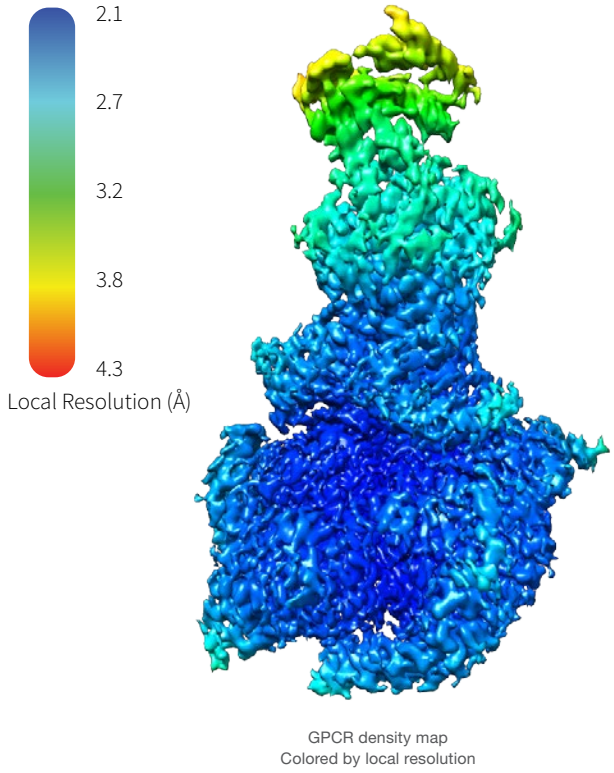
Introduction

Sample vitrification is a critical step in achieving high-resolution imaging and reconstruction for all protein samples. Membrane proteins require special considerations for solubilization and purification to preserves their functional state. A range of approaches has been applied to enable structure determination for G protein-coupled receptors (GPCRs). The most common to date has been solubilization in detergents; this application note is specifically related to cryo-EM grid preparation for GPCR complexes solubilized in detergent [1, 2]. Optimized sample preparation conditions, which ensure sample and TEM grid homogeneity combined with high particle density, allow for efficient imaging by conventional defocus TEM data collection and subsequent single particle image analysis (SPA).

1. The protein sample should be optimized before moving to cryo-EM

As with all structure determination projects, the sample for imaging should be optimized for purity, homogeneity, stoichiometry, stability and concentration, with the quality of protein assessed by size exclusion chromatography (SEC), Western blot, Coomassie stain and negative stain single particle analysis prior to moving to cryo-EM [3]. An example of a GPCR complex suitable for imaging is illustrated in Figure 2. This step has proven to be most critical in achieving the best possible resolution from a single particle cryo-EM experiment.

- Assuming it is possible to achieve a stable complex as determined by a monodispersed SEC elution profile, in combination with SDS-PAGE analysis (both by Western blotting with appropriate antibodies as well as Coomassie Blue staining), the next step would be to prepare a negatively stained specimen.



GPCR density map
Colored by local resolution

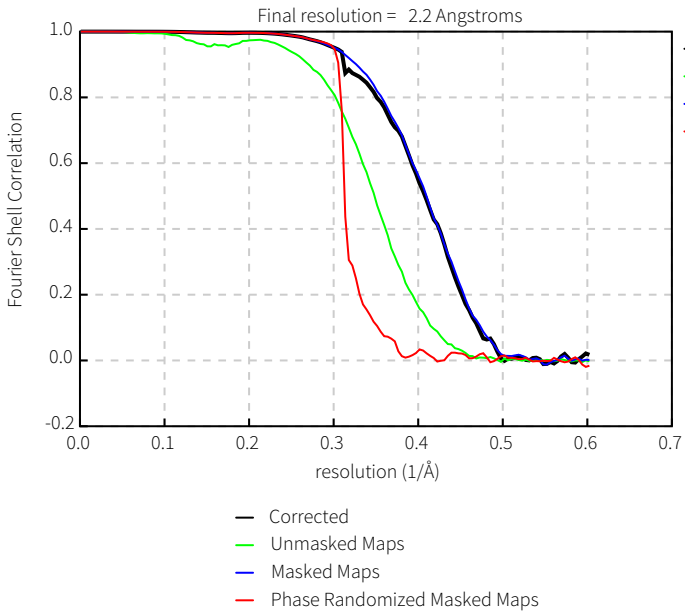


Figure 1. Example of a high-resolution structure of a GPCR determined by single particle cryo-EM on a Thermo Scientific Krios Cryo-TEM. Left: Density map colored by local resolution of a GPCR. Right: FSC curve of the final 3D reconstruction showing a nominal resolution of 2.2 Å (0.143 FSC, gold standard).

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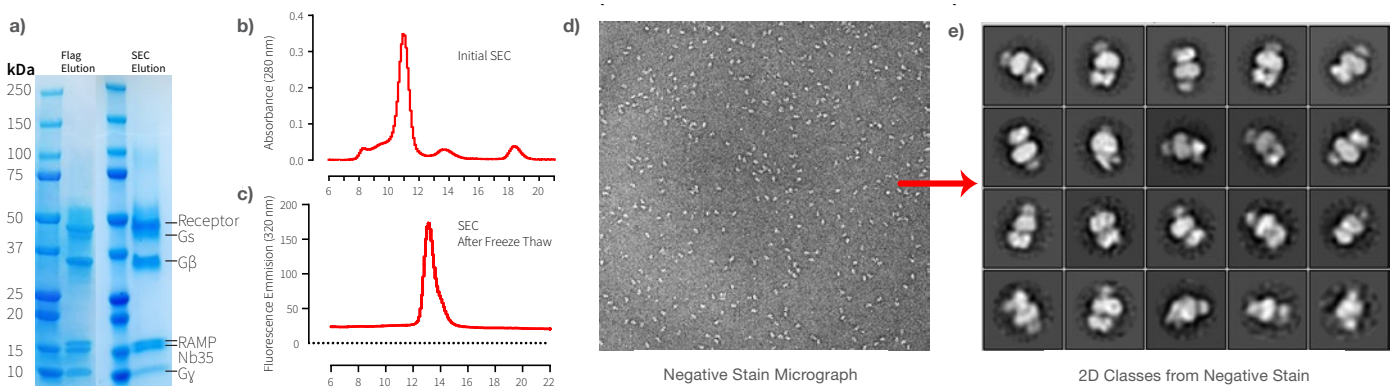


Figure 2. Assessing protein quality and suitability for grid preparation. **a)** Coomassie stain of two SDS-PAGE gels, one after FLAG affinity chromatography (left) and one after SEC (right). By Coomassie staining, only the components of the GPCR complex are visible. **b)** Typical SEC chromatogram during the purification of a GPCR; the center of the peak is collected and pooled and subjected to a freeze thaw cycle to ensure complex stability **c)** SEC chromatogram after a freeze thaw cycle, showing a monodispersed, Gaussian distributed elution profile. **d)** A representative micrograph from a Thermo Scientific Talos L120C TEM. The specimen is negatively stained with uranyl formate at a concentration of 0.08 mg/mL (assuming 1 AU_{280}=1 mg/mL). **e)** 2D class averages from the same negative stain images. In this case 65% of selected particles were clearly GPCR complexes.

- To standardize preparations, assume that 1 $AU_{280\ nm}$ = 1 mg/mL (as measured by a Thermo Scientific NanoDrop Spectrophotometer) then dilute the sample in the buffer that the GPCR is stable in (without any detergent) to a concentration of 0.1-0.2 mg/mL immediately before grid preparation.
- For GPCRs we have found that the stain that yields the best results is uranyl formate.

Protocol for preparation of negatively-stained grids

1. Preparation of uranyl formate (UF) (cat no# 24762-1 from Bioscientific) stain
   - Bring water to a boil on hot plate, weigh out 37 mg of UF powder in a 5 mL beaker
   - Add 5 mL boiling water to the beaker containing UF; stir for 5 min
   - Add 5 µL of 5M NaOH into solution, stir for another 5 min
   - Filter stain through a 0.2 µm filter and keep stain in dark
2. Continuous carbon grids (cat no# CF100H-Cu-50 from EMS) are glow-discharged for 30 sec
3. Keep all samples on ice. Dilute sample with detergent-free buffer to a concentration of 0.1-0.2 mg/mL immediately before applying on grid.
4. Add 3.5 µL of diluted sample onto grid and allow it to sit for 1 min
5. Remove excess fluid by touching the edge of the grid to a Whatman filter paper
6. Prepare 3 drops of UF stain (~10 µL each) on clean parafilm.
   - Gently hold grid to first drop of stain and immediately remove excess fluid
   - Repeat
   - Lastly, allow grid to touch to third drop of stain for 30 sec and blot off. If grids look heavily stained, duration of third drop may be reduced.
7. Blot the final drop and leave the grid to dry
8. Grid is ready for EM

- For protein preparations confirmed to contain intact and homogeneous complexes by image analysis of the negatively stained specimen, the next step is vitrification. We found best results come from GPCR preparations where more than 60% of particles represent complexes of interest.

2. Vitrification of detergent-solubilized GPCRs

2.1 List of prerequisites and things to check

- Thermo Scientific L120C, Glacios or Krios Cryo-TEM
- Thermo Scientific Vitrobot Mk IV System
- Plasma discharger (Quorum GloCube Plus https://www.quorumtech.com/products/glow-discharge-for-tem-and-surface-modification, or Ion Bombardier https://www.shinkuu.co.jp/plasma-treatment-device/pib-10/)
- 200 or 300 mesh Quantifoil R1.2/1.3 Cu grids or 300 mesh Ultrafoil R1.2/1.3 Au mesh
  - Grids are prewashed with acetone, usually a day before vitrification
- Dilution buffer (same used for SEC)
- GPCR sample at concentration greater than 3 mg/mL

2.2 Plasma discharge conditions

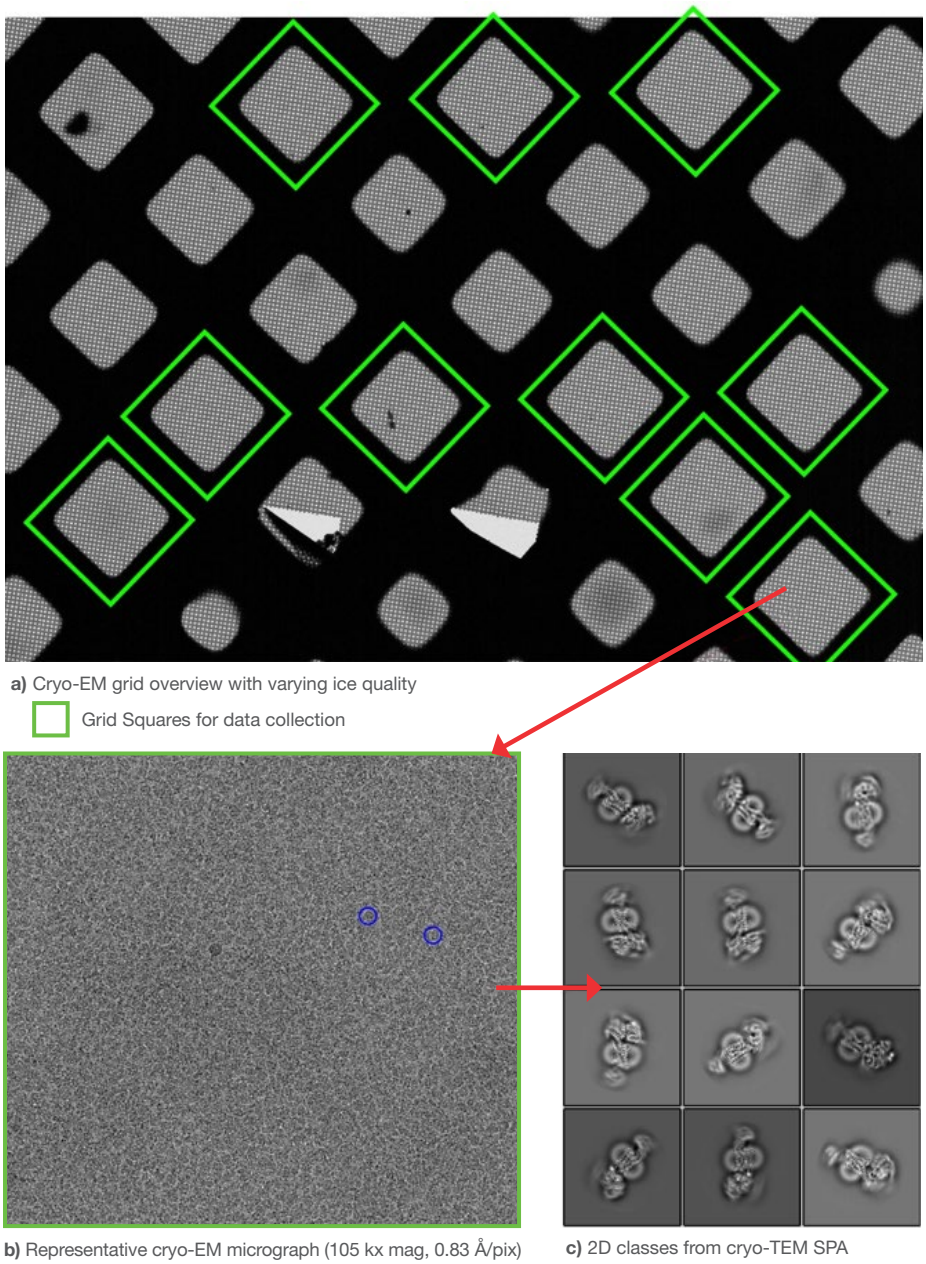
We have noted that the conditions used to plasma discharge the TEM grids prior to sample application and vitrification have a profound effect on the resulting quality of the specimen embedding in vitreous ice. In general, longer plasma discharge times and optimized plasma current have yielded the best results.

- For the GloCube Plus
  - 15-20 mA plasma current with the polarity making the air positively charged
  - Discharge time: 60-90 seconds
- For the Ion Bombardier
  - 9 mA plasma current with the polarity making the air positively charged
  - Discharge time: 90 seconds

2.3 Sparse screening of sample concentration and blotting conditions

It is necessary to screen for the optimal blotting conditions that yields the thinnest possible ice with good particle distribution in the grid holes. This is achieved by a sparse screening of both sample concentration and blot time or sample concentration and blot force. We have found that grids that have highly dense, monodispersed particles give the best results as shown in Figure 3b.

- It should be noted that the buffer has lower surface tension due to the presence of detergent, requiring much higher than "usual" blotting forces on the Vitrobot System.
- To save sample and reduce the time needed to screen grids, generally only blot time or blot force (along with sample concentration) should be varied in any individual screening session.
- An example of a sparse screening strategy can be seen in Table 1.
- Sample is "flash" thawed at 30°C, swiftly mixed by pipetting, and immediately placed on ice until application to a TEM grid.
- Vitrobot System with fresh, dry filter papers is pre-cooled to 4°C, with the humidity set to 100% (during sample application, humidifier should be disabled avoiding wetting of sample or filter paper).



a) Cryo-EM grid overview with varying ice quality

Grid Squares for data collection

b) Representative cryo-EM micrograph (105 kx mag, 0.83 Å/pix)

Particle Position

c) 2D classes from cryo-TEM SPA

Figure 3: Inspection and screening for optimal vitrification. These optimal vitrification conditions led to the 3D reconstruction in Figure 1. The figure in (b) shows an example of densely packed particles that yielded a high-resolution structure (sub 2.5 Å). Slightly lower density can also yield high quality data but we do not recommend collecting on grids with sparse particle density in thin ice.

- If dilutions need to be made, they are prepared with matched buffer that was used for the final purification step (usually SEC buffer). Typically, only one or two dilutions are made (i.e. 7 mg/mL and 3 mg/mL).
- 3 µL of sample is applied to the grid.

2.4 Screening for optimal vitrification conditions

Once a series of grids has been vitrified, optimal grids are identified by screening on either a 200 kV Thermo Scientific Arctica or Glacios Cryo-TEM, prior to SPA data collection on a 300 kV Thermo Scientific Krios Cryo-TEM. As both the Arctica and Glacios Cryo-TEM have a grid autoloader, Thermo Scientific EPU Software can automatically collect an atlas for each grid in the cassette, as shown in Figure 3a.

- Grids that appear to have consistent, thin ice are further investigated. Figure 3a shows a TEM grid with optimal grid squares, featuring both thin ice and many areas to collect data.
- Grids that appear consistent with high quality data collection are highlighted and further screened at high magnification to identify regions suitable for imaging particles. These are homogenous and feature regions with a uniform (and highly dense) distribution of particles (see Figure 3b).

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Blot time (sec)	Blot force	Drain time (sec)	Sample conc. (mg /mL)
10	20	0	4
10	20	0	4
7	20	0	2

Table 1. Example of our Vitrobot settings for sparse screening of blotting conditions, with blot times and blot force settings higher than conventionally used.

Best 3D reconstructions have been achieved with Quantifoil UltraFoil gold grids due to the minimal beam induced motion that they impart. However, blot conditions that have been found for the holey carbon grids for the same specimen are not necessarily transferable to gold grids.

Blot time (sec)	Blot force	Drain time (sec)	Sample conc. (mg /mL)
5-10	15-20	0-0.5	1-8

Table 2. Some other blot conditions that have yielded good vitrification

3. Concluding remarks

As active GPCRs are relatively small molecules to be analyzed by cryo-EM, thin ice is key for optimal signal-to-noise ratio and successful image analysis. Optimally thin ice can be promoted by highly dense packing of particles, which we found still allows for automated imaging even at low defocus.

Final note: The quality of data recovered from single particle imaging is critically dependent on the quality of the protein used for vitrification.

4. Troubleshooting

4.1 Ice is too thick

- Provided particle distribution looks to be sufficiently dense, then more grids need to be prepared at higher blot force/ blot time.
- Promote thin ice by increasing protein concentration of sample and longer blot times.

4.2 Sample looks clumped/patchy

This is a common problem with preparation of GPCR grids.

- A first step would be to check a series of sample dilutions. Smaller molecular weight GPCRs tend to need much higher concentration (even up to 8 mg/mL) and much higher blot forces (typically 18-20 on the Vitrobot System) to yield a monodispersed layer of particles in ice.
- If the problem still persists, increasing blot time and adding a small drain time can be helpful (see table 1 after combining with table 2)
- After trying both points above, revisit glow discharging. Try a longer plasma discharge regime (90-120 seconds) at both higher and lower plasma currents.

4.3 Sample concentration too low

- If possible, increase sample concentration.
- Adjusting glow discharge conditions can be helpful to “encourage” the GPCRs into the grid holes.
- Try different buffer conditions, *eg.* changing from Tris to HEPES or MOPS and adjusting the ionic strength of the sample.
- Double application of the sample to the grid prior to plunging. This can be done either manually, prior to placing the sample in the Vitrobot System, or by blotting on the Vitrobot System without the ethane container mounted on the robot. In the latter, one would need to ensure the humidity is appropriately high around the sample to avoid drying.

5. References

1. Zhao *et al.*, Activation of the GLP-1 receptor by a non-peptidic agonist, Nature 2020
2. Liang *et al.*, Structure and Dynamics of Adrenomedullin Receptors AM1 and AM2 Reveal Key Mechanisms in the Control of Receptor Phenotype by Receptor Activity-Modifying Proteins, ACS Pharmacology & Translational Science
3. Booth *et al.*, Visualizing Proteins and Macromolecular Complexes by Negative Stain EM: from Grid Preparation to Image Acquisition, Journal of Visualized Experiments

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