FFT before alignment

FFT after alignment
Homogeneity
Good distribution
Most orientations
Use whichever buffer keeps your protein happy

**BUT KEEP IN MIND**

Contrast between the protein/complex density and its surrounding is affected by buffer composition. Try to avoid:

- Strong scattering elements (high atomic numbers)
- High salt concentrations (better stay under 300 mM)
- Crowding agents, e.g. glycerol
Invest in thorough biochemical and biophysical characterization

SEC/MALLS
AU

Thermal shift assays

Functional assays

Negative stain EM

The complexity of macromolecular assemblies and their variable stability during in vitro preparations requires adequate characterization before embarking on single-particle EM analysis.
Superose6 Inc. 10/300 after Ni-NTA Buffer: 20mM Hepes pH:7.4 300mM NaCl

- 0 hour
- 24 hours
- 25 hours (+1mM TCEP after 24 hours)

An example
Reduced sample

Oxidized sample

Negative Stain EM
to assess homogeneity and gross structural variability and to obtain low resolution 3D maps
Negative stain EM

metallic grid (mesh)
A number of different stains with different characteristics. Uranyl formate is generally a good choice due to its finer grain.
Negative stain EM
Selecting the area of proper stain thickness is critical

Sample evaluation by negative stain EM

Classification and averaging of NS data

Cascio et al., 2002, EMBO J. 21
3D map of negative stained sample
Finer sample evaluation by NS EM

Size exclusion chromatography
SEC
Finer sample evaluation by NS EM

Size exclusion chromatography
SEC
Embedding in vitreous ice

cryo-EM
Is there a correlation between NS and CryoEM? A bad sample by NS will be bad by CryoEM but the opposite is not always true.
A: Grid hole with ideal single particle and ice behavior

B: Grid holes with areas of ideal single particle and ice behavior

Particles adsorb to the air water interface

A: HIV-1 Trimer Complex 1 (sample #5)

B: Rabbit Muscle Aldolase (sample #22)

C: DnaB Helicase-helicase Loader (sample #33)

D: T20S Proteasome (sample #43)

Noble et al. *Elife*, 2018 May 29;7
Adsorption to the air water interface increases over time

Spot-to-plunge time: 500 ms

Spot-to-plunge time: 170 ms

A GPCR-G protein complex

Zhang et al, Nature 2017
A few things to consider

Different grids (material, hole size)

Variations in grid surface hydrophilicity

Use of support layers

Surfactants

Cross-linking
A typical Quantifoil grid

Vitrified specimen
Render grids hydrophilic

Beware of variations in plasma cleaners or glow dischargers

PELCO easiGlow at 20 mA for 60 s

Cressington 208 at 10 mA for 90 s
A typical Quantifoil grid

A lacey grid
A lacey grid

Qu et al, Cell 2018 Aug 23;174(5):1117-1126

Drulyte et al.

Volume 74 | Part 6 | June 2018 | Pages 560–571 | 10.1107/S2059798318006496
Addition of a support layer

Amorphous carbon most commonly used.

Use of graphene oxide gaining traction due to its relative electron transparency

Palovcak et al., Journal of Structural Biology, V204-1, October 2018, 80-84
Addition of a support layer

Amorphous carbon
Improving preferred orientation with graphene oxide
**Surfactants**

Add detergents or amphipols at low concentration (below CMC) to create a protective monolayer at the air water interface.

Often used reagents: DDM, GDN, A8-35, OG, CHAPS

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(Crosslinking)

- Engineer Cysteines for disulfide bridges (if structural information at hand)

- Chemical cross-linking with lysine reactive compounds or other bifunctionalities

A couple of different ways:
- glycerol/cross-linker gradient centrifugation [GraFix].
- ‘on-column’ glutaraldehyde cross-linking during SEC runs

Requires caution, both in execution and interpretation of a structure. Necessitates some form of understanding of native conformation
A few things to consider

Different grids (material, hole size)

Variations in grid surface hydrophilicity

Use of support layers ★

Surfactants ★

(Cross-linking)