Workshop on Addressing Challenges in CryoEM Grid Preparation

What works, what does not

New York Structural Biology Center
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Workshop on Addressing Challenges in CryoEM Grid Preparation

What works, what does not

Everything works some times, nothing works every time
Why do we need specimen preparation? (vitrification)

**Biological specimens consist of up to 80% of water**
→ Dehydration in EM vacuum
  → COLLAPSE OF STRUCTURE
    → Vitrification prevents structural collapse and preserves the specimen in a near-native environment

**Biological specimens consist of light atoms (C, N, O, H)**
→ $\sigma_{el}/\sigma_{in} = Z/19$ (~ 2 inelastic per elastic scattering event)
  → BEAM DAMAGE
    → Vitrification does not prevent beam damage, but minimizes the observable effects of beam damage
Traditional vitrification procedure

EM grid (copper)
Traditional vitrification procedure

EM grid (copper)

Holey film (carbon)
Traditional vitrification procedure

EM grid (copper)

Holey film (carbon)

Glow discharge
Traditional vitrification procedure

- EM grid (copper)
- Holey film (carbon)
- Glow discharge
- Apply sample (pipette)
- Vitrification (plunger)
Vitrification

Acceleration
to prevent ice crystal formation: temperature has to decrease at a rate of $10^5$-$10^6$ K/s

Dubochet et al. (1988) 
_Q. Rev. Biophys._ **21**: 129-228

thermal conductivity of sample
– water is poor thermal conductor
→ sample has to be thin
distance

wetting characteristics of coolant
– LN$_2$ bad (Leidenfrost effect)
→ ethane cooled by LN$_2$

Adrian et al. (1984) 
_Nature_ **308**: 32-36
Vitrification

FEI Vitrobot

Gatan Cryoplunge

Leica EM GP

Semi-automated
Control of many parameters
The vitrified specimen

Thin layer of perfectly amorphous ice
Many particles that are well separated and adopt randomly distributed orientations

But the reality is often:

No particles
Aggregation
Preferred orientations
Traditional vitrification procedure

- EM grid (copper)
- Holey film (carbon)
- Glow discharge
- Apply sample (pipette)
- Vitrification (plunger)
Specimen preparation

- EM grid (copper)
- Gold grids
- Self-blotting grids
Specimen preparation

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  - Gold grids
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- Holey film (carbon)
  - Gold film
  - Pacify surface with PEG
Specimen preparation

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Thin support film
- Thin carbon film
- Graphene film
Specimen preparation

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Glow discharge
Specimen preparation

- **EM grid** (copper)
  - Gold grids
  - Self-blotting grids
- **Holey film** (carbon)
  - Gold film
  - Pacify surface with PEG
- **Thin support film**
  - Thin carbon film
  - Graphene film
- **Glow discharge**
- **Apply sample** (pipette)
  - Multiple applications
  - Ink jet – Spotiton
- **Vitrification** (plunger)
  - Blotting time (physical water removal)
  - Single- or double-sided blotting
  - Waiting time (water evaporation)
  - Temperature & humidity
Problems with vitrified specimens

1. Ice layer

- Crystalline ice (hexagonal)
- "Leopard skin" ice
- Ice contamination
Problems with vitrified specimens
2. No particles

Optimized purification → Beautiful particles in negative-stain EM → Empty holes in cryo-EM

Possible reasons:
- Sample concentration too low
- Image contrast too low
- Ice layer too thin
- Particles do not go to holes
- Sample denatures at air/water interface

Possible solutions:
- Particle density does not change linearly with protein concentration
- Small or elongated particles
- High-density buffer (e.g., glycerol)
- Mostly for viruses and other large specimens
- Glow-discharge, grid material, coating
- Multiple sample application, blotting technique
- Thin support film (carbon, graphene)
- Transport/shipping (freeze)
- Chemical cross-linking

Spotiton!
Problems with vitrified specimens

3. Aggregation

Optimized purification → Beautiful particles in negative-stain EM → Aggregates in cryo-EM

Potential reason: Protein partially denatures upon contacts with the air/water interface

Possible solutions:
- try chemical cross-linking (stabilizes the protein)
- try to adsorb to a substrate (prevents interactions with air/water interface and keeps the proteins separated)
- try buffer additives (e.g., lysine for DNA-binding proteins) (may compensate charges)
Problems with vitrified specimens

3. Aggregation

- Target protein aggregates
- Sometimes the target protein JUST DOES NOT aggregate...
- Adding “decoy protein” prevents aggregation of target protein
Problems with vitrified specimens
3. Aggregation

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Spotiton!
4. Preferred orientations

Beautiful particles in vitrified ice

Class averages show only (or mostly) one projection

Particles align to air/water interface

Particles adsorb to the support film

Particles align to only one of the two air/water interfaces

Adsorption to support film is less “orienting” than air/water
Problems with vitrified specimens

4. Preferred orientations

Potential solutions:

- try thicker ice
  (lower likelihood to hit air/water interface)

- try to add some detergent
  (lower surface tension of the air/water interface)

- try to adsorb to a substrate
  (substrate may have a lesser orienting effect)

- try to change the buffer composition
  (change surface characteristics of the protein)

- try to add tags to the protein
  (change surface characteristics of the protein)

- try to change glow discharge conditions, e.g., use amyl amine
  (change the "ice characteristics")
Problems with vitrified specimens
4. Preferred orientations

Don’t be fooled by elongated particles!

Cryo-EM image – “normal” ice

Class averages
Problems with vitrified specimens

4. Preferred orientations

Don’t be fooled by elongated particles!

Cryo-EM image – thin ice

Class averages
Problems with vitrified specimens

4. Preferred orientations

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Tilt the grid!