Application and fabrication of monolayer graphene grids in cryo-EM

Xiao Fan, Ph.D.
NCCAT & NRAMM workshop. Aug 24th, 2021
Through the lens of an electron microscope

### Cryo-EM sample preparation

1. The sample is transferred to a metal mesh and excess material is removed.

2. The sample forms a thin film across the holes in the mesh when it is shot into ethane at about -190°C.

3. The water vitrifies around the sample, which then is cooled by liquid nitrogen during the measurements in the electron microscope.

### Data collection and processing

1. Randomly oriented proteins are hit by the electron beam, leaving a trace on the image.

2. The computer discriminates between the traces and the fuzzy background, placing similar areas in the same group.

3. Using thousands of similar traces, the computer generates a high-resolution 2D image.

4. The computer calculates how the different 2D images relate to each other and generates a high-resolution structure in 3D.

### Raw particles

Streptavidin

52 kDa at 2.6 Å

Illustration: © Johan Jarnestad/The Royal Swedish Academy of Sciences

http://www.lander-lab.com/aboutem.php
Resolution revolution of single particle cryo-EM

From “Blob-ology” To “Atom-ology”

Significantly boosted throughput of data collection and more automated data processing pipeline
Key steps towards high resolution

3D reconstruction requires projections from different orientation

- **Structurally homogenous biological samples**
  No continuously flexible region/component.
  ✔️

- **Random distributed orientations in full 3D space**
  No preferred orientation.
  ✗

- **Image quality (SNR) of individual projection**
  Protein size, ice thickness, DQE of the camera, radiation damage.
  ✗

- **Number of particles**
  Concentration, number of micrographs.
  ✗

- **Alignment accuracy**
  Beam induced motion, angular assignment accuracy.
  ✔️

[Link](http://people.csail.mit.edu/gdp/cryoem.html)
**Particle distribution in cryo-sample**

**Ideal cryo-EM sample**
- Evenly distributed & randomly oriented
- Ice thickness is comparable to particle size

**Not enter the hole**

**Severe preferred orientation**

Illustration of common problems in cryo-EM samples

Protein-ality

### Cross-sectional schematic diagrams of particle and ice behaviors

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Name</th>
<th>Example cross-sectional schematic diagram</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32 kDa Kinase</td>
<td><img src="image1.png" alt="Diagram" /></td>
<td>14</td>
<td>Neural Receptor</td>
<td><img src="image2.png" alt="Diagram" /></td>
<td>27</td>
<td>IDE</td>
<td><img src="image3.png" alt="Diagram" /></td>
<td>36</td>
<td>Apoferritin (0.5 mg/mL)</td>
<td><img src="image4.png" alt="Diagram" /></td>
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<tr>
<td>4</td>
<td>Hemaggulatin</td>
<td><img src="image5.png" alt="Diagram" /></td>
<td>17</td>
<td>Protein with Bound Lipids (deglycosylated)</td>
<td><img src="image6.png" alt="Diagram" /></td>
<td>30</td>
<td>GDH</td>
<td><img src="image7.png" alt="Diagram" /></td>
<td>39</td>
<td>Apoferritin with 0.5 mM TCEP</td>
<td><img src="image8.png" alt="Diagram" /></td>
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<tr>
<td>5</td>
<td>HIV-1 Trimer Complex 1</td>
<td><img src="image9.png" alt="Diagram" /></td>
<td>18</td>
<td>Protein with Bound Lipids (glycosylated)</td>
<td><img src="image10.png" alt="Diagram" /></td>
<td>31</td>
<td>GDH</td>
<td><img src="image11.png" alt="Diagram" /></td>
<td>40</td>
<td>Protein with Carbon Over Holes</td>
<td><img src="image12.png" alt="Diagram" /></td>
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<tr>
<td>6</td>
<td>HIV-1 Trimer Complex 1</td>
<td><img src="image13.png" alt="Diagram" /></td>
<td>19</td>
<td>Lipa-protein</td>
<td><img src="image14.png" alt="Diagram" /></td>
<td>32</td>
<td>GDH + 0.001% DDM (2.5 mg/mL)</td>
<td><img src="image15.png" alt="Diagram" /></td>
<td>41</td>
<td>Protein and DNA Strands with Carbon Over Holes</td>
<td><img src="image16.png" alt="Diagram" /></td>
</tr>
<tr>
<td>7</td>
<td>HIV-1 Trimer Complex 2</td>
<td><img src="image17.png" alt="Diagram" /></td>
<td>20</td>
<td>GPCR</td>
<td><img src="image18.png" alt="Diagram" /></td>
<td>33</td>
<td>Drab Helicase-helicase Leader</td>
<td><img src="image19.png" alt="Diagram" /></td>
<td>42</td>
<td>T200 Proteosome</td>
<td><img src="image20.png" alt="Diagram" /></td>
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<tr>
<td>10</td>
<td>Stick-like Protein 1</td>
<td><img src="image21.png" alt="Diagram" /></td>
<td>21</td>
<td>Rabbit Muscle Actinase (trimeric)</td>
<td><img src="image22.png" alt="Diagram" /></td>
<td>34</td>
<td>Apoferritin</td>
<td><img src="image23.png" alt="Diagram" /></td>
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<td>T200 Proteosome</td>
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<tr>
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<td>Stick-like Protein 2</td>
<td><img src="image25.png" alt="Diagram" /></td>
<td>22</td>
<td>Rabbit Muscle Actinase (dimers)</td>
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<td>35</td>
<td>Apoferritin</td>
<td><img src="image27.png" alt="Diagram" /></td>
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<td>T200 Proteosome</td>
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<tr>
<td>13</td>
<td>Neural Receptor</td>
<td><img src="image29.png" alt="Diagram" /></td>
<td>25</td>
<td>Protein in Nannobac (6.5 mg/mL)</td>
<td><img src="image30.png" alt="Diagram" /></td>
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<td>Apoferritin</td>
<td><img src="image31.png" alt="Diagram" /></td>
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<td>MB Proteosome</td>
<td><img src="image32.png" alt="Diagram" /></td>
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<tr>
<td>37</td>
<td>Apoferritin (1.25 mg/mL)</td>
<td><img src="image33.png" alt="Diagram" /></td>
<td>46</td>
<td>Protein on Streptavidin</td>
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</tbody>
</table>

### Common tricks to improve a bad cryo-sample

- **Different types of grids**
  - (Cu/Au, Quantifoil/UltrAuFois)
- **Support films**
  - (Continuous carbon, GO)
- **Glow discharge conditions**
  - (air, H₂, special chemicals)
- **Polylysine**
- **Additive/detergent**
- **Multiple application**

Continuous carbon support film has been widely used in large complex study.

Average ice thickness at
- center: 56 ± 35 nm
- edge: 99 ± 24 nm

For protein targets with **MW > 600 kDa**, using a carbon support film is very helpful!


Cryo-sample with support film

Continuous carbon support film has been widely used in large complex study

Signal: protein MW
Noise: thickness of ice and support film

Given the same support film
MW ↓ SNR ↓

For support film: the thinner, the better!
You would gain SNR/contrast when the noise from reduced ice thickness is greater than the noise of the support film.

Continuous carbon film
Large protein
Small protein
Thinner film

Average ice thickness at
center: 56 ± 35 nm
edge: 99 ± 24 nm


For protein targets with MW > 600 kDa, using a carbon support film is very helpful!
Graphene support film for cryo-sample

Different types of 2D materials

- Graphene
- hBN
- MoS$_2$
- WSe$_2$

Illustration of graphene

A single layer of carbon atoms, near transparent to electron.

Properties of graphene

- Excellent electroconductibility
- Excellent thermal conductivity
- Strongest material
- Hydrophobic surface

For 200 kV electron beam

2010 Nobel Prize in Physics

<table>
<thead>
<tr>
<th>Material</th>
<th>MFP (nm)</th>
<th>$I/I_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold (50 nm)</td>
<td>101</td>
<td>61%</td>
</tr>
<tr>
<td>A-carbon (20 nm)</td>
<td>250</td>
<td>92%</td>
</tr>
<tr>
<td>Monolayer graphene</td>
<td>250</td>
<td>99.9%</td>
</tr>
</tbody>
</table>

Tests on the new graphene grids

2.2 Å apo-ferritin (450 kDa) on graphene grids

2.6 Å streptavidin (52 kDa) on graphene grids

Yimo Han, Xiao Fan, et al., PNAS, 2020 117 (2).
Graphene reduce particle local motion

益处：

统计上，粒子在石墨烯网格上的局部运动更小。这可能是因为石墨烯可以减少充电效应和电子束诱导的运动。

Graphene grids can enrich different samples

Yimo Han, Xiao Fan, et al., *PNAS*, 2020 117 (2).

Membrane protein

Soluble protein

Proteoliposome

Particle distribution on graphene grid

with graphene

w/o graphene

Top view vs Side view

Strong preferred orientation without graphene!

Pros and cons of graphene grids

Pros:

- Suitable for very small proteins (> 50 kDa).
- Reduce particle motion.
- More orientations available.
- Preventing air-water-interface damage.
- Enrich particle density/concentration.
- Diffraction pattern as a good index.

Cons:

- The yield of ultra-clean single crystalline graphene grid is very low.
- Requirement of expensive instruments.
- No high-quality commercial alternatives.

Is there a way to produce high-quality monolayer graphene grids easily and robustly with low cost & high yield?
High-yield monolayer graphene grids for cryo-EM

Yimo Han, Xiao Fan, et al., *PNAS*, 2020 117 (2).

We have a video of full production process in our paper!
Protect graphene with MMA

- Graphene on both side of cooper foil (Cu substrate).
- Keep the side for future supporting face-up.
- Spin-coat the upside graphene with MMA (2,500 rpm for 1 min).
Remove backside graphene

- Flip the MMA coated graphene on copper foil with backside graphene up.

- **Argon/Oxygen glow-discharge/plasma cleaner for 30s – 60s** to remove backside graphene.
Etch out Cu substrate by Ammonium persulfate

- Cut “MMA/graphene/Cu stack” into small pieces.
- Float the small pieces on 0.5-1M APS (MMA side up). 20 – 30 min

Yimo Han, Xiao Fan, et al., PNAS, 2020 117 (2).
Clean MMA/graphene bilayer film

• Use glass-slide to transfer the MMA/graphene film (MMA side up) to DI water.

• Wash for 10-min. Repeat this process twice.
MMA-coated graphene grid

- Scoop out the MMA/graphene film using Quantifoil Au R1.2/1.3 300 mesh grids with carbon side.
- Air dry with MMA side up.
Remove MMA protect layer

• Annealing: bake the grid on a hot plate at 130°C for 20 mins and cool it down.

• Soak the grid into acetone for 30 mins to dissolve MMA. Repeat once for better clean (optional).
Final clean up

- Transfer the grid to Isopropanol (IPA) to remove the acetone residue for 20 mins.
- Take out the grid one by one with graphene side up.
- Final annealing.

Yimo Han, Xiao Fan, et al., PNAS, 2020 117 (2).
High-yield monolayer graphene grids for cryo-EM

99% yield of suspended monolayer graphene
Clean and uniform graphene surface

SEM image of home-made graphene grid

Contact angles
Raman spectroscopy

UV-Ozone as a mild surface treatment to increase hydrophilicity

Yimo Han, Xiao Fan, et al., PNAS, 2020 117 (2).
Graphene grid is suitable for small proteins
Monolayer graphene support film has a minimum size restriction for near-atomic resolution study of most of the targets. (at least for those > 50 kDa)

Sample distribution could be improved on graphene
Enriched concentration, more orientations, less damage from AWI, smaller motion.

High-yield monolayer graphene grids fabrication
We have established a convenient protocol to produce high-quality monolayer graphene grids at large scale.
UV-Ozone for robust surface functionalization.

However, graphene grids is NOT a cure-all! Again, protein-ality matters!
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