PRINCETON UNIVERSITY

International Human Frontier Science Program Organization

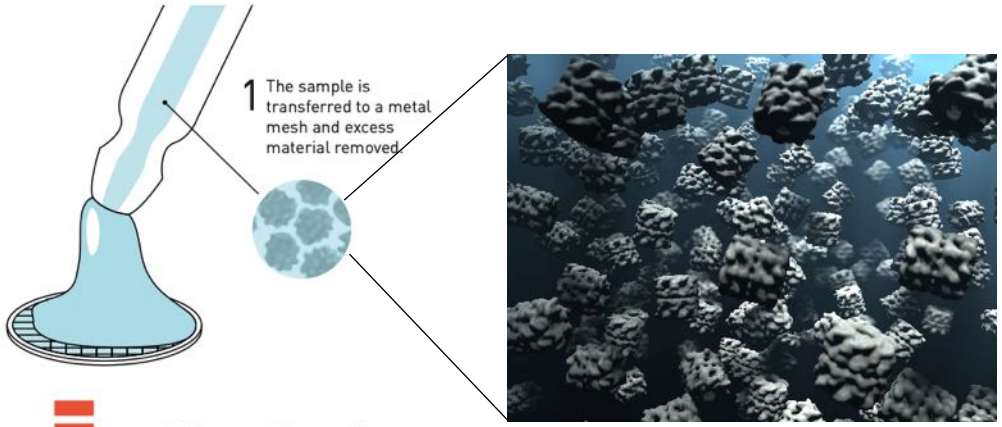
Application and fabrication of monolayer graphene grids in cryo-EM

Xiao Fan, Ph.D.

NCCAT & NRAMM workshop. Aug 24th, 2021

Through the lens of an electron microscope

Cryo-EM sample preparation



Protein particles in solution

Rapid freezing to keep native conformations and random orientations

Protein particles in vitreous ice

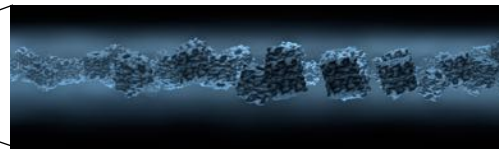


Illustration: ©Johan Jarnestad/The Royal Swedish Academy of Sciences

Data collection and processing

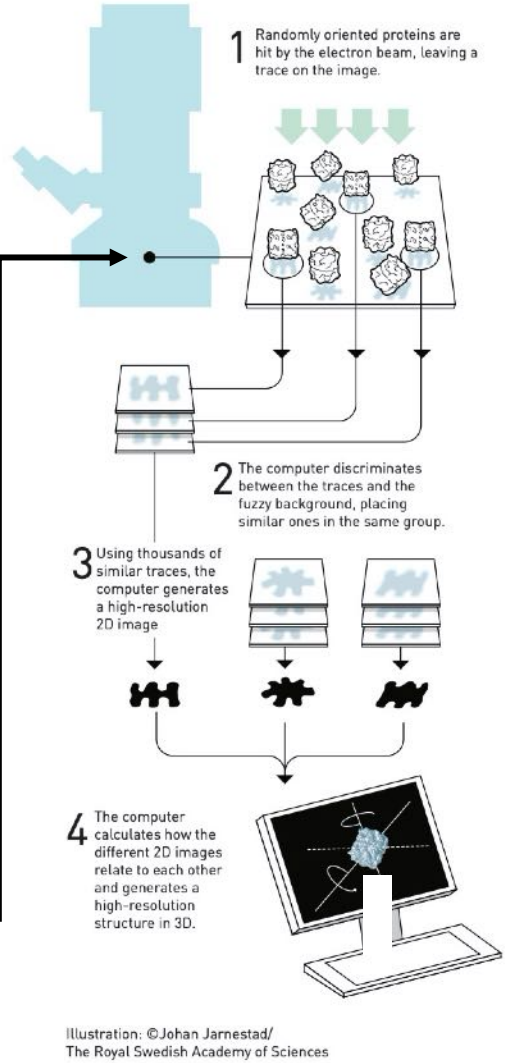
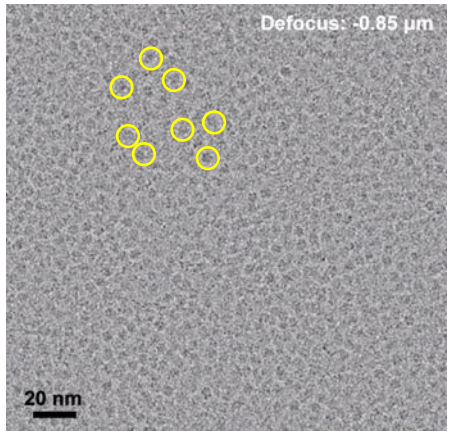
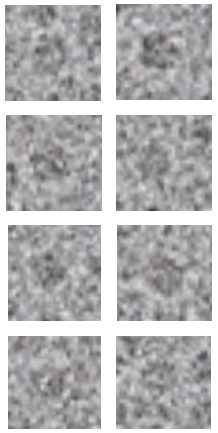
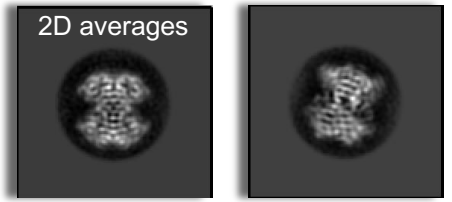


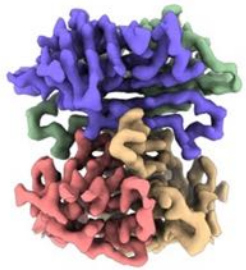
Illustration: ©Johan Jarnestad/
The Royal Swedish Academy of Sciences





Raw image → Raw particles



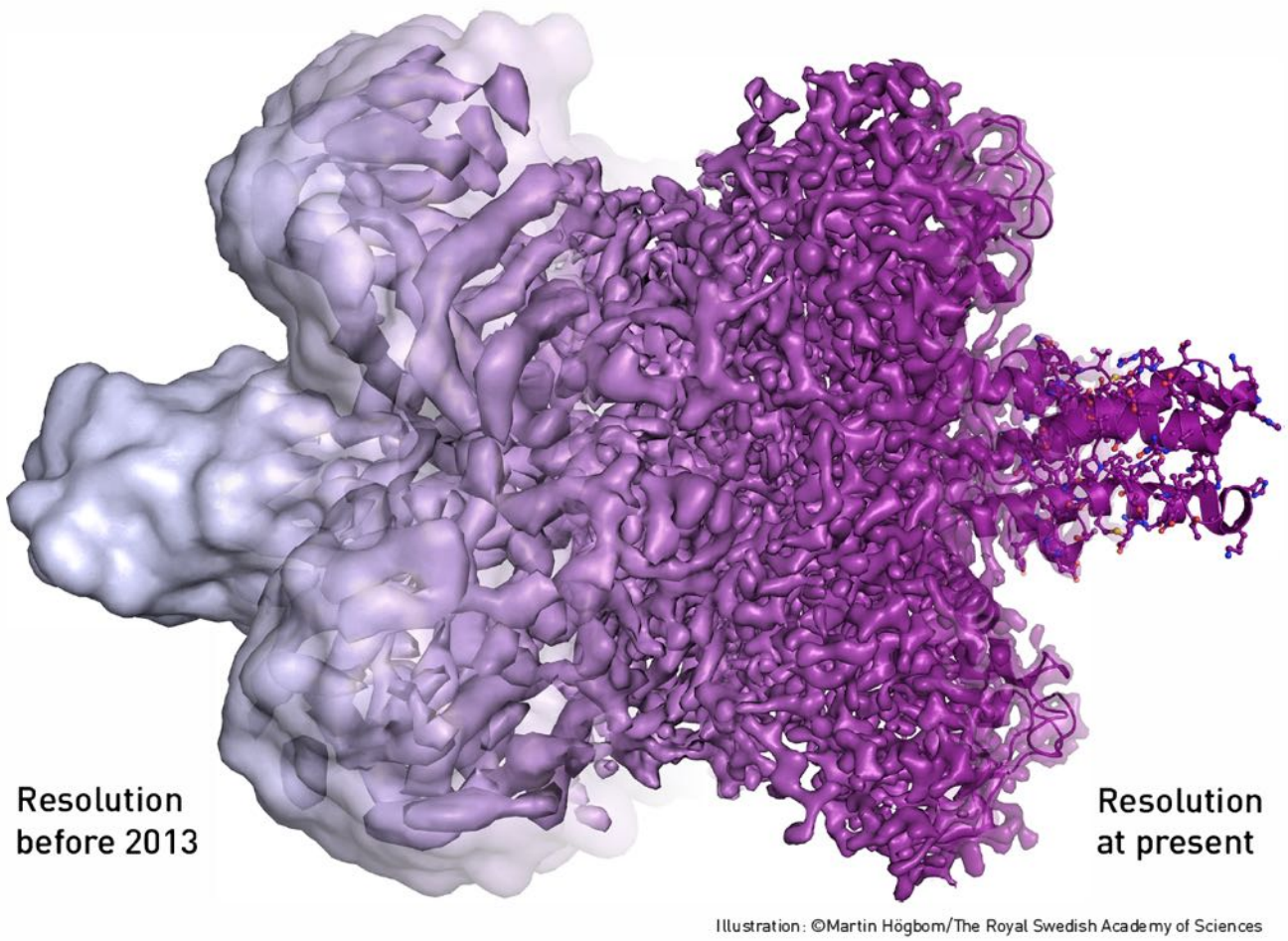


Streptavidin

52 kDa at 2.6 Å

http://www.lander-lab.com/aboutem.php

Resolution revolution of single particle cryo-EM

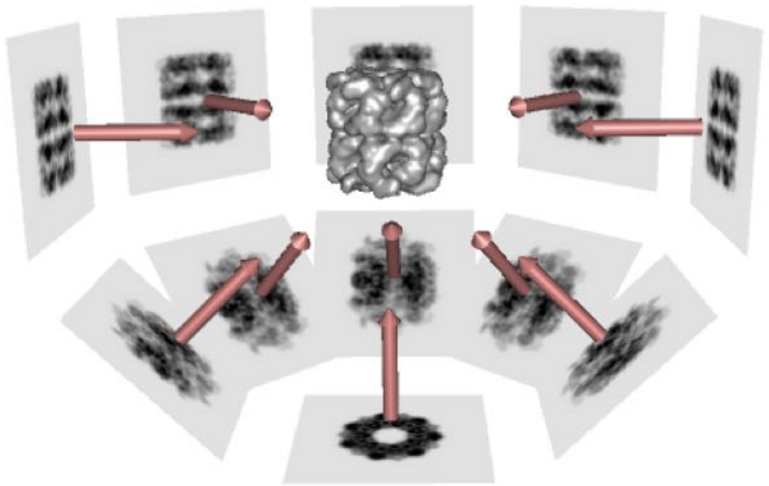


From “Blob-ology” To “Atom-ology”

Resolution	**Size**
High	**Large**
Low	**Small**

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.

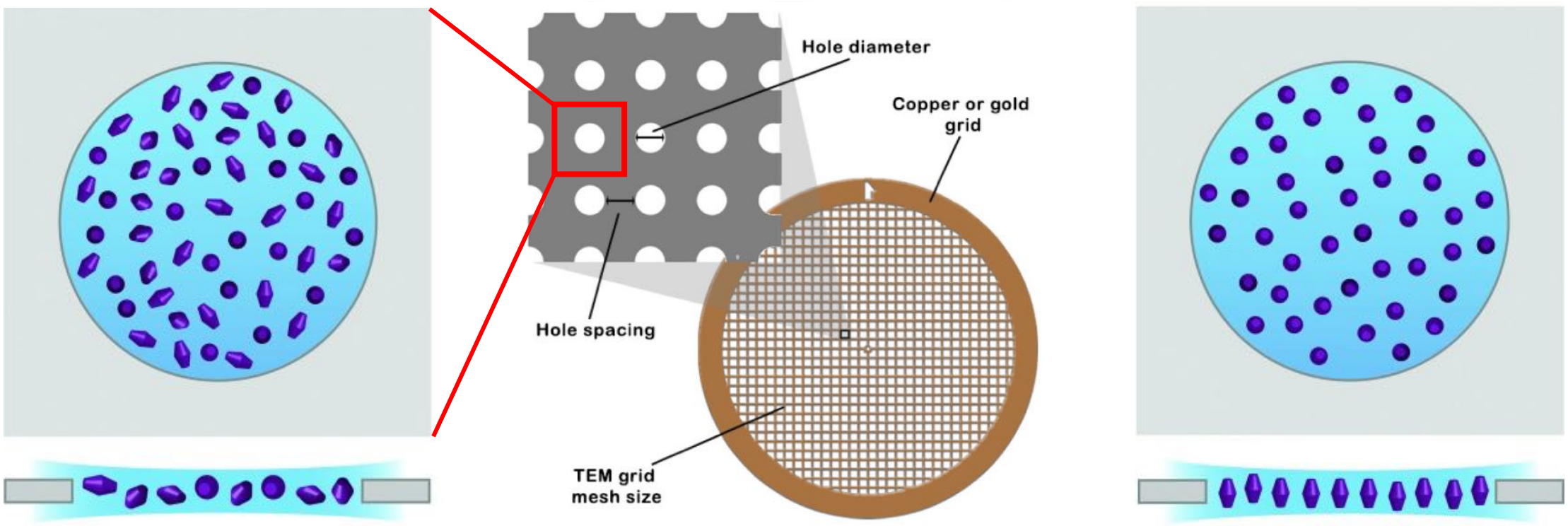
http://people.csail.mit.edu/gdp/cryoem.html

Particle distribution in cryo-sample

Ideal cryo-EM sample

Not enter the hole

Severe preferred orientation



Evenly distributed & randomly oriented

Ice thickness is comparable to particle size

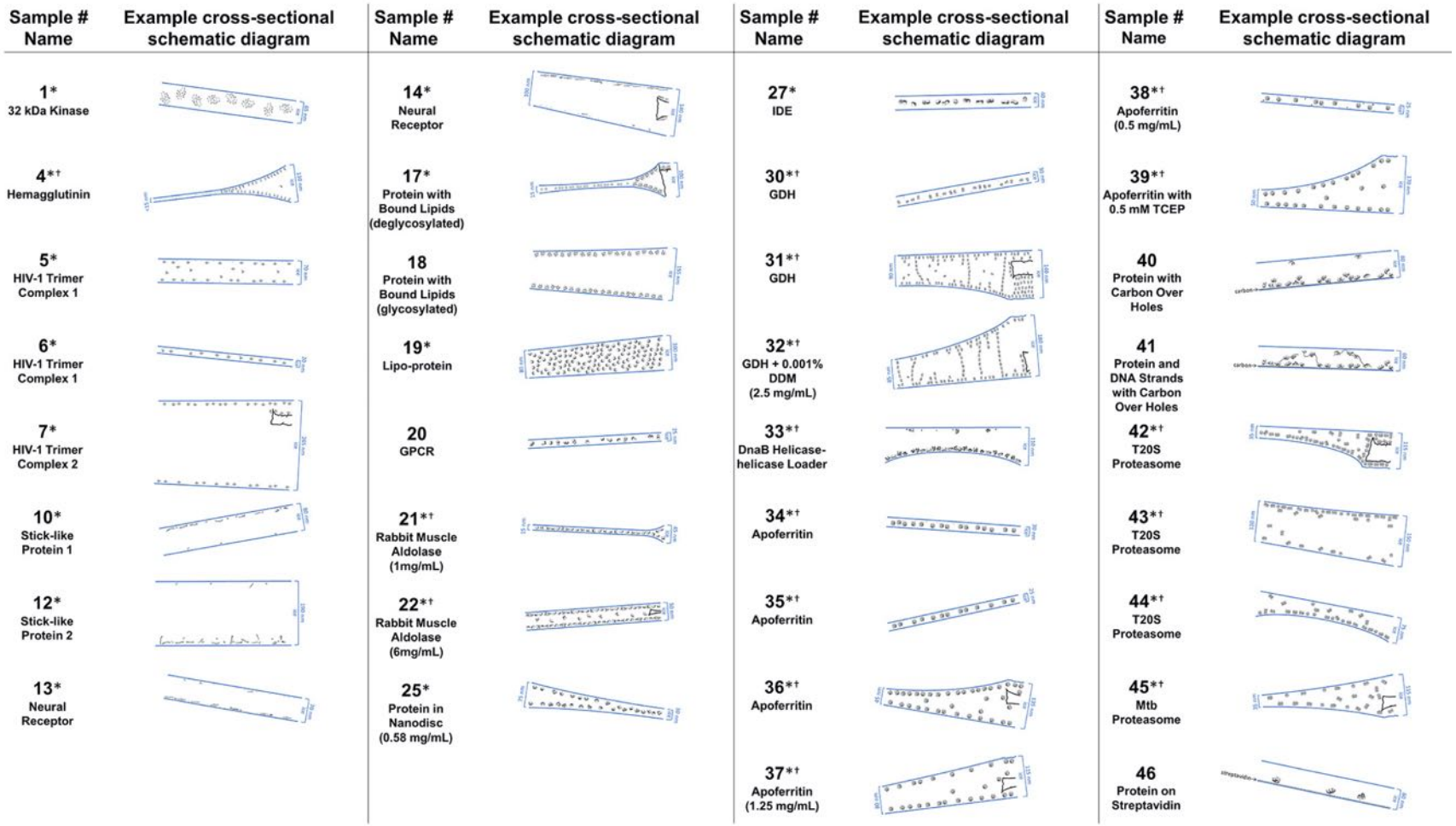
Illustration of common problems in cryo-EM samples

Protein-ality

Drulyte, I. et al. *Acta Crystallogr. D,* 74, 560–571 (2018).

Particle distribution in cryo-sample

Cross-sectional schematic diagrams of particle and ice behaviors



Common tricks to improve a bad cryo-sample

Different types of grids
(Cu/Au, Quantifoil/UltrAuFoils)

Support films
(Continuous carbon, GO)

Glow discharge conditions
(air, H_2, special chemicals)

Polylysine

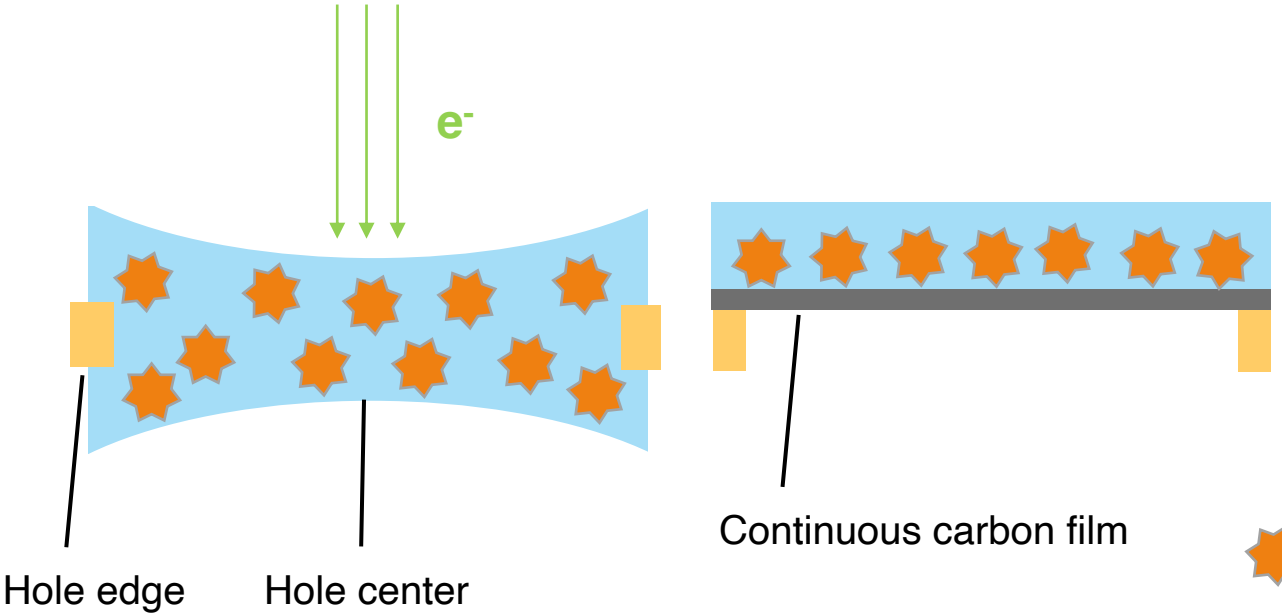
Additive/detergent

Multiple application

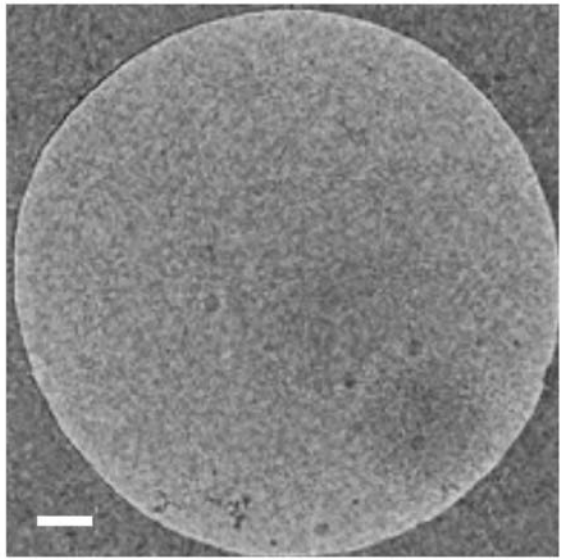
Noble, Alex J., et al. *Elife* 7 (2018): e34257.

Cryo-sample with support film

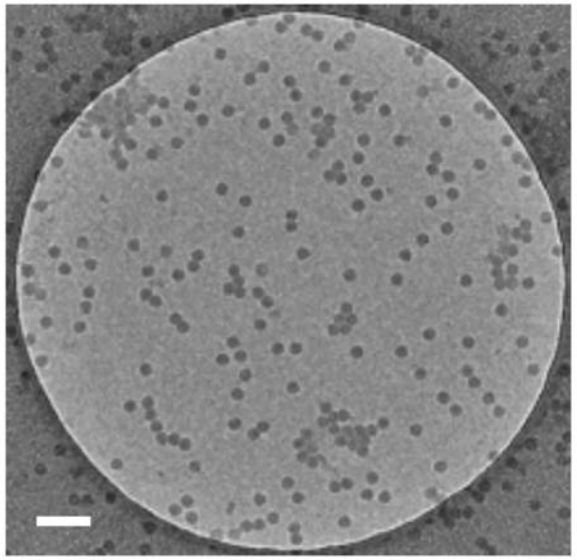
Continuous carbon support film has been widely used in large complex study



w/o carbon support



w/ carbon support



Drulyte, I. et al. *Acta Crystallogr. D,* 74, 560–571 (2018).

Large protein

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

Average ice thickness at

center: 56 ± 35 nm

edge: 99 ± 24 nm

Noble, Alex J., et al. *Elife* 7 (2018): e34257.

Cryo-sample with support film

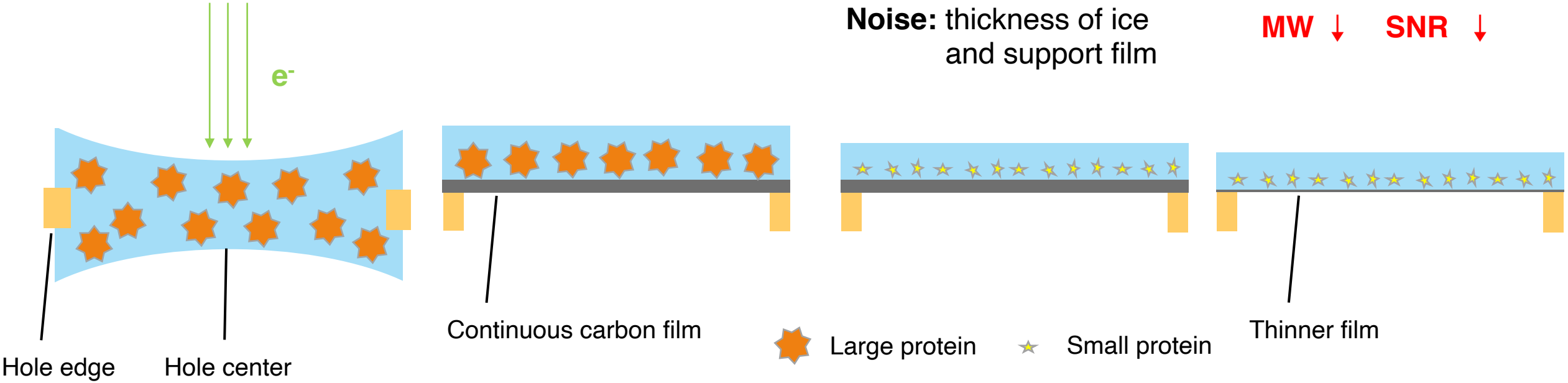
Continuous carbon support film has been widely used in large complex study

Signal: protein MW

Noise: thickness of ice and support film

Given the same support film

MW ↓ SNR ↓



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

Average ice thickness at

center: 56 ± 35 nm

edge: 99 ± 24 nm

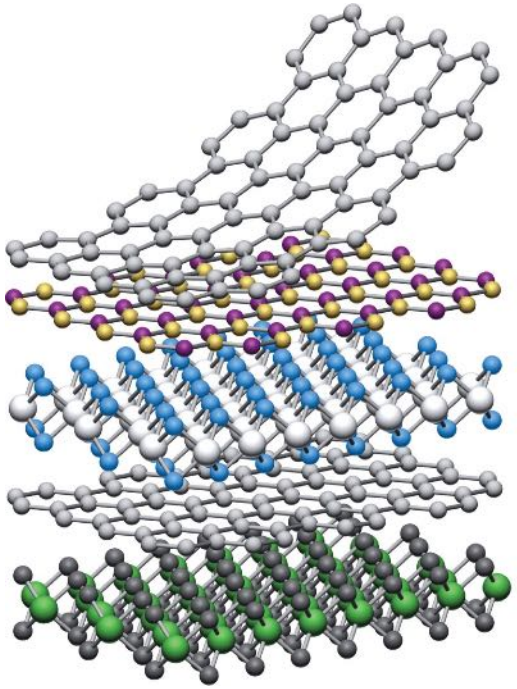
Noble, Alex J., et al. *Elife* 7 (2018): e34257.

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.

Graphene support film for cryo-sample

Graphene support film for protein sample?



graphene

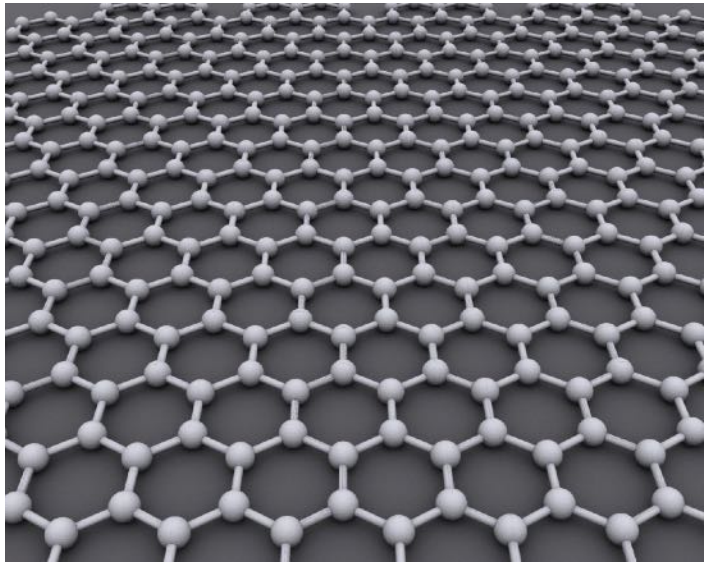
hBN

MoS_2

WSe_2

A.K. Geim,I. V. Grigorieva. *Nature* 499, 419-425 (2013).

Different types of 2D materials



https://en.wikipedia.org/wiki/Graphene

Illustration of graphene

	MFP (nm)	I/I_0
Gold (50 nm)	101	61%
A-carbon (20 nm)	250	92%
Monolayer graphene	**250**	**99.9%**

For 200 kV electron beam

Properties of graphene

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

Excellent electroconductibility

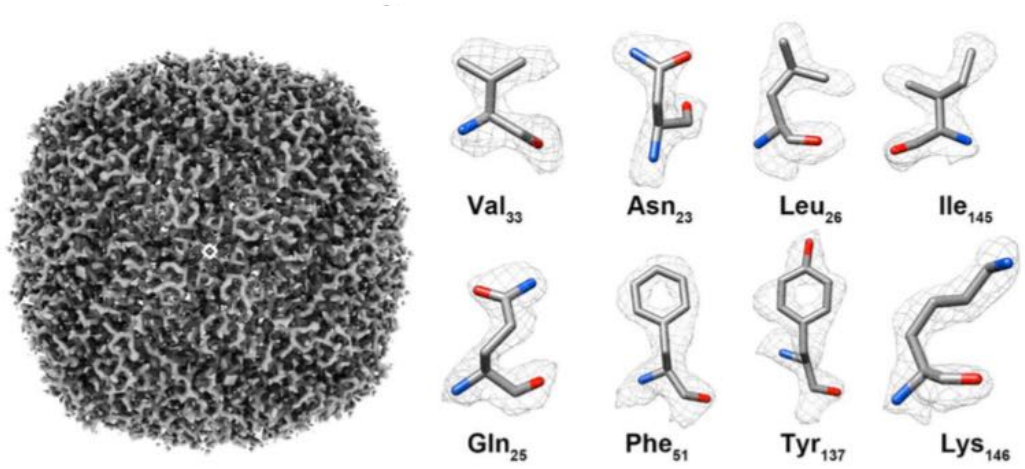
Excellent thermal conductibility

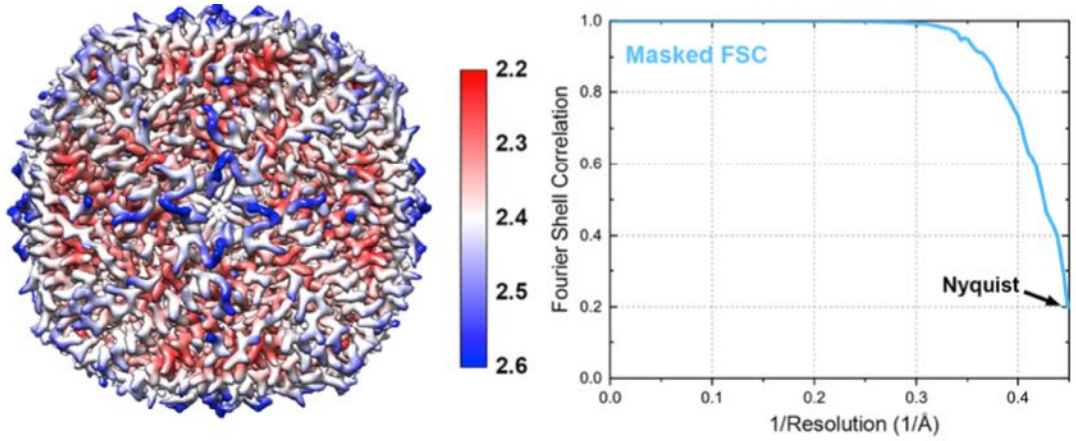
Strongest material

Hydrophobic surface

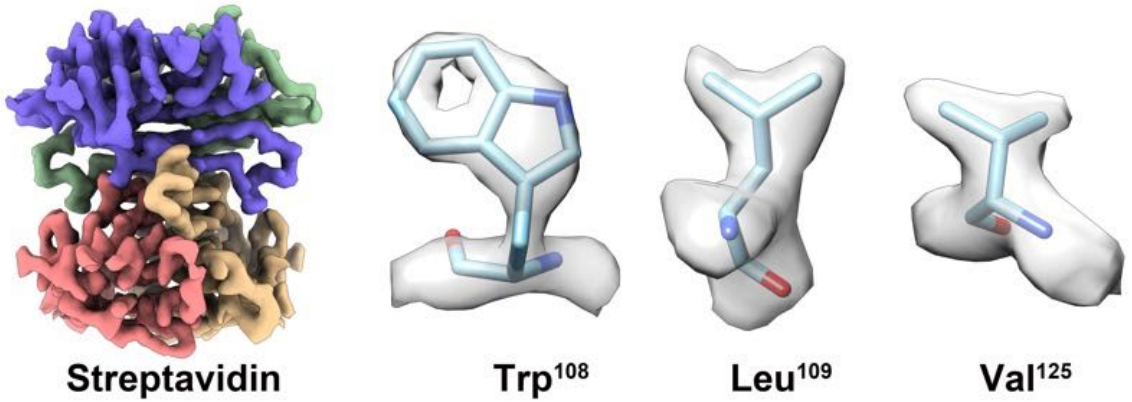
2010 Nobel Prize in Physics

Tests on the new graphene grids

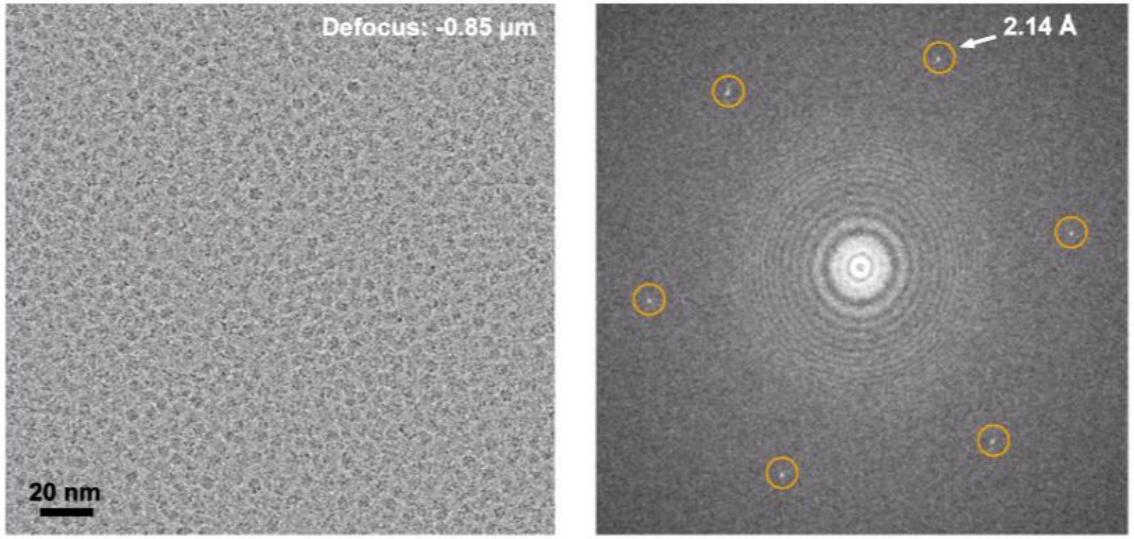




2.2 Å apo-ferritin (450 kDa) on graphene grids



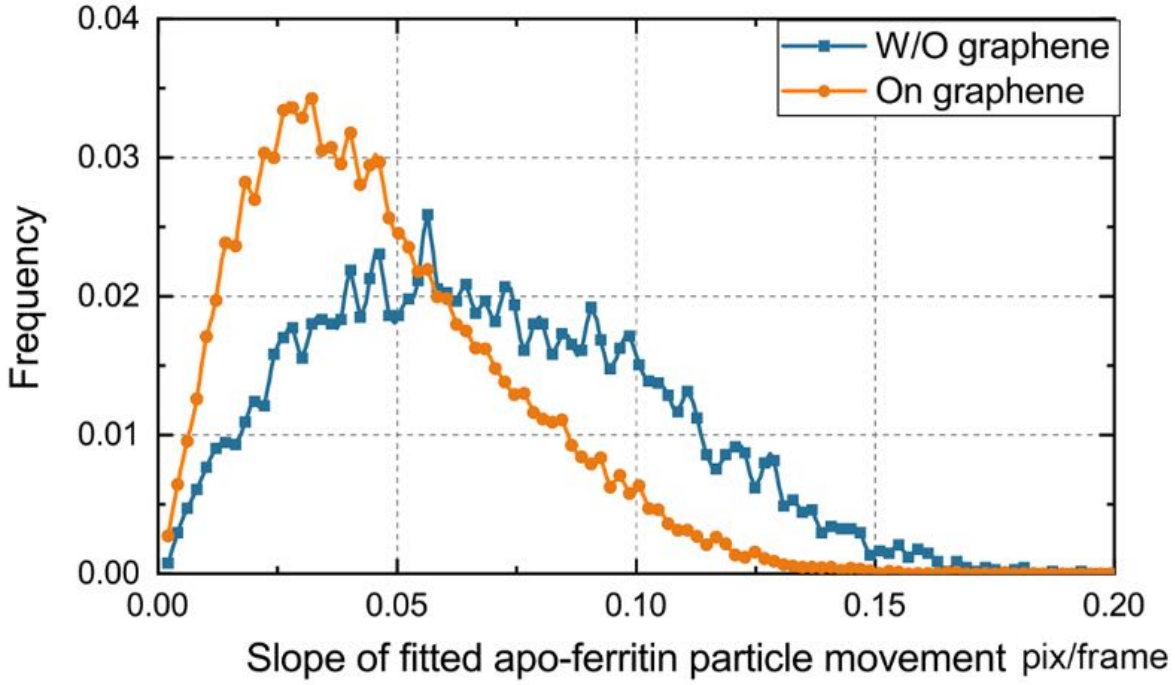
2.6 Å streptavidin (52 kDa) on graphene grids

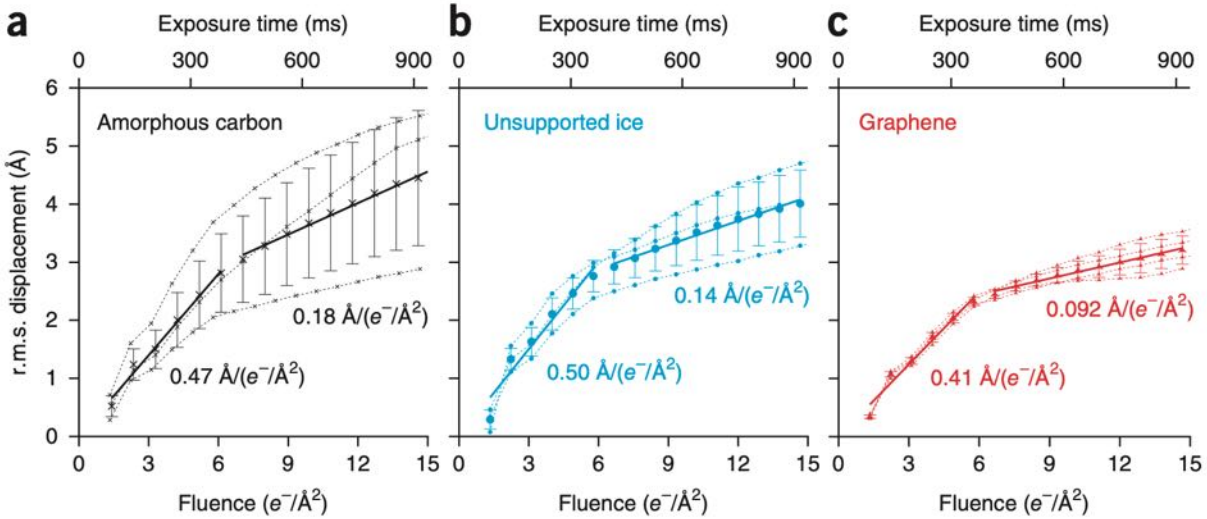


Yimo Han, Xiao Fan, et al. , *PNAS*, 2020 117 (2).

Graphene reduce particle local motion

Local motion (rate) estimated by Particle Polishing





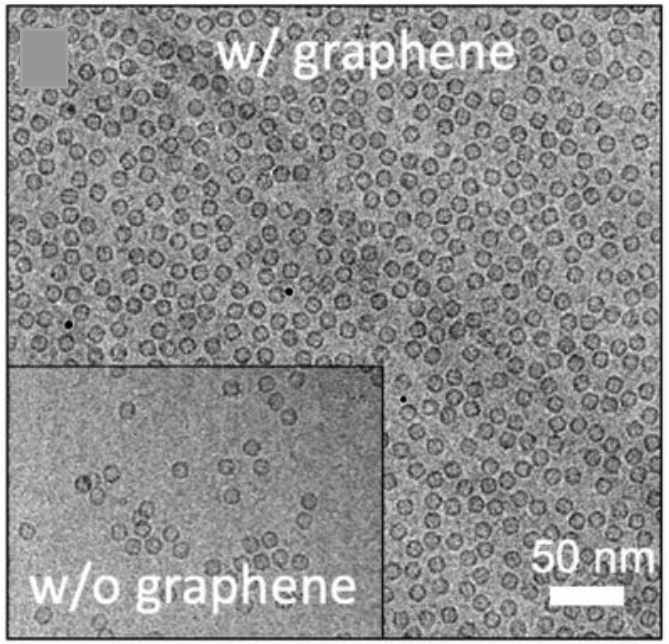
C.J. Russo & L.A. Passmore, *Nature Methods*, 11, 649-652 (2014)

Benefit:

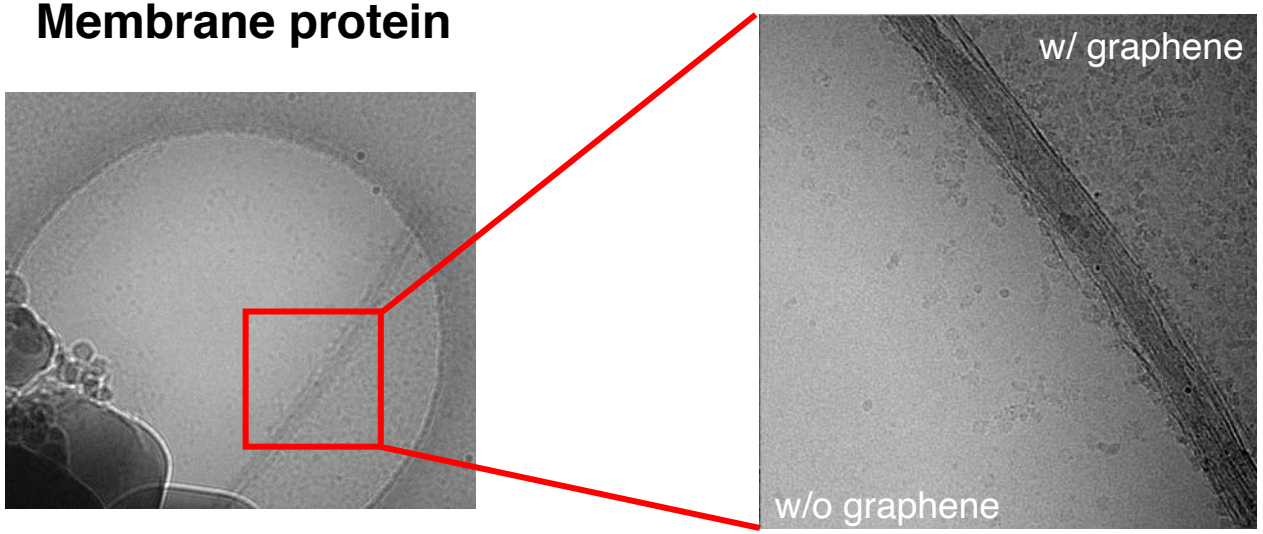
Statistically, particles on **graphene grid** have **smaller local motion**. It might be because graphene can reduce **charging effect** and **beam induced motion**.

Graphene grids can enrich different samples

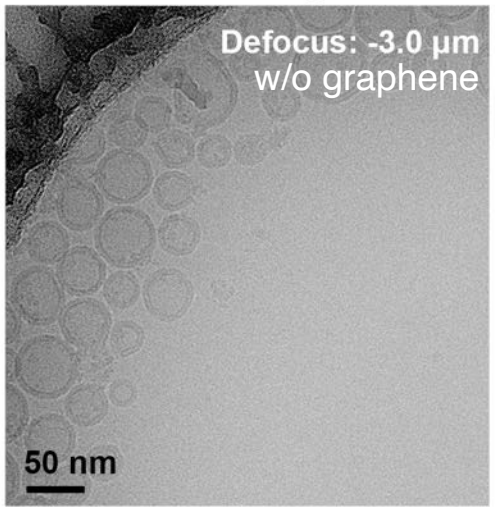
Soluble protein

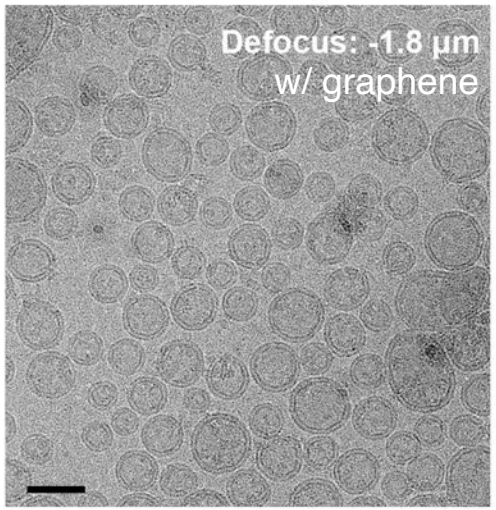


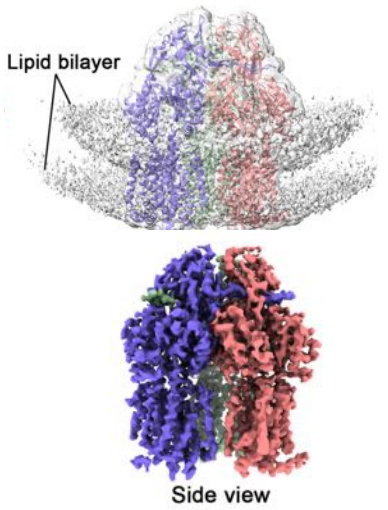
Membrane protein



Proteoliposome







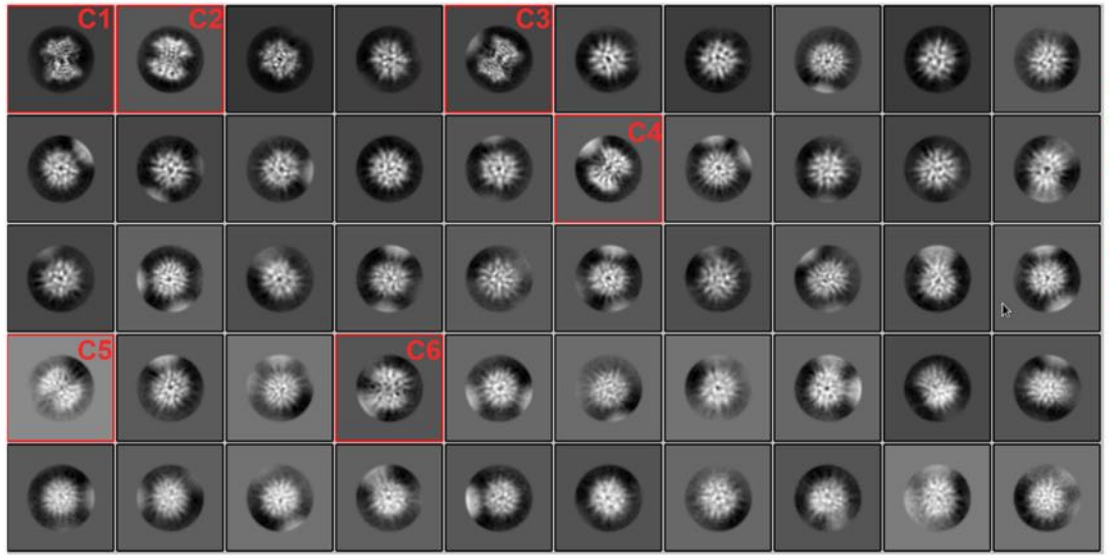
AcrB in liposome

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

Xia Yao, Xiao Fan, et al. , *PNAS,* 2020 117 (31).

Particle distribution on graphene grid

with graphene

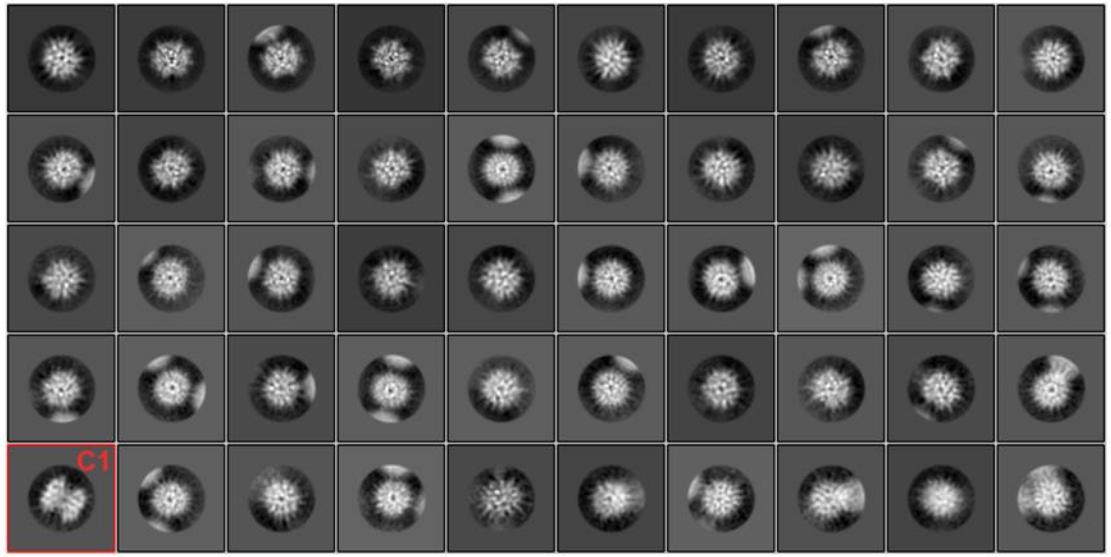


Classes with side view

C1: 8,051 6.0%
C2: 6,562 4.9%
C3: 4,428 3.3%
C4: 3,063 2.3%
C5: 2,205 1.6%
C6: 2,023 1.5%

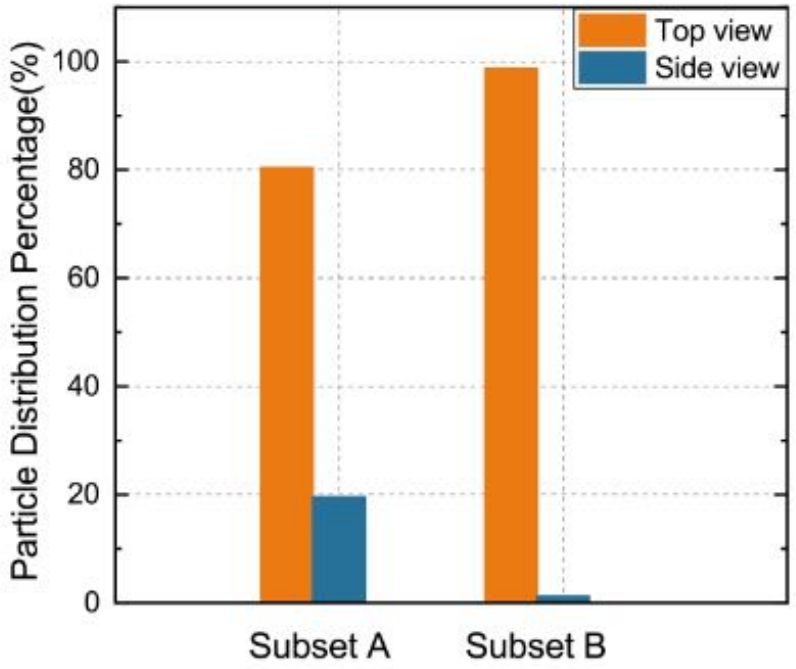
Total:
26,332 19.6%

w/o graphene



Classes with side view

C1: 972 1.2%



Top view vs Side view

Strong preferred orientation without graphene!

Fan, Xiao, et al. *Nature communications* 10.1 (2019): 1-11.

Pros and cons of graphene grids

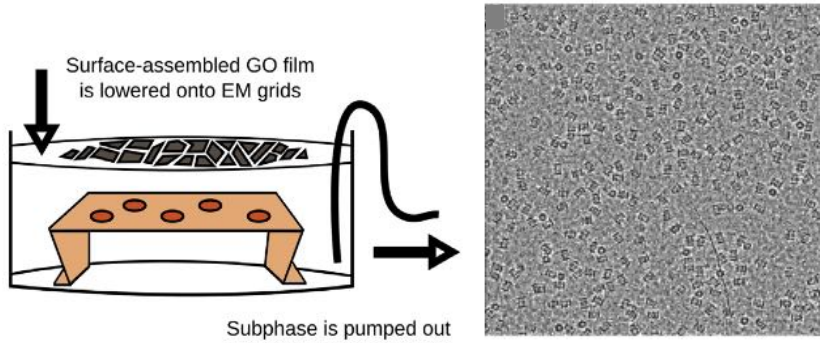
Pros:

- 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.

Graphene oxide (GO) grid is an alternative but not good enough.

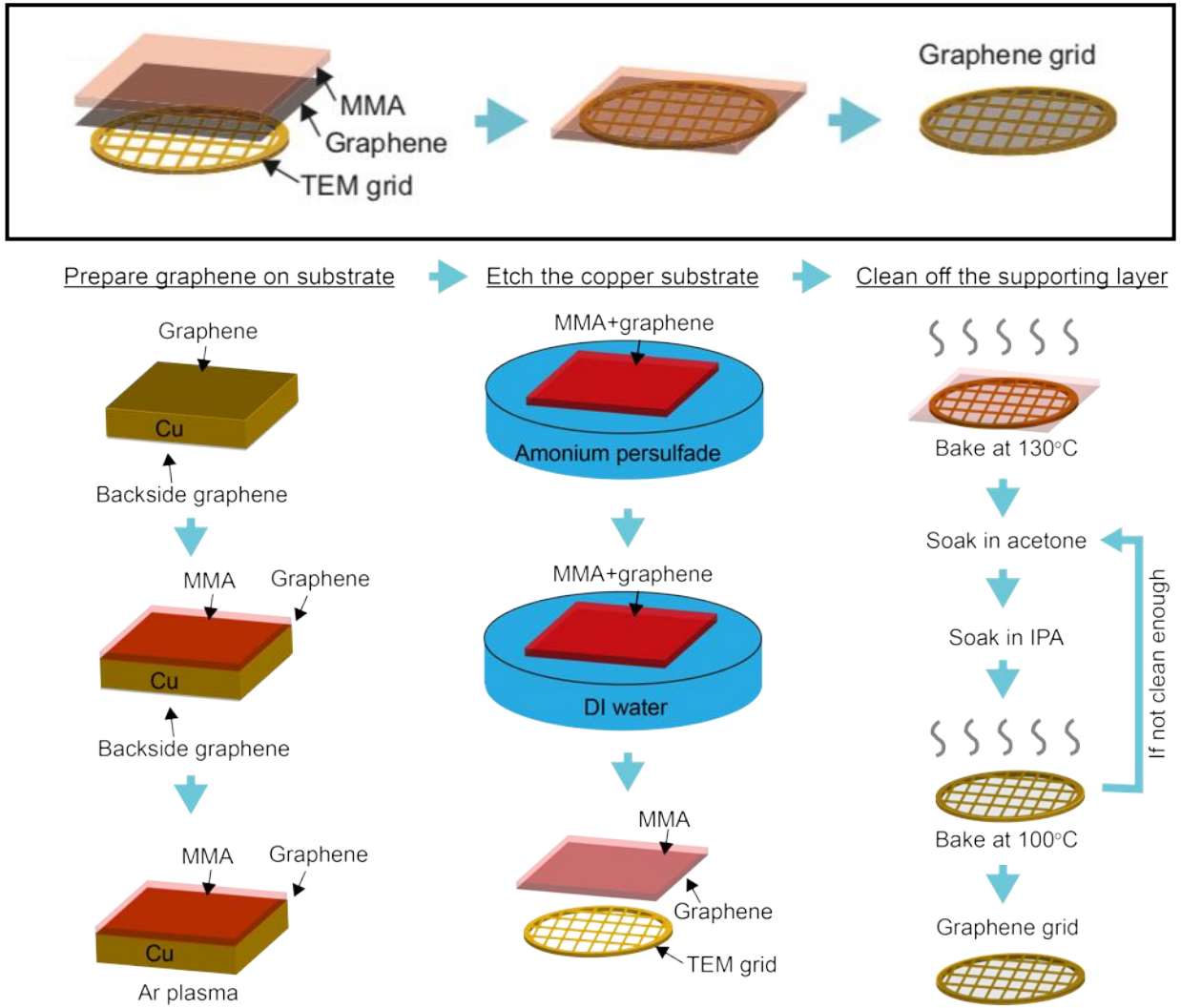


E. Palovcak, et al. *Journal of Structural Biology*. 204, 80-84 (2018)

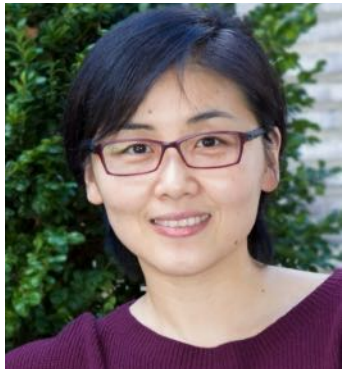
Is there a way to produce high-quality monolayer graphene grids easily and robustly with low cost & high yield?

High-yield monolayer graphene grids for cryo-EM

Methyl methacrylate (MMA) -mediated graphene transfer

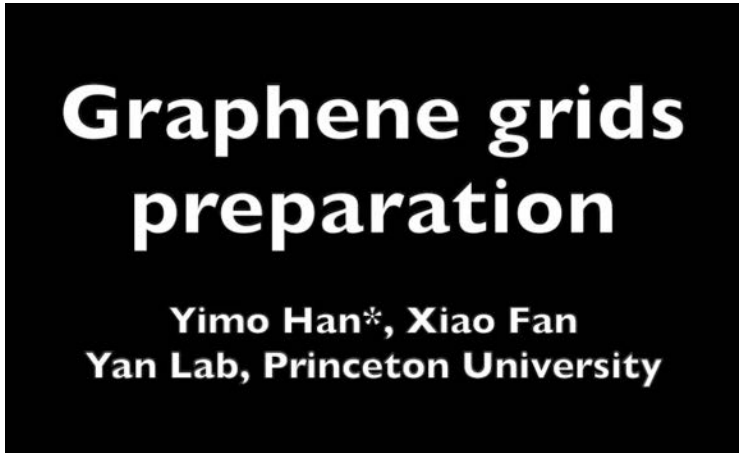


Yimo Han



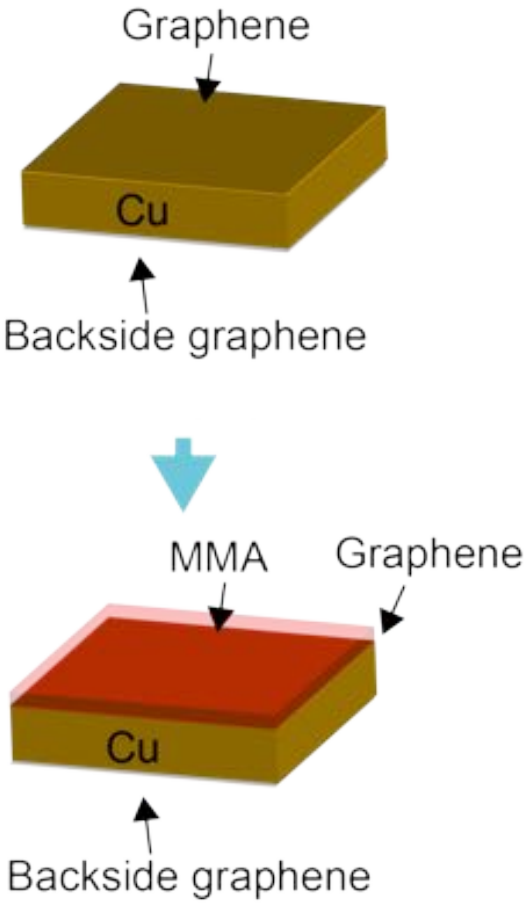
Nieng Yan

We have a video of full production process in our paper!

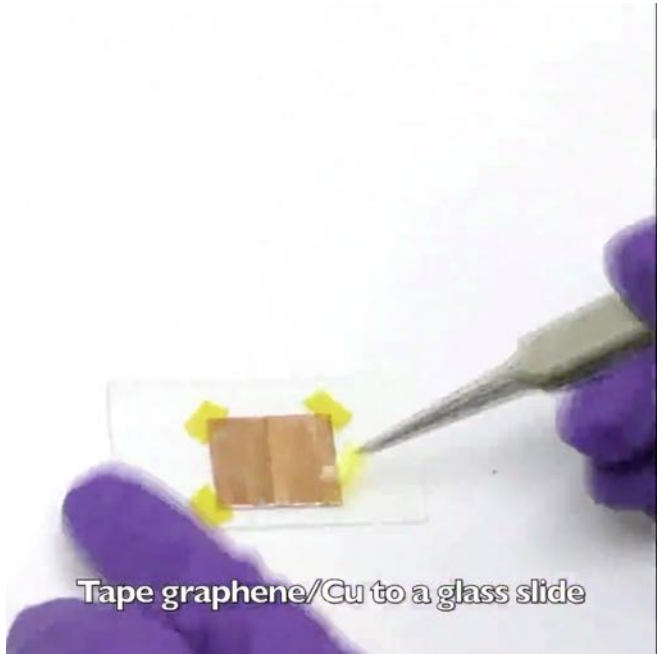


Yimo Han, Xiao Fan, et al. , *PNAS,* 2020 117 (2).

Protect graphene with MMA

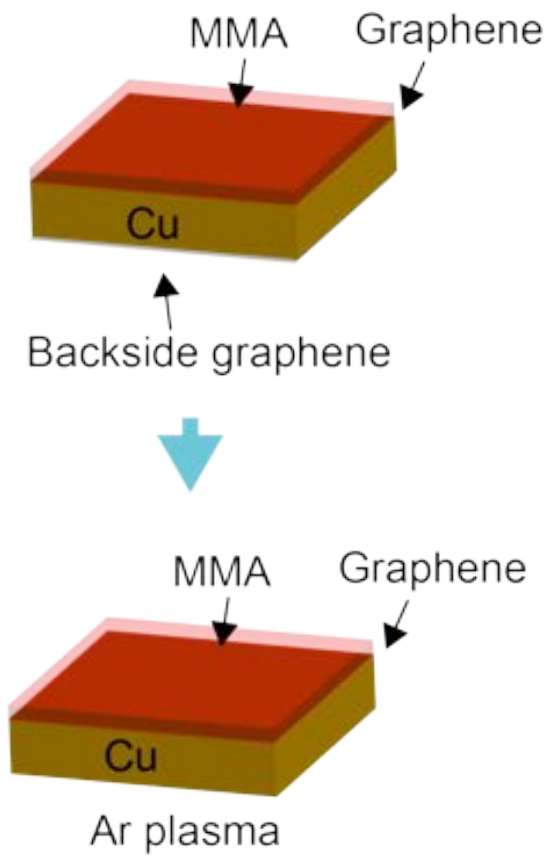


- Graphene on both side of cooper foil (Cu substrate).
- Keep the side for future supporting face-up.
- Spin-coat the upside graphene with MMA (**2,500 rpm for 1 min**).

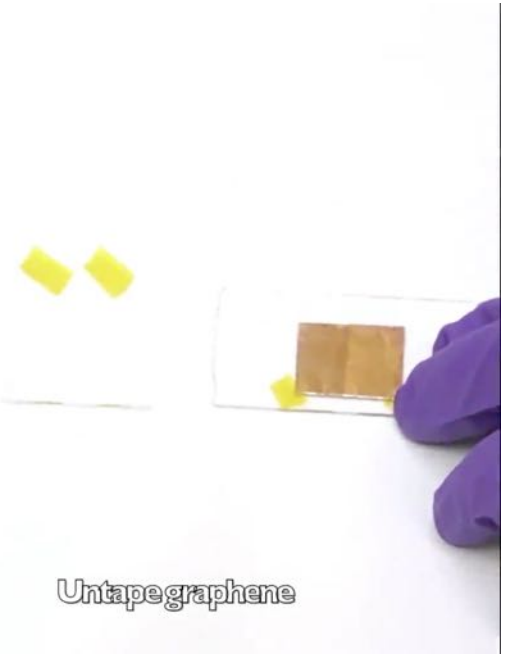


Yimo Han, Xiao Fan, et al. , *PNAS*, 2020 117 (2).

Remove backside graphene

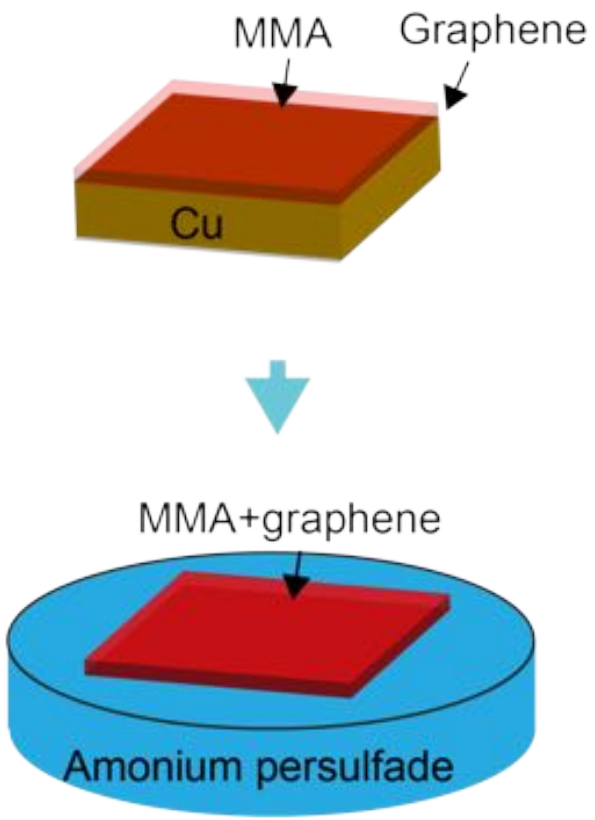


- Flip the MMA coated graphene on copper foil with backside graphene up.
- **Argon/Oxygen glow-discharge**/plasma cleaner for **30s – 60s** to remove backside graphene.



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

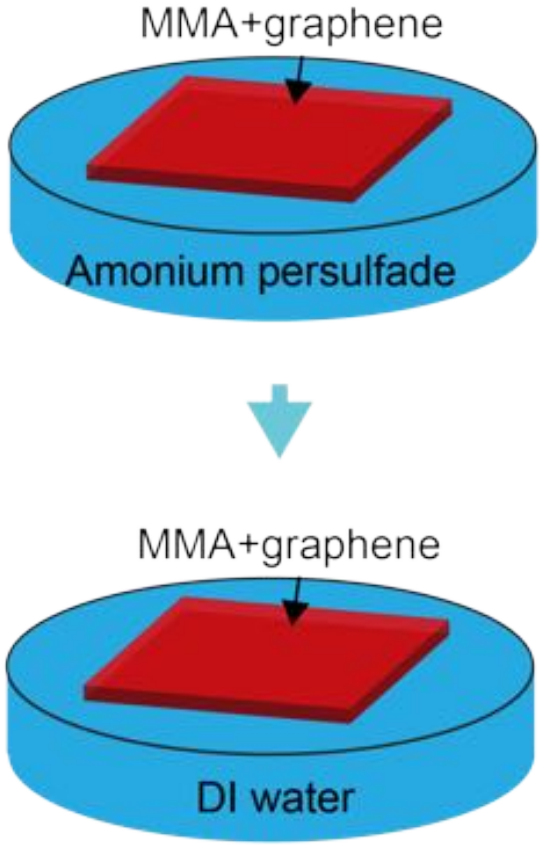
Etch out Cu substrate by Ammonium persulfate



- Cut “MMA/graphene/Cu stack” in to 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).

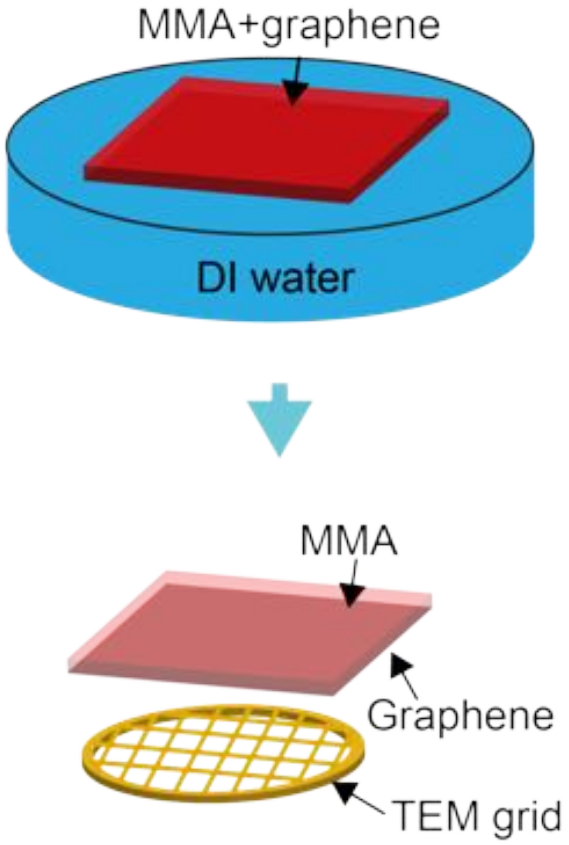
Clean MMA/graphene bilayer film



- Use glass-slide to transfer the MMA/graphene film (**MMA side up**) to DI water.
- Wash for 10-min. Repeat this process twice.

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

MMA-coated graphene grid



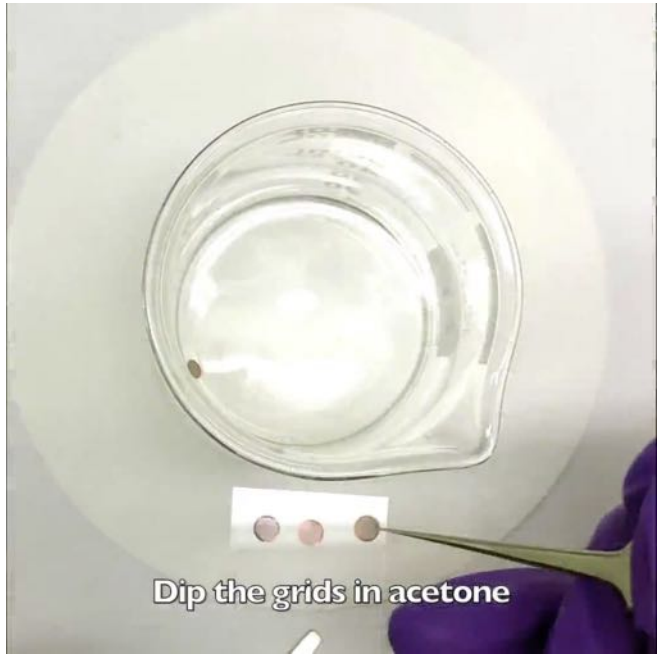
- Scoop out the MMA/graphene film using Quantifoil Au R1.2/1.3 300 mesh grids with carbon side.
- Air dry with MMA side up.

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

Remove MMA protect layer

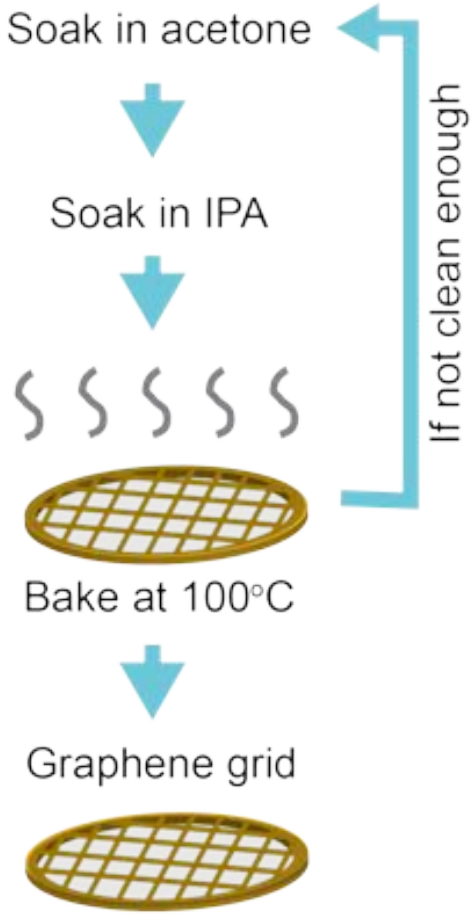
Soak all grids in acetone

- 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).

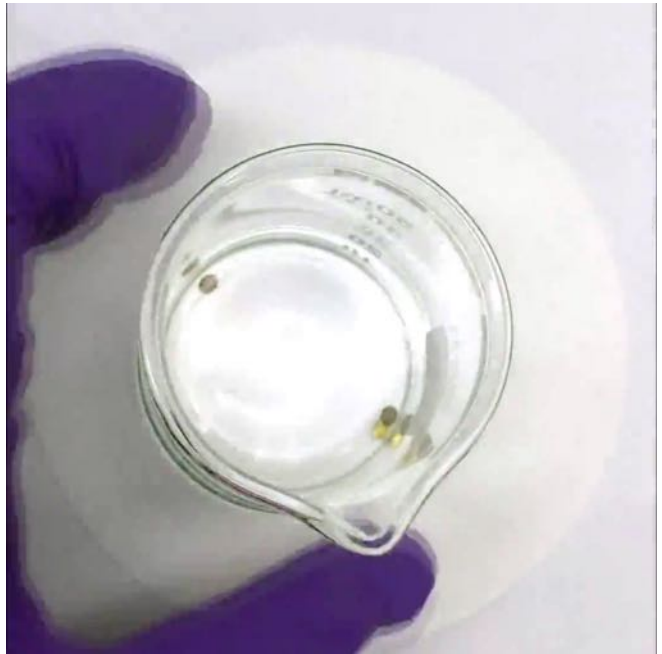


Yimo Han, Xiao Fan, et al. , *PNAS,* 2020 117 (2).

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.

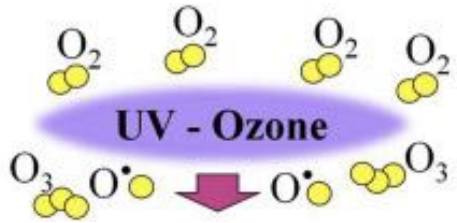


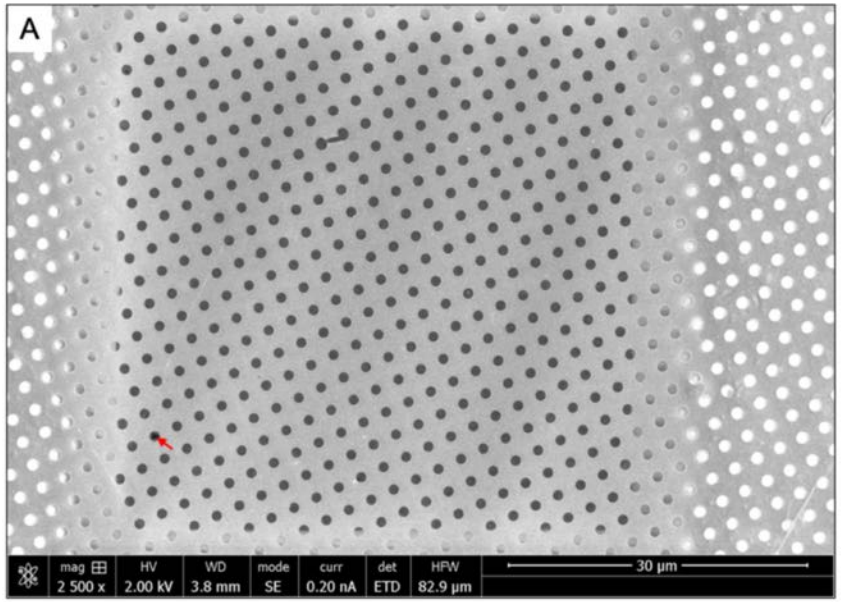
Yimo Han, Xiao Fan, et al. , *PNAS,* 2020 117 (2).

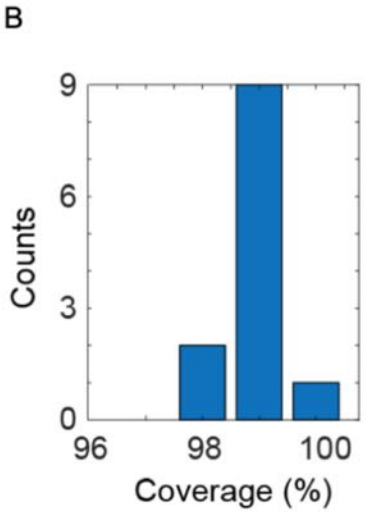
High-yield monolayer graphene grids for cryo-EM

99% yield of suspended monolayer graphene

Clean and uniform graphene surface



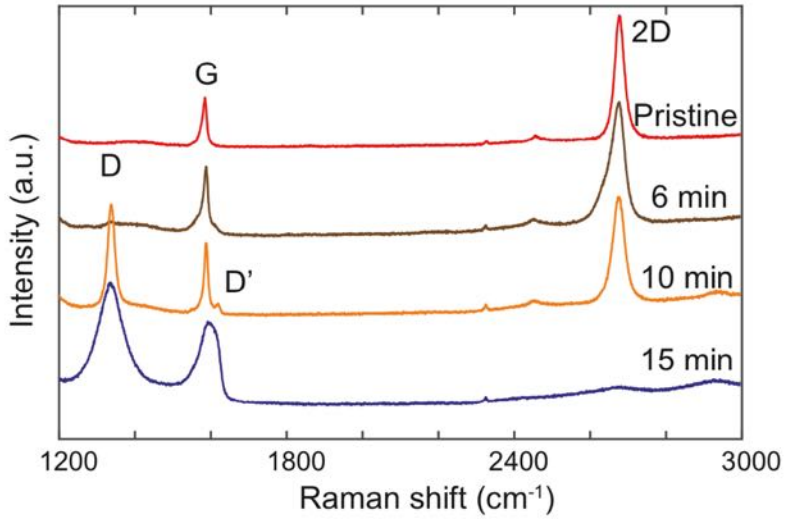




SEM image of home-made graphene grid

Contact angles

Raman spectroscopy



UV-Ozone as a mild surface treatment to increase hydrophilicity

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

Take home message

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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Thank you!

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