Preparation of Graphene-Sandwiched Specimen for Cryo-EM

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Abstract

Cryogenic electron microscopy (cryo-EM) enables high-resolution structural determination of macromolecules at near-atomic resolution through single particle analysis (SPA). However, air-water interface (AWI) effect, beam-induced charging effect and particle motion limit the full potential of this technique. The graphene sandwich method addresses these challenges by encapsulating specimens between two graphene layers, thereby improving cryo-EM data quality. This approach also expands the application prospects of graphene in electron microscopy. To simplify the implementation of this method, the present protocol details the stepwise process for preparing graphene-sandwiched specimens and collecting corresponding cryo-EM data.

Introduction

Single particle cryo-EM has emerged as one of the most powerful techniques for determining the structures of macromolecules and sensitive materials at near-atomic resolution, owing to breakthroughs in associated hardware and software systems[1-5]. Sometimes, a single dataset can capture multiple conformations or complexes of macromolecules, facilitating reliable prediction of molecular mechanisms[1,6-8].

In the traditional cryo-EM specimen preparation workflow, a sample solution of appropriate concentration is applied to a glow-discharged grid (to enhance particle adsorption). Excess solution is then blotted with filter paper in a commercial cryogenic instrument, followed by plunge-freezing to form vitreous ice. Ideally, particles should distribute randomly and uniformly within the ice layer—with a thickness slightly greater than that of the particles—and exhibit sufficient orientational diversity to support structural reconstruction[9-11]. Nevertheless, several challenges persist during cryogenic specimen preparation and data collection, including AWI effects, high background noise, charging effects, and beam-induced motion. These factors collectively reduce the efficiency of structural reconstruction and limit the achievable resolution[12-19].

Atomically thin graphene and its derivatives, characterized by minimal background noise, have been widely adopted as support films in cryo-EM[20-22]. These films facilitate particle adsorption, mitigate AWI effects, and reduce the required sample concentration[23,24]. Additionally, graphene's exceptional mechanical strength and electrical conductivity alleviate beam-induced

motion and charging effects of the specimen[25-27]. Despite these advantages, graphene-coated grids only block AWI on one side, leaving particles vulnerable to adsorption on the exposed AWI. While sandwich structures using materials such as amorphous carbon films or silicon nitride films can theoretically block AWI on both sides[28-30], their high background noise and poor electrical conductivity restrict resolution and practical applicability. Graphene liquid cells (GLCs) effectively encapsulate specimens to avoid the aforementioned issues; however, the fragility and low contrast of graphene make the fabrication of graphene-sandwiched specimens technically challenging[31-34].

Here, we describe a simple and robust method for fabricating graphene-sandwiched frozen specimen for SPA[35]. This method incorporates graphene with self-assembled monolayers (GSAMs) to enhance the contrast and mechanical stability of suspended graphene, thereby lowering the technical barrier for specimen preparation[36]. By following this stepwise protocol, graphene-sandwiched specimens can be reproducibly fabricated.

Protocol

1. Removal of graphene from the backside of graphene/copper

1. Prepare a piece of graphene/copper (with single-layer or few-layer graphene), put it upside down on a glass slide and secure its edges with additional slides. Transfer the assembly to a reactive ion etcher (Pico SLS Diener).
   NOTE: Securing the edges of the graphene/copper substrate prevents damage to the frontside graphene layer. Few-layer graphene exhibits greater mechanical robustness than single-layer graphene.
2. Perform glow discharge at 150 W for 3 min under a 10-sccm airflow to remove the backside graphene of the graphene/copper as graphene is typically grown on both sides of the copper by the chemical vapor deposition (CVD) method.
   NOTE: Glow discharge parameters may require optimization for different instruments; the color of the substrate can serve as a visual indicator of etching efficacy.
3. Take out the graphene/copper and verify complete removal of backside graphene.
   NOTE: Prior to glow discharge, the frontside and backside graphene layers exhibit similar colors. After glow discharge, the backside becomes darker due to graphene removal.

2. Preparation of stearic acid solution

1. Weigh an appropriate amount of stearic acid powder into a glass vial.
   NOTE: Plastic pipettes should be avoided, as they may introduce organic contaminants.
2. Add isopropanol to the vial to achieve a final stearic acid concentration of 0.005%.
   NOTE: This concentration is optimized for the formation of stearic acid monolayers on graphene in subsequent steps.

3. Deposition of stearic acid solution onto graphene/copper

1. Place the glow-discharged graphene/copper (frontside up) on a clean glass slide.
2. Deposit an appropriate volume of stearic acid solution onto the frontside graphene using a glass capillary.
   NOTE: This step forms graphene with self-assembled monolayers (GSAMs), which enhances graphene contrast and mechanical stability during subsequent processing. For a 1.5 cm × 1.5 cm graphene/copper, approximately 10 μL of 0.005% stearic acid solution is sufficient to form a uniform stearic acid monolayer.

4. Optical microscopy inspection

1. Transfer the GSAMs/copper (on the glass slide) to the stage of an optical microscope.
2. Monitor the surface of the graphene/copper to observe the drying process of the stearic acid solution.
   NOTE: A drying time of 20 minutes is typically sufficient. A fume hood can be used to accelerate drying.

5. Preparation of ammonium persulfate (APS) solution

1. Weigh an appropriate amount of APS powder into a beaker.
2. Add double-distilled water (ddH_2O) to the beaker to achieve a final APS concentration of 0.5 M. Stir the solution to ensure complete dissolution.
   NOTE: APS solution should be prepared fresh whenever possible. For short-term storage (approximately two weeks), the solution can be stored in a sealed container to slow decomposition.

6. Etching copper substrate for achieving free-standing GSAMs membrane

1. Use scissor to cut the GSAMs/copper into small patches, each slightly larger than the target cryo-EM grid.
   NOTE: Avoid bending the graphene/copper during cutting. Placing the GSAMs/copper between two glass slides and applying gentle pressure can help maintain flatness.
2. Pour the APS solution into a Petri dish. Using tweezers, place three GSAMs/copper patches (frontside up) on the surface of the APS solution, then cover the Petri dish.
   NOTE: Three patches are prepared to fabricate three specimens. The volume of APS solution is critical: insufficient volume slows etching, while excessive volume may cause the patches to contact the Petri dish lid.
3. Etch the copper substrates for approximately 40 minutes. Avoid agitating the APS solution during etching.

7. Fabrication of graphene-sandwiched specimens

1. Place three graphene grids in a plasma cleaner (Harrick Plasma). After evacuating the chamber for 2 minutes, perform plasma treatment at low power for 15 seconds.
   NOTE: The graphene grid serves as the bottom layer of the graphene-sandwiched specimen.
2. Pour a sufficient volume of buffer into a petri dish. Using a metal mesh, transfer the suspended GSAMs patches onto the buffer surface.
   NOTE: The GSAMs patch serves as the top layer of the graphene-sandwiched specimen. Exercise caution to avoid damaging adjacent GSAMs patches during transfer, and the buffer should be suitable for the sample.
3. Deposit 3 μL of the sample solution onto the plasma-treated graphene grid and incubate for 1 min to ensure adequate macromolecule adsorption.
   NOTE: Graphene grids reduce the required sample concentration, but the subsequent immersion step increases it. The optimal final sample concentration is approximately 1 μM.
4. Using tweezers, immerse the graphene grid (frontside up) beneath the suspended GSAMs patch. Rapidly scoop the GSAMs patch onto the grid, then quickly blot the backside of the grid with filter paper. Transfer the grid to a grid storage box.
   NOTE: An alternative method involves using a loop to scoop the GSAMs patch and place it onto the grid, which reduces macromolecule loss but increases the risk of

GSAMs damage (due to fragility). Blotting time should be approximately 1 s to leave a thin solution layer on the grid—insufficient solution results in an ice layer too thin to accommodate macromolecules.

8. Freezing of graphene-sandwiched specimens

1. Manual freezing method: Prepare liquid ethane pre-cooled with liquid nitrogen. Using tweezers, transfer a grid from the storage box and rapidly immerse it in the liquid ethane. Store the frozen grid in a cryogenic storage box (blue box).
   NOTE: Rapid immersion minimizes the formation of crystalline ice, which degrades specimen quality.
2. Automatic freezing method: Configure the cryogenic instrument (e.g., FEI Vitrobot) according to standard parameters. Perform automated blotting and freezing, then transfer the frozen grid to a cryogenic storage box.
   NOTE: For the FEI Vitrobot, set the blotting time to 0.5 s and blotting force to -2 if the grid was not pre-blotted manually. Omit the instrument-based blotting step if manual blotting was performed. The reproducibility of graphene-sandwiched specimen fabrication is approximately 80%.

9. Cryo-EM quality inspection

1. Load the frozen specimen into the cryo-EM column. At low magnification, identify regions of the grid with appropriate ice thickness.
   NOTE: Successfully encapsulated regions exhibit uneven ice thickness and a characteristic striped black pattern.
2. Capture medium-magnification micrographs to identify holes with optimal ice thickness.
   NOTE: Avoid regions with excessively dark holes, as their boundaries are difficult to distinguish.
3. Adjust the defocus value, then capture high-magnification micrographs.
   NOTE: Micrograph contrast is reduced due to the two graphene layers. The presence of multiple sets of hexagonal diffraction spots confirms successful graphene encapsulation.

Representative results

By following the aforementioned protocol (Figure 1), graphene-sandwiched specimens can be reproducibly fabricated using standard laboratory equipment (Figure 2). Since CVD grows graphene on both sides of copper substrates, glow discharge is essential to remove backside graphene and prevent contaminations. Prior to glow discharge, the frontside and backside of the copper substrate exhibit similar colors (Figures 3A, 3B); after discharge, the backside darkens due to graphene removal (Figure 3D). The concentration and volume of stearic acid solution are critical for GSAMs formation (Figure 3E), and glass capillaries must be used instead of plastic pipettes to avoid organic contamination (Figure 3F).

To fabricate three graphene-sandwiched specimens, three small GSAMs/copper patches are cut and placed on the APS solution surface (Figure 4A). While a single large patch can be used, small patches offer greater flexibility (e.g., enabling preparation of different macromolecule samples by transferring patches to different buffers). The copper substrate is gradually etched (Figure 4B) and fully dissolved after approximately 40 minutes in 0.5 M APS (Figure 4C). A metal mesh or glass slide can be used to transfer the GSAMs patches to the macromolecule-compatible buffer (Figure 4D). After scooping the top GSAMs layer onto the grid,

macromolecules adsorb to both sides of the grid (Figure 4E), and excess solution is blotted from the grid backside with filter paper (Figure 4F).

Both manual (Figure 5A) and automated (Figure 5B) freezing methods are suitable for graphene-sandwiched specimens. Frozen specimens can be stored for subsequent quality inspection. At low cryo-EM magnification, successfully encapsulated regions exhibit uneven ice thickness and a distinct striped black pattern (Figure 5C). Medium-magnification micrographs enable identification of holes with optimal ice thickness (Figure 5D). High-magnification micrographs of these holes reveal clear macromolecule particles (Figure 5E), which are suitable for near-atomic resolution 3D reconstruction. The diffraction pattern of the two graphene layers (Figure 5F) confirms successful encapsulation. This method has been validated for high-resolution structural determination of various macromolecules with different molecular weights.

Discussion

The air-water interface poses significant challenges for cryo-EM specimens, as particles adsorbed to the AWI often exhibit preferred orientations, aggregation, or partial/complete denaturation. Graphene grids effectively capture particles and mitigate AWI effects on one side; however, particles remain vulnerable to adsorption on the exposed AWI. While continuous carbon films or silicon nitride films can be used to sandwich specimens and block both AWIs, their high background noise and poor electrical conductivity limit their utility.

Graphene liquid cells are widely used in life sciences and materials science to observe dynamic processes. In this study, we adapt GLC technology for cryo-EM to address AWI-related issues and provide a stepwise protocol to simplify graphene-sandwiched specimen preparation. To enhance the contrast and stability of suspended graphene, we use GSAMs (instead of bare graphene) as the top layer of the sandwich structure. Several key details are critical to improving fabrication success rates.

Preparation and adding of stearic acid solution, etching of the copper, fabricating and freezing graphene sandwiched sample are critical during sample preparation. It is suitable to drop 10 μL 0.005% stearic acid solution for a patch which is about 1.5 cm × 1.5 cm, and higher concentration or larger volume results in contaminations in micrographs and therefore interferes with the observation and reconstruction of the macromolecules. It's practicable to remove self-assembled or extra stearic acid on the graphene by washing with isopropanol if the stearic acid is no longer needed for the final sample. Ensure the front side of graphene/copper is facing up during etching of the copper as opposite direction slows down the etching process and causes the scroll of GSAMs. During fabrication of graphene sandwiched sample, immersing of the grid may cause the movement of the suspending GSAMs. For macromolecules needing thick ice, automatic method with higher speed should be better to avoid the formation of crystal ice.

The thickness of the graphene-sandwiched specimen (critical for subsequent inspection and reconstruction) is primarily controlled by blotting. While manual blotting is feasible, automated blotting (via instruments) offers greater adjustability. Using this protocol, we have achieved high-resolution structural reconstructions of the 20S proteasome, viral spikes, and apoferritin. This method also holds promise for observing dynamic processes of macromolecules in TEM.

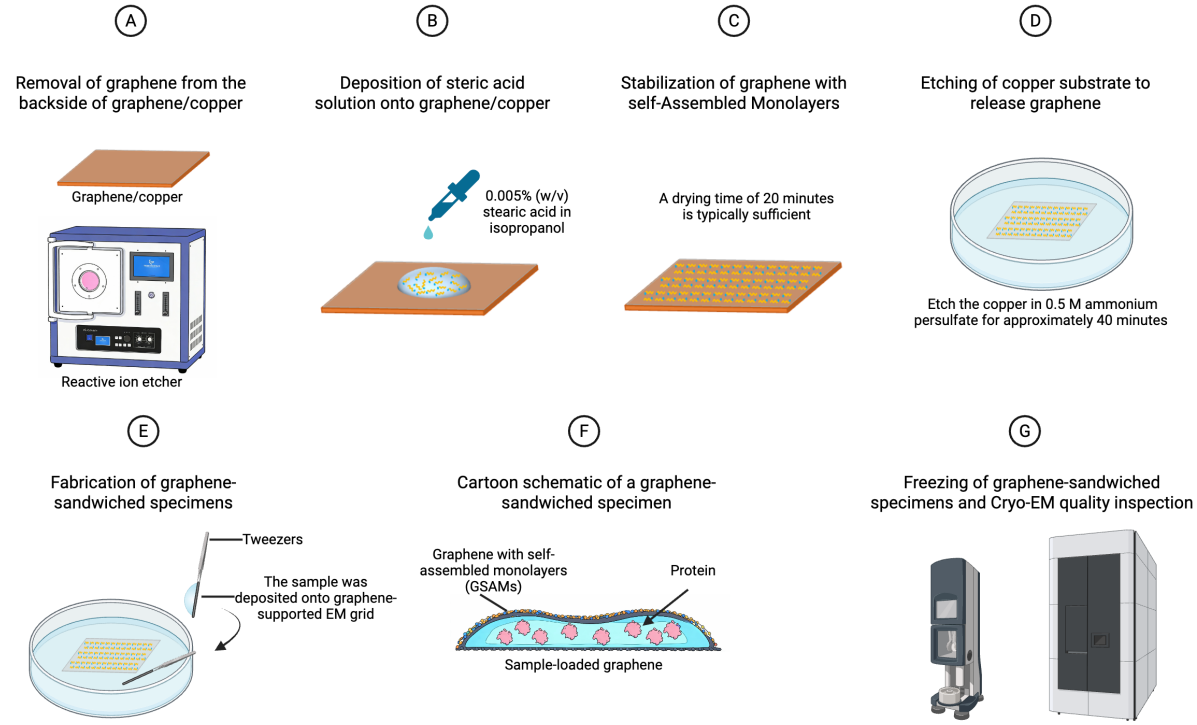

Figure 1: Schematic illustration for preparing graphene-sandwiched specimen. (A) The backside graphene on the copper was removed using a reactive ion etcher. (B) For a 1.5 cm × 1.5 cm graphene/copper, approximately 10 μL of 0.005% stearic acid solution is sufficient to form a uniform stearic acid monolayer. (C) The sample was placed in a fume hood and allowed to dry for approximately 20 min to obtain GSAMs/copper. (D) The copper substrates were etched in ammonium persulfate (APS) solution for approximately 40 min. Avoid agitating the APS solution during etching. (E) After depositing the sample onto the graphene grid, use tweezers to immerse the grid (frontside up) beneath the suspended GSAMs patch. Rapidly scoop the GSAMs patch onto the grid and immediately blot the backside with filter paper. (F) Specimen preparation by the graphene sandwich approach, in which an additional layer of graphene film with stearic acid coating is placed onto the sample-loaded graphene grid, forming an aqueous film before vitrification for cryo-EM analysis. (G) Cryo-EM samples were prepared using a Vitrobot, and the vitrified specimens were examined and data were collected using a cryo-electron microscope.

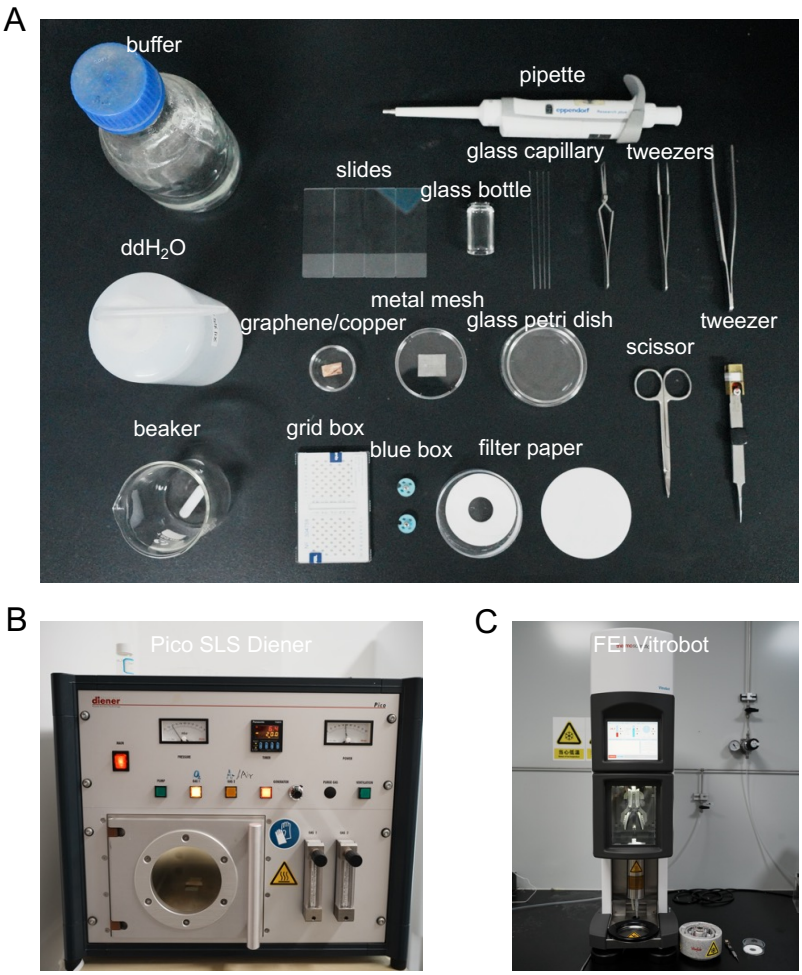

Figure 2: Required materials and equipment for graphene sandwich method. (A) Main materials for fabricating graphene-sandwiched sample. (B) The reactive ion etcher for removing the backside graphene of the graphene/copper. (C) The instrument (FEI Vitrobot) for freezing of graphene-sandwiched sample.

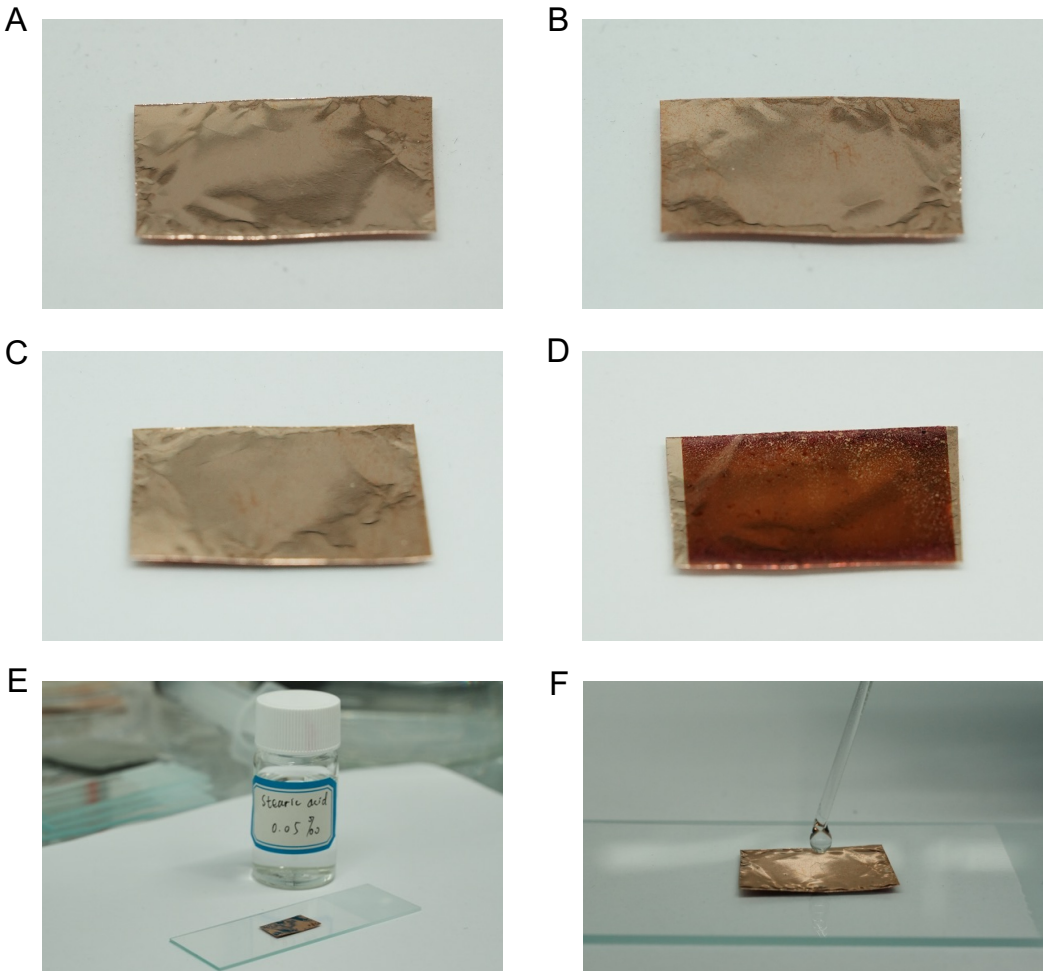

Figure 3: Removal of the backside graphene and preparation of GSAMs/copper. (A) Frontside of graphene/copper before glow discharge. (B) Backside of graphene/copper before glow discharge. (C) Frontside of graphene/copper after glow discharge. (D) Backside of graphene/copper after glow discharge. (E) Stearic acid solution with the concentration of 0.005%. (F) Dropping of stearic acid solution onto the graphene/copper after glow discharge.

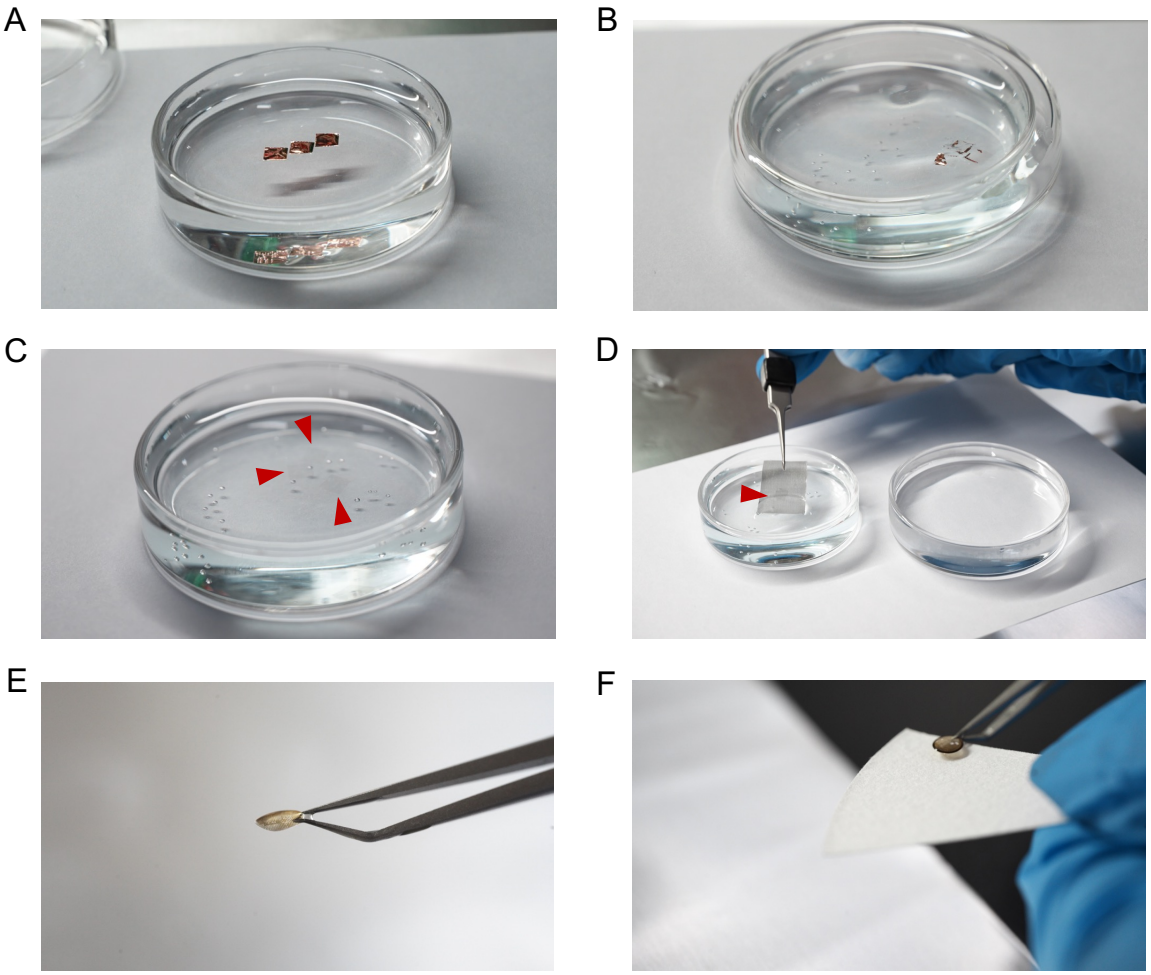

Figure 4: Preparation of graphene-sandwiched specimen. (A) Three patches of GSAMs/copper before etching. (B) Three patches of GSAMs/copper after partial etching. (C) Three patches of GSAMs/copper after complete etching. (D) Transfer of the suspending GSAMs onto the buffer. (E) Both sides of the grid are coverd with solution. (F) Blot the grid at the backside to remove extra solution.

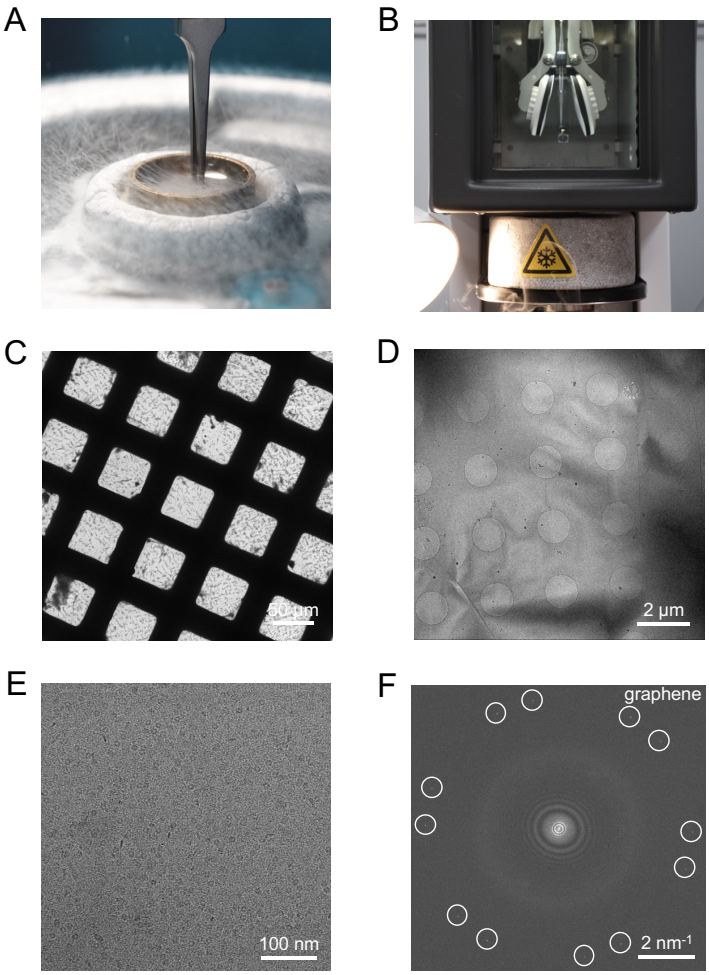

Figure 5: Freeze and inspection of graphene-sandwiched specimen. (A) Freeze the graphene-sandwiched specimen manually. (B) Freeze the graphene-sandwiched specimen automatically. Inspection of the graphene sandwiched specimen at low magnification (C), medium magnification (D), and high magnification (E). (F) Representative Fourier transform image of graphene sandwiched area shows two layers of graphene.

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