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Preparation of Tunable Microchips to Visualize Native Protein Complexes for Single-Particle Electron Microscopy

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Abstract

Recent advances in technology have enabled single-particle electron microscopy (EM) to rapidly progress as a preferred tool to study protein assemblies. Newly developed materials and methods present viable alternatives to traditional EM specimen preparation. Improved lipid monolayer purification reagents offer considerable flexibility, while ultrathin silicon nitride films provide superior imaging properties to the structural study of protein complexes. Here, we describe the steps for combining monolayer purification with silicon nitride microchips to create a tunable approach for the EM community.

Keywords

Electron microscopy; Single-particle analysis; Affinity capture; Silicon nitride; Microchips; Protein assemblies

1 Introduction

Single-particle electron microscopy (EM) is a valuable tool for investigating the structural properties of biological complexes [1]. With this technique, structural information embedded in the images is extracted to computationally build a 3D density map of the examined assemblies. One recurring challenge for single-particle methods is obtaining a homogeneous sample that facilitates downstream imaging and computational analysis. Conventional biochemical purification is often employed to help isolate protein assemblies but can sometimes fall short of the desired sample concentration and purity. A second bottleneck in preparing single-particle EM specimens is the inherent limitations introduced by traditional materials and processes. These combined shortcomings have created an opportunity for improvement in generating better EM specimens.

Lipid monolayers present a different approach for single-particle specimen preparation [2]. The amphipathic nature of the lipid is well suited for both the adherence to a solid EM support and the capture of protein complexes. The versatility of lipid monolayers comprised of functionalized Ni-NTA moieties enables His-tagged proteins to bind to the lipid with increased specificity [3]. This “monolayer purification” platform can be used to recruit His-tagged proteins from cell lysates, nuclear fractions, or pre-fractionated samples directly onto the support film in a single step [4–7]. The method further evolved to include non-His-

tagged protein complexes when His-tagged Protein A adaptors were introduced, bridging the Ni-NTA lipid with a target antibody [8]. This modification makes the method tunable and robust, lending itself to study proteins and assemblies for which antibodies are readily available [9–11].

Carbon-coated support films are traditionally used in the preparation of biological specimens for EM imaging purposes. Amorphous carbon supports can have a great deal of variability and defects from production that can ultimately impact resolution. With the advent of in situ EM, silicon nitride (SiN) microchips provide an alternative support material that is transparent to the electron beam [12–16]. As SiN membranes can be more consistently manufactured, their very flat surface renders them an attractive tool. The hydrophobic nature of SiN membranes also provides a compatible surface for use with lipid monolayers. Pairing SiN microchips with the monolayer purification approach has shown to be a valuable tool, including recent reports describing BRCA1-transcriptional complexes [17–19].

In this chapter, we present in detail how to make a tunable microchip specimen to visualize protein complexes derived from breast cancer cells grown under stressful conditions. The protocol describes (1) the preparation of the silicon nitride microchip, (2) the proper setup and transfer of a monolayer to the microchip, (3) the procedure for creating the “tuned” EM specimen, and (4) recommendations for data collection and image processing. Image information and a representative 3D structure of BRCA1-transcriptional complexes are shown.

2 Materials

1. Glass volumetric vial with stopper (1 mL).
2. Chloroform.
3. Parafilm.
4. Buffer A: 20 mM HEPES (pH 7.2), 140 mM NaCl, 2 mM MgCl₂, and 2 mM CaCl₂.
5. Ultrapure water.
6. Hot plate.
7. 5 N sodium hydroxide solution.
8. 5 mL syringe with Luer-Lok tip.
9. 33 mm syringe filter (0.2 µm).
10. 15 mL conical tube.
11. Aluminum foil.
12. Silicon nitride microchips (Protochips).
13. Carbon fiber tweezers (Pelco).
14. HPLC-grade acetone.

15. HPLC-grade methanol.
16. Dish or beaker for solvent.
17. Whatman 1 circular filter paper (90 mm).
18. Compressed air.
19. Glass petri dish with cover (100 × 15 mm).
20. Disposable glass cell culture tubes (6 × 50 mm).
21. Hamilton 10 µL syringe.
22. Pipet.
23. BRCA1 antibody (C-20, 0.2 mg/mL, Santa Cruz Biotechnology).
24. Glass Pasteur pipet.
25. Clear PVC vacuum tubing.
26. House vacuum system.
27. Ni-NTA lipid and DLPC lipid (Avanti Polar Lipids). Prepare by weighing 1 mg of powdered lipid into a 1 mL glass volumetric vial, and add chloroform to the 1 mL mark on the vial. Cap the vial and seal with parafilm. Store in a −20 °C freezer for up to 3 months. Lipids used in the preparation of monolayers are dissolved in chloroform. Chloroform is a carcinogen. Please review the MSDS and consult with EHS for its safe use and disposal.
28. His-tagged Protein A (10 mg, 50 mg/mL, Abcam) has been optimized for IgG binding and includes a 6× His tag at its N-terminus. It was pre-diluted to a working concentration of 0.1 mg/mL (1/500) in Buffer A, aliquotted, and stored at −20 °C. Buffer A can be substituted for the user's preferred buffer.
29. Uranyl formate solution (0.75%). Boil 3 mL of ultrapure water in a 10 mL beaker on a hot plate. Using tongs, transfer the beaker to a stir plate in a chemical fume hood. Add 22.5 mg of uranyl formate and a small stirbar. Stir for 5 min. Add 4.2 µL of 5 N NaOH. Stir another 5 min. Draw the solution up into a 5 mL syringe. Filter the mixture through a 0.2 µm PVDF filter to remove the undissolved uranyl formate and collect in a 15 mL conical tube. Cover the tube with aluminum foil. Uranyl formate solution is light sensitive. Covering the tube in aluminum foil minimizes the exposure to light. If stored in this manner, the uranyl formate solution can be kept up to 48 h without a decline in quality. Uranyl formate is toxic if inhaled or ingested. Please review the MSDS and consult with EHS for safe use and proper disposal.
30. Negatively stained BRCA1-RNAP II complexes were examined using a FEI Spirit Bio-Twin TEM equipped with a LaB₆ filament and operating at 120 kV.
31. Images are recorded using a FEI Eagle 2k HS CCD camera having a 30 µm pixel size and employing low-dose conditions (~1–5 electrons/Å²).

32. SPIDER is a software package [20] used for 2D classification of procedures through a multivariate data analysis approach. Individual protein complexes (particles) are selected from micrographs. After selection, all particles are extracted then processed through several iterations of multi-reference alignment implementing a K-means classification routine. The parameters in each step are modulated by the user; however, the routines are standard.
33. RELION is a software package [21] used to perform reconstruction and refinement calculations using an empirical Bayesian methodology.

3 Methods

3.1 Cleaning the Microchips

Silicon nitride membranes can be as thin as 5 nm and should be handled with care to avoid fracture (*see Note*¹). Microchips are supplied in Gel-Paks with the membrane side facing upward (Fig. 1a). The manufacturer recommends handling the microchips at the edges with carbon fiber tip tweezers to avoid damaging the silicon frame (*see Note*²). Before use, the microchips should be cleaned in a low-dust environment with HPLC-grade acetone and methanol to avoid surface contamination (*see Note*³).

1. In a dish or beaker containing acetone, submerge and release the microchip (Fig. 1b). Wash by gently swirling the dish in a circular motion for 1–2 min.
2. Promptly move the microchip to a second dish containing methanol. Do not allow the microchip to dry during transfer. Swirl to wash for 1–2 min.
3. Remove the microchip from the methanol and gently wick off the excess fluid by touching the edge to Whatman 1 filter paper.
4. To prevent residue or contamination from dust particles, the microchips may be dried using residue-free compressed air (Fig. 1c). With the microchip still in the tweezers, direct a gentle flow of air across the surface until it is dry (*see Note*⁴).

3.2 Preparation of Microchip Surface

The hydrophobicity of the silicon nitride membrane can be enhanced to facilitate proper binding of the nonpolar lipid tail domains. A simple way to do this is by heating the microchip on a hot plate (Fig. 1d; *see Note*⁵).

¹The carbon fiber tip tweezers are non-scratching. The microchips used in this study are diced, which provides flat edges and allows the edges to be gripped easily, without contacting the surface of the silicon nitride membrane. With a magnification device, regularly check the integrity of the silicon nitride membrane from cleaning through specimen preparation.

²The acetone/methanol rinse steps in this procedure are employed to remove a protective photoresist coating that is applied to the surface of the microchip during the fabrication process. Note that some types of silicon nitride microchips may be supplied by the manufacturer without this protective coating. In this case, the cleaning step may be omitted, but it is still recommended as a precaution to remove contaminants or debris that may be present on the membrane surface. Cleaning the microchip should require less than 10 min.

³Direct the airflow across, rather than perpendicular to, the microchip surface to avoid damaging the SiN membrane. The laminar airflow also prevents dust particles in the air from drying onto the membranes during solvent evaporation.

⁴If the ambient conditions are relatively dry, heating the microchip may not be necessary. Otherwise, this step takes ~2.5 h.

⁵The ratio of Ni-NTA to DLPC can be increased or decreased as necessary depending on the specimen preparation. DLPC filler lipid acts to spatially disperse the Ni-NTA lipid. Increase the dilution of Ni-NTA if the particle density is too high and vice versa. As a rule of thumb, 5% Ni-NTA is a good starting place for a negatively stained specimen and 25% Ni-NTA for a cryo-EM specimen.

1. With the membrane side up, place the microchip on a clean glass petri dish or glass slide.
2. Preheat the hot plate to 150 °C. Place the dish containing the microchip onto the hot plate for 1.5 h.
3. With forceps or hot gloves, carefully remove the dish from the hot plate and place on a heat-resistant surface. Allow the microchip to cool to room temperature.

3.3 Preparation of Ni-NTA Lipid Monolayers

Individual lipid components are solubilized in chloroform, and monolayers consist of a combination of Ni-NTA (active binding) lipids and DLPC (inactive, filler) lipids. The concentration of active binding sites present in the monolayer can be adjusted by modifying the ratio of the Ni-NTA to DLPC lipids (*see Note* ⁶). The resulting lipid mixture is added over a drop of water, forming a thin monolayer film, which can then be transferred to the hydrophobic surface of a microchip (Fig. 2a; *see Note* ⁷).

1. In advance, remove solubilized lipids from storage and allow them to warm to room temperature (*see Note* ⁸).
2. Place a piece of circular Whatman 1 filter paper in a glass petri dish. Wet the filter with ultrapure water, and then place a 2-in. by 2-in. piece of parafilm on top of the wetted filter. Place the cover back on the dish.
3. Obtain three disposable glass tubes to be used for the preparation of the lipid mixture. Label one with the percentage of lipid to be used (e.g., “5% Ni-NTA”) and one “rinse.” The remaining tube will contain chloroform to be used for dilution and can be labeled “CHCl₃” (*see Note* ⁹). Using a Pasteur pipet, add ~0.5 mL chloroform to the “CHCl₃” and “rinse” tubes.
4. Rinse a Hamilton syringe by aspirating and dispensing several times with chloroform in the “rinse” tube.
5. Aspirate 10 µL of chloroform from the “CHCl₃” source tube and dispense into the tube labeled “5% Ni-NTA.”
6. From the DLPC filler lipid source vial, aspirate and dispense 28 µL lipid into the “5% Ni-NTA” tube. Rinse the syringe.
7. From the Ni-NTA lipid source vial, aspirate 2 µL of lipid and dispense into the “5% Ni-NTA” tube. Rinse the syringe. Seal all tubes and vials with a small piece of parafilm until use to prevent evaporation (*see Note* ¹⁰).

⁶Even subtle vibration can disturb the lipid interface. Prepare and store the monolayer on a vibration-free surface to ensure proper formation. Total preparation time will be ~1.5 h.

⁷Minimize the time solubilized lipid is at room temperature.

⁸Chloroform should be handled in a chemical fume hood. Please review the chloroform MSDS regarding its safe use and proper disposal.

⁹For a 20% Ni-NTA lipid solution, increase the Ni-NTA lipid to 8 µL and decrease the DLPC lipid to 22 µL. The volume of lipids is 30 µL in a total of 40 µL. Theoretically, up to 40 monolayers can be prepared.

¹⁰When properly stored on ice, we have found that monolayers are stable up to 24 h.

8. Pipet 15 μL of ultrapure water onto the parafilm in the humid petri dish. Do this for as many microchips as is necessary for the experimentation. Separate the water drops by ~ 1 cm.
9. Using the Hamilton syringe, add 1 μL of the 5% Ni-NTA lipid mixture over the apex of each water drop. After addition of the lipid, the drop will flatten and spread (Fig. 2a).
10. Put the lid on the petri dish. To keep the lipid monolayers hydrated, seal them in a humid environment by wrapping parafilm around the petri dish.
11. Incubate the dish at room temperature for 10 min before placing on ice for at least 1 h (*see Note* ¹¹).

3.4 Preparation of Tunable Microchip Specimen

Here we describe the transfer of the monolayer to the microchip followed by step-by-step addition of His-tagged Protein A adaptor, target antibody, and sample. Though the procedure for negative staining is described, tunable microchips also work well in cryo-EM applications [15, 17–19]. Uranyl formate stain should be prepared in advance. All filter paper used in the preparation of EM specimens is Whatman 1. Each solution will be removed from the microchip by wicking with the edge of a small piece of filter paper unless otherwise indicated. All incubations are performed at room temperature (~ 23 °C; *see Note* ¹²).

1. Carefully remove the parafilm sealing the glass dish containing the monolayers, and return the dish to the ice.
2. Using carbon fiber tip tweezers, place each microchip membrane side down on the surface of a lipid monolayer (Fig. 2b). The microchip typically will come to rest on the side of the water droplet. The nonpolar tail adheres to the hydrophobic surface of the microchip. Incubate for 1 min (*see Note* ¹³).
3. Gently lift the microchip off the monolayer, and place it membrane side up on a free space in a Gel-Pak (Fig. 2c). Using a pipet, add 3 μL of His-tagged Protein A and incubate for 1 min (*see Note* ¹⁴).
4. Carefully remove the Protein A solution by wicking it off the microchip with the edge of filter paper (Fig. 2d). Promptly add 3 μL of antibody solution (*see Note* ¹⁵). Incubate for 1 min.

¹¹Preparation of a tunable microchip specimen will take ~ 15 min for each specimen.

¹²While 1 min is the recommended time, we have found that microchips can be left on the monolayer longer without effect. To save time, all microchips can be added to the monolayers at the same time.

¹³Make sure the antibody for your target is suitable for Protein A binding. There are also His-tagged versions of Protein G adaptors commercially available for antibodies with poor Protein A affinity.

¹⁴Typically, the antibody is diluted 1/1000 (0.2–1 ng/ μL) in Buffer A as a starting point. The antibody's optimal dilution in Western blotting is a reasonable guide. Buffer A can be substituted for the user's preferred buffer. For the initial experimentation, additional antibody dilutions (higher or lower) can be tested to find the optimal concentration. When troubleshooting, consider the antibody epitope including the benefits of using a polyclonal or monoclonal antibody. Polyclonal rabbit antibody against the BRCA1 C-terminus (1/1000, 0.2 ng/ μL) was used for our representative data.

¹⁵We typically use a sample concentration of 0.01–0.02 $\mu\text{g}/\mu\text{L}$ when preparing negatively stained specimens. Sample concentration and incubation times can be determined empirically. We used 0.02 $\mu\text{g}/\mu\text{L}$ partially purified nuclear extract from oxidatively stressed breast cancer cells as the protein source for the representative data [25].

5. Remove the antibody solution using a Hamilton syringe. Immediately add the protein sample and incubate on the microchip for 2 min at room temperature (*see Note* ¹⁶).
6. Remove the sample solution and immediately rinse with 3 μ L ultrapure water (*see Note* ¹⁷).
7. Remove the water and immediately add 3 μ L of 0.75% uranyl formate to wash.
8. Remove the uranyl formate wash, and immediately add another 3 μ L of uranyl formate to stain the specimen. Incubate for 10–30 s (*see Note* ¹⁸).
9. Remove the uranyl formate wash and carefully aspirate excess stain with a Pasteur pipet connected by PVC tubing to a gentle house vacuum (*see Note* ¹⁹). Store the microchip on filter paper in a clean, covered glass dish or Gel-Pak to protect from debris until imaging.

3.5 TEM Image Collection

1. Allow the scope to fully evacuate the column and cool.
2. Load the microchip sample (Fig. 3a) containing tunable components (Fig. 3b) into a single-tilt EM specimen holder at room temperature. The microchip is loaded in a similar manner to copper supports. If possible, align the imaging window to the center of the specimen holder.
3. With the beam engaged, find the window on the microchip. The sample must be aligned along the axis of the beam line, in order for proper defocus to be attained. This is done at the center point of the specimen holder. The sample is now ready for imaging.
4. Once an area of good particle occupancy has been identified, digital images are acquired at a defocus value of $\sim$ –1.5 μ m (Fig. 3c; *see Note* ²⁰).
5. Save images in 16-bit.tiff format for downstream image processing procedures.

¹⁶The number of washes can be increased as needed for optimal staining.

¹⁷Staining time duration should be determined empirically. Generally, incrementally decrease the staining time if the stain is too heavy and increase if too light.

¹⁸An in-line vacuum trap flask should be present to collect uranyl formate waste. The pipet with vacuum should not come in contact with the microchip. Alternatively, the stain can be allowed to air-dry.

¹⁹Silicon nitride microchips suitable for TEM applications contain a viewing window composed of ultrathin, amorphous silicon nitride supported by a silicon frame. The silicon nitride films are available in a range of thicknesses with films of either 30 or 50 nm typically providing an ideal ratio of both membrane strength and electron transparency. The viewing windows may be either a flat film of constant thickness or contain integrated features. In this study, the membrane was composed of 200 nm thick silicon nitride, into which were etched an array of microwells (10 $\times$ 10 μ m square) across the surface. The depth of these wells was 170 nm, resulting in a membrane thickness of only 30 nm at the bottom of each microwell. Microchips are available with viewing windows of different sizes; however, as a general rule of thumb, the thinner the membrane thickness, the smaller the window region due to the increased fragility of the film. Suppliers of silicon nitride microchips for TEM include, among others, Protochips (used in this study), Norcada, and TEM Windows. These suppliers offer a wide range of different features and window options. Users familiar with semiconductor processing may also elect to manufacture their own microchips.

²⁰The range of defocus values generally is smaller as a result of the consistent flatness of the SiN membrane in comparison to carbon-coated supports. The same TEM conditions are used to collect images of negatively stained and ice-embedded specimens except for varying the defocus range.

3.6 Data Analysis and Representative Results

1. Prior to particle selection, the original images are normalized using the standard routines in SPIDER software package. Individual complexes from the images are manually selected using the WEB interface of the SPIDER software, employing a box size that is approximately twice the diameter of the particles of interest. Multi-reference alignment routines are implemented outputting representative 2D class averages (Fig. 3c, right panel) as previously described [19].
2. A previous map of RNA polymerase II [22] was used as a reference to reconstruct the current complexes in the RELION software package. We implemented 25 refinement iterations using an angular sampling interval of 7.5°. Other parameters input into RELION include a pixel size of 4.4 Å and a regularization parameter of $T = 4$. The angular distribution plot shows a lack of strongly preferred orientations for ~2000 particles (Fig. 3d). A representative 3D reconstruction of the BRCA1-RNAP II complex is shown (Fig. 4a, b).

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Fig. 1.

The preparation of SiN microchips for single-particle EM. **(a)** The microchips are supplied in a protective Gel-Pak. Carbon fiber-tipped forceps are ideal for handling microchips. **(b)** In a shallow dish or beaker, solvents remove the protective coating and clean the microchip surface. **(c)** Compressed air (red straw) helps to dry the surface and keep it residue-free after cleaning. **(d)** Heating to 150 °C for 60 min prior to use removes the remaining moisture from the SiN membrane and enhances its hydrophobicity

Fig. 2.

Decorating tunable microchips with lipid monolayers. **(a)** The lipid mixture is applied over a water drop. Note the flattening of the drop (top left) after addition of lipid. **(b)** With the microchip inverted and the SiN membrane facing the drop, the microchip is carefully lowered onto the monolayer enabling transfer of the lipid layer to the microchip surface. **(c)** Returning the microchip to the Gel-Pak stabilizes it for adding solutions during specimen preparation. **(d)** Whatman 1 filter paper is used to gently wick away excess solutions

Fig. 3. EM imaging results for BRCA1-RNAP II assemblies. **(a)** The top view of a SiN microchip shows the centrally located imaging window. **(b)** Schematic to show the SiN membrane (light gray) coated with a lipid mono-layer containing Ni-NTA moieties (Ni, light blue). His-tagged Protein A adaptors (red) bind to the Ni-NTA head groups and provide a docking site for IgG antibodies (dark blue) to recruit protein complexes. **(c)** A representative micrograph (left) of the BRCA1-RNAP II complexes captured on the tunable microchip. Class averages (right) were calculated using the SPIDER software package. The scale bar is 25 nm. **(d)** An angular distribution plot generated by RELION indicates particle orientations were not limited in the 3D reconstruction

Fig. 4. 3D reconstruction of BRCA1-RNAP II assemblies isolated from breast cancer cells. **(a)** The EM density map (white) is shown in different orientations with the RNAP II core and DNA (green and blue, respectively; pdbcode 5IYA, [22]) fit in the map. The BRCA1-BARD1 N-terminal RING domain (pink; pdbcode 1JM7, [23]) and C-terminal BRCT domain (gray; pdbcode 1JNX, [24]) are also shown within the density map based on a previously determined structure [19]. The scale bar is 5 nm. **(b)** Sections (1–4) through the EM density map indicate the overall fit of the atomic models with the map. The scale bar is 2.5 nm