Published in final edited form as:

Methods Mol Biol. 2017 ; 1647: 185–196. doi:10.1007/978-1-4939-7201-2_12.

Preparation of Disease-related Protein Assemblies for Single Particle Electron Microscopy

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Abstract

Electron microscopy (EM) is a rapidly growing area of structural biology that permits us to decode biological assemblies at the nanoscale. To examine biological materials for single particle EM analysis, purified assemblies must be obtained using biochemical separation techniques. Here we describe effective methodologies for isolating histidine (his)-tagged protein assemblies from the nucleus of disease-relevant cell lines. We further demonstrate how isolated assemblies are visualized using single particle EM techniques and provide representative results for each step in the process.

Keywords

recombinant proteins; histidine tag; Fibrocystin; electron microscopy; single particle analysis

1 Introduction

Electron microscopy (EM) allows us to examine and characterize biological entities ranging from relatively large, uniform virus structures to smaller, non-symmetrical proteins [1,2]. Determining the intricate details of disease-related proteins using high-resolution EM imaging can reveal new targets for rational drug design. The presented protocols here are adapted from our recent EM structural studies on native BRCA1 protein assemblies formed in human cancer cells [3,4]. A new aspect of the presented work, is the application of these methods for disease-related protein assemblies beyond human cancer. Here we demonstrate the utility of our established protein separation techniques for instances in which the target

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protein is expressed in low abundance. Specifically, we provide detailed methods to produce and characterize protein assemblies incorporating the C-terminal domain (CTD) of the Fibrocystin/polyductin protein (FPC) [5,6].

There are many known mutations in the FPC protein that are implicated in autosomal recessive polycystic kidney disease. At the cellular level, the longest mRNA product of the FPC gene encodes a membrane-bound protein that undergoes Notch-like proteolytic cleavage to generate a functional carboxy-terminal domain (CTD-FPC). The CTD-FPC translocates to the cell's nucleus to carry out a variety of gene-related functions. As the FPC protein is naturally expressed in low abundance in the kidney epithelia, this presents a technical barrier to understand the molecular basis of disease-related mechanisms [7].

To address this issue and facilitate structural analysis on FPC assemblies, we employed a protein enrichment strategy by over-expressing a his-tagged (6x-His) version of the CTD-FPC in mouse kidney cells (mIMCD-3 line; ATCC; [8]). Nuclear assemblies that incorporated the His-tagged recombinant protein were isolated and imaged using previously established single particle EM techniques [3]. The overall steps of the process that we describe include: 1) a nuclear extraction step, 2) a nickel chromatography isolation procedure, 3) EM specimen preparation and data collection, and 4) processing the image data. Overall, this work demonstrates a means to assess disease-related protein assemblies for structural analysis while providing new avenues to explore protein interfaces for drug discovery purposes.

2 Materials

All reagent solutions described in our procedures work optimally when prepared with ultrapure water. In general, working reagents should be prepared and stored on ice or at 4°C. When working to obtain structural information of protein complexes it is important to avoid excessive procedures that can create “bubbles” in solution or at the air-water interface, as these disturbances can affect protein integrity.

2.1 Cytoplasmic and Nuclear Extraction

Cellular nuclear material is obtained by using a commercially available kit, NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). For this procedure, we use a recommended packed cell volume of 100 μ L. For different cell volumes, please consult manufactures recommendations. Reagents are prepared according to the following steps, and we recommend following the procedures described by the manufacturer to obtain the nuclear fraction.

1. Protease inhibitor: 1 tablet of cOmplete mini protease inhibitor 100x (Roche Diagnostics). Using forceps, place 1 tablet of cOmplete mini protease inhibitor 100x on a piece of weigh paper, then crush the tablet. Carefully transfer the crushed tablet to the 600 μ L polypropylene tube. Add 100 μ L of water to the crushed tablet. Pipette up and down gently to dissolve into solution.
2. Cytoplasmic extraction reagent one (CER 1) solution: Add 1000 μ L of CER 1 reagent to a labeled, pre-chilled 1.5 mL polypropylene tube, followed by 10 μ L

of the protease inhibitor solution and 10 μ L of Halt phosphatase inhibitor 100x (Thermo Scientific). Pipette up and down gently to mix the reagents.

3. Cytoplasmic extraction reagent two (CER 2) solution: Add 55 μ L of CER 2 reagent to a labeled, pre-chilled 600 μ L polypropylene tube.
4. Nuclear extraction reagent (NER) solution: Add 500 μ L of NER solution into a pre-chilled 1.5 mL polypropylene tube, followed by 5 μ L of protease inhibitor solution (*see* Note 1).

2.2 Nickel Chromatography Purification

Nickel chromatography utilizes the affinity of a recombinant protein with a polyhistidine (His) tag to associate with nickel cations. The protein of interest is then eluted off using buffers containing imidazole (*see* Note 2). To perform these steps, prepare the following buffers in 50 mL conical tubes using ultrapure water and chilled to 4°C:

1. HEPES buffer: Add 10 mL ultrapure water to a 50 mL graduated cylinder. Weigh 0.2383 g HEPES and transfer to the 50 mL cylinder. Add ultrapure water to a volume of 50 mL. Transfer to a beaker, mix and adjust the pH to 7.4 with 5N NaOH. Store on ice in a 50 mL conical tube (*see* Note 3).
2. HEPES Buffer with salts: 0.2383 g HEPES (20 mM), 0.4091g NaCl₂ (140 mM), 100 μ L of 1M MgCl₂ (2mM), 11.098mg CaCl₂ (2 mM), fill to 50 mL with ultrapure water.
3. Imidazole: 0.34g imidazole (1 M), fill to 5mL with ultrapure water.
4. Wash Buffer: 10 mL HEPES Buffer with salt, 50 μ L 1M imidazole (5 mM imidazole).
5. Elution Buffer: 4 mL HEPES Buffer with salt, 1 mL of 1M imidazole (200mM imidazole).
6. Ni-NTA agrose slurry (QIAGEN): Pipette 400 μ L of the Ni-NTA agarose storage buffer solution into a 1.5 mL polypropylene centrifuge tube (*see* Note 4).

2.3 Preparing TEM Grids

2.3.1. Coating TEM grids—The follow method describes how to prepare EM grids for sample application using conventional methods. Alternatively, carbon-coated grids may be purchased from commercial suppliers (e.g., Ted Pella, Inc). All filter paper used in the preparation of EM specimens is Whatman Filter Paper 1 (GE Healthcare).

1. Fill a clean PYREX 190 x 100 with ultrapure water to the brim. Water should be free of any dust or particles.

¹Halt Phosphatase inhibitor is not used when doing a nickel column purification, as it may interfere with the nickel resin's binding capacity.

²Imidazole is a salt, which competes with the polyhistidine (His) tag for the Nickel-NTA resin. All buffers and samples must be kept at 4°C or on ice to maintain protein integrity.

³This buffer is used to dilute the sample. It should be fully chilled to 4°C before using it.

⁴The slurry is a 50% resin and buffer mixture. Pipetting a volume of 400 μ L results in a 200 μ L bed volume of Ni-NTA resin. Before we begin the purification the Ni-NTA slurry is separated from the storage buffer and equilibrated with the wash buffer.

2. Using a pasture pipette, place 1 drop of collodion 2% in amyl acetate (Electron Microscopy Sciences) into water.
3. Using forceps, place copper grids (glider grids 200 mesh, Ted Pella, Inc.) dark side down on the liquid (*see* Note 5)
4. Cut a piece of linen paper matching the size of the layout of the grids.
5. Place paper onto of grids. Allow the liquid to fully soak.
6. In a petri dish place a piece of filter paper.
7. Use forceps to cut the polymer film around paper.
8. Smoothly and quickly lift paper with grids off of the water. Use forceps to grab the corners furthest from self then sweeping the paper up so the grids face away from oneself. Place the grids facing up on the petri dish. Leave petri dish lid ajar to allow for drying.
9. Dry grids for 1–2 days for optimal results.
10. Following the polymer coating of the copper grids, a fine layer of carbon is applied. Place the sheet of paper holding the grids under the dome of a DV502-A vacuum evaporator (Denton Vacuum, Inc.).
11. Pump the vacuum down to a pressure of 5×10^{-6} torr.
12. Once a stable vacuum is established, pass 40 amps is through a conical graphite tip for about 30 seconds. This method creates a thin and even layer of atomized carbon on the grids.

2.3.2. Glow Discharge Procedure for Continuous Carbon Grids

1. Place continuous carbon coated copper grids on glass slide wrapped in parafilm to keep the grids in place.
2. Open dome of Pelco easiGlow (Ted Pella, Inc.)
3. Insert slide with attached carbon-coated grid into Pelco easiGlow assembly.
4. Wipe rubber rim of the dome and machine to remove any dust.
5. Replace glass dome.
6. Use stylist to tap auto run (*see* Note 6)
7. After the cycle is completed, open the dome and remove glow-discharged grids

2.3.3. Preparing uranyl formate heavy metal stain

1. Boil 3 mL of ultrapure water in a 10 mL beaker on a hot plate.
2. Using tongs, transfer the beaker to a stir plate and add 22.5 mg of uranyl formate and a stir bar. Stir for 5 minutes.

⁵The rim of edge of the copper grid is darker on one side. Grids can be placed close together, but should not overlap.

⁶Auto run should be set at 15 mA, glow 1 minutes and hold 10 seconds.

3. Add 4.2 μ L of 5N NaOH. Stir another 5 minutes.
4. Draw-up in a 5 mL syringe.
5. Filter through a 0.2 μ m PVDF filter (Fisher) into a 15 mL conical tube to remove and undissolved uranyl formate, then cover tube with aluminum foil (*see* Note 7).

2.4 Microscope and Camera

1. Negatively stained CTD-FPC complexes can be examined using a FEI Spirit Bio-Twin TEM equipped with a LaB₆ filament and operating at 120 kV.
2. Images are recorded using an Eagle 2k HS CCD camera employing low-dose conditions ($\sim$ 1–5 electrons per $\text{\AA}^2$)

2.5 Software

1. SPIDER is a software package [2] which allows for 2D classification of protein complexes through a multivariate data analysis approach. Individual protein complexes (particles) are selected from micrographs. After selection, all particles are subjected to a low-pass filter. Particles are then aligned through several iterations of vector alignment. The final step before classification is masking the particles, to remove the background noise. The parameters in each step are modulated by the user, however, the routines are standard.
2. RELION is a software package [9] which performs reconstruction and refinement calculations using an empirical Bayesian methodology.

3 Methods

3.1 Cytoplasmic and Nuclear Extraction

The following information is based on the guidelines provided by Thermo Scientific for optimal use of their NE-PER Nuclear and Cytoplasmic Extraction Reagents.

1. Collect cells by scraping and transfer cell suspension to 50 mL conical tubes.
2. Centrifuge the tubes at 500 x g for 5 minutes at 4°C.
3. Wash cells by resuspending the cell pellet with 1.5 mL PBS with phosphatase inhibitor.
4. Transfer cells to 2 pre-chilled 2 mL polypropylene tube and pellet by centrifugation at 500 x g for 2 minutes.
5. Add ice-cold CER 1 to the resulting cell pellet.
6. Vortex the sample for 15 seconds followed by a 10-minute incubation on ice.
7. Add 55 μ L ice-cold CER 2 buffer to the sample solution.

⁷Uranyl 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 hours.

8. Vortex the tube for 5 seconds then incubate on ice for 1 minute.
9. Vortex the tube for 5 seconds on the highest setting. Centrifuge the tube for 5 minutes at 16,000 x g in a microcentrifuge at 4°C. (see Note 8)
10. Carefully decant the supernatant containing the cytoplasmic components to a waste beaker. (see Note 9)
11. Resuspend the insoluble pellet containing nuclear components in ice-cold NER solution.
12. Vortex for 15 seconds then place on ice. Repeat the vortexing-incubation cycle in 10 minute intervals for a total of 40 minutes (see Note 10).
13. Centrifuge at 16,000 x g for 10 minutes at 4°C.
14. Carefully transfer the supernatant containing the nuclear extract (NE) to a pre-chilled 1.5 mL polypropylene tube and place on ice. (see Note 11)

3.2 Nickel Chromatography Purification

Immobilized-metal affinity chromatography (IMAC) exploits the principles of specific protein interactions with chelated metal groups, held in place by immobilized surfaces or beads. One popular association is formed by the interaction of polyhistidine residues (His tag) with functionalized Nickel- Nitrilotriacetic acid (Ni-NTA) resin. Below we describe procedures for isolating His-tagged CTD-FPC protein assemblies from nuclear extracts prepared according to the protocol described in **section 3.1**.

1. Dilute the NE in 20 mM HEPES buffer without salts and store on ice. (see Note 12)
2. Centrifuge the mixture Ni-NTA agarose slurry for 2 minutes at 700 g.
3. Carefully remove the supernatant and discard.
4. Add 1 mL of the wash buffer containing 20mM HEPES buffer, and 5mM imidazole to the resin and invert to mix.
5. Centrifuge for 2 minutes at 700 x g.
6. Remove the supernatant and discard.
7. Resuspend the Ni-NTA resin into 1 mL of wash buffer and centrifuge at 700 x g for 2 minutes. Remove supernatant.
8. Add the diluted NE to Ni-NTA resin.
9. Gently mix the material on a clinical rotator at 4°C for 60 minutes. (see Note 13)

⁸Place the tube in a specific orientation, for example, so that the lid hinge is located outside of rotator. This helps to locate the pellet in the tube since the pellet created is not always easily distinguishable.

⁹Our protein of interest is a nuclear protein therefore we do not need keep the cytoplasmic fraction.

¹⁰The intermittent vortexing allows for a more complete release of the soluble nuclear components.

¹¹Extracts can be stored at -80°C for 30 days or used immediately.

¹²This dilution step lowers the salt concentration of the sample, bringing it closer to a physiological level.

¹³The amount of volume remaining is a small quantity. The meniscus of the buffer solution should be just above the resin.

10. Wash the Ni-NTA resin using the wash buffer to remove background proteins. (see Note 14). Collect the wash buffer that flows through the resin in a 15 mL conical tube.
11. Elute the His-tagged proteins of interest using the elution buffer. The high concentration of imidazole in this buffer will compete off the His-tagged FPC complexes. Typically, the eluted material is collected in multiple fractions. Each fraction is roughly equal to 1 bed volume, which is ~200 μ L in our experiments.
 - a. Add 1 mL of the elution buffer, carefully pipetting it along the column wall.
 - b. Collect increments of 200 μ L in pre-chilled 1.5 mL polypropylene tube. A total of 5 fractions are usually collected. The His-tagged CTD-FPC protein often elutes in fraction 2 and 3 (See Figure 1).
12. All sample collected should be stored on ice and analyzed for total protein concentration using a standard Bradford assay.

3.3 Preparation of Negatively Stained EM Specimens

1. Using parafilm, place 3– 200 μ L drops of ultrapure water and 2– 200 μ L drops of 1% uranyl formate in a row. These drops will be used for specimen washing and staining steps (see Note 15).
2. Carefully, pick-up a fresh glow-discharged grid with forceps. The tips of the forceps, should only touch the edge of the grid (see Note 16).
3. Place 3 μ L of sample on the grid and incubate at room temperature for 1 minute (Figure 2a).
4. Blot off the excess sample onto filter paper (Figure 2b).
5. Wash the face of the grid 3 times with ultrapure water. Gently touch the face of the grid on each water droplet then again blot off the ultrapure water using filter paper. Use a new drop for each wash step. Do not fully immerse the grid into the water droplet or wet the back of the grid.
6. Wash the face of the grid 1 time with 1% uranyl formate, utilizing the same method as the water wash.
7. Stain with 1% uranyl formate for 30 seconds (Figure 3a). Keeping the sample on the stain for 30 seconds is what differs this from the previous wash step.
8. Blot away excess solution using a vacuum hose. Be cautious not to touch the vacuum hose directly to the grid as it may damage the sample (Figure 3b). Store the samples in a labeled petri dish until ready to image.

¹⁴This volume is dependent upon the bed volume. The wash volume is ~15 times the bed volume.

¹⁵Negative stain refers to the fact proteins are embedded by the uranyl salt solution. This step produces amplitude contrast that indicates the presence of white particles on a dark background.

¹⁶The grids are delicate and easy to crack with forceps. Keeping the forceps along the edge of the grid maximizes the potential imaging regions of the grid free of damage.

3.4 TEM Image Collection

1. Allow the scope to fully evacuate the column and cool.
2. Insert the sample via a single-tilt EM specimen holder at room temperature.
3. With the beam engaged the sample must be aligned along the axis of the beamline, in order for proper defocus to be attained. This is done at the center point of the sample.
4. The sample is now ready for imaging. A typical technique for this is to obtain an unbroken, well stained grid square with wide ridges formed by the coating process. These appear as dark lines throughout the square.
5. The edge of one of these ridges are then magnified and the defocus value is changed until a clear focus is obtained (*see Note 17*). Scanning along the edge of a ridge generally gives a good contrast region to visualize the biological particles.
6. Once an area of good particle occupancy has been identified digital images are acquired (Figure 4a). Save images in 16-bit.tiff format for downstream image processing procedures.

3.5 Data Analysis and Representative CTD-FPC Results

1. Prior to particle selection, the original images are normalized using the standard routines in SPIDER software package [2].
2. Individual complexes from the images are manually selected using the WEB interface of the SPIDER software package [2], and employing a box size that is approximately twice the diameter of the particles of interest.
3. Multi-reference alignment routines are implemented outputting 2D classes (Figure 4b) as previously described [3].
4. Image stacks containing selected particles are imported into the RELION software package [9].
5. A spherical structure can be used as a reference map to reconstruct CTD-FPC complexes through 25 refinement iterations using an angular sampling interval of 7.5°. Other parameters input into RELION [9] include a magnification of 68,000x, a pixel size of 4.4 Å, and a regularization parameter of $T=4$. A representative 3D reconstruction of the His-tagged CTD-FPC complex is shown in Figure 4C.

Acknowledgments

This work was supported by NIH/NCI grant R01CA193578 to D.F.K.

¹⁷For FPC a nominal magnification of ~68,000 x was used, giving a final sampling at the specimen level of 4.4 Å per pixel. FPC images were acquired using a defocus value of -1.5 µm.

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Figure 1. Western blot probed with anti-His antibodies (Abcam) shows samples obtained from the Ni-NTA purification procedure. Lanes include the nuclear extract, column flow-through, wash and eluted fractions (1–5). His-tagged CTD-FPC elutes in fractions 2 and 3.

Figure 2. Biological material was carefully added to glow-discharged continuous carbon grids. **(a)** Aliquots (3 μ L each) of sample was applied to glow discharged EM grids with the grid secured in forceps. **(b)** When washing each sample, turn the grid to the side to blot off excess solution onto Whatman #1 filter paper.

Figure 3.

(a) To negatively stain the samples with 1% uranyl formate, touch the face of the grid onto uranyl formate droplet without submersing the grid into the stain. (b) Dry the stained specimens with a vacuum hose from the backside of the grid. The vacuum hose should not come in contact with the grid as it may damage the sample.

Figure 4.

(a) A representative micrograph of purified CTD-FPC assemblies taken at 68,000x magnification. Scale bar is 20 nm. (b) Class averages obtained from multi-reference alignment procedures implement in the SPIDER software package [2]. (c) 3D reconstruction of the CTD-FPC complex calculated using RELION software package [9] with each view rotated 90°.