Additional File 1

Detailed protocols for the preparation of agarose–acrylamide composite gels and the electroelution procedure.

[Introduction]

The agarose–acrylamide composite gel is well suited for separating ultra-large protein complexes. It offers several advantages over conventional polyacrylamide gradient gels, including rapid preparation using a lower acrylamide concentration, shorter separation time, and higher resolution for large complexes exceeding 700 kDa, such as photosystems (PSs) in photosynthetic organisms, including the PSI-light-harvesting complex (LHC)I (PSI–LHCI; ~700 kDa) and PSII–LHCII (~1,400 kDa).
The protein complexes within the agarose–acrylamide composite gel were suitable for fluorescence measurements. Complexes separated by gel electrophoresis and subsequently extracted by electroelution were used for negative staining and cryoelectron microscopy (cryo-EM) analyses.

[Chemical components]

[Agarose–acrylamide composite gel]

Agarose concentration	0.5%	1%
Agarose	0.09 g	0.18 g
30% acrylamide solution*1	2.25 mL	
3 × gel buffer*2	6 mL	
Milli-Q water	10 mL	
10% APS*3	60 µL	
TEMED	15 µL	
Total	18 mL	

[Sample gel]

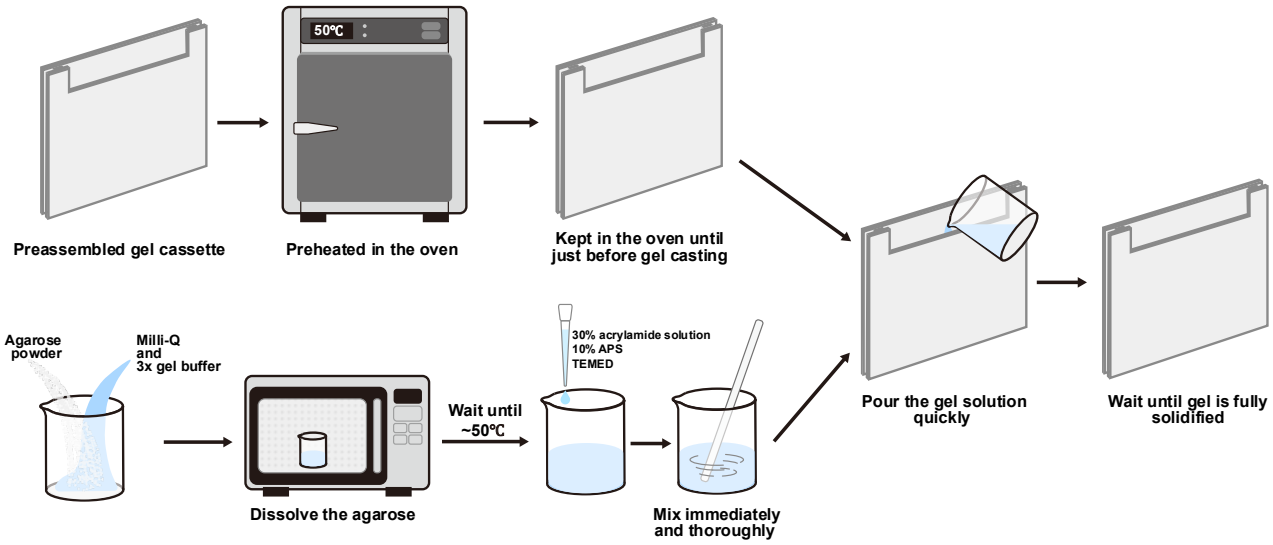
Acrylamide concentration	3.6%
30% acrylamide solution	0.72 mL
3 × gel buffer	2 mL
Milli-Q water	3.28 mL
10% APS	50 µL
TEMED	5 µL
Total	6 mL

*1 30% (w/v) acrylamide/bisacrylamide solution (37.5:1)

*[2] 75 mM imidazole-HCl (pH 7.0; 4°C), 1.5 M 6-amino caproic acid
*[3] Ammonium peroxodisulfate

Agarose–acrylamide composite gel preparation

[Gel preparation workflow]



[Gel cassette assembly]

The gel casting cassette consists of two glass plates, a sealing gasket, and clips.

1. Clean the glass plates with 70% ethanol. Assemble the plates using a sealing gasket and secure them with clips.
2. Prewarm the assembled cassette in an oven at around 45–50°C.

[Casting and solidifying the agarose–acrylamide composite gel]

1. Mix 3 × gel buffer, Milli-Q water, and agarose powder (Seakem Gold Agarose, Lonza)[*3] according to the separation gel composition table in a container (e.g., 50-mL beaker or centrifuge tube).
2. Heat the mixture in a microwave until the agarose powder is completely dissolved and the solution becomes transparent. *Note:* Heat at short intervals to avoid sudden boiling or overheating.
3. Allow the agarose solution to cool to approximately 50°C, mixing occasionally to maintain uniform temperature, prevent solidification, and avoid over-viscosity.
4. Remove the prewarmed cassette from the oven.
5. Quickly add 30% acrylamide solution, 10% APS, and TEMED in the amounts listed in the sample gel composition table to the agarose solution. If using a beaker, stir with a glass rod. *Note:* As acrylamide is a neurotoxin and suspected carcinogen, make sure always wear gloves and a mask during handling.
6. Immediately pour the agarose–acrylamide composite solution between the glass plates, leaving space for the sample gel.

7. Allow the gel to solidify at room temperature for at least 20 min.
8. Fill the remaining space above the gel with Milli-Q water and store at 4°C for at least overnight.
9. Proceed to the next step or store the gel at 4°C sealed and hydrated during storage. The gel remains usable for up to one month under these conditions.

[*3] We used SeaKem® Gold Agarose from Lonza. Switching to a different agarose product may alter electrophoretic mobility considerably.

[Sample gel casting and polymerization]

1. After rinsing the separation gel with Milli-Q water, discard the rinse.
2. Prepare the sample gel mixture by combining 30% acrylamide, 3 × gel buffer, Milli-Q water, 10% APS, and TEMED according to the sample gel composition table.
3. Pour the mixture onto the separation gel.
4. Insert the comb and allow polymerization at room temperature for at least 30 min. In cooler environments, extend polymerization time to ensure completion.

[Preparation for vertical electrophoresis]

1. After polymerization, carefully remove the clips and sealing gaskets from the cassette.
2. For gels with high agarose concentrations, apply tape to the glass plate corners to prevent the gel from slipping out.
3. To prevent damage to the wells, submerge the gel cassette in distilled water or running buffer and carefully remove the comb.
4. Place the gel cassette into the electrophoresis apparatus and pre-cool to 4°C by keeping it in a cold room or refrigerator.
5. Pour 250 mL of pre-cooled (4°C) 1 × anode buffer[*4] into the bottom of the lower chamber.
6. Gently remove any air bubbles trapped beneath the gel.
7. Prepare 100 mL pre-cooled (to 4°C) solution of 1× cathode buffer[*5], supplemented with 0.05% DOC and 0.02% α-DDM.
8. Pour the cathode buffer into the upper chamber, and ensure the wells are completely filled.

[*4] Anode buffer: 25 mM imidazole-HCl (pH 7.0 at 4°C)
[*5] Cathode buffer: 50 mM Tricine, 7.5 mM imidazole (pH 7.0 at 4°C)

[Clear-native polyacrylamide gel electrophoresis (CN-PAGE)]

1. Rinse each well with the running buffer by pipetting up and down several times.
2. Load the prepared samples into the wells.
3. Run electrophoresis at a constant current of 5 mA (maximum 300 V) for 120 min at 4°C.

After electrophoresis, excise the band of interest from the gel. The band can be used immediately for further experiments or stored in 50% (w/v) glycerol at –80°C for future use.

[Troubleshooting: Weak or distorted bands]

1. **Ensure complete dissolution of agarose:** Partial dissolution or premature solidification of agarose during cooling creates uneven gel matrices, resulting in distorted or smeared bands. Heat and stir until the solution is completely clear.
2. **Pour at the correct temperature:** Allow the agarose solution to cool before adding acrylamide. Pouring above 60°C may cause uneven polymerization or reagent degradation.
3. **Ensure complete sample solubilization:** Fully solubilize the protein sample and remove insoluble material by centrifugation before loading to obtain sharp bands.

Electroelution

The electroelution experiment was adapted from the Bio-Rad Model 422 electro-eluter manual[*6] with modifications to the buffer system.

[*6] (https://www.bio-rad.com/en-jp/product/model-422-electro-eluter?ID=c92365e7-8ca6-4772-bc9b-92940fc31016)

Modified contents:

- **Section 4: Model 422 electro-eluter assembly**

1. Pre-activate the membrane caps in 1× cathode buffer*[5] at 60°C for at least 1 h. Wear gloves when handling the membrane caps to prevent contamination. When reusing, store them in 1× cathode buffer containing 0.05% sodium azide (NaN_3) at 4°C. Reheating or resoaking is unnecessary for reuse. *Note*: Sodium azide is highly toxic. Always use eye protection, double gloves, and handle in a fume hood. Use dilute solutions (≤5%) when possible.
2. Place the required number of glass tubes on a clean paper towel and insert the frit. Push the frit firmly to the bottom edge. Wet the grommet with buffer before inserting the glass tube into the electro-eluter to ease assembly. Fill empty grommet holes with stoppers.
3. Set a pre-wetted membrane cap at the bottom of each silicone adaptor while wearing gloves. Rinse the adaptor space three times with 1× cathode buffer containing 0.05% α-DDM and fill the space with the same buffer. Pipette buffer to remove bubbles around the membrane.
4. Slide the silicone adaptor with the membrane cap onto the frosted end of the glass tube with the frit. Remove air bubbles by partially pulling the adaptor on and off several times. *Note*: Air bubbles in the frit reduce current and yield.
5. Fill the glass tube with gel slices and 1× cathode buffer. Gel bands may be minced to increase recovery, keeping the gel height ≤1 cm. Taller height requires longer elution times.
6. Place the module in the chamber. Fill the lower buffer chamber with ~500 mL of 1× anode buffer*[4] and ensure the buffer level is above the top of the silicone adaptors. Remove bubbles at the membrane base. Fill the upper chamber with ~100 mL of 1× cathode buffer containing 0.05% DOC and 0.02% α-DDM. Dry banana plugs before connecting to prevent short circuits.
7. Place a stir bar in the lower buffer tank and stir vigorously to prevent bubbles from adhering to the membrane during the run.
8. Attach the lid, with red cables to the anode and black to the cathode.
9. Run electroelution at 5 mA, 110 V, and 4°C for 2 glass tubes.
   Note: Elution time does not exceed 3 h when gel height is ≤1 cm. Consider increasing the number of glass tubes during electroelution or decreasing the band slices within the glass tube.

[*5] Cathode buffer: 50 mM Tricine, 7.5 mM imidazole (pH 7.0 at 4°C)

*4 Anode buffer: 25 mM imidazole-HCl (pH 7.0 at 4°C)

[Protein sample concentration after electroelution]

After electroelution, the extracted proteins were directly concentrated and washed without further purification.

1. After electroelution, carefully and quickly remove the silicone adaptor along with the membrane cap, and collect the protein samples from the membrane cap.
2. Transfer the collected protein sample into an Apollo*7 concentrator tube using a pipette.
3. To wash the protein sample, add 3 mL of 25 mM Bis-Tris buffer (pH 7.5) containing 0.05% α-DDM, and centrifuge at 2,000 ×*g* for 20 min at 4°C.
4. Discard the filtrate and repeat the washing step three times.
5. Transfer the concentrated sample into a sterile Eppendorf tube using a pipette.
6. If not used immediately for electron microscopy, store the sample at –80°C.

*7 Apollo 7 mL centrifugal concentrators (150 kDa MWCO; Orbital Biosciences, Topsfield, MA, USA)

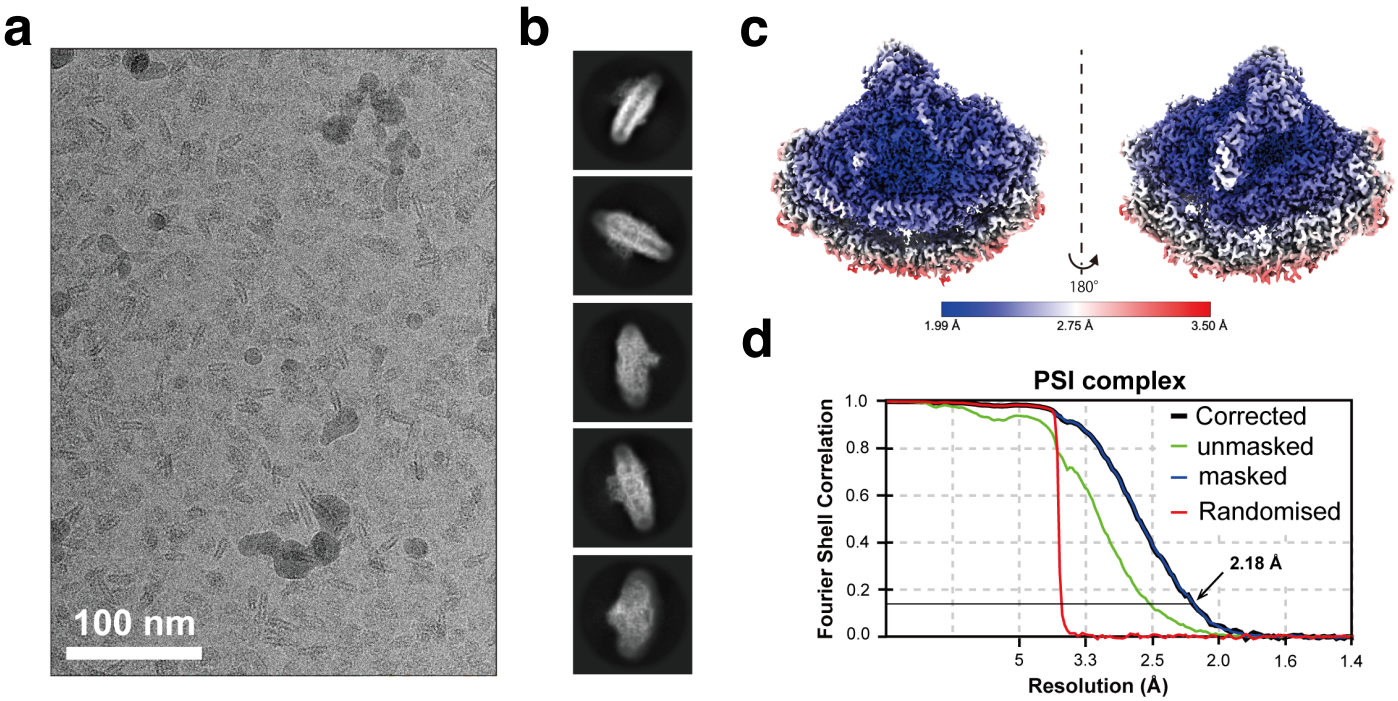
[Troubleshooting: Abnormal voltage/current or low efficiency]

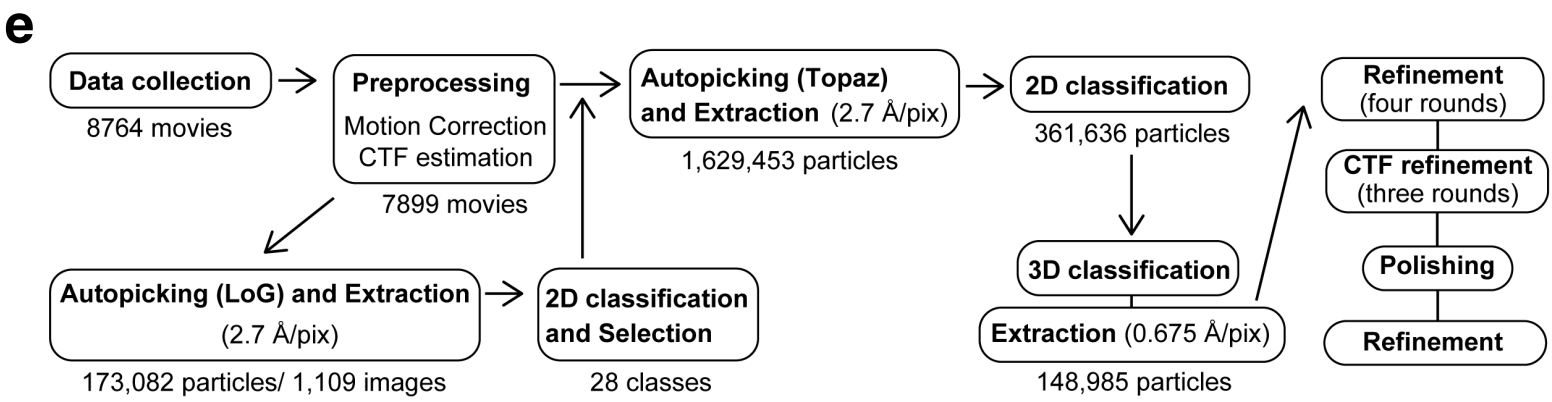
1. **Ensure complete removal of air bubbles:** Air trapped in the frit or beneath the membrane cap can impede current flow, reducing elution efficiency. Inspect the apparatus carefully to remove bubbles before starting.
2. **Adjust the number of glass tubes:** If stacked bands in a glass tube exceed 1 cm in height, upper proteins may not elute completely. Increase the number of glass tubes or reduce the amount of gel in each tube to improve recovery.

Additional file 2
Cryo-EM data collection and refinement statistics.

	***Arabidopsis thaliana* PSI WT**
EMDB ID	**EMD-66073**
PDB ID	**9WLS**
Data collection and processing	
Magnification	105,000
Voltage (kV)	300
Electron exposure ($e^-/Å^2$)	40
Defocus range (mm)	-0.5 to -1.0
Pixel size (Å)	0.675
Symmetry imposed	C1
Initial particle images (no.)	1,629,453
Final particle images (no.)	148,985
Map resolution (Å)	2.18
FSC threshold	0.143
Map resolution range (Å)	1.99-2.59
Map sharpening *B* factor ($Å^2$)	-40.0832
Refinement	
Initial model used	8j7b
Model resolution (Å)	2.1
FSC threshold	0.5
Model composition	
Non-hydrogen atoms	36141
Protein residues	3267
Ligands	206
***B* factors ($Å^2$)**	
Protein	14.26
Ligand	16.22
R.m.s deviations	
Bond lengths (Å)	0.010
Bond angles (Å)	1.049
Validation	
MolProbity score	1.28
Clashscore	5.18
Poor rotamers (%)	0.94
Ramachandran plot	
Favored (%)	98.20
Allowed (%)	1.80
Disallowed (%)	0

Additional file 3
Summary of cryo-EM data acquisition and image processing.

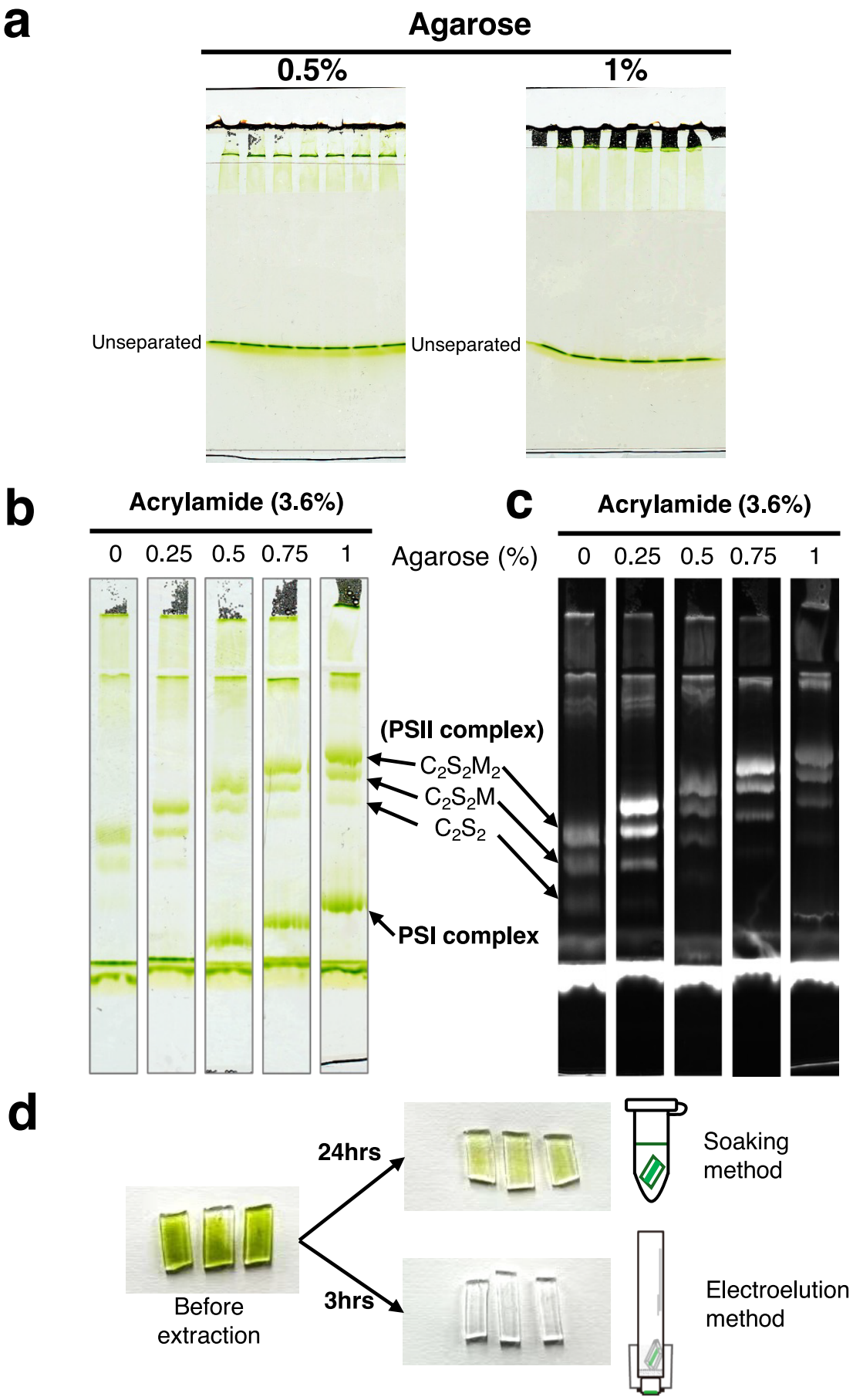




(a) Cryo-EM image of purified photosystem (PS)I complex of *Arabidopsis thaliana* isolated using DOC-based clear-native polyacrylamide gel electrophoresis (CN-PAGE) method. **(b)** 2D class images of the PSI complex. **(c, d)** Local resolution and fourier shell correlation curve of the final cryo-EM map of the PSI complex. **(e)** Flow chart of cryo-EM image processing.

Additional file 4

CN-PAGE separation of Arabidopsis thaliana thylakoid membrane complexes and comparison of protein yields obtained by soaking and electroelution.



(a) *Arabidopsis thaliana* thylakoid membrane complexes were separated by clear-native polyacrylamide gel electrophoresis (CN-PAGE) on 0.5% and 1% agarose gels. **(b)** CN-PAGE and **(c)** chlorophyll fluorescence images obtained at room temperature. Notably, the chlorophyll yield from photosystem (PS)I was much lower than that from PSII and was not clearly visible. **(d)** Chlorophyll pigments remaining after extraction using the conventional soaking method or electroelution.