Supplementary Protocols

A. Expression of cNWs proteins in *E. coli*

1) Transform 20 µl *E. coli* BL21-Gold (DE3) cells (or derivatives) with 20 ng of pET28a-NWs plasmid DNA.
2) Plate the cells on LB agar plate containing 50 µg/mL Kanamycin and grow colonies at 37°C overnight.
3) Inoculate a colony into a sterile flask containing 70 mL LB supplemented with 50 µg/mL Kanamycin.
4) Grow seed culture overnight at 37°C, shaking at 200 rpm.
5) Inoculate 6L LB medium supplemented with 50 µg/mL Kanamycin and shake at 200 rpm at 37°C until OD_{600} reaches 0.6.
6) Induce at OD_{600} of 0.6 with 1 mM IPTG (1 M stock in H_2O, filtered).
7) Grow cells for another 3 hours at 37°C (for NW9 and NW11) or another 16 hours at 18°C (for NW30 and NW50).
8) Harvest cells by centrifugation (7000x*g*, 15 minutes, 4°C).
9) Remove supernatant and store cell pellet at -80°C.

B. Purification of NW9 and NW11

1) Thaw cells at room temperature (RT) for 40 minutes then resuspend in 5 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 % Triton X-100) per gram of cell. Make sure you add one protease inhibitor cocktail tablet per 50 ml of cell suspension. Sometimes, we supplement lysis buffer with 1 mM EDTA In addition to the protease inhibitor tablet (optional).
2) Add ~1 mg lysozyme per mL of cell suspension
3) Stir in the cold room for 30-40 minutes.
4) Sonicate cells on ice (20 min net sonication, 30% duty cycle, 1 second on - 3 seconds off)
5) Add 5 mM $MgCl_2$ (if you supplemented the lysis buffer with EDTA) and 100U Benzonase Nuclease then stir on ice or at 4°C for 45 minutes.
6) Spin down cell debris and insoluble materials at 35000 g and 4°C for 50 min.

7) Apply (3 times) supernatant to Ni-NTA column equilibrated with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl (= buffer A) +1 % Triton X-100.
8) Wash with 10 column volumes (CV) buffer A+ 1% TritonX-100.
9) Wash with 10 CV buffer A + 50 mM Cholate.
10) Wash with 10 CV buffer A
11) Wash with 10 CV buffer A + 20 mM imidazole.
12) Elute protein with 3-5 CV buffer A+ 500 mM imidazole
13) Check protein purity with SDS-PAGE then dialyze against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl using a 12-14 kD MWCO membrane.
14) Add 2 mM DTT and TEV protease (His6-tagged; produced in-house), incubate at 4°C for ~ 8-16 hours. Add TEV at a protease to NW protein ratio of 1: 50 (w/w).
15) Run SDS-PAGE to make sure the N- terminal His tag cleavage is complete.
16) After TEV cleavage is complete, concentrate up to 500-600 μM, flash freeze and store at -80°C. Alternatively, move on directly to the circularization reaction (preferred).

Optional

As a lot of NW9 and NW11 proteins can be found in the insoluble fraction after cell lysis, we perform purification under denaturing conditions as follows;

1) Dissolve the pellet in 6 M guanidine hydrochloride (Gn-HCl), 50 mM Tris-HCl, pH 8.0, 500 mM NaCl.
2) Centrifuge (35000 g, 50 min, 4°C) to remove insoluble debris.
3) Apply (3 times) onto NiNTA equilibrated with 6 M Gn-HCl buffer
4) Wash with 10 CV 6 M Gn-HCl buffer
5) Wash with 10 CV buffer A to refold the NW9 and NW11 on the NiNTA column.
6) Wash with buffer A + Triton X-100, buffer A + cholate, buffer A, and buffer A + 20 mM Imidazole as described above in the standard protocol.
7) Elute with buffer A + 500 mM Imidazole.
8) Perform dialyses and TEV cleavage as mentioned above.

C. Purification of NW30 and NW50

NW30 and NW50 were purified under denaturing conditions and refolded as follows:

1) Dissolve the cell pellet in 6 M Gn-HCl, 50 mM Tris-HCl, pH 8.0, 500 mM NaCl.
2) Centrifuge (35000 g, 50 min, 4°C) to remove insoluble debris.
3) Apply (3 times) onto NiNTA equilibrated with 6 M Gn-HCl buffer
4) Wash with 10 CV 6 M Gn-HCl buffer.
5) Wash with 10 CV buffer A to refold the NW30 and NW50 on the NiNTA column.
6) Wash with buffer A + Triton X-100, buffer A + cholate, buffer A, and buffer A + 20 mM Imidazole as described above under section **B** (steps 8-11).
7) Elute with buffer A + 500 mM Imidazole.
8) Perform dialyses and TEV cleavage as described above under section **B** (steps 13-16).
9) NW30 and NW50 (still containing a C-terminal His_6 tag) were further purified by size exclusion chromatography (SEC; Superdex 200 16/60 [GE Healthcare] equilibrated in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM sodium cholate, 0.5 mM EDTA).
10) SEC fractions containing NW30 and NW50 were further purified over Ni^{2+}-NTA resin to remove truncation products (which lack a C-terminal His_6 tag). (Follow steps 7-13 from section **B**)
11) Purified proteins were concentrated, flash frozen and stored at -80°C. Alternatively, move on directly to the circularization reaction (preferred).

General Comments about NWs productions.

- In order to minimize the truncation products (for NW30 and NW50), expression is optionally being done at lower temperatures (20°C or below) and an additional His purification step is added after SEC.
- Make sure the TEV reaction is complete before moving on to the next step.
- We do not remove TEV after N terminal His tag cleavage. Instead, we remove it together with the sortase enzyme at a later step.

D. MSP circularization

1) Dilute the purified proteins into sortase reaction buffer (300 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$) so that the final NWs concentration is less than 15 µM.

2) Add freshly made evolved sortase (Addgene plasmid # 75144) so that the final sortase: protein molar ratio is 1: 2. Continue incubation at 37°C while stirring for 3-4 hours (preferred) or at 4°C overanight.
3) Monitor the reaction by SDS-PAGE. Take aliquot every 1-hour and mix it with the SDS-PAGE sample buffer.
4) Add the covalent sortase inhibitor, AAEK2, to a final concentration of 500 µM once sortase reaction is complete (usually after 3-4 hours at 37°C).
5) Incubate the reaction for another 30 min at room temperature while gently shaking.
6) Apply (3 times) the reaction mixture onto NiNTA equilibrated with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl buffer. **Please note** that we perform this Ni purification step to remove TEV, sortase, cleaved C-terminal His tag and the un-circularized NWs. We recommend that you use excess Ni resin in this step.
7) Wash with 3 CV of buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole.
8) Combine flow-through and wash fractions (containing the circularized products) then add 50-100 mM EDTA (optional).
9) Concentrate the circularized products using centricon concentrators. Use 10 kDa MW cutoff concentrators for NW9 and NW11, and use 30 and 50 kDa MW cutoff for NW30 and NW50 respectively.
10) Inject cNWs into a Superdex 75 16/60 column equilibrated in buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl plus 50 mM sodium cholate or 1 mM dodecyl-β-D-maltoside (DDM).
11) Collect fractions containing cNWs and check the purity using SDS-PAGE.
12) Exchange the buffer into buffer A_{ix} (20 mM Tris, pH 8.2, 1 mM DDM) using centricon concentrators or dialysis.
13) Concentrate the protein solution then apply it to a Resource Q column equilibrated with buffer A_{ix}. A linear salt gradient from 0-60 % buffer B_{ix} (20 mM Tris, pH 8.2, 1 mM DDM, 1M NaCl) was applied. Circularized proteins usually elute around 150-200 mM NaCl.
14) Collect the fractions containing cNWs and concentrate. Use fresh preparation to make nanodiscs (preferred) or store at -80°C for future use.

E. Example of nanodiscs assembly reaction using circularized NW9 (cNW9)

V = 300 μL

- 135 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 70 mM sodium cholate buffer. Final cholate concentration: 31.5 mM.
- 45 μL POPC/POPG (3:2) (80 mM stock) → final concentration: 12 mM.
- 120 mL cNW9 (500 μM stock) → final concentration: 0.2 mM.
- Incubate for 1 hour on ice.
- Add ~ 0.3-0.5 g of wet Biobeads SM-2 and continue incubation over ice for another 30-60 min.
- Shake on orbital shaker for 4-12 h at 4°C.
- Remove Biobeads by filtration (or use the centrifuge tube filters Spin-X (Costar)) and then apply reaction mixture to Superdex 200 size exclusion column.

F. Example of nanodiscs assembly reaction using circularized NW11 (cNW11)

V = 300 μL

- 159 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM sodium cholate buffer. Final cholate concentration: 31.8 mM.
- 45 μL POPC/POPG (3:2) (80 mM stock) → final concentration: 12 mM.
- 96 μL cNW11 (500 μM stock) → final concentration: 0.16 mM.
- Incubate for 1 hour on ice.
- Add ~ 0.3-0.5 g of wet Biobeads SM-2 and continue incubation over ice for another 30-60 min.
- Shake on orbital shaker for 4-12 h at 4°C.
- Remove Biobeads by filtration and then apply reaction mixture to Superdex 200 size exclusion column.

G. Example of nanodiscs assembly reaction using circularized NW30 (cNW30)

V = 300 μL

- 175 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM sodium cholate buffer. Final cholate concentration: 35 mM.

- 25 μL POPC/POPG (3:2) (60 mM stock) → final concentration: 5 mM.
- 100 μL cNW30 (100 μM stock) → final concentration: 33.3 µM.
- Incubate for 1 hour on ice.
- Add ~ 0.4-0.5 g of wet Biobeads SM-2 and continue incubation over ice for another 30-60 min.
- Shake on orbital shaker for 3-4 h at 4°C.
- Remove Biobeads by filtration and then apply reaction mixture to Superose 6 size exclusion column.

  - **Please note** that we also performed the assembly reaction successfully in the presence of excess lipids at NW30: lipids ratio of 1:1000 (**Supplementary Figure 7**)

H. Example of nanodiscs assembly reaction using NW50

V = 300 μL

- 183 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM sodium cholate buffer. Final cholate concentration: 36.6 mM.
- 42 μL POPC/POPG (3:2) (80 mM stock) → final concentration: 11.25 mM.
- 75 μL cNW50 (10 μM stock) → final concentration: 2.5 µM.
- Incubate for 1 hour on ice.
- Add ~ 0.4-0.5 g of wet Biobeads SM-2 and continue incubation on ice for another 30-60 min.
- Shake on orbital shaker for 3-4 h at 4°C.
- Remove Biobeads by filtration then apply reaction mixture to Superose 6 size exclusion column.