Native purification of GFP-tagged proteins and protein complexes

Citations:

Pleiner, T. et al. (2020) Structural basis for membrane insertion by the human ER membrane protein complex. *Science*

Plasmids:

Addgene ID	Plasmid name	ORF
149336	pTP396	His14-Avi-SUMO^{Eu1}-anti GFP nanobody
149334	pTP264	His14-*bd*NEDD8-Biotin ligase BirA
149333	pAV286	His14-Tev-SENP^{EuB} protease

Protocol:

The following protocol outlines a single-step strategy for the native purification of any GFP-tagged protein or protein complex and includes protocols for producing the necessary proteins from plasmids that are available from Addgene. This protocol yields GFP-tagged proteins of very high purity and in excellent yield. Such purified complexes can be used for downstream applications like mass spectrometry and structure determination e.g. via cryo-EM.

The strategy relies on an anti-GFP nanobody (Nb) - expressed from pTP396 - equipped with a multifunctional tag as shown below:

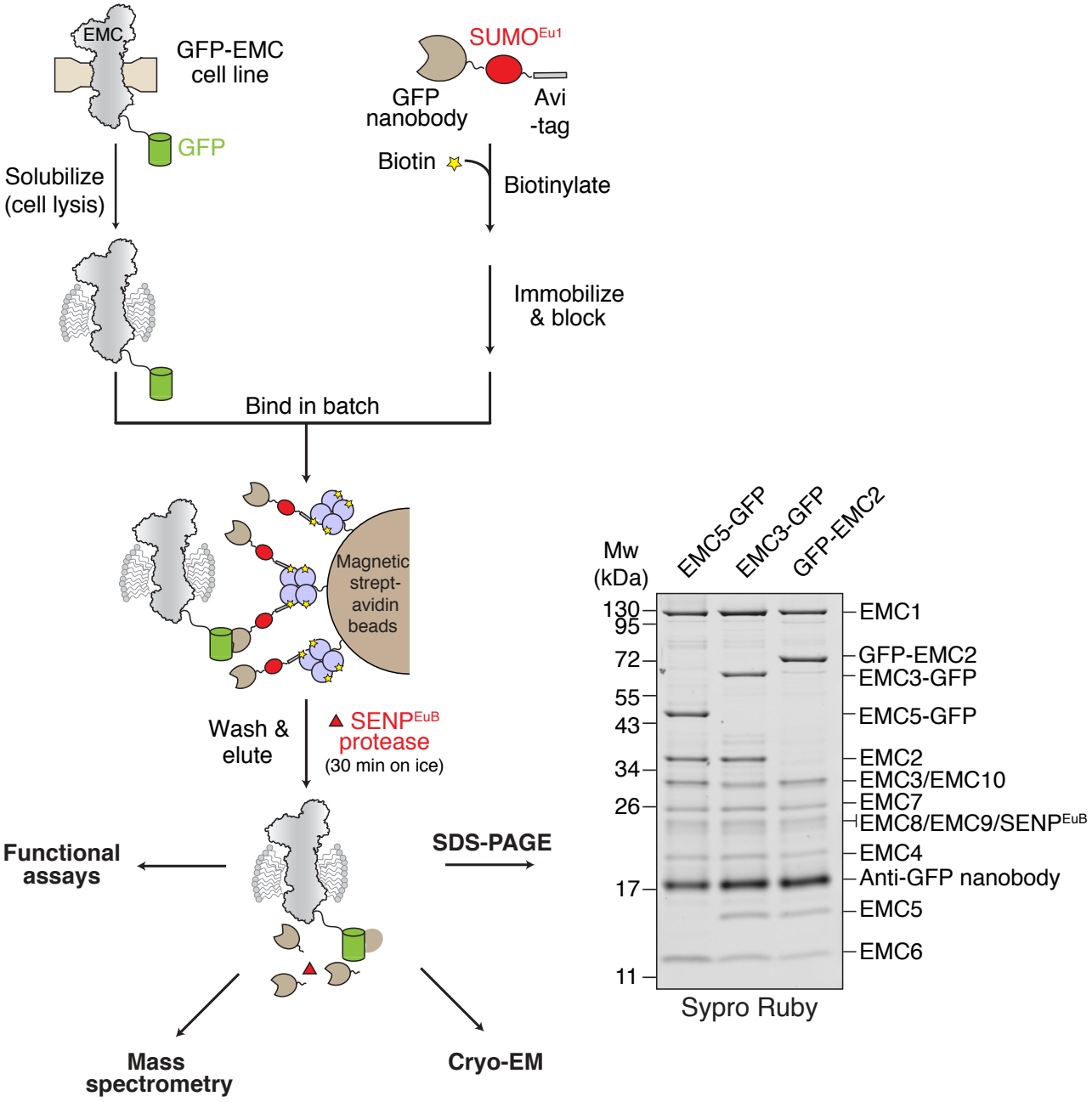
The His14-tag enables Ni^{2+}-chelate affinity chromatography purification of the fusion protein after bacterial expression (described below). The Avi-tag is a 15 amino acid peptide with a single lysine that can be specifically biotinylated using purified *E. coli* biotin ligase BirA (Beckett et al., 1999; Fairhead and Howarth, 2015). After biotinylation the nanobody can be immobilized onto magnetic Streptavidin beads. The engineered SUMO^{Eu1} module serves as a protease recognition site that is cleaved by the corresponding SENP^{EuB} protease and thus allows gentle release of the nanobody along with its bound target complex on ice and in physiological buffer, while avoiding elution of unspecific background binders. SUMO^{Eu1}, in contrast to the more widely used yeast *sc*SUMO, is resistant to cleavage by endogenous deSUMOylases in eukaryotic cell lysates, enabling stable isolation of nanobody:target complexes from eukaryotic extracts (Vera Rodriguez et al., 2019). The engineered SENP^{EuB} protease cleaves SUMO^{Eu1} very efficiently at low concentration (˜250 nM) on ice within only 30 minutes.

The anti-GFP nanobody “GBP1/Enhancer” was previously crystallized in complex with GFP and shown to have very high affinity for GFP (K_D ˜ 0.59 nM)(Kirchhofer et al., 2010). Its binding to GFP is stable under a wide range of harsh conditions, including high salt and detergent concentrations. A resin-bound version, which cannot easily be used for native purification, has

been widely used as “GFP-Trap” (Chromotek, Germany). A detailed characterization of the interaction is provided by Chromotek here: ‘https://www.chromotek.com/products/detail/product-detail/gfp-trap’.

For the final purification, magnetic Streptavidin beads are preferred over porous beads, as they show higher capacity for larger protein complexes and also enable concentrated elution in a very small volume.

The following schematic illustrates the described workflow and shows the purity of single-step purified EMC complexes via different GFP-tagged EMC subunits:



An example protocol from Pleiner *et al.*, 2020:

This protocol was aimed at generating enough purified (membrane protein) complex for structure determination by cryo-EM. It can easily be up- or down-scaled for regular IPs e.g. for mass spectrometry. The solubilization/lysis buffer was optimized for purification of the ER membrane protein complex and can easily be replaced.

2 L of HEK293 cells stably expressing GFP-tagged EMC2 were grown in suspension and induced for 48 hours with 1 µg/ml doxycycline (DOX). Cells were harvested, washed with 1x PBS, weighed and resuspended with 6.8 ml solubilization buffer per 1 g cell pellet (50 mM HEPES/KOH pH 7.5, 200 mM NaCl, 2 mM $MgAc_2$, 1% (w/v) DDM [Anatrace, USA], 1 mM DTT, 1x complete EDTA-free protease inhibitor cocktail [#1187358000, Roche, Germany]). After 1 hour of head-over-tail incubation with solubilization buffer, the lysate was cleared by centrifugation for 40 min at 4˚C and 30,000 x g in a Sorvall RC6+ centrifuge (SS-34 rotor). In parallel, beads with immobilized nanobody were prepared. Briefly, 60 µl resuspended Pierce Streptavidin magnetic beads (#88816, Thermo Fisher Scientific, USA) per 1 g cell pellet were pre-equilibrated in wash buffer (solubilization buffer with 0.015% (w/v) DDM). Then 20 µg biotinylated His_{14}-Avi-$SUMO^{Eu1}$-tagged anti-GFP nanobody (see below for expression protocol) per 60 µl beads were immobilized for 20 min on ice with occasional mixing. After this, all remaining biotin binding sites were blocked by incubation with 100 µM biotin in 50 mM HEPES/KOH pH 7.5 for 5 min on ice with occasional mixing. The beads were then washed with wash buffer and incubated with the cleared detergent cell lysate for 1 hour, binding head-over-tail at 4˚C. The beads were washed four times with 1 ml wash buffer and finally, the anti-GFP nanobody along with all bound proteins was released under native conditions and in minimal volume (~20-30 µl for 60 µl beads) by cleavage with 250 nM $SENP^{EuB}$ in wash buffer for 30 min at 4˚C with occasional mixing.

Expression and purification of biotinylated anti-GFP nanobody

The nanobody fusion protein was expressed from pTP396 in *E. coli* NEB Express I^q cells. A 100 ml SuperBroth pre-culture was grown overnight at 28˚C and diluted to 1 L after ~18 hours. After dilution, the culture was incubated at 18˚C until an OD_{600} of ~2.0 (2-4 hours). Expression was then induced by addition of 0.2 mM IPTG for 18-20 hours at 18˚C. Cells were harvested and the pellet was resuspended in 120 ml lysis buffer (50 mM Tris/HCl pH 7.5, 300 mM NaCl, 20 mM imidazole, 1 mM DTT, 1 mM PMSF) and lysed by freeze-thaw followed by sonication (4x 1 min pulsing in thin-walled metal containers in an ice-water bath, Macro-Tip at 100% amplitude, Branson Sonifier). The lysate was cleared by centrifugation for 45 min at 17,000 rpm and 4˚C in an SS-34 rotor. Half of the lysate was frozen after addition of 250 mM sucrose for later purifications. The other half (60 ml) was incubated in batch with ~4 ml bed volume Ni^{2+}-NTA agarose (Thermo Fisher Scientific, USA) for 1 hour at 4˚C and then transferred to a gravity flow column. The resin was washed with three column volumes of lysis buffer before stepwise elution with imidazole elution buffer (50 mM Tris/HCl pH 7.5, 300 mM NaCl, 500 mM imidazole, 1 mM DTT, 250 mM sucrose). The buffer of the eluate was then exchanged to storage buffer (50 mM Tris/HCl pH 7.5, 200 mM NaCl, 1 mM DTT and 250 mM sucrose) using a PD-10 desalting column (GE Healthcare, USA). The purified protein was aliquoted, snap-frozen in liquid nitrogen, and stored at -80˚C until further use.

Purification of *E. coli* biotin ligase BirA

The expression of biotin ligase BirA from *E. coli* and its use for biotinylation of Avi-tagged proteins has been described extensively before (Beckett et al., 1999; Fairhead and Howarth, 2015) (commercial products by Avidity, USA). His_{14}-*bd*NEDD8-tagged BirA Biotin ligase was expressed from pTP264 (Addgene ID 149334) in *E. coli* NEB express I^q (New England Biolabs, USA) for 18-20 hours at 18˚C in 1 L SuperBroth using 0.2 mM IPTG for induction. After cell harvest, lysis and binding to Ni^{2+}-resin as described above, tagged BirA was eluted with imidazole elution buffer. The buffer was exchanged to storage buffer as described above. The purified protein was aliquoted, snap-frozen in liquid nitrogen, and stored at -80˚C until further use.

Biotinylation of purified nanobody

An in-solution biotinylation reaction contained 300 µl 5x biotinylation buffer (250 mM Tris/HCl, 500 mM NaCl, 62.5 mM $MgCl_2$, 50 mM ATP, 50 mM biotin), at least 50 µM purified Avi-tagged anti-GFP nanobody (from pTP396), a 1:50 molar ratio of purified His-tagged BirA, and water to add up to 1.5 ml final volume. The reaction was incubated for 3 hours at 25˚C and then applied to a PD-10 desalting column (GE Healthcare, USA) equilibrated in storage buffer to remove excess biotin. Fractions with normal 260/280 ratio (comparable to starting ratio of the prep, usually ~0.6-0.7) were pooled and quantitative biotinylation was assessed by test binding to Streptavidin beads. If fully biotinylated and added below bead capacity, all of the nanobody should be in the bound fraction and almost none should remain in the unbound fraction.

Expression of $SENP^{EuB}$ protease

His_{14}-Tev-tagged $SENP^{EuB}$ protease (Addgene ID 149333) (Vera Rodriguez et al., 2019) was expressed in *E. coli* NEB express I^q for 6 hours at 18˚C in 1 L SuperBroth using 0.2 mM IPTG for induction. After cell harvest, lysis and binding to Ni^{2+}-resin as described above, tagged protease was eluted with imidazole elution buffer. The buffer was exchanged to storage buffer as described above. The purified protein was aliquoted, snap-frozen in liquid nitrogen, and stored at -80˚C until further use.

References

Beckett, D., Kovaleva, E. & Schatz, P.J. (1999) A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci*, **8**, 921-929.

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