

Fluorescence-detection size-exclusion chromatography utilizing nanobody technology for expression screening of membrane proteins

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Abstract

Membrane proteins play numerous physiological roles and are thus of tremendous interest in pharmacology. Nevertheless, stable and homogeneous sample preparation is one of the bottlenecks in biophysical and pharmacological studies of membrane proteins because membrane proteins are typically unstable and poorly expressed. To overcome such obstacles, GFP fusion-based Fluorescence-detection Size-Exclusion Chromatography (FSEC) has been widely employed for membrane protein expression screening for over a decade. However, fused GFP itself may occasionally affect the expression and/or stability of the targeted membrane protein, leading to both false-positive and false-negative results in expression screening. Furthermore, GFP fusion technology is not well suited for some membrane proteins depending on their membrane topology. Here, we developed an FSEC assay utilizing nanobody (Nb) technology, named FSEC-Nb, in which targeted membrane proteins are fused to a small peptide tag and recombinantly expressed. The whole-cell extracts are solubilized, mixed with anti-peptide Nb fused to GFP and applied to a size-exclusion chromatography column attached to a fluorescence detector for FSEC analysis. FSEC-Nb enables one to

evaluate the expression, monodispersity and thermostability of membrane proteins without the need of purification by utilizing the benefits of the GFP fusion-based FSEC method, but does not require direct GFP fusion to targeted proteins. We applied FSEC-Nb to screen zinc-activated ion channel (ZAC) family proteins in the Cys-loop superfamily and membrane proteins from SARS-CoV-2 as examples of the practical application of FSEC-Nb. We successfully identified a ZAC ortholog with high monodispersity but moderate expression levels that could not be identified with the previously developed GFP fusion-free FSEC method. Consistent with the results of FSEC-Nb screening, the purified ZAC ortholog showed monodispersed particles by both negative staining EM and cryo-EM. Furthermore, we identified two membrane proteins from SARS-CoV-2 with high monodispersity and expression level by FSEC-Nb, which may facilitate structural and functional studies of SARS-CoV-2. Overall, our results show FSEC-Nb as a powerful tool for membrane protein expression screening that can provide further opportunity to prepare well-behaved membrane proteins for structural and functional studies.

Introduction

Biophysical and biochemical studies, especially the structural determination of membrane proteins, require stable and homogeneous sample preparations, the acquisition of which is often hindered by the poor expression and unstable nature of membrane proteins¹⁻³.

To overcome this issue, various methods have been developed⁴⁻¹⁶. In particular, following the pioneer works on the application of GFP fusion techniques for membrane protein expression screening¹²⁻¹⁴, GFP fusion-based Fluorescence-detection Size-Exclusion Chromatography (FSEC) has been widely utilized for rapid evaluation of the expression status and thermostability of membrane proteins from both eukaryotes and prokaryotes^{15,16}.

In GFP fusion-based FSEC, recombinantly expressed GFP-fused proteins can be detected by a fluorescence detector following size-exclusion chromatography. The resulting fluorescence chromatography profiles allow one to rapidly analyze the expression level, monodispersity, and stability of both unpurified and purified membrane proteins at a scale on the order of nanograms. GFP fusion-based FSEC,

which is suited for the high-throughput screening of panels of orthologs, mutations and membrane proteins under different biochemical conditions, has been shown to be powerful in determining the structure of eukaryotic and prokaryotic membrane proteins by both cryo-EM and X-ray crystallography¹⁷⁻²⁵.

Nevertheless, several significant disadvantages of this method have also been recognized.

First, because GFP is a highly stable, soluble protein, its fusion sometimes causes false-positive hits by FSEC screening. In the case of such false-positive hits, GFP fusion proteins exhibit monodispersity by FSEC, but target membrane proteins may immediately aggregate or precipitate after the removal of GFP due to the instability of the target membrane protein alone²⁶. Second, in addition to the issue of false positivity, GFP fusion also causes a false negativity because it sometimes negatively affects the expression level^{27,28}. Finally, depending on the membrane topology of the target membrane protein, the GFP fusion technique may be difficult to apply. For instance, GFP fusion technology is not well suited for application with bacterial membrane proteins whose both N- and C-terminal ends are located at the periplasm because GFP

tends to fail to fold properly at the periplasm and thus does not show its fluorescence^{29,30}. Likewise, eukaryotic Cys-loop receptors are also known to be unsuitable for either N- or C-terminal GFP fusion^{31,32}. Thus, the insertion of GFP into the cytoplasmic loop is required for the application of GFP technology^{31,32}. This finding indicates that the simple strategy of N- or C-terminal GFP fusion is not applicable to some eukaryotic membrane proteins; thus, the application of GFP fusion-based FSEC may need optimization of the position at which GFP is inserted.

To overcome such disadvantages, a GFP fusion-free FSEC method would be ideal, and a multivalent nitrilotriacetic acid (NTA) fluorescent probe called P3NTA was developed as a pioneer work of the GFP fusion-free FSEC method⁹. The P3NTA probe can bind the poly-histidine tag fused to a target membrane protein for detection by FSEC without the need for purification. However, since interactions of the P3NTA probe with poly-histidine-tagged proteins are relatively weak and nonspecific, endogenous proteins from host cells with multiple accessible histidine residues may seriously affect the detection of target proteins³³. In particular, expression constructs of membrane proteins with high stability and monodispersity but relatively moderate

expression are hard to identify from FSEC screening by P3NTA due to its relatively weak and nonspecific detection ability. However, such expression constructs would now still be promising since structure determination by cryo-EM requires much less purified protein than that by X-ray crystallography³⁴.

To make further practical use of GFP fusion-free FSEC, we hypothesized that the application of other types of small peptide tags with high affinity and specificity would be ideal and that recent advances in nanobody (Nb) technologies for small peptides would meet such demands for GFP fusion-free FSEC.

Nb technology has been broadly utilized in laboratory research, clinical diagnosis and potential therapies³⁵. Nbs, which are derived from the antigen-specific variable domain of the camelid heavy-chain antibody, have a molecular weight of 12-15 kDa and can be recombinantly expressed in bacteria with high yield.

Recently, the peptide tags ALFA and BC2 and the corresponding specific Nbs we refer to here as NbALFA and NbBC2, respectively, were developed^{33,36}. The ALFA tag (SRLEEELRRRLTE), designed de novo, forms a stable, hydrophilic and electroneutral α -helix in solution with an extremely high affinity of ~26 pM for NbALFA^{33,37}. The de

novo designed sequence of ALFA is absent in common model organisms, which makes its recognition by NbALFA unique³³. The BC2 tag (PDRKAAVSHWQQ), derived from residues 16-27 of β -catenin, is unstructured in solution, and has a high affinity of ~1.4 nM for NbBC2³⁶.

Here, we developed a new type of FSEC utilizing Nb technology named FSEC-Nb. A membrane protein fused to the small peptide tag ALFA is recombinantly expressed in bacterial or eukaryotic cells. The whole-cell extracts are then solubilized and mixed with the NbALFA Nb, which is specific for the ALFA tag, fused to mEGFP³⁸ for FSEC analysis (Fig. 1).

To validate the method, we applied FSEC-Nb to the expression of bacterial and eukaryotic membrane proteins and showed that FSEC-Nb can be applied to ortholog screening and a thermostability assay.

Notably, we applied FSEC-Nb to orthologs of the zinc-activated ion channel (ZAC) family, a member of the Cys-loop receptor superfamily, which are unsuitable for either N- or C-terminal GFP fusion, and identified a ZAC ortholog from *Oryzias latipes* (OlZAC). However, we were not able to detect the expression of OlZAC by P3NTA, a

previously developed GFP fusion-free FSEC method. Consistent with the FSEC-Nb results, the negative staining EM and cryo-EM of the purified ZAC ortholog showed the monodispersity of the particles. Furthermore, we screened the expression of membrane proteins from SARS-CoV-2 by FSEC-Nb and identified two of them with a high level of expression and monodispersity, which could facilitate further structural and functional studies of SARS-CoV-2. Overall, our results showed FSEC-Nb as a powerful tool for expression screening of membrane proteins.

Results

Establishing the FSEC-Nb method

To overcome the disadvantages of the conventional FSEC method, we designed FSEC-Nb, which utilizes short peptides as fusion tags and Nbs specific to these peptides fused to monomerized EGFP proteins as a probe (Fig. 1).

We first applied our method to a prokaryotic ortholog of Zrt/Irt-like protein (ZIP) in the *E. coli* expression system. ZIPs function as metal transporters and are conserved from prokaryotes to eukaryotes, including humans³⁹. Among the ZIP family, the

structure of the bacterial ZIP protein from *Bordetella bronchiseptica* (BbZIP) was determined by crystallography³⁹; we chose to utilize BbZIP to establish our FSEC-Nb system because both its N- and C-terminal ends are located at the periplasm³⁹, which is not well suited for application of the GFP fusion-based FSEC method in bacterial expression systems.

In our experiment, BbZIP was fused to the peptide tags ALFA and BC2 at its C-terminus and recombinantly expressed in *E. coli*. The whole-cell extract was solubilized with detergents and mixed with mEGFP-fused Nbs specific for either the ALFA or BC2 tag (Fig. 2A and 2B). After removal of the pellet by ultracentrifugation, the sample was applied to a SEC column connected to a fluorescence detector (Fig. 1). When BbZIP was probed with mEGFP-tagged NbALFA, the FSEC plots presented peaks for both the mEGFP-tagged NbALFA in complex with the ALFA peptide-tagged BbZIP and free mEGFP-tagged NbALFA (Fig. 2A), but the corresponding complex peak was not observed when BbZIP was probed with mEGFP-tagged NbBC2 (Fig. 2B). These results showed that mEGFP-NbALFA specifically recognized the ALFA peptide-tagged BbZIP protein for the detection of BbZIP expression. The reason for the

failure of mEGFP-tagged NbBC2 and the BC2 tag is unknown but may have been due to the difference between tags in terms of their affinities for their Nbs (ALFA: ~26 pM, BC2: ~1.4 nM)^{33,36}. Furthermore, we tested expression of the C-terminally mEGFP-tagged BbZIP by FSEC but did not detect its expression (Fig. 2C), consistent with the finding that the C-terminal end of BbZIP is located at the periplasm³⁹. We also tested expression of BbZIP C-terminally fused to muGFP⁴⁰, a derivative of superfolder GFP⁴¹, since superfolder GFP is more suitable for its folding at the periplasm^{42,43}. However, we still did not detect the expression of the muGFP-tagged BbZIP by FSEC (Fig. 2C).

Overall, based on the results from BbZIP, we decided to employ the ALFA peptide tag and mEGFP-tagged NbALFA with our FSEC-Nb system for further experiments.

Thermostability assay by FSEC-Nb

We next applied the FSEC-Nb method to check membrane protein expression in mammalian cells and tested whether the FSEC-Nb system can be employed for thermostability assays of membrane proteins (Fig. 3A and 3B). We chose the human

P2X3 (hP2X3) protein, a member of the P2X receptor superfamily with known structures⁴⁴⁻⁴⁶.

ALFA-tagged hP2X3 was transiently expressed in HEK293 cells, which were solubilized for further FSEC-Nb experiments. The FSEC profiles of ALFA-tagged hP2X3 labeled with mEGFP-fused NbALFA showed peaks for both the mEGFP-fused NbALFA in complex with the ALFA-tagged hP2X3 and free mEGFP-fused NbALFA (Fig. 3C), showing that the FSEC-Nb technique can be applied in HEK293 cells.

In the thermostability assay of hP2X3 by FSEC-Nb, solubilized samples were incubated at their respective temperatures for 10 minutes using a thermal cycler, and the precipitated materials were then removed by ultracentrifugation before labeling with mEGFP-tagged NbALFA (Fig. 3A and 3B). The FSEC-Nb profiles clearly showed a thermal shift of the main peaks from the samples incubated at temperatures near and above 55 °C (Fig. 3D), with estimates of the T_m of 56.6 °C.

We then tested the thermostabilizing effects of ATP on hP2X3 (Fig. 3E). ATP is an endogenous ligand of P2X receptors that typically increases the thermostability of P2X receptors¹⁶. Consistently, in the thermostability assay carried out by FSEC-Nb, ATP

showed a clear stabilizing effect, increasing the estimated T_m by 15 °C. These results showed that FSEC-Nb can be employed to assay the thermostability of membrane proteins without the need for purification steps.

Expression screening of ZAC orthologs and SARS-CoV-2 membrane proteins

As examples of the practical application of FSEC-Nb, we then applied FSEC-Nb to screen ZAC family proteins and membrane proteins from SARS-CoV-2 (Fig. 4 and 5).

ZACs belong to the Cys-loop ligand-gated ion channel (LGIC) superfamily, which also includes nicotinic acetylcholine (nACh), 5-HT₃, GABA_A and glycine receptors^{47,48}.

In addition to Zn²⁺, the gating of ZACs, nonselective cation channels that are widely expressed in the human body, is activated by Cu²⁺ and protons⁴⁸. Since ZACs were the last members of the Cys-loop LGIC superfamily to be discovered⁴⁷⁻⁴⁹, their function and structure are poorly characterized.

To facilitate structural and biophysical studies of ZAC proteins, we utilized FSEC-Nb to overcome the difficulty imposed by heterogeneous ZAC expression and purification. We chose to apply FSEC-Nb to ZACs because Cys-loop LGIC superfamily proteins

were reported to be unsuitable for either N- or C-terminal GFP fusion^{31,32}.

ZAC genes from *Homo sapiens* (HsZAC), *Danio rerio* (DrZAC), *Oryzias latipes* (OlZAC) and *Oreochromis niloticus* (OnZAC) were synthesized with ALFA and octa-histidine tags at the C-terminus, and recombinantly expressed in HEK293 cells. The expressed ZAC orthologs were probed by mEGFP-tagged NbALFA for detection by the FSEC-Nb method. FSEC-Nb screening of ZAC orthologs showed that the profile for OlZAC exhibited a higher and sharper peak than those for other ZAC orthologs (Fig. 4A). In contrast, we could not detect the expression of the C-terminally muGFP-tagged OlZAC by FSEC (Fig. 4B). Furthermore, we could not detect the expression of OlZAC by P3NTA-based FSEC, the previously developed GFP fusion-free FSEC method (Fig. 4C), showing the improved sensitivity of FSEC-Nb over P3NTA-based FSEC.

SARS-CoV-2 is a pathogen that causes coronavirus disease 2019 (COVID-19)⁵⁰⁻⁵². Using FSEC-Nb, we screened the expression of a series of membrane proteins from SARS-CoV-2 (Fig. 5). We identified ORF3a and ORF7b with high monodispersity and high expression level, comparable to those of hP2X3 (Fig. 5). ORF3a is an ion channel and potential target for COVID-19 therapy⁵³. A mutation on ORF7b reportedly showed

higher replicative fitness⁵⁴. Consistent with the sharp peak from FSEC-Nb, the cryo-EM structure of ORF3a was recently reported on bioRxiv⁵³. Overall, our FSEC-Nb screening results may facilitate structural and functional studies of SARS-CoV-2.

Detergent screening of OlZAC

Purification of membrane proteins requires detergents to extract the proteins from the biological membrane. The type of detergent used often affects the monodispersity and stability of a membrane protein in purification; thus, detergent screening is beneficial for establishing purification protocols for membrane proteins. Furthermore, the addition of lipids and lipid-like compounds, such as cholesteryl hemisuccinate (CHS), which was shown to be useful for the purification and crystallization of various GPCRs⁵⁵⁻⁵⁷, could also affect the stability of membrane proteins¹⁶. In our assay of OlZAC thermostability by FSEC-Nb, we tested multiple types of detergents for OlZAC; among these detergents were n-dodecyl- β -D-maltoside (DDM); DDM additive with CHS at a ratio of 5:1 (w:w), referred to as DDM-CHS; lauryl maltose neopentyl glycol (LMNG); and glyco-diosgenin (GDN). The FSEC-Nb profiles of OlZAC solubilized with DDM

showed a thermal shift of the main peaks from the samples incubated at temperatures near and above 60 °C (Fig. 6A), with estimates of the T_m of 60.6 °C (Fig. 6B). Unpurified ALFA-tagged OlZAC samples solubilized with the respective detergents were heat-treated at 60 °C for 10 minutes, and FSEC-Nb was applied to both heated and unheated samples for comparison (Fig. 6C). Compared to DDM, LMNG conferred better thermostability to OlZAC, whereas DDM-CHS solubilized OlZAC similarly as with DDM (Fig. 6C). The performance of GDN was similar to that of LMNG (Fig. 6C). Based on the results of detergent screening with OlZAC by FSEC-Nb, we decided to employ either DDM or DDM-CHS for protein extraction from the membrane and either LMNG or GDN for the subsequent purification steps.

Large-scale culture and purification of OlZAC

For large-scale culture in HEK293S cells, we then generated bacmid DNA for OlZAC, which was used to transfect Sf9 insect cells to prepare BacMam virus. To optimize the expression conditions, using FSEC-Nb, we performed small-scale expression screening in HEK293S cells by testing different amounts of P2 virus, incubation times, and cell

culture temperatures at 16 hours after the addition of P2 virus (Fig. 7A and 7B) and decided to choose one set of conditions for large-scale culture (1% volume P2 virus addition, 88 hours of culture at 37 °C after the addition of virus).

OlZAC was purified as described in the Methods section. Briefly, the membrane collected from cell lysates was solubilized in DDM-CHS, and the detergent was then exchanged to LMNG in the affinity chromatography steps. During size-exclusion chromatography in SEC buffer containing LMNG, the UV absorbance plot showed a symmetric peak for OlZAC and a prior void peak (Fig. 7C). A total of 2.4 liters of HEK293 cell culture yielded approximately 0.5 mg of purified OlZAC protein. Trp-based FSEC verified the monodispersity of the pooled fractions constituting the main SEC peaks (Fig. 7D), and the purity of the pooled fractions was validated by SDS-PAGE (Fig. 7E).

Negative staining EM and cryo-EM of OlZAC

To evaluate the sample quality of OlZAC, which was identified by FSEC-Nb, we performed negative staining EM and preliminary cryo-EM of OlZAC (Fig. 8).

Pioneering structural studies of other eukaryotic pLGIC members by crystallography and cryo-EM have elucidated their fundamental architecture: a pentamer comprised of an extracellular component for ligand gating, a transmembrane component for ion permeation and an intracellular component^{32,58-64}. ZACs possess low amino acid sequence identity with other pLGIC members, with the closest matches exhibiting ~20% identity with ZACs^{47,48}. Accordingly, little is still known about the ZAC structure.

The OlZAC purified under the apo conditions was reconstituted into amphipol by mixing with amphipols at a mass ratio of 1:20, and the detergent was removed by Bio-Beads. We tested the reconstitution of NAPol on a small scale by Trp-FSEC, which resulted in a high and symmetric peak for the amphipol-reconstituted OlZAC (Fig. S1A). NAPol is a nonionic amphipol that is soluble across a wide pH range and compatible with multivalent cations^{65,66}; thus, we chose NAPol for ZACs since both pH and the presence of divalent cations are relevant to the functional status of ZACs. On a large scale, we further reconstituted OlZAC into NAPol and separated the amphipol-reconstituted OlZAC by SEC (Fig. S1B).

The amphipol-reconstituted OlZAC was then stained by uranyl acetate and observed

under an electron microscope. The images taken by the EM-CCD camera showed monodispersed OlZAC particles (Fig. 8A). Because of the high contrast after negative staining, the particles were easily recognized from the images (Fig. 8B). The particles extracted from over one hundred images were classified into several 2D classes, which validated the stoichiometry and constitution of ZACs (Fig. 8C, 8D and 8E). Similar to other pLGIC members, ZACs form a pentamer (Fig. 8E) and is composed of extracellular, transmembrane and intracellular components (Fig. 8D).

We then performed preliminary cryo-EM single-particle analysis of OlZAC with a K3 direct detection camera (Fig. 8F and 8G), which also showed monodispersed particles.

These results showed the sample quality of OlZAC identified by FSEC-Nb, which would be suitable for structural studies.

Discussion

In this work, we developed a new type of FSEC assay, named FSEC-Nb, utilizing the ALFA peptide tag and anti-ALFA peptide Nb NbALFA. In FSEC-Nb, targeted membrane proteins are tagged by the peptide tag ALFA and recombinantly expressed in

either prokaryotic or eukaryotic cells before being probed by mEGFP-tagged NbALFA for FSEC analysis (Fig. 1). We first tested two peptide tags, ALFA and BC2, and found that the peptide tag ALFA was more suitable for the detection of BbZIP by FSEC-Nb in a bacterial expression system (Fig. 2). We then applied the FSEC-Nb method for a thermostability assay (Fig. 3). As a demonstration of the practical application of FSEC-Nb, we then applied FSEC-Nb for the screening of orthologs of ZAC, a member of the Cys-loop LGIC superfamily without a known 3D structure, as well as membrane proteins from SARS-CoV-2 (Fig. 4 and 5). We then further screened different types of detergents for the purification of OlZAC (Fig. 6). Finally, using purified OlZAC (Fig. 7), we performed negative staining EM and preliminary cryo-EM, which showed the monodispersity of the purified OlZAC sample (Fig. 8).

FSEC-Nb confers the advantage of conventional GFP fusion-based FSEC but avoids the following disadvantages of GFP fusion-based FSEC.

First, GFP fusion-based FSEC is not well suited for some membrane proteins, depending on their membrane topology²⁹⁻³². To be noted, the membrane topology prediction from 29 organisms by TransMembrane Hidden Markov Model (TMHMM)⁶⁷

showed ~20% of multispinning membrane proteins possess both their N- and C-terminal ends at the extracellular or periplasmic sides. Furthermore, even when applicable, GFP fusion may occasionally affect the expression and/or stability of targeted membrane proteins, potentially leading to both false-positive and false-negative results²⁶⁻²⁸. In our recent worst case, we screened over 60 homologs of MgtC, a virulence factor in *Salmonella enterica*⁶⁸, by GFP fusion-based FSEC with the C-terminally mGFPuv-tagged expression constructs (Table S1), because the C-terminal end of MgtC is located at the cytoplasm. We identified only two of them with high monodispersity and expression level (Fig. S2). However, both two proteins aggregated and precipitated after the removal of the GFP tag. In addition, compared to the P3-NTA method, a previously developed GFP fusion-free FSEC method utilizing poly-histidine tag, FSEC-Nb showed better performance in the screening of ZAC protein orthologs (Fig. 5). Overall, FSEC-Nb would be useful for expression screening of both types of membrane proteins to which the conventional GFP fusion-based FSEC is applicable and is not applicable.

On the other hand, representing a disadvantage of our FSEC-Nb assay over the

conventional GFP fusion FSEC method, purified GFP-fused NbALFA, which acts as a probe, needs to be prepared in each laboratory that wishes to use this method. However, the purification of mEGFP-fused NbALFA would be easy for most biochemistry and structural biology laboratories, as its *E. coli* expression level is quite high (more than 10 mg of purified protein from 1 liter of *E. coli* culture), and 1 mg of mEGFP-fused NbALFA is enough for 1,000 FSEC-Nb experiments and would thus last for a couple of years with conventional laboratory usage. Furthermore, to improve access for FSEC-Nb, we have deposited the expression vectors for mEGFP- and mCherry-fused NbALFAs as well as template vectors with the ALFA tag for expression in *E. coli* (pETNb-nALFA and pETNb-cALFA) and insect (pFBNb-cALFA) and mammalian (pBMNb-cALFA) cells to the Addgene plasmid repository (Fig. 9). We have also deposited BbZIP gene in pETNb-cALFA and hP2X3 gene in pBMNb-cALFA as positive controls for FSEC-Nb. Thus, FSEC-Nb can be easily introduced to most biochemistry and structural biology laboratories, particularly to labs those with the experience with the conventional GFP fusion-based FSEC method, which has already been widely used. Notably, all of these vectors can be used for not only a small-scale expression check by FSEC-Nb but also

large-scale protein expression.

Overall, FSEC-Nb can be used for expression screening and thermostability assays on a small scale with high sensitivity and specificity without the need for GFP fusion to target proteins. Such advantages of FSEC-Nb will enable us to explore further opportunities to prepare target proteins for structure determination as well as other biophysical and pharmacological studies.

Materials and Methods

Purification of mEGFP-tagged Nbs

With an interval of GSGSGS, the NbALFA sequence was fused in frame with an N-terminal His₈-mEGFP affinity tag and subcloned into the pET28b vector. The protein was overexpressed in *E. coli* Rosetta (DE3) cells in LB medium containing 30 µg/ml kanamycin at 37 °C by induction at an OD₆₀₀ of ~0.5 with 0.5 mM isopropyl D-thiogalactoside (IPTG) for 16 hours at 18 °C. The *E. coli* cells were subsequently harvested by centrifugation (6,000 × g, 15 minutes) and resuspended in buffer A (50 mM Tris-HCl (pH 8.0), 150 mM NaCl) supplemented with 0.5 mM

phenylmethylsulfonyl fluoride (PMSF). All purification steps were performed at 4 °C. The *E. coli* cells were then disrupted with a microfluidizer, and debris was removed by centrifugation (70,000 × g, 60 minutes). The supernatant was loaded onto equilibrated Ni-NTA resin pre-equilibrated with buffer A and mixed for 1 hour. The column was then washed with buffer A containing 30 mM imidazole, and the protein was eluted with buffer A containing 300 mM imidazole. The imidazole was removed by dialysis in buffer B (20 mM HEPES (pH 7.0), 150 mM NaCl) overnight. Finally, the purified mEGFP-tagged NbALFA was concentrated to 1 mg/ml using an Amicon Ultra 30K filter (Merck Millipore) and stored at -80 °C before use. mEGFP-tagged NbBC was similarly expressed and purified.

FSEC-Nb in the *E. coli* expression system

In the *E. coli* expression system, BbZIP tagged with either the ALFA or BC2 peptide at its C-terminus was synthesized and subcloned into the pET28b vector and overexpressed with a protocol similar to that for the expression of mEGFP-tagged NbALFA described above. The *E. coli* cell pellets from 5 ml of LB culture were

suspended in 400 μ l of buffer A and sonicated, and the cell debris was removed by centrifugation ($20,000 \times g$, 10 minutes). The lysates were solubilized by mixing 500 μ l of buffer A containing 2% (w:v) DDM and 0.5 mM PMSF for 1 hour, and were ultracentrifuged ($200,000 \times g$, 20 minutes). Unless noted, 1 μ g of mEGFP-tagged NbALFA was added to the supernatant and incubated for 30 minutes. Considering the reported affinity values of NbALFA binding to an ALFA-tag (k_{on} ($M^{-1}s^{-1}$): $3.6(\pm 0.1) \times 10^5$, k_{off} (s^{-1}): $9.4 (\pm 0.2) \times 10^{-6}$, K_D 26 (± 1) pM)³³, 30 minutes incubation would be enough for the saturation of the binding. After centrifugation ($20,000 \times g$, 10 minutes), 50 μ l of the sample was applied to a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated with buffer A containing 0.05% (w:v) DDM for the FSEC assay. GFP fusion-based FSEC was performed similarly but without the labeling step with mEGFP-tagged NbALFA. In the FSEC assay, fluorescence was detected using the RF-20Axs fluorescence detector for HPLC (Shimadzu, Japan) (for mEGFP, excitation: 480 nm, emission: 512 nm) (for muGFP, excitation: 480 nm, emission: 508 nm) (for mGFPuv, excitation: 395 nm, emission: 507 nm). FSEC-Nb experiments with mEGFP-tagged NbBC2 were performed with a similar protocol.

FSEC-Nb in the HEK293 expression system

hP2X3, ZAC orthologs and membrane proteins from SARS-CoV-2 containing ALFA and His₈ tags at their C-terminus were synthesized and subcloned into a derivative of the Bac-to-Bac system vector with the CMV promoter and WPRE motif. Using 3 µl of Lipofectamine 2000 (Thermo Fisher Scientific, US), 1 µg of each plasmid was transfected into 1 ml of HEK293S cells in adherent culture at a density of 0.5 million cells/ml in DMEM supplemented with 10% FBS. Cells were incubated in a CO₂ incubator (37 °C, 5% CO₂) for 48 hours after transfection and solubilized with 200 µl of buffer A containing 2% (w:v) DDM supplemented with 0.5 mM PMSF, 5.2 µg/ml aprotinin, 2 µg/ml leupeptin, and 1.4 µg/ml pepstatin A (all from Sigma-Aldrich) for 1 hour. After ultracentrifugation (200,000 × g, 20 minutes), 1 µg of mEGFP-tagged NbALFA or 2 µl of 0.5 µM P3NTA was added to and mixed into 100 µl of the supernatant for 30 minutes. Then, after centrifugation (20,000 × g, 10 minutes), 50 µl of the sample was applied to a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated with buffer A containing 0.05% (w:v) DDM for the FSEC assay. In the

FSEC assay, fluorescence was detected as described above. The P3NTA peptide was prepared and used for FSEC as previously described (excitation: 480 nm, emission: 520 nm)⁹.

Thermostability assay by FSEC-Nb

ALFA peptide-tagged hP2X3 was expressed in HEK293 cells and solubilized as described above. Cells from 4 ml of culture were resuspended in 1.2 ml of buffer A (50 mM TRIS (pH 8.0), 150 mM NaCl) containing 2% DDM by the addition of either ATP at a final concentration of 1 mM (ATP-bound conditions) or 0.6 unit of apyrase (Sigma, USA) to remove endogenous ATP (apo conditions), rotated at 4 °C for 1 hour, and then ultracentrifuged (200,000 g, 10 minutes). One hundred microliters of the supernatant was dispensed into 1.5-ml Eppendorf tubes and incubated at the respective temperature for 10 minutes using either a thermal cycler or heat block bath. After ultracentrifugation (200,000 g, 10 minutes), the supernatant was mixed with 1 µg of mEGFP-tagged NbALFA and then centrifuged (20,000 g, 10 minutes). Then, 50 µl of the supernatant was applied to a Superdex 200 Increase 10/300 GL column (GE Healthcare)

equilibrated with buffer A containing 0.05% (w:v) DDM for the FSEC assay. We estimated the melting curves based on the peak heights and determined the melting temperatures by fitting the melting curves to a sigmoidal dose-response equation because the melting curves based on the peak heights were known to be consistent with the melting curves based on the peak area estimated by Gaussian fitting¹⁶.

Detergent screening by FSEC-Nb

HEK293S cells expressing ALFA-tagged OIZAC were prepared as described above. The collected cells were solubilized in buffer A containing different types of detergents: 2% (w:v) DDM, 2% (w:v) DDM-CHS, 1% (w:v) LMNG, and 1% (w:v) GDN. FSEC-Nb and thermostability assays by FSEC-Nb were conducted as described above.

Expression and purification of OIZAC

OIZAC tagged with ALFA and His₈ was expressed in HEK293S GnTI⁻ cells using a baculovirus-mediated gene transduction system in mammalian cells⁶⁹.

Small-scale expression screening to determine large-scale culture conditions was

performed by FSEC-Nb (Fig. 7A and 7B). FSEC-Nb was carried out with the protocol described above. A 800-ml culture of HEK293S GnTI⁻ cells was grown to a density of $2.5 \times 10^6 \text{ ml}^{-1}$ and infected with 8 ml of P2 BacMam virus. After 16 hours of culture at 37 °C, 10 mM sodium butyrate was added, and the temperature was maintained at 37 °C for another 72 hours of culture. Then, the cells were harvested and washed with buffer A. All purification steps were performed at 4 °C. Cells were broken by sonication with protease inhibitors (1 mM PMSF, 5.2 µg/ml aprotinin, 2 µg/ml leupeptin, and 1.4 µg/ml pepstatin A, all from Sigma-Aldrich). Membrane fractions were collected by ultracentrifugation ($200,000 \times g$, 60 minutes). The membrane was solubilized in buffer A containing 2% (w:v) DDM-CHS and supplemented with protease inhibitors (1 mM PMSF, 5.2 µg/ml aprotinin, 2 µg/ml leupeptin, and 1.4 µg/ml pepstatin A, all from Sigma-Aldrich) for 2 hours. The debris was removed by ultracentrifugation ($200,000 \times g$, 60 minutes). The supernatant was loaded onto equilibrated TALON resin (Clontech) and then washed with buffer A containing 0.01% (w:v) LMNG and 10 mM imidazole. Protein was eluted with buffer A containing 300 mM imidazole. The eluted protein was loaded on a Superdex 200 10/300 GL column and subjected to SEC in buffer B

containing 0.01% (w:v) LMNG. The main peak fractions were pooled and concentrated to ~1 mg/ml using an Amicon Ultra 100K filter (Merck Millipore).

Amphipol reconstitution

All steps were performed at 4 °C. On a small scale, 10 µg of OlZAC (10 µl) was mixed with 200 µg of NAPol (Anatrace, dissolved in 2 µl of buffer B) and incubated for 16 hours. The detergent was removed by incubation with Bio-Beads SM-2 (Bio-Rad) for 4 hours, after which the beads were removed over a disposable Poly-Prep column. Twenty microliters of the eluent was diluted to 200 µl, and 50 µl of the sample was applied to a Superdex 200 10/300 GL column equilibrated with buffer A for Trp-based FSEC (excitation: 280 nm, emission: 325 nm). At a large scale, 500 µg of OlZAC (500 µl) was mixed with 10 mg of NAPol (Anatrace, dissolved in 100 µl of buffer B) and incubated for 16 hours. The detergent was removed with Bio-Beads SM-2 (Bio-Rad) for 4 hours, and the beads were subsequently removed over a disposable Poly-Prep column. The eluent was applied to a Superdex 200 10/300 GL column equilibrated with buffer B, and the main fractions consisting of the amphipol-reconstituted OlZAC were pooled and

concentrated to ~3 mg/ml using an Amicon Ultra 100K filter for electron microscopic analysis.

Negative staining and electron microscopy

Gilder 400 square mesh grids (AG400) were glow discharged in a PELCO easiGlow apparatus at a current of 25 mA for 30 seconds. Five microliters of protein solution (~20 µg/mL) was dropped onto the grid and allowed to remain on the grid for 1 minute.

The residual protein solution was blotted from the grid edge with a piece of filter paper.

The grid was covered with 2% uranyl acetate, blotted immediately, covered again with 2% uranyl acetate for 30 seconds and blotted again. After drying, the grid was observed under a Talos L120C microscope at 120 kV. In total, 133 micrographs were taken with a Ceta CCD camera at a nominal magnification of 92,000× at a pixel size of 1.55 Å. The micrographs were processed in RELION 3.0 for particle picking, extraction and 2D classification⁷⁰.

Cryo-EM data acquisition

A total of 2.5 μl of OlZAC in NAPol was applied to a glow-discharged holey carbon film grid (QUANTIFOIL, R1.2/1.3, 100 Holey Carbon Films, Au 300 mesh) blotted with a Vitrobot (FEI) system using a 3.0-s blotting time with 100% humidity at 9 °C and plunge-frozen in liquid ethane. Cryo-EM images were collected on a Titan Krios (FEI) electron microscope operated at an acceleration voltage of 300 kV. The specimen stage temperature was maintained at 80 K. Images were recorded with a K3 Summit direct electron detector camera (Gatan Inc.) set to super-resolution mode with a pixel size of 0.41 Å (a physical pixel size of 0.82 Å) and a defocus ranging from -1.3 μm to -2.0 μm . The dose rate was 20 $\text{e}^- \text{s}^{-1}$, and each movie was 1.76 seconds long, dose-fractioned into 40 frames, with an exposure of 1.3 $\text{e}^- \text{Å}^{-2}$ for each frame.

Gene synthesis

The gene fragments for mEGFP, muGFP, mCherry, ALFA and BC2 tags, NbALFA, NbBC2, BbZIP, ZAC, hP2X3, and membrane proteins from SARS-CoV-2 used for this research were synthesized by Genewiz (Suzhou, China).

Data availability

All data and materials are available from the authors upon reasonable request. The plasmids shown in Fig. 9 (mEGFP-NbALFA, mCherry-NbALFA, pETNb-nALFA, pETNb-cALFA, pFBNb-cALFA, pBMNb-cALFA) have been deposited into Addgene (<http://www.addgene.org/>) (Addgene IDs: 159986, 159987, 159988, 159989, 159990 and 159991). We have also deposited BbZIP gene in pETNb-cALFA and hP2X3 gene in pBMNb-cALFA as positive controls for FSEC-Nb (Addgene IDs 160498 and 160499).

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Author contributions

F.J. performed experiments with assistance from S.S., M.W. and Y.W. F.J. and M.H. wrote the manuscript. M.H. supervised the research. All authors discussed the manuscript.

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1 FSEC-Nb designation and verification

(A) Flow chart of FSEC-Nb for membrane protein expression and purification. (B) Cartoon diagram of the FSEC system for FSEC-Nb. (C) Cartoon of a representative FSEC trace from FSEC-Nb.

Figure 2 Establishing the FSEC-Nb method

(A) FSEC traces of unpurified ALFA peptide-tagged BbZIP with mEGFP-tagged NbALFA, as detected by mEGFP fluorescence. A close-up view of the main peak profiles for the complex of ALFA-tagged BbZIP and mEGFP-tagged NbALFA is also shown. (B) FSEC traces of unpurified BC2 peptide-tagged BbZIP with mEGFP-tagged NbBC2, as detected by mEGFP fluorescence. (C) FSEC traces of C-terminally mEGFP-tagged and muGFP-tagged BbZIP, as detected by mEGFP and muGFP fluorescence, respectively.

Figure 3 Thermostability assay by FSEC-Nb

(A) Flow chart of the thermostability assay by FSEC-Nb. (B) Cartoon diagram of the thermostability assay by FSEC-Nb. (C) FSEC-Nb traces of unpurified ALFA-tagged hP2X3, as detected by mEGFP fluorescence. (D) FSEC-Nb traces of unpurified ALFA-tagged hP2X3 preheated at the indicated temperatures. A close-up view of the

main peak profiles for the complex of the ALFA-tagged hP2X3 and mEGFP-tagged NbALFA is also shown. (E) Melting curves of hP2X3 in the presence and absence of ATP, as detected by FSEC-Nb. The fitted curves are shown as blue (apo) and green (with ATP) lines.

Figure 4 Expression screening of ZAC orthologs

(A) FSEC-Nb traces of unpurified ALFA peptide and His8-tagged ZAC orthologs with mEGFP-tagged NbALFA, as detected by mEGFP fluorescence. A close-up view of the main peak profiles for the complex of the ALFA-tagged ZAC and mEGFP-tagged NbALFA is also shown. The expression of ZAC orthologs from *Homo sapiens* (GI: 206725456), *Danio rerio* (528523664), *Oryzias latipes* (765127633), and *Oreochromis niloticus* (542233486) was screened by FSEC-Nb. (B) FSEC traces of C-terminally muGFP-tagged OlZAC, as detected by muGFP fluorescence. (C) FSEC traces of unpurified ALTA peptide and His8-tagged ZAC orthologs with P3NTA, as detected by fluorescein fluorescence.

Figure 5 Expression screening of membrane proteins from SARS-CoV-2

FSEC-Nb traces of unpurified ALFA peptide and His8-tagged membrane proteins from SARS-CoV-2 with mEGFP-tagged NbALFA, as detected by mEGFP fluorescence. A

close-up view of the main peak profiles is also shown. The expression of ORF3a (UniProt ID: P0DTC3), E (P0DTC4), M (P0DTC5), ORF7a (P0DTC7), and ORF7b (P0DTD8) was screened by FSEC-Nb.

Figure 6 Detergent screening for OlZAC purification

(A) FSEC-Nb traces of unpurified ALFA-tagged OlZAC preheated at the indicated temperatures. A close-up view of the main peak profiles is also shown. (B) Melting curves of OlZAC, as detected by FSEC-Nb. The fitted curve is shown as a black line. (C) Normalized peak heights of ALFA-tagged OlZAC preheated at 60 °C for 10 minutes solubilized with the indicated detergents. The peak heights were normalized to that from the sample solubilized with DDM at 4 °C. Error bars represent standard error of the mean (N=6).

Figure 7 Large-scale expression and purification of OlZAC

(A) FSEC profiles of OlZAC, as detected by FSEC-Nb for the optimization of cell culture conditions. (B) Time course curves of the main peak heights, as detected by FSEC-Nb. HEK293S cells were infected with P2 BacMam virus for OlZAC expression at a 1% or 2% volume. At 16 hours after virus addition, cell culture temperatures were

maintained at 37 °C or shifted to 30 °C. (C) Size-exclusion chromatography of OlZAC, as detected by UV absorbance. (D) FSEC trace of purified OlZAC, as detected by Trp fluorescence. (E) SDS-PAGE of the purified OlZAC after SEC.

Figure 8 Negative staining EM and cryo-EM of OlZAC

(A, B) Negative staining EM images of OlZAC particles. (C-E) Selected 2D class averages, as calculated using RELION. (E, F) Cryo-EM image at 1.8 µm defocused with OlZAC particles.

Figure 9 Vector maps for FSEC-Nb

(A, B) Maps of the expression vectors for mEGFP-tagged (E) and mCherry-tagged (F) NbALFA. (C-F) Maps of the expression vectors for FSEC-Nb in *E. coli* (C, D), insect cells (E), and mammalian cells (F).

Figure S1 Amphipol reconstitution of OlZAC

(A) FSEC trace of NaPol-reconstituted OlZAC on a small scale, as detected by Trp

fluorescence. (B) Size-exclusion chromatography of NaPol-reconstituted OlZAC, as detected by UV absorbance.

Figure S2 Expression screening of MgtC by GFP-fusion FSEC

(A, B) FSEC traces of C-terminally mGFPuv-tagged TpMgtC (Accession Number: WP_038038224.1) and AtMgtC (WP_043965058.1), as detected by mGFPuv fluorescence.

Table S1 MgtC orthologs for GFP fusion-based FSEC screening

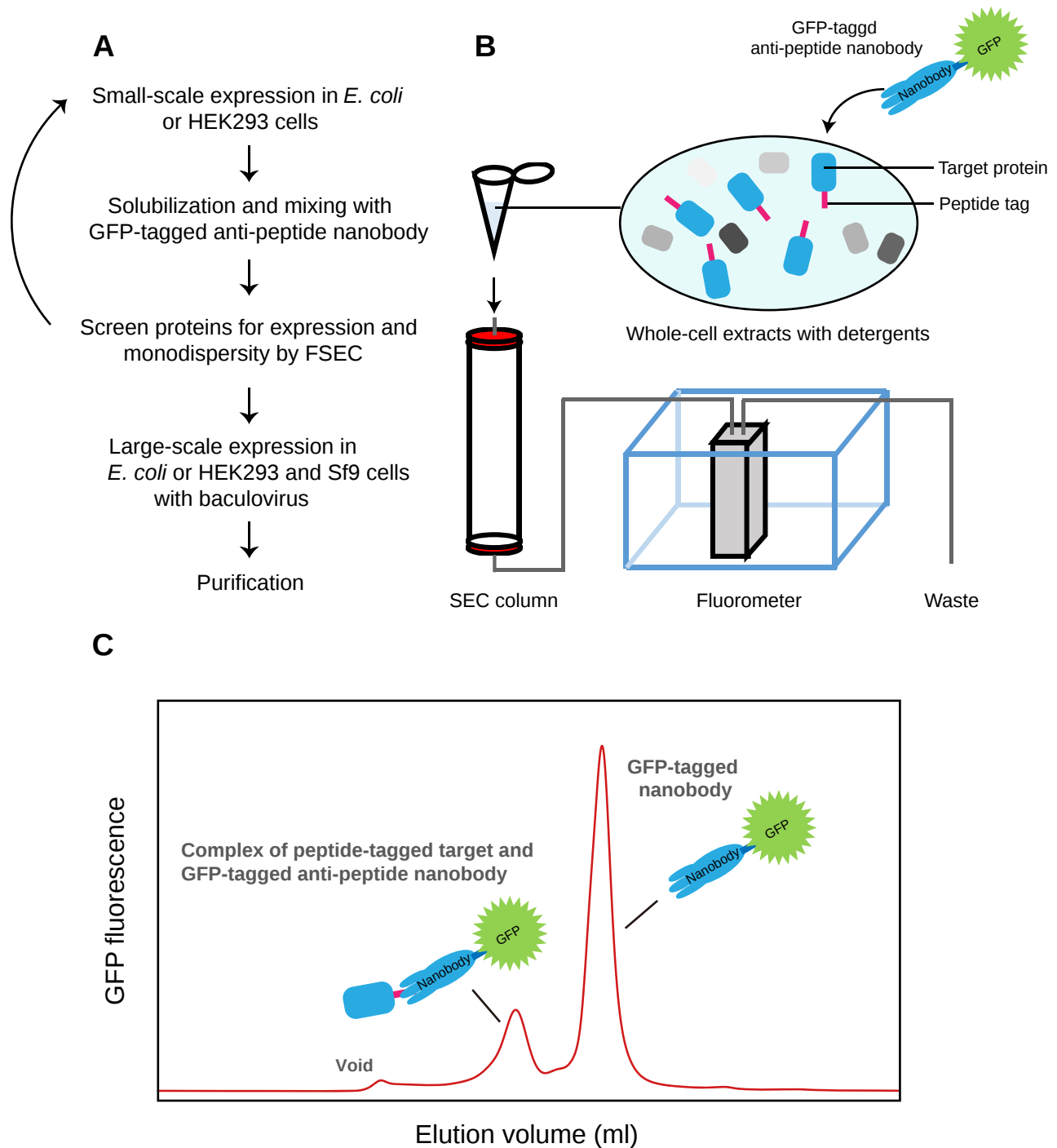


Figure 1

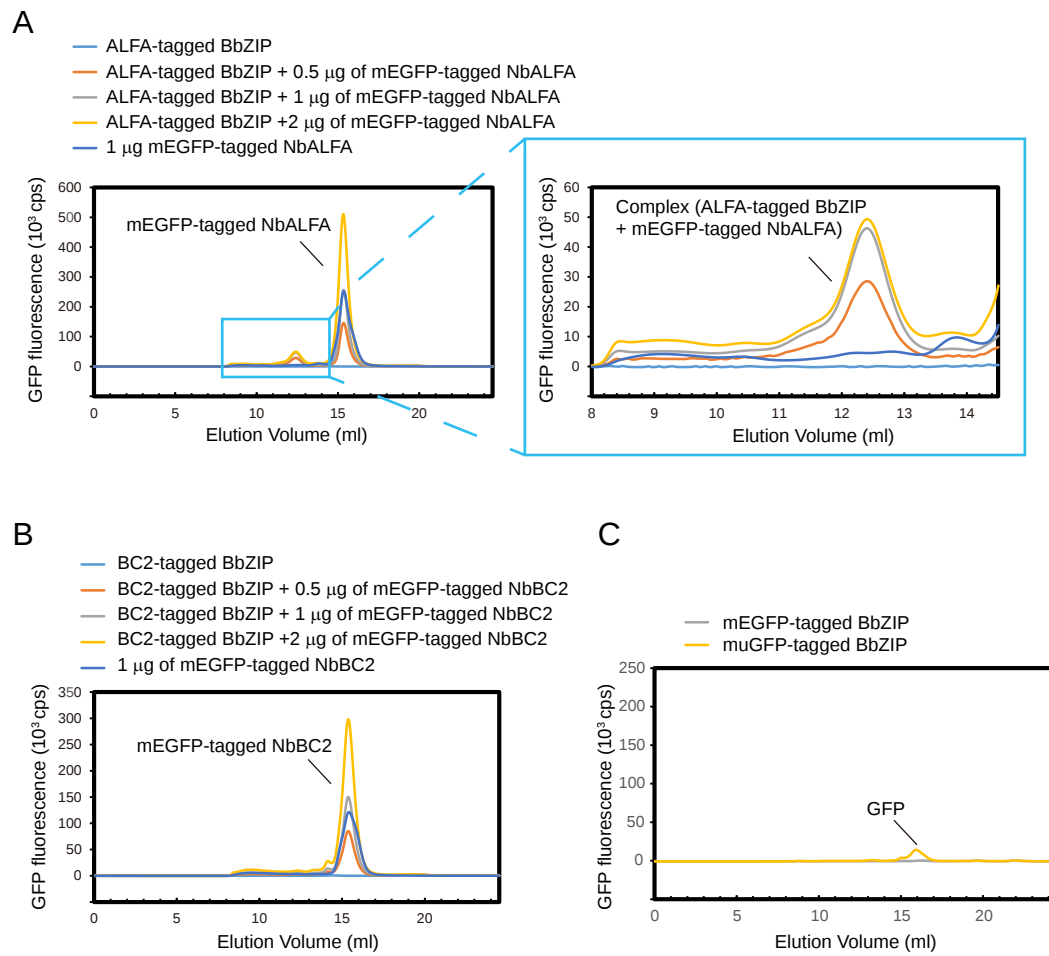


Figure 2

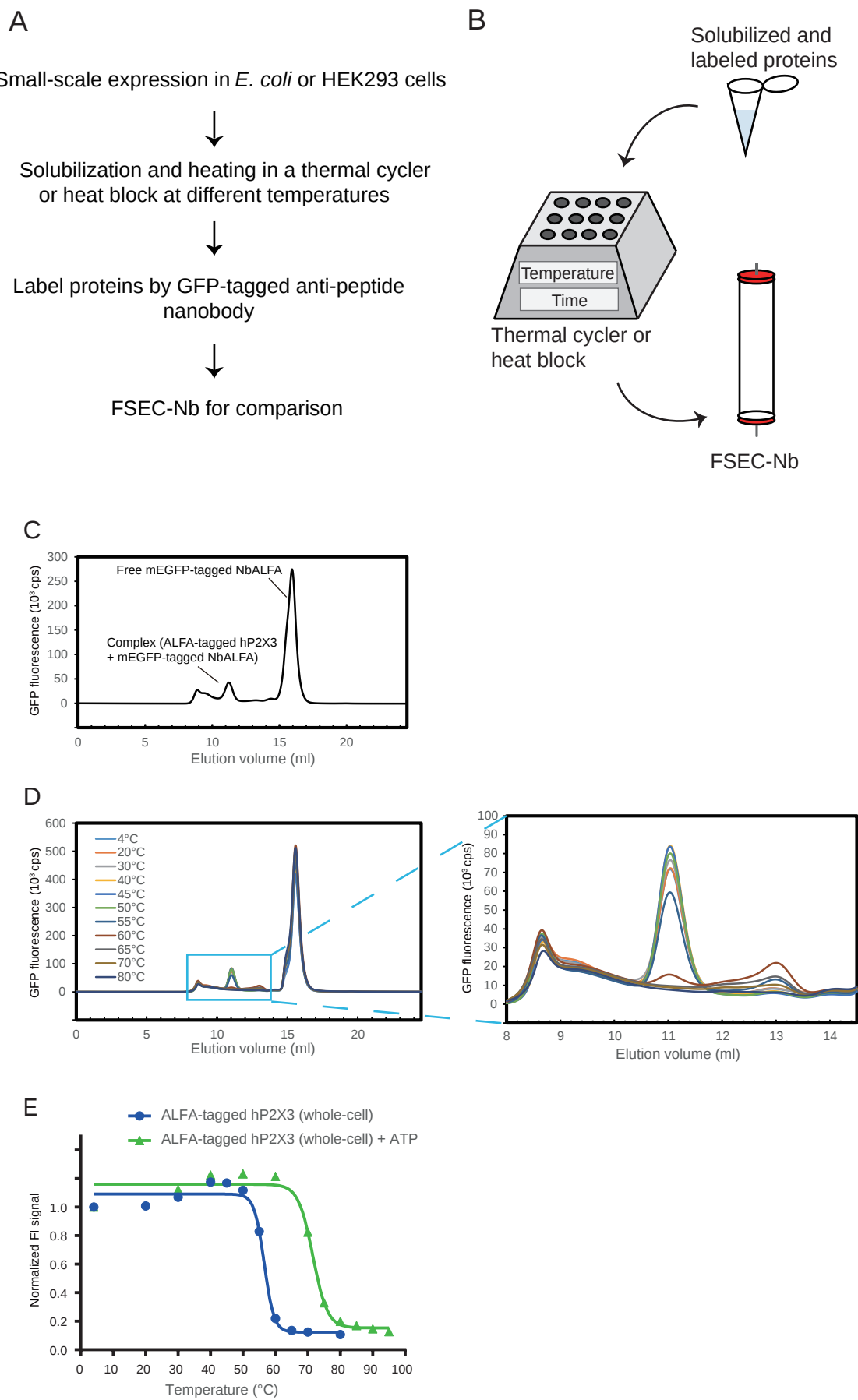


Figure 3

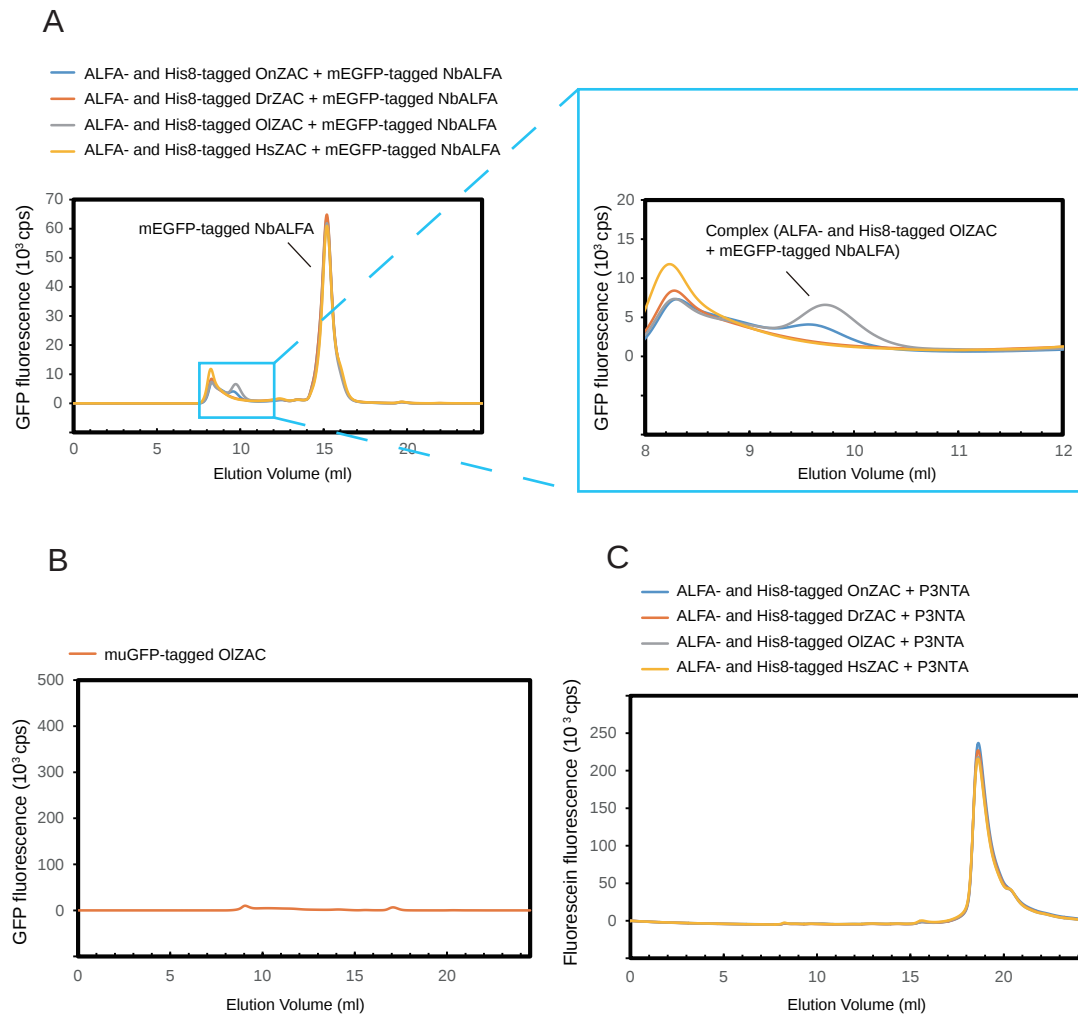


Figure 4

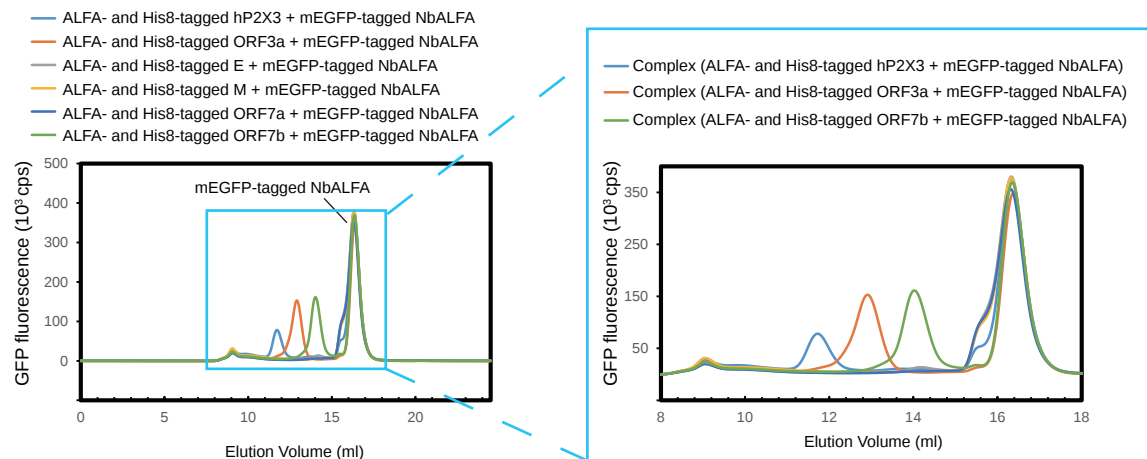


Figure 5

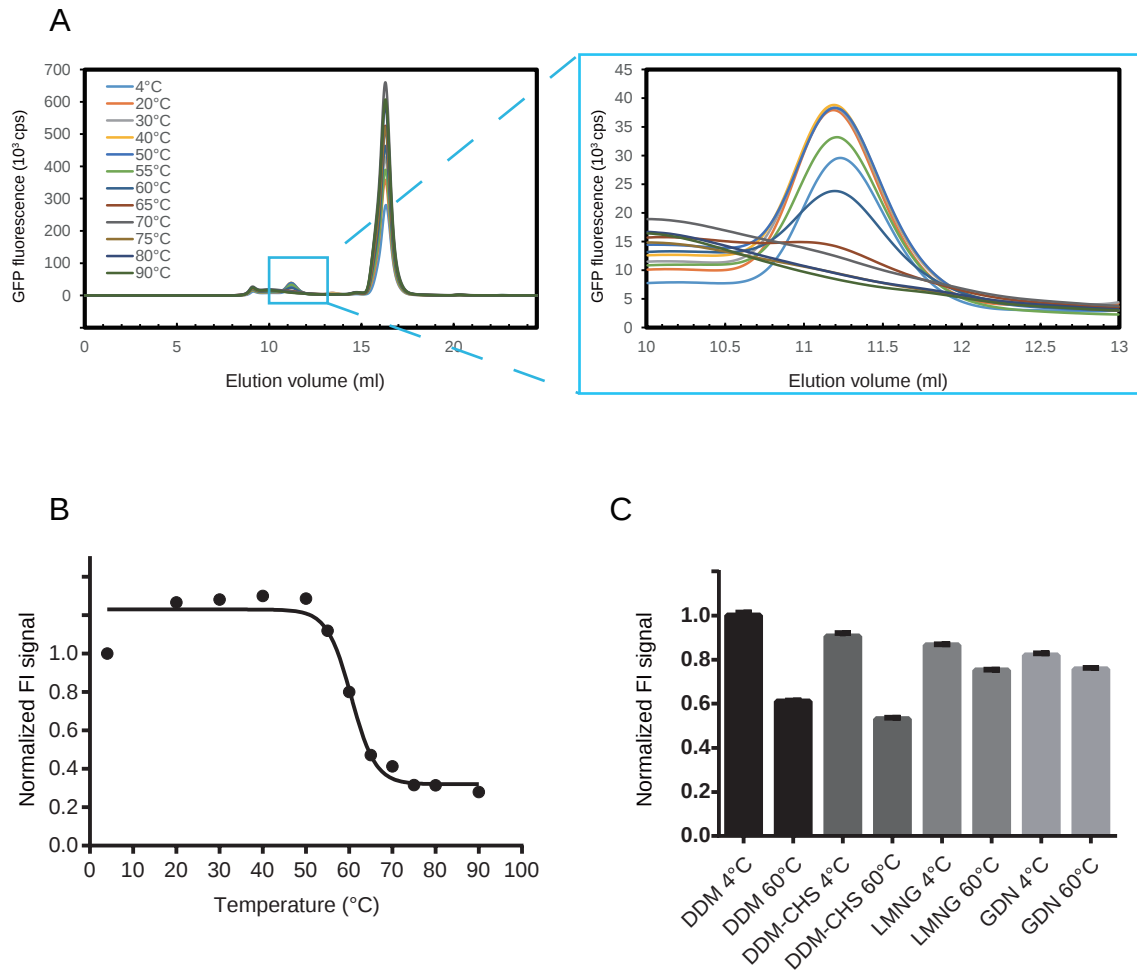


Figure 6

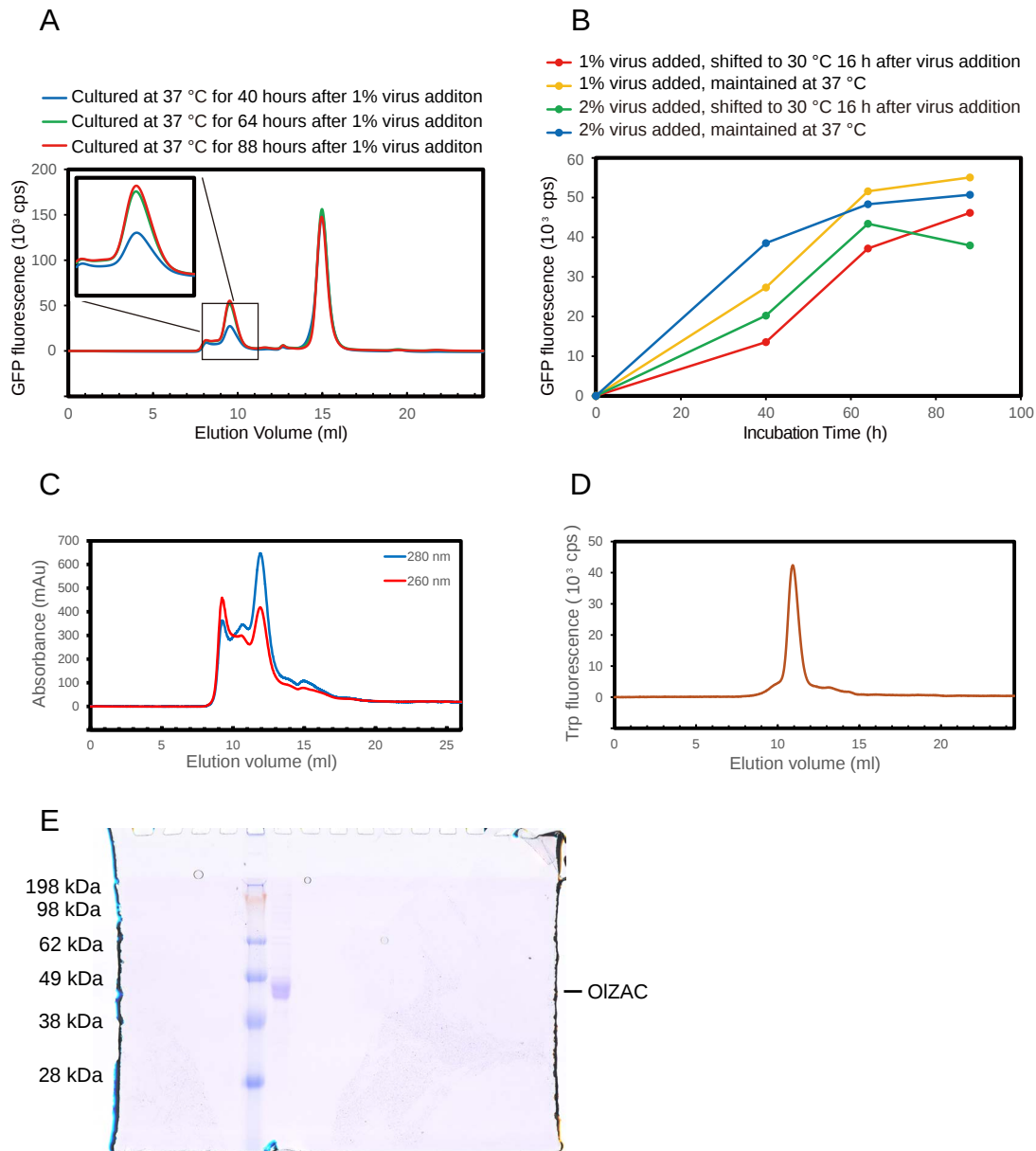


Figure 7

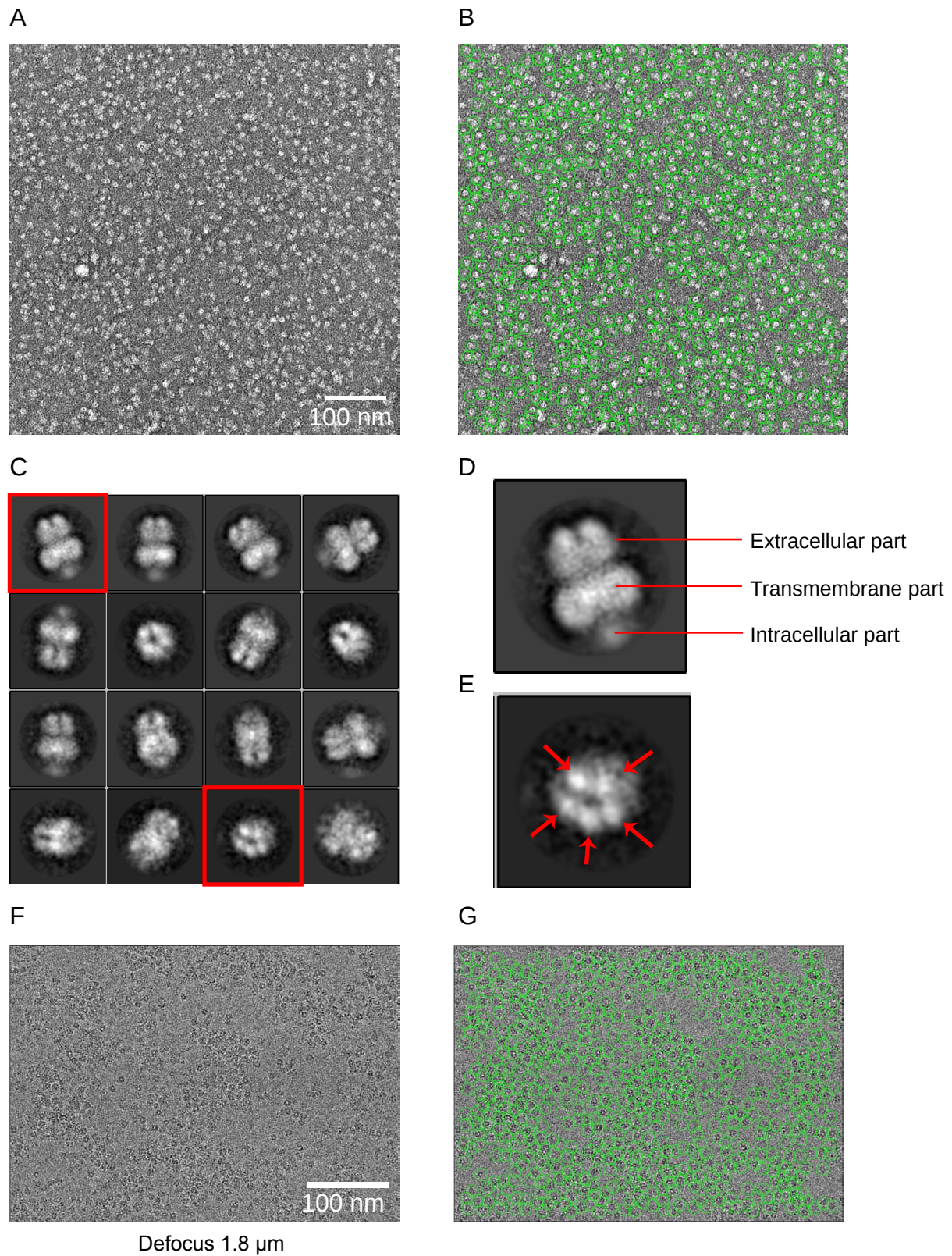


Figure 8

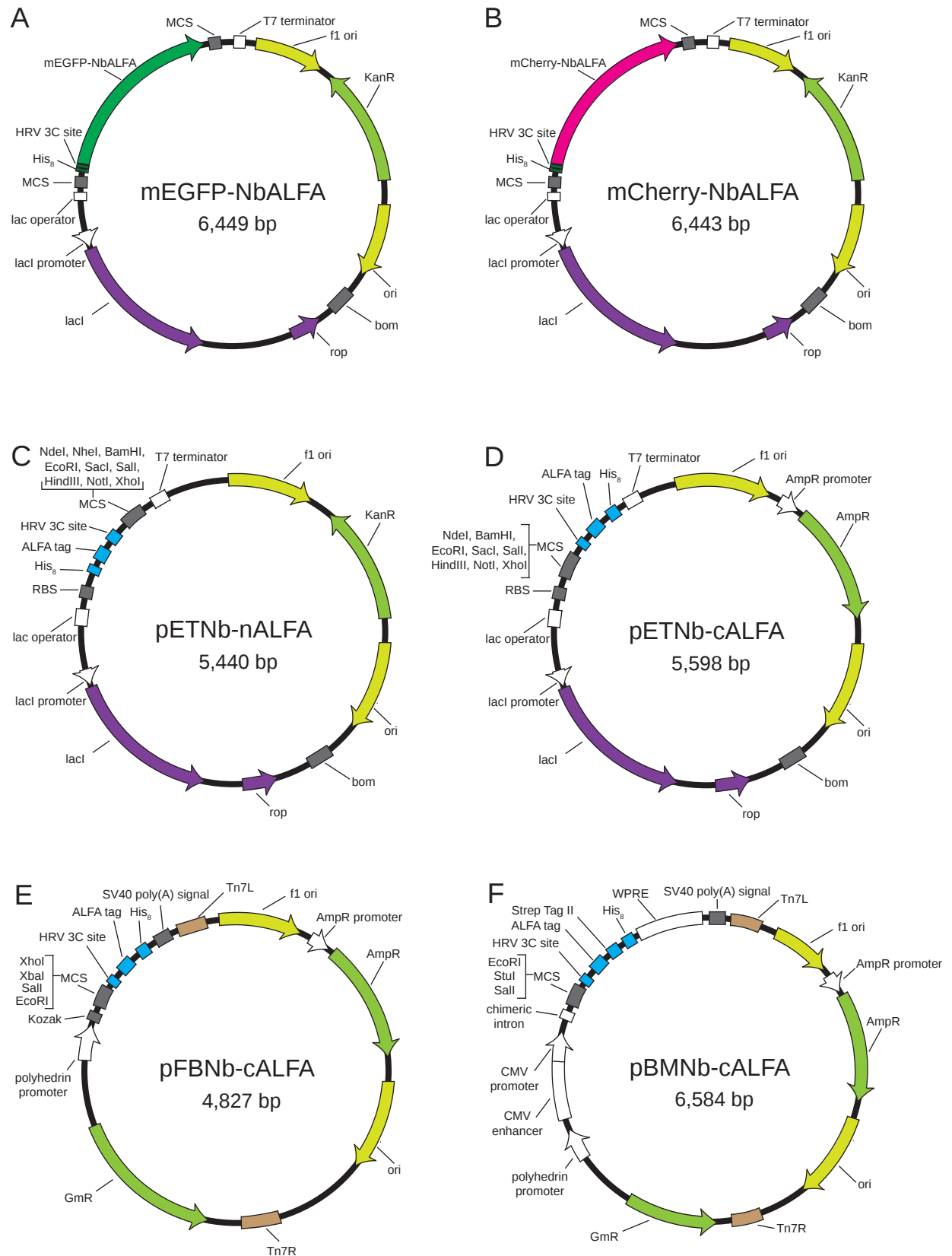


Figure 9

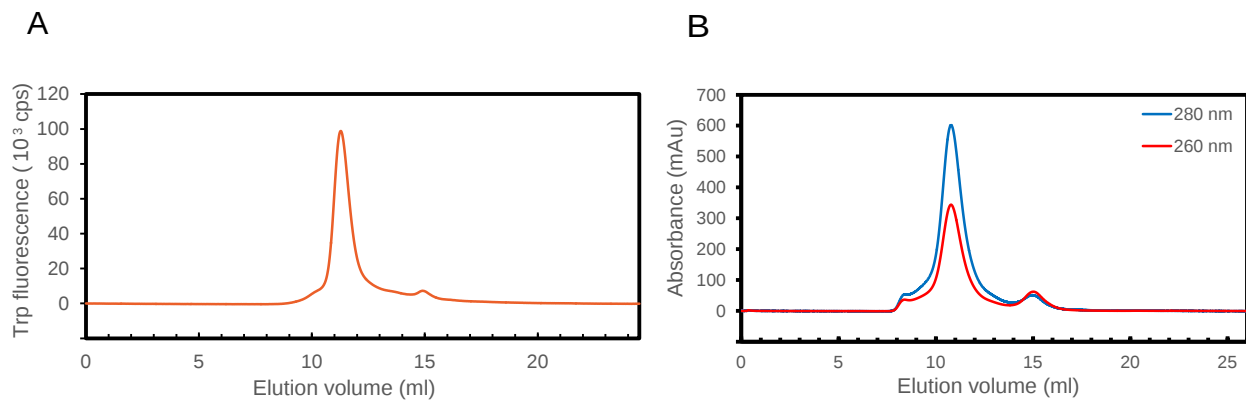


Figure S1

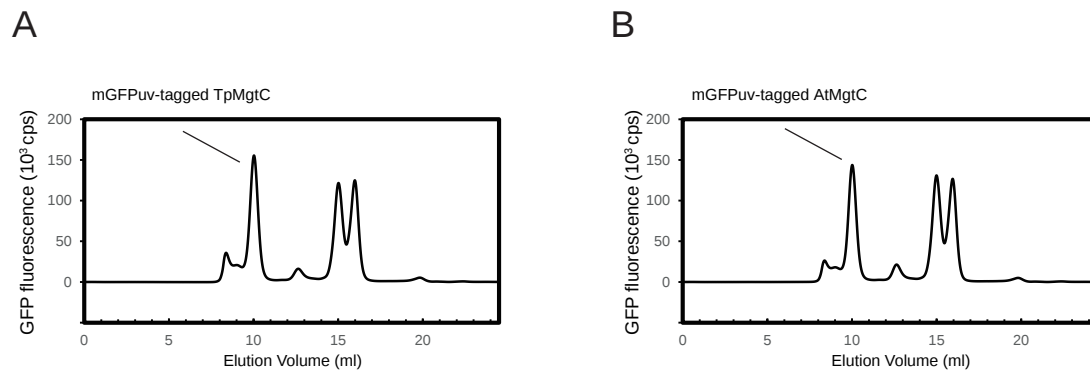


Figure S2

No	Species	Accession Number	Abbreviation
1	<i>Halobacillus halophilus</i>	WP_014641389.1	HhMgtC
2	<i>Lactococcus lactis</i>	WP_021722089.1	LiMgtC
3	<i>Mycobacterium aromaticivorans</i>	WP_051660484.1	MaMgtC
4	<i>Virgibacillus halodenitrificans</i>	WP_060678220.1	VhMgtC
5	<i>Klebsiella oxytoca</i>	WP_049112851.1	KoMgtC
6	<i>Yersinia ruckeri</i>	WP_004722955.1	YrMgtC
7	<i>Elizabethkingia meningoseptica</i>	WP_019051280.1	EmMgtC
8	<i>Methylophilus</i> sp.	WP_049639727.1	MsMgtC
9	<i>Pedobacter agri</i>	WP_010599226.1	PaMgtC
10	<i>Thermobacillus composti</i>	WP_015253117.1	TcMgtC
11	<i>Thermobispora bispora</i>	WP_013130974.1	TbMgtC
12	<i>Brevibacillus brevis</i>	WP_017246836.1	BbMgtC
13	<i>Vibrio vulnificus</i>	WP_045614446.1	VvMgtC
14	<i>Clostridium acetobutylicum</i>	WP_010966920.1	CaMgtC
15	<i>Xanthobacter autotrophicus</i>	WP_012113199.1	XaMgtC
16	<i>Bacillus cereus</i>	KYQ01271.1	BcMgtC
17	<i>Gracilibacillus boracitolerans</i>	GAE93792.1	GbMgtC
18	<i>Lactobacillus paracasei</i>	WP_016383475.1	LpMgtC
19	<i>Rubrobacter xylanophilus</i>	WP_011565853.1	RxMgtC
20	<i>Lysinibacillus boronitolerans</i>	WP_016994404.1	LbMgtC
21	<i>Desulfurispora thermophila</i>	WP_018084380.1	DtMgtC
22	<i>Thermanaerothermophilus daxensis</i>	WP_054522013.1	TdMgtC
23	<i>Thermocrinis albus</i>	WP_012992317.1	TaMgtC
24	<i>Sphaerobacter thermophilus</i>	ACZ39839.1	StMgtC
25	<i>Thermorudis peleae</i>	WP_038038224.1	TpMgtC
26	<i>Rubellimicrobium thermophilum</i>	WP_040645344.1	RtMgtC
27	<i>Quasibacillus thermotolerans</i>	WP_039233714.1	QtMgtC
28	<i>Thermincola ferriacetica</i>	WP_083436703.1	TfMgtC
29	<i>Caldicellulosiruptor naganensis</i>	WP_045165679.1	CnMgtC
30	<i>Anoxybacillus thermarum</i>	WP_043965058.1	AtMgtC
31	<i>Desulfofundulus</i>	WP_027355452.1	DeMgtC
32	<i>Thermodesulfobium narugense</i>	WP_013756617.1	TnMgtC
33	<i>Serratia</i>	WP_006319255.1	SeMgtC
34	<i>Bacillus mannanilyticus</i>	WP_025026422.1	BmMgtC
35	<i>Alkalihalobacillus akibai</i>	WP_035663104.1	AaMgtC
36	<i>Hungateiclostridium thermocellum</i>	WP_003513884.1	HtMgtC
37	<i>Stenotrophomonas pictorum</i>	WP_054658310.1	SpMgtC
38	<i>Prevotella maculosa</i>	WP_019967744.1	PmMgtC
39	<i>Prevotella salivae</i>	EFV04256.1	PsMgtC

40	<i>Prevotella veroralis</i>	WP_018911053.1	PvMgtC
41	<i>Prevotella</i> sp.	WP_177216065.1	PspMgtC
42	<i>Peptococcaceae</i> bacterium	KJS46311.1	PbMgtC
43	<i>Sphingobacteriales</i> bacterium	OJV97509.1	SbMgtC
44	<i>Yersinia pestis</i>	WP_015683614.1	YpMgtC
45	<i>Natronincola peptidivorans</i>	WP_090446121.1	NpMgtC
46	<i>Myxococcus hansupus</i>	WP_021781415.1	MhMgtC
47	<i>Obesumbacterium proteus</i>	WP_046459523.1	OpMgtC
48	<i>Limihaloglobus sulfuriphilus</i>	WP_146682549.1	LsMgtC
49	<i>Lactobacillus kefir</i>	WP_054769137	LkMgtC
50	<i>Dictyoglomus</i> sp.	PMQ01502.1	DspMgtC
51	<i>Bacillus</i> sp.	WP_094032362.1	BspMgtC
52	<i>Sphingobacterium detergens</i>	WP_120259343.1	SdMgtC
53	<i>Chryseobacterium culicis</i>	WP_105683769	CcMgtC
54	<i>Lactobacillaceae</i>	WP_021357857.1	LaMgtC
55	<i>Bacteroides eggerthii</i>	WP_118363478.1	BeMgtC
56	<i>Acidaminococcus</i>	WP_016459447.1	AcMgtC
57	<i>Tissierella</i> sp. P1	WP_094904138.1	TspMgtC
58	<i>Mucilaginibacter</i> sp.	WP_067187481.1	MspMgtC
59	<i>Erwinia typographi</i>	WP_034897147.1	EtMgtC
60	<i>Clostridium tepidiprofund</i>	WP_066821746.1	CtMgtC
61	<i>Arthrobacter</i> sp.	WP_155850019.1	AsMgtC
62	<i>Chitinophagaceae</i> bacterium	WP_157444983.1	CbMgtC
63	<i>Risunghinella massiliensis</i>	WP_044641850.1	RmMgtC
64	<i>Deltaproteobacteria</i> bacterium	OGR23206.1	DbMgtC
65	<i>Carnobacterium iners</i>	WP_085559504.1	CiMgtC
66	<i>Lactobacillus farraginis</i>	KRM01365.1	LfMgtC
67	<i>Thermosyntropha lipolytica</i>	WP_073089568.1	TlMgtC
68	<i>Acidobacteria</i> bacterium	PYX87356.1	AbMgtC

Table S1. MgtC orthologs for GFP fusion-based FSEC screening