Fluorescence-Detection Size-Exclusion Chromatography for Precrystallization Screening of Integral Membrane Proteins

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Summary

Formation of well-ordered crystals of membrane proteins is a bottleneck for structure determination by X-ray crystallography. Nevertheless, one can increase the probability of successful crystalization by precrystallization screening, a process by which one analyzes the monodispersity and stability of the protein-detergent complex. Traditionally, this has required microgram to milligram quantities of purified protein and a concomitant investment of time and resources. Here, we describe a rapid and efficient precrystallization screening strategy in which the target protein is covalently fused to green fluorescent protein (GFP) and the resulting unpurified protein is analyzed by fluorescence-detection size-exclusion chromatography (FSEC). This strategy requires only nanogram quantities of unpurified protein and allows one to evaluate localization and expression level, the degree of monodispersity, and the approximate molecular mass. We show the application of this precrystallization screening to four membrane proteins derived from prokaryotic or eukaryotic organisms.

Introduction

X-ray crystallography is currently the most powerful technique for determining atomic resolution structures of biological macromolecules (Hendrickson, 2000). The resulting atomic structures, in turn, not only provide insight into mechanism, but they may also accelerate the discovery of therapeutic agents (Blundell et al., 2002; Kuhn et al., 2002). In particular, the structures of membrane proteins, including receptors, channels, and transporters, are especially important because these molecules are the targets of most drugs currently prescribed (Zambrowicz and Sands, 2003). Unfortunately, atomic resolution structural information on membrane proteins is limited, as they are more difficult to express and crystallize compared to water-soluble proteins (Tate, 2001; Walian et al., 2004). In order to increase the likelihood of obtaining crystals of a membrane protein, it is advantageous to not only optimize the expression conditions, but to also characterize the homogeneity of the protein prior to crystallization.

In a typical scenario, one embarks on an effort to crystallize a new membrane protein by first examining differential expression systems and purification strategies, with the aim of obtaining milligram quantities of purified protein. With purified protein in hand, one may then assess the homogeneity of the sample, both in terms of chemical composition and oligomerization state, by a variety of techniques that might include SDS-PAGE, mass spectrometry, light scattering, and size-exclusion chromatography (SEC). If the protein proves to be homogenous, then one proceeds to crystallization. However, if the protein is not homogenous or fails to crystallize, then one may vary a number of parameters that might include the detergent and/or lipids used in purification and crystallization (Garavito and Ferguson-Miller, 2001; le Maire et al., 2000; Long et al., 2005; Seddon et al., 2004) and the nature of the protein construct (Cohen et al., 1995), perhaps also exploring the properties of proteins from other species (Kendrew, 1948, 1950; Kendrew and Parrish, 1956; Kendrew and Pauling, 1956). Here, we refer to the aforementioned experiments as “precrystallization screening” (Figure 1).

In traditional precrystallization screening, one typically monitors the presence of the protein by absorbance at 280 nm and by staining on SDS-PAGE gels (Figure 1A). To do this, however, microgram to milligram quantities of the protein are required for reliable detection. Because almost all proteins, as well as nucleic acids, absorb at 280 nm, the target protein must also be free of major contaminants. Thus, a substantial investment in time and resources must be made in order to bring a target molecule from the cloning stage to precrystallization screening. Moreover, because many target molecules fail precrystallization screening, due to polydispersity or instability, the time and resources invested in the moderate- to large-scale expression and purification of these proteins are wasted.

To obviate the requirement for moderate- to large-scale expression and purification, we have developed approaches that allow one to carry out precrystallization screening on nanogram quantities of unpurified protein obtained from whole-cell lysates or crude membrane preparations of prokaryotic or eukaryotic cells (Figure 1B). Here, the target proteins are covalently fused to green fluorescent protein (GFP) (Chalfie et al., 1994; Shimomura et al., 1962; Zhang et al., 2002). The resultant fusion proteins are monitored first for expression level and pattern in whole cells by epifluorescence microscopy (eukaryotic cells) or batch fluorescence measurements (prokaryotic cells). After solubilization of whole cells or crude membranes, SEC profiles are monitored by fluorescence spectroscopy. SEC is one of the most useful tools for monitoring the monodispersity and stability of the target protein; a monodisperse and folded protein will generally yield a single symmetrical Gaussian peak, while a polydisperse, unstable, or unfolded protein will typically yield multiple asymmetric peaks (Barth et al., 1994; Ricker and Sandoval, 1996).

In this article, we present the methodology of precrystallization screening by epi- and batch fluorescence, together with FSEC, followed by application of these methods to a eukaryotic, oligomeric ion channel protein...
and a bacterial transport protein. Subsequently, we describe the application of FSEC to the screening of tag position and of stability in different detergents. Finally, we evaluate Gaussian peak fitting of FSEC peak profiles as a method for quantitatively evaluating the mono- or polydispersity of a protein sample.

Results and Discussion

Covalent GFP Fusions

Our precrystallization screening methodology has two facets. The first involves a series of new expression vectors, for bacterial and mammalian cells, in which the target gene is covalently linked to GFP. Fused to the terminus of GFP is a polyhistidine tag for affinity purification, and inserted between the target protein and GFP is a thrombin site for proteolytic cleavage of the target protein from GFP (Figure 2). Enhanced green fluorescent protein (EGFP) is chosen for eukaryotic expression, and GFPuv (Crameri et al., 1996) is chosen for bacterial expression in order to (1) maximize the stability of the chromophore in each expression system, (2) exploit the stronger fluorescence signals in comparison to the wild-type counterparts, and (3) utilize genes that have codons optimized for each expression system (Crameri et al., 1996; Haas et al., 1996; Heim et al., 1995). To reduce GFP-mediated dimerization that might confound FSEC analysis, alanine 206 in both GFP variants is mutated to lysine (Zacharias et al., 2002). In all expression vectors, multiple cloning sites (MCSs) are located at the 5’ or 3’ side of the GFP coding sequence so that the target protein can be tagged with GFP at its N or C terminus, respectively. Because compatible MCSs are used for both N- and C-terminal GFP fusion vectors for each expression system, one can readily screen N- and C-terminal-tagged constructs by using the same PCR product.

The covalently fused GFP constructs allow one to visually inspect subcellular localization of proteins in eukaryotic cells by fluorescence microscopy and to determine protein expression in bacterial cells by batch fluorescence. Moreover, polyhistidine and thrombin sites in the GFP fusion vectors allow one to purify and characterize the proteins from a small number of cells and take advantage of the robust fluorescence from GFP. These features profoundly benefit precrystallization screening of integral membrane proteins whose expression levels are usually significantly lower than those of soluble proteins.

Figure 2. Maps of the GFP Fusion Vectors
(A) Eukaryotic expression vectors (pNGFP-EU and pCGFP-EU). Transcription is driven by a cytomegalovirus promoter (CMV) and terminated by SV40 polyadenylation sequences. Sequences encoding a polyhistidine tag, a thrombin proteolysis site (Th), and enhanced green fluorescent protein (EGFP) are located at either the 5’ end or the 3’ end of the multiple cloning sites (MCS). The restriction sites in the MCS, the stop codons, the A206K mutation in EGFP, and the translation initiation site (Kozak-ATG) are indicated.
(B) Bacterial expression vectors (pNGFP-BC and pCGFP-BC). For the bacterial expression vectors, transcription is directed by a T7 promoter and terminated by a T7 terminator sequence. The coding sequence is designed in the same way as for the eukaryotic expression vectors, except that a variant of uvGFP, which has codons optimized for bacterial expression, is used instead of EGFP.
Fluorescence-Detection Size-Exclusion Chromatography

The second facet of our precrystallization screening methodology involves a chromatography system fitted with an SEC column and a fluorescence detector. With this setup, one can monitor the elution of GFP fusion proteins in the context of whole-cell lysates or solubilized crude membranes (Figure 3). We call this method fluorescence-detection SEC (FSEC). Here, we will focus our attention on integral membrane proteins, although one can carry out FSEC precrystallization screening on water-soluble proteins as well.

For membrane proteins, crude membranes from bacterial cells or intact tissue culture cells are solubilized in a detergent-containing solution, followed by a high-speed centrifugation step (Figure 3A). The supernatant is then directly applied to an SEC column equilibrated in a detergent-containing solution, and the column is connected to a fluorometer fitted with a flow cell (Figure 3B). FSEC is a powerful screening method because the peak areas, profiles, and elution volumes provide information on (1) the expression level, (2) the degree of monodispersity, and (3) the approximate molecular mass of the fusion protein, respectively. Because FSEC exploits the unique fluorescence signal of GFP, neither protein purification nor large-scale culture is required; readily obtainable fluorometers can detect ~10 ng of GFP. In the following sections, we describe the application of our precrystallization screening strategy to four different membrane proteins expressed in bacterial or eukaryotic cells.

Precrystallization Screening of P2X Receptors

P2X receptors are eukaryotic integral membrane proteins that form ion channels gated by ATP (Khakh, 2001; North, 2002). There are seven P2X receptor subtypes (P2X<sub>1</sub>-<sub>7</sub>), and all subtypes except P2X<sub>6</sub> form functional channels when expressed in human embryonic kidney (HEK) 293 cells. To determine whether any of the rat P2X receptor subtypes would be suitable for crystallization trials, we carried out precrystallization screening with GFP fusion constructs. PCR products of P2X<sub>1</sub>-<sub>7</sub> genes were subcloned into either pC GFP-EU or pNGFP-EU, and the resulting plasmids were transfected into HEK293 cells. Two days after transfection, the subcellular localizations of the P2X receptors were checked by fluorescence microscopy (Figure 4A). On the basis of visual inspection, the C-terminally-tagged P2X constructs (C-P2Xs) expressed more robustly compared to the N-terminally-tagged variants (N-P2Xs). In the case of P2X<sub>2</sub>, both the N- and C-terminal variants resulted not only in fluorescence at the plasma membrane, but also in diffuse fluorescence in the cytoplasm, the latter of which was presumably due to free GFP generated by adventitious proteolysis. For P2X<sub>4</sub>, the C-P2X<sub>4</sub> variant appeared to express at the highest level and was found in intracellular puncta, while the N-P2X<sub>4</sub> construct was found primarily on the cell surface. Although it is possible that fusion of GFP to the receptor’s termini alters trafficking or ion channel function, we note that the approximate subcellular localizations of the C-terminally-tagged P2X receptors observed in the present study are consistent with previous observations (Bobanovic et al., 2002). Moreover, electrophysiological and ligand binding experiments have shown that the P2X<sub>4</sub>-GFP fusions possess essentially wild-type behavior (Kawate, 2005). Therefore, these data indicate that GFP fusions are relatively benign perturbations to the receptor.

To more quantitatively evaluate the expression level and the degree of monodispersity of the P2X constructs, the N- and C-terminal GFP fusions were expressed in transiently transfected HEK293 cells; one 35 mm dish was used for each construct. The cells were solubilized in a buffer containing the nonionic detergent n-dodecyl-β-D-maltoside (C<sub>12</sub>HM), and the resulting supernatant was analyzed by FSEC. As shown in Figure 4B, the fluorescence peak associated with the C-P2X<sub>4</sub> construct was much larger than the peaks from the other constructs, thus confirming the initial observation that C-P2X<sub>4</sub> expressed at a higher level than the other
constructs. For the C-P2X4 construct, the fluorescence peak was nearly symmetric, the elution position of the peak was suggestive of an oligomer, and there was only a small peak at the void volume of the column. The expression level of C-P2X4 was estimated as \( 1 \, \mu g \) per 10^6 cells by using a standard curve derived from known concentrations and fluorescence yields of recombinant GFP.

The other fusion proteins, C-P2X5, C-P2X7, N-P2X4, and N-P2X5, had reasonably symmetrical peak shapes, but they all expressed at much lower levels (Figure 4B). The C-P2X2 construct gave rise to a small but significant peak at the void volume, suggesting that it had a tendency to form high-molecular weight aggregates. Interestingly, the C-P2X3 supernatant contained a substantial amount of free GFP (Figure 4B, bottom panel), which was consistent with the previous fluorescence microscopy (Figure 4A). Taken together, inspection of transfected cells by fluorescence microscopy and analysis of solubilized cells by FSEC suggest that the rat P2X4 receptor is a promising molecule for crystallization trials.

Precrystallization Screening of Bacterial Homologs of Na⁺/Cl⁻-Dependent Neurotransmitter Transporters
Na⁺/Cl⁻-dependent neurotransmitter transporters (NSS) are integral membrane proteins that use ion gradients to drive the uptake of a broad array of substrates, including the biogenic amines, amino acids, and osmolytes (betaine and creatine), into cells (Masson et al., 1999). In an effort to obtain crystals of a bacterial homolog of an NSS protein, genes corresponding to orthologs from six prokaryotic organisms (genes 1–6) were subcloned into either pCGFP-BC (C-1, C-2, ..., C-6) or pNGFP-BC (N-1, N-2, ..., N-6) vectors and screened by FSEC after solubilization of crude membranes in a buffer containing C₁₂M. As shown in Figure 5A, the expression levels and the degree of monodispersity of the C-terminal GFP fusions varied substantially. Moreover, the expression levels of constructs C-2 and C-6 were much greater than those of C-1, C-3, C-4, and C-5. Interestingly, the proteins that were more abundantly expressed yielded more symmetric peaks, whereas those that were poorly expressed gave less symmetric peaks, suggestive of aggregation, misfolding, or heterogeneity in subunit stoichiometry. The differences of the calculated molecular masses of the fusion proteins are within 10%, yet proteins C-1 and C-3 eluted significantly later than the other proteins. One explanation for this behavior is that proteins C-1 and C-3 were binding to the resin, perhaps due to misfolding or partial unfolding. The FSEC traces for the N-terminal fusions showed lower expression levels compared to the C-terminal variants, suggesting that tagging at the N terminus had a deleterious effect on expression (data not shown).

On the basis of the FSEC screening, the target proteins of the C-2 and C-6 constructs were subjected to crystallization trials with proteins that were expressed as non-GFP fusions. After affinity purification, the purified C-6 product was subjected to SEC detected by absorbance at 280 nm (Figure 5B). The qualitative similarity of the FSEC (Figure 5A) and the SEC profiles indicates that the GFP tag, as well as the purity and concentration of the target protein, did not substantially affect the
monodispersity of the C-6 construct. In fact, the target protein from the C-6 construct crystallized readily, yielding crystals that diffracted beyond 1.7 Å resolution (Figures 5C–5E) (Yamashita et al., 2005).

Screening Tag Position by FSEC

Using traditional approaches to precrystallization screening, we had previously obtained diffraction-quality crystals of the trimeric glutamate transporter homolog from Pyrococcus horikoshi (GltPh) expressed with a C-terminally-tagged construct (Figures 6B and 6C) (Yernool et al., 2004). Interestingly, N-terminally-tagged variants of GltPh did not express as well as C-terminally-tagged constructs and did not yield crystals (data not shown). At the molecular level, inspection of the GltPh crystal structure shows that the C terminus is projecting away from the protein; thus, it appears that the protein can accommodate a C-terminal tag. A molecular understanding of the difficulties encountered with N-terminal fusions is less clear, in part because the first few residues of the protein cannot be reliably positioned in electron density. However, there are electron density features that suggest that the N terminus makes contact with the protein core, and this may be why N-terminal fusions are not tolerated.

To test whether FSEC can provide data to determine optimal tag location, we cloned the GltPh gene into the pCGFP-BC and pNGFP-BC E. coli expression vectors, yielding the C-GltPh and N-GltPh constructs. Analysis of crude solubilized membranes by FSEC (Figure 6A) showed that while C-GltPh had a narrow and symmetric peak, N-GltPh yielded a smaller and asymmetric peak, suggestive of heterogeneity in subunit stoichiometry and/or incomplete assembly. These results are consistent with our previous observations made prior to the development of FSEC technology. Therefore, the studies of the N- and C-terminal fusions of GltPh highlight the importance of screening fusions at both ends of the target protein, and they emphasize how FSEC precrystallization analysis can provide important information rapidly and easily.

Detergent Screening by FSEC

Successful crystallization of a membrane protein is often critically dependent on the detergent, and, in many cases, the most well-ordered crystals are formed in...
the presence of the detergent that forms the smallest micelles but that nevertheless maintains the protein in a monodisperse and stable state (Michel, 1983; Ostermeier and Michel, 1997). The traditional approach to screening detergents involves purification of the membrane protein of interest, exchange of the protein into a panel of detergents, and subsequent evaluation of the degree of monodispersity and stability. This traditional approach is exceptionally time and resource consuming, and, therefore, identifying optimal detergents is one of the major technical obstacles for membrane protein crystallization. However, one can instead use FSEC to determine the degree of monodispersity and stability of the target protein, without purification, by using whole-cell lysates.

Here, we show a typical example of detergent screening by FSEC with the eukaryotic integral membrane protein J, a member of the ENaC/DEG family of ion channels (Kellenberger and Schild, 2002). It was expressed in Sf9 insect cells by recombinant baculovirus infection as a C-terminal GFP fusion (C-J). The baculovirus DNA was created by site-specific transposition in E. coli cells with a plasmid containing the entire coding region of the fusion protein. C-J-expressing Sf9 cells were solubilized in six detergents (C12M, n-decyl-β-D-maltoside [C10M], n-octyl-β-D-glucoside [β-OG], octaethylene glycol monododecylether [C12E8], lauryl dimethylamine-N-oxide [LDAO], and CHAPS), and after high-speed centrifugation, the supernatants were analyzed by FSEC by using a column equilibrated in C12M. In our experience, we have found that the deleterious action of a destabilizing detergent is not “rescued” by a mild and typically non-denaturing detergent such as C12M in the mobile phase of the SEC column.

As shown in Figure 7, peak profiles from the solubilized samples, except for those in β-OG and CHAPS, were sharp and symmetrical, suggesting that the protein was monodisperse in these detergents. In C12M, C12E8, and LDAO, only a small fraction of the protein migrated in the void volume of the column, thereby indicating that the protein did not tend to aggregate under these conditions. Significantly, the major peak of the C-J eluted at a position that is consistent with a trimer or tetramer, and previous studies have suggested a tetrameric subunit stoichiometry for this class of proteins (Firsov et al., 1998). In C10M, however, a significant fraction eluted in the void volume despite the fact that the major peak was still sharp and symmetrical. This observation suggests that although C-J could be solubilized in this detergent without disruption of its native association state, it was only marginally stable in C10M. In fact, when the peak fraction was reanalyzed by FSEC after 3 days at 4°C, most of the protein eluted in the void volume (data not shown). When C-J was analyzed after solubilization in β-OG and CHAPS, the major fluorescence...
peaks were broad and asymmetric, indicating that these two detergents were not suitable for maintaining C-J in a stable, monodisperse state. In conclusion, these studies suggested that C12M, C12E8, and LDAO were the most promising detergents for purification and crystallization of the C-J.

Gaussian Peak Fitting as a Tool for FSEC Peak Validation
In many cases, simple visual inspection of FSEC profiles can allow one to estimate the expression level and degree of monodispersity of a target protein. However, there are instances when visual inspection is inadequate. Since peak shapes of monodisperse proteins eluting from an ideal size-exclusion chromatography column obey Gaussian distributions, the peak(s) corresponding to the target protein can be fit to Gaussian functions. Depending on the extent to which more than one function is required to fit the peak in question, one can then estimate whether a particular peak is composed of more than one species (Barth et al., 1994). For example, if a given peak from a FSEC trace requires multiple Gaussians to achieve a reasonable fit, then it is likely that the target protein is correspondingly heterogeneous in aggregation or association state and is thus not suitable for crystallization trials. Although Gaussian fitting of chromatographic peaks may not always be ideal due to broadening processes reflective of extra-column volumes, axial diffusion, dispersion, and solute and resin interactions (Popovici et al., 2004; Stulik et al., 2003), it is a robust and rapid way to visualize and quantify the possible peaks underlying a specific FSEC trace.

As one example, we fit the profiles of C-GltPh and N-GltPh, with multiple Gaussian functions. As shown in Figure 8 and Table 1, the C-GltPh trace was adequately fit to three Gaussians (peaks 1 and 2 and GFP), while the N-GltPh trace required four (peaks 1–3 and GFP). Therefore, the major difference between the two constructs is that the N-GltPh construct has a substantial third peak that likely corresponds to monomeric subunits of the transporter (Figure 8B, peak 3). Interestingly, peak fitting of both the N-GltPh and C-GltPh profiles suggests that the major peak may have a second component (Figures 8A and 8B, peak 2). At this point, it is not clear whether peak 2 actually represents a discrete entity or whether it is merely a reflection of nonideal migration on the column. Nevertheless, this analysis demonstrates that the C-GltPh construct is less polydisperse than the N-GltPh construct; therefore, it is a more promising species for crystallization trials.

FSEC versus SEC
For a variety of proteins we have tested so far, the FSEC profiles of the fusion proteins were similar to the SEC profiles of the purified nonfusion proteins (e.g., Figures 5 and 6). However, we have encountered a few situations where fusion with GFP does alter the behavior of the target protein, thereby compromising the utility of the FSEC approach. Additionally, when SEC is performed on more concentrated purified proteins, the profiles may differ from those of FSEC because this latter technique is typically carried out on diluted samples. Nevertheless, in our experience, these occurrences are rare, and they do not outweigh the great advantages of a fluorescence-based precrystallization screening strategy. Therefore, in the initial steps of a crystallography project, FSEC is an efficacious and practical method for precrystallization screening of integral membrane proteins.

Conclusions
X-ray crystallography of integral membrane proteins is problematic because finding a membrane protein that will yield diffraction-quality crystals requires a substantial investment in time and resources. However, we have shown that unproductive large-scale protein expression and purification can be minimized by fluorescence-detection size-exclusion chromatography (FSEC), a rapid precrystallization screening method in which monodispersity and stability of the target protein are characterized with only nanogram quantities of unpurified protein. In this method, the target protein is covalently fused to GFP, and the resulting unpurified fusion protein is analyzed by SEC. Although the GFP fusion technique has been used previously to monitor bacterial membrane protein expression and to screen detergents used for solubilization (Drew et al., 2001, 2005), to our knowledge it is novel to combine covalent GFP fusion and SEC techniques to analyze monodispersity and stability of the fusion protein for protein crystallization. Moreover, FSEC-based precrystallization screening may be exploited with the previously described GFP fusion approach to establish cell lines possessing high expression levels of promising constructs (Mancia et al., 2004).

In this report, the advantages and significance of covalent GFP fusion proteins and FSEC precrystallization screening were demonstrated in examples with eukaryotic and prokaryotic membrane proteins. In these experiments, small amounts of unpurified target membrane proteins were rapidly and easily evaluated for solubility and molecular mass and stability in detergent conditions. The utility of this approach is further emphasized by its successful application to a bacterial integral membrane transport protein that yielded crystals that diffracted beyond 1.7 Å resolution.

Experimental Procedures

GFP Fusion Vector Construction
The eukaryotic GFP fusion vectors (pCGFP-EU and pNGFP-EU) were created by using standard molecular biology techniques starting with the pEGFP-C1 vector obtained from Clontech. Briefly, the

| Table 1. Peak Fitting Summary of C-GltPh and N-GltPh |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | c Fit standard error, SE = sqrt(SSSE/DOF) |                             |                             |                             |
|                             | a Degree of freedom-adjusted coefficient of determination, DOF r^2 = (1 − SSE/n − 1)/SSM/DOF − 1) |
|                             | a r^2 coefficient of determinant, r^2 = 1 − SSE/SSM |
|                             | b SSE, sum of squared errors; SSM, sum of squares about the mean; n, total number of data values; m, the number of coefficients in the model |

GFP Fusion Vector Construction
The eukaryotic GFP fusion vectors (pCGFP-EU and pNGFP-EU) were created by using standard molecular biology techniques starting with the pEGFP-C1 vector obtained from Clontech. Briefly, the
original multiple cloning site (MCS) was replaced with either an octahistidine coding sequence (His6) for pCGFP-EU or with a thrombin recognition site coding sequence (TRS) followed by a new MCS (XhoI-HindIII-EcoRI-Pst-Sal) for pNGFP-EU. The NheI and NotI sites at the 5' side of the EGFP sequence were used to introduce either the new MCSs and TRSs for pCGFP-EU or the His6 sequence for pNGFP-EU. To minimize EGFP dimerization, alanine 206 was mutated to a lysine residue by PCR.

The N-terminal bacterial GFP fusion vector (pNGF-BC) was created by inserting GFPuv into the pET22c (Novagen) vector, together with the His6 and TRS described above. The original GFPuv coding sequence was obtained from a plasmid containing GFPuv kindly gifted from Dr. John Hunt, and it was modified by PCR to (1) change alanine 206 to a lysine, (2) remove XhoI, BamHI, HindIII, and NcoI sites, and (3) add a polyasparagin linker at the 3' end (GFPuv-β). The SpeI site in the pET22c vector was knocked out by PCR, and a His6-TRS was inserted into the pET22c vector between the NdeI and NcoI sites (pET22c-β). Subsequently, the MCS in pET22c-β between the BamHI and XhoI sites was replaced by a pair of synthetic oligos in order to introduce a stop codon after the XhoI site (pET22c-γ). Finally, GFPuv-β was inserted into pET22c-γ between the Agel and SpeI sites to produce pNGF-BC.

The C-terminal bacterial GFP fusion vector (pGFP-BC) was created by using GFPuv-β and pET22b (Novagen). Briefly, a plb leader peptide sequence was removed from pET22b by a pair of synthetic oligos (pET22b-β). TRS and His6 coding sequences were added to GFPuv-β at its 5' and 3' ends, respectively, by PCR, and inserted between the XhoI and NheI sites of pET22b-β. In order to make the MCS of pGFP-BC compatible with that of pNGF-BC, a NcoI site was knocked out, and the NdeI site was converted to a new NcoI site by PCR. The resulting MCS includes NcoI, BamHI, EcoRI, SacI, HindIII, NotI, and XhoI sites. Finally, the first Met in GFPuv was mutated to Val to minimize internal translation initiation.

**FSEC for P2X Receptors**

HEK293 cells were cultured to ~90% confluency in a 6-well plate (Corning) and transiently transfected with the specific expression constructs (1 μg/well) by using Lipofectamine 2000 (3–5 μl Invitrogen) as instructed by the manufacturer. After incubating for 24–48 hr, the cells were harvested by gentle pipetting, washed with PBS, and resuspended in 500 μl solubilization buffer (PBS pH 8.0, 20 mM C₂₀M, and 1 μl protease inhibitor cocktail set III [Calbiochem]). The resulting suspension was rotated for 1 hr at 4°C, followed by centrifugation at 66,000 × g for 40 min. A fraction of the supernatant (200 μl) was loaded onto a Superose 6 column (10/30, Amersham Biosciences) equilibrated with SEC buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, and 1 mM C₂₀M) and run at a flow rate of 0.5 ml/min. The eluent was detected by a fluorometer as described above, with the fit standard error, and the F value.

**FSEC for Bacterial Proteins**

The desired expression vector was transformed into BL21(DE3) pLysS-competent cells by using standard methods, and the resulting cells were plated onto LB agar plates supplemented with ampicillin and chloramphenicol. After incubation for ~16 hr at 37°C, a single colony was picked and used to inoculate 10 ml LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. Cells were cultured in a shaker at 37°C. When the OD₆₀₀ reached ~0.6, expression was induced by the addition of 1 mM IPTG, and the cells were grown for an additional 3 hr. The cells were collected by centrifugation, resuspended in 500 μl sonication buffer (50 mM Tris-HCl pH 8.0, 190 mM NaCl, 10 mM KCl, 15 mM EDTA, 10 μM lysozyme, and 100 μM PMSF), and disrupted by sonication on ice. Sonication was repeated four times with 1 min intervals by using VirSonic475 (Virtis) in which each cycle was programmed as follows: sonication time, 1 s; interval, 1 s; total sonication time, 10 s. The sonicated sample was first centrifuged at 10,000 × g for 15 min to pellet unbroken cells, and then the membranes were collected by a second centrifugation at 200,000 × g for 20 min. The membrane pellet (~5–20 μg) was solubilized with 500 μl solubilization buffer 1 (50 mM Tris-HCl [pH 8.0], 190 mM NaCl, 10 mM KCl, 15 mM EDTA, 100 μM PMSF, and 40 mM C₁₂M) and gently mixed at 4°C for at least 1 hr, followed by centrifugation at 200,000 × g for 20 min. A fraction of the supernatant (200 μl) was loaded onto a Superose 6 10/30 column preequilibrated in running buffer (20 mM Tris-HCl [pH 8.0], 190 mM NaCl, 10 mM KCl, 1 mM C₁₂M) and run at the flow rate of 0.5 ml/min. The eluent was detected by a fluorometer as described above, with the only difference being the excitation wavelength (395 nm) and emission wavelength settings (507 nm).

**Detergent Stability Screening of a Eukaryotic Membrane Protein J**

Eukaryotic membrane protein J was expressed in Sf9 cells by baculovirus infection for 72 hr at 27°C by using standard methods. The baculovirus was created from pFastBac (Invitrogen) in which the entire coding sequence of C-terminally-tagged protein J from pCGFP-EU vector was inserted between the BamHI and HindIII sites. Cells from 1 ml of culture were collected by centrifugation and solubilized in 300 μl PBS (Cellgro) containing one of the following detergents, where the final detergent concentration is given in parentheses: C₁₂M (20 mM), C₁₀M (20 mM), i-OG (250 mM), C₂₀E₈ (20 mM), LDAO (20 mM), and CHAPS (125 mM). Solubilization was carried out for 1 hr at 4°C with gentle rotation. After centrifugation at 66,000 × g for 40 min, 200 μl of the soluble fraction was loaded onto a Superose 6 10/30 column preequilibrated with running buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM C₂₀M), and FSEC was carried out as described above.

**Gaussian Peak Fitting**

Fluorescence values between 1000 s and 2500 s on the FSEC traces of C-Gltm and N-Gltm were imported to PeakFit software (SasSolve Software, Inc). Initial peak detection and fitting were done by the residual method, and the Gaussian functions were further fitted to the original peaks by using a least squares minimization algorithm with iteration cycles of 61 (C-Gl) and 34 (N-Gl). The resulting Gaussian peaks were validated by the r² coefficient of the determinant, the degree of freedom-adjusted coefficient of determination, the fit standard error, and the F value.

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