Expression Screening of Integral Membrane Proteins by Fusion to Fluorescent Reporters

1

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Abstract

The production of recombinant integral membrane proteins for structural and functional studies remains technically challenging due to their relatively low levels of expression. To address this problem, screening strategies have been developed to identify the optimal membrane sequence and expression host for protein production. A common approach is to genetically fuse the membrane protein to a fluorescent reporter, typically Green Fluorescent Protein (GFP) enabling expression levels, localization and detergent solubilisation to be assessed. Initially developed for screening the heterologous expression of bacterial membrane proteins in *Escherichia coli*, the method has been extended to eukaryotic hosts, including insect and mammalian cells. Overall, GFP-based expression screening has made a major impact on the number of membrane protein structures that have been determined in the last few years.

Keywords

Integral membrane protein • Green fluorescent protein • Insect cells • *Escherichia coli* • *Saccharomyces cerevisiae* • *Pichia pastoris* • HEK 293 cells

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1.1 Introduction

The production of recombinant integral membrane proteins (IMPs) for structural and functional studies is technical challenging due to low levels of expression often limited by toxicity to the expression host cells. To overcome these limitations screening of sequence variants either engineered or exploiting the natural sequence diversity of orthologues, has been successfully used to improve the production of many mem-

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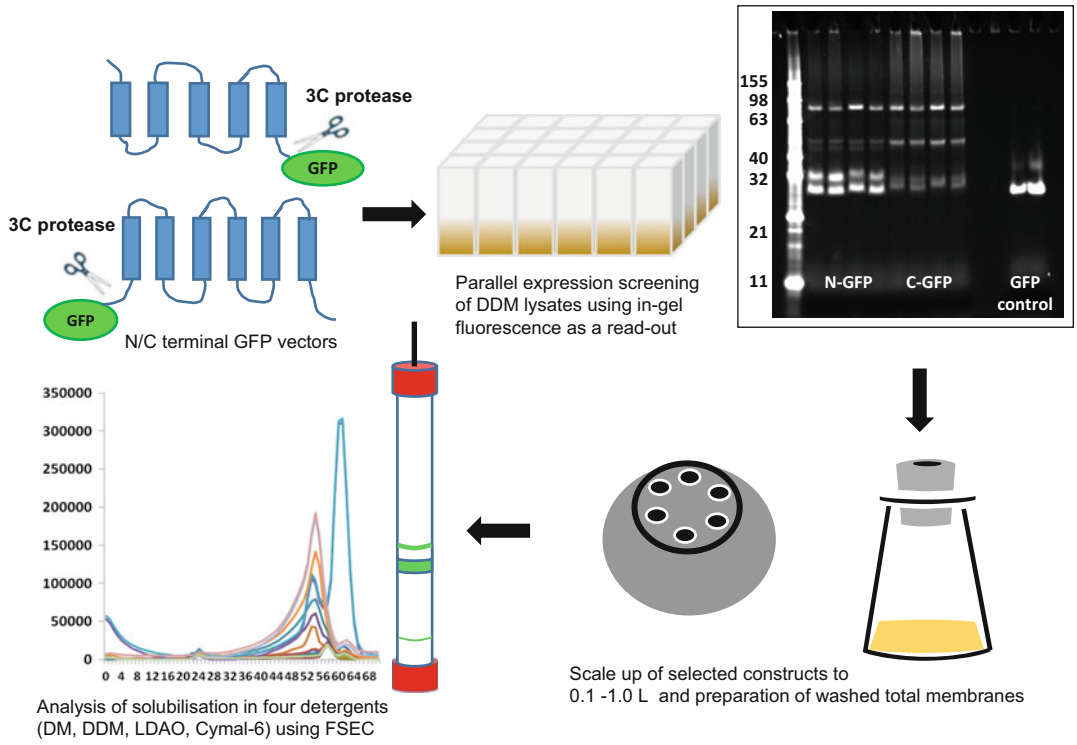

Fig. 1.1 Schematic diagram of workflow for screening for expression of integral membrane proteins

brane proteins. This approach has been greatly facilitated by genetic fusion to a fluorescent reporter protein, typically Green fluorescent protein (GFP). This enables rapid expression screening and hence identification of proteins that are stably inserted into the membrane without the need to purify the membrane protein (Drew et al. 2005). Once a well expressed stable protein is identified the GFP moiety can also be used to monitor purification and for pre-crystallization screening (Drew et al. 2006; Kawate and Gouaux 2006). A generic workflow for this method is shown in Fig. 1.1. In this chapter the use of GFP as a reporter for the expression of membrane proteins in different heterologous hosts will be reviewed.

1.2 Bacteria

Escherichia coli is the most commonly used prokaryotic host for overexpression of IMPs, followed by the Gram positive bacterium, *Lactococcus lactis* (Kunji et al. 2003; Drew et al. 2006; Gordon et al. 2008; Frelet-Barrand et al. 2010; Chen 2012; King et al. 2015). Bacterial hosts have obvious advantages for the over-expression of recombinant proteins with rapid growth rates, inexpensive growth media and the ease of genetic manipulation. Moreover, the biology of transcription, translation and insertion into membranes are also well characterised, allowing manipulation of the host cell to facilitate heterologous expression of proteins. Nevertheless, the expression of membrane proteins in bacteria can be problematical for a number of reasons. The expressed protein may prove to be toxic to the host cell (Kunji et al. 2003) or saturate the membrane insertion machinery (Loll 2003; Wagner et al. 2006). Rare codons in the protein or insufficient amino acid availability (Angov et al. 2008; Marreddy et al. 2010; Bill et al. 2011) or insufficient membrane capacity (Arechaga et al. 2000) may all limit the expression of membrane proteins in bacteria. Therefore, screening for correctly folded protein

is critical, with fusion to GFP at either the N or C-terminus now being widely used as a reporter of insertion into the bacterial membrane (Drew et al. 2001; Sonoda et al. 2011; Lee et al. 2014a, b, c). The combination of (1) high-throughput cloning strategies to construct fusion GFP fusion vectors with (2) screening in *E. coli* using in gel-fluorescence of detergent lysates of whole cells, enables the expression of large numbers of IMPs to be evaluated at small scale (Sonoda et al. 2011; Schlegel et al. 2012; Lee et al. 2014a; Bird et al. 2015). For example, in one study, 47 orthologues of bacterial SEDS (shape, elongation, division, and sporulation) proteins were cloned and candidate proteins rapidly identified for further analysis (Bird et al. 2015). Typically an affinity purification tag, for example octa-histidine, is included with the GFP reporter so that fluorescence can be used to monitor the mono-dispersity and integrity of the membrane proteins during purification by size exclusion chromatography (Fluorescence detected Size Exclusion Chromatography, FSEC) (Drew et al. 2006; Bird et al. 2015). Thus, fusion to GFP has facilitated purification to homogeneity and subsequent crystallization of many IMPs expressed in *E. coli*, for example, *Pseudomonas aeruginosa* lysP, *E. coli* sodium-proton NhaA and the *Streptococcus thermophilus* peptide transporter $PepT_{St}$ (Lee et al. 2014b; Nji et al. 2014).

Fusion of IMPs to GFP is useful for comparing expression in different strains of bacteria (see Fig. 1.2 for an example). The *E. coli* strain BL21(DE3) and related strains are most commonly used for heterologous protein production. In these strains, the bacteriophage T7 RNA polymerase is expressed from the mutant *lac*UV5 promoter resulting in high-level expression of a polymerase that is more processive than the native *E. coli* RNA polymerase (Iost et al. 1992). Driving transcription generally leads to higher levels of heterologous protein production. How-

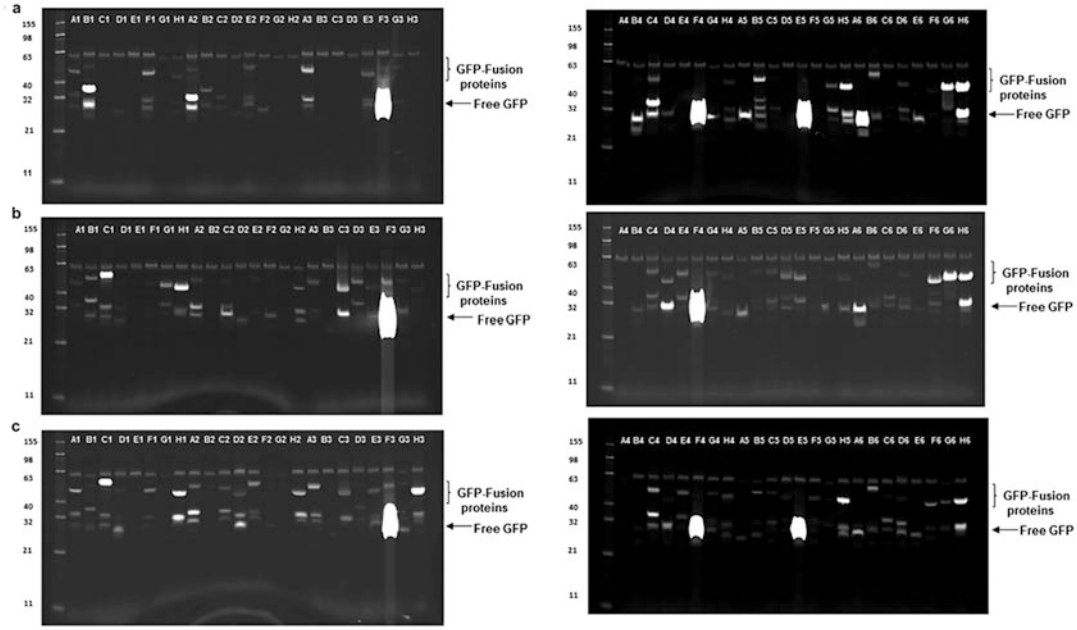

Fig. 1.2 Screening expression in *E.coli* of 47 SED (Sporulation Elongation Division) proteins from a wide range of bacteria, by in-gel fluorescence. Strains were grown in Powerbroth (Molecular Dimensions) and expression induced at 20 °C overnight. (**a**) C41(DE3) plysS, induced with 1 mM IPTG. (**b**) Lemo21 (DE3), grown in the presence of 0.625 mM rhamnose and induced with 1 mM IPTG. (**c**) KRX, induced with 2.5 mM rhamnose and 1 mM IPTG. Detergent lysates of *E. coli* cells were analysed by SDS-PAGE and gels imaged using Blue Epi illumination and a 530/28 filter. A GFP control is shown in lane F3 and the numbers to the *left* refer to the sizes in kDa of molecular weight markers run in parallel

ever, for membrane proteins this can result in saturation of the *Sec* translocon and subsequent misfolding of much of the expressed membrane protein (Wagner et al. 2006, 2007; Klepsch et al. 2011). To avoid this problem, Miroux and Walker isolated strains of BL21(DE3) that survived the over-expression of membrane proteins by an unknown mechanism (Miroux and Walker 1996). These strains, C41(DE3) and C43(DE3), known as the Walker strains, are used pragmatically to express a membrane proteins, though high levels of expression are not seen for all membrane proteins (Miroux and Walker 1996; Wagner et al. 2008). Analyses of the Walker strains, using the bacterial membrane protein YidC fused to GFP (Wagner et al. 2007), showed that mutations in the *lac*UV5 promoter are responsible for the often improved membrane protein expression (Drews et al. 1973; Wagner et al. 2008). The mutations that were found, result in lower levels of mRNA production and hence a slower rate of protein synthesis. This presumably ensures that membrane protein translocation machinery is not saturated.

These data suggested that to optimize expression levels of folded and functional inserted IMPs, it is important to match the rate of transcription /translation with the capacity of the *Sec* translocon. The Lemo21(DE3) strain has been specifically engineered according to this principal and incorporates the gene for T7 lysozyme on a plasmid under the control of the highly titratable rhamnose promoter (Giacalone et al. 2006; Wagner et al. 2008). T7 lysozyme is an inhibitor of T7RNA polymerase, and Schlegel et al. showed that the expression level of a number of membrane proteins could be optimised by varying the level of rhamnose in the cell media (Schlegel et al. 2012). However, not all IMPs express well in Lemo21(DE3) and screening *E. coli* strains with different expression kinetics is important for achieving expression (Schlegel et al. 2012; Bird et al. 2015).

Fusion of IMPs with GFP at the C-terminus of the protein in tandem with the erythromycin resistance protein (23S ribosomal RNA adenine *N*-6 methyl transferase, ErmC) has been used to evolve both *E coli* and *L. lactis* strains for improved production of membrane proteins (Linares et al. 2010; Gul et al. 2014). In both cases the protein is under the regulation of a titratable promoter, the arabinose inducible pBAD promoter in *E. coli* and the NICE (nisin-inducible controlled gene expression) promoter in *L. lactis*. In this approach, the optimum inducer concentration, induction time and temperature of induction are established using readout from the GFP reporter. The cells are then exposed, under these conditions, to increasing levels of erythromycin, since the GFP and ErmC are at the C-terminus, cells that have evolved to express higher levels of the functional protein will be resistant to a higher concentration of erythromycin. The strains are then plated on erythromycin at the highest concentration used and the most fluorescent colonies are analysed. The strains can be cured of the selection plasmid and it was shown that expression is increased for proteins other than the test plasmid (Linares et al. 2010; Gul et al. 2014). The evolved *E. coli* when compared with the parental strain showed up to a tenfold increase in fluorescence levels and when compared to the Walker strains had increased levels of expression per unit of biomass (Gul et al. 2014). Interestingly, deep sequencing of four evolved *E. coli* strains revealed that all had mutations were in the gene encoding DNA-binding protein, H-NS, which is involved in chromosome organization and transcriptional silencing, although the exact mechanism causing the elevated expression is unclear (Gul et al. 2014). In *L. lactis* the strain selection led to a two to eightfold increases in the expression levels of a variety of proteins. In contrast to *E. coli*, deep sequencing of the genome of the evolved strains identified point mutations in a single gene, *nisK*, which is the histidine kinase sensor protein of the two component regulatory system that directs nisin-A mediated expression. It seems likely that the mutations enhance phosphoryl transfer to NisR and increase transcription from the nisin-A promoter (Linares et al. 2010).

Most IMPs have been produced in *E. coli*, which reflects its popularity as a host for heterologous expression of soluble proteins. However other bacterial species may be more suitable

for IMP production. For example, Gram positive bacteria, such as *L. lactis*, express two copies of the IMP chaperone YiDC and thus may be better than *E. coli* at translocating heterologous proteins and hence may be less susceptible to saturation of the integration machinery (Zweers et al. 2008; Funes et al. 2009; Funes et al. 2011; Schlegel et al. 2014) A number of other features of *L. lactis*, like the slower growth rate and reduced proteolytic activity when compared to *E. coli*, may also facilitate IMP production in this bacterium (Schlegel et al. 2014).

1.3 Yeast

Like *E. coli*, yeast require relatively low cost of media, have fast growth rates and can be easily genetically modified, making them attractive expression host for IMP production. Moreover, the post translational modifications and lipid environment of yeast cells may be more appropriate for the expression of eukaryotic IMPs. The two yeast strains that have been widely used for IMP production are *Saccharomyces cerevisiae* and *Pichia pastoris* and less commonly, *Schizosaccharomyces pombe* (Yang and Murphy 2009; Yang et al. 2009; He et al. 2014). It is important to note that protein glycosylation in yeast is not typical of higher eukaryotic cells with N-linked glycosylation sites in *S. cerevisiae* hyper-glycosylated with high mannose glycoforms. In *P. pastoris*, the N-linked glycans are shorter than in *S. cerevisiae* and strains have been engineered that add glycoforms more typical of human glycoproteins (Hamilton et al. 2006; Darby et al. 2012).

The GFP screening pipeline used with *E. coli* has been adapted to both *S. cerevisiae* and *P. pastoris* (Drew et al. 2008; Drew and Kim 2012b; Brooks et al. 2013; Scharff-Poulsen and Pedersen 2013). There are, however, some differences, for example, as part of the screening process it can be useful to include a confocal microscope image to confirm the localization of the IMP-GFP fusion protein (Newstead et al. 2007; Drew et al. 2008) (Fig. 1.3). Additionally, *S. cerevisiae* cloning can be carried out by *in vivo* homologous recombination of PCR products into 2 μ based episomal vectors (Drew and Kim 2012a; Scharff-Poulsen and Pedersen 2013). The inducible *GAL1* promoter is often used to drive expression as the yields are generally higher compared to constitutive promoters (Newstead et al. 2007). The induction of the IMP-GFP fusion can be optimized by varying parameters, such as, the timing of induction, using non-selective media, the addition of chemical chaperones such as DMSO, glycerol

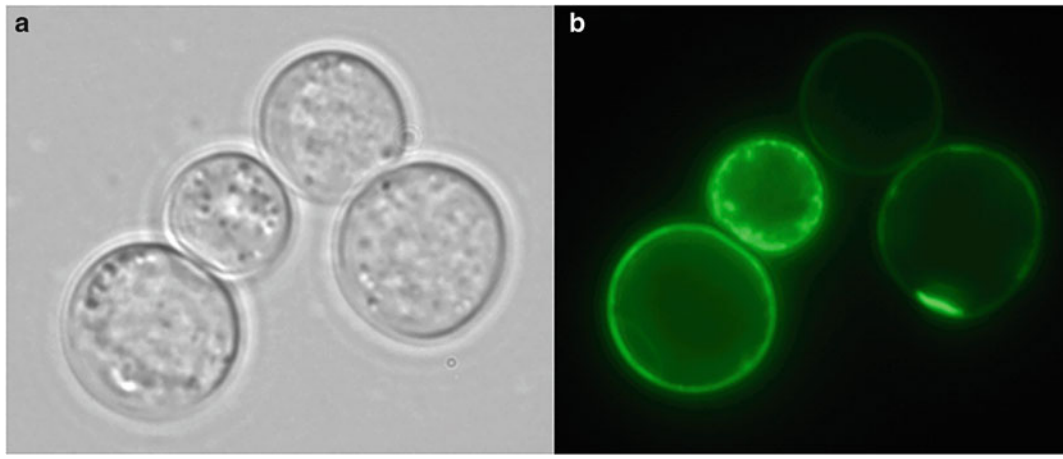

Fig. 1.3 *S. cerevisiae* expressing a recombinant *Candida albicans* TOK1 GFP fusion protein observed under (**a**) white light (**b**) fluorescence optics (Image courtesy of Prof. Per Pedersen, University of Copenhagen)

and histidine and also by lowering the temperature (Drew and Kim 2012c). Furthermore, the levels of expression of IMP-GFP fusions can be improved by the choice of strain and by plasmid engineering (Pedersen et al. 1996; Drew and Kim 2012a; Scharff-Poulsen and Pedersen 2013; Molbaek et al. 2015). For example, Molbaek *et al*. produced functional full-length human ERG K^+-GFP fusions by utilizing the strain PAP1500, which overexpresses the *GAL4* transcriptional activator. This was combined with a vector that has a strong hybrid CYC-GAL promoter and the compromised *leu2-d* gene, which elevates the episomal copy number to between 200 and 400 plasmids per cell in response to leucine starvation (Romanos et al. 1992; Molbaek et al. 2015).

For *P. pastoris*, strain development is more complicated. Since genes to be expressed have to be integrated into the yeast genome using a resistance marker such as zeocin and typically use the methanol inducible AOX1 promoter (Logez et al. 2012). This means that a shuttle vector has to be constructed and different *P. pastoris* transformants have to be characterised to identify the best recombinant strain for IMP expression. Again, fusion to GFP enables the expression screening of integrated clones using a plate based assay. For example, using this methodology Brooks et al. isolated a clone of mouse PEMT (ER associated phosphatidyl ethanolamine N-methyl transferase) that gave a final yield of 5 mg/L of purified protein (Brooks et al. 2013). In an interesting development, Parcej et al. reported the use of fusions to different fluorophores to monitor the expression of the human heterodimeric ATP binding cassette (ABC) transporter associated with antigen processing (TAP) in *P. pastoris*. The subunits were tagged with either monomeric venus and a HIS_{10} tag or monomeric cerulean with a strepII tag, dual wavelength monitoring was then used to monitor expression of individual subunits and purification of the complex (Parcej et al. 2013). This approach could clearly be applied to the expression of multi-subunit IMPs in other cell hosts.

Yeast is clearly a very useful host for expression of IMPs, however in a study of 43 eukaryotic membrane proteins Newstead et al. showed that while 25 out of 29 yeast membrane proteins were produced to greater than 1 mg/L in *S. cerevisiae*, only 4 of the 14 membrane proteins from higher eukaryotic organisms were produced at this level, suggesting that a higher eukaryotic heterologous expression systems is often necessary for higher eukaryotic proteins (Newstead et al. 2007).

1.4 Insect and Mammalian Cells

Insect cells are widely used for the production of eukaryotic recombinant proteins, including IMPs. The cells are easy to handle and in general give higher yields of recombinant proteins than transfected mammalian cells. The main cell lines in use are from *Spodoptera frugiperda* (Sf9 and Sf21) and *Trichoplusia ni* (High Five) with the gene of interest typically introduced using the baculovirus expression vector system (BEVS) (Zhang et al. 2008; Mus-Veteau 2010; Milic and Veprintsev 2015). Transient transfection with plasmid vectors has also been reported for rapid screening of IMP expression using GFP fusion proteins (Chen et al. 2013). In addition, *Drosophilia melanogaster* S2 cells in combination with inducible plasmid vectors have been used for the expression of recombinant IMPs (Brillet et al. 2010). However, it is important to note that the lipid composition of insect cell membranes differs from those of mammalian and bacterial cells. For example, the main sterol in mammalian cells is cholesterol, whereas it is ergosterol in insect cells (and yeast): there are no sterols in bacterial cell membranes (Lagane et al. 2000; Eifler et al. 2007). In addition, N-glycosylation in insect cells consists of short so-called pauci-mannose glycoforms, which are not found on mammalian IMPs.

GFP-tagging can be used for expression screening in insect cells in the same way as for *E. coli* and yeast. However in contrast to *E. coli* cells, there is evidence of GFP-tagged proteins produced in insect cells that are misfolded but still show GFP fluorescence (Thomas and Tate 2014). Fusion to GFP remains a convenient way for screening many constructs in parallel at

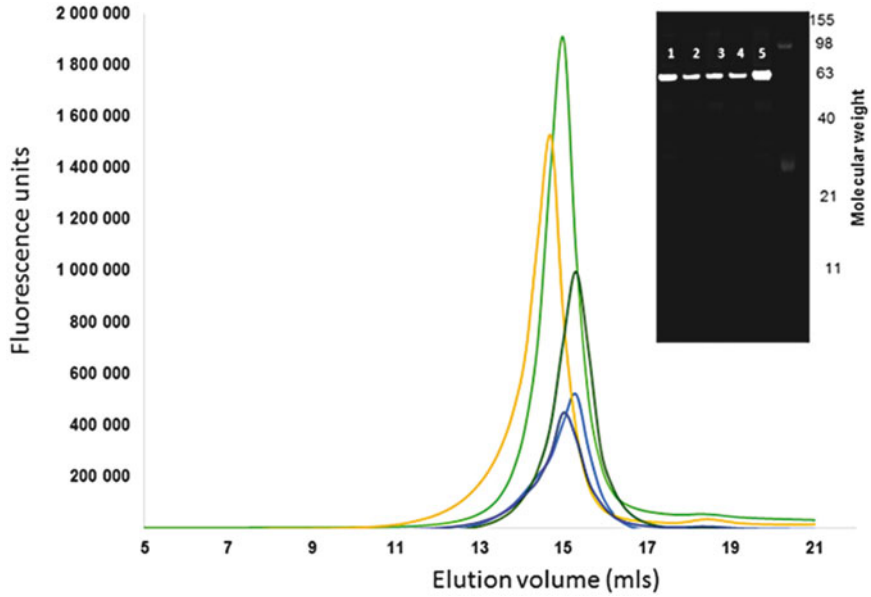

Fig. 1.4 Fluorescence detected size exclusion profiles (FSEC) and in-gel fluorescence (inset) of detergent extracts of the total membrane fraction from SF9 insect cells expressing *Caenorhabditis elegans* GTG1 fused to GFP. Membranes were extracted in the following detergents (1 % final concentration plus 0.2 % cholesterol): n-Decyl-β-D-Maltoside (DM: lane 1, *dark blue trace*); n-Dodecyl-β-D-Maltoside (DDM: lane 2, *dark green trace*); Lauryldimethylamine-N-Oxide (LDAO: lane 3, *yellow trace*); 6-Cyclohexyl-1-Hexyl-β-D-Maltoside (cymal-6: lane 4, *blue trace*); n-Dodecylphosphocholine (FC12; lane 5, *green trace*)

small scale, particularly different orthologues, in order to identify the best expressed candidate for purification and crystallization (Lee and Stroud 2010; He et al. 2014; Hu et al. 2015). Analysis of the subsequent products by FSEC (see Fig. 1.4 for an example) enables the optimal detergent for solubilisation to be identified and any misfolded fusion proteins to be detected.

Transient expression in Human Embryonic Kidney cells (HEK293) provides a rapid way of screening protein expression, including IMPs and has become the system of choice for the production of secreted/cell surface glycoproteins for structural biology (Aricescu and Owens 2013). In particular HEK-293 cells deficient in N-acetylglucosamine tranferase I (HEK Gnt1 −/−) are used to produce proteins containing only a high mannose glycoform, which can be removed by endoglycosidase treatment following purification. Simplifying the N-glycosylation of proteins appears to favour crystallization since sample heterogeneity is reduced (Chang et al. 2007). This approach is equally relevant for modifying the N-glycans of IMPs which may in turn aid crystallization.

The use of GFP fusions in combination with transient expression in HEK cells was introduced by Gouaux and co-workers (Kawate and Gouaux 2006) for optimizing the expression of the ATP-gated ion channel P2X4. Protein production for crystallization was subsequently transferred to insect cells (Kawate et al. 2009). For IMP production in mammalian cells, inducible stable cell lines are usually required to generate sufficient biomass without the problem of toxicity from constitutive expression (Chaudhary et al. 2011, 2012). Although this requires more time and effort than using insect cells, there are now a number of structures of membrane proteins produced in this way. In all cases, multiple constructs were initially screened by transient expression using fusion to GFP as a reporter of protein expression and stability by FSEC analysis. Although recombinant protein yields from mammalian cells are generally lower than either microbial or insect cell over-expression systems, there may be a sig-

nificant advantage in using mammalian cells for the production of human/mammalian IMPs. The proteins will be produced in a cellular context with native post-translational modifications and lipid environment, it is becoming increasingly apparent that this leads to improved protein quality due to lower levels of misfolded aggregates (Yamashita et al. 2005; Chaudhary et al. 2011).

An alternative to the production of stable cell lines for IMP production is the use of baculovirus mediated gene transduction for large-scale production of IMPs in mammalian cells, typically HEK Gnt1 −/− (Goehring et al. 2014). The so-called BacMam system (Dukkipati et al. 2008) involves the inclusion of a mammalian cell transcription unit(s) within a baculovirus transfer vector so that on generation of a recombinant virus, the inserted gene can be expressed in mammalian cells. The same plasmid vector can be used for small-scale transient transfection of HEK cells to identify the optimal construct and then to generate a BacMam baculovirus for scaling up of protein production by bulk transduction of HEK cells for further characterization (Goehring et al. 2014). Using this protocol, sample preparation can be accomplished in 4–6 weeks, which is at least half the time required to generate and scale-up stable cell lines. The approach has been used by the Gouaux group to produce a number of IMPs for structural determination (Althoff et al. 2014; Baconguis et al. 2014; Dürr et al. 2014; Lee et al. 2014c; Wang et al. 2015).

1.5 Summary and Conclusions

Initially developed for screening the expression of bacterial membrane proteins in *Escherichia coli*, the use of GFP fusions has been successfully extended to eukaryotic hosts, including insect and mammalian cells. Although *E. coli* and yeast are useful tools for the over-expression of recombinant membrane proteins, there is a marked difference in the lipid compositions of membranes from prokaryotes and eukaryotes. This in turn may affect the quality and quantity of heterologous proteins inserted into the host membrane. Given that the host cell determines the nature of post-translational modifications, such as glycosylation and phosphorylation, in choosing an expression host for screening, it may the appropriate to match the host cell to the recombinant product for example, human IMPs in mammalian cells.

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