Review

Lisa Maria Kögler, Jan Stichel and Annette G. Beck-Sickinger*

Structural investigations of cell-free expressed G protein-coupled receptors

<https://doi.org/10.1515/hsz-2019-0292>

Received June 18, 2019; accepted September 2, 2019; previously published online September 20, 2019

Abstract: G protein-coupled receptors (GPCRs) are of great pharmaceutical interest and about 35% of the commercial drugs target these proteins. Still there is huge potential left in finding molecules that target new GPCRs or that modulate GPCRs differentially. For a rational drug design, it is important to understand the structure, binding and activation of the protein of interest. Structural investigations of GPCRs remain challenging, although huge progress has been made in the last 20 years, especially in the generation of crystal structures of GPCRs. This is mostly caused by issues with the expression yield, purity or labeling. Cell-free protein synthesis (CFPS) is an efficient alternative for recombinant expression systems that can potentially address many of these problems. In this article the use of CFPS for structural investigations of GPCRs is reviewed. We compare different CFPS systems, including the cellular basis and reaction configurations, and strategies for an efficient solubilization. Next, we highlight recent advances in the structural investigation of cell-free expressed GPCRs, with special emphasis on the role of photo-crosslinking approaches to investigate ligand binding sites on GPCRs.

Keywords: cell-free protein synthesis (CFPS); crosslinking; G protein-coupled receptor (GPCR); mass spectrometry (MS); neuropeptide Y; soluble expression.

Introduction

The superfamily of G protein-coupled receptors (GPCR) consists of about 800 members and thus is one of the

largest families of proteins in the human genome. After extracellular activation, they transduce signals over the cell membrane, resulting in very diverse functions throughout the whole body. Based on sequence similarities, mammalian GPCRs can be subdivided into five classes (Fredriksson et al., 2003). Class A or rhodopsin-like GPCRs, is the biggest class with about 700 members. Class B1 consists of secretin receptors, and class C of the metabotropic glutamate receptors, both defined by a long N terminus, which is, in the case of class C receptors, also the site for ligand recognition. The last two classes are the Class F (Frizzled) and the Class B2 (Adhesion) receptors.

As GPCRs are involved in the regulation of such diverse processes, they have a high potential as pharmaceutical targets. Indeed, by the end of 2017, about 35% of all approved drugs were directed against GPCRs (Sriram and Insel, 2018). Still there is huge potential left – only about 134 GPCRs have been established as pharmaceutical targets, leaving a high number of receptors that have the potential of becoming new addressees. In order to find new drugs, expanding the number of druggable GPCRs is a demand. This can be achieved by targeting, for example, currently orphaned GPCRs that have highly selective expression or activation patterns related to diseases. Examples are GPR 22, which might have a protective role in heart disease (Adams et al., 2008) or GPR3, which has the potential in becoming a target in the treatment of Alzheimer's disease (Huang et al., 2015). In addition, new drugs are designed, which are directed against established targets and activate or inactivate them differentially or allow for fine-tuning of GPCR activation. Both approaches benefit from a rational drug design, which is based on knowledge about the structure or structure-activity relationships of the target.

In general, GPCRs receptors consist of seven trans-membrane (TM) helices that are connected by alternating intra- and extracellular loops (ICL/ECL) with an extracellular N terminus and an intracellular C terminus. The latter often contains a short, eighth helix that is oriented perpendicular to the membrane. Despite their otherwise diverse structure and sequence, a highly conserved disulfide bridge is present between the ECL2 and TM3. This disulfide

*Corresponding author: Annette G. Beck-Sickinger, Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Leipzig University, Brüderstr. 34, D-04103 Leipzig, Germany, e-mail: abeck-sickinger@uni-leipzig.de

Lisa Maria Kögler and Jan Stichel: Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Leipzig University, Brüderstr. 34, D-04103 Leipzig, Germany

bridge has an important contribution to the structure and/or function of many GPCRs (Wheatley et al., 2012).

The crystal structure of bovine rhodopsin was resolved in 2000 (Palczewski et al., 2000) and the first crystal structure of a human GPCR, the β_2 adrenergic receptor (β_2AR), in complex with a ligand was discovered 7 years later (Cherezov et al., 2007). Huge progress has been made in the determination of GPCR structures by X-ray diffraction (Xiang et al., 2016; Lee et al., 2018; Shimada et al., 2018). In 2018 crystal structures of 46 receptors in more than 250 complexes with agonists, antagonists, effector proteins, etc. were available in the Protein Data Bank and summarized in the GPRCdb (Pándy-Szekeres et al., 2018). To date (June 2019), this number has increased to 62 unique receptors (Figure 1), represented in 321 structures (gpcrdb.org/structure/statistics) and reflecting the demand of structural insights into GPCRs.

GPCRs are highly flexible proteins that can adapt many distinct conformations in the basal, as well as in the agonist bound and antagonist bound states (Katritch et al., 2013; Manglik et al., 2015; Latorraca et al., 2017). Therefore, the information that can be obtained by crystallography, which always reflects one static conformation, is limited and complementary *in vitro* and *in vivo* methods are often used to generate additional knowledge. For example, nuclear magnetic resonance (NMR) spectroscopy can be used to obtain information about protein structures and dynamics of a receptor-bound ligand (Luca et al., 2003; Lopez et al., 2008; Catoire et al., 2010; Stehle et al., 2014; Kaiser et al., 2015; Isogai et al., 2016; Eddy et al., 2018; Joedicke et al., 2018). Crosslinking approaches are used to map interaction sites between ligands and receptors or different receptors (Coin et al., 2013), while *in vivo* studies by mutagenesis with subsequent functional assays can be used to identify important sites and direct interactions. Furthermore, binding assays provide information about affinities of labeled ligands in a direct way or of unlabeled ligands indirectly by competition binding experiments (Flanagan, 2016). The findings generated by these experiments can be used as basis for computational approaches, like modeling and molecular dynamic simulations, which further serve drug development through virtual screening approaches (Kooistra et al., 2013) and help in prediction of binding sites for other receptors.

Cell-free protein synthesis

For structural investigations *in vitro*, like X-ray diffraction, NMR spectroscopy or cross-linking approaches,

cell-free protein synthesis (CFPS) has become an increasingly important research area with high potential also for other fields, including the industrial protein production (Carlson et al., 2012).

The basis was set 60 years ago, when Hoagland et al. synthesized a peptide using a cell-free extract from rat liver and moreover demonstrated, that protein synthesis takes place on the ribosome, requiring ATP, GTP and tRNA (Hoagland et al., 1958). In 1961, the first CFPS system based on an extract from *Escherichia coli* (*E. coli*) was developed to study translational processes (Matthaei and Nirenberg, 1961). Besides the capability for *in vitro* production of proteins, these systems helped to increase our understanding of biological systems, exploit and expand them (Swartz, 2011; Carlson et al., 2012).

Cell-free protein synthesis in general describes the synthesis *in vitro* without using intact, living cells and is based on cellular extracts that contain the core protein expression machinery including ribosomes and aminoacyl-tRNA synthetases (aaRS), while energy restoring systems, essential precursors like amino acids, NTPs, tRNA and DNA have to be supplied (Spirin et al., 1988; Schwarz et al., 2007). This has several advantages over recombinant protein production.

Advantages and disadvantages of cell-free protein synthesis

One of the biggest advantages of CFPS is the possibility to express proteins or peptides, which would harm living cells, including toxic proteins (Orth et al., 2011; Villate et al., 2012), vaccines or membrane integrated proteins (Tsuboi et al., 2010). The latter can be cytotoxic in eukaryotic cells, when the overexpression leads to an oversaturation of the cellular machinery and intracellular accumulation. Prokaryotic cells, like *E. coli*, solve this by the formation of inclusion bodies, which circumvent cytotoxic effects. As inclusion bodies are mechanically stable and resistant against proteolytic degradation, this can be advantageous, but this characteristic may impede solubilization and purification, especially in the case of membrane proteins (Banères et al., 2011). The purification after cell-free expression is, in contrast to the purification from inclusion bodies, reduced to one or two affinity purification steps and the extract after expression can be directly applied on columns (Henrich et al., 2015b). In total, many time-consuming and critical steps are eliminated during CFPS. Furthermore, problems like degradation, mistargeting or insoluble expression can be directly

addressed in this open system, allowing for an optimization towards protein production independently of cell-viability or growth (Schwarz et al., 2007; Bernhard and Tozawa, 2013; Henrich et al., 2015b). This enables, for instance, the addition of substances that support or modulate co-translational folding, including detergents, lipids, chaperones, or the adjustment of the surrounding pH or redox-potential, as is discussed later. The addition of protease inhibitors and RNase inhibitors to avoid degradation also fits CFPS (Shin and Noireaux, 2010), which facilitates the production of unstable proteins or small, bioactive peptides. Further optimization can be performed with respect to energy regeneration systems, reaction times, temperatures or buffer systems. This optimization procedure has been adjusted for high-throughput screening platforms for the production of proteins, including membrane proteins, DNA regulatory elements and enzymes (Calhoun and Swartz, 2005; Swartz, 2011; Catherine et al., 2013; Chappell et al., 2013). Moreover, automated high-throughput systems are in development for optimization of protein production (Sawasaki et al., 2002; Spirin, 2004; Aoki et al., 2009; Quast et al., 2015). This makes CFPS attractive for the industrial protein production, as well as for the search of novel proteins or proteins containing non-natural amino acids.

Beside the advantages of CFPS and the high potential for industrial protein production, there are only very few examples of industrial applications. CFPS systems often result in high protein yields, but because of the small reaction scales, the amount of total protein is substantially lower than in recombinant expression systems. While scale up is indeed possible, this is not fully established yet. The established reaction scales allow for the protein production for clinical application or for research purposes. In fact, some cell-free produced proteins, like GM-CSF, a human granulocyte-macrophage colony-stimulating factor (Zawada et al., 2011), anti-CD19-lymphoma idiotype diabody, a vaccine directed to B-cells (Ng et al., 2012), and MR1-1^[11C], a single chain variable fragment antibody for immune-positron emission tomography (Matsuda et al., 2012), were in preclinical phases, in 2013. Another drawback of cell-free systems is the use of costly precursors, such as NTPs, leading to rather high costs for the production of proteins.

Posttranslational modifications (PTMs) have to be considered when expressing proteins. They are often of importance for the structure and function and are key players in the regulation of proteins on different levels (Knorre et al., 2009). PTMs are hard to address by cell-free approaches and are deeply dependent on the extract source. A lack of PTMs by using a prokaryotic source leads

to a reduced stability or activity, but a more homogenous sample. This can be an advantage for structural investigations requiring uniform proteins (Junge et al., 2008, 2010). In recombinant expression systems, PTMs are also an issue. Here, prokaryotic hosts often express eukaryotic proteins with insufficient or no PTMs. The usage of eukaryotic systems can cause incomplete PTMs when overexpressing proteins, as the cellular synthesis machinery is oversaturated (Tate, 2001), which results in heterogeneous samples.

However, CFPS systems allow the efficient incorporation of non-standard amino acids (NSAA) by different strategies. The advantages of CFPS for protein labeling is based on two of its key features – the open system and the precise control over the reaction environment. The lack of membranes in CFPS eliminates the necessity for the transport of NSAAs inside the cell; they can simply diffuse to the ribosomes. This enables an efficient incorporation of NSAAs with limited cellular uptake. Furthermore, no modifications by cellular enzymes take place and the addition of NSAAs cannot lead to cytotoxicity. The only limit is that they should not inhibit the protein synthesis itself. As all aspects of protein expression aim to the expression of the protein of interest (POI), no insertion of NSAAs in other proteins is possible, which minimizes the amount of unnatural amino acids that has to be added. This reduces the costs dramatically, when dealing with, for example, isotopically labeled amino acids. The control over the reaction environment further enables the inhibition of amino acid modifying enzymes, as well as certain adjustments to the surrounding that stabilize these amino acids and circumvent the use of amino acid precursors. The full control of the amino acid pool furthermore eliminates the need of handling special strains or cell lines and minimal media. In addition, it ensures a complete insertion of amino acids, which are recognized by cellular aaRS, into the expressed protein. This holds true for selectively or isotopically labeled amino acids. In addition, orthogonal systems can be used by CFPS approaches. Here, the orthogonal-aaRS (o-aaRS), o-tRNA and NSAA can be added directly to the expression. As o-aaRS have been shown to have a low efficiency of loading the respective amino acid to the o-tRNA compared to natural aaRS (Tanrikulu et al., 2009; Nehring et al., 2012; Umehara et al., 2012; Albayrak and Swartz, 2013b), the amounts can be enriched in CFPS systems. By expressing the o-tRNA in the cells, which are used for extract preparation, the Swartz group greatly improved this system (Goerke and Swartz, 2009; Bundy and Swartz, 2010). This leads to a high yield in NSAA incorporation. Another approach to expand o-tRNA levels was made by

the Swartz group by co-expression of o-tRNA and the POI in the CFPS reaction (Albayrak and Swartz, 2013a; Hong et al., 2014).

To sum this up, the CFPS system has advantages over recombinant systems due to its open nature, which allows for a precise control of the environment including additives and amino acids and easy access to the expressed proteins. Furthermore, these systems promote a straightforward optimization procedure suitable for high-throughput screens, which makes it attractive for automated processes and allows for the efficient incorporation of unnatural amino acids, but to date only in relatively small scales.

Cell-free reaction configuration

CFPS reactions can be set up in mainly two different modes – in a batch reaction or in a continuous exchange/flow cell-free (CECF/CFCF) reaction.

The compartment batch reaction uses microplates as reaction containers, with small volumes. By optimization procedures yields within mg/ml range can be obtained (Kim et al., 2006b). This system offers advantages by easy handling and scalability (Kim and Swartz, 1999) and fits high-throughput applications (Nirenberg and Matthaei, 1961; Schwarz et al., 2010). Furthermore, the small volumes are of advantage when using expensive additives or labeled amino acids. A drawback of this system is the short expression time of only a few hours, which is restricted by the small volumes and the consumption of precursors, while inhibitory byproducts, like pyrophosphate, accumulate (Nirenberg and Matthaei, 1961; Kim and Swartz, 1999; Hovijitra et al., 2009). This results in moderate protein yields, especially for larger proteins. Thus, the batch mode expression is often not suitable for structural investigations, despite extensive optimization procedures.

To enhance reaction times, two compartment set-ups were developed. These allow the continuous supply with fresh precursors and the removal of inhibitory byproducts by passive diffusion (CECF) or a continuous flow (CFCF). One compartment contains the reaction mix and is the place of protein expression. It consists of the high-molecular weight components, which are necessary for transcription/translation. The feeding mix contains low-molecular weight substances, which are consumed during expression, like amino acids, NTPs or protease inhibitors. The two compartments are separated by a semi-permeable membrane, which allows the diffusion of low-molecular weight substances. Therefore, precursors are refilled in the reaction mix while inhibitory by-products are removed.

As this allows increased reaction times for up to 24 h, the protein yield is increased. It has been reported that, compared to batch expression, a two component setup leads to 5–10 times more protein resulting in a yield of several mg/ml (Spirin et al., 1988; Kigawa et al., 1999; Kigawa, 2010; Klammt et al., 2011). A disadvantage of this expression mode is the higher amount of low-molecular weight substances. Every substance that is smaller than the molecular weight cut-off of the dialysis membrane, has to be added to both compartments, which results in higher costs. Nevertheless, the increased protein yield makes this system more suitable for proteins used for structural investigations.

Cell-free lysates

The cellular extract is the basis for CFPS reactions, and the source has to be chosen carefully, dependent on the expressed protein and the application. The cell extracts contain the core protein expression machinery – the ribosomes, the aaRS, translation factors, as well as some residual membranes (Spirin et al., 1988). The preparation of cell extracts in general consists of cell growth, harvesting, disruption and removal of the cell walls or membranes. During the preparation low-molecular weight substances are removed, including amino acids, precursors, as well as DNA and mRNA. This gives the operator full control over the reaction environment and ensures, that only the POI is synthesized.

To date, cell extracts from different organisms are in common use and new ones are being developed. They vary in efficiency and costs of lysate preparation, expression yields, PTMs and accepted additives. Furthermore, different nucleic acid templates are accepted. Using a DNA template results in a coupled transcription/translation system, whereas for mRNA only the translation takes place during CFPS.

The first aspect to consider is whether to use a eukaryotic or prokaryotic source (Spirin, 2004). Prokaryotic extracts have, in general, higher translation rates, resulting in higher productivity. As they are highly compatible with coupled transcription/translation systems, DNA based genetic constructs can be used. These vectors are well established, as prokaryotic organisms are commonly used in recombinant expression. For the same reason, strains with reduced degradative activities or other characteristics that suit protein expression are available. In prokaryotic extracts, these degradative activities lead to a comparable higher rate of degradation with respect to genetic messengers, proteins or energy suppliers.

Furthermore, prokaryotic extracts result in shorter reaction times and, especially for eukaryotic proteins, protein aggregates or insoluble expression. In addition, no or only certain PTMs are inserted. This differs in eukaryotic extracts, which are in general more suitable for eukaryotic proteins. Furthermore, they are more stable and are better compatible with eukaryotic mRNAs. However, the required genetic constructs are more complex and less well established as compared to prokaryotic systems.

The most commonly used cell extracts are derived from different *E. coli* strains, like BL21, A19, C41 or C43. *Escherichia coli* cell extracts are well established, in use for more than 50 years (Matthaei and Nirenberg, 1961) and the extract production is efficient and cost effective. It includes fermentation until the mid of the log phase, cell harvesting by centrifugation, disruption and processing by multiple centrifugation and incubation steps, as well as extensive dialysis (Kigawa et al., 2004; Schwarz et al., 2007; Shrestha et al., 2012). As the proteome is fractionated during lysate preparation, the extract composition is dependent on the centrifugal force applied during extract processing, which they are also named after. Commonly used extracts are the so-called S12 (12 000 g), S30 (30 000 g) or S60 (60 000 g) extracts (Zubay, 1973; Kim et al., 2006a). Advantages of *E. coli*-based cell extracts include high protein yields in the mg/ml scale (Focke et al., 2016), as well as the compatibility with the T7 polymerase/T7 promoter system. In this system, the transcription is under the control of the Lac repressor, which inhibits the protein expression until the inducing agent isopropyl β -D-1-thiogalactopyranoside is added. Therefore, residual chromosomal DNA fragments will be ignored during expression, resulting in a higher specificity (Zubay, 1973; Schwarz et al., 2007). Cell extracts from *E. coli* furthermore tolerate a number of diverse additives including membrane mimicking molecules, which is of importance for the expression of membrane-integrated proteins. Detergents, lipids or nanodiscs can be added during synthesis, leading to a soluble expression (Ryabova et al., 1997; Junge et al., 2011; Niwa et al., 2012). The folding of proteins, which is problematic for eukaryotic proteins in *E. coli*, can be additionally supported by the addition of chaperons, chemical stabilizers or redox systems (Ryabova et al., 1997; Niwa et al., 2012; Lyukmanova et al., 2012a) and co-translational screening for these additives is possible (Reckel et al., 2010; Rath et al., 2011). Indeed, the disulfide-containing eukaryotic proteins, human and mouse prion-like Doppel protein and mouse interleukin-22, have been successfully synthesized using an extract from *E. coli* by addition of a glutathione shuttle (Michel and Wüthrich, 2012). The *E. coli* system results in no or only little PTMs, which on the one

hand, might be desired, but might also be problematic on the other hand, as discussed before. As the *E. coli*-based CFPS system is very old, several optimization processes were performed leading to an increased yield, lower costs or higher protein quality. Some notable optimizations include the use of different, cheaper ATP regeneration systems (glucose-6-phosphate or fructose-1,6-phosphate instead of phosphoenol pyruvate) and the application of purified components. The latter led to the development of the PURE system (Shimizu et al., 2001), which uses pre-purified proteins, tRNAs, aaRS and translation factors, leading to an expression yield of ~160 μ g/ml after 1 h reaction time in the batch format.

Archae extracts were developed very early (Elhardt and Böck, 1982), but they are not commonly used, as they result in low proteins yields without exhibiting the advantages of eukaryotic extracts, like a soluble expression or PTMs. Nevertheless, a number of coupled transcription/translation systems was developed based on *Sulfolobus solfataricus* (Ruggero et al., 1993) or *Thermococcus kodakaraensis* (Endoh et al., 2006), which are both thermophilic organisms. This allows for the expression of thermostable proteins at high temperatures.

Yeast extracts from *Saccharomyces cerevisiae* were developed nearly 40 years ago (Sissons, 1974). As yeast is often used for the recombinant expression of proteins, it is well-known and methods regarding the modification of nucleic acid templates and cell strains are well established, allowing for the production of extracts with certain features. Furthermore, the relative costs for cell cultivation are low, the cultivation itself is fast and the yeast system allows for some PTMs (Rothblatt and Meyer, 1986). Overall, protein yield is lower compared to *E. coli*-based cell extracts and is in the range of μ g/ml. Efforts in optimizing this system have been made in recent years, for example, with respect to extract preparation (Hodgman and Jewett, 2013) and an ATP regeneration system (Anderson et al., 2015), as yeast extracts offer great possibilities for an industrial scale production, like the production of bio-ethanol (Ullah et al., 2015).

The development of wheat germ extracts started in 1973 (Roberts and Paterson, 1973). This well-known system results in high yields of complex proteins, although the overall protein yield is lower compared to *E. coli*-based cell extracts. As the wheat germ extract has a eukaryotic source, it is consistent with the expression of eukaryotic proteins and often results in soluble expression and correct folding. To further promote folding and stabilize disulfide bridges, the translation conditions can be modified by, for example, removal of dithiothreitol from the expression buffer or by addition of disulfide isomerases (Kawasaki

et al., 2003). As the extract contains no endogenous membrane structures, detergents, lipids or nanodiscs have to be added for the expression of membrane proteins and a diverse set is well accepted in this system (Shadiac et al., 2013). With respect to PTMs, only a limited number is introduced into proteins by wheat germ extracts, as the endoplasmic reticulum is removed during extract preparation. This preparation is, compared to the preparation of *E. coli*-based cell extracts, more complex and expensive. The wheat germ endosperm contains several nucleases and proteases and hence has to be removed during extract preparation, which is a critical step (Madin et al., 2000). Wheat germ extracts have been applied for the production of several proteins. Using this system isotopically labeled ubiquitin, as well as a cold-regulated RNA-binding protein was expressed and NMR spectroscopy with these proteins was performed (Morita et al., 2004). Furthermore, proteins with a DNA-binding tag on a chip were expressed (Sawasaki et al., 2008). The wheat germ system is compatible with high-throughput applications (Sawasaki et al., 2002; Endo and Sawasaki, 2004) and is applied in malaria research for the production of malaria proteins that are used to characterize vaccine candidates (Tsuboi et al., 2010).

Another very old system is a mammalian extract from rabbit reticulocytes, which was developed 60 years ago. It was used to express radioactively labeled hemoglobin (Schweet et al., 1958). The system is well established and has, as other mammalian extracts, the major advantage of producing proteins with mammalian-like PTMs (Zhu, 2012). This helps in the folding and promotes the function of many proteins. However, to achieve PTMs by a rabbit reticulocyte extract microsomes have to be added to the extract (MacDonald et al., 1988). These microsomes are endoplasmic reticulum derived vesicles, which can be transformed into giant unilamellar vesicles that are used as a membrane model system (Shaklee et al., 2010; Fenz et al., 2014). Drawbacks of this system include the low yield of synthesized protein, despite optimization (Anastasina et al., 2014), as well as the necessity of working with living animals.

Only recently, the moth *Spodoptera frugiperda* has been used as an extract source (Tarui et al., 2000). The lysate preparation is easy and fast, beside the high cultivation costs for insect cells. During preparation, parts of the endoplasmic reticulum remain as microsomes. This provides certain PTMs, as well as a soluble expression of proteins, which can be directly inserted into microsomes (Merk et al., 2012). This approach is of special interest for the synthesis of membrane-integrated proteins and covers investigations in a more natural membrane-like

environment after transformation of the microsomes into giant unilamellar vesicles. This direct insertion during expression without the need of additives makes insect cell extract a potential candidate for the automated production of membrane proteins (Quast et al., 2015). In 2014, Stech et al. demonstrated the capability of this system by performing optimization screens in an insect CECF system. Furthermore, they expressed various proteins, including enhanced yellow fluorescent protein, proheparin-binding epidermal growth factor-like growth factor, a transmembrane protein, as well as bacteriorhodopsin, human endothelin-B receptor (ETBR) and human erythropoietin, a glycoprotein (Stech et al., 2014).

Extracts from other sources have been prepared as well, but are new and/or not commonly used, as only few applications have been reported so far. Extracts from tobacco BY-2 yield relatively high amounts of proteins and allow for certain PTMs, but they also contain endogenous amino acids, which makes it, to date, not suitable for labeling approaches (Komoda et al., 2004; Buntru et al., 2014). Other extracts are based on cultured mammalian cells, including mouse embryonic fibroblasts (Zeenko et al., 2008), CHO cells (Brödel et al., 2013), HEK293 cells (Bradrick et al., 2013) or HeLa cells (Goldstein et al., 1974; Weber et al., 1975). In general, these cell-lines are well known and highly characterized. Furthermore, they enable mammalian PTMs and facilitate the production of membrane proteins. Nevertheless, the cultivation is more laborious and the costs are higher. Furthermore, these systems result, until now, only in very low protein yields. Beside these drawbacks, as soon as they are established mammalian-based cell extracts offer high potential for investigations of eukaryotic proteins.

In conclusion, the best-established systems for CFPS are, to date, based on *E. coli* and wheat germ cellular extracts. These systems have been greatly investigated and optimized over the last decades and therefore offer a stable core for the production of proteins. Still, the *E. coli*-based CFPS has the highest protein yield, accepts a wide variety of additives and has been used for structural and functional investigations of several membrane proteins.

Soluble expression of membrane integrated proteins by *E. coli*-based CFPS

As expression and solubilization techniques for membrane proteins advance, available structures have increased over the last years. However, it is still a challenging task to obtain these proteins in purities and amounts sufficient for structural investigation. Cell-free expression platforms

offer an effective alternative for the fast production of membrane proteins. Membrane proteins can be expressed in different modes by CFPS – as precipitate (P-CF), in the presence of detergents (D-CF) or with lipids (L-CF).

In the P-CF mode, the expression is performed without the addition of any hydrophobic support, which leads to the precipitation and accumulation of membrane proteins that still possess some of their secondary structure elements (Maslennikov et al., 2010). After expression, the proteins are solubilized and refolded in detergents, which can be exchanged to another hydrophobic support that fits the method for biochemical and biophysical characterization. Using the P-CF mode, the voltage gated potassium channel MVP, as well as amino acids transporter LeuT were obtained as active proteins. Furthermore, a crystal structure of the potassium channel KcsA at 2.85 Å resolution was obtained. The received crystal structure was comparable to the structure of cellularly expressed KcsA (Focke et al., 2016). In addition, the backbone structure of the TM domains of three *E. coli* histidine kinase receptors was determined by NMR spectroscopy after P-CF expression (Maslennikov et al., 2010).

Escherichia coli extracts accept a large variety of hydrophobic compounds, including detergents, lipids or nanoparticles (Lyukmanova et al., 2012a) (Figure 2). This allows co-translational insertion of many membrane proteins in these structures.

In the D-CF mode the hydrophobic support are detergent micelles, which differ in length, structure, charge and flexibility. As all these characteristics have influence on the protein structure (Seddon et al., 2004), screens for the optimal detergent in the expression of the POI are recommended. The accepted detergents include alkyl glucosides, like *n*-dodecyl β-D-maltoside or octyl-beta-glucoside, polyoxyethylene alkyl ether (Brij- and Tween derivatives), steroid derivatives (Digitonin, Chaps), long-chain phosphoglycerols, mono- and bi-chain phosphocholines and polyethylene glycol derivatives, like Triton X-100 (Berrier et al., 2004; Klammt et al., 2005). The D-CF

mode has been used for the soluble expression with subsequent NMR spectroscopy of the voltage-sensing domain of the potassium channel KvAP from archaeon *Aeropyrum pernix* (Shenkarev et al., 2010), as well as for the soluble expression of a number of GPCRs, rhodopsin from a marine alga (Wada et al., 2011) and, most recently, the photosystem II subunit S (Krishnan et al., 2019).

Liposomes and bicelles consist of a lipid bilayer and are more closely related to native membranes than detergents, which supports the folding and stability of membrane proteins. They are often used after D-CF expression, but a co-translational insertion of membrane proteins into lipids is also possible. Problems in this mode often occur due to the precipitation of liposomes together with the synthesized membrane proteins. Nevertheless, using liposomes, the subunits of the SecYEG translocon were synthesized parallel in one reaction. The subunits assembled to a functional protein, which exhibited transport mechanism, as well as signal peptidase activity (Matsubayashi et al., 2014).

Nanodiscs are more often used in the L-CF mode than liposomes. These discs consist of a lipid bilayer that is surrounded by a membrane scaffold protein (Denisov et al., 2004; Hagn et al., 2013). Nanodiscs are highly soluble and can have different sizes, spanning from 6 to 20 nm in diameter. In contrast to liposomes, nanodiscs do not tend to form aggregates during expression and are tolerated in high concentrations in *E. coli*-based CFPS systems (Katzen et al., 2008; Roos et al., 2012; Lyukmanova et al., 2012a). Another advantage is the possibility of purification without the need to attach a purification tag to the POI. This tag can be incorporated into the membrane scaffold protein, which allows the purification of POIs that are inserted into nanodiscs. In 2013, Proverbio et al. expressed the human endothelin A receptor (ETAR) and ETBR in the presence of various hydrophobic supports, including nanodiscs (Proverbio et al., 2013). The expressed receptors were able to bind ligands and the proteolytic processing based on conformational recognition was examined.

Figure 2: Type of hydrophobic support that can be added during CFPS for a soluble membrane protein expression.

Furthermore, the *E. coli* MraY translocase, as well as proteorhodopsin were successfully expressed in a soluble manner using nanodiscs (Roos et al., 2012).

Solubilization by other substances demonstrates the flexibility of the CFPS system. By using non-natural membrane amphiphiles, namely diblock copolymer membranes, the mechanosensitive channel of large conductance (Jacobs et al., 2019) and the C-X-C receptor type 4 (CXCR4) (de Hoog et al., 2014) were expressed in a properly folded manner. Furthermore, a number of GPCRs was successfully synthesized in the presence of a polyfructose-based uncharged NV10 polymer (Klammt et al., 2011).

In addition to a proper hydrophobic support, additives can further enhance folding of membrane proteins. Disulfide bridge formation in *E. coli* lysates can be effectively promoted by the adjustment of redox conditions, directly in the reaction (Bundy and Swartz, 2011; Keller et al., 2011; Shingaki and Nimura, 2011; Zawada et al., 2011; Michel and Wüthrich, 2012). Furthermore, redox shuffling systems, chaperones or iodacetamide can be added during expression to help in the correct formation of disulfide bridges (Kim and Swartz, 2004; Yin and Swartz, 2004; Yang et al., 2004; Michel and Wüthrich, 2012; Proverbio et al., 2013).

A sufficient optimization with respect to the hydrophobic support and the redox potential of the environment is suggested as protocols for screens and a systematic already exist (Shenkarev et al., 2010; Isaksson et al., 2012; Quast et al., 2015; Henrich et al., 2015a; Rues et al., 2018).

Cell-free expression of class A GPCRs

The expression of GPCRs with their seven TMs is challenging by CFPS approaches, as it is performed with only partial regulation of protein folding. As they are of great interest in the field of drug development, efforts have been made to fit CFPS systems to the expression of GPCRs. This led to a number of receptors that were synthesized in the last 15 years (Table 1).

Most receptors have been expressed in *E. coli*-based CFPS systems. Using the detergents Brij-35 and digitonin human β 2AR, human muscarinic acetylcholine receptor M2 and rat neurotensin receptor were solubly expressed in 2005, and binding was confirmed for β 2AR (Ishihara et al., 2005). Expression of rat vasopressin type 2 receptor was carried out in the presence of a variety of detergents (Klammt et al., 2005). Digitonin, Brij-35, -58, -78 and -98 were found to effectively solubilize the receptor with no or only little impact on protein expression. A similar screen was performed for the human melatonin 1B receptor, the

human ETBR, the human and porcine vasopressin receptor type 2, the rat corticotropin releasing factor and the human neuropeptide Y (NPY) receptor type 4 (Y_4 R). Brij-78 effectively solubilized all receptors (Klammt et al., 2007). This detergent was additionally found to be efficient in the expression of ETAR and ETBR. In this approach, also liposomes and nanodiscs were used (Proverbio et al., 2013). After reconstitution into proteoliposomes ligand binding, complex formation and conformation-specific proteolysis was confirmed for the expressed receptors. The detergent Brij-35 has been used for the expression of nine different olfactory receptors, human formyl peptide receptor 3, human vomeronasal receptors 1 and 5 (Corin et al., 2011), the human trace amine-associated receptor 5 (Wang et al., 2013) and CXCR4 (Chi et al., 2016). For all these receptors secondary structure and/or ligand binding abilities were confirmed. In addition, a thermostabilized neurotensin receptor 1 was expressed as precipitate and in the presence of Brij-58. For both variants, the secondary structure of the receptor was confirmed by circular dichroism and NMR spectra were obtained (Shilling et al., 2017).

In the P-CF mode, human β 2AR, muscarinic acetylcholine receptor M1 and somatostatin receptor type 5 were expressed and the amount of expressed protein was measured (Lyukmanova et al., 2012b). Furthermore, C-C chemokine receptor type 1 and 5, somatostatin receptor type 2 and 5, GPCR family C group 5 member B, retinoic acid-induced protein 3, as well as corticotropin-releasing factor receptor type 1 and 2 β were expressed as precipitate, with subsequent reconstitution, or in the presence of a polyfructose-based, uncharged NV10 polymer. For the last two receptors, ligand binding assays and NMR spectroscopy were performed (Klammt et al., 2011).

Using the L-CF mode, the dopamine D2 receptor was expressed, properly folded and functional in bilayers (Basu et al., 2013). Neurokinin 1 receptor, dopamine D1 receptor and β 2AR were also co-translationally inserted into nanodiscs, where they maintained their ligand binding profiles (Gao et al., 2012). β 2AR was furthermore expressed in nanodiscs with its ICL3 being replaced by T4 lysozyme (Yang et al., 2011) and ligand binding was determined for a β 1 adrenergic receptor expressed in nanodiscs (Rues et al., 2016).

Only few other sources for the preparation of the cellular extract for GPCR expression have been reported. By wheat germ extracts, HRH1 in glycosomes (Suzuki et al., 2018), dopamine D2 receptor in bilayers (Basu et al., 2013) and CXCR4 in diblock copolymer membranes (de Hoog et al., 2014) were expressed as active proteins. Some receptors have been reported to be successfully inserted into microsomes by CFPS with an insect cell extract.

Table 1: GPCRs expressed by different CFPS modes.

Mode	Hydrophobic support	GPCR	Class	Analysis	Ref.	
<i>E. coli</i>	P-CF	β2 adrenergic receptor	A		Lyukmanova et al. (2012b)	
		Muscarinic acetylcholine receptor M1	A			
	Reconstituted in DDM	Somatostatin receptor type 5	A	Secondary structure, NMR spectra	Shilling et al. (2017)	
		Thermostabilized neurotensin receptor 1	A			
	Reconstituted in polyfructose-based, uncharged NV10 polymer	C-C chemokine receptor type 1	A	Ligand binding, NMR spectroscopy	Klammt et al. (2011)	
		C-C chemokine receptor type 5	A			
		Somatostatin receptor type 2	A			
		Somatostatin receptor type 5	A			
		Corticotropin-releasing factor receptor type 1	B1			
		Corticotropin-releasing factor receptor type 2β	B1			
		Retinoic acid-induced protein 3/	C			
		GPCR class C group 5 member A				
		GPCR class C group 5 member B	C			
	D-CF	Brij-35	β2 adrenergic receptor	A	Ligand binding	Ishihara et al. (2005)
			Muscarinic acetylcholine receptor M2	A		
			Neurotensin receptor (rat)	A		Klammt et al. (2005, 2007)
			Vasopressin type 2 receptor (human and rat)	A		
			Trace amine-associated receptor 5	A	Secondary structure, ligand binding	Corin et al. (2011), Wang et al. (2013)
			C-X-C chemokine receptor type 4	A	Ligand binding	Chi et al. (2016)
			Formyl peptide receptor 3	A	Secondary structure, ligand binding	Corin et al. (2011)
			Vomeronasal receptor 1	A	Secondary structure, ligand binding	
			Vomeronasal receptor 5	A	Secondary structure, ligand binding	
			Nine different olfactory receptors	A	Secondary structure, ligand binding	
		Brij-58	Vasopressin type 2 receptor (rat)	A	Secondary structure, NMR spectroscopy	Klammt et al. (2005)
			Endothelin B receptor	A		Klammt et al. (2007)
			Thermostabilized neurotensin receptor 1	A		Shilling et al. (2017)
		Brij-78	Vasopressin type 2 receptor (human, rat and porcine)	A	Reconstitution in proteoliposomes, ligand binding, complex formation, conformation-specific proteolysis	Klammt et al. (2005, 2007)
			Melatonin 1B receptor	A		Klammt et al. (2007)
			Neuropeptide Y receptor type 4	A		
			Endothelin A receptor	A		Proverbio et al. (2013)
			Endothelin B receptor	A		Klammt et al. (2007) and Proverbio et al. (2013)
			Corticotropin releasing factor (rat)	B1		Klammt et al. (2007)
			Brij-98 Digitonin	Vasopressin type 2 receptor (rat)		A
		β2 adrenergic receptor		A		
		Muscarinic acetylcholine receptor M2		A		
		Neurotensin receptor (rat)		A		
		Vasopressin type 2 receptor (rat)		A	Klammt et al. (2005)	

Table 1 (continued)

Mode	Hydrophobic support	GPCR	Class	Analysis	Ref.
L-CF	Liposomes	Endothelin A receptor	A	Reconstitution in proteoliposomes	Proverbio et al. (2013)
		Endothelin B receptor	A	ligand binding, complex formation, conformation-specific proteolysis	
	Bicelle Nanodiscs	Dopamine D2 receptor	A	Secondary structure	Basu et al. (2013)
		Endothelin A receptor	A	Reconstitution in proteoliposomes,	Proverbio et al. (2013)
		Endothelin B receptor	A	ligand binding, complex formation, conformation-specific proteolysis	
		Neurokinin 1 receptor	A	Ligand binding	Gao et al. (2012)
		Dopamine D1 receptor	A	Ligand binding	
		β1 adrenergic receptor	A	Ligand binding	Rues et al. (2016)
		β2 adrenergic receptor	A	Ligand binding	Gao et al. (2012)
		β2 adrenergic receptor with T4 lysozyme replacing ICL3	A		Yang et al. (2011)
		Other	Polyfructose-based uncharged NV10 polymer	Corticotropin-releasing factor receptor type 1	B1
Corticotropin-releasing factor receptor type 2β	B1				
Wheat germ					
L-CF	Bicelle	Dopamine D2 receptor long isoform	A	Ligand binding, protein sequencing	Basu et al. (2013)
Other	Diblock copolymer membrane	C-X-C chemokine receptor type 4	A	On chip immobilized, ligand binding	de Hoog et al. (2014)
	Glycerosomes	Histamine H1 receptor	A	Ligand binding	Suzuki et al. (2018)
Insect cells					
L-CF	Microsomes	Histamine H1 receptor	A	Ligand binding, protein sequencing	Sansuk et al. (2008)
		Endothelin receptor B	A	Ligand binding	Zemella et al. (2017) Sonnabend et al. (2017)
		μ opioid receptor	A	Ligand binding	
		C-X-C chemokine receptor type 4	A		
		C-X-C chemokine receptor type 5	A		
		Thyrotrophic receptor	A		
		Glucagon-like peptide 1 receptor	B1		
		G-protein coupled receptor 56	B2		
		Metabotropic glutamate receptor 1	C		

Brij-35, polyoxyethylene(23)laurylether; Brij-58, polyoxyethylene(20)cetyllether; Brij-78, polyoxyethylene(20)stearyl-ether; Brij-98, polyoxyethylene(20)oleylether; CFPS, cell-free protein synthesis; DDM, n-dodecyl β -D-maltoside; GPCR, G protein-coupled receptors.

These include the HRH1 (Sansuk et al., 2008), ETBR (Zemella et al., 2017), the μ opioid receptor, metabotropic glutamate receptor 1, glucagon-like peptide 1 receptor, G-protein coupled receptor 56, thyrotrophic receptor, and CXCR4 and 5 (Sonnabend et al., 2017).

It is important to note that, as shown in Table 1, the structure and/or function was not verified for all expressed receptors. Furthermore, only a limited number of assays

are available to verify the structure of GPCRs, like circular dichroism (CD)-spectroscopy (Corin et al., 2011; Wang et al., 2013; Shilling et al., 2017). Also the number of assays to verify the function of GPCRs is limited and mostly based on ligand binding like microscale thermophoresis (Corin et al., 2011), surface plasmon resonance spectroscopy and fluorescence measurements (Proverbio et al., 2013), radioactive and nonradioactive competition (Klammt et al.,

2011; Basu et al., 2013) and saturation binding assays (Ishihara et al., 2005; Sonnabend et al., 2017; Zemella et al., 2017; Suzuki et al., 2018). Assays for intracellular effector binding and activation are still missing. Therefore, the success of cell-free synthesis is mostly based on expression success and extracellular binding.

Overall, a number of GPCRs has been expressed using CFPS to date and ligand binding and structure has been verified for some of them. Furthermore, biophysical characterization by NMR spectroscopy was successfully applied for corticotropin-releasing factor receptor type 1 and 2 β and a thermostabilized neurotensin receptor 1. However, most efforts have been made in the expression of GPCRs by the D-CF mode.

Characterization of cell-free expressed membrane proteins

CFPS has high potential for the expression of membrane proteins for structural investigation. As screens for optimal expression and solubilization conditions can be performed fast and in parallel, the protein yield can be increased to levels that suit most methods. Commonly used for structural investigations are X-ray diffraction of protein crystals, NMR spectroscopy and mass spectrometry (MS)-based methods, like crosslinking.

Many of the problems, that arise in the crystallization of GPCRs, can be easily addressed by cell-free methods and the purification effort is minimal, as cell-free expressed protein are usually relatively pure (Boland et al., 2014). Still, as the amounts of expressed protein in these systems are rather low, no GPCR crystal structure was obtained from cell-free systems. Advances have been made in the last years to increase the protein yield by CFPS, leading to the crystallization of some membrane proteins from *E. coli*-based cell-free systems. In 2007, the crystal structure of the multidrug transporter EmrE from *E. coli* at resolutions of 3.8 Å (Chen et al., 2007) and in 2014, the structure of the diacylglycerol kinase, an integral membrane kinase, at resolutions of 2.28 Å (Boland et al., 2014), were obtained. Furthermore, Wada et al. successfully determined a crystal structure of rhodopsin from *Acetabularia acetabulum* at a resolution of 3.2 Å (Wada et al., 2011). As rhodopsin contains seven transmembrane α -helices similar to GPCRs, crystallization of these proteins might also be achieved in the next years.

Cell-free methods offer advantages in receiving proteins for NMR spectroscopy. Nevertheless, so far only

a few examples of NMR spectra received from cell-free expressed GPCRs have been reported, for example, the corticotropin-releasing factor receptor type 1 and 2 β (Klammt et al., 2011) and a thermostabilized neurotensin receptor 1 (Shilling et al., 2017).

The third approach is crosslinking, combined with MS. By crosslinking approaches, a covalent link is created either intra- or intermolecularly. The combination with MS allows for the study of interaction partners and surfaces, as well as conformational changes in the protein under different conditions (Fischer et al., 2013; Schmidt et al., 2013). Crosslinking with subsequent MS can be used complementary to structural investigations by NMR spectroscopy or X-ray diffraction (Ryan and Matthews, 2005) and has gained importance in the last years with advantages in instruments and software.

Crosslinking methods can provide additional structural information on GPCRs. By photo-crosslinking, the binding of urocortin-I on the corticotropin releasing factor receptor type 1 was studied *in vivo* (Coin et al., 2013) and the binding of secretin on the secretin receptor was studied in membrane preparations. Furthermore, the complex of β 2AR with the GPCR kinase 5, extracted from insect cells, was analyzed by a combinatorial approach that included crosslinking studies with a homobifunctional and a zero-length crosslinker (Komolov et al., 2017). To the best of our knowledge, no cross-linking approaches with cell-free expressed GPCRs were performed.

Photo-crosslinking studies with cell-free expressed class A GPCRs

As GPCRs are important targets for drugs, a detailed knowledge of their binding modes is necessary for rational drug design. Thus, it is aimed to combine *E. coli*-based cell-free expression with photo-crosslinking, enzymatic digestion, affinity purification, MS and tandem MS methods to identify the binding site of three different class A GPCRs.

The family of NPY receptors is formed by four receptors in humans (Pedragosa-Badia et al., 2013), which are all involved in the regulation of food intake and the circadian rhythm. Furthermore, they have roles in memory retention, angiogenesis and anxiety (Brothers and Wahlstedt, 2010). These receptors have high potential in serving as targets in the development of drugs for the treatment of diverse diseases like metabolic and cardiovascular diseases (Tan et al., 2018; Yi et al., 2018), disorders of the central nervous system (Duarte-Neves et al., 2016; Göttsche and Woldbye, 2016), as well as certain

cancer types (Li et al., 2015; Tilan and Kitlinska, 2016). Despite their close relation, the NPY receptors display different tissue presence and signaling properties. Furthermore, they have divergent ligand binding profiles (Brothers and Wahlestedt, 2010). The endogenous ligands NPY and peptide YY bind to the NPY receptor type 1 (Y_1R), 2 (Y_2R) and 5 (Y_5R) with high affinities, besides their relatively low sequence similarity and partially opposite functions. Understanding the subtype selectivity is important for the generation of specific drugs with no side effects (Pedragosa-Badia et al., 2013).

For the Y_2R , the C-terminal part (25–36) of NPY is crucial for activation, while the N-terminal part can be truncated without any loss in affinity or potency (Kirby et al., 1993; Beck-Sickinger and Jung, 1995). The structure of Y_2R has been intensively studied by NMR spectroscopy of recombinantly expressed and *in vitro* refolded receptors (Schmidt et al., 2009, 2017). A combination with mutagenesis studies and molecular modeling (Kaiser et al., 2015) revealed the binding mode of the C terminus of NPY. The resulting binding mode was modeled, suggesting a steep binding pose of NPY with the C-terminal

part binding deep in the TM helix bundle (Figure 3). The helix of NPY is suggested to remain flexible, following the motion of the ECL2. This was investigated by photo-crosslinking studies between cell-free expressed Y_2R and an NPY variant (Kögler et al., 2019). The workflow is depicted schematically in Figure 4. The receptor was expressed by a CECF system, which was optimized with respect to detergents, buffer, pH and additives to promote disulfide bridge formation. The photoactivatable amino acid *p*-benzoyl-phenylalanine (Bpa) has been incorporated into NPY at position 27 by solid-phase peptide synthesis, an exchange that is well tolerated (Beck-Sickinger et al., 1994; Cabrele and Beck-Sickinger, 2000). Furthermore, a biotin tag was added at position 22 for purification and visualization, using the high affinity binding of biotin to streptavidin and avidin ($K_D \sim 10^{-14}$ M) (Wilchek and Bayer, 1988; Dundas et al., 2013). As biotin binds deep in the binding pocket of avidin and streptavidin, a spacer arm was added to avoid steric hindrance (Finn et al., 1984).

The binding mode of NPY at Y_1R is not that well characterized. NPY binds mostly at the upper part of TMs and ECL1 (Walker et al., 1994; Sautel et al., 1995, 1996; Du et al., 1997; Sylte et al., 1999; Sjödin et al., 2006) with a direct interaction between D^{6.59} and R³⁵ in NPY (Merten et al., 2007). In contrast to Y_2R , Y_1R needs the N terminus of NPY and the shortage of the first amino acids already leads to a strongly reduced affinity (Beck-Sickinger and Jung, 1995). This effect is even more pronounced for longer truncations (Pedragosa-Badia et al., 2013). The receptor N terminus is not necessary for activation, but truncation lead to a significant reduced binding affinity, suggesting a role of the receptor N terminus in the recognition and positioning of the ligand (Lindner et al., 2009).

By performing photo-crosslinking studies with an NPY variant, bearing Bpa at position 1, an exchange that is tolerated (Beck-Sickinger et al., 1994; Cabrele and Beck-Sickinger, 2000), and biotin at position 4, we investigated the binding of the N terminus of NPY at Y_1R (Figure 4). In combination with mutagenesis, NMR spectroscopy, crystallography and molecular modeling, the binding mode of NPY to Y_1R has been solved (Yang et al., 2018).

The ECL2 plays a crucial role for Y_2 and Y_5 receptors, which has been demonstrated by the generation of receptor chimeras (Lindner et al., 2008). For Y_1R , the role of ECL2 is only partly investigated. Based on the binding mode of NPY at Y_1R , an interaction between the receptor ECL2 and the α -helix of NPY is suggested. This was proven by photo-crosslinking experiments with the NPY variant, used for photo-crosslinking studies on Y_2R ($K^{22}[(Ahx)_2\text{-biotin}]\text{Bpa}^{27}\text{NPY}$).

Figure 3: Model of NPY bound to Y_2R . NPY is shown in blue and Y_2R in gray. NPY has a steep binding mode, with the C terminus binding deep inside the TM helix bundle of Y_2R . This C terminus of NPY unwinds upon binding to the TM helix bundle, while L²⁴ and I²⁸ of NPY form hydrophobic contact towards the ECL2 of Y_2R . The sidechains of residues involved in binding are shown in the respective colors and are labeled. ECL, extracellular loop; TM, transmembrane helix. Modified from Kaiser et al. (2015).

Figure 4: Schematic depiction of the photo-crosslinking workflow between NPY and the $Y_{1/2}R$.

Cell-free expressed receptor in Brij-58 micelles is purified by ligand affinity chromatography using a biotinylated ligand immobilized on avidin agarose beads. Elution is performed with the crosslinking ligand, containing Bpa and a biotin tag. After photo-activation, the complex is enzymatically digested, purified by affinity chromatography and the resulting crosslinked segments are analyzed by MS.

Conclusion

Cell-free expression methods offer some advantages over recombinant expression. These are mainly based on the open nature of this system, which enables the addition of substances. This promotes expression and solubilization, allows rapid screens for optimal expression conditions. There are still problems to obtain a sufficient amount of protein for structural investigations by X-ray diffraction and NMR spectroscopy. Nevertheless, huge progress has been made in recent years, especially in the expression of functional GPCRs. Crosslinking approaches, however, need only small amounts of protein, which makes these approaches the methods of choice for the investigation of ligand receptor interaction with cell-free expressed receptors, as demonstrated for the Y_1R and Y_2R .

Acknowledgments: The financial contribution of the DFG (SFB 1052/A3), (CRC 1052/A3), the German

Federal Ministry of Education and Research, Funder Id: <http://dx.doi.org/10.13039/501100002347> (031A239B) and the SMWK/SAB, Funder Id: <http://dx.doi.org/10.13039/501100006114> (100316655) is kindly acknowledged. We acknowledge the use of the GPCRdb database (<http://www.gpcrdb.org>).

Conflict of interest statement: The authors declare that there are no competing interests associated with this article.

References

- Adams, J.W., Wang, J., Davis, J.R., Liaw, C., Gaidarov, I., Gatlin, J., Dalton, N.D., Gu, Y., Ross, J. Jr., Behan, D., et al. (2008). Myocardial expression, signaling, and function of GPR22: a protective role for an orphan G protein-coupled receptor. *Am. J. Physiol. Heart Circ. Physiol.* 295, H509–H521.
- Albayrak, C. and Swartz, J.R. (2013a). Cell-free co-production of an orthogonal transfer RNA activates efficient site-specific

- non-natural amino acid incorporation. *Nucleic Acids Res.* **41**, 5949–5963.
- Albayrak, C. and Swartz, J.R. (2013b). Using *E. coli*-based cell-free protein synthesis to evaluate the kinetic performance of an orthogonal tRNA and aminoacyl-tRNA synthetase pair. *Biochem. Biophys. Res. Commun.* **431**, 291–295.
- Anastasina, M., Terenin, I., Butcher, S.J., and Kainov, D.E. (2014). A technique to increase protein yield in a rabbit reticulocyte lysate translation system. *Biotechniques* **56**, 36–39.
- Anderson, M.J., Stark, J.C., Hodgman, C.E., and Jewett, M.C. (2015). Energizing eukaryotic cell-free protein synthesis with glucose metabolism. *FEBS Lett.* **589**, 1723–1727.
- Aoki, M., Matsuda, T., Tomo, Y., Miyata, Y., Inoue, M., Kigawa, T., and Yokoyama, S. (2009). Automated system for high-throughput protein production using the dialysis cell-free method. *Protein Expr. Purif.* **68**, 128–136.
- Banères, J.L., Popot, J.L., and Mouillac, B. (2011). New advances in production and functional folding of G-protein-coupled receptors. *Trends Biotechnol.* **29**, 314–322.
- Basu, D., Castellano, J.M., Thomas, N., and Mishra, R.K. (2013). Cell-free protein synthesis and purification of human dopamine D2 receptor long isoform. *Biotechnol. Prog.* **29**, 601–608.
- Beck-Sickinger, A.G. and Jung, G. (1995). Structure-activity relationships of neuropeptide Y analogues with respect to Y1 and Y2 receptors. *Biopolymers* **37**, 123–142.
- Beck-Sickinger, A.G., Wieland, H.A., Wittneben, H., Willim, K.D., Rudolf, K., and Jung, G. (1994). Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **225**, 947–958.
- Bernhard, F. and Tozawa, Y. (2013). Cell-free expression – making a mark. *Curr. Opin. Struct. Biol.* **23**, 374–380.
- Berrier, C., Park, K.H., Abes, S., Bibonne, A., Betton, J.M., and Ghazi, A. (2004). Cell-free synthesis of a functional ion channel in the absence of a membrane and in the presence of detergent. *Biochemistry* **43**, 12585–12591.
- Boland, C., Li, D., Shah, S.T.A., Haberkstock, S., Dötsch, V., Bernhard, F., and Caffrey, M. (2014). Cell-free expression and in meso crystallisation of an integral membrane kinase for structure determination. *Cell. Mol. Life Sci.* **71**, 4895–4910.
- Bradrick, S.S., Nagyal, S., and Novatt, H. (2013). A miRNA-responsive cell-free translation system facilitates isolation of hepatitis C virus miRNP complexes. *RNA* **19**, 1159–1169.
- Brödel, A.K., Raymond, J.A., Duman, J.G., Bier, F.F., and Kubick, S. (2013). Functional evaluation of candidate ice structuring proteins using cell-free expression systems. *J. Biotechnol.* **163**, 301–310.
- Brothers, S.P. and Wahlestedt, C. (2010). Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* **2**, 429–439.
- Bundy, B.C. and Swartz, J.R. (2010). Site-specific incorporation of p-propargyloxyphenylalanine in a cell-free environment for direct protein-protein click conjugation. *Bioconjug. Chem.* **21**, 255–263.
- Bundy, B.C. and Swartz, J.R. (2011). Efficient disulfide bond formation in virus-like particles. *J. Biotechnol.* **154**, 230–239.
- Buntru, M., Vogel, S., Spiegel, H., and Schillberg, S. (2014). Tobacco BY-2 cell-free lysate: an alternative and highly-productive plant-based *in vitro* translation system. *BMC Biotechnol.* **14**, 37.
- Cabrele, C. and Beck-Sickinger, A.G. (2000). Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J. Pept. Sci.* **6**, 97–122.
- Calhoun, K.A. and Swartz, J.R. (2005). Energizing cell-free protein synthesis with glucose metabolism. *Biotechnol. Bioeng.* **90**, 606–613.
- Carlson, E.D., Gan, R., Hodgman, C.E., and Jewett, M.C. (2012). Cell-free protein synthesis: applications come of age. *Biotechnol. Adv.* **30**, 1185–1194.
- Catherine, C., Lee, K.H., Oh, S.J., and Kim, D.M. (2013). Cell-free platforms for flexible expression and screening of enzymes. *Biotechnol. Adv.* **31**, 797–803.
- Catoire, L.J., Damian, M., Giusti, F., Martin, A., van Heijenoort, C., Popot, J.L., Guittet, E., and Baneres, J.L. (2010). Structure of a GPCR ligand in its receptor-bound state: leukotriene B4 adopts a highly constrained conformation when associated to human BLT2. *J. Am. Chem. Soc.* **132**, 9049–9057.
- Chappell, J., Jensen, K., and Freemont, P.S. (2013). Validation of an entirely *in vitro* approach for rapid prototyping of DNA regulatory elements for synthetic biology. *Nucleic Acids Res.* **41**, 3471–3481.
- Chen, Y.J., Pornillos, O., Lieu, S., Ma, C., Chen, A.P., and Chang, G. (2007). X-ray structure of EmrE supports dual topology model. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 18999–19004.
- Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K., et al. (2007). High-resolution crystal structure of an engineered human β 2-adrenergic G protein-coupled receptor. *Science* **318**, 1258–1265.
- Chi, H., Wang, X., Li, J., Ren, H., and Huang, F. (2016). Chaperonin-enhanced *Escherichia coli* cell-free expression of functional CXCR4. *J. Biotechnol.* **231**, 193–200.
- Coin, I., Katritch, V., Sun, T., Xiang, Z., Siu, F.Y., Beyermann, M., Stevens, R.C., and Wang, L. (2013). Genetically encoded chemical probes in cells reveal the binding path of urocortin-I to CRF class B GPCR. *Cell* **155**, 1258–1269.
- Corin, K., Baaske, P., Ravel, D.B., Song, J., Brown, E., Wang, X., Geissler, S., Wienken, C.J., Jerabek-Willemsen, M., Duhr, S., et al. (2011). A robust and rapid method of producing soluble, stable, and functional G-protein coupled receptors. *PLoS One* **6**, e23036.
- de Hoog, H.P., Lin JieRong, E.M., Banerjee, S., Décaillot, F.M., and Nallani, M. (2014). Conformational antibody binding to a native, cell-free expressed GPCR in block copolymer membranes. *PLoS One* **9**, e110847.
- Denisov, I.G., Grinkova, Y.V., Lazarides, A.A., and Sligar, S.G. (2004). Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size. *J. Am. Chem. Soc.* **126**, 3477–3487.
- Du, P., Salon, J.A., Tamm, J.A., Hou, C., Cui, W., Walker, M.W., Adham, N., Dhanoa, D.S., Islam, I., Vaysse, P.J., et al. (1997). Modeling the G-protein-coupled neuropeptide Y Y1 receptor agonist and antagonist binding sites. *Protein Eng.* **10**, 109–117.
- Duarte-Neves, J., Pereira de Almeida, L., and Cavadas, C. (2016). Neuropeptide Y (NPY) as a therapeutic target for neurodegenerative diseases. *Neurobiol. Dis.* **95**, 210–224.
- Dundas, C.M., Demonte, D., and Park, S. (2013). Streptavidin-biotin technology: improvements and innovations in chemical and biological applications. *Appl. Microbiol. Biotechnol.* **97**, 9343–9353.

- Eddy, M.T., Lee, M.Y., Gao, Z.G., White, K.L., Didenko, T., Horst, R., Audet, M., Stanczak, P., McClary, K.M., Han, G.W., et al. (2018). Allosteric coupling of drug binding and intracellular signaling in the A_{2A} adenosine receptor. *Cell* 172, 68–80.
- Elhardt, D. and Böck, A. (1982). An *in vitro* polypeptide synthesizing system from methanogenic bacteria: sensitivity to antibiotics. *Mol. Gen. Genet.* 188, 128–134.
- Endo, Y. and Sawasaki, T. (2004). High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *J. Struct. Funct. Genomics* 5, 45–57.
- Endoh, T., Kanai, T., Sato, Y.T., Liu, D.V., Yoshikawa, K., Atomi, H., and Imanaka, T. (2006). Cell-free protein synthesis at high temperatures using the lysate of a hyperthermophile. *J. Biotechnol.* 126, 186–195.
- Fenz, S.F., Sachse, R., Schmidt, T., and Kubick, S. (2014). Cell-free synthesis of membrane proteins: tailored cell models out of microsomes. *Biochim. Biophys. Acta* 1838, 1382–1388.
- Finn, F.M., Titus, G., Horstman, D., and Hofmann, K. (1984). Avidin-biotin affinity chromatography: application to the isolation of human placental insulin receptor. *Proc. Natl. Acad. Sci. U.S.A.* 81, 7328–7332.
- Fischer, L., Chen, Z.A., and Rappsilber, J. (2013). Quantitative cross-linking/mass spectrometry using isotope-labelled cross-linkers. *J. Proteomics* 88, 120–128.
- Flanagan, C.A. (2016). GPCR-radioligand binding assays. *Methods Cell. Biol.* 132, 191–215.
- Focke, P.J., Hein, C., Hoffmann, B., Matulef, K., Bernhard, F., Dötsch, V., and Valiyaveetil, F.I. (2016). Combining *in vitro* folding with cell free protein synthesis for membrane protein expression. *Biochemistry* 55, 4212–4219.
- Fredriksson, R., Lagerstrom, M.C., Lundin, L.G., and Schiöth, H.B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.* 63, 1256–1272.
- Gao, T., Petrova, J., He, W., Huser, T., Kudlick, W., Voss, J., and Coleman, M.A. (2012). Characterization of de novo synthesized GPCRs supported in nanolipoprotein discs. *PLoS One* 7, e44911.
- Goerke, A.R. and Swartz, J.R. (2009). High-level cell-free synthesis yields of proteins containing site-specific non-natural amino acids. *Biotechnol. Bioeng.* 102, 400–416.
- Goldstein, E.S., Reichman, M.E., and Penman, S. (1974). The regulation of protein synthesis in mammalian cells VI. Soluble and polyribosome associated components in controlling *in vitro* polypeptide initiation in HeLa cells. *Proc. Natl. Acad. Sci. U.S.A.* 71, 4752–4756.
- Götzsche, C.R. and Woldbye, D.P. (2016). The role of NPY in learning and memory. *Neuropeptides* 55, 79–89.
- Hagn, F., Etzkorn, M., Raschle, T., and Wagner, G. (2013). Optimized phospholipid bilayer nanodiscs facilitate high-resolution structure determination of membrane proteins. *J. Am. Chem. Soc.* 135, 1919–1925.
- Henrich, E., Dötsch, V., and Bernhard, F. (2015a). Screening for lipid requirements of membrane proteins by combining cell-free expression with nanodiscs. *Methods Enzymol.* 556, 351–369.
- Henrich, E., Hein, C., Dötsch, V., and Bernhard, F. (2015b). Membrane protein production in *Escherichia coli* cell-free lysates. *FEBS Lett.* 589, 1713–1722.
- Hoagland, M.B., Stephenson, M.L., Scott, J.F., Hecht, L.I., and Zamecnik, P.C. (1958). A soluble ribonucleic acid intermediate in protein synthesis. *J. Biol. Chem.* 231, 241–257.
- Hodgman, C.E. and Jewett, M.C. (2013). Optimized extract preparation methods and reaction conditions for improved yeast cell-free protein synthesis. *Biotechnol. Bioeng.* 110, 2643–2654.
- Hong, S.H., Ntai, I., Haimovich, A.D., Kelleher, N.L., Isaacs, F.J., and Jewett, M.C. (2014). Cell-free protein synthesis from a release factor 1 deficient *Escherichia coli* activates efficient and multiple site-specific nonstandard amino acid incorporation. *ACS Synth. Biol.* 3, 398–409.
- Hovijitra, N.T., Wu, J.J., Peaker, B., and Swartz, J.R. (2009). Cell-free synthesis of functional aquaporin 2 in synthetic liposomes. *Biotechnol. Bioeng.* 104, 40–49.
- Huang, Y., Skwarek-Maruszewska, A., Horré, K., Vandeweyer, E., Wolfs, L., Snellinx, A., Saito, T., Radaelli, E., Corthout, N., Colombelli, J., et al. (2015). Loss of GPR3 reduces the amyloid plaque burden and improves memory in Alzheimer's disease mouse models. *Sci. Transl. Med.* 7, 309ra164.
- Isaksson, L., Enberg, J., Neutze, R., Göran Karlsson, B., and Pedersen, A. (2012). Expression screening of membrane proteins with cell-free protein synthesis. *Protein Expr. Purif.* 82, 218–225.
- Ishihara, G., Goto, M., Saeki, M., Ito, K., Hori, T., Kigawa, T., Shirouzu, M., and Yokoyama, S. (2005). Expression of G protein coupled receptors in a cell-free translational system using detergents and thioredoxin-fusion vectors. *Protein Expr. Purif.* 41, 27–37.
- Isogai, S., Deupi, X., Opitz, C., Heydenreich, F.M., Tsai, C.J., Brueckner, F., Schertler, G.F., Veprintsev, D.B., and Grzesiek, S. (2016). Backbone NMR reveals allosteric signal transduction networks in the β 1-adrenergic receptor. *Nature* 530, 237–241.
- Jacobs, M.L., Boyd, M.A., and Kamat, N.P. (2019). Diblock copolymers enhance folding of a mechanosensitive membrane protein during cell-free expression. *Proc. Natl. Acad. Sci. U.S.A.* 116, 4031–4036.
- Joedicke, L., Mao, J., Kuenze, G., Reinhart, C., Kalavacherla, T., Jonker, H.R.A., Richter, C., Schwalbe, H., Meiler, J., Preu, J., et al. (2018). The molecular basis of subtype selectivity of human kinin G-protein-coupled receptors. *Nat. Chem. Biol.* 14, 284–290.
- Junge, F., Schneider, B., Reckel, S., Schwarz, D., Dötsch, V., and Bernhard, F. (2008). Large-scale production of functional membrane proteins. *Cell. Mol. Life Sci.* 65, 1729–1755.
- Junge, F., Luh, L.M., Proverbio, D., Schäfer, B., Abele, R., Beyer-mann, M., Dötsch, V., and Bernhard, F. (2010). Modulation of G-protein coupled receptor sample quality by modified cell-free expression protocols: a case study of the human endothelin A receptor. *J. Struct. Biol.* 172, 94–106.
- Junge, F., Haberstoch, S., Roos, C., Stefer, S., Proverbio, D., Dötsch, V., and Bernhard, F. (2011). Advances in cell-free protein synthesis for the functional and structural analysis of membrane proteins. *N. Biotechnol.* 28, 262–271.
- Kaiser, A., Müller, P., Zellmann, T., Scheidt, H.A., Thomas, L., Bosse, M., Meier, R., Meiler, J., Huster, D., Beck-Sickinger, A.G., et al. (2015). Unwinding of the C-terminal residues of neuropeptide Y is critical for Y(2) receptor binding and activation. *Angew. Chem. Int. Ed.* 54, 7446–7449.
- Katritch, V., Cherezov, V., and Stevens, R.C. (2013). Structure-function of the G protein-coupled receptor superfamily. *Annu. Rev. Pharmacol. Toxicol.* 53, 531–556.
- Katzen, F., Fletcher, J.E., Yang, J.P., Kang, D., Peterson, T.C., Cappuccio, J.A., Blanchette, C.D., Sulchek, T., Chromy, B.A., Hoepflich, P.D., et al. (2008). Insertion of membrane proteins into discoid-

- dal membranes using a cell-free protein expression approach. *J. Proteome Res.* 7, 3535–3542.
- Kawasaki, T., Gouda, M.D., Sawasaki, T., Takai, K., and Endo, Y. (2003). Efficient synthesis of a disulfide-containing protein through a batch cell-free system from wheat germ. *Eur. J. Biochem.* 270, 4780–4786.
- Keller, T., Egenberger, B., Gorboulev, V., Bernhard, F., Uzelac, Z., Gorbunov, D., Wirth, C., Koppatz, S., Dötsch, V., Hunte, C., et al. (2011). The large extracellular loop of organic cation transporter 1 influences substrate affinity and is pivotal for oligomerization. *J. Biol. Chem.* 286, 37874–37886.
- Kigawa, T. (2010). Cell-free protein production system with the *E. coli* crude extract for determination of protein folds. *Methods Mol. Biol.* 607, 101–111.
- Kigawa, T., Yabuki, T., Yoshida, Y., Tsutsui, M., Ito, Y., Shibata, T., and Yokoyama, S. (1999). Cell-free production and stable-isotope labeling of milligram quantities of proteins. *FEBS Lett.* 442, 15–19.
- Kigawa, T., Yabuki, T., Matsuda, N., Matsuda, T., Nakajima, R., Tanaka, A., and Yokoyama, S. (2004). Preparation of *Escherichia coli* cell extract for highly productive cell-free protein expression. *J. Struct. Funct. Genomics* 5, 63–68.
- Kim, D.M. and Swartz, J.R. (1999). Prolonging cell-free protein synthesis with a novel ATP regeneration system. *Biotechnol. Bioeng.* 66, 180–188.
- Kim, D.M. and Swartz, J.R. (2004). Efficient production of a bioactive, multiple disulfide-bonded protein using modified extracts of *Escherichia coli*. *Biotechnol. Bioeng.* 85, 122–129.
- Kim, T.W., Keum, J.W., Oh, I.S., Choi, C.Y., Park, C.G., and Kim, D.M. (2006a). Simple procedures for the construction of a robust and cost-effective cell-free protein synthesis system. *J. Biotechnol.* 126, 554–561.
- Kim, T.W., Kim, D.M., and Choi, C.Y. (2006b). Rapid production of milligram quantities of proteins in a batch cell-free protein synthesis system. *J. Biotechnol.* 124, 373–380.
- Kirby, D.A., Koerber, S.C., Craig, A.G., Feinstein, R.D., Delmas, L., Brown, M.R., and Rivier, J.E. (1993). Defining structural requirements for neuropeptide Y receptors using truncated and conformationally restricted analogues. *J. Med. Chem.* 36, 385–393.
- Klammt, C., Schwarz, D., Fendler, K., Haase, W., Dötsch, V., and Bernhard, F. (2005). Evaluation of detergents for the soluble expression of alpha-helical and beta-barrel-type integral membrane proteins by a preparative scale individual cell-free expression system. *FEBS J.* 272, 6024–6038.
- Klammt, C., Schwarz, D., Eifler, N., Engel, A., Piehler, J., Haase, W., Hahn, S., Dötsch, V., and Bernhard, F. (2007). Cell-free production of G protein-coupled receptors for functional and structural studies. *J. Struct. Biol.* 158, 482–493.
- Klammt, C., Perrin, M.H., Maslennikov, I., Renault, L., Krupa, M., Kwiatkowski, W., Stahlberg, H., Vale, W., and Choe, S. (2011). Polymer-based cell-free expression of ligand-binding family B G-protein coupled receptors without detergents. *Protein Sci.* 20, 1030–1041.
- Knorre, D.G., Kudryashova, N.V., and Godovikova, T.S. (2009). Chemical and functional aspects of posttranslational modification of proteins. *Acta Nat.* 1, 29–51.
- Kögler, L.M., Stichel, J., Kaiser, A., and Beck-Sickinger, A.G. (2019). Cell-free expression and photo-crosslinking of the human neuropeptide Y2 receptor. *Front. Pharmacol.* 10, 176.
- Komoda, K., Naito, S., and Ishikawa, M. (2004). Replication of plant RNA virus genomes in a cell-free extract of vacuolated plant protoplasts. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1863–1867.
- Komolov, K.E., Du, Y., Duc, N.M., Betz, R.M., Rodrigues, J., Leib, R.D., Patra, D., Skinotis, G., Adams, C.M., Dror, R.O., et al. (2017). Structural and functional analysis of a β 2-adrenergic receptor complex with GRK5. *Cell* 169, 407–421 e416.
- Kooistra, A.J., Roumen, L., Leurs, R., de Esch, I.J., and de Graaf, C. (2013). From heptahelical bundle to hits from the Haystack: structure-based virtual screening for GPCR ligands. *Methods Enzymol.* 522, 279–336.
- Krishnan, M., de Leeuw, T., and Pandit, A. (2019). Cell-free soluble expression of the membrane protein PsbS. *Protein Expr. Purif.* 159, 17–20.
- Latorraca, N.R., Venkatakrishnan, A.J., and Dror, R.O. (2017). GPCR dynamics: structures in motion. *Chem. Rev.* 117, 139–155.
- Lee, Y., Basith, S., and Choi, S. (2018). Recent advances in structure-based drug design targeting class A G protein-coupled receptors utilizing crystal structures and computational simulations. *J. Med. Chem.* 61, 1–46.
- Li, J., Tian, Y., and Wu, A. (2015). Neuropeptide Y receptors: a promising target for cancer imaging and therapy. *Regen. Biomater.* 2, 215–219.
- Lindner, D., Stichel, J., and Beck-Sickinger, A.G. (2008). Molecular recognition of the NPY hormone family by their receptors. *Nutrition* 24, 907–917.
- Lindner, D., Walther, C., Tennemann, A., and Beck-Sickinger, A.G. (2009). Functional role of the extracellular N-terminal domain of neuropeptide Y subfamily receptors in membrane integration and agonist-stimulated internalization. *Cell. Signal.* 21, 61–68.
- Lopez, J.J., Shukla, A.K., Reinhart, C., Schwalbe, H., Michel, H., and Glaubitz, C. (2008). The structure of the neuropeptide bradykinin bound to the human G-protein coupled receptor bradykinin B2 as determined by solid-state NMR spectroscopy. *Angew. Chem. Int. Ed.* 47, 1668–1671.
- Luca, S., White, J.F., Sohal, A.K., Filippov, D.V., van Boom, J.H., Grishammer, R., and Baldus, M. (2003). The conformation of neurotensin bound to its G protein-coupled receptor. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10706–10711.
- Lyukmanova, E.N., Shenkarev, Z.O., Khabibullina, N.F., Kopeina, G.S., Shulepko, M.A., Paramonov, A.S., Mineev, K.S., Tikhonov, R.V., Shingarova, L.N., Petrovskaya, L.E., et al. (2012a). Lipid-protein nanodiscs for cell-free production of integral membrane proteins in a soluble and folded state: comparison with detergent micelles, bicelles and liposomes. *Biochim. Biophys. Acta* 1818, 349–358.
- Lyukmanova, E.N., Shenkarev, Z.O., Khabibullina, N.F., Kulbatskiy, D.S., Shulepko, M.A., Petrovskaya, L.E., Arseniev, A.S., Dolgikh, D.A., and Kirpichnikov, M.P. (2012b). N-terminal fusion tags for effective production of g-protein-coupled receptors in bacterial cell-free systems. *Acta Nat.* 4, 58–64.
- MacDonald, M.R., McCourt, D.W., and Krause, J.E. (1988). Post-translational processing of alpha-, beta-, and gamma-prepro-tachykinins. Cell-free translation and early posttranslational processing events. *J. Biol. Chem.* 263, 15176–15183.
- Madin, K., Sawasaki, T., Ogasawara, T., and Endo, Y. (2000). A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 559–564.

- Manglik, A., Kim, T.H., Masureel, M., Altenbach, C., Yang, Z., Hilger, D., Lerch, M.T., Kobilka, T.S., Thian, F.S., Hubbell, W.L., et al. (2015). Structural insights into the dynamic process of β_2 -adrenergic receptor signaling. *Cell* 161, 1101–1111.
- Maslennikov, I., Klammt, C., Hwang, E., Kefala, G., Okamura, M., Esquivies, L., Mors, K., Glaubitz, C., Kwiatkowski, W., Jeon, Y.H., et al. (2010). Membrane domain structures of three classes of histidine kinase receptors by cell-free expression and rapid NMR analysis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 10902–10907.
- Matsubayashi, H., Kuruma, Y., and Ueda, T. (2014). Cell-free synthesis of SecYEG translocon as the fundamental protein transport machinery. *Orig. Life Evol. Biosph.* 44, 331–334.
- Matsuda, T., Furumoto, S., Higuchi, K., Yokoyama, J., Zhang, M.R., Yanai, K., Iwata, R., and Kigawa, T. (2012). Rapid biochemical synthesis of ^{13}C -labeled single chain variable fragment antibody for immuno-PET by cell-free protein synthesis. *Bioorg. Med. Chem.* 20, 6579–6582.
- Matthaei, H. and Nirenberg, M.W. (1961). The dependence of cell-free protein synthesis in *E. coli* upon RNA prepared from ribosomes. *Biochem. Biophys. Res. Commun.* 4, 404–408.
- Merk, H., Gless, C., Maertens, B., Gerrits, M., and Stiege, W. (2012). Cell-free synthesis of functional and endotoxin-free antibody Fab fragments by translocation into microsomes. *Biotechniques* 53, 153–160.
- Merten, N., Lindner, D., Rabe, N., Römpler, H., Mörl, K., Schöneberg, T., and Beck-Sickinger, A.G. (2007). Receptor subtype-specific docking of Asp6.59 with C-terminal arginine residues in Y receptor ligands. *J. Biol. Chem.* 282, 7543–7551.
- Michel, E. and Wüthrich, K. (2012). Cell-free expression of disulfide-containing eukaryotic proteins for structural biology. *FEBS J.* 279, 3176–3184.
- Morita, E.H., Shimizu, M., Ogasawara, T., Endo, Y., Tanaka, R., and Kohno, T. (2004). A novel way of amino acid-specific assignment in ^1H - ^{15}N HSQC spectra with a wheat germ cell-free protein synthesis system. *J. Biomol. NMR* 30, 37–45.
- Nehring, S., Budisa, N., and Wiltshi, B. (2012). Performance analysis of orthogonal pairs designed for an expanded eukaryotic genetic code. *PLoS One* 7, e31992.
- Ng, P.P., Jia, M., Patel, K.G., Brody, J.D., Swartz, J.R., Levy, S., and Levy, R. (2012). A vaccine directed to B cells and produced by cell-free protein synthesis generates potent antilymphoma immunity. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14526–14531.
- Nirenberg, M.W. and Matthaei, J.H. (1961). The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 47, 1588–1602.
- Niwa, T., Kanamori, T., Ueda, T., and Taguchi, H. (2012). Global analysis of chaperone effects using a reconstituted cell-free translation system. *Proc. Natl. Acad. Sci. U.S.A.* 109, 8937–8942.
- Orth, J.H., Schorch, B., Boundy, S., Ffrench-Constant, R., Kubick, S., and Aktories, K. (2011). Cell-free synthesis and characterization of a novel cytotoxic pierisin-like protein from the cabbage butterfly *Pieris rapae*. *Toxicon* 57, 199–207.
- Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., et al. (2000). Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* 289, 739–745.
- Pándy-Szekeres, G., Munk, C., Tsonkov, T.M., Mordalski, S., Harpsoe, K., Hauser, A.S., Bojarski, A.J., and Gloriam, D.E. (2018). GPCRdb in 2018: adding GPCR structure models and ligands. *Nucleic Acids Res.* 46, D440–D446.
- Pedragosa-Badia, X., Stichel, J., and Beck-Sickinger, A.G. (2013). Neuropeptide Y receptors: how to get subtype selectivity. *Front. Endocrinol. (Lausanne)* 4, 1–13, Article 5.
- Proverbio, D., Roos, C., Beyermann, M., Orbán, E., Dötsch, V., and Bernhard, F. (2013). Functional properties of cell-free expressed human endothelin A and endothelin B receptors in artificial membrane environments. *Biochim. Biophys. Acta* 1828, 2182–2192.
- Quast, R.B., Kortt, O., Henkel, J., Dondapati, S.K., Wustenhagen, D.A., Stech, M., and Kubick, S. (2015). Automated production of functional membrane proteins using eukaryotic cell-free translation systems. *J. Biotechnol.* 203, 45–53.
- Rath, P., Demange, P., Saurel, O., Tropis, M., Daffe, M., Dötsch, V., Ghazi, A., Bernhard, F., and Milon, A. (2011). Functional expression of the PorAH channel from *Corynebacterium glutamicum* in cell-free expression systems: implications for the role of the naturally occurring mycolic acid modification. *J. Biol. Chem.* 286, 32525–32532.
- Reckel, S., Sobhanifar, S., Durst, F., Löhr, F., Shirokov, V.A., Dötsch, V., and Bernhard, F. (2010). Strategies for the cell-free expression of membrane proteins. *Methods Mol. Biol.* 607, 187–212.
- Roberts, B.E. and Paterson, B.M. (1973). Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. U.S.A.* 70, 2330–2334.
- Roos, C., Zocher, M., Müller, D., Münch, D., Schneider, T., Sahl, H.G., Scholz, F., Wachtveitl, J., Ma, Y., Proverbio, D., et al. (2012). Characterization of co-translationally formed nanodisc complexes with small multidrug transporters, proteorhodopsin and with the *E. coli* MraY translocase. *Biochim. Biophys. Acta* 1818, 3098–3106.
- Rothblatt, J.A. and Meyer, D.I. (1986). Secretion in yeast: reconstitution of the translocation and glycosylation of α -factor and invertase in a homologous cell-free system. *Cell* 44, 619–628.
- Rues, R.B., Dötsch, V., and Bernhard, F. (2016). Co-translational formation and pharmacological characterization of β_1 -adrenergic receptor/nanodisc complexes with different lipid environments. *Biochim. Biophys. Acta* 1858, 1306–1316.
- Rues, R.B., Dong, F., Dötsch, V., and Bernhard, F. (2018). Systematic optimization of cell-free synthesized human endothelin B receptor folding. *Methods* 147, 73–83.
- Ruggero, D., Creti, R., and Londei, P. (1993). *In vitro* translation of archaeal natural mRNAs at high temperatures. *FEMS Microbiol. Lett.* 107, 89–94.
- Ryabova, L.A., Desplancq, D., Spirin, A.S., and Plückthun, A. (1997). Functional antibody production using cell-free translation: effects of protein disulfide isomerase and chaperones. *Nat. Biotechnol.* 15, 79–84.
- Ryan, D.P. and Matthews, J.M. (2005). Protein-protein interactions in human disease. *Curr. Opin. Struct. Biol.* 15, 441–446.
- Sansuk, K., Balog, C.I., van der Does, A.M., Booth, R., de Grip, W.J., Deelder, A.M., Bakker, R.A., Leurs, R., and Hensbergen, P.J. (2008). GPCR proteomics: mass spectrometric and functional analysis of histamine H1 receptor after baculovirus-driven and *in vitro* cell free expression. *J. Proteome Res.* 7, 621–629.
- Sautel, M., Martinez, R., Munoz, M., Peitsch, M.C., Beck-Sickinger, A.G., and Walker, P. (1995). Role of a hydrophobic pocket of the human Y1 neuropeptide Y receptor in ligand binding. *Mol. Cell. Endocrinol.* 112, 215–222.
- Sautel, M., Rudolf, K., Wittneben, H., Herzog, H., Martinez, R., Munoz, M., Eberlein, W., Engel, W., Walker, P., and

- Beck-Sickinger, A.G. (1996). Neuropeptide Y and the nonpeptide antagonist BIBP 3226 share an overlapping binding site at the human Y1 receptor. *Mol. Pharmacol.* 50, 285–292.
- Sawasaki, T., Ogasawara, T., Morishita, R., and Endo, Y. (2002). A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. U.S.A.* 99, 14652–14657.
- Sawasaki, T., Kamura, N., Matsunaga, S., Saeki, M., Tsuchimochi, M., Morishita, R., and Endo, Y. (2008). Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate. *FEBS Lett.* 582, 221–228.
- Schmidt, P., Lindner, D., Montag, C., Berndt, S., Beck-Sickinger, A.G., Rudolph, R., and Huster, D. (2009). Prokaryotic expression, *in vitro* folding, and molecular pharmacological characterization of the neuropeptide Y receptor type 2. *Biotechnol. Prog.* 25, 1732–1739.
- Schmidt, C., Zhou, M., Marriott, H., Morgner, N., Politis, A., and Robinson, C.V. (2013). Comparative cross-linking and mass spectrometry of an intact F-type ATPase suggest a role for phosphorylation. *Nat. Commun.* 4, 1985.
- Schmidt, P., Bender, B.J., Kaiser, A., Gulati, K., Scheidt, H.A., Hamm, H.E., Meiler, J., Beck-Sickinger, A.G., and Huster, D. (2017). Improved *in vitro* folding of the Y₂ G protein-coupled receptor into bicelles. *Front. Mol. Biosci.* 4, 100.
- Schwarz, D., Junge, F., Durst, F., Frölich, N., Schneider, B., Reckel, S., Sobhanifar, S., Dötsch, V., and Bernhard, F. (2007). Preparative scale expression of membrane proteins in *Escherichia coli*-based continuous exchange cell-free systems. *Nat. Protoc.* 2, 2945–2957.
- Schwarz, D., Daley, D., Beckhaus, T., Dötsch, V., and Bernhard, F. (2010). Cell-free expression profiling of *E. coli* inner membrane proteins. *Proteomics* 10, 1762–1779.
- Schweet, R., Lamfrom, H., and Allen, E. (1958). The synthesis of hemoglobin in a cell-free system. *Proc. Natl. Acad. Sci. U.S.A.* 44, 1029–1035.
- Seddon, A.M., Curnow, P., and Booth, P.J. (2004). Membrane proteins, lipids and detergents: not just a soap opera. *Biochim. Biophys. Acta* 1666, 105–117.
- Shadiac, N., Nagarajan, Y., Waters, S., and Hrmova, M. (2013). Close allies in membrane protein research: cell-free synthesis and nanotechnology. *Mol. Membr. Biol.* 30, 229–245.
- Shaklee, P.M., Semrau, S., Malkus, M., Kubick, S., Dogterom, M., and Schmidt, T. (2010). Protein incorporation in giant lipid vesicles under physiological conditions. *Chembiochem* 11, 175–179.
- Shenkarev, Z.O., Lyukmanova, E.N., Paramonov, A.S., Shingarova, L.N., Chupin, V.V., Kirpichnikov, M.P., Blommers, M.J., and Arseniev, A.S. (2010). Lipid-protein nanodiscs as reference medium in detergent screening for high-resolution NMR studies of integral membrane proteins. *J. Am. Chem. Soc.* 132, 5628–5629.
- Shilling, P.J., Bumbak, F., Scott, D.J., Bathgate, R.A.D., and Gooley, P.R. (2017). Characterisation of a cell-free synthesised G-protein coupled receptor. *Sci. Rep.* 7, 1094.
- Shimada, I., Ueda, T., Kofuku, Y., Eddy, M.T., and Wüthrich, K. (2018). GPCR drug discovery: integrating solution NMR data with crystal and cryo-EM structures. *Nat. Rev. Drug Discov.* 18, 59–82.
- Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001). Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* 19, 751–755.
- Shin, J. and Noireaux, V. (2010). Study of messenger RNA inactivation and protein degradation in an *Escherichia coli* cell-free expression system. *J. Biol. Eng.* 4, 9.
- Shingaki, T. and Nimura, N. (2011). Improvement of translation efficiency in an *Escherichia coli* cell-free protein system using cysteine. *Protein Expr. Purif.* 77, 193–197.
- Shrestha, P., Holland, T.M., and Bundy, B.C. (2012). Streamlined extract preparation for *Escherichia coli*-based cell-free protein synthesis by sonication or bead vortex mixing. *Biotechniques* 53, 163–174.
- Sissons, C.H. (1974). Yeast protein synthesis. Preparation and analysis of a highly active cell-free system. *Biochem. J.* 144, 131–140.
- Sjödin, P., Holmberg, S.K., Akerberg, H., Berglund, M.M., Mohell, N., and Larhammar, D. (2006). Re-evaluation of receptor-ligand interactions of the human neuropeptide Y receptor Y1: a site-directed mutagenesis study. *Biochem. J.* 393, 161–169.
- Sonnabend, A., Spahn, V., Stech, M., Zemella, A., Stein, C., and Kubick, S. (2017). Production of G protein-coupled receptors in an insect-based cell-free system. *Biotechnol. Bioeng.* 114, 2328–2338.
- Spirin, A.S. (2004). High-throughput cell-free systems for synthesis of functionally active proteins. *Trends Biotechnol.* 22, 538–545.
- Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y., and Alakhov, Y.B. (1988). A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* 242, 1162–1164.
- Sriram, K. and Insel, P.A. (2018). G protein-coupled receptors as targets for approved drugs: how many targets and how many drugs? *Mol. Pharmacol.* 93, 251–258.
- Stech, M., Quast, R.B., Sachse, R., Schulze, C., Wüstenhagen, D.A., and Kubick, S. (2014). A continuous-exchange cell-free protein synthesis system based on extracts from cultured insect cells. *PLoS One* 9, e96635.
- Stehle, J., Silvers, R., Werner, K., Chatterjee, D., Gande, S., Scholz, F., Dutta, A., Wachtveitl, J., Klein-Seetharaman, J., and Schwalbe, H. (2014). Characterization of the simultaneous decay kinetics of metarhodopsin states II and III in rhodopsin by solution-state NMR spectroscopy. *Angew. Chem. Int. Ed.* 53, 2078–2084.
- Suzuki, Y., Ogasawara, T., Tanaka, Y., Takeda, H., Sawasaki, T., Mogi, M., Liu, S., and Maeyama, K. (2018). Functional G-protein-coupled receptor (GPCR) synthesis: the pharmacological analysis of human histamine H1 receptor (HRH1) synthesized by a wheat germ cell-free protein synthesis system combined with asolectin glycosomes. *Front. Pharmacol.* 9, 38.
- Swartz, J.R. (2011). Transforming biochemical engineering with cell-free biology. *AIChE J.* 58, 5–13.
- Sylte, I., Andrianjara, C.R., Calvet, A., Pascal, Y., and Dahl, S.G. (1999). Molecular dynamics of NPY Y1 receptor activation. *Bioorg. Med. Chem.* 7, 2737–2748.
- Tan, C.M.J., Green, P., Tapoulal, N., Lewandowski, A.J., Leeson, P., and Herring, N. (2018). The role of neuropeptide Y in cardiovascular health and disease. *Front. Physiol.* 9, 1281.
- Tanrikulu, I.C., Schmitt, E., Mechulam, Y., Goddard, W.A., 3rd, and Tirrell, D.A. (2009). Discovery of *Escherichia coli* methionyl-tRNA synthetase mutants for efficient labeling of proteins with azidonorleucine *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15285–15290.
- Tarui, H., Imanishi, S., and Hara, T. (2000). A novel cell-free translation/glycosylation system prepared from insect cells. *J. Biosci. Bioeng.* 90, 508–514.

- Tate, C.G. (2001). Overexpression of mammalian integral membrane proteins for structural studies. *FEBS Lett.* 504, 94–98.
- Tilan, J. and Kitlinska, J. (2016). Neuropeptide Y (NPY) in tumor growth and progression: Lessons learned from pediatric oncology. *Neuropeptides* 55, 55–66.
- Tsuboi, T., Takeo, S., Sawasaki, T., Torii, M., and Endo, Y. (2010). An efficient approach to the production of vaccines against the malaria parasite. *Methods Mol. Biol.* 607, 73–83.
- Ullah, M.W., Khattak, W.A., Ul-Islam, M., Khan, S., and Park, J.K. (2015). Encapsulated yeast cell-free system: a strategy for cost-effective and sustainable production of bio-ethanol in consecutive batches. *Biotechnol. Bioprocess Eng.* 20, 561–575.
- Umehara, T., Kim, J., Lee, S., Guo, L.T., Soll, D., and Park, H.S. (2012). N-acetyl lysyl-tRNA synthetases evolved by a CcdB-based selection possess N-acetyl lysine specificity *in vitro* and *in vivo*. *FEBS Lett.* 586, 729–733.
- Villate, M., Merino, N., and Blanco, F.J. (2012). Production of meganucleases by cell-free protein synthesis for functional and structural studies. *Protein Expr. Purif.* 85, 246–249.
- Wada, T., Shimono, K., Kikukawa, T., Hato, M., Shinya, N., Kim, S.Y., Kimura-Someya, T., Shirouzu, M., Tamogami, J., Miyauchi, S., et al. (2011). Crystal structure of the eukaryotic light-driven proton-pumping rhodopsin, *Acetabularia* rhodopsin II, from marine alga. *J. Mol. Biol.* 411, 986–998.
- Walker, P., Munoz, M., Martinez, R., and Peitsch, M.C. (1994). Acidic residues in extracellular loops of the human Y1 neuropeptide Y receptor are essential for ligand binding. *J. Biol. Chem.* 269, 2863–2869.
- Wang, X., Cui, Y., and Wang, J. (2013). Efficient expression and immunoaffinity purification of human trace amine-associated receptor 5 from *E. coli* cell-free system. *Protein Pept. Lett.* 20, 473–480.
- Weber, L.A., Feman, E.R., and Baglioni, C. (1975). A cell free system from HeLa cells active in initiation of protein synthesis. *Biochemistry* 14, 5315–5321.
- Wheatley, M., Wootten, D., Conner, M.T., Simms, J., Kendrick, R., Logan, R.T., Poyner, D.R., and Barwell, J. (2012). Lifting the lid on GPCRs: the role of extracellular loops. *Br. J. Pharmacol.* 165, 1688–1703.
- Wilchek, M. and Bayer, E.A. (1988). The avidin-biotin complex in bioanalytical applications. *Anal. Biochem.* 171, 1–32.
- Xiang, J., Chun, E., Liu, C., Jing, L., Al-Sahouri, Z., Zhu, L., and Liu, W. (2016). Successful strategies to determine high-resolution structures of GPCRs. *Trends Pharmacol. Sci.* 37, 1055–1069.
- Yang, J., Kanter, G., Voloshin, A., Levy, R., and Swartz, J.R. (2004). Expression of active murine granulocyte-macrophage colony-stimulating factor in an *Escherichia coli* cell-free system. *Biotechnol. Prog.* 20, 1689–1696.
- Yang, J.P., Cirico, T., Katzen, F., Peterson, T.C., and Kudlicki, W. (2011). Cell-free synthesis of a functional G protein-coupled receptor complexed with nanometer scale bilayer discs. *BMC Biotechnol.* 11, 57.
- Yang, Z., Han, S., Keller, M., Kaiser, A., Bender, B.J., Bosse, M., Burkert, K., Kögler, L.M., Wifling, D., Bernhardt, G., et al. (2018). Structural basis of ligand binding modes at the neuropeptide YY1 receptor. *Nature* 556, 520–524.
- Yi, M., Li, H., Wu, Z., Yan, J., Liu, Q., Ou, C., and Chen, M. (2018). A promising therapeutic target for metabolic diseases: neuropeptide Y receptors in humans. *Cell. Physiol. Biochem.* 45, 88–107.
- Yin, G. and Swartz, J.R. (2004). Enhancing multiple disulfide bonded protein folding in a cell-free system. *Biotechnol. Bioeng.* 86, 188–195.
- Zawada, J.F., Yin, G., Steiner, A.R., Yang, J., Naresh, A., Roy, S.M., Gold, D.S., Heinsohn, H.G., and Murray, C.J. (2011). Microscale to manufacturing scale-up of cell-free cytokine production – a new approach for shortening protein production development timelines. *Biotechnol. Bioeng.* 108, 1570–1578.
- Zeenko, V.V., Wang, C., Majumder, M., Komar, A.A., Snider, M.D., Merrick, W.C., Kaufman, R.J., and Hatzoglou, M. (2008). An efficient *in vitro* translation system from mammalian cells lacking the translational inhibition caused by eIF2 phosphorylation. *RNA* 14, 593–602.
- Zemella, A., Grossmann, S., Sachse, R., Sonnabend, A., Schaefer, M., and Kubick, S. (2017). Qualifying a eukaryotic cell-free system for fluorescence based GPCR analyses. *Sci. Rep.* 7, 3740.
- Zhu, J. (2012). Mammalian cell protein expression for biopharmaceutical production. *Biotechnol. Adv.* 30, 1158–1170.
- Zubay, G. (1973). *In vitro* synthesis of protein in microbial systems. *Annu. Rev. Genet.* 7, 267–287.