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High throughput platforms for structural genomics of integral membrane proteins

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Abstract

Structural genomics approaches on integral membrane proteins have been postulated for over a decade, yet specific efforts are lagging years behind their soluble counterparts. Indeed, high throughput methodologies for production and characterization of prokaryotic integral membrane proteins are only now emerging, while large-scale efforts for eukaryotic ones are still in their infancy. Presented here is a review of recent literature on actively ongoing structural genomics of membrane protein initiatives, with a focus on those aimed at implementing interesting techniques aimed at increasing our rate of success for this class of macromolecules.

Keywords

Membrane proteins; high-throughput; structure; structural genomics

Introduction

Membrane proteins comprise approximately 30% of all polypeptides in both prokaryotic and eukaryotic organisms. However, they make up but a fraction of a percent of those with known structure. The discrepancy with soluble proteins in successful structure determination arises primarily from their poor performance in over-expression systems, and in their instability in the detergent-containing solutions required for the membrane extraction and purification steps.

The advent of recombinant DNA technologies first, and of high-throughput methodologies later has shifted the *modus operandi* from one in which procedures were endlessly tuned and tailored to a protein of interest, most often derived from natural sources, to one in which experimental conditions are first optimized, then set, as a close to invariant standard. In this way, different proteins are screened to select the subset with a biophysical profile such as to maximize the probability of successful crystallization. The molecules analyzed are typically variants of a gene of interest belonging to the same family, selected using bioinformatics in the form of orthologues or homologues. Such an approach constitutes the essence of structural genomics, first considered for soluble proteins in the mid 1990's and loosely defined as the large-scale determination of proteins structures using high-throughput,

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automated platforms. The utility of screening natural variants has been clearly demonstrated in membrane protein structural biology, for prokaryotes and eukaryotes, using different expression systems [1–3]. Furthermore, the approach is suited for operation in high throughput mode [4–6].

For membrane proteins, true structural genomics initiatives taking a project from gene to structure in full automation still reside in the realm of science fiction. This is mainly because of the iterative rounds of optimization requiring extensive manual intervention that are typically needed at the crystallization stage to produce diffraction quality crystals. Therefore, this adds to the importance of utilizing a robust screening platform to identify well-expressing candidates that are stable in detergents compatible with crystallization.

In number of unique structures solved, and in increment in the yearly rate of structures determined, membrane proteins seem to be lagging 20 years behind their soluble counterparts, and just now entering the exponential phase of growth. The same appears to hold true for the development of structural genomics approaches, only with a shorter lag time. Indeed, 15 years from its nascence, the field of structural genomics has matured and clear paths for the development of methods with application to integral membrane proteins are finally being implemented. Not surprisingly, platforms for prokaryotic integral membrane proteins, or those relying on bacterial expression systems are at a more advanced stage than those for eukaryotic ones, but efforts are being made on all fronts. As to be expected, structural genomics methods are not necessarily implementable in most individual laboratories due to the large capital outlay of equipment often necessary to gain the economies of scale, so most of the efforts are focused around centers or groups of collaborating laboratories. Also, many reports have suggested methodologies that could be used for high throughput efforts, as opposed to having been tested on considerable numbers (>100) of proteins. This review will describe methodologies used, and progress made, especially by larger scale initiatives. The focus will be on those platforms tailored for x-ray crystallographic analysis rather than for other structure determination methods such as electron crystallography and NMR, for which however target selection procedures are similar, and progress is being made [7, 8].

***Escherichia coli*-based high-throughput platforms**

Escherichia coli (*E. coli*) is an ideal host for structural genomics approaches. Bacterial expression systems can be readily and easily genetically manipulated, and are simple, rapid and cost-effective to use, and the resulting yields of recombinant material can be very large. Furthermore, methods for the incorporation of labels for NMR studies or for crystal structure determination are well established [9–11] and can be performed in high-throughput fashion [12,13]. Therefore and not surprisingly, structural genomics efforts on integral membrane proteins from prokaryotic sources have relied almost exclusively on this expression host.

Structural genomics platforms utilize a fixed experimental platform and a ‘funnel’ approach (Fig. 1). To maximize the probability of a successful outcome, many targets need to be processed rapidly and cost-effectively as at each stage of the pipeline there are significant losses due in part to its invariant nature. The larger the number and the quicker the rate, the higher the probability of success in identifying candidates with increased probability of success for in-depth crystallization efforts. Indeed, several groups have developed pipelines with the capability of processing hundreds or even thousands of integral membrane protein targets, and these are essentially all based on recombinant expression in *E. coli* [14, 15], with *Lactococcus lactis* being a viable alternative host [16], which however, has yet to be utilized in a full structural genomics approach.

The key elements that need to be considered in designing a platform for structural biology of prokaryotic membrane proteins are, at a minimum, target selection, cloning, expression and purification in detergents, and finally detection methods. Throughput, automation, speed, cost-effectiveness and scalability are the parameters that need to be optimized at each stage and intrinsic to this approach are standardized “one size fits all” procedures.

As a start, any structural genomics based initiative requires a bioinformatics analysis to generate a suitable list of targets for cloning and initial expression screens. Target selection for membrane proteins has benefited from the experience with soluble proteins in terms of predictions of disordered regions, methods to identify similar macromolecules or macromolecular assemblies in different species and grouping these into distinct clusters or families [17–19]. However, membrane protein specific adaptations to selection protocols have also had to be made to account for the presence of the transmembrane regions [20, 21].

At the cloning stage, high-throughput methods to construct expression vectors have utilized state-of-the art techniques developed in part, and widely used by the structural genomics efforts on soluble proteins such as ligation-independent (LIC), polymerase incomplete primer extension (PIPE) and the GATEWAY cloning methods [22–24]. These methods can and are routinely handled in a fully automated manner [22–24].

Several groups have devoted considerable efforts to establishing generically applicable expression and purification conditions, a key aspect of any structural genomics platform. For example, Lewinson *et al.* expressed and purified 36 members of this prokaryotic P-type transporters family [25] and varied promoter, growth temperature post induction, location of the affinity tag, and detergents used for solubilization and during purification [26]. Interestingly, they reported that the identity and location of the affinity tag has a dramatic effect on the yields, up to a 100-fold. Furthermore, a single detergent type, n-dodecyl- β -D-maltopyranoside (DDM) was suggested as being sufficient for solubilization and purification of a large majority of their test proteins. In agreement with this observation, Willis *et al.* in a review of membrane protein structures deposited in the protein data bank showed that many proteins are solubilized and purified in just a few detergents with DDM being the most frequently used, followed by OG [27]. Eshaghi *et al.*, profiled the expression of 49 *E. coli* membrane proteins [28]. They varied similar parameters as Lewinson *et al.* [26] and optimized experimental conditions to obtain a success rate of over 70% at the expression stage, as could be judged by dot blot on detergent-soluble fractions [28]. Many of the experiments conducted in this study were developed in a multi well assay plate format, a prerequisite to a fully automated approach.

Increase in speed, cost-efficiency and high-throughput are inevitably coupled to efforts to miniaturize the experimental platform. However, this highlights the importance of a robust detection strategy. Indeed, several groups have investigated different techniques to assay expression levels and purification yields. For example, Hammon *et al.* [29] designed and implemented a high throughput assay to survey over 300 prokaryotic membrane proteins using a simple approach consisting of engineering constructs fused at their C terminal to green fluorescent protein (GFP) for detection, followed by a poly-histidine tag for metal affinity chromatography based purification. The authors showed that GFP fusions are a suitable method to identify detergent-stable, well-expressing membrane proteins. Furthermore, they show that the behavior of the fusion is a good mimic of that of the membrane protein absent GFP. Removal of the GFP can be accomplished by proteolysis, a step that can be problematic, especially in detergent containing solutions [30]. Alternatively, proteins that pass the initial selection process can be reengineered for expression absent GFP. This somewhat cumbersome but reliable approach was proposed by Fan *et al.* in a recent report of a screening platform tested on 75 *E. coli* membrane proteins [30].

Hammon *et al.* also highlighted the importance of benchmarking expression levels against known standards to decide upon reasonable thresholds for selection of candidates for optimization [29]. The importance of benchmarking is also borne out by a comparison of “expression” levels as detected by coomassie staining or western blot analysis [31]. The authors demonstrated a doubling in expression levels if western blotting is used over coomassie staining. This may explain, at least in part, large variations in percentage expression level between studies.

Fully-fledged production structural genomics pipelines for integral membrane proteins have also been described. For example, Dobrovetsky *et al.* demonstrated a pipeline for membrane protein expression utilizing a single expression vector, a single extraction and purification detergent combined with ion-exchange chromatography or SEC as a final purification step [14, 32]. The authors screened 280 *E. coli* and *Thermotoga maritima* membrane proteins and showed that an invariant pipeline approach, analogous to those used for structural genomics of soluble proteins will succeed, but not without substantial and unexpected losses at each stage. Love *et al.* [15] have designed, implemented and optimized a high throughput pipeline that has been used to process ~8000 prokaryotic integral membrane proteins. The authors utilized a single extraction and purification detergent (DDM), poly-histidine tags at the N or C termini for metal affinity chromatography, and a size exclusion chromatography assay to determine detergent selection and stability [33]. This brute force pipeline encompasses many of the technologies developed for soluble membrane proteins, as have also been adopted by other studies [14, 26, 32] but is perhaps the first to test thousands as opposed to hundreds of targets, and clearly demonstrates that this entire approach will work [4–6, 34, 35]. Integral to these pipelines are the detergent choice in which the membrane protein is extracted, purified and eventually crystallized. Experiments have shown that while use of a single detergent throughout the entire process is desirable, it is often necessary to vary detergent or introduce detergent mixtures to improve extraction and purification, and aid in crystallization or NMR experiments [9]. Indeed, instability of membrane proteins in detergents represents the biggest hurdle of the entire process, and this needs to be monitored. To this end, Postis *et al.* [36] have developed a useful multi-well plate based screen for identification of stabilizing conditions, which involves microdialysis of proteins into various detergent-containing buffers, followed by absorption spectroscopy at 340nm at different time points to monitor aggregation. Alexandrov *et al.* have developed a method to fluorescently monitor thermal stability of membrane proteins [37], and this has recently been adapted to high throughput stability screening [30]. Size exclusion chromatography is the most recently used readout for performance of membrane proteins in detergent-containing solutions [33, 38]. This chromatographic step is very informative, but it is also rather slow, inevitably reducing the throughput of the pipeline. To circumvent this hindrance, Vergis *et al.* developed an ingenious analytical size-exclusion chromatography mimetic, consisting of a series of filtration plates with differing molecular weight cutoffs [39]. By passing the membrane proteins through these plates and assaying what flows through and what is retained, the authors could predict, to some extent, what the gross behavior of the membrane protein would be on a conventional size exclusion chromatography based assay. The authors used this method to assay 96 different detergent extractions in parallel, but it could be readily adaptable to analyzing different membrane proteins in a single mobile phase in a high throughput format.

High-throughput approaches for eukaryotic membrane proteins

Yeast expression systems (including *Saccharomyces cerevisiae*, *Pichia pastoris* and *Schizosaccharomyces pombe*) are well suited for the expression of membrane proteins, and have been used for “50% of heterologously produced eukaryotic membrane protein structures. Interestingly, Gelperin *et al.* [40] showed that 88% of yeast membrane proteins

express in yeast, a percentage comparable to that of soluble proteins (95%). Yeast systems have also been adapted to utilize GFP tagging of integral membrane proteins for rapid identification of expressing proteins [41]. Various high throughput adapted vector systems are also available and complete pipelines have been established [42]. For example, Li *et al.* presented data in support of a medium throughput pipeline with impressive statistical success for expressing and purifying a variety of yeast membrane proteins in *S. cerevisiae* for structural studies [43!!]. The authors used high throughput LIC cloning [22] followed by a simple pipeline consisting of small scale expression screens (500 mL culture volumes) assayed by immuno-dot blots, followed by solubilization in a single detergent (DDM) and size-exclusion chromatography to determine prioritization for further scale up. A recent update from the same group of investigators on this *S. cerevisiae* based system is presented by Hays *et al.* [44]. *P. pastoris* has also been utilized to screen a large number of GPCRs and in an interesting study, it was used comparatively with *E. coli* and Semliki Forest Virus expression vectors to test more than 100 targets [45]. The results showed that *E. coli* may not be the best host for expression of this class of proteins, and that *P. pastoris* could be optimized to produce increased amounts of functional GPCR, whereas expression levels of different GPCRs varied dramatically in different insect cell types [45–47].

Insect expression systems have also been adapted to high throughput methodologies [48!!], 49]. System-based on the infection of insect cells with recombinant baculoviruses often produce more proteins per litre, more economically, than mammalian cell expression systems. While the expression levels attainable might be comparable or even inferior to their mammalian counterparts, insect cells have the advantage of being readily adaptable to growth in suspension in economical media and require less specialized equipment for their maintenance. Not surprisingly, baculovirus infected insect cells have displayed their utility in the solving of a number of landmark eukaryotic membrane protein structures [2,50–56]. All the necessary methodologies, such as LIC cloning vectors and dedicated microscale incubators are in place to make substantial progress in the near future utilizing this expression system for membrane proteins. Indeed, some groups are already in a functional high throughput phase of production using these systems.

Other approaches

Savage *et al.* [57] conducted a useful study to see if cell free systems could complement the expression of membrane proteins in *E. coli*. The authors reported an "20% gain in expression success by using this dual approach. Furthermore, Beebe *et al.*, building on earlier work of Makino *et al.* [58] in establishing a high throughput cell free synthesis of soluble proteins for NMR, demonstrated how cell free expression systems are automatable and scalable for membrane proteins [59]. Samples for NMR based structural studies are likely to benefit the most from progress in cell free expression methods. This is because of the high levels of label incorporation and smaller reagent volumes necessary for this approach [60].

Summary and Outlook

The field of structural studies on membrane proteins is tremendously exciting at present, and the growth in the number of publications and groups working on membrane proteins demonstrates that these once seemingly intractable targets are being forced to yield their secrets. High throughput methodologies are being increasingly utilized for structural biology of membrane proteins, as they provide clear advantages. They reduce costs and greatly increase the probability of finding targets that are tractable, and that are likely to crystallize. Finally, we look forward to witnessing firsthand an increase in structures of eukaryotic

membrane proteins due to implementation of high-throughput, structural genomics methodologies to this class of macromolecules.

Highlights

- > Structural genomics techniques have been developed for membrane proteins
- > Structures of Prokaryotic and eukaryotic membrane proteins can be determined
- > High throughput methods increase chances of finding a "well-behaved" membrane protein

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Figure.

A two-dimensional funnel scheme for high throughput structural studies on membrane proteins. The throughput of the entire process decreases from cloning to crystallization. The last step of the process is not shown in the scheme. This involves progression from crystallization to structure determination and tends to require extensive rounds of labour-intensive optimization to obtain diffraction quality crystals, and cannot typically be automated. Throughput also diminishes as the complexity of the expression system increases. Not surprisingly most progress with structural genomics of membrane proteins has involved bacterial expression systems.