A collaborative gene-to-structure workflow using cryoEM

Improving the speed and efficiency of fully native membrane protein structure determination with cryoEM

The Challenge

Cryogenic electron microscopy (cryoEM) is a powerful method for high-resolution three-dimensional (3D) protein reconstruction. This method, which does not require 3D crystals, can observe organic macromolecules in multiple conformations in their native environment; allowing researchers to carry out structural imaging on samples that are difficult to analyze with methods like X-ray crystallography and nuclear magnetic resonance.

However, cryoEM is not without its challenges. It requires access to expensive infrastructure and specialized expertise, creating barriers of use for many research groups and contract research organizations (CROs). Moreover, it requires high-quality and stable samples for high-resolution protein reconstruction, as well as optimized end-to-end workflows to reduce project running times, which can be challenging in collaborations. This has led to the impression that the pipeline of structural analysis using cryoEM can be slow or unfeasible.

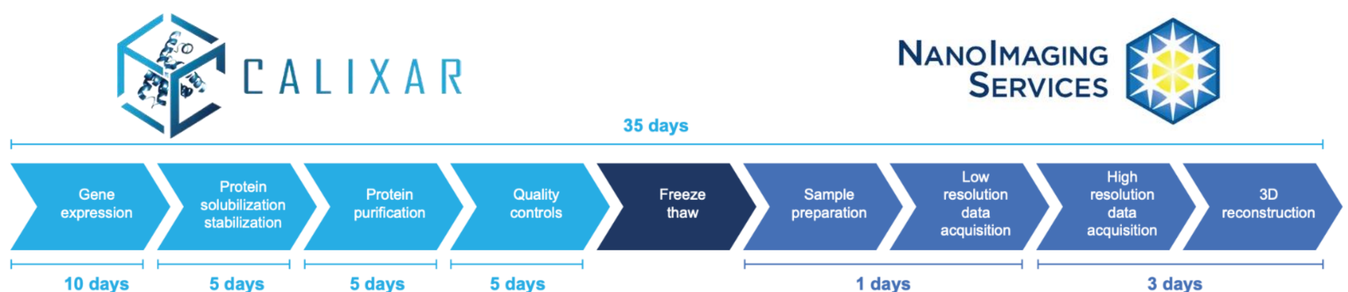
The Solution

The combination of cryoEM technologies and expertise in protein sample preparation delivers protein structures at higher resolutions in shorter timescales. The development of new technologies like CryoSPARC Live, for example, processes structural information during collection and allows real-time go/no-go decision making about sample imaging, drastically reducing imaging times. Another major development has been the implementation of efficient structural imaging pipelines by cryoEM service providers like NanoImaging Services. Finally, continuous innovation in the field of fully native membrane protein isolation, provided by companies such as CALIXAR, ensures the access to high-quality protein sample for structural biology studies.

Collaborations with NanoImaging Services & CALIXAR are enabling Pharmas, Biotechs, and CROs to perform structural analysis of high-value drug targets in a short time without having to purchase cryoEM equipment or recruiting experts in protein sample production and optimization, while providing access to cutting-edge technologies.

The Results

Here, we present results from a collaborative project between NanoImaging Services and CALIXAR, a company who specializes in delivering high-quality membrane proteins for drug discovery. This project combined CALIXAR's production of the fully native AcrB protein from *AcrB* gene with NanoImaging Services' rapid 3D structure determination using cryoEM. In total, this project returned the 3D reconstruction of AcrB protein at 3.1Å from *AcrB* gene expression in only 35 days.



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CALIXAR

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The Results Part One: CALIXAR – AcrB protein isolation

In the first stage of this project, CALIXAR's patented technology platform achieved a gene-to-protein workflow of AcrB, a bacterial multidrug efflux pump subunit, in 25 days. Here, AcrB protein was expressed in *E. coli*, solubilized, and purified with a high yield, with up to 100% solubilization using a combination of DDM and CALX detergents (Figure 1A). AcrB was purified by IMAC, which recovered over 90% of pure protein (Figure 1B), and then submitted to freeze/ thaw cycles for cryostability (Figure 1C) enabling to ship stable and homogenous frozen protein sample.

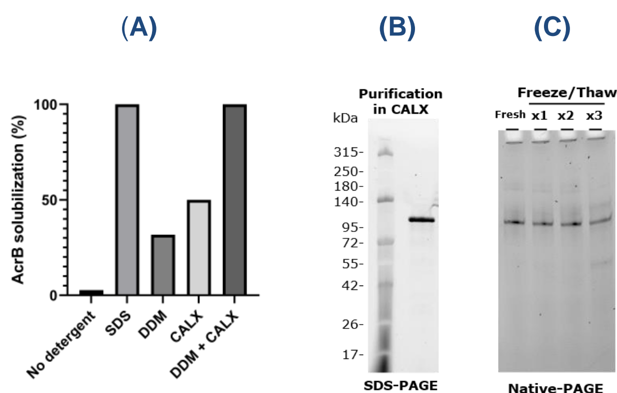
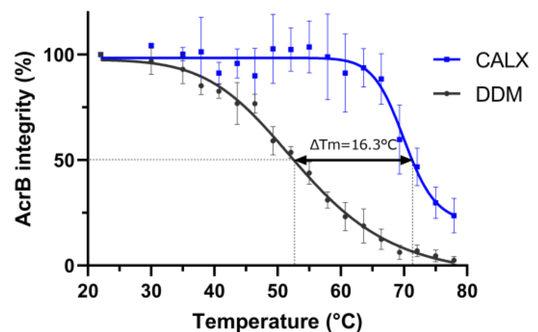


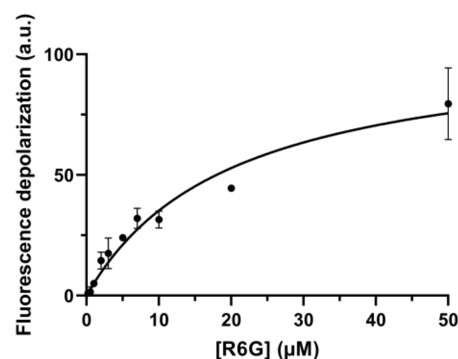
Figure 1: CALX improves AcrB solubilization in combination with DDM (A), and IMAC purification of AcrB in CALX buffer yields a highly pure (B) and cryostable (C) protein with no additive.

Next, CALIXAR evaluated the stabilizing effect of CALX molecules on purified AcrB. Thermalshift assay revealed a melting temperature gain of +16°C in CALX compared with DDM detergent (Figure 2A). Rhodamine 6G ligand binding assay showed that CALX detergent maintains AcrB in its functional conformation (Figure 2B).

Purified AcrB was then shipped to NanoImaging Services for cryoEM processing as part of the coordinated gene-to-structure workflow.



(A)



(B)

Figure 2: CALX improves the thermostability of AcrB (A), which remains in its active, druggable, conformation (B).

The Results Part Two: NanoImaging Services – CryoEM

In the second stage of this project, NanoImaging Services performed 3D reconstruction of AcrB in 10 days using cryoEM. In the first part of the pipeline, AcrB protein material was shipped frozen with an international courier to the NanoImaging Services east facility where cryoEM sample preparation and low-resolution data acquisition was performed. Here, several grids were prepared varying the sample concentration, the type of grid used, and vitrification parameters. Initial screening was performed on a TFS Glacios microscope operating at 200 KeV to identify the best conditions for high resolution structure determination.

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Suitable grids were then shipped to the San Diego facility for one day of Krios acquisition, followed by high-resolution data acquisition and 3D reconstruction using CryoSPARC Live, which performs real-time image processing. Figure 3 shows a subset of the 2D classes calculated by Cryosparc and used in the 3D reconstruction.

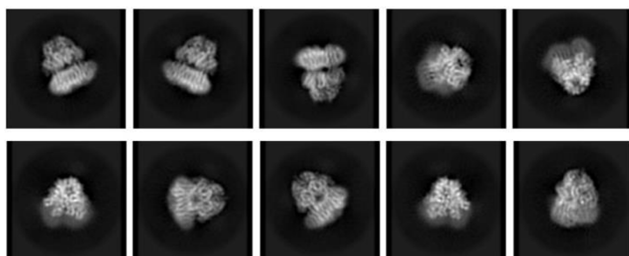

Figure 3: Subset of the final 2D classes calculated by Cryosparc. Box size ~265Å. The 2D classes are sharp, with secondary structure elements clearly visible, and show different views of the sample

The 3D reconstruction of AcrB can be seen in Figure 4. The cryoEM imaging and data processing returned a 3.1Å structure of AcrB and showed that AcrB is present as a trimer after solubilization with CALX (Figure 4). Extraction of membrane proteins into isolated particles is needed for biochemical and structural analyses, and the native structure of the sample must be retained. Different detergents or other extraction methods can affect the structure and biological activity of the protein in question. This structure of AcrB is very similar (the rootmean-square-deviation (RSMD) on Cα carbons is 1.85Å, and 1.95Å, respectively with 6csx and 6sgs, two other AcrB extracted and purified using different detergents) suggesting that the use of CALX is not detrimental to the protein. Most of the AcrB chain is visible, except for 500-512, 868-872, and the last few residues. An example of the high-quality visibility of side chains can be seen in Figure 5.

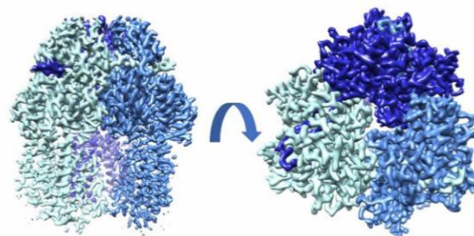

Figure 4: Overall view of the cryoEM map obtained for AcrB. Left Panel, side view of the trimer; right panel: top view of the trimer. The three monomers are colored in three shades of blue.

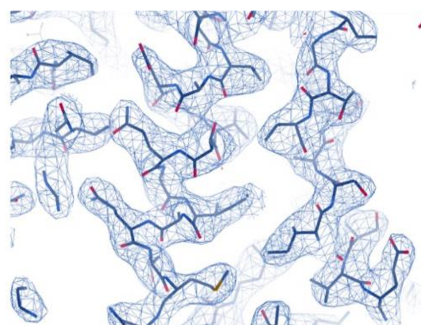

Figure 5: Example of the electron density obtained for AcrB. The density of high quality and side chains are clearly visible and identifiable.

This collaborative project between CALIXAR and NanoImaging Services solved the 3D structure of AcrB in just 35 days. The combination of CALIXAR's expertise in fully native membrane protein isolation and NanoImaging Services' expertise in rapid high-resolution structure determination with cryoEM led to an efficient gene-to- structure coordinated workflow for AcrB, even when the project split over two contract research organizations where protein material must be isolated, purified, shipped, and analyzed. Combining optimized workflows like these show the potential for cryoEM to resolve protein structures rapidly and efficiently.

One of the key reasons for the rapid imaging pipeline was first the use of high-quality membrane protein sample and second the use of CryoSPARC Live, which returned real-time processing and 3D reconstruction of AcrB in three days of start of data acquisition. Future cryoEM projects with CALIXAR & NanoImaging Services also have the potential to be even faster and more cost effective when screening stages are skipped.

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Conclusion

New technologies are enabling cryoEM to deliver high-quality 3D membrane protein structures in rapid timescales. Here, AcrB protein 3D structure was solved ultimately from fully native AcrB purified and stabilized protein provided by CALIXAR in a total of 35 days. This project demonstrates the possibility of seamless gene-to-structure workflows, even as part of international collaborations.

The collaborative approach of this project provided a target-optimized pipeline, leveraging the unique strengths of the participating CROs. Outsourcing membrane protein production and cryoEM services is therefore a viable and attractive approach for gene-to-structure studies, especially for a project which requires access to expensive infrastructure and specialized expertise.

About AcrB product

CALIXAR expertise

NanoImaging Services expertise

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