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Application Note

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Evaluation of Membrane Protein Properties by Fluorescence-Detection Size-Exclusion Chromatography (FSEC) Using an Nexera™ lite inert system

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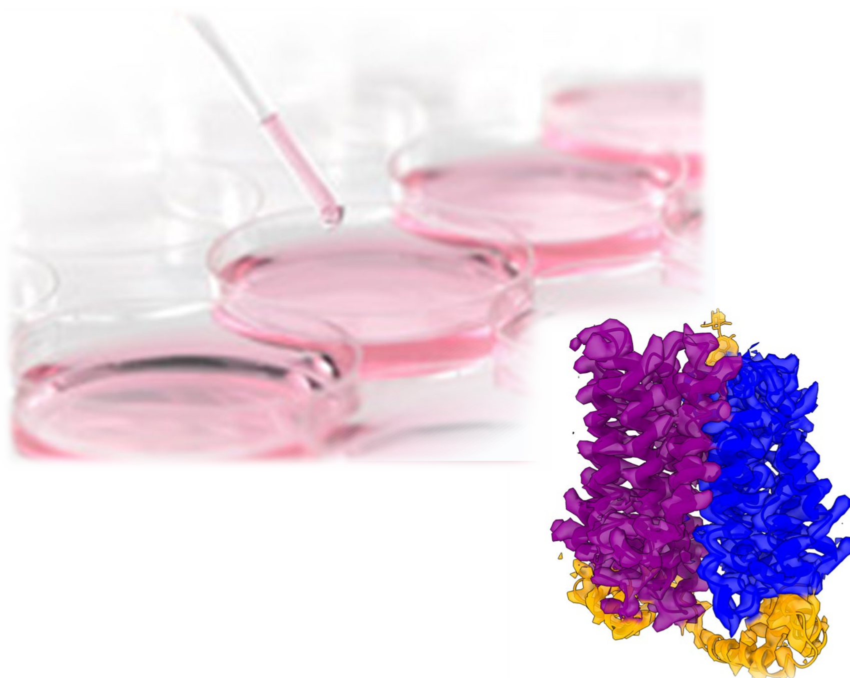

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■ Abstract

In order to understand the function of membrane proteins based on the three-dimensional structure, it is important to obtain highly purified membrane proteins through purification. Therefore, rapid evaluation of experimental system that provides stable and efficient expression of target membrane proteins is essential.
Fluorescence-detection Size-Exclusion Chromatography (FSEC)[1)] enables the evaluation of expression levels and uniformity by specifically detecting GFP (Green Fluorescent Protein) fusion membrane proteins in unpurified samples. In this article, an application of FSEC to the evaluation of the thermal stability of eukaryotic membrane transporters is introduced.

1. Introduction

Cells, the smallest unit comprising the human body, are covered by a membrane consisting of lipids. Various membrane proteins are located in the cell membrane, receiving information from outside the cell and transporting substances inside and outside the cell. These membrane proteins have garnered attention not only because they are associated with important biological phenomena, but also as target molecules for drugs.
To understand the functions of membrane proteins and how they bind and respond to drugs, their three-dimensional structural information is extremely useful. Even so, a major bottleneck in structural determination is the preparation of enough amount of highly purified protein samples.
However, when membrane proteins are transiently expressed using cultured cells, in many cases, sufficient expression levels cannot be obtained, and as a result, it is not possible to secure a sufficient amount of purified samples. Moreover, although the purification of membrane proteins requires their extraction from lipid bilayers using detergents, membrane proteins are generally unstable and easily denatured during solubilization. Due to these technical difficulties, the number of membrane protein structures actually determined has been limited until recently.
To overcome these difficulties, it is crucial to evaluate the expression level and stability of numerous samples in a simple and convenient manner, and select “highly expressed” and “highly stable” membrane proteins suitable for structural analysis. FSEC has been developed for rapid and convenient screening of those proteins. In this technique, a GFP tag is fused to a target membrane protein. The protein behavior is then evaluated by detecting the GFP fluorescence in gel filtration chromatography, which allows analysis of the target protein in unpurified status. An autosampler and a highly sensitive fluorescence detector installed in an HPLC system enable continuous analysis of numerous samples and detection of samples in minute quantities, respectively.
This Application Note introduces the advantages of the HPLC system and its application by presenting examples of specific FSEC applications which are used at the Nishizawa Lab., Graduate School of Medical Life Science, Yokohama City University.

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2. Overview of FSEC

The principle of FSEC will be first explained by offering the most common example utilizing GFP-fused proteins (Fig. 1). Refer to the cited paper[1)] for details not described in this article.
In structural analysis research, Escherichia coli or cultured cells (such as mammalian HEK293 cells and insect Sf9 cells) are generally used for transient expression of target proteins. During designing of the expression, a GFP tag is fused to either the N or C terminus of the target protein. The GFP-fused proteins are then expressed in cells, and the cells are harvested and subjected to FSEC analysis.
In analysis, the target proteins are extracted from lipid membranes by mixing the cells of GFP-fused proteins with a solution containing a detergent. This process is known as "solubilization." Note that the solubilized sample here contains not only the transiently expressed GFP-fused proteins but also various contaminating proteins from cells. In FSEC, the sample is analyzed in unpurified status using gel filtration chromatography.
Although UV absorption (wavelength: 280 nm) is typically utilized to detect proteins in gel filtration chromatography, here the evaluation with UV absorption is impossible since the signal from the target protein in unpurified samples is hindered by the large "noise" originating from other contaminating proteins. To avoid the difficulty, FSEC utilizes a fluorescence detector to detect a specific signal from GFP-fused proteins, which allows evaluation of the elution profile of the target even in unpurified samples.
The expression level and solubilization efficiency of the GFP-fused protein can be evaluated from the height of elution peaks in gel filtration chromatography. Moreover, the homogeneity of the GFP-fused protein can be evaluated from the peak shape. For example, a broad peak or multiple peaks suggests aggregation of the GFP-fused protein. Conversely, a sharp single peak suggests stable, uniform solubilization of the GFP-fused protein. Namely, a very sharp and high single peak indicates a high potential for structural studies. A large number of target protein candidates are analyzed in FSEC, and only samples suitable for structural determination are screened based on the status of obtained profiles.
After cell harvesting, this technique requires only two steps, solubilization and removal of the insoluble fraction, to proceed to acquisition of profiles. Thus, FSEC is an easy, convenient technique for rapid screening of target proteins.

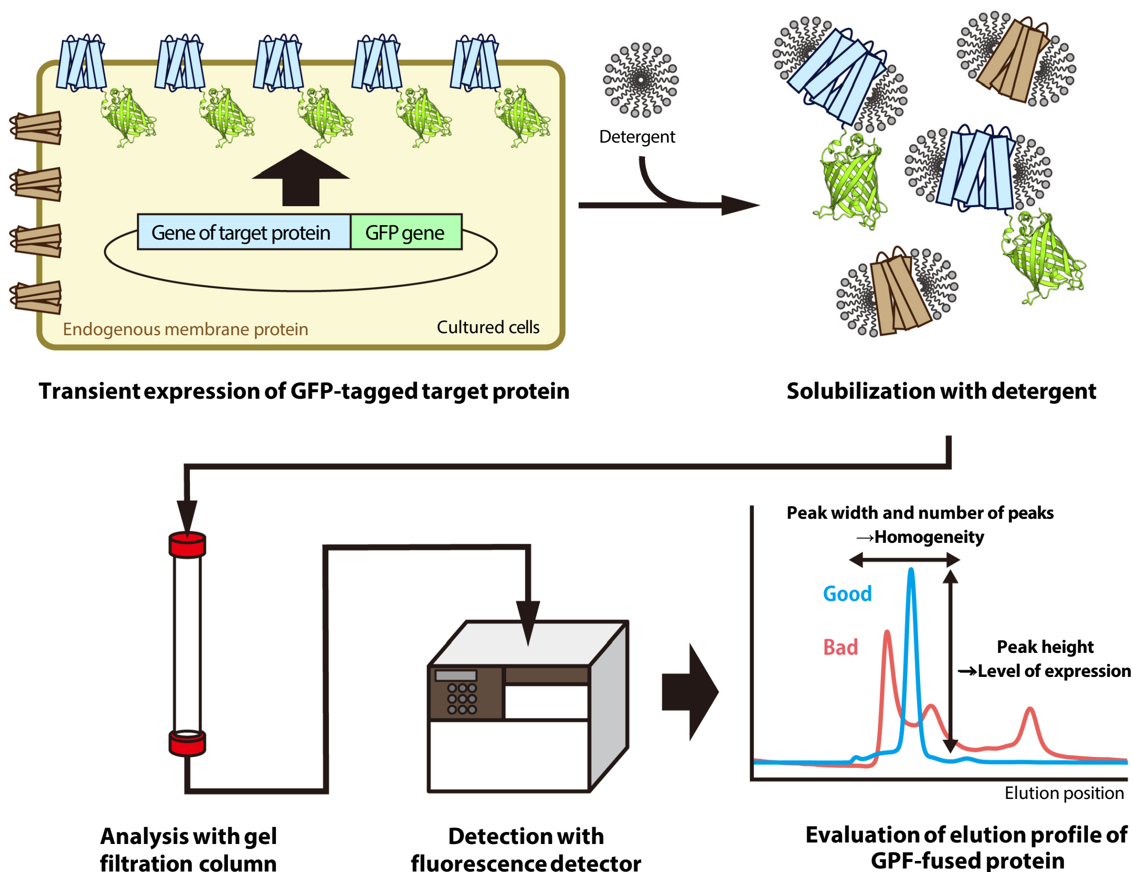

Fig. 1 Fluorescence-Detection Size-Exclusion Chromatography: FSEC

3. Analytical Conditions

Fig. 2 shows the HPLC configuration employed in this study, and Table 1 shows the analytical conditions.
It is possible to reduce the risk of corrosion of HPLC caused by mobile phase containing high concentration of salt by using Nexera lite inert, which eliminates metal materials from the flow path. In addition, different selectable columns can be set using 6-port high pressure column switching valve (FCV-14AHi).
Switching between these six ports, it is possible to execute analyses using columns suited to the analytical purpose and sample amount without manually changing the columns (Fig.3).
The fluorescence detector monitors the fluorescence attributed to GFP (Ex 480 nm/Em 512 nm). Since the detector supports the monitoring of two sets of excitation and emission wavelengths, the fluorescence associated with tryptophan (Trp) in proteins (Ex: 280 nm/Em: 350 nm) can be monitored in addition to GFP fluorescence.
Furthermore, the target protein can be fractionated with high purity and high recovery rate by connecting a fraction collector (FRC-10A). It is also suitable for fractionation in small volumes such as microtubes and 96-well microplates.

Table 1 Analytical Conditions

System	: Nexera lite inert (with FRC-10A)
Mobile phase	: 10 mM HEPES-NaOH pH 7.0, 150 mM NaCl. 0.03% DDM
Flow rate	: 0.4 mL/min
Column	: Size exclusion chromatography column (4.6 x 300 mm, 5 µm, 300Å,)
Column temp.	: 6 ˚C*1
Injection volume	: 50 µL
Detection	: Ex at 480 nm、Em at 512 nm (for GFP) Ex at 280 nm、Em at 350 nm (for Trp)

*1 Analyzed in a chromatography chamber

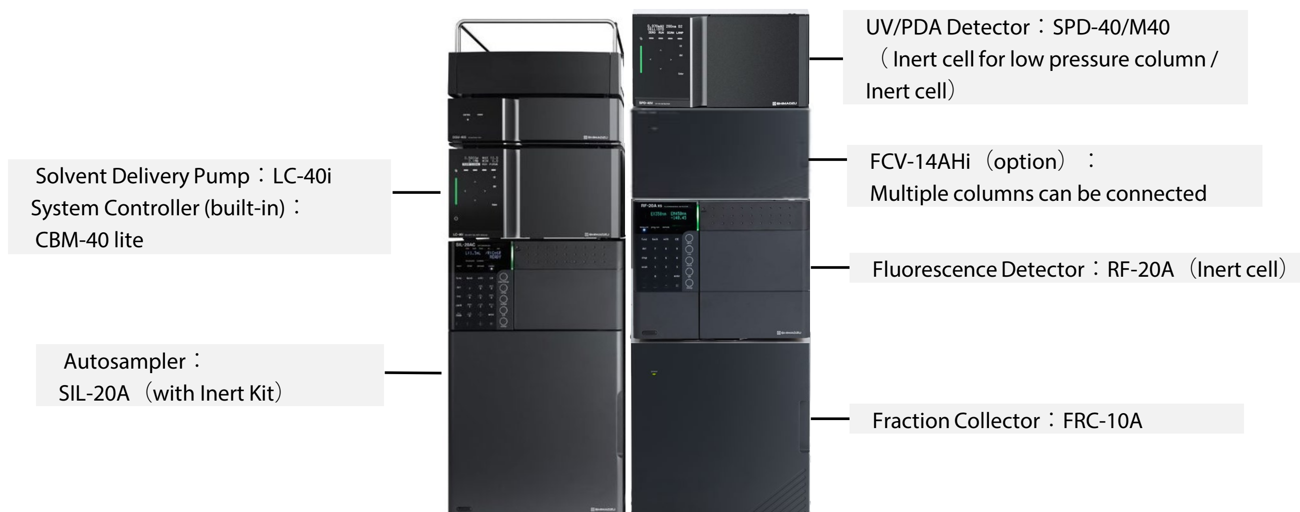

Fig.2 Nexera™ lite inert (with Fraction Collector)

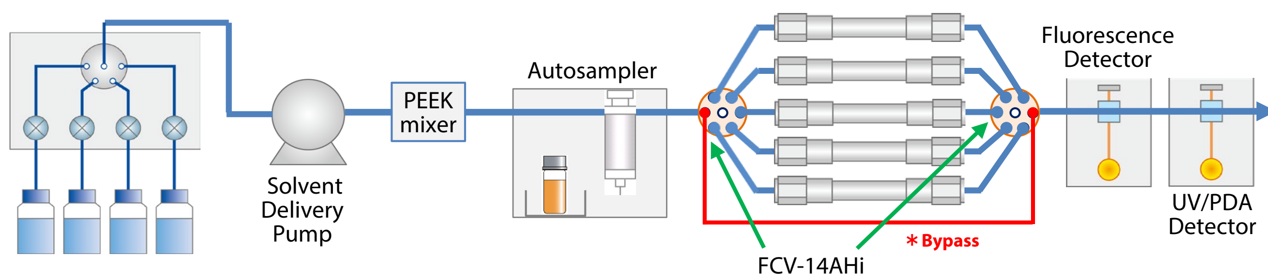

Fig.3 Flow Diagram

4. Sample Preparation

Recent researches on structural analysis of membrane proteins have primarily focused on the membrane proteins from higher eukaryotes such as humans and mice. Along with this trend, HEK293 cells and Sf9 cells have been utilized as expression systems for transient expression of recombinant membrane proteins, since these cells are suitable for expression of eukaryotic proteins. Taken those cultured cells as an example, this section describes the procedures from harvesting of cells expressing membrane proteins to FSEC analysis (Fig.4).

1) Sample Fraction （Required Time: 10 Min Max.）
Small quantities of cells expressing the membrane protein are collected and pelletized. For Sf9 cells grown in a free-floating state, approximately 1 mL of culture solution is normally collected. For HEK293 cells grown in adherent conditions, cells from one well on a 6-well or 12-well plate are collected. The collected cell suspension is centrifuged at a low speed to eliminate the culture supernatant and acquire a cell pellet.

2) Solubilization with Detergent
(Required Time: 30 to 60 Min)
About 200-300 µL of a solution containing a buffer, salt, and a detergent is added to the cell pellet to suspend the pellet in the solution. The suspension is then mixed by inversion for 30 to 60 min at a low temperature to solubilize the membrane proteins. Simply suspending the pellet without physical cell-disruption by sonication is sufficient for solubilization since the cell membranes of both HEK293 and Sf9 are weak, but sonication is sometimes used to reduce the viscosity derived from genomic DNA. Since dodecyl maltoside (n-Dodecyl-β-D-maltoside) is often chosen as the solubilizing detergent, the possibility of other detergents is also examined, taking it as the starting point.

3) Removal of Insoluble Materials (Required Time: 20 Min)
The sample solution after solubilization often contains cell remnants and protein aggregates produced in the presence of detergent. Since injecting those materials as-is into the HPLC system may clog its flow lines and columns, the solubilized sample solution is ultracentrifuged for approximately 20 min to pelletize and eliminate the insoluble materials from the solution. After ultracentrifugation, the supernatant is transferred to a vial and placed on the sample rack of the autosampler.

4) Analysis (Required Time: 20 to 60 Min/Sample)
The autosampler is used to inject 30 to 100 µL of the sample into the gel filtration column, and the sample is eluted with a solution containing a buffer solution, salt, and detergent. The analytical time and flow rate of the mobile phase vary depending on the type of gel filtration column used. If too many samples are analyzed in one sequence, some samples can denature while awaiting injection, thus hampering the interpretation of results. For this reason, the number of samples in one continuous batch analysis should be limited to that which can be completed overnight at most (12 h max.).
As described above, the total time required for pretreatment from sample collection to analysis start is about 2 hours. The procedures are extremely simple and easy, and since few steps are involved, the effects of procedures on proteins can basically be eliminated, allowing the initial properties of the target protein to be evaluated. Since the buffer solution, salt concentration, and detergent used in solubilization must be optimized for each sample, an optimal combination is evaluated based on the elution profiles obtained by FSEC.

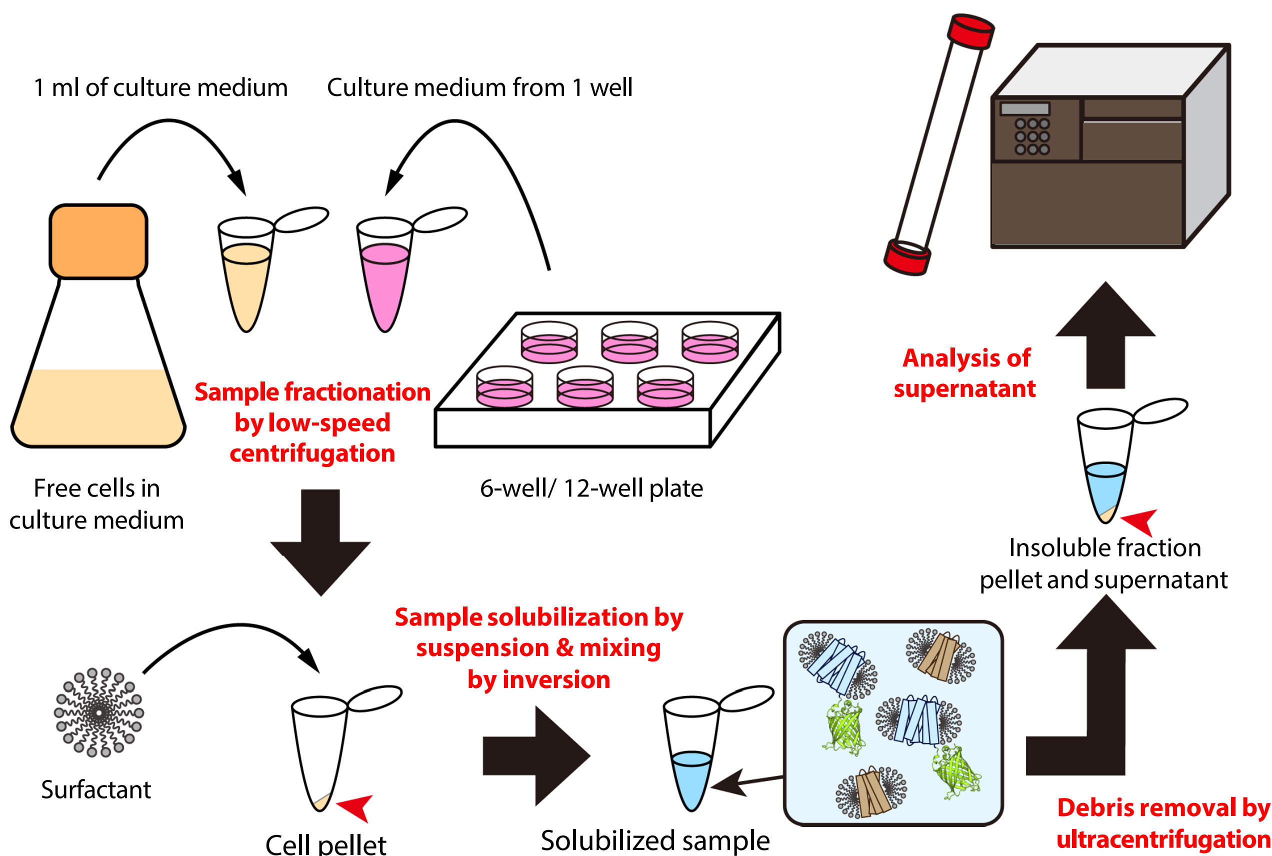

Fig. 4 Sample Preparation Procedure

5. Examples of FSEC Applications
—FSEC-TS: Evaluation of Thermal Stability—

In this technique, a solubilized GFP-fused membrane protein is heated for a certain time and analyzed by FSEC, and the changes in the peak shapes with and without heating are observed to evaluate the thermal stability of the sample[2].
The eukaryotic membrane transporter A was expressed in a C-terminal GFP fusion state and analyzed without purification. In this case, it is common to estimate the denaturation temperature using the peak height decreased by heating as an index (Fig. 5). A sample is heated with a thermal cycler or heat block for 10 min, and aggregates are removed by ultracentrifugation. The pretreated sample is then analyzed with the HPLC system. Since GFP denatures in about 10 min when heated to 80 ˚C, this FSEC-TS can be widely used for samples that degenerate in the temperature range of 80 ˚C or lower.

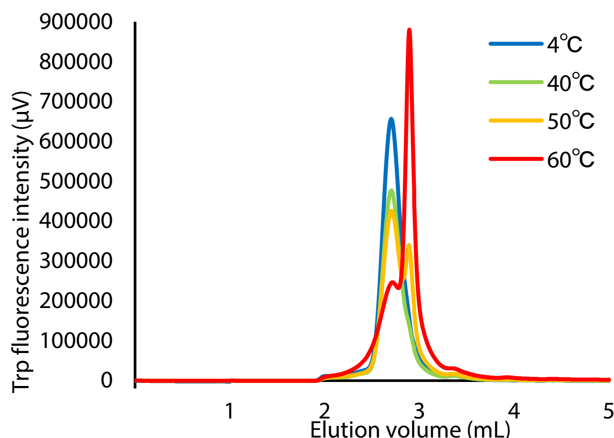

Fig. 5 Sample Data from FSEC-TS

6. Conclusion

The basic idea of FSEC is simple: to separate and monitor a fluorescently labeled sample by gel filtration column chromatography. However, it is possible to detect fluorescent proteins with high sensitivity and specificity. In addition, consecutive analyses of multiple samples can be done using autosampler and examination of multiple columns is possible as well using column switching valves. Consequently, reduction of work amount on experimenters and rapid analytical measurement can be simultaneously accomplished. With this convenience, FSEC has become well-known and widely used around the world. In fact, numerous papers on structural analysis have reported various types of FSEC applications, such as species screening, variant screening, and confirmation of multimerization[2) 3) 4) 5)].

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＜Acknowledgements＞

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<Related application>

・Evaluation of Membrane Protein Properties by Fluorescence-Detection Size-Exclusion Chromatography (FSEC) Using an HPLC System, Application Note No. 53

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