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Application Note

No. 53

Life Science

Evaluation of Membrane Protein Properties by Fluorescence-Detection Size-Exclusion Chromatography (FSEC) Using an HPLC System

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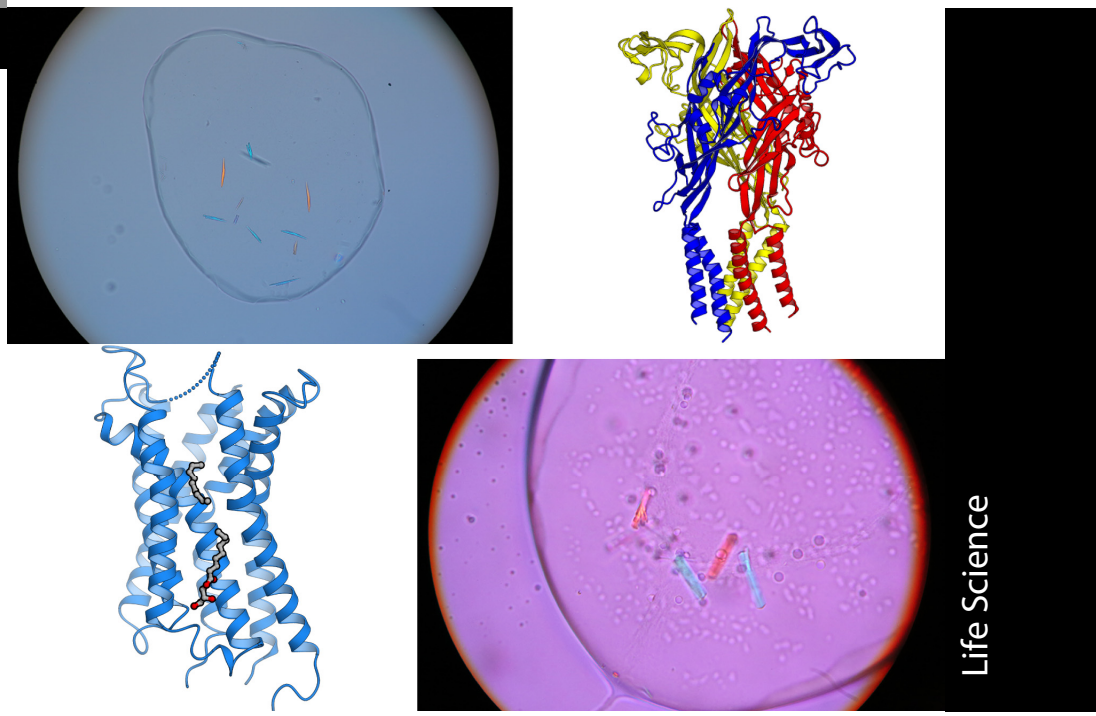

Life Science

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, required for X-ray crystallographic and cryo-electron microscopic analysis. 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. Fluorescence-detection size-exclusion chromatography (FSEC) [1] has been developed for rapid and convenient screening of those proteins. In this technique, a green fluorescent protein (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. FSEC is an indispensable experimental technique, and the HPLC system customized for FSEC is now the most extensively used experimental instrument.

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 Nureki Laboratory, Department of Biological Sciences, Graduate School of Science, the University of Tokyo.

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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.

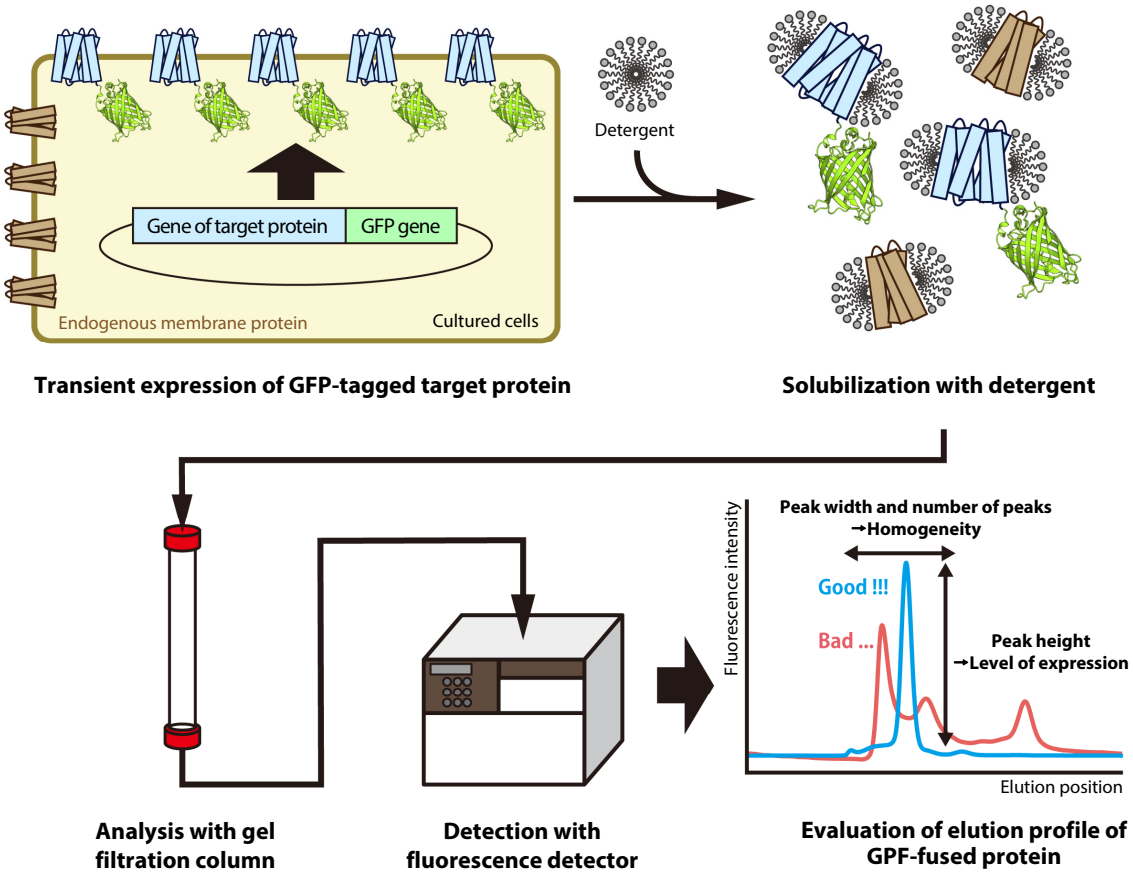

Fig. 1 Fluorescence-Detection Size-Exclusion Chromatography: FSEC

3. Description of Instrumentation

The configuration of the HPLC system actually used in the Nureki laboratory is as shown in the figure below. HPLC control, pumping, and detection are performed in the following steps.

1) CBM-20A: System Controller

The system controller controls the analytical instruments described below and serves as a link between a controlling PC and the analytical instruments. Remote operation is possible by setting on the controlling PC various parameters such as target samples, solvent delivery lines used, as well as optimized pump flow rate for the target samples.

2) LC-20AD: Solvent Delivery Pump

A buffer for use in analysis is delivered to the system via each of four lines A-D. Utilizing multiple lines enables analysis with multiple mobile phases. After analysis, the flow lines can be washed automatically with ultrapure water by the solvent switching mechanism.

3) SIL-20AC: Autosampler

The solubilized samples can be set on a rack of 70 or 105 vials, which fits the autosampler. Specifying the vial number on the controlling PC allows suction of a designated volume of the sample for analysis.

4) FCV-14AHi: Column Switching Valve

This valve unit allows switching of the flow lines up to six ports. Each port connects to a different column (see Table 1). Switching ports makes it possible to select the suitable column for the analytical target or the scale of the sample volume without manually reconnecting the column.

5) RF-20Axs: Fluorescence Detector

This detector monitors the fluorescence attributed to GFP (Ex: 480 nm/Em: 510 nm). Furthermore, 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. This technique is utilized in the Trp-FSEC method (described below) for analysis of samples from which contaminating proteins have been removed.

The description above is an overview of the HPLC system. The entire system above is installed in a cold chamber under 4 °C. This system is operated by the controlling PC located outside the cold chamber. Configuring a batch sequence on the controlling PC then allows continuous analysis of multiple samples. Normally, a batch sequence to continuously analyze 10 to 20 samples is executed to acquire the data automatically.

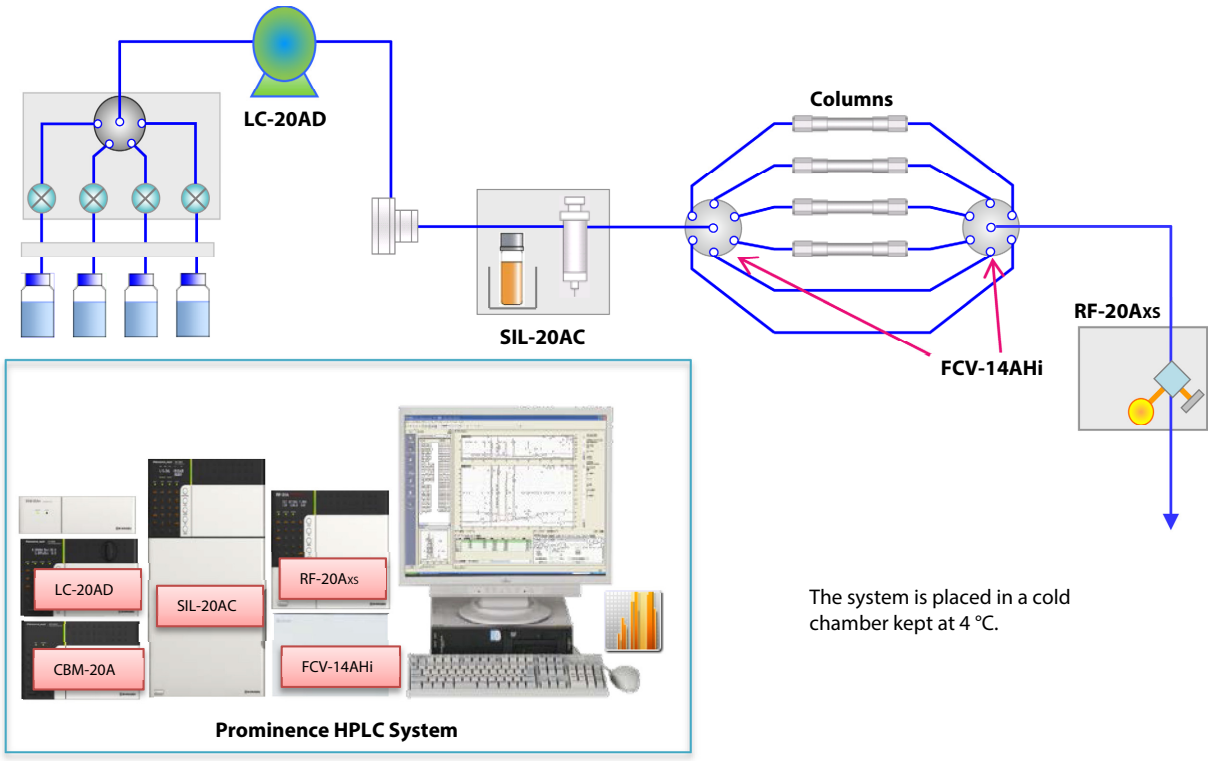

Fig. 2 Analytical Instruments and Schematic Flow Diagram

Table 1 Column Used (Examples)

Column	Manufacturer	Column volume	Flow rate	Notes (fractionation range and applications)
ENrich™ SEC 650 10 × 300	Bio-Rad	24 mL	0.6 to 0.7 mL/min	5 to 650 kDa; used for membrane proteins no larger than about 200 kDa.
Superdex 200 Increase 5/150	GE Healthcare	3 mL	0.2 mL/min	10 to 600 kDa; used to roughly verify peak shapes of multiple samples.
Superose 6 Increase 10/300	GE Healthcare	24 mL	0.5 mL/min	5 to 5000 kDa; used for large target proteins such as membrane protein complexes.

Table 2 Analytical Conditions (Examples)

Mobile phase (example)	:	20 mmol/L Tris-HCl (pH8.0), 150 mmol/L NaCl, 0.03 % DDM
Column temperature	:	4 °C (system is installed in cold chamber under 4 °C)
Detection	:	Ex at 480 nm, Em at 510 nm (for GFP) Ex at 280 nm, Em at 350 nm (for Trp)
Injection volume	:	50 µL

* Optimal conditions such as the pH of the buffer and the detergent used in the mobile phase are evaluated in accordance with samples, and optimized solvents are used for analysis.

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.

1) Sample Fractionation (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.

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.

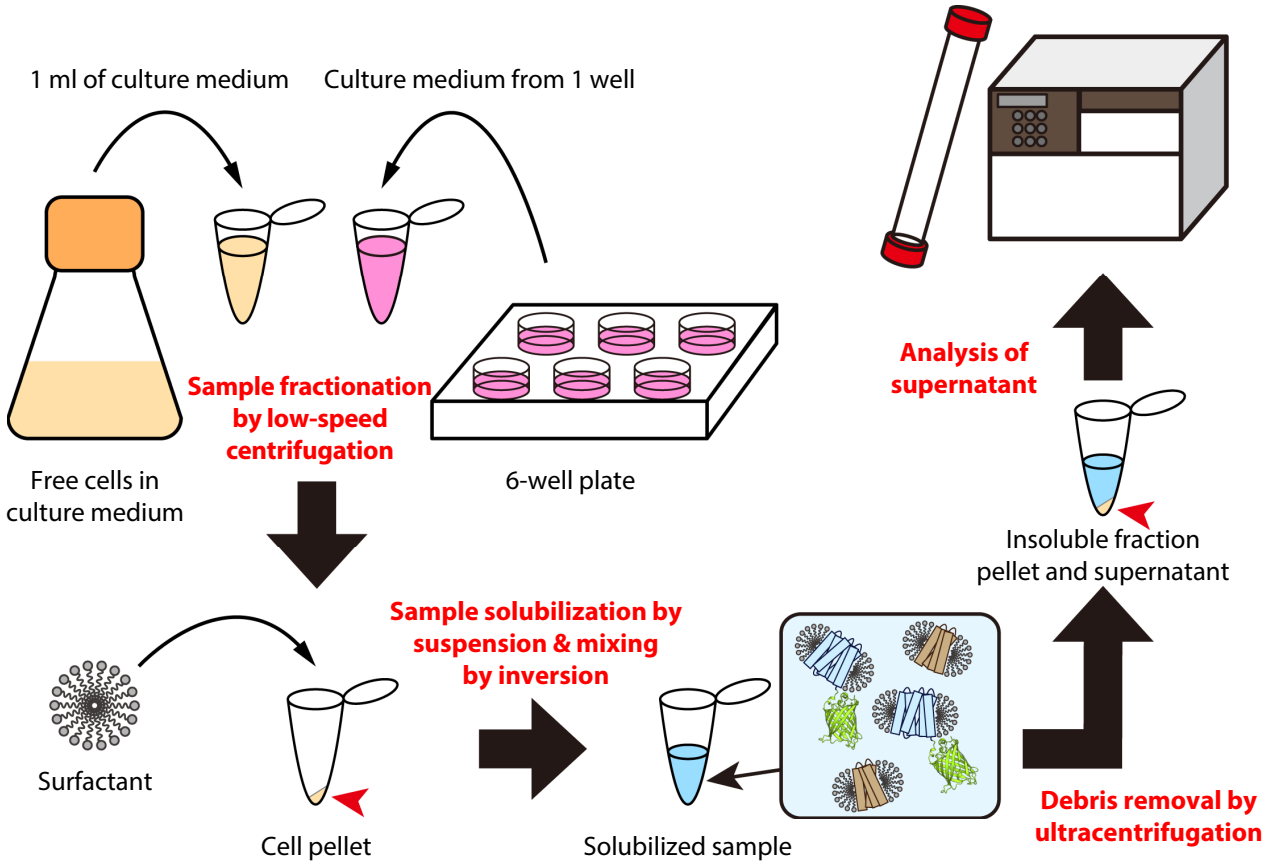

Fig. 3 Sample Preparation Procedure

5. Actual Data

An actual example of the data obtained by GFP-FSEC is shown here. In the experiment shown on the right, membrane transporters A from different species were transiently expressed in HEK293 cells, and their properties were evaluated by the GFP-FSEC method, under the aim of structural determination of eukaryotic membrane transporter A. The cell pellet from one well on a 12-well plate was suspended in 250 µL of a solubilization buffer, pretreated, and analyzed using the ENrich™ 650 column from Bio-Rad, under the injection volume of 30 µL.

As shown in Fig. 4, the profile from species 1 shows a high single peak. In contrast, the profile from species 2 shows two peaks, indicating that two different states are involved in the sample. From the profile from species 3, it is considered that some heterogeneous aggregation occurred in the sample since the profile widely spreads. Since the profile from species 4 does not show an obvious peak, it can be assumed that the expression level was low or the solubilization was insufficient. From the observation above, the sample from species 1 can be deemed optimal for structural analysis research, and the subsequent experimentation can proceed. Although the example here features a comparison among different species, a similar procedure can apply for a comparison of the same sample with different mutations introduced, or that of the same sample solubilized in solutions with different components.

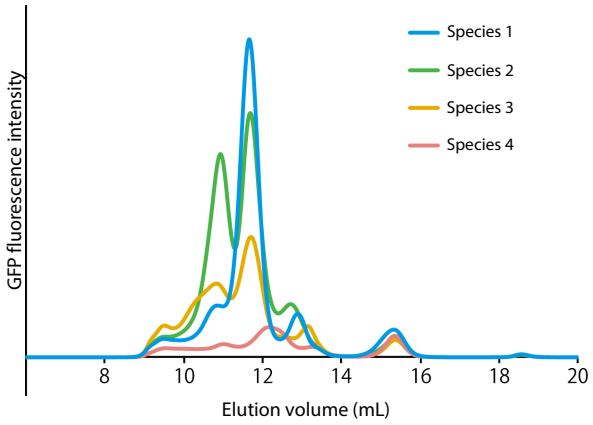

Fig. 4 Sample Data from GFP-FSEC

Membrane transporters A from different eukaryotes were expressed with a C-terminal GFP tag and analyzed by GFP-FSEC in unpurified conditions.

6. Examples of FSEC Applications

The description up to this point has introduced a technique solubilizing a GFP-fused membrane protein derived from cells and analyzing that protein as-is. The same HPLC system, however, can also be used to evaluate the properties of samples other than GFP-fused membrane proteins by changing the sample preparation or signal detection method. The following description introduces the three techniques most frequently used among those applications. For details of the principle of each technique, refer to the description in the papers cited.

1) FSEC-TS: Evaluation of Thermo 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 thermo stability of the sample [2]. 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.

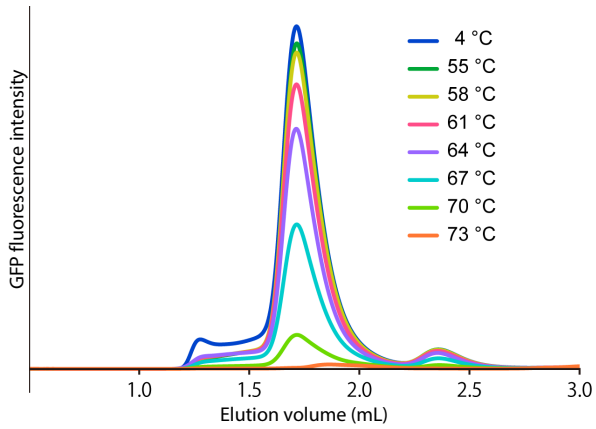

Fig. 5 Sample Data from FSEC-TS

Unpurified GFP-tagged membrane receptor B is heated at a temperature shown in the figure for 10 min, followed by analysis with a Superdex 200 Increase 5/150 column.

2) Trp-FSEC: Identification and Evaluation of Purified Samples

Most proteins contain tryptophan (Trp), one of the amino acid residues, as their component. Trp has an aromatic structure, and when exposed to excitation light at a wavelength of 280 nm, it fluoresces at a wavelength of 350 nm [3]. This technique uses the Trp-derived fluorescence as an index, and detects a sample far smaller (e.g. 500 ng to 1 µg) than that of UV monitoring at wavelength of 280 nm [2], utilizing the high sensitivity of the RF-20Axs fluorescence detector on the HPLC system. This approach is mainly used to check the elution profile of a purified sample, or to be combined with the FSEC-TS method for a purified sample. The technique is also useful for investigating whether a complex is formed in a sample or not, by analyzing mixed purified samples by Trp-FSEC and checking the peak position in the elution profile (Fig. 6).

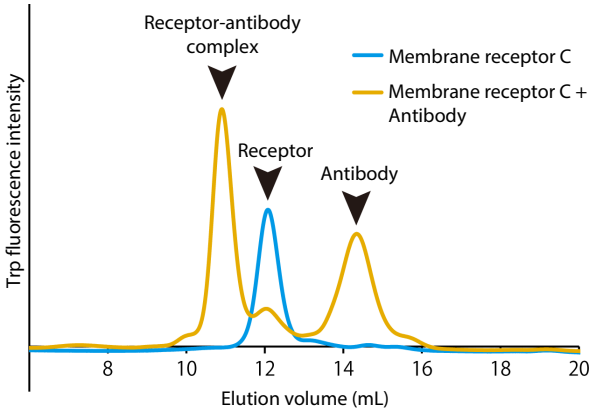

Fig. 6 Sample Data from Trp-FSEC

Purified membrane receptor C was mixed with a specific antibody which has the capacity to bind to membrane receptor C, and was analyzed by Trp-FSEC.
A peak derived from the complex of the receptor and antibody is observed in the high molecular weight region.

3) NTA-Probe FSEC: Detection of His-Tagged Samples

Fluorescent peptides (NTA-probe), binding to His tags widely used for protein purification, are added to solubilized samples to detect objective proteins with a His tag [4].

As shown in Fig. 7, by obtaining the elution profile of a sample based on the fluorescence from the peptides, the sample properties can be evaluated in the same manner as in the GFP-FSEC technique.

For some membrane proteins, GFP fusion may inhibit proper membrane integration. This NTA-probe FSEC is an effective technique for analyzing samples for which application of GFP fusion is technically difficult, by using the FSEC method. Note that in this approach, the peak heights may vary depending on the abundance of contaminating proteins or the quantity ratio of the objective proteins and the probe. Careful attention is therefore necessary when using the method for strict comparison of expression levels or for combining with the FSEC-TS. Even so, simple evaluations on existence/non-existence of expressed proteins are amply possible by this technique.

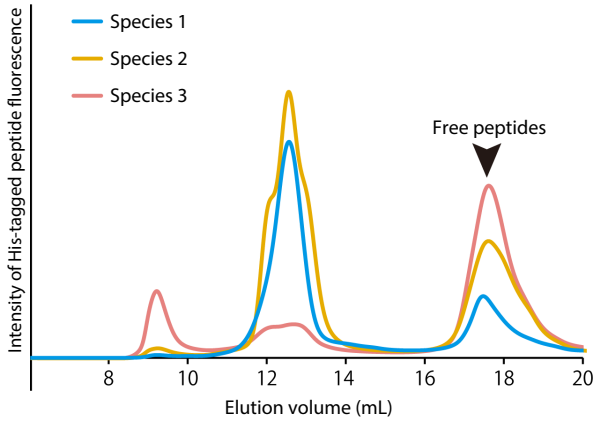

Fig. 7 Sample Data from FSEC Using Fluorescent Peptides

His-tagged membrane transporters D were solubilized, mixed with fluorescent peptides, and analyzed by FSEC.
Species 1 yielded a relatively sharp peak, while species 2 produced a broad peak, and species 3 did not obtain an obvious peak, indicating a low level of expression.

7. Conclusion

The basic idea of FSEC is simple: to separate and monitor a fluorescently labeled sample by gel filtration column chromatography. As described in this article, however, FSEC has a wide range of potential applications, allowing for evaluating the properties of not only membrane proteins but also a wide range of target proteins in each step from expression to purification. In addition, continuous analysis of numerous samples by using an autosampler enables rapid data acquisition, while the burden on chemists is reduced. 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 [5) 6) 7)]. Nureki Laboratory will take the utmost advantage of FSEC for experimental purposes, and continuously work on purification and structural determination of highly challenging membrane proteins.

Acknowledgements

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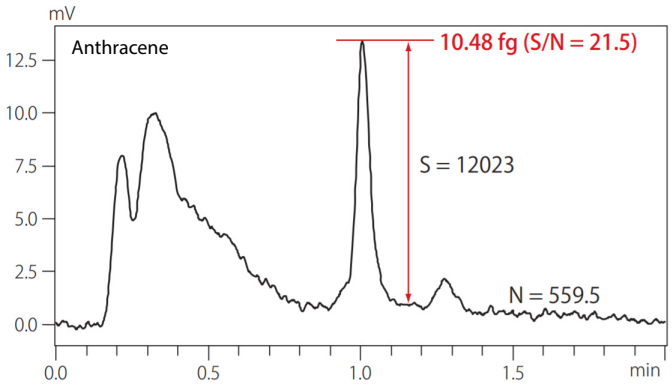
RF-20Axs Fluorescence Detector

RF-20Axs uses newly developed optics to provide the world's highest level* of sensitivity. This instrument demonstrates its outstanding performance in analyses requiring detection of ultratrace components.

*Based on in-house investigation as of August 2015

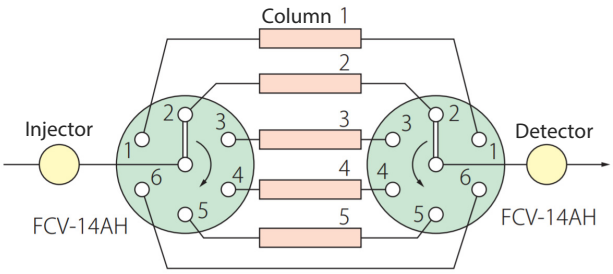
◇An example of high-sensitivity analysis of anthracene (RF-20Axs)

Injection of 10.48 fg of anthracene results in an S/N of 21.5 (RF-20Axs). This corresponds to a detection limit (S/N: 3) of about 1.5 fg, indicating exceptional sensitivity. This instrument sets a new standard for analysis requiring detection of ultratrace-level components.



High-Sensitivity Analysis of Anthracene

FCV-14AH/FCV-14AHi Column Switching Valve



A column switching valve equipped with a 7-port, 6-position high pressure valve. This allows automated multi-column switching (using 2 units). The valve can be controlled by a system controller or workstation.

*Components of the FCV-14AHi model in contact with liquids have bio-inert specifications.

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