Automated Analysis System of Biomolecular Interaction by Index-Matching Fluid-Mediated Fluorometry

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ABSTRACT In this post-genome era, analysis of the functions of proteins and other biomolecules is becoming an important area of study, creating a need for highly versatile systems capable of the high-sensitivity measurement and analysis of biomolecular interactions. In the research reported here, we have developed a system for the automated analysis of biomolecules by "fluid-mediated fluorometry", which is a new technique of measurement. Due to the approximate doubling of measurement sensitivity and the anti-drying effect offered by this measurement technique, we have achieved real-time measurement of minute samples. Using this system we have evaluated interactions between synthetic peptides and proteins and agonist measurement of yeast as a biomolecular tool, confirming its efficacy as a high-versatility, high-throughput technique for biomolecular interaction analysis.

1. INTRODUCTION

Proteins which constitute genetic products are directly involved in the life and living of the human body and are targets for the majority of drug actions. Today, with genome analysis completed for many species, amino acid sequences of proteins have been predicted from the coding genes, however it is impossible to identify their functions by genetic information alone since proteins are always modified after translation by many biochemical processes such as phosphorylation, glycosidation, acetylation and methylation. Thus the detection and functional analysis of proteins have become an important area of research that aims to tie these masses of genomic information to the design of drugs and tailor-made therapies.

To this end, peptides that are low in molecular weight and easy to synthesize are extensively used to construct the libraries of peptides for approaches to identification and analysis of casual proteins of diseases and improvement of the screening efficiency for lead compounds that constitute bottlenecks in terms of the time and money required to develop drugs.

Accordingly there is an urgent need for a highly versatile system capable of high-throughput, high-sensitivity measurement and analysis of biomolecular interactions. In the present study, we have developed a system for the automated analysis of biomolecules--from sample acquisition to reaction and measurement--by "fluid-mediated fluorometry", a novel technique of measurement (see Figure 1).

Figure 1 Automated analysis system.

2. EVALUATION OF FLUID-MEDIATED FLUOROMETRY

2.1 Principle of Fluid-Mediated Fluorometry

In fluorometry using an optical detection head, clearance must be provided between the detection head and the object of measurement in order to prevent contamination, and the Fresnel reflection loss, etc. of the excitation light and fluorescent light at the refraction interfaces between the tip of the optical detection head and the air, and between the object of measurement and the air constitute factors in the decrease of the fluorescent acquisition efficiency.

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Further, most of the organic samples usually exert their physiological functions in a liquid endogenous environment. Loss of function due to drying during the detection becomes a problem.

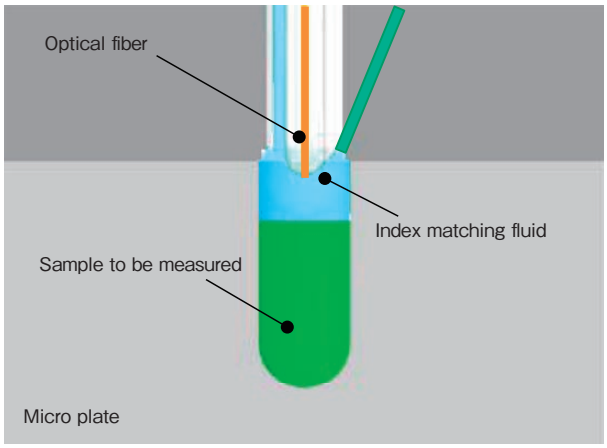

Figure 2 Fluid-mediated fluorometry.

Our fluid-mediated fluorometry technique (see Figure 2), is characterized by the space between the optical detection head and the object of measurement being filled with droplets (hereinafter referred to as fluid) thereby eliminating the layer of air that contributed to a decrease in sensitivity as well as to drying. By selecting as the fluid a material that has an index of refraction close to that of the optical detection head or the object to be measured it becomes possible to achieve high-sensitivity measurement. And if a fluid having low affinity with the measurement samples is used, contamination does not occur even if the detection head comes into contact with the fluid. If, in addition, the fluid is non-volatile, an anti-evaporation effect on the biological samples can be expected.

2.2 Numerical Study of the Role of Fluid Mediation in Improving Sensitivity

We carried out a simple numerical study of two factors in the improvement in sensitivity in fluid-mediated fluorometry, namely (1) reduction in the Fresnel reflection loss, and (2) enlargement of the numerical aperture (NA).

2.2.1 Reduction in Fresnel Reflection Loss

If light impinges on an interface at which the index of refraction changes discontinuously, part of the advancing power is reflected. The reflectance is dependent on the index of refraction, an incidence angle, and polarization of incident light. However, as long as an incidence light is limited to perpendicular incidence, it will not depend on polarization. In this case, the Fresnel coefficient of perpendicular incidence (power reflection coefficient) may be shown as

$$r^2 = \left(\frac{n_1 - n_2}{n_1 + n_2}\right)^2 \qquad (1)$$

Here, n_1 and n_2 represent the indexes of refraction of medium 1 and 2, respectively; r indicates the strength ratio of catoptric light to incident light, and r^2 expresses the ratio of power. Thus, for example, when n_1 is 1.515 and n_2 is 1.000, r^2 will be 0.0419, and when laser light is coupled to an optical fiber with no coating, a reflection loss of approximately 4 % is unavoidable.

Even in fluorometry using microplates, Fresnel reflection constitutes a major obstacle. If we consider a case in which excitation and received power are both carried out by an optical glass fiber probe, the excitation light will undergo a loss of approximately 6 %--about 4 % at the fiber-air interface and about 2 % at the air-sample interface. This will be analogous for the fluorescent light that under the irradiation of excitation light is emitted to impinge on the fiber, so that added together more than 10% of the overall sensitivity will be lost due to interface reflection. When components other than perpendicular incidence are also included, the loss becomes even greater. In fluid-mediated fluorometry, the intervening gaps are filled with oil having a high index of refraction so that, in effect, the hetero interfaces are only from optical fiber to sample and back and total reflection loss can be held to less that 1 %. This is advantageous not only in terms of sensitivity, but also in eliminating stray light in the measurement of faint fluorescent light.

2.2.2 Enlargement of Numerical Aperture (NA)

Effect of the fluid on enlargement of the numerical aperture (NA) was then investigated. Just as in the oil-immersed objective lens of a microscope, where the gap between the sample and the objective lens is filled with oil, fluid-mediated fluorometry uses an oil having an index of refraction much higher than that of water or glycerin, making it possible to obtain a larger numerical aperture.

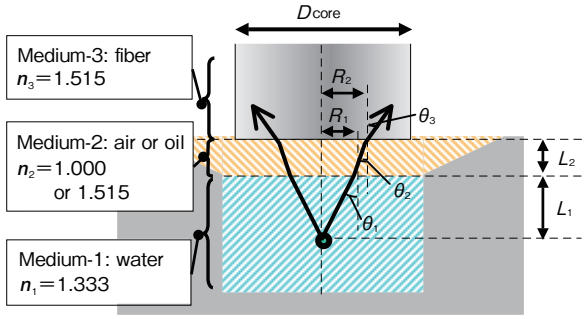

Figure 3 Effect of enlargement of numerical aperture by fluid-mediation.

Figure 3 shows a cross-section of the apparatus for fluid-mediated fluorometry using microplates. The fluorescent light emitted from the sample under excitation is refracted at the interface and detected by the optical fiber. The fluorescent light is emitted isotropically in all directions, and polarization may be taken as negligible. For simplicity it is assumed that the fluorescent samples are point light sources which have no size and are positioned vertically under the optical fiber. Medium 1 is a fluid that contains the sample, with an index of refraction of 1.333, equal to that of water. Medium 2 is the intervening layer which, in the fluid-mediated method is assumed to be sili-

cone oil having an index of refraction of 1.515, but in the conventional measurement method is taken to be an air gap with an index of refraction of 1. Medium 3 is the index of refraction of the optical fiber core, or 1.515 in this case.

The angles of refraction at the interface (θ_1 - θ_3) are linked by Snell's Law so that θ_3 and R_2 may be represented using θ_1 as

$$\theta_3(\theta_1) = \sin^{-1}\left(\frac{n_1}{n_3}\sin(\theta_1)\right) \quad (2)$$

$$R_2(\theta_1) = L_1\tan\theta_1 + L_2\tan\left(\sin^{-1}\left(\frac{n_1}{n_3}\sin(\theta_1)\right)\right) \quad (3)$$

Here, $n_1 \sim n_3$ represent the index of refraction of each medium, respectively; θ_1 stands for the incident angle of light from medium 1 to medium 2, θ_2 and θ_3 for the angles of incidence and refraction from medium 2 to 3, respectively. R_1 and R_2 indicate the distance between the light beam and the central axis of the optical fiber core when it passes through the medium interface. L_1 expresses the depth of fluorescent light source within medium 1, and L_2 shows the gap between the interface of medium 1, 2 and the optical fiber (medium 3). The conditions under which the light beam is coupled to the optical fiber are such that the point of intersection of the light beam and the fiber end face is within the core, and the angle of incidence is less than the NA of the fiber, that is, both

$$R_2(\theta_1) \leqq D_{\text{core}}/2 \quad (4)$$

$$\sin\theta_3(\theta_1) \leqq \frac{n_2}{1.000}\ NA \quad (5)$$

are satisfied. Note also that the well aperture of the microplate shall be sufficiently large, and that there shall be no loss due to blocking of light by the brim of the well. Fluorescence sensitivity shall be proportional to $I_1^{\max}$ which is obtained by integrating, after solid angle conversion, the maximum value of θ_1 that satisfies the conditions of Equations (4) and (5) simultaneously, with respect to fluorescent light source depth L_1.

Figure 4 shows $I_1^{\max}$ when gap L_2 at the interfaces between the fiber and media 1~2 is changed. Since the wider L_2 is, the narrower will be the solid angle at which light can be detected, the graph as a whole shows a tendency to descend to the right. It can be seen that in a case in which a specialty fiber of high NA is used for the probe, the angle at which light can be detected will be approximately an order of magnitude greater that with the conventional multimode fiber.

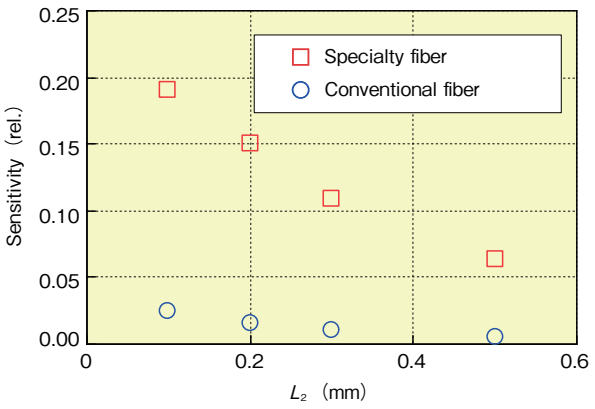

Figure 4 Dependence of measurement sensitivity on gap thickness.

To estimate the ratio of enlargement of photodetection sensitivity due to the fluid, we find $I_1^{\max}$ with respect to the cases in which medium 2 is oil and air respectively, and define the value of the ratio as the figure of merit (FOM):

$$\text{FOM} = \frac{I_1^{\max\_oil}}{I_1^{\max\_air}} \quad (6)$$

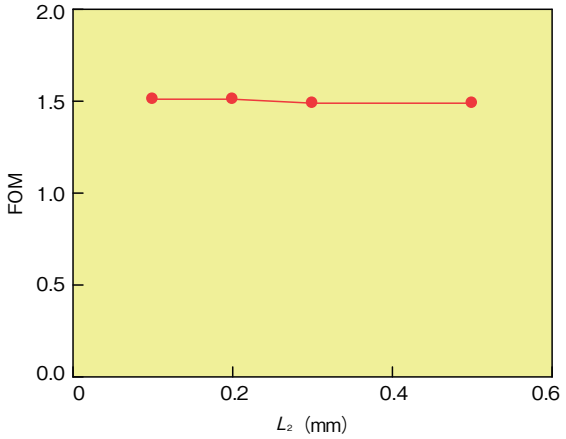

Figure 5 Sensitivity figures of merit (using specialty fiber).

Figure 5 is a graph plotting FOM versus gap L_2 when using a special optical fiber. It was found that a sensitivity merit of approximately 1.5 folds could be obtained, largely independent of changes in L_2.

Based on the discussion above, it can be seen that through the reduction in Fresnel reflection loss and NA enlargement, an improvement in sensitivity of up to double can be anticipated.

2.3 Implementation of Fluid-Mediated Fluorometry

2.3.1 Samples and Fluid Conditions

As shown in Figure 6, we brought the optical fiber head that emits the excitation light down from above into proximity with a well that has been filled with a fluorescent reagent and the fluid, and obtained the detection intensity of the resultant fluorescence and reflected light. The fluorescent reagents were diluted with N, N, N', N'-carboxytetramethylrhodamine (TAMRA) and used. The well used was of substantially cylindrical configuration with a diameter of 1.4 mm, having 8 μL of fluorescent reagent on which index-matching silicone oil was dripped. The measured value was taken as the fluorescent intensity at the point at which the optical fiber head approached to a position of 0.3 mm with respect to the fluorescent pigment surface.

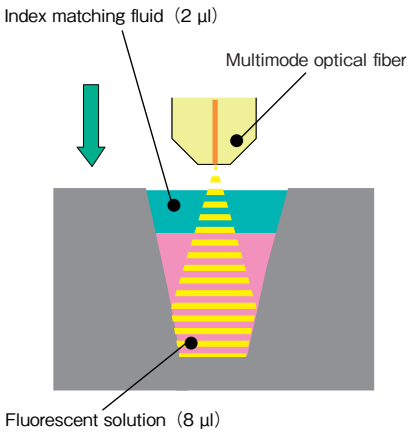

Figure 6 Schematic of conditions for evaluation of fluid-mediated fluorometry.

2.3.2 Fluorescence Optical System

Figure 7 depicts the fluorescence optical system used in fluid-mediated fluorometry.

In order to support high-sensitivity fluorometry, a monochrome laser source was used for excitation light, with a spectroscope using dichroic mirrors, bandpass filters and PMTs (photomultiplier tube) for detection. The sensing optical fiber, for purposes of high-sensitivity fluorometry, had a large NA and was designed based on MFD (mode field diameter) suited to the requisite level of measurement gap resolution, and the lens system was optimized to match this.

In addition to being designed with the capability of polychromic spectrometry, measurement of fluorescence pigments for a variety of applications can be supported by changing the excitation light source and optical elements. Measurement of backscattered light is also supported to allow data compensation through status recognition and detection of status error. In this way it is also possible for the optical fiber head to approach or come into contact with the fluid plane, as recognition of the measurement status.

2.3.3 Results of Fluorometry Monitored by Fluorescence and Reflection Channels

Figure 8 shows an example of results of measurement of

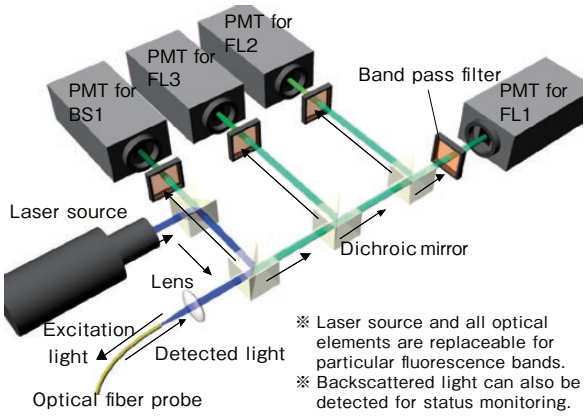

Figure 7 Optical system used in fluid-mediated fluorometry.

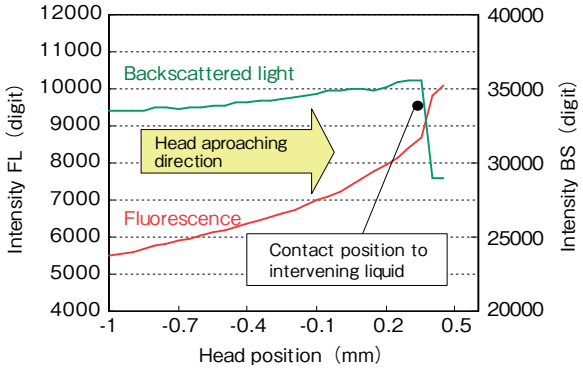

Figure 8 Fluid-mediated fluorometry monitored 2-channel signals.

one well. The main factors affecting reflected light are caused by: (1) the end face of the optical fiber, (2) the fluid plane of the sample measured; and (3) the reflection of excitation light at the plate surface. There is a gradual increase in the reflection light signal as the tip of the optical fiber approaches the well, but since, if it comes into contact with the fluid, the reflection due to factors (1) and (2) above decreases significantly, detection sensitivity will decline.

The fluorescence signal also increased as the optical fiber approaches, but unlike the reflected light, when it comes into contact with the fluid, the intervening air gap disappears, so that it rises significantly.

2.4 Effect of Improvement in Sensitivity by Fluid-Mediated Fluorometry

2.4.1 Improvement in Measurement Sensitivity

In order to obtain direct confirmation of the effect of sensitivity improvement achieved by fluid-mediated fluorometry, we first measured without using fluid, and carried out comparative measurements in that condition with fluid supplied. As a result measurement sensitivity was

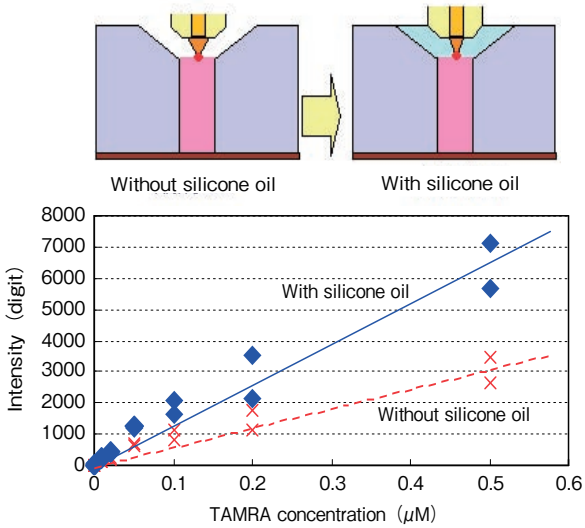

Figure 9 Effect of improvement in measurement sensitivity by fluid-mediated fluorometry.

improved by two folds (see Figure 9), agreeing with the results of the numerical analysis presented above. This is thought to be due to the reduction of Fresnel reflection loss in the refractive index interface and by the increase in the photodetection NA due to fluid mediation.

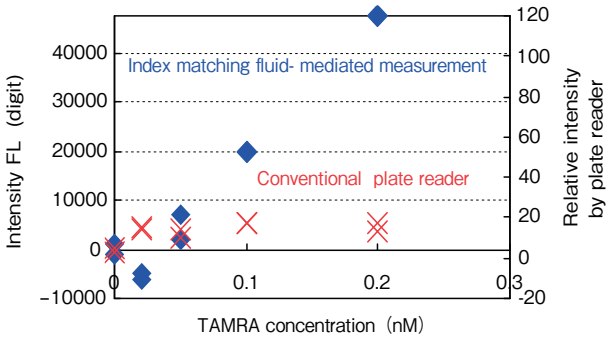

Figure 10 Detection of fluorescent signal using low-concentration samples.

Figure 10 shows the result of measurements carried out at low levels of concentration. It was possible to obtain adequate measurement provided the concentration was in the order of 0.1 nM. To detect feeble signals from particularly low-contration samples requires not only improvements in the efficiency of condensation of the fluorescent light but also reduction in background noise. Since interface reflection of excitation light is a major factor in background noise, it is a major advantage in terms of detecting feeble fluorescence that fluid-mediated fluorometry enables the reflected light together with its deviation to be reduced for each detection event.

2.4.2 Anti-drying Effect of Samples by Fluid-mediated Fluorometry

We also conducted a measurement evaluation with respect to the anti-drying effect, which is a further advantage of fluid-mediated fluorometry (see Figure 11). The well had a deep-hole configuration measuring 1.4 mm in diameter and 4 mm in depth, but even so, when no fluid was used there was a marked drop in detected fluorescent intensity due to drying. This makes it impossible to disregard the passage of time after sorting or the effect of the measurement procedure, and makes quantitative evaluation difficult. In contrast, it was found that when a non-volatile fluid was used, drying was prevented, stable measurements could be taken even for minute samples, and changes in the properties of the object of measurement could be determined over long periods in real time.

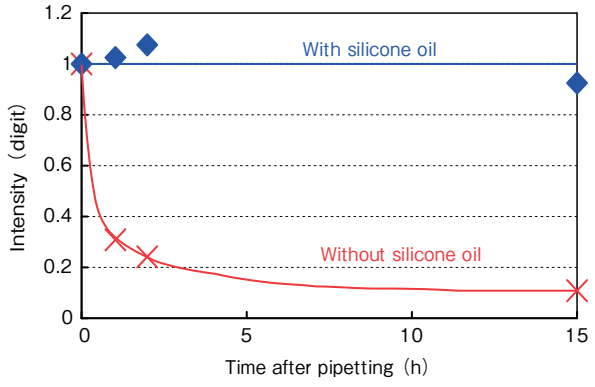

Figure 11 Anti-drying effect by fluid-mediated fluorometry.

3. DEVELOPMENT OF AUTOMATED ANALYSIS SYSTEM AND ITS APPLICATION TO BIOMOLECULAR MEASUREMENT

3.1 Automated Analysis System

In accordance with the concept of an automated analysis system, we have developed a system (see Figure 12) capable of performing the series of operations--from acquisition of multiple samples through sorting, mixing, and real-time reaction measurement, to the following processes for the purpose of measurement (cleaning and drying of the optical fiber tip, replacing of the pipette tip, etc.)--in a fully automated manner.

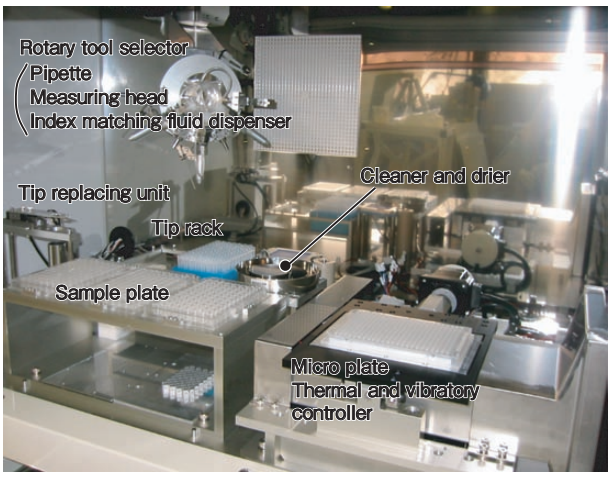

Figure 12 Core units of the automated analysis system.

3.2 Evaluation of Peptide-Protein Interactions

3.2.1 Background

A system that could provide simple and efficient detection of proteins, and was also capable of analyzing their physical and chemical properties and their functional nature would be enormously useful. Dr. Mihara's Group at the Tokyo Institute of Technology has focused on the configuration recognition properties of proteins, designed and synthesized peptides with a variety of 2- and 3-dimensional structures, and is engaged in developing a novel assay method for detection of protein phosphorylation as well as constructing protein chip for detecting and analyzing proteins [1)~8)].

Peptides are of lower molecular weight than proteins, and it is possible to design and synthesize many different 2- and 3-dimensional molecular configurations, including α-helix, β-sheet, loop structure and so on. By means of protein fingerprinting using fluorescent-modified peptide arrays, it is possible to characterize large numbers of pro-

teins. In addition to the detection of specific proteins, this method also holds promise of functional analysis of proteins in the form of analysis of the bonding characteristics of proteins, and the investigation of peptide ligands that can control the function of proteins. In fact Dr. Mihara's Group, taking as a model the calmodulin-calcineurin (CaM-Cn) system shown in Figure 13, is configuring systems such as one for investigating a peptide ligand that can control the dephosphorylation activity of Cn by bonding with CaM [9].

In the following section we shall discuss an evaluation, carried out jointly with Dr. Mihara's Group, that utilized the advantages of fluid-mediated fluorometry is the stable, high-sensitivity measurement of the status of solutions in its applicability to the functional analysis of proteins.

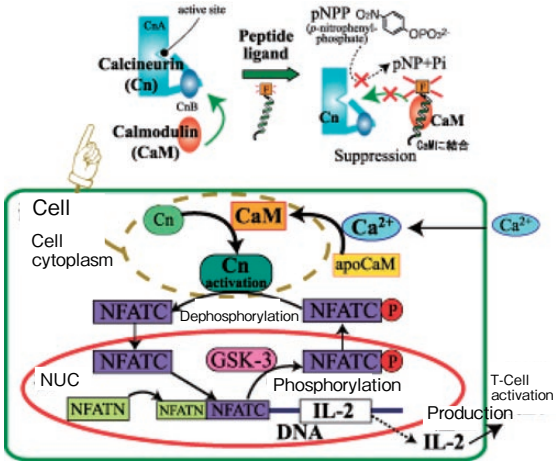

Figure 13 Immune signaling system using calmodulin and calcineurin.

3.2.2 Evaluation of Peptide-protein Interactions

(1) Measurement of CaM- L8K6 combination

First of all, to evaluate the suitability of fluid-mediated fluorometry for measuring combination of proteins and peptides, we conducted evaluations of the binding between CaM and L8K6 peptide, which is already known, and of the binding to L8K2E4 peptide, which has low combination ability.

4 μL each of 2 μM peptide and a CaM solution with different concentration levels were mixed and sorted, and fluorescent intensity was measured by fluid-mediated fluorometry.

As shown in Figure 14, the well has an aperture for the fluid above the portion for holding the sample. Since measurements were made for each well individually the rate of measurement was 1 well per second, but scanning of the optical fiber head enables measurement of 50 wells per second.

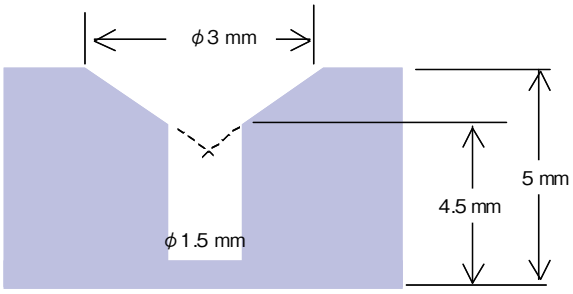

Figure 14 Schematic structure of the well for peptide-protein measurement.

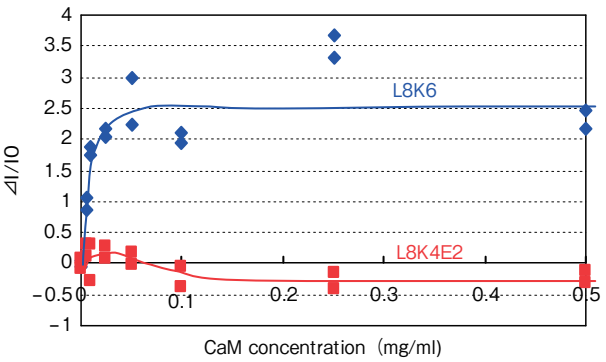

Figure 15 Evaluation of magnitude of peptide-protein binding.

Figure 15 shows the results of the measurements. For L8K2E4 peptide, which has low combination ability with CaM, virtually no increase was observed in fluorescent intensity, but for L8K6 peptide, an increase in fluorescent intensity was observed as CaM concentration increased. Under conditions of high CaM concentration measurement discrepancies occurred attributable to differences in the amount of sorting due to adhesion to the pipette tip, but the curve to the extent of saturation could be fitted to the binding constant Ka of 6.8 x 10^6 M^{-1} between CaM and L8K6 peptide, confirming that it is possible to evaluate binding between protein and peptide.

(2) Matrix evaluations of peptide-protein interactions

Next, in order to evaluate its applicability to protein analysis using the peptide library, a matrix evaluation was carried out using several peptides and proteins. As shown in Table 1, we used a total of five standards--four proteins and a buffer solution--for evaluations of four types of synthetic peptides, and made a relative evaluation of the increase in fluorescent intensity due to binding. Concentrations were 3 μM for the proteins and 1 μM for the peptides; the conditions of well and capacity were similar to those of the previous section.

Table 1 Measurement matrix elements for evaluation of peptide-protein interactions.

		Synthetic peptide (1 μM)			
		TAMRA-L8K6	TAMRA-L8K2E4	TAMRA-L6A2K4E2	TAMRA-A8K2E2
Protein (3 mol/l)	Calmodulin (CaM)	○	○	○	○
	Alpha amylase (α-ami)	○	○	○	○
	Protein kinaseA (PKA)	○	○	○	○
	Insulin (Ins)	○	○	○	○
	Buffer	○	○	○	○

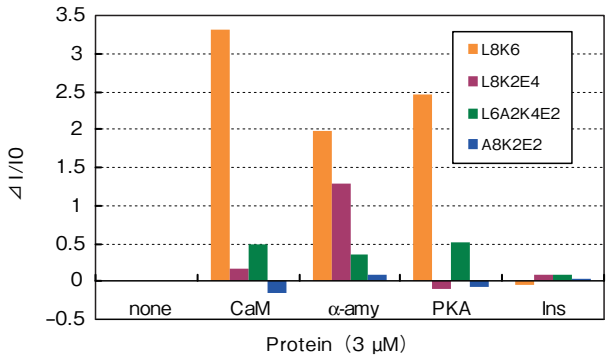

Figure 16 Results of matrix measurement of peptide-protein interactions.

Figure 16 shows the results. With respect to L8K6 peptide, binding with CaM was the strongest, and binding also occurred with α-amylase and protein kinase A but not with insulin. These results suggest that the magnitude of binding can be evaluated by fluorescent intensity. With respect to L8K2E4 peptide, limited to the protein standards used here, we may say that binding occurred only with α-amylase, suggesting that specific binding can be detected. These results indicate that it is possible, by using multiple species of synthetic peptides, to screen ligands acting with specific proteins, and by means of protein chips using synthetic peptides, to detect proteins.

3.3 Development and Evaluation of Ligand Screening System using Yeast

3.3.1 Background

On the cell membrane of the a-type cells of the yeast, there are G protein conjugate receptors (GPCRs) known as Ste2p, to which binds α-factor, a binding factor secreted by α-type cells. Dr. Kondo's Group at Kobe University, one of the participants in the present development work, took GPCRs as the molecular target for drug design and are developing a ligand screening system utilizing signal transmission of the a-type cells of the yeast.

By means of genetic methods, the target receptors were expressed on the yeast cell surface and peptide ligands were presented on the yeast surface. By transfection of library genes, different types of peptide ligands could be presented on the various yeasts. Whenever in a cell presented peptide ligand bound to the expressed receptors as an agonist, signaling would be induced within the cell. At the final stage of the signaling pathway, under the control of a *FUS1* promoter whose transcription activity was induced, reporters genes such as fluorescent protein were expressed which in turn make more change in the profiles of yeast genes so that positive agonists could be screened (see Figure 17). That is, the yeasts expressing positive ligands give off fluorescent light and are easily detected or separated, giving promise of an extremely effective method of large-scale screening.

Dr. Kondo's Group is developing a cell system that is optimally suited to this screening. Especially, they are working towards construction of such a system using

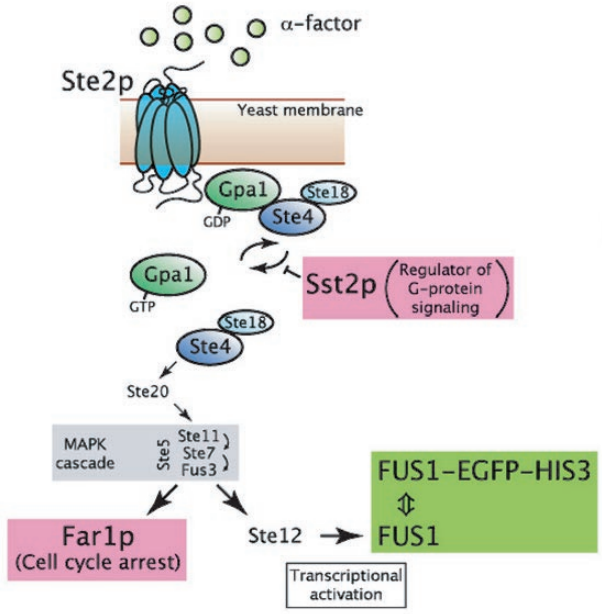

Figure 17 Screening system using EGFP.

human receptors and its application to high-throughput drug design screening.

In the following section we report on measurement of yeasts for agonist detection by fluid-mediated fluorometry carried out in conjunction with Kobe University.

3.3.2 Evaluation by Fluorescent Detection using Peptide-presented Cells

The yeast cells were present dispersed within a medium, and since they settled to the bottom, a measurement method was adopted in which 0.5 μL of a yeast cell suspension was sorted in a conical well as shown in Figure 18, approximately 10 μL of the fluid (index-matching silicone oil) was dripped from above, and the optical fiber head was scanned 2-dimensionally. Analysis of the 2-dimensional data thus obtained yielded readings of fluo-

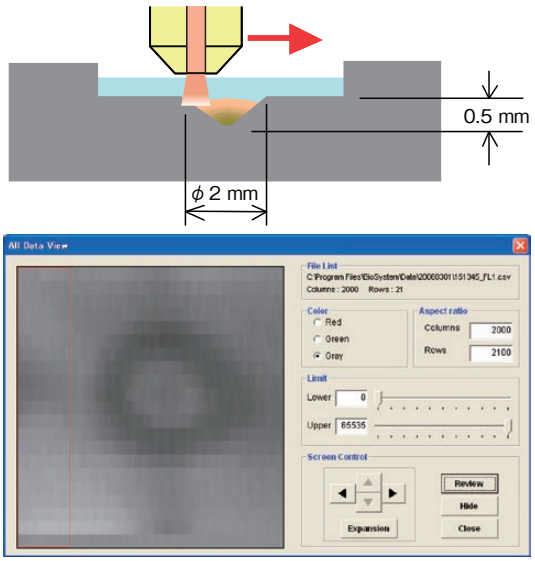

Figure 18 Measurement of unevenly localized objects using planar scanning head.

rescence values for the cell portion.

We used a mutant of Saccharomyces cerevisiae BY4741 [*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bar1Δ::LEU2 far1Δ::kanMX4 sst2Δ::AUR1-C* P_{fus1}*-FUS1-EGFP-HIS3*] as the yeast host (hereinafter referred to as IMGH-8) in our system. Screening sensitivity was improved since this strain is deficient in the following inhibitory protein of targeted signaling as well as the proteins involved in other signaling pathways.

1. The Bar1p protease gene for degradation of α-factor has been destroyed.
2. The Sst2p gene whose protein product impedes the signaling through inhibiting separation reaction of heterotrimer G-protein which works with the receptors binding to α-factor, has been destroyed.
3. The Far1p gene whose protein product will be activated by phosphorylation with the expression of Fus1p at the final step of signaling and inhibit cell cycle progress, has been destroyed.

As a high-expression reporter plasmid, a multi-copy type pMHRS-pFUS1 which expresses Fus1-EGFP under *FUS1* promoter was used. pUESCα-FLO42 which encodes and presents α-factor to the cell surface under *GAL1* promoter was used for presentation of α-factor, and an empty vector pESC-URA was used as a negative control. Through transfection of these plasmids into the host cells, IMGH-8/pESC-URA and IMGH-8/pUESCα-FL042 cell clones were obtained. These clones were then cultured in an SD-Ura medium. Fifteen hours in an SG-Ura medium, cells were started to amplify. To obtain a cell suspension with an intensity of OD_{600} = 10, cells were diluted and measured at various phases.

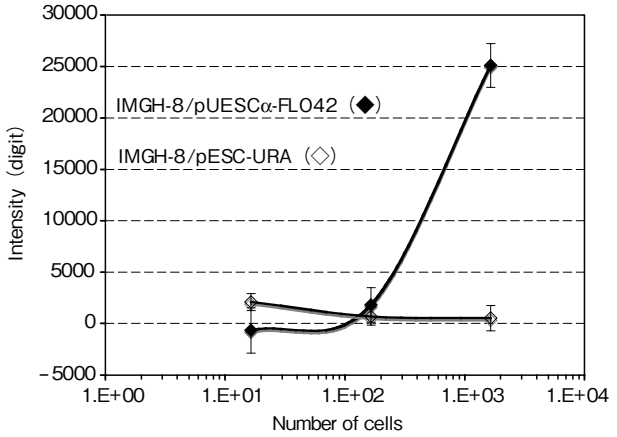

Figure 19 Evaluation of IMGH-8 with peptides presented on the surface by detection of fluorescence.

Figure 19 shows the results for measurement of IMGH-8/pESC-URA and IMGH-8/pUESCα-FL042. At cell counts of approximately 10^3 or more a clear difference could be observed. These results indicate that presentation on the cell surface is extremely useful in the study of ligand binding, and that resultant fluorescence of EGFP can be detected by our system.

4. CONCLUSION

We have developed a novel method called fluid-mediated fluorometry to measure minute biomolecular interactions using proteins and peptides as the main targets. This has made it possible, by means of a fundamental analysis using fluorescent pigment, to achieve approximately double the sensitivity together with an anti-drying effect, and to obtain real-time measurements of minute fluorescent samples while in solution. We have also developed a highly versatile automated analysis system to carry out this measurement, and have confirmed its efficacy by evaluating biological contents.

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