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Detection of Protein Phosphorylation by Open-Sandwich Immunoassay

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1. Introduction

Understanding the post-translational modifications of proteins represents a next major challenge in post-genomic era. Intricate cascades of phosphorylation reactions regulate cell proliferation, differentiation, migration and so on. For example, Olsen et al recently applied high-resolution mass spectrometry-based proteomics to phosphoproteome of human cell cycle, quantifying 6,027 proteins and 20,443 unique phosphorylation sites [Olsen, 2010]. Phosphorylation-dependent signals participate in several diseases, such as cancers, immune diseases, and Alzheimer's. The method to produce phosphorylation-site and phosphorylation state-specific antibodies was established in 1990's [Nishizawa, 1991; Yano, 1991; Nagata, 2001; Goto, 2007] after a discovery of phosphorylated cytoskeletal protein-specific antibodies [Sternberger, 1983]. Since then, investigators have successfully observed spatio-temporal dynamics of particular phosphorylation *in vitro* or *in situ* using the antibodies. Recently, these antibodies are also proven useful as a live cell imaging probe *in vivo* [Hayashi-Takanaka, 2009; Kimura, 2010; Hayashi-Takanaka, 2011], clinical diagnosis, and drug screening [Brumbaugh, 2011].

Here we show a couple of applications of phosphorylation-specific antibodies to proteomic studies, more specifically focusing on a novel immunoassay approach, which we call open-sandwich immunoassay.

2. Open-sandwich immunoassay

2.1 An innovative immunoassay: Open-sandwich immunoassay

The open-sandwich immunoassay (OS-IA) was developed in a rather fortuitous way as follows [Ueda, 1996; Ueda, 2002]. Previously, one of the authors had been interested in the regulation of the tyrosine kinase activity of epidermal growth factor (EGF) receptor by the ligands other than the natural one (EGF), as a prototype molecular biosensor [Ueda, 1992]. Since the EGF receptor was found activated by ligand-induced dimerization [Schlessinger, 1986], a possible way to realize such regulation was to replace the EGF-binding domain of EGF receptor with a pair of specific binding domains that dimerize upon addition of their ligand. Pairs of such binding domains are known to exist in nature. For example, FKBP12 and FRAP are known to associate in the presence of an antibiotic rapamycin [Rossi, 1997] and the two erythropoietin (Epo) binding domains of EPO receptor homodimerize upon binding

with EPO [Lacombe, 1999]. As an alternative of such domains, one of the authors was looking for antibody variable regions (V_H and V_L), wherein the association between V_H and V_L is strengthened by the antigen. In other words, at the beginning we had no intention to devise a novel immunoassay. We only wanted to determine the V_H/V_L interaction strength and the effect of an antigen on this interaction to devise such a hybrid receptor (Fig. 1A).

Theoretically, there might be a range of immunoglobulin variable regions that show (i) weak and (ii) strong V_H/V_L interaction irrespective of antigen binding, (iii) weak V_H/V_L interaction in the absence of an antigen, which is strengthened by antigen binding, and (iv) strong V_H/V_L interaction in the absence of an antigen, which is weakened by antigen binding. Before that time there were very few reports on the strength of the interaction between V_H and V_L fragments and its antigen dependency, we tried to measure the interaction between V_H and V_L of anti-hen egg lysozyme (HEL) antibody, HyHEL-10 using a surface plasmon resonance biosensor Biacore (Fig. 1B).

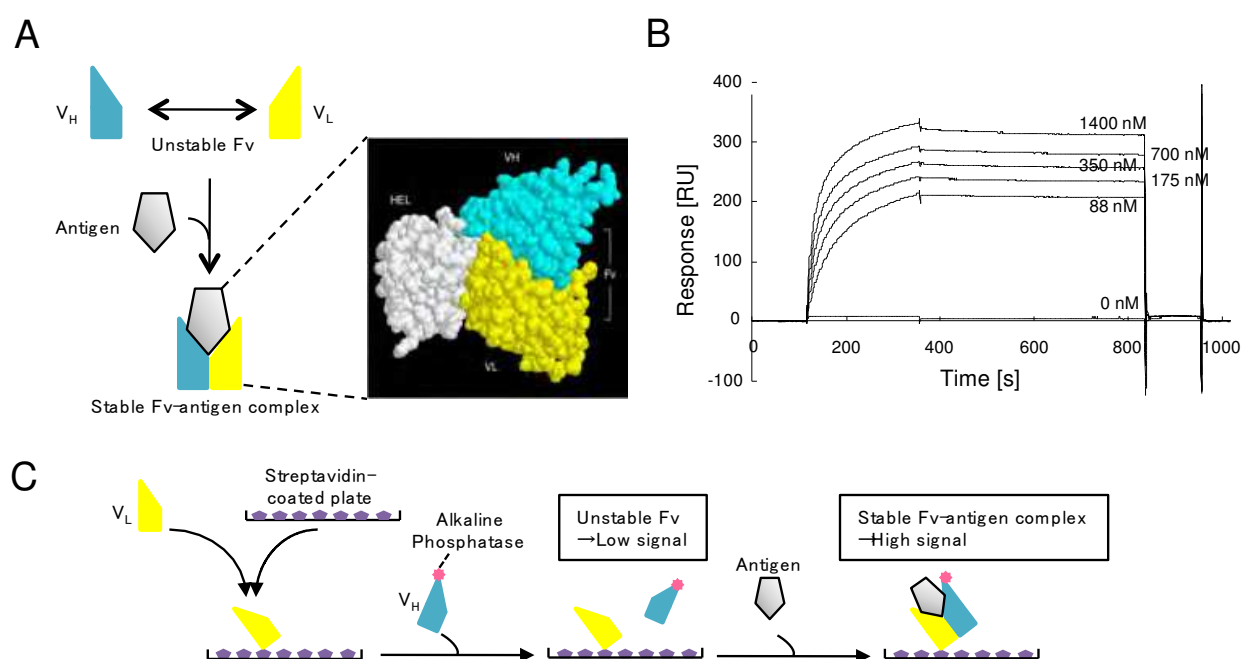


Fig. 1. Principle of open-sandwich immunoassay (OS-IA). (A) V_H/V_L /antigen ternary complex. While the intrinsic binding affinity of V_H and V_L is low, when they are added with antigen, that of V_H/V_L /antigen complex becomes high. (B) A measurement of V_H/V_L complex stability by Biacore. The association curve of V_H on immobilized V_L is significantly influenced by the co-existing antigen concentration as shown. (C) A basic procedure of OS-IA. V_L (or V_H) is immobilized, and labeled V_H (or V_L) is put together with a sample. To determine the affinity of V_H and V_L as a function of antigen concentration in a sample, the amount of labeled V_H (or V_L) bound onto the well is quantified.

When the binding of soluble V_H fragment to the immobilized V_L fragment was optically monitored, the interaction was calculated to be very weak in the absence of the antigen ($K_a < 10^5/M$), and markedly strengthened in the presence of the antigen ($K_a \sim 10^9/M$). The principal reason for this was found to be a remarkable reduction of dissociation rate k_{off} of the complex. This antigen-induced equilibrium shift was also observed in an ELISA, where the binding of phage particles displaying the V_H fragment to the V_L fragments immobilized

on microplate wells was measured by horseradish peroxidase (HRP)-labeled anti-phage antibody (Fig. 1C). A reproducible standard curve could be drawn for the antigen HEL concentration in the sample (Fig. 2A). The results indicated such an assay could be utilized as a novel means to measure antigen concentration in a sample. We termed this type of assay an open-sandwich immunoassay (OS-IA) because the antigen to be measured was bound to two fragments of antibody, V_H and V_L like an open sandwich. Further studies using other many antibodies revealed that all four types of variable regions shown in the beginning of this paragraph have been identified, however, many anti-hapten antibodies exhibit the property categorize in (iii) described above which makes them suitable for OS-IA [Suzuki, 1999; Lim, 2007; Suzuki, 2007; Ihara, 2009; Islam, 2011].

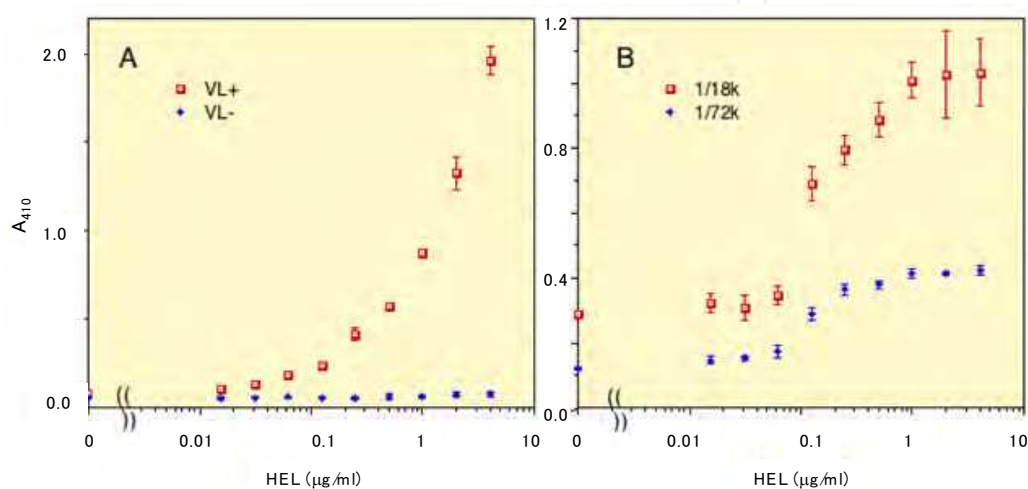


Fig. 2. Dose-response curves obtained with OS-IA and conventional sandwich immunoassay. (A) Association of phage-displayed V_H with immobilized V_L in the presence of hen egg lysozyme (HEL) was probed with labeled anti-phage antibody. A control without immobilized V_L is also shown. (B) Sandwich ELISA with HyHEL10 scFv and rabbit anti HEL serum at two dilutions as indicated. The signal was obtained with HRP-labeled anti rabbit IgG.

OS-IA has several advantages compared with conventional immunoassays. Sandwich immunoassay is one of conventional noncompetitive immunoassay with high sensitivity and a wide working range of more than three orders of magnitude. The principle of sandwich ELISA is to detect antigen in a sample captured first antibody immobilized on microplate by enzyme-linked second antibody. However, sandwich immunoassay has a fundamental limitation that the antigen to be measured must be large enough to have at least two epitopes to be captured; therefore, small monovalent antigens are not suitable for the assay. Another conventional immunoassay, competitive immunoassay is useful to measure monovalent antigens. The principle of assay is based on the competitive binding of labeled antigen and non-labeled antigen in sample, when captured by an antibody immobilized on a surface such as microplate wells. While competitive assay enables measurement of monovalent antigen, careful optimization of the reaction conditions is necessary to attain suitable sensitivity and working range, and a large amount of antigen is required. Furthermore, while the sensitivity is theoretically approximately $1/100$ of K_d , the affinities of antibodies are limited by the surface area interacting antigens and paratopes of antibodies, hence, small antigens is generally undetectable with high sensitivity (Fig. 2B).

As a way to circumvent these limitations of conventional immunoassay, using OS-IA less than 10 ng/ml antigen is detected in a shorter time period than by a conventional sandwich assay, due to the omission of an incubation/washing cycle. In addition, OS-IA using HyHEL-10 resulted in better sensitivity than that obtained with the corresponding sandwich immunoassay. Also, the applications of OS-IA to small antigens could attain a similar or lower detection limit as well as wider working range than attained with the corresponding competitive assay. Why can OS-IA detect small antigens with a higher sensitivity and a wider working range? It may be concerted with that Pellequer *et al.* categorized the changes in compactness of the V_H/V_L interface between bound and unbound antibodies on the size of the antigen and found that small antigens or haptens cause a closure of the interface, whereas larger protein antigens have little effect of the compactness of V_H/V_L interface [Pellequer, 1999]. This is also in accordance with previous observations that anti-hapten antibodies recognize their antigen between the V_H/V_L interfaces, whereas anti-protein antibodies do it on the upper surface of V_H/V_L dimers.

2.2 Application to homogeneous assays

In the field of healthcare, food safety and environmental monitoring, homogeneous assays are available for rapid and simple screening of components in samples. It is necessary for automation techniques for high-throughput screens. Here, we show applications of OS-IA to homogeneous assays.

First, we employed fluorescence resonance energy transfer (FRET), in which, one fluorophore as donor transfers its excited-state energy to another fluorophore as acceptor, resulting in emitting fluorescence of a different color. FRET generally occurs when the donor and acceptor are in approximate distance (10-100 Å) [Selvin, 1994]. It has been applicable to a homogeneous immunoassay by labeling antibody and antigen with donor and acceptor of fluorescence respectively. However, this is a competitive immunoassay with less sensitivity than that of noncompetitive immunoassay, and requires a large amount of labeled antigen [Pradelles, 1994]. Furthermore, endogenous antigens cannot be detected in the assay.

We performed open-sandwich fluoroimmunoassay (OS-FIA) using fluorescein-labeled V_H and rhodamine-labeled V_L [Ueda, 1999] (Fig. 3A). A principle of OS-FIA is as follows; 1) Without antigen, the two fusion Fv fragments remain monomeric, so FRET between them is negligible. 2) The addition of antigen induced heterodimerization of the two chains, accompanied by the FRET. When the labeled fragments were added to the sample solution, the antigen concentration could be measured by monitoring V_H/V_L interaction with FRET. The each detection takes within only 2 minutes. However, a site-specific fluorolabeling for the assay is needed several laborious trials. Then we next use GFP variants fused V_H and V_L to obtain site-specific labeled probes [Arai, 2000] (Fig. 3B). The V_H and V_L fused to GFP variants are expressed in cytoplasm of mutant strains that have oxidized cytoplasmic environments to make proper S-S bonds in V_H or V_L . Using the purified V_H and V_L from *E. coli*. OS-FIA could be carried out without significant loss of sensitivity.

The second application makes use of bioluminescence resonance energy transfer (BRET) [Arai, 2001] (Fig. 3C). In BRET donor is a bioluminescence, and acceptor is a fluorescent protein. When donor and acceptor are in an approximate distance, luminescence of donor is transferred to acceptor, resulting in emitting fluorescence from acceptor. When V_H -Rluc and V_L -eYFP were mixed with a sample reagent, an antigen in the sample dependent increase in BRET was measured. Compared with our comparable OS-FIA, the sensitivity is a 10-fold higher.

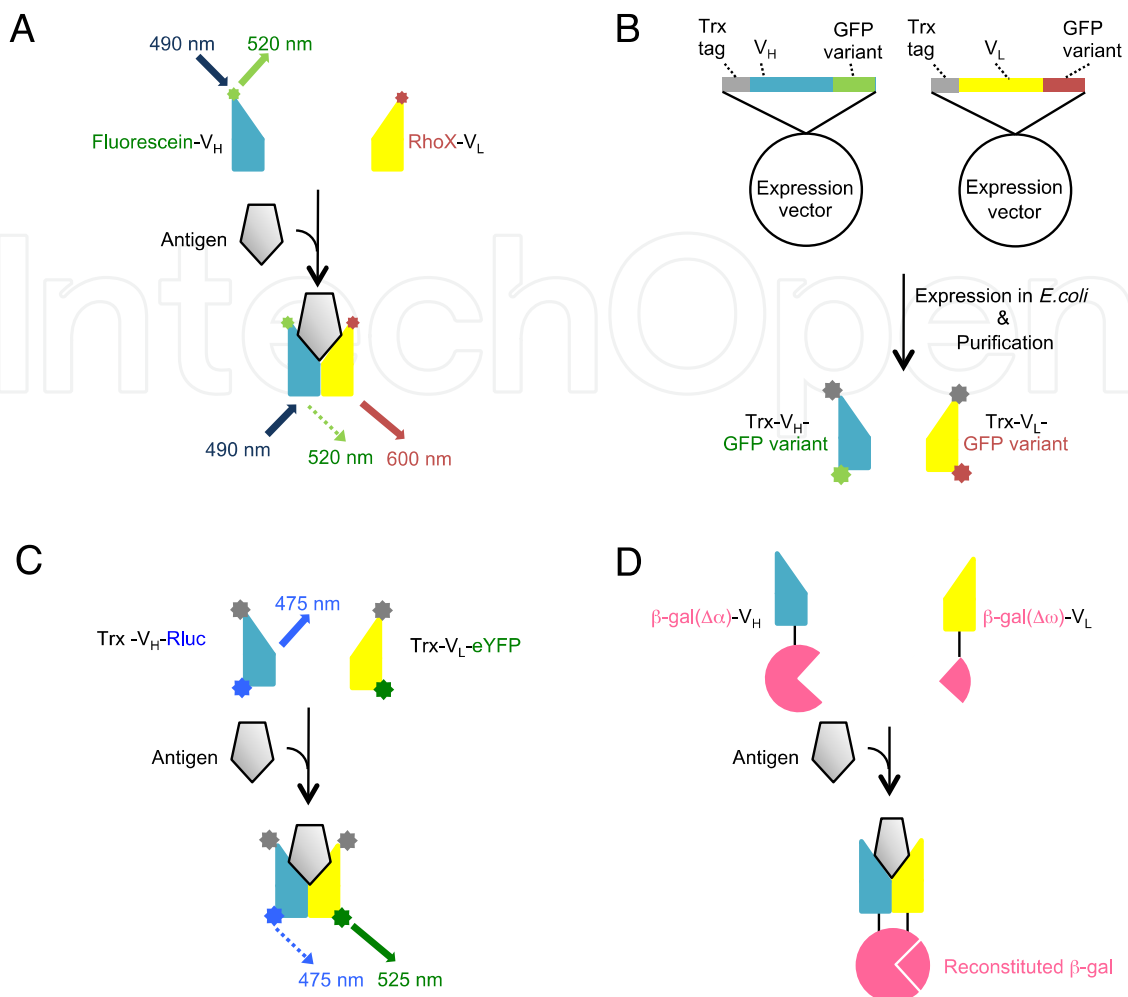


Fig. 3. Scheme of OS-IA applied to various homogeneous assays. (A) FRET-based OS-IA. Addition of antigen leads to decreased donor-derived (green) emission as well as increased acceptor-derived (red) emission. (B) A procedure to obtain site-specifically labeled Fv for OS-FIA. GFP variants are used as a label for the FRET-based assay. (C) BRET-based OS-IA. (D) Enzymatic complementation-based OS-IA. The two complementing fragments of β -galactosidase was used as a reporter for V_H / V_L association.

Thirdly, to obtain higher sensitivity we utilized β -galactosidase (β -gal) complementation [Yokozeki, 2002; Ueda, 2003] (Fig. 3D). Because of backgrounds of FRET due to relatively high protein concentrations of labeled V_H and V_L compared with dissociation constant, K_d of V_H/V_L interaction, we decided to employ β -gal complementation to reduce the amounts of proteins. A protein-protein interaction assay *in vivo* had been developed using enzymatic complementation between the two deleted mutants of β -gal fused to the respective interacting proteins. In our assay, an enzymatic complementation of β -gal was monitored between the antigen dependent interaction of V_H tethered to an N-terminal deletion mutant ($\Delta\alpha$) of β -gal and V_L tethered to another deletion mutant ($\Delta\omega$) of β -gal. Without antigen, the almost deleted mutants of β -gal are detached from each other due to monomeric Fv, resulting in low enzymatic activity. The addition of antigen induced stabilization of the ternary complex, thus the mutants came close and reconstructed its enzyme activity. With the use of HyHEL10, the measurable concentration range was relatively broad from 0.1

ng/ml to more than 10 $\mu\text{g/ml}$. The lowest measurable concentration was almost 1000-fold less than those of OS-FIA and BRET based OS-IA, while an incubation for 40 minutes are needed to enzymatically amplify the signal in contrast to the cases of FRET and BRET, where the emitted light is readily measured.

Here we showed four examples of homogeneous OS-IA. Users are advised choose an appropriate format to consider both of their merits and demerits. For instance, the sensitivity of OS-IA based on enzyme complementation is much higher than that of OS-FIA, however, an enzyme complementation is generally irreversible and require rather long incubation time. OS-FIA needs the unconventional device in biological laboratories, while it has several merits; a high sensitivity and a facile standardization of signals. On the other hand, OS-FIA and OS-IA based on BRET are applicable for real-time imaging probes in live cells.

2.3 Split-Fv system

To rapidly evaluate and select antibody variable region (Fv) fragments that are suitable to OS-IA, we devised two phage-based systems. The first one is "split-Fv system". Phage display is a powerful method for screening functional antibody fragments retaining a high affinity to the antigen [Winter, 1994; Gao, 1999]. Although the method needs some technical training and some disadvantages compared with conventional monoclonal antibody technology exist, such as inability to display all the antibody fragments cloned from hybridoma cells for several reasons, it still has a power especially the use of recombinant antibody is essential. For the screening of the antigen binding ability of V_H/V_L by phage display, a simultaneous display of both fragments in close proximity on the same phage is necessary. On the other hand, to perform OS-ELISA, that measures V_H/V_L interaction rather than the antigen-antibody interaction, separate expression of V_H/V_L is necessary; for example, phage display of a V_H fragment and production of a soluble V_L fragment is desired.

To enable the facile switch of these two display formats, we adopted a filamentous phage p7-p9 display system to individually display formats V_H and V_L fragments as a functional Fv on the tip of the phage (Fig. 4A).

However, we put a modification into the reported system that an amber codon was placed between V_L and gene 7, which makes to enable/disable display of V_L (tethered with His-myc tag) by changing the *sup* phenotype of host *E. coli*. When *sup*⁺ strains are used for phage production, V_L -(His-myc)-p7 is expressed, resulting in displaying Fv on the phage. When *sup*⁻ strains are used, soluble V_L -(His-myc) along with a V_H -displaying phage is expressed. Thus, OS-IA can be performed in a plate in which V_L is immobilized by anti myc antibody or V_L -specific ligand protein L coated on the plate. Thorough the two-step selection the first selection of highest affinity binders to antigen give a higher possibility of spotting most suitable candidates to OS-IA [Aburatani, 2003].

While the split-Fv system was successfully used to select and clone many Fvs that are suitable for OS-IA, some other Fvs did not show positive antigen binding, or the level of secreted V_L fragment was too low to perform OS-IA, possibly due to limited stability of the isolated V_L domain. Compared with the scFv fragment that is known to have a high tendency to form multimers, the antibody Fab fragment is reported to stay monomeric, allowing selection for affinity in contrast to selection for avidity. Recently, we devised a Fab display system that can perform OS-IA [Dong, 2009] (Fig. 4B).

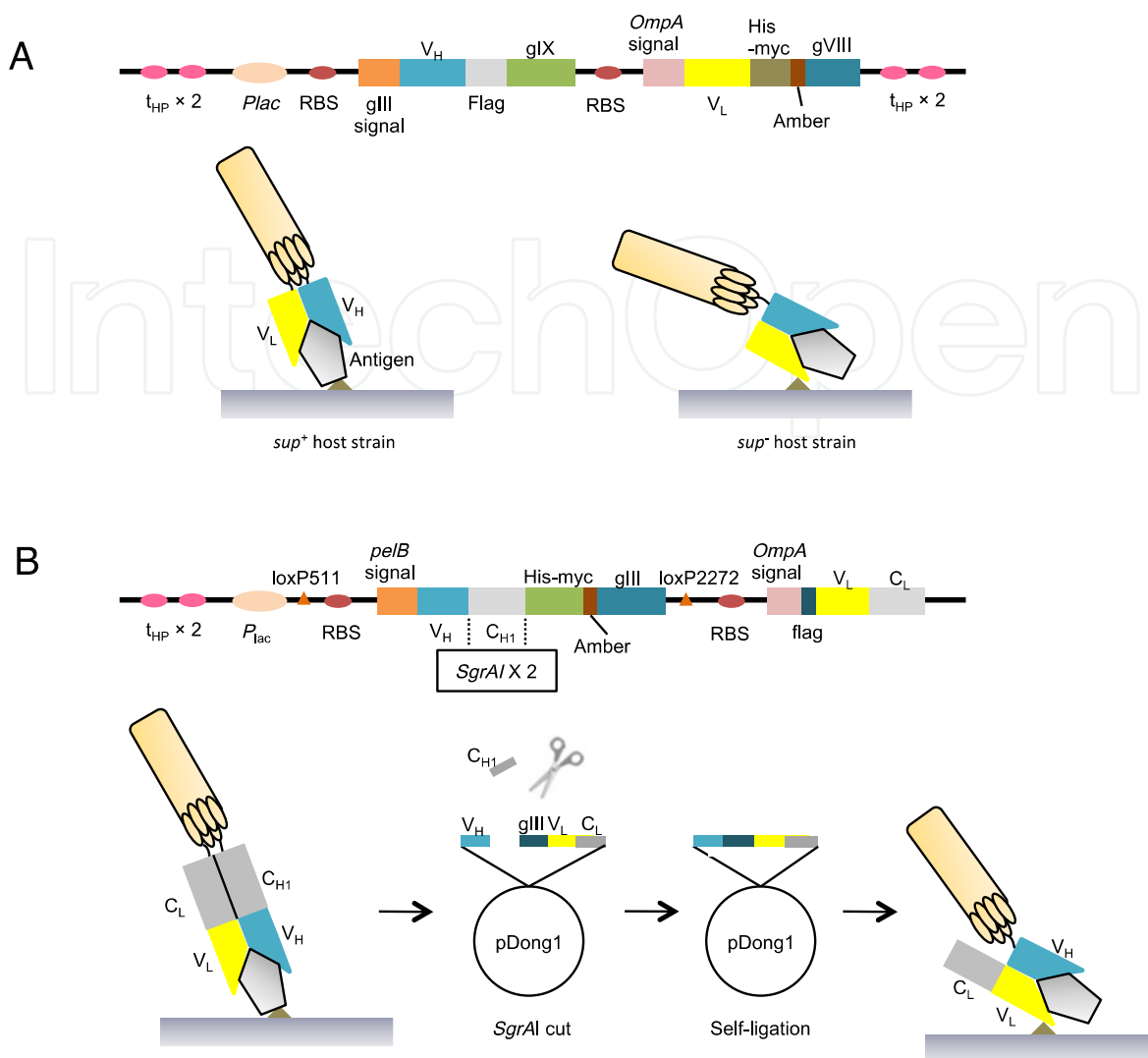


Fig. 4. Phage display systems to select antibodies for OS-IA. (A) Split Fv system. An amber codon was placed between V_L and gene 7, which makes to enable/disable display of V_L (tethered with His-myc tag) by changing the *sup* phenotype of host *E. coli*. When *sup+* strains are used for phage production, V_L -(His-myc)-p7 is expressed, resulting in displaying Fv on the phage. When *sup-* strains are used, soluble V_L -(His-myc) along with a V_H -displaying phage is expressed. Thus, OS-IA can be performed in a plate in which V_L is immobilized by anti-myc antibody or V_L -specific ligand such as protein L coated on the plate. (B) pDong1 system. Human IgG1 C_{H1} and C_k domains were included in pDong1 to regard selecting human antibody using the system. An amber codon was placed between Fd gene and gene III, therefore, Fab fragment is displayed on the surface of phage using *sup+* host *E. coli* to check the affinity of Fab to antigen. On the other hand, two restriction sites for *SgrAI* are incorporated at the both ends of C_{H1} gene to convert antigen-selected vectors for Fab display (Fab vector) to the vector for V_H display with simultaneous secretion of L chain (OS vector). After digestion with *SgrAI* and self-ligation, the culture supernatant of resultant phage-containing culture contains secreted L chain fused to flag tag and V_H -displaying phage. Thus, V_H -displaying phage is detected with enzyme-labeled anti-phage antibody on a plate in which L chain is immobilized by anti-flag or anti-L chain antibody coated on the plate to perform OS-IA.

A phagemid vector named pDong1 is shown in Fig 4B. pDong1 was designed to display Fab on a minor protein pIII of M13 phage. The genes for human IgG₁ C_H1 and C_k domains were included in pDong1 to regard selecting human antibody using the system. An amber codon was placed between Fd gene and gene III, therefore, Fab fragment is displayed on the surface of phage using sup⁺ host *E. coli* to check the Fab affinity. On the other hand, a rare cutting restriction site, *SgrAI* is incorporated at both end of C_H1 gene to convert antigen-selected vectors for Fab display (Fab vector) to the vector for V_H display with simultaneous secretion of L chain (OS vector). After cutting by *SgrAI* the culture supernatant contains secreted L chain fused to flag tags and V_H-displaying phage. Thus, V_H-displaying phage is detected using HRP-labeled anti-phage antibody on a plate in which V_L is immobilized by anti flag or His or myc antibody or V_L-specific ligand protein L coated on the plate to perform OS-IA. In addition, pDong1 is encoding one each of two recombination sites, LoxP2272 and LoxP511, which were placed at the upstream and the downstream of Fd-FIII ORF, respectively, that enable increased diversity of phage library due to Fd/L chain exchange. It is useful to make a library with Cre recombinase.

These systems are very powerful to select suitable antibody fragments for OS-IA from natural and engineered libraries and allow detailed analysis on the molecular bases of variable V_H/V_L interaction strength and its antigen dependency [Masuda, 2006; Sasajima, 2006; Lim, 2007; Suzuki, 2007; Islam, 2011]

3. Application of anti-phosphotyrosine antibody to open-sandwich immunoassay

A most important and unique character of OS-IA is that it can noncompetitively detect small molecules including organic chemicals. Phosphate addition to a target amino acid affects very small change in molecular weight. As a way to rapidly assay protein phosphorylation events, here we show the use of OS-IA [Sasajima, 2006].

Phosphorylation of tyrosine is known to be very important in intracellular signal transduction events [Ullrich, 1990], and a number of good polyclonal [Ross, 1981] and monoclonal anti phosphotyrosine (PY) antibodies are reported [Frackelton, 1983; Glenney, 1988]. We decided to synthesize PY20 Fv gene based on the published amino acid sequence [Ruff-Jamison, 1991; Ruff-Jamison, 1993a], because PY20 Fv was reported to bind PY with high affinity ($K_d = 1.55 \times 10^{-7}$ M) [Ruff-Jamison, 1993b].

For OS-IA to detect phosphotyrosine we use the split Fv system. When the culture supernatant containing V_H-displaying phage and soluble V_L fragment was applied to the microplate wells immobilized with antigen conjugate (PY-BSA) for phage ELISA, a strong and specific binding of the phage to PY-BSA wells and a clear competitive inhibition by added PY during incubation were observed. The PY concentration that gave half-maximal inhibition was 10 µg/ml, indicating sufficient affinity (Fig. 5A). The V_H/V_L interaction strength and its PY-dependency of PY20 Fv were investigated using the same culture supernatant and microplate wells immobilized with Penta-His antibody. As a result, a PY-dependent increase with the maximum response of 30 % increment was observed (Fig. 5C). However, we reasoned the increase might not be sufficient when the assay is taken to FRET-based homogeneous format, where increased protein concentration and reduced dynamic range due to spectral overlap of the two fluorophores can limit its sensitivity. So we then improved the response of OS-ELISA by site-directed mutagenesis approach.

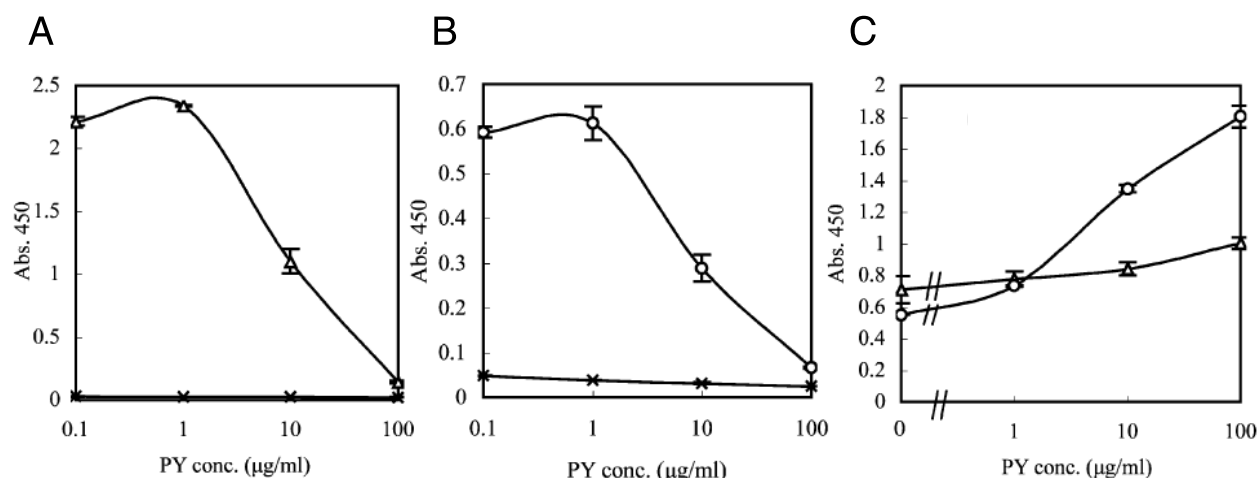


Fig. 5. Detection of phosphotyrosine with PY20. (A) Competitive split Fv-phage ELISA. Culture supernatant was mixed with twice the concentration of PY, and the bindings to immobilized PY-BSA (Δ) or BSA (\times) were evaluated. (B) Competitive split Fv-phage ELISA with $V_H(Q39R)$ mutant. (C) OS phage ELISA with the wild-type (Δ) and the mutant (\circ) culture supernatant.

We knew that a mutation in a V_H residue facing V_L interface (H39) can effectively modulate V_H/V_L interaction strength without affecting antigen-binding affinity [Masuda, 2006]. Since 39_H of PY20 is glutamine, probably making two hydrogen bonds with a corresponding V_L residue Gln(38_L), we introduced random mutation to this residue in order to get mutant(s) with lower V_H/V_L interaction strength in the absence of PY. After screening phage clones, a mutant showing higher response $V_H(Q39R)$ was obtained. When the antigen binding activity of the mutant Fv and also its competition by PY were investigated by phage ELISA, significant binding to PY-BSA and its inhibition by PY similar to wild-type PY20 were observed (Fig. 5B). Similarly, when OS-IA was performed for this mutant, significantly higher PY-dependent signal increase of 200% with reduced background signal corresponding to V_H/V_L interaction was obtained. Surprisingly, the signal in the presence of PY was almost twice that of wild-type PY20, and the resulting sensitivity was higher than that with competitive assay (Fig. 5C).

To conduct FRET-based homogeneous assay, the gene for mutant V_H or the V_L was fused to eCFP or eYFP, respectively (Fig. 6A). V_H -eCFP was excited at 433 nm and the fluorescent spectra at 500-650 nm were recorded in the presence of several PY concentrations. The result shows a slight PY-concentration-dependent decrease in eCFP fluorescence around 475 nm and a significant increase in eYFP fluorescence peaking around 525 nm, resulting in increased eYFP/eCFP fluorescence ratio up to ~30%. The result clearly showed that we could successfully detect PY in a homogeneous solution in a noncompetitive manner.

Next, when pp60 peptide encoding a physiologically tyrosine-phosphorylated protein c-src residue 521-533 containing pY527 was added, a clear increment in FRET was detected (Fig. 6B). Prior dephosphorylation of the peptide by calf intestinal alkaline phosphatase resulted in complete reversal of the spectrum compared to that of the control.

Because of its simple fluorescence ratiometric detection, this OS-FIA will be useful for diagnostics and facile *in situ* visualization of intracellular tyrosine phosphorylation, which includes Alzheimer, malignantly growing cells and immune abnormal cells and so on. In near future, in combination with an appropriate method will be proven to be a powerful

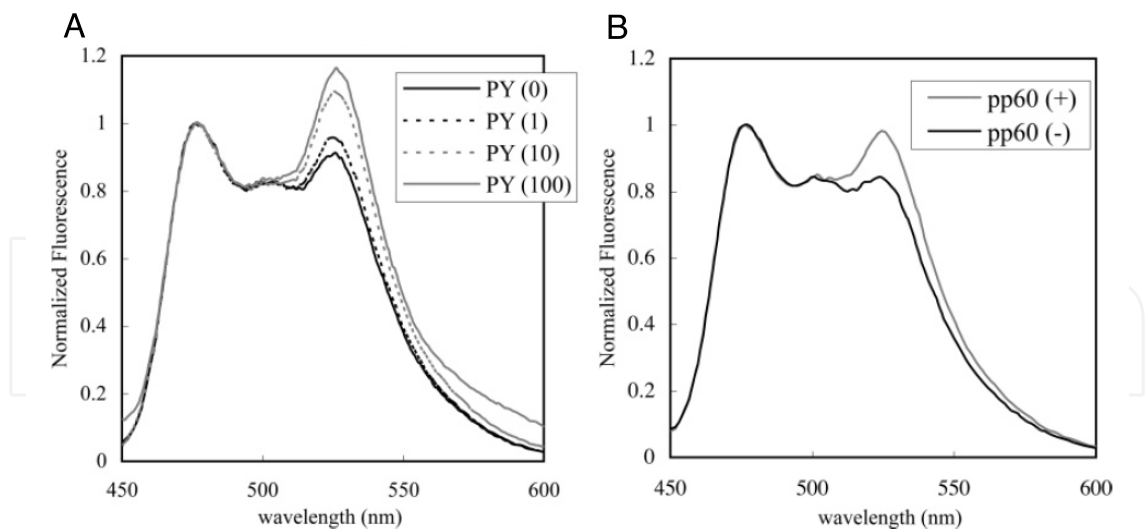


Fig. 6. FRET-based OS-IA for PY and peptide tyrosine phosphorylation (A) OS-FIA for PY. Fluorescence spectra of the probe (500 ng/ml) in the presence of 1 mg/ml BSA and indicated amounts of PY (in $\mu\text{g/ml}$). (B) OS-FIA with pp60 c-src peptide. The same spectra with (A) in the presence (gray line) or absence (black line) of the peptide. Phosphatase treatment of the peptide restored the signal (not shown).

tool to find indicators of several diseases in clinical specimens and to monitor intracellular imaging of protein tyrosine phosphorylation, as in the cases of other useful probes to monitor intracellular calcium concentration [Chung, 2009; Horikawa, 2010].

4. Application of vimentin phospho-specific antibody to open-sandwich immunoassay

4.1 Phosphorylation of intermediate filaments

Next, we used an antibody that recognizes site-specifically phosphorylated vimentin to detect more specific protein phosphorylation. The cytoskeletons are composed of three major groups distinguished by their diameter; microfilaments with 6 nm diameter, microtubules with 24 nm diameter, and intermediate filaments with its diameter between that of microfilaments and that of microtubules (10 nm). Vimentin is one of commonly observed intermediate filament. In 1980's, the mechanism of intermediate filament disassembly was considered to be its degradation by proteases, since intermediate filament has a stable structure with insolubility and chemical unreactivity. Furthermore, intermediate filaments are thought to play only a role to maintain cytoplasmic organization [Ishikawa, 1968; Lazarides, 1980b; Lazarides, 1980a; Lazarides, 1982]. On the other hand, it is known that vimentin exists in a phosphorylation form during mitosis [Celis, 1983; Bennett, 1988; Chou, 1990; Liao, 1997]. In 1987, it was shown that phosphorylation induces disassembly of the filaments *in vitro* [Inagaki, 1987; Inagaki, 1988; Inagaki, 1989; Inagaki, 1990]. The discovery of conformational dynamics of intermediate filaments leads to reconsider the roles in cell function. Recently, phosphorylation-dependent assembly/disassembly of intermediate filaments has been reported to be associated with cell cycle, cell migration and several diseases, while the entire roles are still obscure [Chou, 1990; Chou, 1991; Inagaki, 1994; Tsujimura, 1994a; Tsujimura, 1994b; Goto, 2000; Yasui, 2001; Eriksson, 2004; Yamaguchi, 2005; Izawa, 2006; Helfand, 2011].

Vimentin contains three substructures; head, rod, and tail domains. The head domain is phosphorylated by several kinases, namely, protein kinase A, protein kinase C, CaMKII, PAK, CDK1, Rho kinase, Aurora B, and Plk1. In regard to the phosphorylation sites, phosphorylation of Ser38, Ser55, Ser71, Ser72, Ser82 are necessary to divide normally into two daughter cells in M stage. When a mutant vimentin in which both Ser71 and Ser 72 are changed to Ala is expressed in T24 cells that do not express endogenous vimentin, incomplete cytokinesis is observed (ref.). We decided employ a rat monoclonal antibody TM71 that specifically binds phosphorylated Ser71 of human vimentin. While Ser71 is not phosphorylated during metaphase, the phosphorylation is observed at cleavage furrow, which is a distinguishable narrow part between the two dividing cells from anaphase to telophase.

4.2 OS-IA using TM71

First, antigen-binding affinity as well as specificity of recombinant TM71 Fab was examined. The cDNAs of TM71 V_H/V_L were cloned into pDong1 phagemid, and the Fab fragment was displayed on phages (Fig. 7A). The wells on a microplate were immobilized with BSA conjugated with phosphorylated or unphosphorylated antigen peptides containing native sequence around Ser71. As a result, specific binding of Fab-displaying phage to phosphorylated antigen was observed on the plate. Next, the V_H/V_L interaction strength and its dependency to phosphorylated antigen using the V_H -displaying phage and the secreted L chain in the culture supernatant (Fig. 7B). The V_H/V_L interaction was increased depending on the concentration of phosphorylated peptide, while it was not the case with unphosphorylated peptide. The detection limit was as low as 0.1 ng/ml.

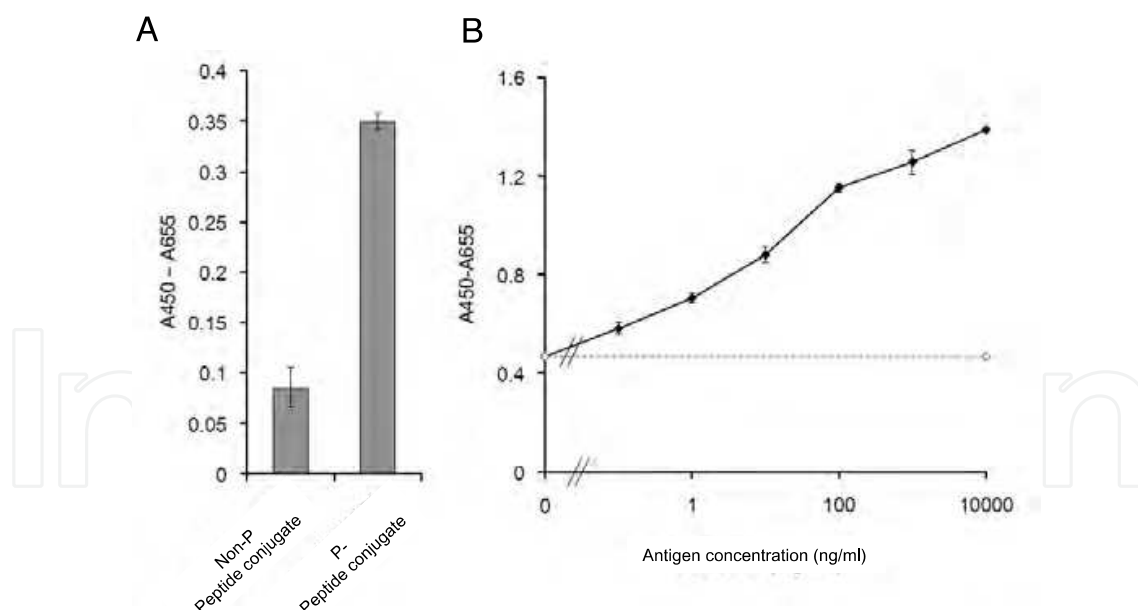


Fig. 7. Conventional phage ELISA and open-sandwich phage ELISA with anti-vimentin P-Ser71 (TM71). (A) Phage ELISA to detect immobilized antigens. (B) Open-sandwich phage ELISA. A solid line indicates the signal in the presence of phosphorylated antigen peptide. A dotted line indicates in the presence of non-phosphorylated peptide.

As described above, when H39 is Gln, the residue probably makes two hydrogen bonds with a corresponding V_L residue L38 (Gln), resulting a strong interaction between V_H and V_L

independent on antigen binding. Since H39 of TM71 is Gln, we randomized the DNA sequence for this residue, and obtained Q39R and Q39D mutants. When OS-IA was performed using these mutants, interaction strengths between V_H and V_L (backgrounds) were significantly decreased, resulting in higher signal/background ratios than that using wild type V_H (Fig. 7B).

Then we applied OS-IA to FRET using Q39R mutant V_H labeled with Rhodamine-X and V_L labeled with Alexa488. The result of the OS-FIA shows a slight phosphorylated antigen-concentration-dependent decrease in Alexa488 fluorescence and a significant increase in Rhodamine-X fluorescence, resulting in increased rhodamine-X/Alexa488 fluorescence ratio up to ~24 %. However, the increased FRET was not observed with unphosphorylated antigen. The result clearly showed that phosphorylated antigen specific detection was performed in a homogeneous solution in a noncompetitive manner.

An immunofluorescence staining of cells using TM71 shows Ser71 is phosphorylated only at cleavage furrow from anaphase to telophase (Fig. 8A). Finally, we try to detect endogenous phosphorylation in live cells. Human glioblastoma U251 cells were electroporated with both $V_H(39R)$ labeled with Rhodamine-X and V_L labeled with Alexa488 by NEON™ Transfection System (Invitrogen, Carlsbad, CA). As a result, the fluorescence of the probe was observed at cleavage furrow, suggesting endogenous phosphorylated antigens were recognized between the V_H/V_L interface (Fig. 8B).

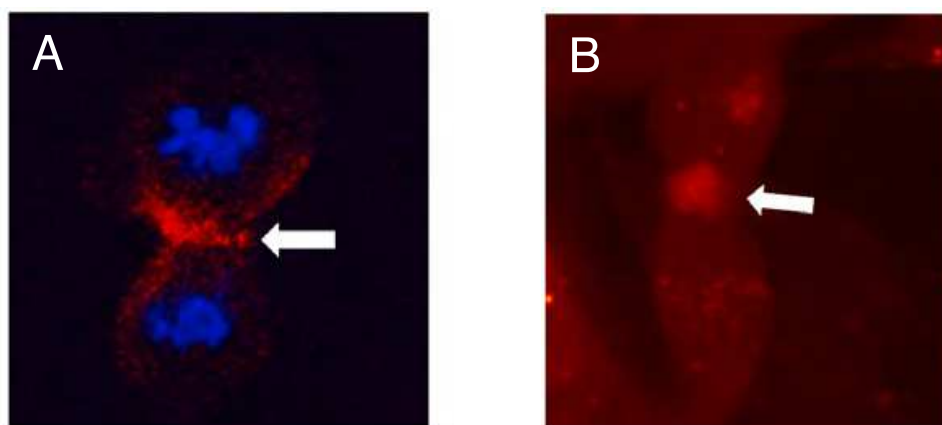


Fig. 8. Recognition of endogenous phosphorylated antigen using the probes based on OS-IA. (A) Immunofluorescence staining of U251 cells with TM71. A cleavage furrow is indicated by an arrow, where the signal from TM71 is observed (red). Nuclei are stained with DAPI (blue). (B) Recognition of endogenous phosphorylated antigen. The mixture of V_H labeled with rhodamine-X and V_L labeled with Alexa488 was electroporated into live U251 cells. Signal of rhodamine-X (red) was observed at a cleavage furrow (arrow).

5. Conclusion

In this chapter, we introduced OS-IA and its application in phosphoproteomics. First, we succeed in detecting general protein tyrosine phosphorylation by FRET approach. Second, the method was extended to site-specific detection of specific protein phosphorylation. Furthermore, it allowed observation of endogenous phosphorylation in a single live cell. This method using OS-IA has the following merits. 1) Once the two appropriate antibody fragments, V_H and V_L are obtained in recombinant forms, many detection methods of

protein-protein interaction, from heterogeneous assays such as ELISA to homogeneous assays such as FRET and PCA could be applied. In this sense, the application of OS-IA will be further expanded in future, including sensitive electrochemical detection [Sakata, 2009] and genetic reporters that allow high throughput screening on yeast [Gion, 2009]. Since most V_H fragments are known to be antigen-specific, future development of multiplexing with multiple reporters will be also possible. 2) Antigen specificity and binding affinity are adjustable by protein engineering with the help of powerful phage display systems. Especially, too strong V_H/V_L interaction in the absence of antigen can be effectively weakened to get larger signal in many OS-IAs. 3) Molecular weight of each antibody fragment is less than 13 kDa, which is small enough to allow fast diffusion in the cell and passing through the nuclear pores.

We are confident that this novel method will be exploited as a live cell imaging probe for phosphorylation and homogeneous assays for clinical diagnosis and drug screening.

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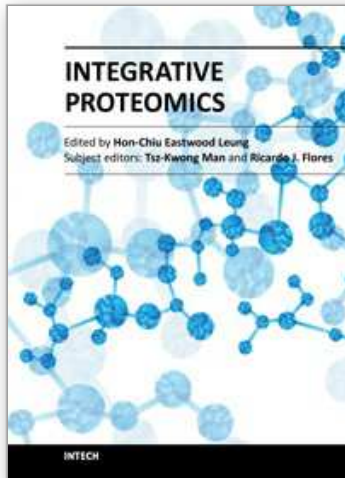
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