Chapter 8

Resonant Waveguide Grating for Monitoring Biomolecular Interactions

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Abstract

Label-free detection technologies have been widely used to characterize biomolecular interactions without having to label the target molecules. These technologies exhibit considerable potential in facilitating assay development and enabling new integrated readouts. When combined with high-throughput capability, label-free detection may be applied to small molecule screens for drug candidates. Based on the resonant waveguide grating biosensors, a label-free high-throughput detection system, the Epic® System, has been applied to monitor molecular interactions. Here we describe a generic label-free assay to quantitatively measure phospho-specific interactions between a trafficking signal—phosphorylated SWTY peptide and 14-3-3 proteins or anti-phosphopeptide antibodies. Compared with the solution-based fluorescence anisotropy assay, our results support that the high-throughput resonant waveguide grating biosensor system has shown the capability not only for high-throughput characterization of binding rank and affinity but also for the exploration of potential interacting kinases for the substrates. Hence, it provides a new generic HTS platform for phospho-detection.

Key words Resonant waveguide grating, Label-free, Epic, High throughput, Phospho-specific interactions, SWTY peptide, 14-3-3 proteins, Kinase

1 Introduction

Label-free detection technologies have been widely used to characterize biomolecular interactions without having to label the target molecules [1, 2]. Different transduction techniques such as surface plasmon resonance (SPR), optical ellipsometry, quartz crystal microbalance, Raman scattering, and calorimetry have been developed. In spite of their diverse applications, most of their compatibility with high-throughput screening remains a challenge, although recent developments, i.e., SPR imaging [3], or incidence-angle dependence of optical reflectivity difference imaging [4], have shown potential for increasing the throughput. Resonant waveguide grating (RWG) sensors have been developed into a high-throughput microplate-based biosensor system, the Epic® System, combining the features of

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label-free detection with high-throughput capability [5]. Among the many applications label-free optical biosensors can be used for, the Epic system offers the possibility of developing a generic assay for evaluating protein-protein interactions specific to phosphorylated target sequences.

1.1 Assay Principle of Biosensors

The Epic® system is based on a RWG biosensor, which exploits the evanescent wave generated by the resonant coupling of light into a waveguide via a diffraction grating. The guided light can be viewed as one or more mode(s) of light that all have directions of propagation parallel with the waveguide, due to the confinement by total internal reflection at the substrate–film and medium–film interfaces. The waveguide has a higher refractive index value than its surrounding medium. Because the guided light mode has a transversal amplitude profile that covers all layers, the effective refractive index N of each mode is a weighted sum of the refractive indices of all layers:

$$N = f_N(n_F, n_S, n_C, n_{ad}, d_F, d_{ad}, \lambda, m, \sigma) \tag{1}$$

Here, n_F, n_S, n_C, and n_{ad} is a refractive index of the waveguide, the substrate, the cover medium, and the protein adlayer, respectively. d_F and d_{ad} are the effective thicknesses of the film and the protein adlayer, respectively. λ is the vacuum wavelength of the light used. $m = 0, 1, 2,$ is the mode number; and σ is the mode type number, which equals 1 for TE (transverse electric or s-polarized) and 0 for TM modes (transverse magnetic or p-polarized). Because of its higher sensitivity, generally the TM ($\sigma = 0$) is used for measuring the binding of biomolecules to the probe proteins immobilized on the surface of the waveguide substrate.

When a laser illuminates the waveguide at varying angles or wavelengths, light is coupled into the waveguide only at corresponding specific angles or wavelengths. This coupling is determined by the effective refractive index of the guided mode, denoted as N. The value of N can be calculated numerically from the mode equation for a given mode of a four-layer waveguide configuration:

$$\begin{aligned} 0 \cong \pi m - k\left(n_F^2 - N^2\right)^{0.5} \left(d_F + d_A \frac{n_A^2 - n_C^2}{n_F^2 - n_C^2} \left[\frac{(N/n_C)^2 + (N/n_A)^2 - 1}{(N/n_C)^2 + (N/n_F)^2 - 1} \right]^{\sigma} \right) \\ + \arctan\left[\left(\frac{n_F}{n_S} \right)^2 \left(\frac{N^2 - n_S^2}{n_F^2 - N^2} \right)^{0.5} \right] \\ + \arctan\left[\left(\frac{n_F}{n_C} \right)^2 \left(\frac{N^2 - n_C^2}{n_F^2 - N^2} \right)^{0.5} \right] \end{aligned} \tag{2}$$

Here, $k = 2\pi/\lambda$.

Since the laser light is coupled to, and propagates parallel to the surface in the plane of a waveguide film, this creates an

electromagnetic field (i.e., an evanescent wave) in the liquid adjacent to the interface. The amplitude (E_m) of the evanescent wave decays exponentially with increasing distance d from the interface:

$$E_m(d) = E_m(0)\exp\left(\frac{-d}{\Delta Z_C}\right) \quad (3)$$

with:

$$\Delta Z_C = \frac{1-\sigma}{k\left(N^2 - n_C^2\right)^{0.5}} + \frac{\sigma\left[(N/n_F)^2 + (N/n_C)^2 - 1\right]^{-1}}{k\left(N^2 - n_C^2\right)^{0.5}} \quad (4)$$

ΔZ_C is the penetration depth (also termed as sensing volume; typically ~150 nm) of the evanescent tail of the waveguide mode that extends into the cover medium. This means that a target or complex of a certain mass contributes more to the overall response when the target or complex is closer to the sensor surface, as compared to when it is further from the sensor surface.

1.2 Resonant Waveguide Grating Biosensor Detection

A beta version of the Corning® Epic® System is comprised of three major components for bioassay applications: an Epic® sensor microplate, an RWG detector, and a liquid handling system. The sensor microplate consists of a glass bottom plate attached to a holey plastic 384-well plate, which enables high-throughput screening. Each well in the 384-well Epic® microplate contains an RWG sensor, which consists of an optical grating and a high index of refraction waveguide coating [6, 7]. When illuminated with broadband light at a fixed angle of incidence, these sensors reflect only a narrow band of wavelengths that is a sensitive function of the effective index of refraction of the waveguide. The sensors are coated with a surface chemistry layer that enables covalent attachment (via peptide bond formation) of peptides/proteins or other biomolecules. Binding of molecular recognition partners to the immobilized target induces a change in the effective index of refraction of the waveguide, and this is manifest as a shift in the wavelength of light that is reflected from the sensor. The magnitude of this wavelength shift is proportional to the amount of analyte that binds to the immobilized target. Unlike most commercial SPR biosensors, which generally use a continuous flow system for the determination of the kinetics of binding, the Epic® System does not utilize flow channels. RWG sensors are evanescent in nature which means that the magnitude of the electric field in the medium adjacent to the sensor surface decays exponentially from the sensor surface. The distance from the sensor surface at which the electric field strength has decreased to $1/e$ of its initial value is the penetration depth. For the Epic® System, the penetration depth is ~150 nm. Thus, the system is selective and sensitive to binding events that take place within this penetration. For most of the

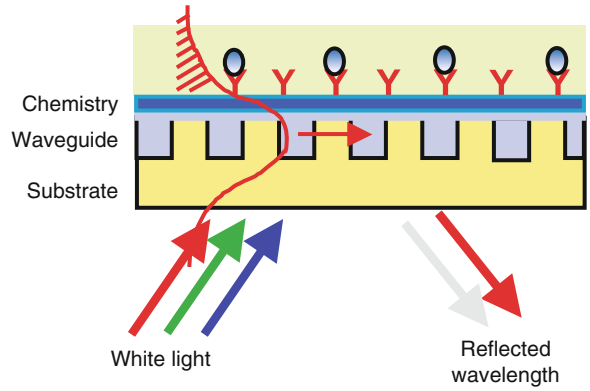

Fig. 1 Detection scheme of RWG biosensor for detecting the binding of biomolecules. The detection scheme of RWG biosensor for detecting the binding of target molecules (●) in a sample to the probe molecules (Y) immobilized onto the surface of a waveguide substrate. The specific binding event is manifested the shift in the wavelength of the reflected light. The waveguide substrate consists of a region within which a grating structure is embedded. The probe molecules are coupled to the derivatized waveguide substrate. From: Wu, M Long S, Frutos AG, Eichelberger M, Li M, Fang Y., *Journal of Receptors and Signal Transduction*, 2009; 29 (3, 4): 202–210, copyright © 2009, Informa Healthcare. Reproduced with permission from Informa

experiments described in this chapter, a well adjacent to the sample well is used as a reference. The most recent version of the Epic® microplate utilizes a self-referencing scheme in which each well of the plate has its own reference region. This is enabled by a plate design that provides protein binding chemistry on only half of the sensor surface so that when a solution of peptide/protein target is added to the well, it only binds to half of the sensor surface, leaving the other half as an in-well or self-reference.

Figure 1 is a schematic drawing for detecting the binding of biomolecules in a sample to the probe molecules immobilized onto the surface of the waveguide substrate. In a particular assay, the probe molecules are pre-coupled to the surface, primarily through covalent-coupling or bio-specific interaction (e.g., biotin–avidin interaction). Alternatively, the immobilization of probe molecules can be monitored in real-time to ensure the efficiency and quality of the coupling. Nonetheless, after the immobilization of probe molecules, the binding of target molecules, in the absence and presence of a modulator, can be directly monitored by the label-free RWG biosensor, as manifested by the shift in wavelengths or angles of the reflected light.

1.3 RWG Biosensor Detection of Phosphorylated Protein-Protein Interactions

Phosphorylation is a key posttranslational process that confers diverse regulation in biological systems involving specific protein-protein interactions recognizing the phosphorylated motifs. Of great interest for ion channels, which represent an important, but underdeveloped class of drug targets, is the surface expression of these membrane proteins modulated by the

posttranslational phosphorylation. A common effect of phosphorylation is a change in protein-protein interactions. 14-3-3 proteins were the first protein modules to be identified as binding specifically to phosphorylated substrates. Evidence from structural studies and sequence analyses indicates that the primary function of 14-3-3 proteins lies in their preferential binding to phosphorylated substrates, through their antiparallel bivalent binding sites. In addition to known canonical binding motifs, several earlier reports have identified interactions between 14-3-3 proteins and the C termini of target proteins. This characteristic binding (i.e., SWpTY motif) has high binding affinity that is comparable to that of the canonical binding motifs. Recently, studies have suggested that 14-3-3 proteins, through binding to inducible phosphorylated motifs, regulate protein expression on the cell surface.

Currently, multiple methods are available for detecting phosphorylation, including antibody-based fluorescence detection, radioactive-ATP, and fluorescent-labeled peptide substrates for FRET or fluorescence polarization detection. Although several choices are available for characterization of phosphorylation and potentially for the development of high-throughput screening assays, such label-based methods are prone to artifacts and other detrimental effects (i.e., label effect on antibody interactions). Here we present a protocol for using a high-throughput label-free optical biosensor system–Epic®—for the interrogation of an example phospho-specific interaction, 14-3-3 with SWpTY motif.

2 Materials

2.1 Reagents

1. Inorganic salts of analytical purity.
2. PEG-amine (*O*,*O*′-Bis(2-aminopropyl)polyethylene glycol 1900).
3. Ethanolamine.
4. Boric acid.
5. Dimethylsulfoxide (DMSO).
6. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS).
7. Octylphenolpoly (ethyleneglycolether) (NP40).
8. Trition X-100.
9. Dithiothreitol (DTT).
10. PBS buffer: a phosphate buffered saline solution with a phosphate buffer concentration of 0.01 M and a sodium chloride concentration of 0.154 M. The solution pH will be 7.4.
11. Anti-SWpTY antibody produced from rabbit.

12. Peroxidase labeled anti-Rabbit IgG (H + G, made in goat).
13. Anti-14-3-3 antibody (made in rabbit).
14. Supersignal ELISA Femto Maximum Sensitivity substrate kit.

All solutions were prepared in a 10 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7.3) unless indicated.

2.2 Preparation and Purification of Recombinant 14-3-3 Proteins

1. 14-3-3ζ protein expressed as a GST-tagged fusion and purified from *E. coli* strain BL21-SI as previously described [8].
2. Recombinant GST-tagged 14-3-3 proteins purified using Glutathione Sepharose 4B beads.

Fluorescence anisotropy measurements were used to determine the binding affinity of 14-3-3ζ for SWpTY [8].

2.3 Synthesis and Preparation of Peptides

1. NH2-SWpTY (NH2AhxAhxFRGRSWpTY-COOH, Ahx: 6-aminohexyl-) peptide and non-phosphorylated NH2-SWTY peptide synthesized by Biomer Technology.
2. SWpTY, RGRSWpTY-COOH; SWTY, RGRSWTY-COOH; SWpTD, RGRSWpTD-COOH; SWpTP, RGRSWpTP-COOH; and SWpTP, RGRSWpTP-COOH peptides from New England Peptides (or another commercial source).

The peptides were prepared as stock solutions by dissolving in water, and if necessary with the addition of a minimal amount of acetonitrile.

3 Methods

3.1 14-3-3 Detection Protocol on the Epic® System

As a common protocol for 14-3-3 detection [9] (Fig. 2), the first step is to immobilize NH2-SWpTY peptide (50 μg/ml, pH 7.5), as a 14-3-3 binding motif, on the Epic® plate. This was followed by the addition of ethanolamine to quench the remaining active sites on the surface. Reference wells were made by the addition of PEG-amine (50 μg/ml, pH 9.2) or the non-phosphorylated peptide NH2-SWTY (50 μg/ml, pH 7.5). The second step is the addition of 14-3-3 protein solutions into both the sample wells and the reference wells. As seen in Fig. 2c, the wavelength shift after the subtraction of the signals from the reference wells is termed the referenced signals for the 14-3-3 binding event. The signal without subtraction of the reference is termed the unreferenced signal. Usually six sample wells and two reference wells were used for each binding reaction unless otherwise described.

To evaluate the specificity of the interaction, 14-3-3 was added into wells modified with either NH2-SWpTY or non-phosphorylated NH2-SWTY (Fig. 3a). Only wells immobilized

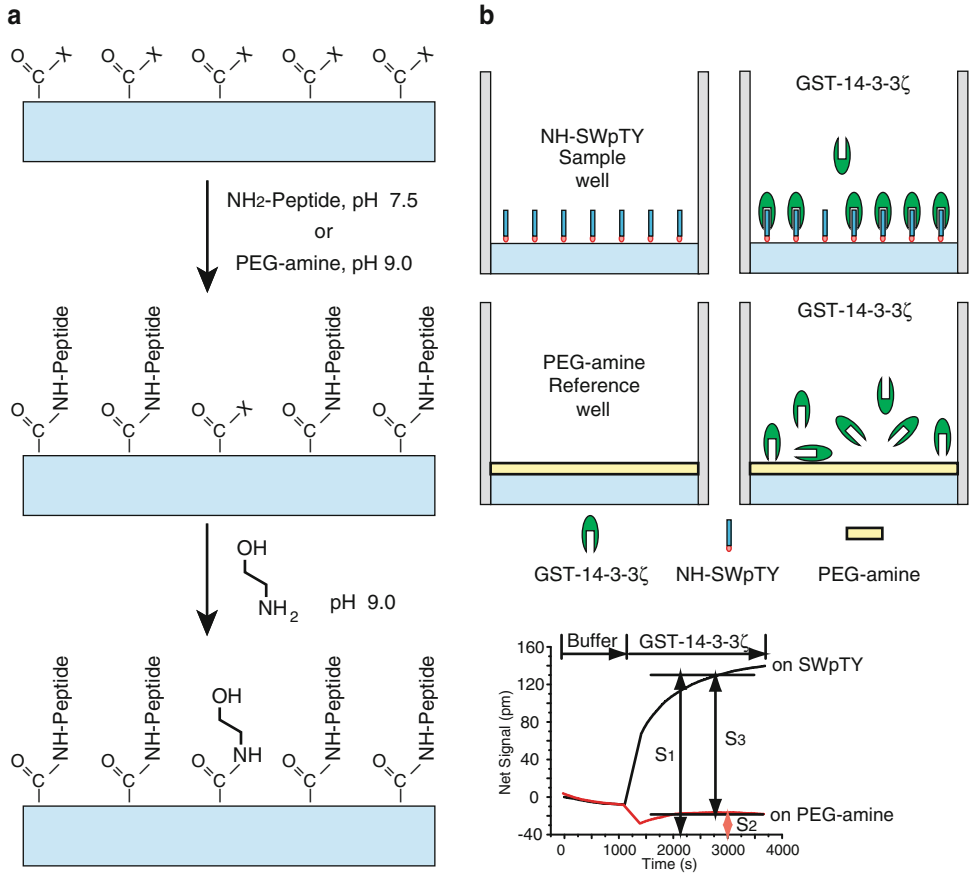

Fig. 2 Scheme of Epic® detection. (**a**) Surface reaction scheme for immobilization of N-terminal amino group modified peptides. X, reactive group; NH2-Peptide, N-terminal amino group modified peptides. (**b**) Binding scheme for 14-3-3. *Left two panels* show the immobilized substrate (NH2-SWpTY) and reference (PEG-amine). *Right two panels* show GST-14-3-3ζ binding. In the sample well, where GST-14-3-3ζ is added, GST-14-3-3ζ binds to the immobilized NH2-SWpTY peptide. In the reference well containing immobilized PEG-amine, there is no binding of GST-14-3-3ζ. (**c**) The time-course charts of the respective responses from sample wells and reference wells, with S1 as an unreferenced signal from GST-14-3-3ζ on NH2-SWpTY (50 μg/ml at pH 5.5. GST14-3-3 at 1 μM) S2 as an unreferenced signal from GST-14-3-3ζ on PEG-amine (50 μg/ml, pH 9.0 GST14-3-3 at 1 μM), and S3 as a referenced signal obtained by subtraction of S1 with S2. From: Wu, M Long S, Frutos AG, Eichelberger M, Li M, Fang Y., *Journal of Receptors and Signal Transduction*, 2009; 29 (3–4): 202–210,

with the phosphorylated peptide NH2-SWpTY gave detectable signals. Furthermore, upon addition of competitive 14-3-3 binding peptides, SWpTY or a nonhomologous 14-3-3 binding peptide R18, the binding signals were significantly reduced (Fig. 3b). In contrast, the non-phosphorylated SWTY peptide did not affect the

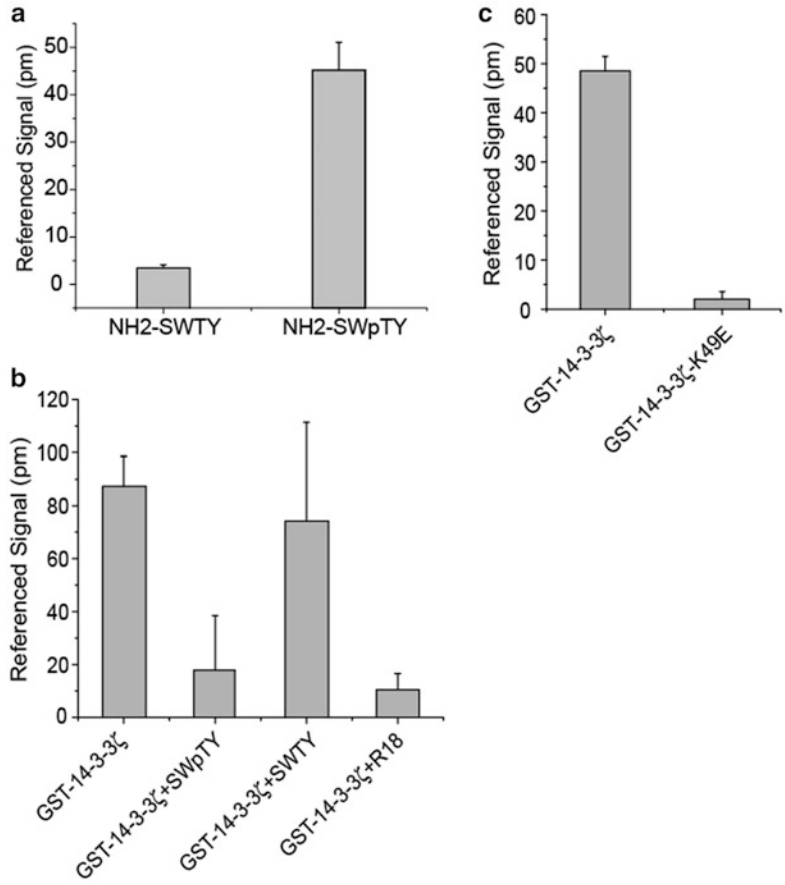

Fig. 3 Specificity of detection. (**a**) The referenced signals for the binding of 14-3-3 to immobilized SWpTY, and SWTY. SWpTY, and SWTY were immobilized at 50 μg/ml, pH 5.5, and GST-14-3-3ζ was added at 0.4 μM. (**b**) Competition assay with competitors and control. 1 μM of competitors (SWpTY and R18) and control peptide (SWTY) were pre-incubated with 0.4 μM GST-14-3-3ζ and applied on the immobilized NH2-SWpTY (50 μg/ml). (**c**) Comparison of the referenced signals from the GST-14-3-3ζ and its validated binding mutant GST-14-3-3ζ (K49E). GST-14-3-3 was used at 0.4 μM

binding of 14-3-3. To be more definitive, the SWpTY peptide binding was tested with wild type 14-3-3 and 14-3-3 K49E, which has a mutation at the binding site [14]. Consistently, the binding signal was obtained only in wild type 14-3-3 proteins (Fig. 3c). Therefore, these experimental results provide the evidence for the specific detection of 14-3-3 interactions with substrate peptides.

3.2 Validation of 14-3-3 Detection by In Situ ELISA

To further validate the 14-3-3 binding event on the Epic® plates, the same Epic® plates above were then subjected to an ELISA assay. After washing five times with PBS buffer, 50 μl of the anti-14-3-3 antibody (1:200 dilution) was added and incubated at 4 °C for 0.5 h. After washing with PBS buffer with 0.1 % Tween, 50 μl of

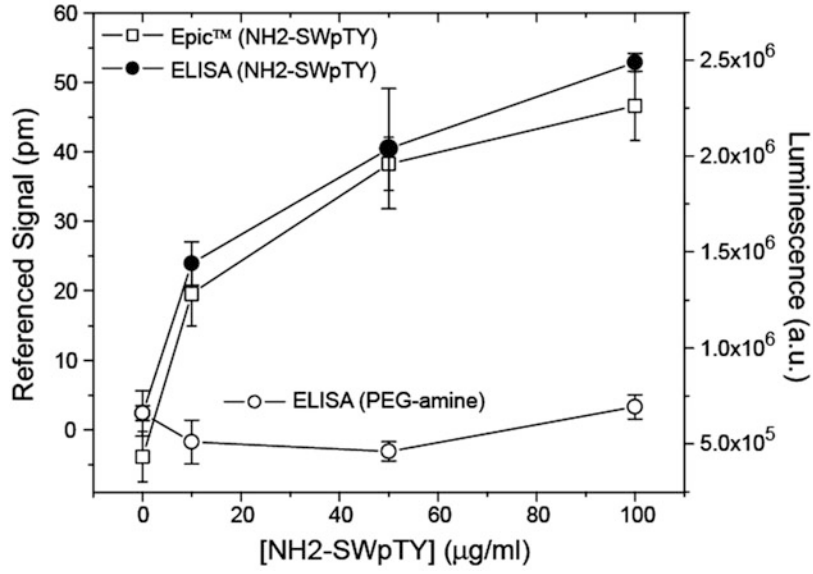

Fig. 4 Binding detection of 14-3-3 to immobilized NH2-SWpTY peptide. NH2-SWpTY in different concentrations (pH 5.5) was immobilized on the Epic™ plate. After addition of GST-14-3-3ζ (0.4 µM), the referenced signals were plotted on the left axis against the concentrations of NH2-SWpTY. The same plate was used for ELISA detection as described in Subheading 3 and the luminescence signals were plotted on the right axis

Peroxidase labeled Anti-Rabbit IgG (1:1,000 dilution) was added and incubated at 4 °C for 0.5 h. After washing with PBS buffer with 0.1 % Tween again, 50 µl of luminescence substrate from Supersignal ELISA Femto Maximum Sensitivity substrate kit was added to each well and subjected to luminescence detection on a multifunctional plate reader.

The ELISA signals proportionally matched those of the Epic™ System (Fig. 4, filled circles). In contrast, reference wells with PEG-amine did not produce any ELISA signal (Fig. 4, open circles). Hence, immobilization of NH2-SWpTY peptide at 50 µg/ml allows for approximately 80 % of the maximal signal and therefore was used for the subsequent experiments.

3.3 Affinity Ranking by Competitive Replacement

The rank order of affinity of the peptide motifs was determined by competition assays. After immobilization of NH2-SWpTY on the Epic® plate, a pre-mixed solution of 14-3-3 and varying concentrations of the competitive peptides were added into the Epic® wells. The referenced signals were applied in Eq. 5 to determine the relative affinities of the corresponding competitive peptides.

$$y = B \times \left\{ \left(\frac{K_{D_{\text{NH-SWpTY}}}}{K_{D_{\text{competitor}}}} x + K_{D_{\text{NH-SWpTY}}} + D_0 + P_0 \right) - \sqrt{\left(\frac{K_{D_{\text{NH-SWpTY}}}}{K_{D_{\text{competitor}}}} x + K_{D_{\text{NH-SWpTY}}} + D_0 + P_0 \right)^2 - 4 \times D_0 \times P_0} \right\} \quad (5)$$

The fitting was done with Eq. 5 using Origin 7.0 (OriginLab), with y, the referenced binding signal; B as a constant; D_0, the concentration of NH2-SWpTY that conferred 90 % of saturated GST-14-3-3ζ response; P_0, the concentration of GST-14-3-3; $K_{D_{\text{NH-SWpTY}}}$, the K_D (=2.1 μM) for NH2-SWpTY binding to GST-14-3-3ζ as determined by the concentration response of GST-14-3-3ζ with 50 μg/ml of NH2-SWpTY immobilized at pH 7.5; $K_{D_{\text{competitor}}}$ as K_D for competitive peptides, and x as the concentration of competitor peptide.

In contrast to the direct detection using the immobilized binding partner, the competition assay uses the binding competitors to measure binding affinity in solution. Affinity detection through competitive replacement has been a routine method [10, 11]. The distinct signals by competitor peptides can be observed with considerably different affinities using the Epic® System. The fitting results for K_D values of the competitors SWpTY, SWTY, SWpTD, and SWpTP are 0.12 ± 0.05 μM, >50 μM, 1.75 ± 0.14 μM, and 6 ± 1.67 μM, respectively. Direct comparison of this method with the previous detection of SWTY-14-3-3 interaction with fluorescence anisotropy suggests a similar rank order of the affinity (Table 1).

Table 1
Comparison of the 14-3-3–SWpTY interaction detection from fluorescence anisotropy and Epic® label-free detection

	Fluorescence Polarization				Epic® System			
Sensitivity (nM)	16				38			
Linear range (nM)	16–700				38–2,000			
Probe K_D (μM) (GST-14-3-3ζ)	1.7 ± 0.3				2.1 ± 0.4			
K_D (μM, competitive)	SWpTY 0.17	SWTY >100	SWpTD 2.2	SWpTP 45	SWpTY 0.12	SWTY >50	SWpTD 1.75	SWpTP 6
Z factor	>0.5				>0.5			
S/*N* ratio	~8.4				~15			

3.4 Characterization for High-Throughput Screening

To characterize the capability of the Epic® System for high-throughput screening of 14-3-3 phospho-specific interactions, 50 μg/ml of NH2-SWpTY at pH 7.5 was immobilized in one set of rows and PEG-amine was immobilized in a second set of rows on the Epic® plate with PEG-amine immobilized wells used as a reference. GST-14-3-3ζ at 1 μM with varying DMSO amounts was added to test the compatibility of adding small molecule library in DMSO solution. GST-14-3-3ζ at 1 μM with or without pre-incubated 20 μM SWpTY (as a positive inhibition control) was added into the wells of one Epic® plate with intrawell self-referencing for the statistic data. The Z factor, Z' factor, and S/N ratio [12] were calculated from Eqs. 6 and 7, respectively, where Av is the average and SD is the standard deviation of the referenced signals.

$$Z = 1 - 3(\mathrm{SD}_{\text{With 14-3-3}} + \mathrm{SD}_{\text{Without 14-3-3}})/(\mathrm{Av}_{\text{With 14-3-3}} - \mathrm{Av}_{\text{Without 14-3-3}}) \tag{6}$$

$$S/N = \left(\mathrm{Av}_{\text{With 14-3-3}} - \mathrm{Av}_{\text{With 14-3-3 and SWpTY}}\right)/ \sqrt{\left(\mathrm{SD}_{\text{With 14-3-3}}\right)^2 + \left(\mathrm{SD}_{\text{With 14-3-3 and SWpTY}}\right)^2} \tag{7}$$

The statistic data of responses of GST-14-3-3ζ, with or without pre-incubated SWpTY, have shown a good signal to noise ratio of ~15. The addition of 20 μM competitor SWpTY peptide in the solution resulted in a 94 % decrease of the referenced signals, with a Z factor of 0.8, indicating the robustness and compatibility of the assay for high-throughput screening.

4 Notes

Common to most of label-free systems is the issue of system prone to nonspecific interactions. This justifies the thorough investigation of the specificity of the current assay by including the following components.

1. Non-phosphorylated SWTY peptide: Only the immobilized phosphorylated peptide SWpTY gave detectable signals, while immobilized non-phosphorylated peptide SWTY gave only minimal background signals.
2. Competitive 14-3-3 binding peptides: Only the competitive peptides added in the solution gave the reduction of RWG signals due to the competitive binding. SWpTY peptide or a nonhomologous 14-3-3 binding peptide R18, the binding signals were significantly reduced, where non-phosphorylated peptide SWTY has shown no such effect.

3. Wild type and mutant 14-3-3 proteins: The assay was tested with wild type 14-3-3 and 14-3-3 K49E, which has a mutation at the binding site. Consistently, the binding signal was obtained only in wild type 14-3-3 proteins.
4. Two different SWpTY binding proteins: Different phosphor-recognizing modules, 14-3-3 protein and an anti-SWpTY antibody, have been applied using the same detection format. Both gave detectable signals, where BSA gave only minimal background signals.
5. Validation with alternative assays: Direct and indirect schemes of the Epic assay have been validated by the alternative assays, by in situ ELISA and fluorescent polarization assay respectively. The ranking and affinity data have further quantitatively verified the specificity of the current Epic detection method.

In contrast to peptides, proteins, and nucleic acids, small molecules are quite challenging for label-free detection, because of the small molecular weight and theoretically much smaller signals. In addition, the nonspecific interactions by small molecules, especially those promiscuous hydrophobic ones, require additional control tests to discriminate artifacts from real signals. To overcome this issue, a self-reference Epic® plate has been designed, as shown in Fig. 5. One half of the sensor was coated with active chemical, good for immobilization; while the other half was not coated, and resistant to the immobilization, and consequently serves as the control. The Epic® system can detect two areas and normalize the

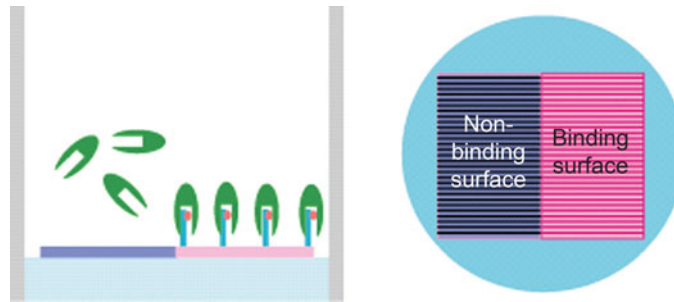

Fig. 5 Schematic representation of the self-referenced biosensor in the well of Epic® plates. The *pink area* denotes the chemical active area good for immobilization; while the *grey area* denotes the area resistant to the immobilization, and consequently serves as the control. The Epic® system can detect two areas and normalize the nonspecific interaction with the surface from the promiscuous small molecules/aggregates. From: Wu, M Long S, Frutos AG, Eichelberger M, Li M, Fang Y., *Journal of Receptors and Signal Transduction*, 2009; 29 (3–4): 202–210, copyright © 2009, Informa Healthcare. Reproduced with permission from Informa

nonspecific interaction with the surface from the promiscuous small molecules/aggregates. For example, as expected, the presence of DMSO did result in a change of bulk refractive index, and, hence, a change in the unreferenced signals of both test wells and reference wells. However, proper use of referencing eliminates this effect, and thus there is no significant difference for the referenced signals between 0 % DMSO up to 10 % DMSO. This is very helpful in the case of competitive format for the detection for screening protein-protein interaction modulators.

5 Conclusion

A label-free assay for the phospho-specific interactions of 14-3-3 proteins has been developed using the resonant waveguide grating Epic® System. When the SWpTY (NH2AhxAhxFRGRSWpTY-COOH) peptide is covalently immobilized to the surface, binding of 14-3-3 proteins can be detected at as low as 38 nM, with adjustable linear ranges depending on the density of immobilized SWpTY. The specificity of detection was validated using competition experiments with a non-phosphorylated SWTY peptide and a binding mutant of 14-3-3 protein. Furthermore, competition assays were performed to determine the rank order of binding affinities of the different peptide motifs. In addition, the assay is compatible with high-throughput screening with a Z factor larger than 0.5 and up to 10 % DMSO tolerance. Therefore the reported assay offers a label-free screening system for phospho-specific interactions applicable at the quantitative level. With minor modifications, the reported assay can be applied for the screening of modulators for 14-3-3 protein-protein interactions. Since the kinase/phosphatase reactions can introduce the phosphorylation and dephosphorylation of the SWTY/SWpTY sequences immobilized on the surface of the sensor plate. The current assay can be further developed into a high-throughput label-free protocol for modulators of kinases and phosphatases. In addition, the assay can be applied into other peptide–protein interactions, i.e., PDZ domains and other extracellular loop sequences of ion channels [13].

The reported assay conditions are tuned for 14-3-3 interactions with the SWpTY peptide. Many aspects of the experimental conditions may be transferable to other interaction systems, especially protein–peptide interactions that are exemplified by phospho-specific antibody to the SWTY peptide. Comparison with solution-based assays such as fluorescence polarization suggests that at least for the interaction between SWpTY and 14-3-3, the Epic® System gave comparable characteristics in terms of sensitivity and dynamic range. Because Epic® allows for quick estimation of binding affinity, it provides an attractive means for quantitative assessment of interactions between different ligands and one

protein or between one ligand and different interacting proteins. This could be applicable to a number of assays such as antibody evaluation and detection of interacting components in cell lysates.

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