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Mini Review

Biosensors on Array Chip by Dual-color Total Internal Reflection Fluorescence Microscopy

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Abstract

The noteworthy progress in biosensing technology during the last few decades has paved the way of the real-time detection, rapid screening, characterization and quantification of biological samples with supreme precision. Scientists in this distinct area have made it possible to enhance biochip-based biosensors using optical transducers. This review is meant to provide an overview of biosensors based on nanoarray biochips using polymer-modified glass surfaces and prism type dual-color total internal reflection fluorescence microscopy (TIRFM). It also endeavors to provide information on how the dual-color TIRFM system has made it possible to monitor the interactions and to detect the colocalization of two different protein molecules (Alexa Fluor® 633 goat anti-rabbit IgG, anti-actin and Alexa Fluor® 488 conjugated actin) at the single-molecular level.

Keywords: Biosensors, Nanoarray biochip, Total internal reflection fluorescence microscopy (TIRFM), Single-molecule detection

Introduction

As time has progressed, the detection and characterization of biomolecules existing in trace amounts in living organisms has attracted increasing attention from researchers. More than ever, the use of bioreceptors in biological recognition processes for the identification and characterization of foreign substances has become a most promising field of study. The development of microarray technique followed by nanoarray technology has provided more effectual technique for the rapid detection and quantification of biological samples such as DNA, proteins, enzymes, carbohydrates, etc.[1-3]. Moreover, by combining biorecognition elements with various transduction methods a potential bioanalysis technology was developed, which is referred to as the biosensor[4]. Clark and Lyons first reported on biosensors in their enzyme electrode patent referring to its potential application to blood glucose monitoring[5]. Typically, a biosensor is a device for the detection of an analyte using a bioreceptor and a transducer[6]. It consists of three basic components; firstly, a sensitive biological material, called a receptor (e.g., Protein, DNA, enzyme, antibody, nucleic acid, tissue, organelle, microorganism, cell and biomimetic).

The second one is the transducer, or the detector, that operates with physicochemical properties based on different optical phenomena, piezoelectric properties, and various electrochemical characteristics. The transducer in biosensors transforms the signal resulting from the interactions of the bioanalytes with the bioreceptors into another form that can easily be quantified and measured. The third part is an electronic or signal processor that is used to record and display the consequences of the analyte-transducer interaction[7]. Figure 1 shows the steps involved in the biosensing process.

Frider in his mini review[8] defined biorecognition systems in more detail, including their biomimetic recognition elements, e.g., aptamers and molecularly imprinted polymers, which are derived from biology. Currently, biosensors are widely used in two major fields of research, biological monitoring and environmental processes control[9-12]. In addition, the demand for home-used biosensors such as glucose meters has significantly increased.

This review focuses on the application of dual-color total internal reflection fluorescence microscopy (TIRFM) based optical transducers for the real-time detection of biological samples of interest at very low concentrations. The full images of individual biomolecules labeled with the different fluorescent dyes were obtained on a polymer-modified nanoarray chip using a Dual-view™ system. There was no mechanical deviation or time-delay, because the images of the single-biomolecules were obtained directly using only one CCD camera.

Results and Discussion

Optical Transducer

In biosensors, transduction mechanisms can be im-

plemented via a wide variety of methods. The most common forms are the electrochemical, optical or mass-based detection methods. Certainly, new types of transducers are being developed endlessly. Simply, any optical or electrical change in the support material, because of the analyte-receptor interaction, (which may lead to the depletion of the analytes or the formation of the products) provides the route for the opto/electronic transduction of the biological recognition process at the sensing surface. Electrochemical biosensors depend on the chemical potential of a particular species (analyte) in the solution and the change of the potential or the current is measured in comparison with a reference electrode. The target analyte takes part in the reaction (usually an enzymatic catalytic reaction that produces or consumes electrons) on the active electrode surface. To produce a signal, the potential (created by the ions produced) is subtracted from the reference electrode[13].

Mass-based detection has been used for the measurement of small changes in mass. The foremost way is the change in the oscillation frequency of a piezoelectric crystal, which depends on the applied electric signal frequency and the mass of the crystal[14,15]. Optical biosensors have been developed based on various kinds of spectroscopy, for example, absorption, fluorescence, phosphorescence, Raman, refraction, and surface plasmon resonance (SPR), with different spectro-chemical properties being recorded, including the amplitude, energy, polarization decay time and phase[4]. Meanwhile, some effective optical biosensors have been reported. Among these, Wolfbeis and co-workers developed an optode for the detection of urea (over the concentration range of 0.3-100 mM) using a pH sensitive dye as the sensor transducer in a lipophilic carboxylated polyvinyl chloride membrane[16]. McCormack *et al.* reported a fiber-optic evanescent wave immunosensor for the detection of lactate dehydrogenase. A polyclonal antibody preparation have been used in both one-step and two-step assays[17].

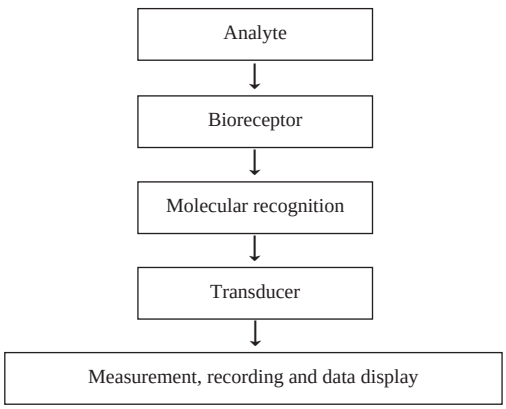

Figure 1. Schematic diagram of the principle of biosensing.

As the decay time of a specific emission signal e.g., fluorescence or phosphorescence is highly dependent upon the excited state of the molecules and their local molecular environment, so, the decay time of a specific emission signal can be used to obtain information about molecular interactions such as protein-protein, protein-ligand and antigen-antibody bindings, etc. For example, Vo-Dinh and coworkers reported a phase-resolved fiber-optics fluoroimmunosensor (PR-FIS) that is able to differentiate between the carcinogen benzo(α)pyrene and its metabolite benzo(α)pyrene tetrol, based on the difference in their fluorescence lifetimes[18].

Evanescent wave based sensors (called SPR based sensors) usually operate using a sensor chip made from a thin layer of gold or other material (such as a

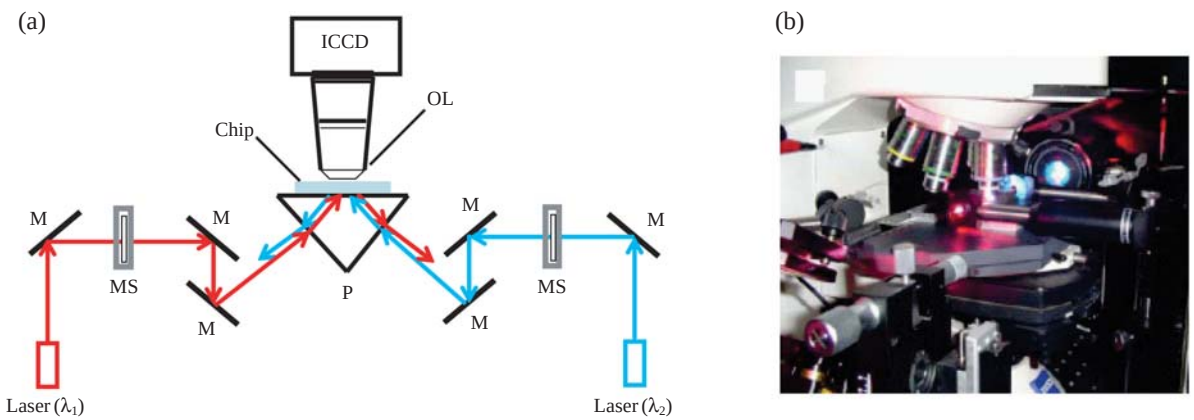

Figure 2. (a) Schematic diagram and (b) image of the experimental setup of the dual-color TIRFM system used for the immobilization and detection of the interactions of the individual single-protein molecules on various polymer-modified glass protein chips. Indications: M, mirror; MS, shutter; λ, Laser source; P, Prism; OL, Objective Lens[49,50].

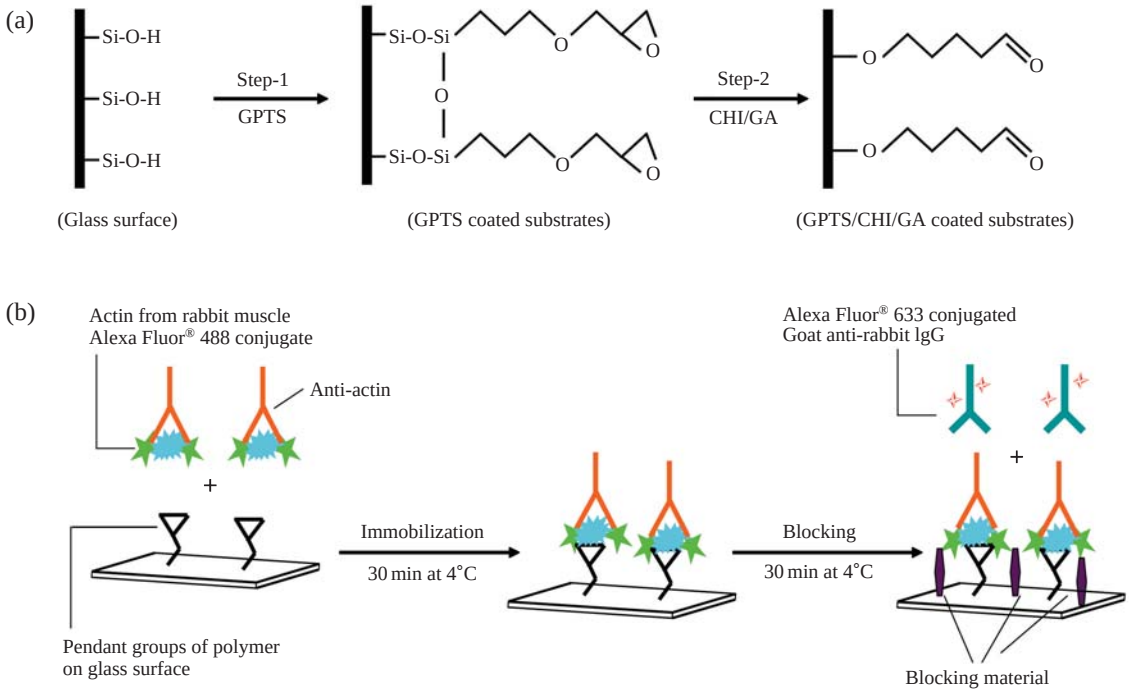

Figure 3. (a) Schematic diagram of the preparation of the polymer-modified glass substrates (step-1 for GPTS and step-2 for CHI/GA-modified glass surfaces). (b) The overall experimental procedure for observing the protein-protein interactions on the polymer-modified glass substrate[48,49].

polymer) on a high refractive index (~1.3) glass surface. The incident light, at a fixed wavelength and angle, reflects off the surface of the chip at the angle of total internal reflection (TIR) and the measurable signal resulting from the binding of a target analyte to a receptor on the substrate surface is detected. In case of TIRFM, an evanescent field area is also produced, which can be utilized in optical transducer based biosensors.

We designed various polymer-coated glass biochips for protein immobilization on the submicron scale. Nearly 90% of the single-protein molecules were detected using the dual-color TIRFM system without any time delay caused by mechanical deviations. The dip-pin nanolithography (DPN) nanoarray technique with an atomic force microscopy (AFM) tip was used to immobilize the protein molecules on the polymer-coated glass surface[40-42]. The protein molecules were adsorbed on the polymer coated-glass substrates by hydrophobic force and electrostatic attraction[44,46,47]. The dual-color prism-type TIFRM system with two individual lasers and one high-sensitivity camera was designed for the direct observation of single-molecule dynamic processes, as well as for the colocalization of different individual proteins on a nanoarray biochip without mechanical movement. Figure 4 shows the dual-color TIRFM images of the interaction between 1 pM Alexa Fluorw® 633 goat anti-rabbit IgG, anti-actin and Alexa Fluor® 488 conjugated actin on the GPTS/CHI/GA-modified nanoarray protein chip with 1% Top-Block as a blocking reagent. The binding of the two different protein molecules on the chip substrate produced a yellow color, due to the colocalization of the various protein molecules with different emission colors (i.e., red and green) (Figure 4). Under the optimum conditions with the blocking reagent (Figure 4b), the binding efficiency of the protein-protein interactions was calculated at a concentration of 1 pM. There were 180,600 molecules in a volume of 300 nL or area of $7.07 \times 10^6\ \mu m^2$. The detection window for dual-color TIRFM was $1.46 \times 10^4\ \mu m^2$; this indicates a precision of $85.5 \pm 0.56\%$[49].

Moreover, when concurrently excited with the dual-wavelength of 488 nm from the Ar^+ laser and 635 nm from the fiber coupled laser and viewed with a Dual-View system™, the fluorescence images of the 500 aM Alexa Fluor® 488-labeled actin/anti-actin antibody complexes at λ_{ex}=488 nm (left in Figure 5a) and the Alexa Flour® 633-labeled goat anti-rabbit IgG antibody at λ_{ex}=635 nm (right in Figure 5b) in 10 mM CHES buffer were monitored on the glass chip without any time-delay. The colocalization of the two different

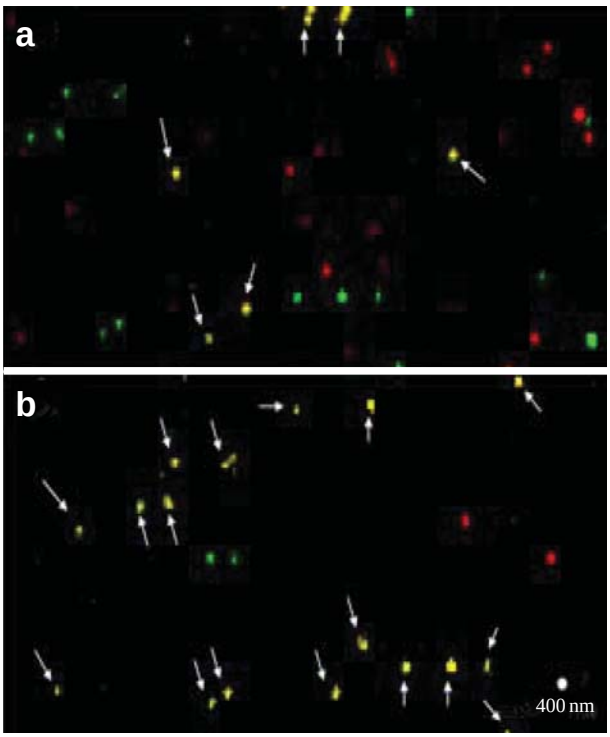

Figure 4. Dual-color TIRFM analyses of the protein-protein interactions (a) without and (b) with the blocking reagent consisting of 1% Top-Block in PBS on the GPTS/CHI/GA-modified glass protein chip. Protein samples, 1 pM Alexa Fluor® 633-goat anti-rabbit IgG (target protein and Alexa Fluor® 488 conjugated actin-anti actin (capture protein); buffer, 10 mM PBS; washing buffer, PBS-Tween 20 (pH 7.4). Indications: Red dot, Alexa Fluor® 633 conjugated goat anti-rabbit IgG; green dot, Alexa Fluor® 488 conjugated actin-anti actin; yellow dot (arrows), binding of the two different protein molecules on the chip[49].

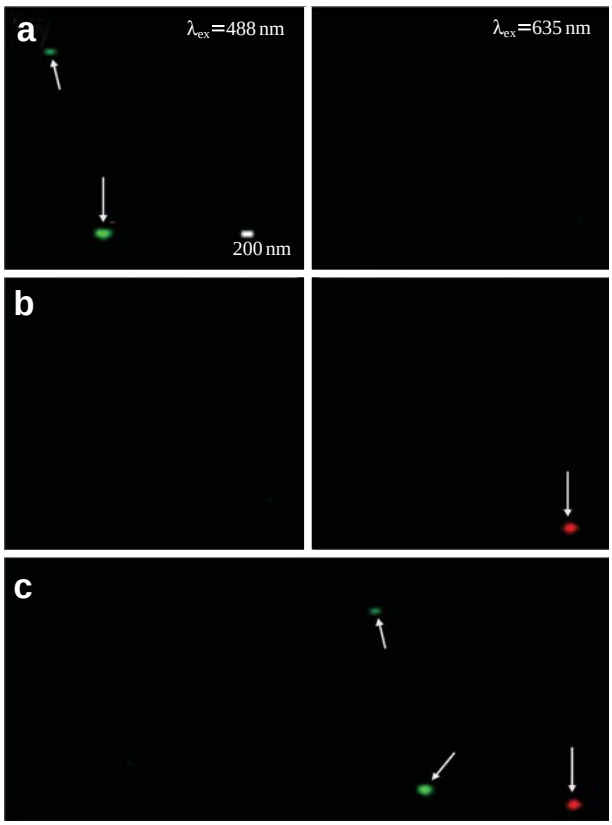

Figure 5. Dual-color TIRFM images of (a) actin from rabbit muscle conjugated with Alexa Fluor® 488 at λ_{ex}=488 nm, (b) Alexa Fluor® 633 goat anti-rabbit IgG at λ_{ex}=635 nm, and (c) overlap of (a) and (b) on a bare cover glass. The sample concentration was 500 aM in 10 mM CHES buffer (pH 10)[50].

protein molecules (Figure 5c) was effectively observed using dual-color TIRFM[50]. The polymer-modified glass substrate showed a 13.5-56.3% higher relative *S*/*N* (signal-to-noise) ratio than the bare glass substrates.

Conclusions

Biosensing technology has become an extensively studied area in recent times. In addition, the demand for biosensors for routine health checking at home (e.g., glucose meters, pregnancy test strips) is increasing day by day. Moreover, integrated biochip systems have advantages in terms of their size, performance and fabrication, as well as for analysis in practical medical diagnostic applications. In spite of the rapid advancements in biosensing technology, more efforts are needed to develop biomimetic recognition systems, optical transducer in biochip devices, nanoscale probes, *etc*., since they can provide rapid and easy analysis in a cost effective manner. We developed a dual-color prism-type TIRFM system and nanoarray biochip on a polymer modified glass surface for the direct detection of single-molecular interactions and for monitoring their colocalization without any time delay or mechanical deviation. This technique can be useful for the direct monitoring of the colocalization of living cells and real-time clinical sample analysis at ultra-low concentrations.

Materials and Methods

Basics of TIRFM System

TIRFM is a well-matched optical technique for the real-time imaging and monitoring of fluorescence labeled single-biomolecules (i.e., DNA and protein molecules). It can be monitored within an evanescent

wave and provides a high signal-to-noise ratio[19,20]. When the incident light from a medium with a high refractive index enters into a second medium with a low refractive index at an angle beyond the critical angle, TIR occurs. The TIR of visible light at the interface of the two different refractive index media (e.g., solid/liquid interface) produces a region called an evanescent field layer that penetrates just a few nanometers (about 200-300 nm) into the lower refractive index medium. Therefore, the only species that can be observed under TIRFM are those that are: (a) intrinsically, or, extrinsically fluorescent and (b) lie close enough to the interface of the two media to be seen within the region illuminated by the evanescent wave[21,22]. The TIR illumination requires a high numerical aperture (~1.4) objective lens to collect the light falling onto the specimens under examination. The emitted light is detected by an imaging system including a cooled CCD camera and digital image process program (WinView/32™ imaging system) is used to control the camera and captured data[47].

Total internal reflection fluorescence (TIRF) has proven to be a compatible technique for investigating interfacial bimolecular interactions. Therefore, many studies have been carried out using TIRFM system coupled with immunoassays[23]. For example, the design, simulation and fabrication of TIR-based chips for highly sensitive fluorescent imaging[24], the monitoring of motor proteins *in vitro*[25], signal transduction in living cells at the single-molecule level[26], ligand binding to a receptor[27], and ultra-sensitive real-time studies of the dynamics of single-DNA molecules at a fused-silica/water interface[28,29]. The development of a microscopic platform for the real-time monitoring of bimolecular interactions[30] and microfluidics-based TIRFM-flow cytometry to examine single cells with evanescent field illumination[31] are also worth mentioning. Moreover, Arakawa *et al.* fabricated a multi-reagent switching micro fluidic system integrated with TIRFM[32].

TIR-based Biochips

A biochip is a collection of miniaturized spots arranged on a solid substrate that permits many tests to be performed at the same time for the purpose of achieving high-throughput screenings[33]. In recent years, chip technology has become the prevalent method for the large-scale characterization of biological samples[34]. The development of a multianalyte analysis system may lead to the practical applicability of biochips such as gene-chips, DNA-chips, protein-chips and cell-chips to point-of-care devices, offering the advantages of the fast quantification of the analyte concentration in the samples and the ability to obtain quick and reliable responses[35,36]. Vo-Dinh and coworkers reported an optical microarray system using a charge-couple-device (CCD) detector and DNA probes[37]. A method of calibrating an antibody-based biochip using two different fluorescent dyes to detect toxic agents, using a planar array of antibody probes was described by Liglar *et al.*[38]. Dempsey *et al.* described a photolithographic technique for the development of a microarray based electrochemical biosensor for the detection of glucose and lactate[39].

Our group reported nanoarray biochips fabricated by the dip-pin nanolithography (DPN) nanoarray technique, using an atomic force microscopy (AFM) tip to immobilize proteins molecules on the polymer-coated glass surface. The DPN array method allows biochips to be fabricated with almost no detectable nonspecific binding of proteins to the passivated-portions of the array, even in a heterogeneous mixture of proteins, and the reaction involving the protein and antigens can be screened by AFM. Therefore, this method provides the opportunity to study a variety of surface-mediated biological recognition process[40-42]. We demonstrated a method of directly monitoring the interactions and detection of the colocalization of two different single-protein molecules labeled with different fluorescent (i.e., Alexa Fluor® 488 and Alexa Fluor® 633) dyes on the polymer, i.e. 3-glycidoxypropyltrimethoxysilane (GPTS), chitosan (CHI, 75-85% deacetylated chitin), glutaraldehyde (GA), coated cover glass.

Dual-color TIRFM Set-up

The basic experimental setup of the dual-color TIRFM system has been described in previous reports with the exception of the two individual lasers and one Dual-View system™[28,29,43]. As shown in Figure 2, two different individual lasers, viz. a wavelength tunable argon ion laser (maximum output power=30 mW at 488 nm; Melles Griot, Irvin, CA, USA) and 635 nm fiber-coupled laser (maximum output power=35 mW at 635 nm; B & W TEKINC, Newark, DE, USA), were used as the excitation light sources. An upright Zeiss Axioskop-2 microscope (Zeiss, Germany) equipped with a Dual-View system™ (Optical Insight, LLC, Tucson) was mounted between the objective lens and the CCD camera. The filter cube consisted of a fixed mirror, a dichroic filter (565dcxr, Chroma technology Corp., Rockingham, USA) and two emission filters (D680/35 and D535/40, Chroma Technology Corp., Rockingham). A Zeiss 100×/1.3 N.A. Plan-Neofluar® microscope objective lens (Zeiss, Germany) was used. A Pentamax 512-EFT/1EA intensified CCD camera (ICCD, Princeton Instruments, Princeton, NJ, USA) was mounted on top of the microscope. A Uniblitz mechanical shutter (model LS2Z2, Vincent Associates, Rochester, NY, USA) was used to block the laser beam

when the camera was switched off, in order to reduce the amount of photobleaching. The experimental timing was controlled using a Stanford Research System model DG-535 four channels digital delay/pulse generator (Stanford Research Systems, Inc., Sunnyvale, CA, USA). The ICCD camera was triggered at time 0 ms with 10 ms duration TTL pulse. The sampling frequency was 10 Hz with the shutter driver set to 10 ms exposure and 90 ms delay. The fluorescence from the individual single-protein molecules was passed through a Dual-View™ filter box, in order to eliminate the unnecessary light and obtain different images of the different single-protein molecules labeled with different fluorescent dyes. The evanescent wave excitation geometry was also similar to that reported elsewhere[28,29,43].

Nanoarray Chip on Polymer Modified Glass Substrate and Observation of Single-protein Molecules

The polymer (GPTS/CHI/GA)-modified cover glass (No.1 Corning, 22 mm square) substrates were prepared using the method reported previously[44,45]. The GA and CHI coatings were coupled to the epoxy groups on the GPTS-modified glass substrates. For the chemical attachment of the protein molecules on the polymer modified glass substrates, the DPN array method was employed using the contact mode of AFM (with AFM tip, silicon nitride cantilever, 0.58 Nm^{-1}, Digital instruments) and the relative humidity was 60%[41,42]. 1% Top-Block was used as a blocking reagent to improve the blocking efficiency and to prevent non-specific binding. The preparation procedure of the protein samples was reported in detail elsewhere[50]. All of the protein samples were prepared at a concentration of 1 μM in 10 mM PBS (phosphate buffered saline) buffer solution and then were further diluted to concentrations ranging from 1 pM to 10 fM with 10 mM PBS solution immediately before beginning the experiments. In addition to these samples, a sample with a concentration of 500 aM was prepared in 10 mM CHES (2-(*N*-cyclohexylamino)ethane sulfonic acid) buffer (pH 10) solution. Figure 3 shows the overall experimental procedures used for the preparation of the polymer-modified glass substrates and the direct observation of the individual protein molecules and immunoassay of the target proteins (i.e., Alexa Flour® 488 actin-antiactin or Alexa Flour® 633 goat anti-rabbit IgG) and their interactions.

The polymer-modified glass substrates were evaluated after washing the chip with 10 mM PBS-Tween 20, followed by drying in a nitrogen atmosphere. The sample on the chip was monitored after introducing 4 μL of 10 mM PBS solution. Direct observation of the individual protein molecules and the immunoassay on the chip were carried out as follows: 2 μL of the target proteins (Alexa Fluor® 633 goat anti-rabbit IgG) were introduced onto the chip surface and incubated for 30 min at 3.5-4.0˚C. The non-bound target protein molecules on the chip surface were removed by washing with 10 mM PBS-Tween 20. Immediately before imaging, the sample was rehydrated with 2 μL of 10 mM PBS and covered with another cover glass. The individual single-protein molecules on the chip were then observed using the dual-color TIRFM system. The substrates in previous studies were characterized at different concentrations of the target protein samples[43]. In addition, the relative signal intensity was measured without considering the non-specific adsorption. The significant nonspecific adsorption and binding were measured at concentrations above 500 fM[48].

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