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High speed digital protein interaction analysis using microfluidic single molecule detection system†

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Abstract

The understanding of protein interaction dynamics is important for signal transduction research but current available techniques prove difficult in addressing this issue. Thus, using the microfluidic approach, we developed a digital protein analytical platform and methodology named MAPS (Microfluidic system Analyzing Protein in Single complex) that can measure the amount of target proteins and protein complexes at the digitally single molecule resolution. By counting protein events individually, this system can provide rough protein interaction ratios which will be critical for understanding signal transduction dynamics. In addition, this system only requires less than an hour to characterize the target protein sample, which is much quicker than conventional approaches. As a proof of concept, we have determined the interaction ratios of oncogenic signaling protein complexes EGFR/Src and EGFR/STAT3 before and after EGF ligand stimulation. To the best of our knowledge, this is the first time that the interaction ratio between EGFR and its downstream proteins has been characterized. The information from MAPS will be critical for the study of protein signal transduction quantitation and dynamics.

Introduction

The development of molecular biology and biochemical techniques for analyzing protein–protein interaction have revealed many important signal transduction pathways, leading to the understanding of many physiological and disease processes at the molecular level. Currently, there are many different types of techniques in detecting protein–protein interactions such as 2D gel electrophoresis-mass spectrometry, immunoprecipitation(IP)/Western blot, fluorescence resonance energy transfer (FRET), and yeast two-hybrid systems.^{1–4} These techniques have been applied in the field of biomedical research and have

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identified many important protein-protein interactions. However, they usually require a long processing time and/or a large amount of sample which limit the application in clinical study. In addition, the issue of interaction ratios and dynamics cannot be addressed by these current techniques.

The detection principle in most of these techniques is like the “analog” type that mostly relies on X-ray film exposure or visual observation, which may limit the sensitivity, specificity, and accuracy. For instance, the bands in a Western blot represent the bulk intensity of target proteins but it is difficult to obtain the ratio of target protein involved in interaction. Using epidermal growth factor receptor (EGFR) as an example, EGFR is frequently over-expressed or mutated in many types of cancer and EGFR signal pathways are responsible for poor prognosis, metastasis and chemoresistance of these cancers.^{5–9} Upon ligand stimulation, multiple molecules are triggered to interact with EGFR. It has been reported that activated EGFR induces the activation of several downstream signal pathways such as PI3K/AKT, Ras/MEK/ERK, JAK/STAT3, Src/FAK, which play important roles in cancer progression.⁷ However, how many EGFR interact with these downstream proteins is still obscure. This information is important because it can not only help the researcher to identify which pathway is more critical in tumorigenesis but also provide the detailed interaction dynamics of these signal pathways. It is difficult to address this kind of question by current techniques; however, the detection of single protein complex can provide a solution. By accumulating results digitally from certain amounts of protein complexes, the analysis of protein interactions ratio and dynamics become feasible.

Fluorescence correlation spectroscopy (FCS) is a powerful tool for detecting and characterizing single molecules. The photon-burst signals are recorded as a function of time and simultaneously calculated for autocorrelation based on photon-bursts.^{10–12} By theoretically fitting the curve to the correlation profile, much information can be obtained such as molecular concentration and mobility. Recently, the FCS technique was incorporated with microchannels that were used to reduce the detection volume.¹⁰ Laser light is focused in the center of the microchannel, and the fluorescence signals from individual molecules are recorded by the avalanche photodiode (APD). Compared to the conventional non-microfluidic based FCS, the microfluidic approach has several important advantages. One of them is that the statistical accuracy of single-molecule characterization is improved because a majority of molecules are counted and contribute to the characterization. The other advantage is the easy control of the detection throughput. In addition, because of the small detection volume, the signal-to-noise-ratio (S/N) is significantly improved.^{13–17}

In this report, we introduce MAPS, a novel digital protein interaction analysis platform and detection system using microchannels with single-molecule detection technique with solid stochastic algorithm. It was designed to provide a rapid method to investigate the detailed protein-protein interactions for potential high throughput applications. The superiority of the MAPS over existing methods is that it can detect the protein interactions down to single-complex level, *i.e.* we can analyze the number of target protein complex individually based on their photon burst profiles. In addition, it only takes less than an hour to analyze one sample, which can significantly reduce the time consumption compared to the conventional approaches. Based on these reasons, it is a powerful technique to understand the mechanisms of signal pathways. Here, we used this technique to determine the interaction dynamics of EGFR/Src and EGFR/STAT3, which play critical roles in tumor progression.^{6–9} The results demonstrated that about 20% to 30% of EGFR interacts with Src or STAT3 upon ligand stimulation. Taken together, MAPS is a novel system to digitally identify protein interaction ratios and dynamics which give us valuable information regarding signal transduction mechanisms.

Experimental

Detection system

We fabricated microchannels with 2 μm widths and 500 nm depths on fused silica substrate using photolithography, reactive ion etching, and thermal bonding. The fabrication process for this device is described in the ESI (Fig. 1[†]). There are 16 microchannels fabricated on one wafer and each microchannel was coated with 0.5% polyethyleneimine (PEI) for 16 h to prevent protein adsorption on the channel surface, and washed out by IP binding buffer (20 mM Hepes-KOH, 0.1 mM KCl, 2 mM MgCl_2 , 15 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 10% glycerol) before applying the cell lysate samples. After loading the sample solution in the channel, a 200 V potential was applied to generate electrokinetic flow. The electrical conductivity of the sample solutions was kept constant in order to maintain a constant flow velocity for all detections. The schematic diagram in Fig. 1a shows the two-color MAPS with single-photon excitation for studying EGFR/Src and EGFR/STAT3 interactions. A 375 nm ultraviolet laser (UV) was focused through 60 $\times$ water immersion objective (NA 1.25) on the center of the microchannel, and time co-incident fluorescence emissions from a single target molecule passing through this spot was detected by two APDs with 525 nm and 605 nm wavelength, respectively. For sample detection, ten sets of independent raw photon burst data recorded for 60 s were plotted on either a 1D histogram or 2D time co-incident photon (2DTP) burst diagram. The number of events distributed in specific regions of a 1D histogram or a 2DTP diagram were then counted, normalized according to the total number of detected quantum dots (QD). This is because the number of QD is approximately one thousand times higher than those of proteins, thus, the number of detected QD is a constant for all experiments. As a result, the digital number of target proteins in sample solution was determined. The ratio of specific target proteins involved in the interaction was then calculated based on these acquired digital numbers. The detection noise level in this photon burst signal was determined by the blank solution (with no QD applied).

Sample preparation

Human embryonic kidney 293 (HEK 293) and HeLa cell lines were maintained in Dulbecco's modified Eagle's medium/F12 medium (Gibco) supplemented with 10% fetal bovine serum and antibiotics. For EGF ligand stimulation, HeLa cells were cultured without fetal bovine serum overnight (at least 12 h) and stimulated with 50 ng/ml EGF for 30 min. Before harvesting of lysates, HeLa cells were treated with dithiobis (succinimidyl propionate) (Pierce) at a final concentration of 1 mg/ml for 5 min to crosslink protein complexes. For preparation of cell lysate samples, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and subsequently lysed in protein lysis buffer (50 mM Tris-HCl, 1% NP-40, 150 mM NaCl, 1 mM EDTA plus protease inhibitors). After sonication of cell lysates, cell debris was removed *via* centrifugation at 12 000g for 10 min. Protein concentrations in the total lysates were measured using a BCA assay kit according to the manufacturer's protocol (Pierce).

Ten micrograms of protein lysates were incubated with quantum dot 605 (QD605) or QD525 *via* specific antibodies (400 ng/ml) in IP binding buffer at 4°C for 4 h. The QD-protein labeling process is described as follows. QD525 and QD605 can be excited by the short wavelength light (<400 nm) and emit in 525 nm and 605 nm fluorescence, respectively. EGFR, Src and STAT3 interacted with specific primary antibodies and were further labeled with QD conjugated secondary antibodies. For instance, EGFR was labeled with a mouse monoclonal antibody (Ab13, which contain two clones of monoclonal

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antibody, Neomarkers) and was further bound with QD605 conjugated anti-mouse secondary antibody (Invitrogen). Because Ab13 recognizes the extracellular domain of EGFR, it does not interfere with Src or STAT3 binding to EGFR within the intracellular domain. Similar to EGFR, Src and STAT3 were labeled with rabbit polyclonal antibodies (Src, 2108, Cell Signaling; STAT3, C-20, Santa Cruz Biotechnology) which bound with QD525 conjugated anti-rabbit secondary antibody (Invitrogen). Because of the recognition in multiple epitopes, individual target proteins can be labeled with multiple QDs which can allow us to distinguish target protein-bound QD events and unbound QD events based on its photon counts. In addition, a single EGFR was recognized by two specific monoclonal antibodies and each primary antibody was further labeled with QD605 conjugated secondary antibody. Thus, each EGFR was identified by the presence of two sets of QD605 signal. It was also expected that if two EGFR were present within the defined detection volume (such as homodimerization), the QD605 signal from such complex would be two times higher than the single EGFR. This principle is also applied to Src and STAT3 detection in which specific polyclonal antibodies can recognize multiple epitopes on the target proteins.

Results and discussion

Single EGFR detection

To prove the concept, we characterized MAPS by detecting exogenous EGFR proteins in HEK 293 cells. We first tested the sample without any EGFR cDNA transfection as a control. The upper panel of Fig. 2a shows a typical photon burst profile as a function of time obtained from the QD605/control IgG sample. There was no interaction with the target protein, therefore, it was to serve as unbound QD605 control. We converted these discrete photon burst profiles into a smooth, continuous photon burst profile using a fast Fourier transform (FFT) filter (Fig. 2a, lower panel).¹⁸ Individual photon burst peaks over the noise level were defined as a signal from QD605 and plotted in a one-dimensional (1D) burst histogram (the number of burst as a function of photon counts.) This histogram showed a Gaussian distribution profile¹⁹ and the maximum expectation value that theoretically covered more than 99% of the events distribution was defined as a boundary between QD605 and QD605/EGFR (Fig. 2b). This threshold value can be determined by $\mu + 3\delta$, where μ and δ are the average QD605/control IgG photon counts and their standard deviation, respectively. Based on this stochastic method, the threshold value between QD605 and QD605/EGFR is determined as 226 photon counts. The number of distributed events higher than 226 photon counts is defined as the number of non-specific background burst. This background number is then normalized by the amount of equal QD605 events and is 147 events per 10^4 events. Meanwhile, in the cells with EGFR cDNA transfection, we labeled EGFR proteins with the specific anti-EGFR antibodies, which were further recognized by QD605 conjugated secondary antibody. Their photon burst signals were obtained and smoothened by the FFT filter described previously (Fig. 2c). All events higher than the noise level obtained from QD605/EGFR sample were plotted in the 1D histogram with the threshold line of 226 photon counts determined by QD605/control IgG sample (Fig. 2d). The events with higher photon burst than the threshold line are defined as EGFR. On the other hand, the events which have less photon counts than the threshold value line are defined as unbound QD605. After normalized by 10^4 total QD605 events, 2628 EGFR events were detected. However, these detected events might contain non-specific background events, which are 147 events per 10^4 QD605. Therefore, after background subtraction, 2481 QD605/EGFR events were obtained by MAPS.

Using the same approach, we detected the number of EGFR proteins in the cells transfected with different amounts of EGFR cDNA, and the analytical results are shown in Table 1. Events in HEK 293 cells without EGFR transfection were detected by control IgG to obtain the background value and by anti-EGFR antibody to measure endogenous EGFR. The

number of detected EGFR events with standard deviation was correlated with the amount of the EGFR cDNA transfected (Fig. 3a). The same EGFR samples were analyzed by the conventional Western blot (Fig. 3b) and the EGFR expression patterns obtained by MAPS were very well correlated to the conventional Western blot analysis.

EGFR/Src and EGFR/STAT3 detection

Subsequently, we characterized two protein interactions of EGFR/Src and EGFR/STAT3 with a single protein complex resolution using the two-color MAPS system based on their time co-incident photon burst diagrams. We labeled EGFR and Src proteins in HeLa cell lysates with QD605 and QD525 respectively by the methods described in the experimental section of this paper and detected their time co-incident photon burst events simultaneously. The dual color photon burst profiles as a function of time obtained from QD605/control IgG and QD525/control IgG sample were shown in Fig. 4a. Each arrow indicates a burst peak from QDs/control IgG. Individual photon bursts are theoretically from the individual proteins because of the low protein concentration comparing to the detection volume. Thresholds for each color were determined based on the stochastic method described previously and the 2DTP diagram obtained from photon bursts of QD525/control IgG and QD605/control IgG sample are shown in Fig. 4b (–EGF) and Fig. 4c (+EGF). Based on the same Gaussian analysis, the threshold values for – EGF were determined as red (QD605) 469 counts and green (QD525) 175 counts. The threshold values for +EGF were red (QD605) 1224 counts and green (QD525) 174 counts. In this figure, events in the area (1), (2), and (3) were considered as non-specific background signals of target protein and protein complex.

The dual color photon burst profiles as a function of time obtained from Src and EGFR labeled samples with EGF stimulation were shown in Fig. 5a. Red, green and purple dotted boxes indicate the detection of EGFR, Src, and EGFR/Src complex, respectively. Time co-incidently detected photon bursts, which are overlapped in time, are defined as the bindings of EGFR and Src. To characterize EGFR and Src interaction, the time co-incident QD525/Src and QD605/EGFR events were plotted on a 2DTP diagram (Fig. 5b) in the absence of EGF stimulation. The areas (1), (2), and (3) indicate the Src-unbound EGFR protein, EGFR/Src complex, and EGFR-unbound Src protein, respectively. The numbers of events in each area were then normalized by the equal amount of total QD events and non specific background subtraction. EGFR and Src interaction with EGF stimulation were also analyzed by the same method (Fig. 5c). The results demonstrated that 10% of EGFR interacted with Src before EGF stimulation and the ratio of interaction rose to 20% after EGF stimulation (Fig. 6a). The pattern of the interaction induction is similar to the conventional method IP/Western blot (Fig. 6c), however MAPS provided the ratio of the interacted EGFR while IP/Western blot did not.

In addition to the EGFR and Src interaction, we also characterized the interactions between EGFR and another downstream protein, STAT3, before and after EGF stimulation by MAPS. 2DTP diagrams with threshold lines were shown in Fig. 5d and 5e. The interaction ratios between EGFR and STAT3, calculated after normalization and background subtraction processes based on the 2DTP diagram, are also shown in Fig. 6b. These results showed that the interaction between EGFR and STAT3 increased almost threefold after EGF stimulation. These results were correlated with the patterns which we obtained from conventional IP/Western blot analysis (Fig. 6c). However, MAPS revealed that 30% of EGFR interacted with STAT3 after EGF stimulation.

Conclusion

Current research of protein–protein interactions has provided important information about the mechanisms of a variety of diseases and critical knowledge for developing novel therapeutic approaches to these diseases. Treatment of cancer is one important example because many anticancer drugs have been developed based on the inhibition of specific cancer pathways. Oncogenes such as EGFR interact with multiple downstream proteins and phosphorylate them triggering various cancer signaling transduction.^{20–22} However, whether all receptors interact with these downstream proteins at the same time or in a dynamic way has been recognized as a critical issue yet virtually impossible to address using conventional technology. As we report here, we developed MAPS that allows us to detect protein–protein interactions at the single-molecule level and also demonstrates the interaction ratio. Using MAPS system, we found that EGFR interacted with downstream molecules Src and STAT3 in a dynamic way before and after ligand stimulation. Moreover, we demonstrated that this system required only a small amount of sample and short running time. Therefore, MAPS is a timely technology that will provide a new avenue for understanding the dynamics of signal networks. In addition, it also can be applied to high-throughput screening for targeting drugs that disrupt specific protein interactions and molecular diagnosis.

Acknowledgments

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Fig. 2.

Detection of single EGFR by MAPS. (a) Upper panel, photon burst signal profile of control QD605/control IgG. Lower panel, QD605/control IgG photon burst signal profile after the FFT filtering process. (b) Histogram of the control QD605/control IgG. The threshold (226 photon count) of unbound QD605 or QD605 bound to a target protein. (c) Upper panel, photon burst profile of QD605/EGFR, which was overexpressed in HEK 293 cell line by transfection of 10 μ g of EGFR plasmid. Lower panel, QD605/EGFR photon burst signal profile after the FFT filtering process. The higher photon count peaks indicate the EGFR events. (d) Histogram of QD605/EGFR. Events with photon counts higher than the threshold were considered as EGFR events.

Fig. 3.
Dose-dependent transfection of EGFR cDNA (0, 4, 6, and 10 μg) in characterizing EGFR detection using (a) MAPS and (b) Western blot analysis.

Fig. 4.

Time co-incident protein interaction analysis. (a) Time co-incident photon burst signals as a function of time for QD605/control IgG and QD525/control IgG sample without EGF stimulation. There is no correlation between QD605 and QD525. (b) and (c) 2DTP diagram from control QD605/control IgG and QD525/control IgG samples with and without EGF stimulation, respectively. These data were used to determine the boundary threshold values. Inside the diagram, (1) indicates the amount of background signal when detecting EGFR, (2) indicates the amount of background signal when detection EGFR/Src or EGFR/STAT3, and (3) indicates the amount of background signal of Src or STAT3.

Fig. 5.

Dynamics of EGFR/Src and EGFR/STAT3 interactions upon ligand stimulation. (a) Time co-incident photon bursts for Src/EGFR sample with EGF stimulation. If the photon bursts were overlapped, two QD events were recognized as co-incident. Thus, these two proteins were defined as binding. 2DTP diagrams showing the event distributions for EGFR (1), Src (3), and the EGFR/Src (2) complex (b) before EGF stimulation and (c) after 30 min of EGF stimulation. 2DTP diagrams showing the event distributions for EGFR/STAT3 (d) before EGF stimulation and (e) after 30 min of EGF stimulation.

Fig. 6.

Results of Src/EGFR and STAT3/EGFR interaction. (a) Ratio bar chart for EGFR and the EGFR/Src complex before and after EGF stimulation. (b) Ratio bar chart for EGFR and the EGFR/STAT3 complex before and after EGF stimulation. (c) Results of IP/Western blot from the sample with the same treatment conditions.

Table 1

Calculation worksheet of exogenous EGFR detection in EGFR cDNA dose-dependent transfection sample

EGFR (μ g)	0	4	6	10
(unit: counts per 10000QD)				
Background (IgG)	147	147	147	147
Mean of EGFR	190	361	1133	2628
EGFR after background subtraction	43	214	986	2481
Standard deviation of EGFR	1	93	559	483