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mMAPS: A Flow-Proteometric Technique to Analyze Protein-Protein Interactions in Individual Signaling Complexes

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Abstract

Signal transduction is a dynamic process that regulates cellular functions through multiple types of biomolecular interactions, such as the interactions between proteins and between proteins and nucleic acids. However, the techniques currently available for identifying protein-protein or protein-nucleic acid complexes typically provide information about the overall population of signaling complexes in a sample instead of information about the individual signaling complexes therein. We developed a technique called “microchannel for multiparameter analysis of proteins in a single complex” (mMAPS) that simultaneously detected individual target proteins either singly or in a multicomponent complex in cell or tissue lysates. We detected the target proteins labeled with fluorophores by flow proteometry, which provided quantified data in the form of multidimensional fluorescence plots. Using mMAPS, we quantified individual complexes of epidermal growth factor (EGF) with its receptor EGFR, EGFR with signal transducer and activator of transcription 3 (STAT3), and STAT3 with the acetylase p300 and DNA in lysates from cultured cells with and without treatment with EGF, as well as in lysates from tumor

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SUPPLEMENTARY MATERIALS

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xenograft tissue. Consistent with the ability of this method to reveal the dynamics of signaling protein interactions, we observed that cells treated with EGF induced the interaction of EGF with EGFR and the autophosphorylation of EGFR, but this interaction decreased with longer treatment time. Thus, we expect that this technique may reveal new aspects of molecular interaction dynamics.

INTRODUCTION

Cellular signaling complexes, which mostly contain proteins and nucleic acids, play a major role in signal transduction by carrying and delivering messages that coordinate basic biological functions. These processes are mainly relayed through protein-protein and protein–nucleic acid interactions. Deregulation of signaling transduction mechanisms is related to many diseases, including cancer (1, 2). For instance, the gene that encodes epidermal growth factor receptor (EGFR), a well-known receptor tyrosine kinase that consists of an extracellular ligand-binding domain and an intracellular kinase domain and is embedded in the cell membrane, is frequently overexpressed in many types of cancer, and this contributes to poor prognosis, metastasis, and drug resistance (3–6). In response to binding of ligands such as EGF, EGFR forms a dimer and induces the activation of an intrinsic tyrosine kinase, triggering autophosphorylation at multiple tyrosine residues in the cytoplasmic domain (7–10). These phosphorylated tyrosine residues in EGFR serve as docking stations for multiple cytoplasmic proteins, including signal transducer and activator of transcription 3 (STAT3). Consequently, activated STAT3 translocates to the cell nucleus, binds to genomic DNA, recruits acetyltransferases such as p300, and activates target gene expression to trigger cell growth and proliferation, which can promote tumorigenesis and cancer progression if not carefully regulated (11, 12).

Delineating the underlying mechanisms of EGFR signal transduction relies highly on the analysis of specific protein-protein and protein–nucleic acid interactions. Currently, various methods are available for analyzing protein-protein and protein-DNA interactions, but most of them cannot analyze individual complexes directly. For instance, immunoprecipitation has been used extensively and serves as a standard for studying protein-protein interaction by pulling down target protein for indirect detection of the bound protein (13). However, sufficient protein lysate sample (usually within the milligram range) is required to pull down a specific protein complex with a large amount of antibody. This procedure requires long processing time, which substantially limits the sensitivity of detection especially when the target protein complex is not abundant. In addition to immunoprecipitation, chromatin immunoprecipitation (ChIP) is also commonly used to immunoprecipitate the associated DNA complex for analyzing protein-DNA interactions (14, 15). Similar to immunoprecipitation, the requirement of a large sample lysate volume and the number of tedious steps in the protocol also restrict its application. Also, most available methods only query one type of interaction at a time and are difficult to probe two or more interactions within the same complex. Another major drawback is that these methods provide only qualitative or semiquantitative data on molecular interaction, limiting the depth of our understanding of critical signal transduction mechanisms.

By directly analyzing individual target molecules, single-molecule detection offers a compelling solution to overcome these limitations and provides the function for absolute qualitative and quantitative assays (16–18). For instance, the use of extremely high sample dilutions has been applied to detect the association of molecules at a single-molecule level by fluorescence correlation spectroscopy and fluorescence resonance energy transfer (19–21), and the microfluidic system is used to study DNA (22), RNA (23), and chromatin (24). The detection of two-protein interactions has also been reported (25), but the limitation of using multiple epitopes to recognize target proteins could mask important multicomponent interactions during analysis. Here, we present a multiplex flow-proteometric technique called “microchannel for multiparameter analysis of proteins in a single complex” (mMAPS) that can directly and simultaneously detect multiple components individually (including endogenous protein and nucleic acids) in a single complex requiring only a small amount of cell or tissue lysate (in the microgram range) and a short period (within a couple of hours) to quantify their interactions in tissue or cultured cell lysates. In this report, we used mMAPS to investigate the dynamics of the interaction between molecules of the EGFR signaling pathways (including EGF, EGFR, STAT3, p300, and DNA) in both cultured cells and animal tumor tissue at the single-complex level.

RESULTS

Development of mMAPS platform for single signaling complex analysis

mMAPS microchannels were integrated into a single-molecule spectroscope equipped with three avalanche photodiodes (APDs) for detecting three different fluorophores simultaneously. The fabrication of microchannels is described in Materials and Methods. Our microchannel is essentially a much smaller capillary that has a minimum cross section of 2 μm in width and 500 nm in depth at the detection spot that focuses single molecules to the same plane that can be detected clearly with APDs (Fig. 1A). The flow driving the target molecules through the detection spot in the microchannel is triggered by electroosmotic force (17, 26) to provide a uniform velocity profile along the width of a channel. Each spectral range for the three detectors was assigned according to the available excitation and emission spectra of current commercial fluorophores. The fluorophores that best suited our applications were green fluorescent protein (GFP), Alexa 488 (A488), Qdot605 (QD605), Alexa 647 (A647), and TOTO3. The system was designed to detect up to three different target molecules simultaneously and distinguish a detected event containing one or multiple targets. We specified the term “event” to describe a detected fluorescent burst that is recognized as a fluorescent molecule passing through the detection spot. During detection, if an event emits three different designated fluorescent signals coincidentally when the sample flows through the detection spot of the microchannel, this indicated that three different target molecules existed in a single complex, whereas two signals indicated a two-molecule complex, and a single fluorescent signal indicated a lone molecule that was not in complex with any of the other target proteins (Fig. 1B). Samples were flowed for a certain period to ensure that a sufficient number of events are captured for analysis. Each event is then plotted on a multidimensional space according to its photon counts of different colors. For example, the x and y values of an event on a two-dimensional fluorescence plot (2D plot) may represent the photon counts of green and red fluorophores tagged to the target molecules. A

dual-fluorophore event indicates a complex containing the two target molecules and is plotted on the *xy* plane (not on the axes), whereas a single-fluorophore event indicates a noninteracting molecule and is plotted on the appropriate axis. Therefore, two target molecules can exist in up to three types of populations—each molecule alone or as a complex, and three target molecules can exist in up to seven populations. The interaction ratio of a complex to a certain target molecule is determined by the quantities of the corresponding populations.

Direct analysis of exogenous protein complexes in cell lysates

Conventional assays to detect biomolecular interactions often involve complicated procedures (such as purification, pull down, electrophoresis, membrane transfer, and antibody incubation), which may affect the detection of target molecules and complexes. In addition, they cannot observe the targets at the single-molecule level, nor can they provide absolute quantification of these targets. Therefore, we tested whether mMAPS could directly analyze target molecules and associated complexes in a fresh cell lysate without any purification and separation processes. We chose the EGF-EGFR interaction as a model because the ligand-receptor interaction between EGF and EGFR has not yet been directly observed at the single-complex level. To detect individual EGF-EGFR complexes, we expressed GFP-tagged EGFR (EGFR^{GFP}) in HeLa cells for 24 hours and then serum-starved the cells overnight before stimulation with A647-conjugated EGF (EGF^{A647}, 100 ng/ml) for 30 min before harvesting (fig. S1). The cell lysate was diluted to 1 µg/ml with detection buffer, and 100 µl of the diluted lysate was loaded to the microchannel for mMAPS analysis. During a 20-min period of data collection, photon bursts of GFP (representing EGFR^{GFP}) and A647 (representing EGF^{A647}) were recorded and analyzed individually; a 15-s photon burst detection for both EGFR^{GFP} and EGF^{A647} is shown in Fig. 2A. To avoid counting the ambiguous bursts that might come from off-target or autofluorescence signals, a green or red photon burst indicated an EGFR^{GFP} (green) or EGF^{A647} (red) molecule if the photon intensity of the burst was three times stronger than the noise (described in Materials and Methods). The binding of EGFR^{GFP} to EGF^{A647} was determined by the coincidence of GFP and A647 signals (Fig. 2B). These events are shown in a 2D plot to demonstrate the distribution and amount of fluorescence detected. As expected, among the several EGFR^{GFP} events identified, no EGF^{A647} or EGF^{A647}-EGFR^{GFP} event was observed in the untreated HeLa lysate (Fig. 2C and fig. S2A). After EGF^{A647} stimulation, additionally both EGF^{A647} and EGF^{A647}-EGFR^{GFP} events were detected and quantified (Fig. 2D and fig. S2B): about 8% of EGFR^{GFP} molecules interacted with EGF^{A647} after treatment (Fig. 2E and table S1A). These findings indicate that the EGF-EGFR interaction from a single complex can be directly observed and quantitated from cell lysate and that mMAPS can be applied to protein-protein interaction for fluorescent-conjugated molecules in cells.

The interaction between protein and DNA is another key element in signal transduction. To test the mMAPS analysis of protein-DNA interactions, we used p53 as a model. p53 is an extensively studied transcription factor that binds predominantly to DNA to activate the expression of many tumor suppressor genes (27–29). To validate that genomic DNA fragments in a cell lysate can be detected by mMAPS, HeLa cell lysates were sonicated to shear genomic DNA, which was then labeled with TOTO3. We then loaded the stained

lysate onto the microchannel to detect individual DNA fragment signals (fig. S3A). The size of the fragmented DNA was determined by electrophoresis to be about 500 base pairs (fig. S3B), which is suitable for traditional ChIP analysis of protein-DNA interactions. To test whether mMAPS could detect DNA-bound p53, we expressed p53^{GFP} in HeLa cells, sonicated the resulting lysates, labeled genomic DNA fragments with TOTO3, and analyzed their simultaneous detection by mMAPS (Fig. 3A). 2D plots of the fluorescence distribution and the quantities of p53^{GFP}, p53^{GFP}-DNA, and DNA-only events (Fig. 3B and fig. S4) showed that, on average, 8.33% of the total p53^{GFP} was in a complex with DNA (Fig. 3C and table S1B).

We were also able to detect posttranslational modifications of individual molecules in cell lysates using mMAPS. In lysates from HeLa cells expressing GFP-labeled p53 (p53^{GFP}), we used antibodies against p53 phosphorylated at Ser¹⁵ (pSer¹⁵) or p53 monomethylated at Lys³⁷² (meLys³⁷²) to detect the total number of pSer¹⁵ or meLys³⁷² molecules (events) that were coincident with p53^{GFP}. Among the total p53^{GFP} events identified, 45.9% were Ser¹⁵-phosphorylated (Fig. 4, A and B, fig. S5A, and table S1C), and in a parallel experiment, 42.94% of total p53^{GFP} events were Lys³⁷²-methylated (Fig. 4, C and D, fig. S5B, and table S1C). We did not detect any nonspecific binding between a control immunoglobulin G (IgG) and p53^{GFP} (Fig. 4, E and F, fig. S5C, and table S1C). Together, these results showed that phosphorylation and methylation of exogenous p53^{GFP} and its interaction with DNA can be detected and quantified rapidly at the single-molecule level by mMAPS. Thus, the method may be applied to detect and quantify protein-DNA interactions and posttranslational modifications for any protein or modification of interest.

Analysis of an endogenous, single signaling complex in cultured cells

Although direct protein-protein and protein-DNA interaction analysis from ectopically expressed GFP fusion proteins in lysates is rapid and specific, it does not reflect the true physiological condition in cells because of exogenous stresses that the expression of GFP-labeled protein can induce (30). Therefore, to analyze endogenous signaling complexes to better capture the physiological context, we developed a methodology similar to the process of immunocytochemistry (fig. S6). We first used EGFR^{GFP} to validate the feasibility of using an EGFR antibody to recognize EGFR in the mMAPS system. HeLa cells expressing EGFR^{GFP} were fixed, permeabilized, and incubated with one of two EGFR antibodies that recognize either the intracellular C-terminal domain (clone D38) or the extracellular N-terminal domain (clone ab13) of EGFR followed by an A647-conjugated secondary antibody; antibody binding was confirmed by immunofluorescence (fig. S7A). Samples were loaded onto mMAPS for analysis, and both GFP and A647 events were quantified (fig. S7B). Where both GFP and A647 signals were emitted coincidentally, EGFR^{GFP} interacted with the antibody (fig. S7C). A substantial number of EGFR^{GFP} events were coincident with those of the antibody (fig. S7D); on average, 57.08% of EGFR^{GFP} proteins were recognized by the D38 antibody (fig. S7E and table S1D), and more than 50% of EGFR^{GFP} were bound by the ab13 antibody (fig. S8 and table S1E). We detected no interaction with an antibody against the Flag tag by immunofluorescence (fig. S9A) and very little by mMAPS analysis (fig. S9, B and C, and table S1F). An antibody against STAT3 also recognized 93.05% of

STAT3^{GFP} expressed in HeLa cells, whereas only 1.16% of STAT3^{GFP} events were recognized by a control IgG (fig. S10 and table S1G).

These results validated the feasibility of using fluorescent immunolabeling in mMAPS analysis. Therefore, we investigated the functional and dynamic interaction between EGF^{A647} and endogenous EGFR in cultured cells using EGF-stimulated autophosphorylation of EGFR at Tyr¹⁰⁶⁸ (pTyr¹⁰⁶⁸) as a marker of EGFR activation. We treated serum-starved HeLa cells with EGF^{A647} for 30 or 120 min and quantified the amount of EGFR alone (phosphorylated or unphosphorylated) or EGFR in complex with EGF^{A647} (Fig. 5A, fig. S11, and table S1H). As expected, neither EGF-EGFR-pTyr¹⁰⁶⁸ nor EGF-EGFR complexes were detected before EGF^{A647} stimulation; on average, only 0.94% of EGFR events were phosphorylated at Tyr¹⁰⁶⁸ (Fig. 5B, left). After EGF^{A647} stimulation for 30 min, both EGF-EGFR and EGF-EGFR-pTyr¹⁰⁶⁸ events were identified, accounting for 4.35 and 2.64% of total detected EGFR, respectively; the proportion of total EGFR phosphorylated at Tyr¹⁰⁶⁸ also increased to 7% (Fig. 5B, middle). After EGF^{A647} stimulation for 120 min, EGF-EGFR, EGF-EGFR-pTyr¹⁰⁶⁸, and EGFR-pTyr¹⁰⁶⁸ slightly decreased to 3.3, 1.27, and 6.09%, respectively (Fig. 5B, right). In the late phase of EGF stimulation, EGF will dissociate from EGFR, and EGFR will either undergo degradation or recycle back to the cell surface (31). Indeed, the ratio of EGF-EGFR-pTyr¹⁰⁶⁸ was lower than the ratio of EGFR-pTyr¹⁰⁶⁸ with EGF unbound (Fig. 5B, right) at the end of stimulation, which is consistent with the internalization and subsequent release of EGF in endosomes. These results demonstrate that the mMAPS platform can detect and quantify individual endogenous proteins and complexes with or without posttranslational modification in cultured cells, which may be used to assess the dynamics of complex formation and protein modifications simultaneously, which cannot be quantitatively detected by conventional colocalization imaging or by coimmunoprecipitation.

Direct single signaling complex analysis of tissue lysates

Analyzing protein complexes directly from tissue is ideal for understanding signal transduction in organisms and reduces the artificial effects introduced by cell culture. However, it has also been challenging to analyze signaling complexes directly from tissue because of the limited amount of sample that can be acquired. To demonstrate that single signaling complexes can be directly detected in lysates from fluorescently labeled frozen tissue sections, we inoculated EGFR-expressing MDA-MB-468 human breast cancer cells into the mammary fat pads of nude mice and collected the tumors formed therein. Tumor tissues were harvested, frozen, sliced into sections, immunolabeled, and lysed in a procedure similar to harvesting endogenous samples from cultured cells (fig. S6). We first detected the EGFR-STAT3 interaction complexes in tumor tissues because STAT3 is an important EGFR downstream transcription factor, as described in the previous section. Tumor tissues were hybridized with antibodies to EGFR and STAT3, followed by A647- and A488-conjugated secondary antibodies, respectively. Endogenous EGFR and STAT3 in the tumor tissue were visible in the fluorescence images (fig. S12A), but the interaction between EGFR and STAT3 in a complex could not be determined and quantified simply by the fluorescence colocalization. Thus, we analyzed the tissue section samples by mMAPS. The individual EGFR (A647) and STAT3 (A488) photon burst events enabled the detection of

EGFR alone, STAT3 alone, or an EGFR-STAT3 complex (fig. S12, B and C). Each individual event was plotted on the 2D plot to determine the quantities of EGFR, STAT3, and EGFR-STAT3 events (Fig. 6A and fig. S12D). Compared to the specific target events, the number of nonspecific binding events by IgG control was detected in relatively low amounts (Fig. 6B and fig. S12E), and no coincident event was identified. These results revealed that, on average, 3.01% of EGFR interacted with STAT3 in xenograft tissue, and these interacting events occupied 10.44% STAT3 that interacted with EGFR (Fig. 6C and table S1I). In addition, considering the labeling efficiencies determined for the antibodies used in the tissue [clone D38 against EGFR and clone ab5073 against STAT3; 57.08 and 93.05%, respectively (figs. S7 and S10)], the numbers of EGFR, STAT3, and EGFR-STAT3 events were normalized to 3.25% of total EGFR events (3.01% before normalization) and 18.36% of total STAT3 (10.44% before normalization) (fig. S13). These results demonstrate that mMAPS can calculate the specific number of protein interactions in tissue with normalization based on the labeling antibody efficiency. It is difficult to measure and normalize the amount of interaction using conventional methods, such as colocalization assays.

STAT3 is a transcription factor that recruits the transcription coactivator p300 and binds to DNA (32–35). It has also been reported that p300-mediated STAT3 acetylation can stimulate STAT3 binding with DNA (36). Therefore, we selected STAT3, p300, and genomic DNA as targets to detect and quantify individual complexes in xenograft tumor tissues. Endogenous STAT3 and p300 in frozen tumor tissue were immunolabeled with A488 and QD605, respectively, and genomic DNA was labeled with TOTO3 (fig. S14A). The photon bursts of STAT3, p300, and genomic DNA fragment events (fig. S14B) and the combinations of typical photon bursts of events detected both the individual signaling molecules (STAT3 alone, p300 alone, and DNA alone; fig. S14C) and the complexes (STAT3-p300, STAT3-DNA, p300-DNA, and STAT3-DNA-p300 interactions; fig. S14C). These data demonstrated that single complexes containing endogenous STAT3, p300, and DNA can be directly detected from a tumor tissue sample. All the detected events were presented in a 3D fluorescence plot, revealing seven different types of events (Fig. 7A and fig. S14D). A minimal number of nonspecific control IgG events were detected, and no interaction events were observed, indicating low background noise (Fig. 7B and fig. S14E). Among all of the STAT3 events, on average 7.04% interacted with both p300 and genomic DNA in the same complex, 2.99% interacted with p300 only, and 15.23% interacted with DNA only (Fig. 7C and table S1J). Thus, in the frozen xenograft tissue section, around 22% of STAT3 (15.23% STAT3-DNA + 7.04% STAT3-p300-DNA) interacted with DNA, and 7.04% of STAT3 interacted with p300 when bound on DNA to activate gene transcription. Looking at the data from the standpoint of p300, on average, 5.88% of total p300 protein molecules interacted with STAT3 and genomic DNA, 2.5% with STAT3 only, and 3.06% with DNA only (Fig. 7C and table S1J). All these data simultaneously obtained in each experiment not only identified STAT3, p300, and DNA in a single complex but also quantified the distribution of lone proteins and population complexes, which cannot be analyzed by conventional methods.

DISCUSSION

Signal transduction plays important roles in cell physiological functions, in which molecules form individual complexes to deliver signals and execute the functions. Therefore, analysis of individual complexes will be the key to dissect the molecular mechanisms of signaling networks. Our development of mMAPS enabled the detection and quantification of individual target proteins and complexes between proteins or between proteins and DNA comprehensively, such as identifying and quantifying seven scenarios from the combination of three different fluorophore-labeled targets. We have shown its application in the analysis of both ligand-receptor and protein-DNA interactions at the single-complex level, using less than 1 μ g of the lysate and without cell immobilization or post-lysis antibody labeling when detecting exogenous proteins, so the sample can be analyzed immediately from harvest. The technique can likely be applied to a cell fraction sample, such as the nucleus, cytoplasm, membrane, and organelles (37).

The use of immunolabeling has enabled the detection and analysis of individual endogenous signaling complexes, but a major concern for immunolabeling is that the target protein labeling efficiency is varied among different antibodies, and there is currently no precise method to assess the labeling efficiency from *in vivo* samples. Through mathematical analysis of fluorescent-tagged and antibody-labeled proteins in mMAPS, we were able to quantify antibody labeling efficiency, enabling a more accurate quantification, rather than estimation, of the number of protein molecules existing in complexes.

We tested the technique by detecting interactions and posttranslational modifications in individual complexes containing EGFR or p53, but mMAPS has the potential to be applied to any target molecule that has an available antibody. One should note that the posttranslational modifications were detected when the target protein complex was still in its native structure. Therefore, it is possible that some modification events were not accessible because of obstruction caused by complex structures. In particular, mMAPS simultaneously detects and quantifies up to three signaling molecules singly or in each possible two-protein or three-protein complex, such as those involving a ligand (EGF), its receptor (EGFR), and an intracellular transducer (STAT3); thus, mMAPS may enable further dissection of the dynamics of signaling transduction. In addition, this technique may also be applied to investigate mutant proteins and their signaling interactions in tumor tissue directly compared with that of wild-type proteins.

Together, our data provide the proof of concept for single signaling complex analysis through the microfluidic system. Because the microchannel that defines the detection volume in mMAPS is based on microfluidics technology, it has the potential to improve throughput, automated data processing, and electronic flow control. It might also be used for clinical sample analysis to identify specific signaling complexes as potential diagnostic markers for cancer. These possibilities suggest that this type of flow-proteomic-based single signaling complex detection platform may be a unique method for in-depth signal transduction analysis and provide opportunities for future high-throughput screening and molecular diagnosis.

MATERIALS AND METHODS

Xenograft mouse model and cell culture

All animals were maintained in an animal facility, and experiments were carried out according to The University of Texas MD Anderson Cancer Center guidelines under protocol #190511133. Nude mice were inoculated with 5×10^6 MDA-MB-468 human breast cancer cells in the mammary fat pad. After the tumor size reached 8 mm in diameter, tumors were harvested, embedded in optimum cutting temperature compound, frozen, and sectioned. HeLa cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium/F-12 medium with 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 µg/ml). For EGF stimulation, HeLa cells were cultured overnight under serum starvation, then incubated with EGF^{A647} (Life Technologies; 100 ng/ml) for 30 min. For EGFR^{GFP}- and p53^{GFP}-expressing cells, EGFR^{GFP} or p53^{GFP} expression plasmid was transfected into HeLa cells using liposomes 24 hours before sample preparation and harvesting (38).

Preparation of cell and tissue lysates

For the analysis of complexes between exogenous, tagged proteins in lysates, culture medium was removed, and cells were washed with phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde (PFA) at room temperature for 10 min, and extracted with radioimmunoprecipitation assay buffer [50 mM tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA] (39). Sample lysates were sonicated to shear the genomic DNA into fragments. One microgram of lysate was diluted in 1 ml of detection buffer [20 mM Hepes-KOH (pH 7.9), 0.1 mM KCl, 2 mM MgCl₂, 15 mM NaCl, 0.2 mM EDTA, 10% glycerol]. If DNA staining was required, sample lysate was stained with 20 nM TOTO3 (Life Technologies) for 20 min to label genomic DNA fragments for analysis (fig. S1). For the analysis of complexes between endogenous proteins in lysates, frozen tissue sections (8 µm in thickness) or cultured cells were fixed in 1% PFA at room temperature for 10 min, washed with PBS, and treated with 2.5% Triton X-100 for 10 min at room temperature. Samples were then blocked with 6% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. After blocking, specific antibodies [STAT3 (ab5073), Abcam; p300 (C20), Santa Cruz Biotechnology; EGFR (D38), Cell Signaling; EGFR (ab13), NeoMarkers; p53 pSer¹⁵ (9284), Cell Signaling; p53 meLys³⁷² (ab16033), Abcam; EGFR-pTyr¹⁰⁶⁸ (1H12), Cell Signaling] or control antibodies (Flag-tag antibody, Roche; normal rabbit IgG, Santa Cruz Biotechnology; normal goat IgG, Santa Cruz Biotechnology) for designated experiments were diluted in BSA and incubated with samples overnight at 4°C. Samples were then washed with PBS to remove the unbound antibodies before incubation with secondary fluorescent antibodies. Secondary antibodies, including donkey anti-goat A488, donkey anti-rabbit QD605, donkey anti-rabbit A647, donkey anti-goat A647, goat anti-mouse A647, goat anti-rabbit A647, goat anti-mouse QD605, and goat anti-rabbit A488 (Life Technologies), were diluted (1:250) and mixed in BSA according to the experimental design and incubated with samples at room temperature for 2 hours. Samples were washed with PBS three times and then stained for genomic DNA with TOTO3 (1:100 in PBS; Life Technologies) at room temperature for 20 min, if analysis for DNA interaction was required. To prevent the possibility of dissociation between antibody and target protein, samples were

subjected to a second fixation process with 1% PFA to fix the interaction between target proteins and antibodies. After samples were imaged by fluorescence microscopy, they were lysed and extracted with radioimmunoprecipitation assay buffer and further diluted in detection buffer. Sample lysates were sonicated with Bioruptor (Diagenode) at high intensity 10 times at 30-s cycles to degrade the cell structure and shear genomic DNA into fragments. Lysates were then centrifuged at 16,000g for 1 min to remove large debris, and the supernatant was used for analysis.

Microchannel fabrication

The microchannels were made on an ultraviolet-grade fused silica wafer using photolithography and wafer bonding as described previously (19). In brief, a wafer (500 μm in thickness) was coated with a thin layer of photoresist, which lithographically defined the microchannel pattern. A 500-nm-deep microchannel pattern was created after CF_4 plasma etching. Subsequently, the photoresist layer was removed, and a cover-fused silica wafer (170 μm in thickness) with reservoir holes (drilled by sand blaster) was thermally bonded to the patterned wafer to seal the entire microchannel. The plastic reservoirs were then glued to the hole as inlet and outlet of samples. The width of the microchannel at the detection spot was 2 μm . Each microchannel was coated with 0.5% polyethyleneimine dissolved in detection buffer for 16 hours to prevent protein adsorption on the channel surface before analysis. The fabricated microchannels in this study were all used only once. The microchannel was potentially reusable with appropriate cleaning process including sample cleaning sonication and high-temperature (1000°C) oven incubation, but its efficiency might be affected by repeated use.

Detection of individual complexes

The sample lysate was loaded onto the reservoirs of the microchannel, and a 150-V potential was applied to generate electroosmotic flow. The electrical conductivity of the sample solutions was maintained to ensure a constant flow velocity for all detections (22). Before analysis, the sample lysate was allowed to flow through the microchannel for 20 min to ensure that the flow was stable for detection. Three excitation lasers with wavelengths of 405, 488, and 637 nm were focused through a 60 $\times$ water immersion objective (numerical aperture, 1.20) at the center of the microchannel. As single protein complex passed through the focus spot, time-coincident fluorescent emissions from the components of the complex were detected with a single-molecule spectroscope equipped with three sensitive avalanche photo diodes (Alba, ISS) for observing green (A488 or GFP), red (QD605), and far-red (TOTO3 or A647) photon signals. Specific optical filters (531/40, 605/15, and 685/40) were installed in the system to prevent signal leakage between different fluorophores during detection. Signals from green fluorescence crossed to detectors for red and far-red fluorescence had not been observed (fig. S15A). Far-red fluorescence also did not leak signals to detectors for green and red fluorescence (fig. S15B), and red fluorescence also has no obvious leakage to other detectors (fig. S15C). For sample detection, three sets of photon burst data were analyzed for quantification of target signaling complexes. Nonspecific interaction between target proteins and normal IgG in each set of experiments served as control of the specificity of mMAPS detection.

Analysis and quantification of complexes

Raw data were collected at a rate of 100 kHz, and each data point represents the photo count within 10 μ s. A custom-made program further compressed the data to 1 kHz to facilitate burst calculation. Before seeking a valid fluorophore signal, a basal noise level had to be defined so that the start and end time of a burst could be located. This noise level was defined by the following steps. The points of a compressed data file were first divided into several 100-point long sections sequentially. For each section, we calculated its average (m), SD (σ), and local noise level ($m + 3\sigma$). The minimum 10% of these local noise levels were selected. The average of the selected noise levels then served as the basal noise level. Another event selection line that was three times higher than the basal noise level was applied to filter out lower and ambiguous bursts. Only the bursts higher than the event selection line were viewed as valid burst signal events and counted. We used the term “event” to specify such burst when it was associated with a molecule. A coincidence of two-color bursts (events) was defined as any overlapping of the bursts (events) of two different colors. Similarly, a three-color coincidence was defined as any overlapping of a two-color coincidence and the third color burst (event). The numbers and ratios of three-color and two-color coincidences were calculated on the basis of the above definitions. One of the colors could serve as the internal control if the absolute number of events comparison among different samples was needed. The internal control could be genomic DNA fragment, tubulin, or actin.

Labeling efficiency and normalization

Target molecules were labeled in cells with GFP tags, and the labeling efficiency of the specific antibody was acquired by measuring the ratio of antibody that recognized the GFP fusion target protein to that which did not. Mathematical formulae were devised to account for the various coincident populations of GFP-tagged proteins and endogenous (non-fluorescent) proteins. These equations and further descriptions are provided in fig. S16.

Supplementary Material

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Fig. 1. Schematic diagram of mMAPS single signaling complex detection

(A) After labeling fluorophores to target molecules, sample lysate was loaded into the microchannel and flowed through the detection spot in the center of the microchannel. Target molecules or complexes were excited by three lasers with wavelengths of 405, 488, and 637 nm. Emission fluorescence was observed by three APDs, which specifically detect three different spectral ranges (531/40 nm for GFP and A488, 685/40 nm for A647 and TOTO3, and 605/15 nm for QD605). The photon burst signals were then analyzed to determine the complex formation. (B) Diagram of photon bursts of the detected complex. Left: All three fluorophores were detected simultaneously, indicating that three target molecules were within a complex. Middle: Two of the three fluorophores showed coincident signals, indicating a two-target molecule interaction. Right: No coincident signal represents individual target molecule alone.

Fig. 2. Direct lysate analysis of EGF-EGFR complex in cells overexpressing EGFR

After HeLa cells overexpressing EGFR^{GFP} were treated with EGF^{A647} (100 ng/ml) for 30 min, the lysate was harvested and directly analyzed by mMAPS. (A) A 15-s photon burst profile showing EGFR^{GFP} and EGF^{A647} signals. (B) Representative zoom-in views of typical events over a 0.5-s time frame. Top: EGFR^{GFP} not bound to EGF^{A647}. Bottom: Coincidence of EGFR^{GFP} and EGF^{A647} signals, indicating the complex. (C and D) Representative 2D plot showing the fluorescence of detected events during a 20-min flow-proteometric analysis without (C) or with (D) EGF treatment. (E) Ratio of EGF^{A647}-bound EGFR^{GFP} ($\frac{\text{“EGF}^{A647}\text{-EGFR}^{GFP}\text{” interaction events}}{\text{total EGFR}^{GFP}\text{ interaction events}} \times 100$; “EGF^{A647}-EGFR^{GFP}” means EGF^{A647} and EGFR^{GFP} interaction complex; total EGFR^{GFP} events included unbound and EGF^{A647}-bound EGFR^{GFP} events) to all detected EGFR in the presence and absence of EGF. Data are means ± SEM of three independent experiments (others shown in fig. S3).

Fig. 3. Detection and quantification of individual p53^{GFP} and genomic DNA interaction
(A) Representative photon bursts of a p53^{GFP}-alone event (left) and an event of p53^{GFP} interacting with genomic DNA (right) in a lysate from HeLa cells expressing p53^{GFP} and stained with the DNA dye TOTO3. **(B)** Representative 2D plot showing the fluorescence distribution and the quantities of p53^{GFP}, genomic DNA fragments, and the interaction complexes. Each plot represents a 20-min flow-proteometric analysis, and the detection was repeated three times. The numbers in parentheses are the quantities of specific events. **(C)** Proportion of genomic DNA-bound and unbound p53^{GFP} among all detected p53^{GFP} ($\frac{\text{"p53}^{\text{GFP}}\text{-DNA" interaction events}}{\text{total p53}^{\text{GFP}} \text{ events}} \times 100$). Data are means $\pm$ SEM of three independent experiments.

Fig. 4. Detection and quantification of individual p53^{GFP} with Ser¹⁵ phosphorylation or Lys³⁷² methylation

(A) Representative 2D plot showing the fluorescence distribution and the quantities of p53^{GFP} and p53^{GFP}-pSer¹⁵ events in HeLa cells expressing p53^{GFP}. Each plot represents a 20-min flow-proteomic detection, and the numbers in parentheses are the quantities of specific events. (B) Ratio of p53^{GFP} labeled with the pSer¹⁵ antibody to all detected p53^{GFP} events ($\frac{\text{p53}^{\text{GFP}} \text{ pSer}^{15} \text{ events}}{\text{total p53}^{\text{GFP}} \text{ events}} \times 100$). (C) Representative 2D plot showing the fluorescence distribution and the quantities of p53^{GFP} and p53^{GFP}-meLys³⁷² events. (D) Ratio of p53^{GFP}-meLys³⁷² among all detected p53^{GFP} ($\frac{\text{p53}^{\text{GFP}} \text{ meLys}^{372} \text{ events}}{\text{total p53}^{\text{GFP}} \text{ events}} \times 100$). (E) Representative 2D plot of p53^{GFP} and control IgG events. (F) Ratio of control IgG-labeled p53^{GFP}. Data are means ± SEM of three independent experiments.

Fig. 5. Analysis of the interaction between endogenous EGFR and EGF^{A647}

(A) 2D plots showing the detection of endogenous phosphorylated (pTyr¹⁰⁶⁸) and nonphosphorylated EGFR in complex with EGF in serum-starved HeLa cells (0 min) and 30 to 120 min after addition of EGF^{A647}. The numbers in parentheses are the quantities of the indicated target-specific events. (B) Ratio of EGFR-pTyr¹⁰⁶⁸ ($\frac{\text{"EGFR-pTyr}^{1068}\text{" events}}{\text{total EGFR events}} \times 100$; yellow), EGF-EGFR ($\frac{\text{"EGF-EGFR" interaction events}}{\text{total EGFR events}} \times 100$; blue), and EGF-EGFR-pTyr¹⁰⁶⁸ ($\frac{\text{"EGF-EGFR-pTyr}^{1068}\text{" interaction events}}{\text{total EGFR events}} \times 100$; black), among all detected EGFR events from (A). EGF stimulated for 0 min (left), 30 min (middle), and 120 min (right). Data are means $\pm$ SEM of three independent experiments.

Fig. 6. mMAPS analysis of endogenous EGFR, STAT3, and associated complexes in tumor tissue sample

(A) 2D plot showing the fluorescence distribution and the quantities of EGFR, STAT3, and EGFR-STAT3 in tumor tissue during a 20-min flow-proteomic analysis. (B) 2D plot of nonspecific binding control by using normal rabbit and goat IgG as the primary antibodies to stain consecutive tissue section samples. (C) Mean ratios $\pm$ SEM of the EGFR-STAT3 interaction to total detected STAT3 ($\frac{\text{"EGFR-STAT3" interaction events}}{\text{total STAT3 events}} \times 100$) or EGFR ($\frac{\text{"EGFR-STAT3" interaction events}}{\text{total EGFR events}} \times 100$). Data are representative or means $\pm$ SEM of three independent experiments.

Fig. 7. Analysis of genomic DNA, endogenous STAT3, p300, and associated complexes in tumor (A) 3D plot of STAT3, p300, DNA, and associated complexes with their quantities and fluorescence distribution during a 20-min flow-proteomic analysis. The numbers in parentheses are the numbers of events. (B) Control samples were stained with normal goat and rabbit IgGs without TOTO3 stain. (C) Left: Mean ratios $\pm$ SEM of STAT3-associated complexes with DNA ($\frac{\text{"STAT3-DNA" interaction events}}{\text{total STAT3 events}} \times 100$), with p300 ($\frac{\text{"STAT3-p300" interaction events}}{\text{total STAT3 events}} \times 100$), or with both ($\frac{\text{"STAT3-p300-DNA" interaction events}}{\text{total STAT3 events}} \times 100$) to total detected STAT3 molecules. Right: Mean ratios $\pm$ SEM of p300-associated complexes with STAT3 ($\frac{\text{"STAT3-p300" interaction events}}{\text{total p300 events}} \times 100$), with DNA ($\frac{\text{"p300-DNA" interaction events}}{\text{total p300 events}} \times 100$), or both ($\frac{\text{"STAT3-p300-DNA" interaction events}}{\text{total p300 events}} \times 100$) to total detected p300 molecules. Data are representative or means $\pm$ SEM of three independent experiments.