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Analysis of Individual Signaling Complexes by mMAPS, a Flow-proteometric System

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Abstract

Signal transduction is essential for maintaining cells' normal physiological functions, and deregulation of signaling can lead to diseases such as diabetes and cancers. Some of the major players in signal delivery are molecular complexes composed of proteins and nucleic acids. This unit describes a technique called **microchannel for Multi-parameter Analysis of Proteins in a Single-complex** or mMAPS for analyzing and quantifying target signaling complexes individually. mMAPS is a flow-proteometric-based system that allows detection of individual proteins or complexes flowing through a microfluidic channel. Specific target proteins and nucleic acids labeled by fluorescent tags are harvested from tissue samples or cultured cells for analysis by the mMAPS system. Overall, mMAPS enables both detection of multiple components within a single complex and direct quantification of different populations of molecular complexes in one setting in a short timeframe, requiring very low sample input.

Keywords

single molecule; single complex; protein-protein interaction; protein- nucleic interaction; flow-proteometry; microfluidic

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Conflicts of Interest

C.-K.C., J.K., and M.-C.H. hold a patent for the methods for detecting molecule-molecule interactions with a single detection channel (US 8586316 B2). All other authors have no conflicts of interest to declare.

Key Reference

Chou, et al., 2014. See above.

Describes the first application of mMAPS in analyzing individual signaling complexes from tissue and culture cell samples.

INTRODUCTION

Proteins and nucleic acids often form complexes that play critical roles in signal transduction by carrying and delivering messages to coordinate fundamental biological functions. Identification of the molecular complexes is crucial to our understanding of signal transduction pathways related to disease progression (Kolch and Pitt, 2010; Papin et al., 2005). Various methods are available to detect protein–protein and protein–nucleic acid interactions, but few are able to analyze individual complexes (Auerbach et al., 2002; Cheng et al., 2004; Collas, 2009; Gavin and Superti-Furga, 2003; Kuo and Allis, 1999; Myers, 1989; Phizicky and Fields, 1995; Stenman et al., 1993; Toby and Golemis, 2001; Williams, 2000). For instance, while conventional immunoprecipitation (IP), which requires a large amount of sample, is currently the standard method for detecting protein-protein interactions, this approach cannot directly detect individual biomolecule complexes or provide an absolute number of complexes present (Jain et al., 2011; Liu et al., 2011; Thompson et al., 2012). Currently, there are several methods for detecting single signaling complexes, including fluorescence correlation spectroscopy (FCS) and fluorescence resonance energy transfer (FRET). Both of these methods can detect the association between molecules at a single molecule level using extremely low sample concentration (high dilutions) (Colyer et al., 2010; Lee et al., 2007; Michalet et al., 2010). The use of low sample input has also been integrated with microfluidic systems to study DNA (Kameoka et al., 2001), RNA (Nolan et al., 2003), protein (Chou et al., 2010), and chromatin (Cipriany et al., 2010). Recently, we reported the development of an optical microfluidic platform termed **microchannel for Multi-parameter Analysis of Proteins in a Single complex or mMAPS**, which can detect and quantify individual biomolecular complexes (protein-protein and/or protein-nucleic acids) *in vivo* directly from samples (Chou et al., 2014). It is also capable of detecting individual proteins with post-translational modifications, such as phosphorylation and acetylation.

Similar to the principles of flow cytometry, which analyzes every single cell in a microfluidic channel, mMAPS detects single molecules (Fig. 1). One of the major advantages of mMAPS is its capacity to detect target complexes in tissues and cultured cells using only a small amount of samples (in the microgram range). In mMAPS, the molecules of interest (proteins and/or nucleic acids) in tissues or cultured cells are briefly cross-linked, fluorescently labeled, lysed, sheared by sonication, and allowed to flow through the focal volume confined by the microfluidic channel device. During the flow, a highly sensitive photon-detecting instrument at the excitation laser-focused detection spot picks up the fluorescence photon burst signal from the target molecule or complex. If a complex (with two or more different target molecules) is detected, coincident photon bursts at different fluorescence wavelengths are then recorded and defined as a target molecular complex. The number of collected target molecules alone or in complexes (events) is plotted against each event's fluorescence photon counts to demonstrate the distribution of the targets and determine the ratio of interactions.

In this protocol, we present the basic design of the mMAPS system and the steps to perform a single complex analysis (see Basic Protocol 1). In addition, we also provide the basic procedures for microchannel fabrication (Support Protocol 1), optical calibration (see

Support Protocol 2) and data normalization (see Basic Protocol 2) based on the labeling antibody efficiency.

STRATEGIC PLANNING

Equipment

The highly sensitive photon-detecting instrument (Alba spectroscopy) is commercially available from ISS, Inc. (Champaign, IL) and can be custom-designed to fit multiple detectors for different fluorescence signals. The laser module for the designated fluorescence excitation is also available from ISS. The instrument requires high numerical aperture (NA) water-immersion objective lenses (preferable with 60X/1.2-NA), dichroic mirror, and optical filters that separate different fluorescence signals to the detectors, and photon detectors (avalanche photon diode, APD) to detect the designated fluorescence signals. It is preferable to apply a pinhole (50 – 70 μm) to enhance the quality of detection. The instrument can collect different fluorescence photon counts simultaneously through the corresponding APDs.

Fluorescent labeling

Since the system detects different designated fluorescence signals simultaneously, selecting the appropriate labeling fluorescence for best signal quality and specificity is critical. One should avoid using fluorophores with a wide range of emission wavelength which could cause signal leakage to non-designated detectors. For instance, a green fluorescent tag, e.g., FITC (for optical filter 525 ± 40 nm), paired with a red fluorescent tag, e.g., Alexa647 (for optical filter 685 ± 40 nm) should be used to analyze a two-molecule interacting complex to give the least signal contamination. Green fluorescence protein (GFP) fusion or chemical dyes, such as FITC and Alexa488, can be used for green fluorescence labeling. Cy5 and Alexa647 for proteins and TOTO3 for nucleic acids can be used for red fluorescence labeling. Using Alexa488 and Alexa555 together is not appropriate for analysis since the photon detector for Alexa555 will most likely also pick up fluorescence signals from Alexa488 and detect it as an Alexa555 signal, which will generate false positive complex signal.

BASIC PROTOCOL 1: SETTING UP mMAPS ANALYSIS FOR SINGLE COMPLEX MEASUREMENTS IN TISSUES AND CULTURED CELLS

This protocol describes general procedures for the use of mMAPS to analyze individual signaling complexes from lysate of tissues or cultured cells. Procedures are described based on the use of the ISS Alba system. The parameters may vary if a different system is used.

Materials

Cells

Culture dishes and media

Highly sensitive photon-detecting instrument (ISS, Alba Confocal Spectroscopy and Imaging Station)

High voltage power supply (Stanford Research Systems, PS350/5000V-25W)

Sonicator (Diagenode, Bioruptor)

NanoDrop 2000 Spectrophotometer (Thermal Scientific)

Gold electrodes (Scientific Instrument Services, W352)

UV-grade quartz microfluidic channel devices, with cross-section of 2 μm (width) by 0.5 μm (depth) at detection points (*described in support protocol 1*)

1% v/v Paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (Electron Microscopy Sciences, EMS 15710)

0.25% v/v Triton X100 in PBS (Sigma, T8787)

6% w/v BSA in PBS (Gemini BioProducts, 700100P1KG)

Lysis buffer, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM ethylenediaminetetraacetic acid

Detection buffer, 20 mM HEPES-KOH (pH 7.9), 0.1 mM KCl, 2 mM MgCl_2 , 15 mM NaCl, 0.2 mM ethylene diaminetetra acetic acid, 10% glycerol

Microchannel coating buffer, 0.5% polyethyleneimine (MW 50~100 kDa) dissolved in detection buffer (Alfa Aesar, 45024-14)

125 mM Glycine in PBS (Fisher BP3815)

CAUTION: Paraformaldehyde is toxic if inhaled or swallowed; is irritating to the skin, eyes, and respiratory system; and may be carcinogenic. Paraformaldehyde should be used with appropriate safety measures such as protective gloves, glasses, clothing, and sufficient ventilation. Waste should be handled according to local hazardous waste regulations. Detailed information can be found in MSDS.

Sample lysate preparation from cultured cells

- 1 Seed appropriate number of cells (~50–80% confluency) in a culture dish or microplate. The following procedures are based on 5×10^4 cells per well cultured in a 24-well microplate.

If using a fluorescent fusion protein or other fluorescent fusion-tagged system, transfections can be performed before or during this step.

Allow transfected cells to grow at least 24 hours to express the fusion proteins. If treatments are needed, perform them before the chemical crosslink (step 2).

- 2 Add 500 μl 1% Paraformaldehyde (PFA) in PBS to each well and incubate for 8 minutes at room temperature (RT) to crosslink the molecules in cells. Add glycine (125 mM in PBS) to stop the crosslinking reaction.

This step is to preserve the intact complexes in cells. The suggested incubation time for crosslinking is 8–10 minutes; longer crosslinking times may hamper the cell lysis process.

- 3** Gently rinse each well with 1 ml PBS for 5 minutes at RT. Repeat the wash 3 times. If the experiment is not designed to use antibody for fluorescence labeling, proceed to step 15 to lyse the cell for analysis.
- 4** Remove PBS and increase the permeability of cells by adding 500 μ L 0.25% Triton X100 in PBS to each well and incubate for 10 minutes at RT.
- 5** Gently wash each well with 1 ml PBS for 5 minutes at RT and repeat the process 3 times.
- 6** Remove PBS and block each well by adding 500 μ L 6% BSA in PBS to each well and incubate at RT for 1 hour.

If using fluorescence conjugated secondary antibody to label the target protein, the addition of normal serum (final concentration 10%) of the antibody's species into the blocking reagent may help to reduce non-specific binding.

- 7** Apply the fluorescence conjugated primary antibody or regular primary antibody (if using fluorescent conjugated secondary antibody) to the sample. The antibody is prepared with 6% BSA with proper dilution. About 250 μ l is sufficient to cover the well. Incubate the sample at 4°C overnight with gentle rotating motion on an orbital shaker. In addition to the experimental and control samples, prepare an additional sample without any fluorescently labeled molecules to serve as a blank sample. The blank sample will be used to determine the background noise level for detection.

The labeling efficiency of each antibody depends on proper dilution to obtain a working concentration (refer to the data sheet provided by the antibody manufacturer for immunostaining). Samples should be protected from light in all subsequent processes after fluorescent materials have been applied.

- 8** Gently wash each sample with 1 ml PBS for 5 minutes at RT. Repeat the wash 3 times. If using fluorescent conjugated primary antibody, proceed to step 11.
- 9** Prepare a 250 μ l fluorescence conjugated secondary antibody solution with proper dilution (typically in the range of 1:500 to 1:1000) in 6% BSA. Add the antibody solution to the sample and incubate at RT for 1 hour or at 4°C overnight.
- 10** After incubation, gently wash each well with 1 ml PBS by rotating motion for 5 minutes at RT. Repeat the wash 3 times. If nucleic acid staining is not required, proceed to step 13.
- 11** Dilute the appropriate amount of nucleic acid staining dye in PBS (e.g., TOTO3 1:500 diluted in PBS). Add the diluted dye to the sample and incubate for 20 minutes at RT.

The dilution of nucleic acid dye may vary depending on the choice of dye and experimental design. This step can also be applied to other dyes, such as lipid dye.

- 12** Gently wash each well with 1 ml PBS for 5 minutes at RT. Repeat the wash 3 times.

- 13** Remove PBS and crosslink the sample by adding 500 μ l 1% PFA to each well and incubate for 8 minutes at RT. Remove PFA and add glycine (125 mM in PBS) to stop the crosslinking reaction.

This step is performed to further preserve the interaction between antibodies and targets as an intact complex for detection.

- 14** Gently wash each well with 1 ml PBS for 5 minutes at RT. Repeat the wash 3 times.

- 15** Remove PBS and add 20 μ l lysis buffer to each sample well and scrape off the cells from the surface of the microplate.

- 16** Add 180 μ l of detection buffer into the well and carefully collect and transfer the sample lysate to a 1.5-ml microcentrifuge tube.

Perform this step carefully and collect the lysate as completely as possible.

- 17** Sonicate (Bioruptor, Diagenode) the sample lysate for ten 30-second cycles at high intensity power.

The setting is based on the use of Bioruptor (Diagenode) with an automatically programmed sonicating/cooling process. The sonication condition may be different when using other sonication systems. Refer to the procedures provided by the manufacture to optimize the sonicating conditions.

- 18** Centrifuge the sample lysate at 16,000 g for 1 minute and transfer the supernatant to a new 1.5-ml microcentrifuge tube.

This step is to remove large particles and aggregates, which may clog the microchannel and produce false positive signals. In order to prevent harvesting aggregates, it is not necessary to collect the whole volume of the supernatant. For a 200- μ l sample, collecting 150 μ l should be sufficient for analysis.

- 19** Adjust the sample lysate protein concentration to 100 μ g/ml by diluting the sample in the detection buffer. The sample is now ready for mMAPS analysis.

NanoDrop 2000 (Thermal Scientific) can be utilized to rapidly measure the protein concentration. Proper dilution is required for mMAPS because samples with high protein concentration may clog the microchannel and generate false positive reading. Generally, higher volume dilution will yield better quality data but with longer

acquisition time to accumulate a sufficient number of events for analysis. Depending on the expression level of target proteins, the sample lysate protein concentration may vary from 20 to 200 µg/ml.

Acquire mMAPS data

- 20 Load the coating buffer into one of the microchannel's reservoirs at least 2 hours before mMAPS data acquisition to reduce adsorption of biomolecules.

The coating buffer should fill the microchannel within 5 minutes. Long coating time ensures fully covering of the coating buffer inside the microchannel. Occasionally a microchannel may have obvious air gaps between coating buffer filled sections even after several hours, then this specific microchannel is marked as a defective one and not used.
- 21 Calibrate of the instrument if it is the first use of the day. (Please refer to Support Protocol 2.)
- 22 Load the sample prepared in step 19 into the microfluidic channel's reservoir with cathode attached. Load the detection buffer to the anode reservoir.

Microchannel fabrication is described in Support Protocol 1.
- 23 Place the microchannel wafer on the microscope stage.

The microchannel wafer should be tightly secured on the stage and avoid any vibration during the detection process.
- 24 Align the microchannel and focus the microchannel center area.
- 25 Apply electric voltage to generate the electroosmotic-flow (EOF).

The suggested voltage is 100–200 V (based on the dimension of microchannel listed in the Materials list), which will generate stable EOF and minimum electric current to avoid potential electrochemical reaction.
- 26 Allow the microchannel to flow for 15 minutes prior to data acquisition.

This step is to allow the sample molecules and complexes to stably flow into the microchannel.
- 27 Start data acquisition by observing the photon bursts when the target molecules/ complexes flow through the detection spot. The detection time for each sample depends on the number of events designed to collect. The software for photon detection, VistaVision 4.0, is commercially available through ISS, Inc.

SUPPORT PROTOCOL 1: FABRICATION OF MICROFLUIDIC CHANNEL DEVICES

The microfluidic channels for mMAPS analysis are made by photolithography (Campbell, 2007). General procedures for microchannel fabrication are described here. The entire process must be conducted in a cleanroom environment.

Materials

Photomask with custom-designed microchannel patterns (Cornell NanoScale Science & Technology Facility (CNF))

Ultraviolet (UV)-grade quartz wafers, one 500- μm in thickness for patterning the microfluidic channels, and one 170- μm in thickness for enclosing the channels; both are 4 inches in diameter (Mark Optics)

Photoresist (Microposit, S1813 positive photoresist)

Developer (Microposit, MF-319 developer)

Piranha solution ($\text{H}_2\text{SO}_4\text{:H}_2\text{O}_2 = 3\text{:}1$ v/v), for cleaning the wafer surface (J.T. Baker, 9681 and 2186)

Spin coater (Laurell, WS-650S Spin Processor)

Mask aligner (Karl Suss, MA6 Mask Aligner)

Reactive ion etcher (RIE) system (Oxford, Plasmalab 100 ICP RIE System)

Drilling facility, for creating 1–2 mm holes on the 500- μm wafer (Ryobi Drilling Press)

Furnace, which can reach 1,000 °C to permanently bond the wafers (Cole-Parmer, EW-33855-30)

Reservoirs (Western Analytical, NanoPort Reservoir Assemblies N-131)

Microchannel Fabrication

1. Wear a cleanroom suit before entering the cleanroom.
2. Clean a brand new 500- μm thick quartz wafer. (step A in Fig. 2).

Hold the wafer near vertically with a tweezers. Rinse both sides of the wafer thoroughly with acetone, followed by isopropanol. Finally rinse the wafer with water, and blow dry the surfaces with pressurized nitrogen.
3. Place the wafer on the chuck of the spin coater, and spin coat 1.5- μm thick photoresist on the wafer at 1,950 rpm for 50 seconds. (step B in Fig. 2)
4. Soft bake the wafer at 115 °C for 3 minutes.
5. Place the photoresist-coated wafer and the photomask on the stages of the mask aligner and align both. Follow the photoresist instruction manual to determine the appropriate dose of UV exposure. (step C in Fig. 2)

The photomask can be made by using an academic fabrication facility in person (e.g. Cornell CNF http://www.cnf.cornell.edu/cnf_process_mask_equipment.html) or by ordering from a photomask manufacturer (e.g. Photo Sciences, Inc. https://dev.photo-sciences.com/how_to_order_photomasks). The microchannel pattern (which is composed by a 2- μm width and 20- μm length central section for detection, and 10- μm width and 5-mm length connection sections on both sides for inlet/outlet) on photomask has to be designed first using software tools,

such as L-Edit (Tanner EDA) or AutoCAD (Autodesk, Inc.). The drawing should follow the design rules provided by the facility or the manufacturer. For the choice of mask material, fused silica is chosen to ensure low thermal expansion and good transmission of UV light. Please refer to the indicated websites and consult with the facility/manufacturer for the photomask construction details.

The dose of UV exposure will determine the quality of the transferred pattern.

6. Immerse the wafer in the developer for 1 minute to remove unwanted photoresist. (step D in Fig. 2)
7. Hard bake the wafer at 150 °C for 10 minutes.
8. Place the wafer in the chamber of the RIE system with the photoresist-coated side up. Etch the wafer using these parameters: 20 mTorr chamber pressure, 150 W RF power, 25 °C, and 20 s.c.c.m (standard cubic centimeters per minute) for 25 minutes to etch 500-nm depth trenches. (step E in Fig. 2)

This step is critical because it will determine the depth of the microchannel.

9. Remove the photoresist by immersing the wafer in clean acetone. (step F in Fig. 2)
10. Rinse the wafer with isopropanol followed by water to remove any photoresist residues.
11. Drill holes for inlets and outlets on the channel devices. (step G in Fig. 2)
12. Clean both 500- μ m and 170- μ m wafers by immersing them in piranha solution twice: 5 minutes for the first immersion to remove any residues on the surface and then 10 minutes for the second immersion to produce hydroxyl group for the bonding surface.
13. Rinse the wafers with water.
14. Immerse the two wafers in water. While still in water, put the two wafers together (starting from the edges to avoid air bubbles) for temporary bonding. Leave the attached wafers in water for 3 days.

This step needs to be performed with extra caution to prevent any air bubbles from forming in the bonded wafer. Any bubbles formed within the microchannel will damage the pattern and cause irregular micro-flow.

15. Move the attached wafers to the furnace, and thermally bond the two wafers at 1050 °C for 10 hours. (step H in Fig. 2)
16. Attach reservoirs at the inlets and outlets after the wafers cool to room temperature. (step I in Fig. 2)
17. The microfluidic channel devices are ready to be used.

Small bubbles may still appear between the bonded wafers; a microchannel connecting to the bubbles is marked defective and will not be used.

SUPPORT PROTOCOL 2: OPTICAL CALIBRATION OF THE INSTRUMENT

This protocol describes the procedures for optical calibration (in the ISS VistaVision environment), which includes aligning the lens and pinholes on the three detectors. These two parameters (lens and pinholes) are mentioned explicitly because their positions may deviate due to minor vibration. It is important to optimize both the lens and pinholes' position before running the instrument for mMAPS analysis. The same principle also applies to different optical systems either by automatic or manual adjustment.

Materials

10 nM of individual Alexa Fluor Dye solution: Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647 in PBS (Life Technologies, A11029, A21434, and A21236)

Nunc Lab-Tek Chambered Coverglass, 8-well (Thermo Scientific, 12565470)

1. Load 300 μ l Alexa Fluor stain solution into one well of the chambered coverglass. Place the chamber on the objective lens and protect from light after this step.
2. Apply the designated excitation laser when optimizing a specific fluorophore. Begin with low power laser to avoid photobleaching the fluorophore and damaging the APDs.
3. On the main operating screen of the system (VistaVision, ISS Inc.), click on Motor Alignment under the Device Control tab.
4. Under the Pinholes & Lens tab, choose the designated fluorescence channel to be calibrated.
5. The steps for calibrating pinholes and lens are similar: first select Pinhole option and decide whether to allow software to perform the optimization automatically (Auto tab), or manually (Manual tab). Both choices involve moving the pinholes/ lens slightly up/down and left/right to identify the best position that maximizes the photon counts. Calibrate pinholes first followed by lens using the same procedures.

For automatic calibration, three items are shown: Steps, Time (msec), and Methods, representing the farthest distance to go from the original position, the time duration to calculate the photon count, and the algorithm to be used, respectively. Default settings work for most conditions.

For manual calibration, specify the increment according to the size of pinhole (or lens), e.g., a 10- μ m increment for a 50- μ m pinhole. While the acquisition is ongoing, move the pinholes (or lens) in different directions incrementally, and observe the changes in signal level. Tune the position of the pinhole and the lens to the center of the maximum photon counts level. Repeats the process a couple of times to optimize calibration.

The calibrated position of pinholes and lenses should result in maximum readings of photon counts emitted from the fluorophores. The optical system is now ready for single complex analysis.

BASIC PROTOCOL 2: ANALYZING mMAPS DATA

In mMAPS, a photon burst appearing on a certain color wavelength channel represents a fluorescently labeled target protein. A coincidence among different color bursts is defined as a complex of different target molecules. mMAPS directly counts bursts from raw data in the time domain without using any complicated mathematical equations. Therefore, the analysis can be performed with common spreadsheet software. This protocol describes the procedures of analyzing data with up to three color channels, e.g., 685 ± 40 , 605 ± 15 , and 525 ± 40 nm in emission wavelength.

Preprocessing acquired data

An output three-channel data file consists of several components, including time points and the photon counts from each color channel. The first step is to extract photon bursts from raw data of individual color channels. The raw data usually contains a large number of data points taken every second, meaning it has very high temporal resolution with many details. For slow-moving fluorescent particles in mMAPS, this high temporal resolution is not necessary, but the bulky data size may slow the analysis. Preprocessing the acquired data reduces the size of data and time spent on analysis.

- 1 Sequentially sum the photon counts from the raw data for each time interval to reduce the temporal resolution. The choice of time interval may depend on the flow speed of fluorescent particles to be probed.

For example, assuming the original temporal resolution of a raw data is 10 μ sec, the temporal resolution can be reduced to 1 msec by summing the photon counts of the raw data for every 100 data points. This function may be programmed into the commercial software supplied with the photon-detecting instrument, e.g., ISS VistaVision.

- 2 Convert the file format to the text file (txt) format if the raw data format cannot be viewed in a spreadsheet application.

Defining photon bursts

mMAPS distinguishes bursts in the time-domain raw data by two thresholds: a lower one for defining the noise threshold and a higher one for validating positive bursts. When the raw data is dissected by a fixed duration, the sections with bursts will have higher average photon counts than the burst-free sections, or the noise-alone sections. Since the photon counts within a noise alone section are randomly distributed, a sub-noise level can be statistically defined by using three standard deviations (3σ) above the mean value (m) of the photo counts. This $m+3\sigma$ value of each section is above about 99.86% of photon counts in the same section. A noise threshold can be defined by averaging the sub-noise levels of several burst-free sections (Fig. 3). The data curves that are higher than the noise threshold are considered as photon bursts.

3 Dissect the time-domain data for every certain data points.

For example, a 120-sec data segment can be continuously sectioned into 1200 sections, and each section is 0.1 sec in duration and consists of 100 data points. The values provided here are experimental, and the duration of section may depend on burst density: a thinner section may be required if the burst density is high.

4 Sort the sections by their sub-noise levels.

5 Select the first 10% of low-photon-count population and average the sub-noise levels. The mean value is used as the noise threshold for the time-domain data.

A value of 10% is adjustable and may depend on the burst density.

6 Compare the time-domain data profile with the noise threshold to distinguish potential bursts.

To this point, bursts can be distinguished from different colors and analyzed to search for coincident events. However, noise fluctuations and bursts from the sample auto-fluorescence may be falsely recognized as potential bursts. Therefore, a second burst-selection threshold is needed to select for the bursts whose photon counts are higher than most of false-positive events. This second threshold is determined by a blank sample, i.e., the same lysate sample without the addition of fluorescently labeled antibody.

7 Directly observe the time-domain raw data of the blank sample, and find a threshold that is right on or above the maximum height of bursts. This custom-found second threshold is defined as the bursts-selection threshold, and shall eliminate the majority of false positive bursts in the blank sample data.

8 Apply the bursts-selection threshold to the test samples to identify individual positive events (Fig. 4).

Once both thresholds are determined, potential burst candidates are verified by comparison to the thresholds. Those validated bursts are recorded in a “burst table”, in which the start time, end time, and the photon counts are recorded. The information associated with bursts is then applied to subsequent tasks, such as plotting the photon counts histogram of the target molecules or identifying the target complexes. (see next section)

Defining and quantifying the complex

For identification of two- or three-target molecule complexes, the start and end time points in individual burst tables are keys to distinguishing the coincidence among different fluorescence bursts. When three target molecules are of interest, the analysis of the burst tables is sequential: two of the three target molecules are analyzed first, and the intermediate results are subsequently processed with the data of the third target molecule for complex analysis. (Fig. 5)

9 For an experiment in which three different fluorescence colors are used, three single-color-burst tables can be created by the method described in steps 1 to 8 under the “Preprocessing acquired data” section..

- 10 From the three burst tables, select two of them (1st and 2nd single-color-burst tables) and search for temporally overlapping bursts by their start and end times.
- 11 Once the coincident bursts are determined, record the start time, end time, photon count of both colors of the coincident bursts in a new intermediate coincidence table (1st–2nd intermediate coincidence table).

In the case that a coincidence composed of the two-color bursts is partially overlapped, the new start time should be the earlier one of the two. Similarly, the new end time should be the later one of the two.
- 12 Remove the old records in both single-color-burst tables that have been recognized as coincident bursts.
- 13 Select the intermediate coincidence table and the 3rd single-color-burst table, and search for three-color coincident bursts by their start and end times.
- 14 Once the coincident bursts are found, record the start time, end time, photon count of three colors of the coincident bursts in a new coincidence table (three-color coincidence table).
- 15 Remove the old records in the two tables (the 1st–2nd intermediate coincidence table and the 3rd single-color-burst tables) that have been recognized as coincident bursts.
- 16 The 1st–2nd intermediate coincidence table is now 1st–2nd two-color coincidence table since it does not contain bursts that overlap with the 3rd color.
- 17 Compare the revised 1st and 2nd single-color-burst tables, which have no coincidence with each other, with the revised 3rd single-color-burst tables separately to search for two-color coincidence of 1st–3rd and 2nd–3rd color bursts.
- 18 Follow same above-mentioned procedures if coincident bursts are found. Two final two-color coincidence tables with recorded start time, end time, and photon counts and three final single-color-burst tables with no association to one another are created.

At the end of comparison analysis, seven different types of events will be identified and quantified.

Data Visualization

Given the link between coordinate axes and color channels, one can plot the analyzed results in a two- or three-dimensional space based on the same principles. For a general case of three-color detection, if the 1st, 2nd, and 3rd color channels are assigned to x-, y-, and z-axes, then the three-color coincidence events are represented as points near the cubic center surrounded by the x-y, y-z, and x-z planes, and two-color coincidences are on the three planes. Lone color events are on the three axes (Fig. 6).

- 19 Analyze the data using the methods mentioned in previous sections. Seven final tables should be generated for three-color detection, which include one three-

color coincidence table, three two-color coincidence tables, and three single-color burst tables.

- 20** Select the three-color coincidence table, and plot the events using their 1st, 2nd, and 3rd photon count values as x, y, and z coordinate values.

Example: The coordinate values are (459, 293, 808) for the data illustrated in Figure 5(A).

- 21** Select the two-color coincidence tables, and plot the events using their 1st, 2nd, and 3rd photon count values as x, y, and z coordinate values. For each event, one of the coordinate values is always zero.

Example: The coordinate values are (675, 0, 1030) for 1st–3rd coincidence, (0, 560, 63) for 2nd–3rd coincidence, and (91, 560, 0) for 1st–2nd coincidence for the data illustrated in Figure 5(B).

- 22** Select the single-color-burst tables, and plot the events using their 1st, 2nd, and 3rd photon count values as x, y, and z coordinate values. For each event, two of the coordinate values are always zeros.

Example: The coordinate values are the remaining rows on the single-color-burst tables, such as (150, 0, 0) for the 1st color, (0, 526, 0) for the 2nd color, and (0, 0, 136) for the 3rd color, for the data illustrated in Figure 5(C).

In these two- or three-dimensional plots, the distribution of coincidence events is shown clearly in the areas that contain multiple fluorescence signals (Fig. 6). Quantitation of complex events is also revealed in these multi-dimensional plots. Once the numbers of individual molecules or complexes are made available by mMAPS, the percentages of a certain target molecule-associated complex among all designated target molecules as well as the target molecular complex ratio in different treatment conditions can be further determined. In addition, each fluorescence color alone can be plotted onto a histogram to demonstrate the distribution of photon counts and event numbers, which may also reveal the homodimer population. Hence, a two-dimensional plot can demonstrate three populations including 1st color alone, 2nd color alone, and 1st – 2nd color complex. A three-dimensional plot can further show a total of seven different populations' distribution and quantitation. These processed data can be used to compare the differences among different treatments.

COMMENTARY

Background Information

mMAPS is a flow-proteomic-based technology which detects individual signaling complexes. It incorporates a laser module that excites fluorescent molecules, a detection module integrated with an inverted microscope that detects emission signals, and a microfluidic module that confines the measurement space and movement of the molecules. The microfluidic module is composed of a microfluidic channel and a power supply, which generates a one-direction electroosmotic flow to transport the solution of fluorescently tagged molecules in the microchannel. The target molecules and complexes are excited

when they pass through the focal spot. Because the flow velocity is a function of the characteristics of the channel wall, the solution, and the applied electric field, the molecules on the cross-section plane are theoretically flowing in the same speed under a fixed electric potential across the channel (Doherty et al., 2003; Liu et al., 2011). We describe the general methods for analyzing cultured cells and fixed tissue samples which could be applied to other biological samples as long as the sample can be fluorescently labeled.

Critical Parameters

Fixation—Proper fixation is highly correlated with the quality of analysis. Although in this protocol we suggest fixing the sample for ~8 minutes, which is sufficient for most cultured cells and single-layer tissue sample, optimization may be necessary because under- or over-fixation may significantly affect the results. For samples such as plants or fungi, longer fixation times or alternative fixation solutions may be needed (Haring et al., 2007; Hogan et al., 2006).

Control sample—Appropriate control samples are critical for mMAPS analysis. A blank sample, without any fluorescence staining, serves as a control for noise background and sample auto-fluorescence level. A negative control sample created by staining with normal IgG (if labeling with antibody) can be used to provide non-specific antibody binding data.

Normalizing mMAPS data based on the fluorescence labeling efficiency—

When a certain target protein is to be recognized by the corresponding antibody, it has been observed that the entire population of the target protein may not be labeled by the antibody. A custom-defined term “labeling efficiency” is used to describe this observation, which is the ratio of target protein successfully bound to its antibody relative to the total target protein. For instance, the labeling efficiency of a target protein (T) by an antibody (A) can be estimating by applying the mMAPS data of the fully associated GFP-fusion protein T (T^{GFP}) and the fluorescently-labeled antibody A (A^{Fl}) to the following equation (Eqn 1):

$$\text{Labeling efficiency of } T \text{ by } A = \frac{\text{coincidence events of } T^{GFP} \& A^{Fl}}{\text{total } T^{GFP} \text{ events}} \times 100$$

The labeling efficiency implies that the true number of target molecules and complexes may be underestimated due to the inadequacy of some fluorescently labeled antibodies to recognize the target proteins.

To address this issue, a labeling efficiency chart is used to illustrate the number of labeled and unlabeled proteins. Using two-protein interaction as an example (Fig. 7):

The efficiency of antibodies labeling target [a] and [b] are defined as P_a and P_b . An S represents target molecule without forming complex, and a C indicates complex formation between targets [a] and [b]. An asterisk indicates that the target molecules have been successfully labeled by a fluorescently labeled antibody, and a minus sign indicates the target molecules that are not labeled with antibody. Therefore, $[C_a^*b^*]$ means both [a] and [b] are successfully labeled by the antibody in [ab] complex and $[C_a^*b^-]$ or $[C_{ab}^*]$ represent the events that only [a] or [b] is been labeled in [ab] complex. The same principle can be

applied to $[C_{a-b-}]$, $[S_a]$, and $[S_b]$. Thus, a partially labeled complex $[C_{a^*b-}]$ or $[C_{a-b^*}]$ is incorrectly detected as a single target protein while a non-labeled complex $[C_{a-b-}]$ is not detected at all.

Given the targeting efficiency $[P_a, P_b]$, detected lone molecules $[N_a, N_b]$, measured coincidence $[C_{a^*b^*}]$, the number of unlabeled, partially labeled, and fully labeled protein and complex can be normalized as follows (Eqn 2–13):

$$\begin{aligned}
 C_{a^*b-} &= C_{a^*b^*} \times \frac{1-P_b}{P_b} \\
 C_{a-b^*} &= C_{a^*b^*} \times \frac{1-P_a}{P_a} \\
 C_{a-b-} &= C_{a^*b^*} \times \left(\frac{1-P_b}{P_b} \right) \times \left(\frac{1-P_a}{P_a} \right) \\
 C_{total} &= C_{a^*b^*} + C_{a^*b-} + C_{a-b^*} + C_{a-b-} = C_{a^*b^*} \times \left(\frac{1}{P_a P_b} \right) \\
 S_{a^*} &= N_a - C_{a^*b-} \\
 S_{a-} &= S_{a^*} \times \left(\frac{1-P_a}{P_a} \right) = (N_a - C_{a^*b-}) \times \left(\frac{1-P_a}{P_a} \right) \\
 S_{a\ total} &= S_{a^*} + S_{a-} = (N_a - C_{a^*b-}) \times \frac{1}{P_a} = \frac{N_a}{P_a} - C_{a^*b^*} \times \left(\frac{1-P_b}{P_a P_b} \right) \\
 S_{b^*} &= N_b - C_{a-b^*} \\
 S_{b-} &= S_{b^*} \times \left(\frac{1-P_b}{P_b} \right) = (N_b - C_{a-b^*}) \times \left(\frac{1-P_b}{P_b} \right) \\
 S_{b\ total} &= S_{b^*} + S_{b-} = (N_b - C_{a-b^*}) \times \frac{1}{P_b} = \frac{N_b}{P_b} - C_{a^*b^*} \times \left(\frac{1-P_a}{P_a P_b} \right) \\
 \text{protein A interaction } \%_{\text{normalized}} &= \frac{C_{total}}{C_{total} + S_{a\ total}} \times 100\% = \frac{1}{P_b} \times \text{protein A interaction } \%_{\text{unnormalized}} \\
 \text{protein B interaction } \%_{\text{normalized}} &= \frac{C_{total}}{C_{total} + S_{b\ total}} \times 100\% = \frac{1}{P_a} \times \text{protein B interaction } \%_{\text{unnormalized}}
 \end{aligned}$$

The calculation will reflect the actual complex formation status without being affected by antibody labeling efficiency.

Troubleshooting

High non-specific binding of antibody—If the issue of non-specific binding is problematic, consider using PBS with 0.5% Tween 20 instead of PBS alone to wash away unbound antibodies. More rinse steps are also helpful in removing excess antibodies. In addition, high concentrations of secondary antibodies may occasionally increase the level of non-specific binding. Therefore, titration to determine an appropriate concentration of secondary antibody is important for reducing non-specific binding. Another option is to perform secondary blocking by using 6% BSA in PBS after the primary antibody washing step.

Poor sonication efficiency—One can consider using TPX microtubes (Diagenode) to improve sonication and shearing efficiency, following the manufacturer's recommendations. In addition, the sonication conditions may vary, depending on the sample and sonicator. A pre-test of sonication condition will be helpful for optimizing the shearing process.

Sample concentration too high or too low—Before the sample is loaded onto the microchannel device for analysis, it can be loaded into the 8-well Nunc Lab-Tek Chambered Coverglass to confirm that the dilution is sufficient to observe the fluorescence bursts, which will show distinct and countable bursts above the background over a 10-sec duration. If the concentration is too high, dilute the sample with an appropriate volume of buffer. If the

concentration is too low, prepare the sample again with appropriate buffer volume from the original sample solution.

Protein adhesion on microchannel wall—Protein adhesion to the microchannel sometimes occurs when the sample lysate protein concentration is too high. This may interfere with microfluidic flow and affect fluorescence detection. Further sample dilution can reduce the possibility of protein adhesion.

Low photon count reading—One possible source of low photon counts is misalignment of the optics. Practice **Support Protocol 2** to re-align the optical elements. If misalignment is not the cause, then the problem may be due to insufficiency in photon intensity of the fluorophore for detection. For example, the photon intensity of GFP is usually reduced when fused with proteins of large molecular weight. Therefore, instead of using GFP, consider using holo-tag or other antibody tags, such as HA or Flag, to label the target protein.

Anticipated Results

A successful mMAPS analysis is expected to yield quantitative results of target molecules and complexes from culture cells or tissue lysates. Here, we provide two examples using these working parameters:

488 nm and 635 nm lasers with the power about 300 μ W at the focus of objective lens;

Pinholes of the three detectors 100 μ m;

Bandpass of optical filters for the three photon detectors at 531 ± 40 , 605 ± 15 , and 685 ± 40 nm;

Electric voltage across the 2-cm microfluidic channel device at 150 V, with electric current below 1 μ A.

Example 1: The ligand-receptor interaction of epidermal growth factor (EGF) and its receptor (EGFR) in HeLa cells can be quantified using GFP-tagged EGFR (EGFR^{GFP}) and Alexa 647-conjugated EGF (EGF^{A647}). Based on the results shown in Figure 8A, one can calculate the interaction ratio accordingly (Eqn 14)

$$\begin{aligned} & \text{interaction \% of "EGF \& EGFR" complex to EGFR} \\ &= \frac{\text{coincidence events of "EGF}^{A647} \& \text{EGFR}^{GFP}\text{"}}{\text{total EGFR}^{GFP} \text{ events}} \\ & \times 100\% = \frac{42}{42+470} \times 100\% = 8.20\% \end{aligned}$$

Example 2: The interaction of endogenous protein STAT3, p300, and genomic DNA in frozen tumor tissue quantified by using Alexa 488-conjugated STAT3 (STAT3^{A488}), Qdot 605-conjugated p300 (p300^{Q605}), and TOTO3-stained DNA fragments (DNA^{TOTO3}). The photon count distribution of the total seven conditions of interaction, including complexes and lone molecules, are shown in Figure 8B. One can identify the interaction ratio of STAT3-associated complex among all detected STAT3 as follows (Eqn 15–18):

$$\text{interaction \% of STAT3 associated complex to STAT3} = \frac{\text{coincidence events of "STAT3}^{A488} \text{ associated complex"}}{\text{total STAT3}^{A488} \text{ events}} \times 100\%$$

$$\text{For "STAT3\&p300": } \frac{8}{186+37+8+22} \times 100\% = 3.16\%$$

$$\text{For "STAT3\&DNA": } \frac{37}{186+37+8+22} \times 100\% = 14.62\%$$

$$\text{For "STAT3\&p300\&DNA": } \frac{22}{186+37+8+22} \times 100\% = 8.70\%$$

Time Considerations

For sample preparation, the time may vary from half a day to two days depending on the incubation time for primary and secondary antibodies. The time for data acquisition may take 1.5 hours or shorter per sample if the concentration of the fluorophore is sufficient. The time for data analysis depends on the number of events collected.

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Literature Cited

- Auerbach D, Thaminy S, Hottiger MO, Stagljar I. The post-genomic era of interactive proteomics: facts and perspectives. *Proteomics*. 2002; 2:611–623. [PubMed: 12112840]
- Campbell, SA. The science and engineering of microelectronic fabrication. 2. New Delhi: Oxford University Press; 2007.
- Cheng Y, Ho E, Subramanyam B, Tseng JL. Measurements of drug-protein binding by using immobilized human serum albumin liquid chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004; 809:67–73.
- Chou CK, Jing N, Yamaguchi H, Tsou PH, Lee HH, Chen CT, Wang YN, Hong S, Su C, Kameoka J, Hung MC. High speed digital protein interaction analysis using microfluidic single molecule detection system. *Lab Chip*. 2010; 10:1793–1798. [PubMed: 20498894]
- Chou CK, Lee HH, Tsou PH, Chen CT, Hsu JM, Yamaguchi H, Wang YN, Lee HJ, Hsu JL, Lee JF, Kameoka J, Hung MC. mMAPS: A Flow-Proteometric Technique to Analyze Protein-Protein Interactions in Individual Signaling Complexes. *Sci Signal*. 2014; 7:rs1. [PubMed: 24595109]
- Cipriany BR, Zhao R, Murphy PJ, Levy SL, Tan CP, Craighead HG, Soloway PD. Single molecule epigenetic analysis in a nanofluidic channel. *Anal Chem*. 2010; 82:2480–2487. [PubMed: 20184350]
- Collas P. The state-of-the-art of chromatin immunoprecipitation. *Methods Mol Biol*. 2009; 567:1–25. [PubMed: 19588082]
- Colyer RA, Scalia G, Kim T, Rech I, Resnati D, Marangoni S, Ghioni M, Cova S, Weiss S, Michalet X. High-throughput multispot single-molecule spectroscopy. *Proc Soc Photo Opt Instrum Eng*. 2010; 7571:75710G–75710G75711.
- Doherty EA, Meagher RJ, Albarghouthi MN, Barron AE. Microchannel wall coatings for protein separations by capillary and chip electrophoresis. *Electrophoresis*. 2003; 24:34–54. [PubMed: 12652571]
- Gavin AC, Superti-Furga G. Protein complexes and proteome organization from yeast to man. *Curr Opin Chem Biol*. 2003; 7:21–27. [PubMed: 12547422]

- Haring M, Offermann S, Danker T, Horst I, Peterhansel C, Stam M. Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Methods*. 2007; 3:11. [PubMed: 17892552]
- Hogan GJ, Lee CK, Lieb JD. Cell cycle-specified fluctuation of nucleosome occupancy at gene promoters. *PLoS Genet*. 2006; 2:e158. [PubMed: 17002501]
- Jain A, Liu R, Ramani B, Arauz E, Ishitsuka Y, Ragunathan K, Park J, Chen J, Xiang YK, Ha T. Probing cellular protein complexes using single-molecule pull-down. *Nature*. 2011; 473:484–488. [PubMed: 21614075]
- Kameoka J, Craighead HG, Zhang HW, Henion J. A polymeric microfluidic chip for CE/MS determination of small molecules. *Analytical Chemistry*. 2001; 73:1935–1941. [PubMed: 11354473]
- Kolch W, Pitt A. Functional proteomics to dissect tyrosine kinase signalling pathways in cancer. *Nat Rev Cancer*. 2010; 10:618–629. [PubMed: 20720570]
- Kuo MH, Allis CD. In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. *Methods*. 1999; 19:425–433. [PubMed: 10579938]
- Lee NK, Kapanidis AN, Koh HR, Korlann Y, Ho SO, Kim Y, Gassman N, Kim SK, Weiss S. Three-color alternating-laser excitation of single molecules: monitoring multiple interactions and distances. *Biophys J*. 2007; 92:303–312. [PubMed: 17040983]
- Liu C, Qu Y, Luo Y, Fang N. Recent advances in single-molecule detection on micro- and nano-fluidic devices. *Electrophoresis*. 2011; 32:3308–3318. [PubMed: 22134976]
- Michalet X, Colyer RA, Scalia G, Kim T, Levi M, Aharoni D, Cheng A, Guerrieri F, Arisaka K, Millaud J, Rech I, Resnati D, Marangoni S, Gulinatti A, Ghioni M, Tisa S, Zappa F, Cova S, Weiss S. High-throughput single-molecule fluorescence spectroscopy using parallel detection. *Proc Soc Photo Opt Instrum Eng*. 2010; 7608
- Myers JD. Development and application of immunocytochemical staining techniques: a review. *Diagn Cytopathol*. 1989; 5:318–330. [PubMed: 2477206]
- Nolan RL, Cai H, Nolan JP, Goodwin PM. A simple quenching method for fluorescence background reduction and its application to the direct, quantitative detection of specific mRNA. *Anal Chem*. 2003; 75:6236–6243. [PubMed: 14616007]
- Papin JA, Hunter T, Palsson BO, Subramaniam S. Reconstruction of cellular signalling networks and analysis of their properties. *Nat Rev Mol Cell Biol*. 2005; 6:99–111. [PubMed: 15654321]
- Phizicky EM, Fields S. Protein-protein interactions: methods for detection and analysis. *Microbiol Rev*. 1995; 59:94–123. [PubMed: 7708014]
- Stenman UH, Bidart JM, Birken S, Mann K, Nisula B, O'Connor J. Standardization of protein immunoprecipitation procedures. Choriogonadotropin (CG). *Scand J Clin Lab Invest Suppl*. 1993; 216:42–78. [PubMed: 8378750]
- Thompson MA, Lew MD, Moerner WE. Extending microscopic resolution with single-molecule imaging and active control. *Annu Rev Biophys*. 2012; 41:321–342. [PubMed: 22577822]
- Toby GG, Golemis EA. Targeting proteins to specific cellular compartments to optimize physiological activity. *Methods Enzymol*. 2001; 332:77–87. [PubMed: 11305119]
- Williams NE. Immunoprecipitation procedures. *Methods Cell Biol*. 2000; 62:449–453. [PubMed: 10503210]

Figure 1. Schematic Diagram of mMAPS Flow-proteomic Analysis. (A) Frozen tissue sections or cultured cells undergo fixation. (B) Target molecules are labeled with fluorescent tags, which can be a fluorescent fusion protein, antibody, or chemical dye. (C) Cells are lysed to release molecular complexes (D) Lysate is passed through the microchannel for analysis.

Figure 2.

Steps of photolithography for fabricating microfluidic channel devices with quartz wafers. For a wafer of 500- μm in thickness: (A) clean; (B) spin coat the photoresist; (C) expose to UV with a photomask; (D) develop the pattern; (E) etch to form channel pattern on the wafer; (F) remove the photoresist; (G) drill holes for the inlets and outlets. The processed wafer is then thermal bonded to a 170- μm thick flat wafer to form channels (H, flipped and transparent view) and attached to the reservoirs (I).

Figure 3.

Determination of noise threshold for defining bursts. The time-domain raw data is cut into sections with a fixed duration (middle panel). The sections are re-arranged according to their $m+3\sigma$ values of photon counts. The lowest 10% of the total sections are averaged to determine the base noise threshold of the burst profile (lower panel).

Figure 4.

Determination of second threshold to identify positive photon bursts. The threshold is adjusted to be above most of the false-positive (auto-fluorescence) bursts in the blank sample and this level of threshold is applied to the test sample to identify the positive events.

Figure 5.

Finding coincident bursts from tables of color-labeled bursts in the case of three target molecules. (A) The 1st and 2nd single-color-burst tables are analyzed first. The temporally overlapping bursts are moved to a new 1st – 2nd coincidence table for comparison with the 3rd color burst data to generate the final three-color coincidence table. (B) The revised 1st and 2nd single-color-burst tables, in which the 1st – 2nd coincidence bursts are removed, are compared with the 3rd color burst table to identify two-color coincidences. (C) After the removal of coincident bursts, the three single-color-burst tables now contain only lone color

bursts. At the end of analysis, seven final data tables (in thick black rectangles) are generated.

Figure 6.

Visualization of the results from a three-color analysis. Coincident and lone bursts are plotted respectively in histograms (one-dimensional), on planes (two-dimensional), and inside a cube (three-dimensional) for three-target molecules, using photon counts of different color labels as coordinates. Complexes composed of three-target molecules (black) are located in the center of the cube; complexes of two-target molecules (cyan, orange, and pink) are on the planes; lone-target molecules (green, red, and blue) are on the axes, with their histograms shown in the right panels.

Figure 7.

A targeting efficiency chart for two-protein interactions. The symbols in each region are defined as follows: *, fluorescently labeled; -, unlabeled; C, numbers of coincident events; S, numbers of single-color events; P_a , fluorescence targeting efficiency of protein A; P_b , fluorescence targeting efficiency of protein B. The dotted rectangles (red and pink) represent events showing single color; the dotted square (yellow) represents events showing two colors.

Figure 8.

Examples of complex analysis by mMAPS. Photon counts of different labeling colors are used as coordinates, in the cases of (A) two-target molecules, EGF and EGFR, and (B) three-target molecules, endogenous STAT3, p300, and genomic DNA. The numbers in parentheses are the counts of specific molecules or complexes.