Single-molecule pull-down for studying protein interactions

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This protocol describes a single-molecule pull-down (SiMPull) assay for analyzing physiological protein complexes. The assay combines the conventional pull-down assay with single-molecule total internal reflection fluorescence (TIRF) microscopy and allows the probing of single macromolecular complexes directly from cell or tissue extracts. In this method, antibodies against the protein of interest are immobilized on a passivated microscope slide. When cell extracts are applied, the surface-tethered antibody captures the protein together with its physiological interaction partners. After washing away the unbound components, single-molecule fluorescence microscopy is used to probe the pulled-down proteins. Captured proteins are visualized through genetically encoded fluorescent protein tags or through antibody labeling. Compared with western blot analysis, this ultrasensitive assay requires considerably less time and reagents and provides quantitative data. Furthermore, SiMPull can distinguish between multiple association states of the same protein. SiMPull is generally applicable to proteins from a variety of cellular contexts and to endogenous proteins. Starting with the cell extracts and passivated slides, the assay requires 1.5–2.5 h for data acquisition and analysis.

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INTRODUCTION

Nearly all vital cellular processes are performed by the coordinated action of proteins acting in multimeric assemblies or complex formations[1]. Cellular processes are also dynamically regulated by signaling networks, wherein the interaction between biomolecules links physical or chemical stimuli to the effector molecules[2,3]. Thus, analysis of protein interactions is central to understanding cell function and regulation. One of the most widely used techniques for studying interacting proteins is the pull-down or coimmunoprecipitation assay[4,5]. In the classical pull-down assay, the protein of interest or bait is selectively isolated from cell or tissue extracts. The physiological binding partners of the bait protein, or the prey proteins, co-purify with the bait protein. The identity of the prey proteins is determined using western blotting or mass spectrometry.

Many proteins participate in different types of complexes and have diverse functionality. Though they are instrumental in discovery of new binding partners, the data from ensemble pull-down assays represent an average of the protein complexes. The true composition of physiological complexes is not easily revealed, because of the multiplicity of protein interactions[5]. Furthermore, no information about the stoichiometry of protein interactions is obtained. Similarly, one can determine the pairwise interacting partners using yeast two-hybrid[6] or complementation assays[7], but the physiological assembly of these interactions cannot be deciphered. Single-molecule experiments can provide additional insight into the molecular architecture of protein assemblies[8], but they have been typically limited to reconstituted complexes with purified proteins[9–12].

We recently described a SiMPull[13] assay that combines the classical pull-down assay with single-molecule fluorescence imaging and enables direct visualization of cellular protein complexes at the single-molecule level. In this paper, we describe the detailed procedure for the SiMPull assay. The assay requires a single-molecule TIRF microscope and can be performed using the same reagents as required for western blot analysis. In one incarnation, an antibody against a bait protein is immobilized on a polymer-passivated flow chamber. Cell extracts are made to flow through the chamber so that the bait protein is captured. If the bait is in complex with other proteins or nucleic acids, the antibody will capture these additional biomolecules. After washing out the unbound proteins, multicolor fluorescence imaging with single-fluorophore sensitivity is used to analyze the composition of the protein complexes. We describe the assay for pull-down with biotin-labeled primary or secondary antibodies. The pulled-down proteins are visualized through fluorescent protein-fused chimeras or by immunofluorescence labeling, as depicted in **Figure 1.**

Several advantages stem from the single-molecule imaging of cellular protein complexes. SiMPull can discriminate between multiple association states of a protein. It provides quantitative data on the bait and prey protein populations. SiMPull also allows us to determine the stoichiometry of the complexes by photobleaching step analysis[14]. The prepared complexes may also be used for biochemical analysis of their activities at the single-molecule level[13,15,16]. Thus, SiMPull can be used as a preparatory tool to study the functional activity of protein complexes that are not accessible through recombinant methods. In addition, SiMPull promises improvement by orders of magnitude in cost, time and sensitivity over conventional western blotting. The method is generally applicable to a wide variety of cellular contexts and can be tailored to any alternative pull-down or fluorophore-labeling schemes. Although we describe the assay for detecting prey proteins with fluorescent protein tags or antibodies, other suitable specific labeling schemes should be compatible with the assay.

However, unlike western blot analysis, this method does not separate biomolecules on the basis of their size, and it relies solely on fluorophore-based detection. Hence, appropriate controls are necessary for correct interpretation of the data. In its current form, SiMPull is applicable when one already knows the anticipated binding partners. Lysates are typically diluted to obtain a sufficiently low protein concentration for single-molecule imaging. Accordingly,

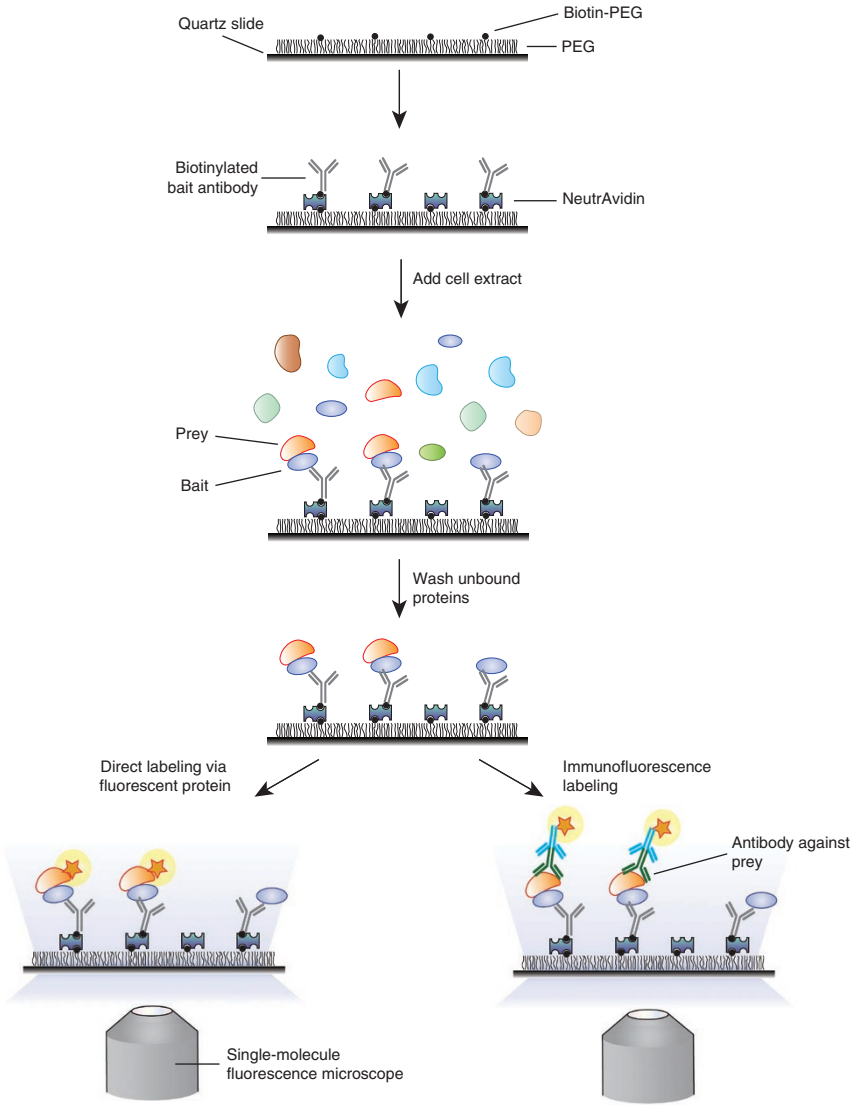

Figure 1 | Schematic for single-molecule pull-down. Microscope slides and coverslips are passivated with PEG and doped with biotin-PEG. Antibodies against the bait protein are immobilized using NeutrAvidin. When the cell extract is added to the chamber, the surface-immobilized antibody captures the bait protein together with the prey. Other cellular components do not bind and are washed away. When the prey protein bears a fluorescent protein tag, it can be directly visualized using a single-molecule fluorescence microscope. Alternatively, the pulled-down complexes can be immunolabeled for prey-protein detection.

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weak interactions with dissociation rate constants of >0.01 s^{-1} may not be suitable for the method[13].

Experimental design

Surface passivation and construction of flow chambers. The key aspect of the pull-down assays is the selective immobilization of the protein of interest or bait on a solid matrix, which will bring along its interacting partners. The bait-recruiting surfaces must specifically bind to the protein of interest while rejecting the nonspecific adsorption of biomolecules that exist in cell extracts. We achieve this by using methoxypolyethylene glycol (mPEG)-coated microscope slides and coverslips. The PEG coating substantially reduces the binding of proteins to the glass surfaces[17,18], as shown in **Figure 2**. A small amount (~2%) of biotinylated PEG is added during the slide preparation; this functionalizes the surface and allows the specific immobilization of biotinylated biomolecules with an avidin linker. Flow chambers are constructed using a passivated slide and coverslip for rapid and convenient exchange of contents (**Fig. 3**). A detailed protocol for surface passivation with mPEG and construction of flow chambers is also described in previous publications[19,20].

Sample preparation. SiMPull can be performed using the same samples (purified proteins, tissue or cell lysates) as those used for a conventional pull-down analysis. Extracts are typically prepared by lysing cultured cells or tissues with detergents. We have tested a variety of detergents and cell types for SiMPull analysis[13]. A protocol for lysis of cultured cells is included in REAGENT SETUP; preparation of animal tissue extracts is described in the **Supplementary Methods**. As in conventional pull-down assays, it is crucial to use nondenaturing conditions to preserve the physiological interactions that may occur. We avoid SDS and other strong ionic detergents for lysis, as they can potentially denature the immobilized antibodies or disassemble protein complexes.

Antibody immobilization. The selective capture of the bait protein is achieved using surface-immobilized antibodies. The biotin-doped surfaces are saturated with NeutrAvidin, and biotinylated antibody against the bait protein is immobilized at a concentration

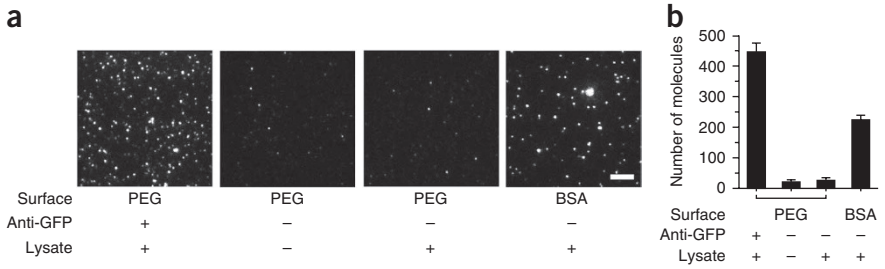

Figure 2 | PEG passivation hinders nonspecific protein adsorption. (**a**) A typical TIRF image of YFP-tagged protein pulled down using polyclonal anti-GFP antibody from the lysate (**a**, left). The nonspecific binding of the protein to the PEG-passivated surface (**a**, second from right) is comparable to the blank (**a**, second from left). Substantial nonspecific adsorption of YFP is observed when the same amount of lysate is added to surface passivated with 1 mg ml^{-1} BSA (**a**, right). Scale bar, 5 µm. (**b**) Bar graph with average number of fluorophores per image (2,500 $µm^2$). Error bars represent s.d. of the mean across >20 images.

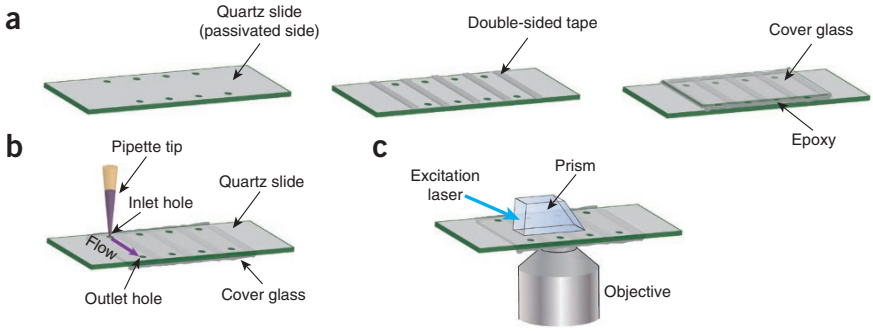

Figure 3 | Preparation of flow chambers. (**a**) To assemble the flow chamber, place the quartz slide with the passivated side facing up. Put the double-sided tape between the successive holes to create channels. Place the cover glass on top of the tape and seal the edges with epoxy. (**b**) The holes on the slides are used for flowing solutions through the chamber using a pipette. (**c**) The prism is placed on top of the slide to create an evanescent excitation field at the quartz-aqueous buffer interface.

of 10–20 nM. This should yield a surface density of about 20–40 antibody molecules per μm^2.

Often it is difficult to label the primary antibody with biotin. Many primary antibodies are supplied either as sera or in buffer with BSA as a stabilizer, and are thus not suitable for labeling reactions. For immobilizing unlabeled antibodies, we use a biotinylated secondary antibody on the surface that can recruit the primary antibody against the bait protein. Secondary antibodies against one species can often cross-react with other species unless they have been specifically adsorbed to minimize cross-reactivity. For assays involving multiple antibodies, we use cross-adsorbed and affinity-purified antibodies.

Single-molecule pull-down. For imaging single molecules, the protein of interest must be immobilized at sufficiently low density such that the bait molecules are well separated on the slide surface. Cell lysates typically require dilution to obtain this low density. As the concentration of the protein of interest in the lysates is not known a priori and it also varies across preparations, it is difficult to predict the appropriate dilution factor for the lysate. To this end, we use an iterative approach: we choose a suitable starting point and then titrate the concentration of the lysate to obtain 0.1–0.2 molecules per μm^2 of imaging area upon 20-min incubation on the antibody-coated surface. In this regime, the density of antibody molecules on the surface is at least 100-fold higher than the concentration of immobilized bait/prey molecules, and hence the protein capture is not limited by the availability of binding sites. **Figure 4** illustrates how the optimal dilution factor is determined for pull-down of a yellow fluorescent protein (YFP)-fused protein.

Fluorophore labeling. We describe two approaches for fluorescence detection of pulled-down proteins. First, the proteins of interest can be expressed as fluorescent protein-fused chimera. Proteins expressed as fluorescent-protein fusions can be directly visualized under a TIRF microscope with single-molecule sensitivity.

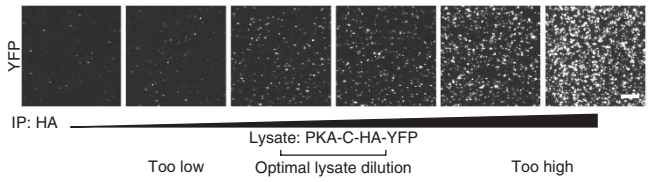

Figure 4 | Determination of lysate dilution factor for PKA tagged with YFP and HA tags. The lysate concentration is serially increased starting with a 5,000-fold dilution (second from left) to a 250-fold dilution (right). The image on the left depicts the background. The optimal fluorophore density for resolving single fluorophores is 0.1–0.2 fluorophores per μm^2 as depicted in the two center images. Scale bar, 5 μm.

This approach ensures a one-to-one labeling of the protein and thus can be used for determining the stoichiometry of the protein in the complex[8,14]. Enhanced YFP is our probe of choice, owing to its superior photophysical properties[8] as compared with other green and red fluorescent protein variants.

Alternatively, one can detect the prey protein using an immunofluorescence labeling scheme. This approach involves an antibody against the prey protein that is orthogonal to the bait-capturing antibody and a corresponding secondary antibody labeled with fluorophores. We demonstrate this scheme through pull-down of endogenous protein complexes from mouse brain tissue (**Fig. 5**). Caution should be taken when you use multiple antibodies for pull-down or detection, as secondary antibodies can cross-react with antibodies from other animals and lead to false positives.

Single-molecule microscopy. We use a prism-type TIRF microscope equipped with an electron-multiplying charge-coupled device (EM-CCD) for single-molecule imaging. The TIR illumination creates an evanescent field of excitation light that extends only 100–200 nm from the surface and hence almost exclusively excites the fluorophores tethered to the surface. This markedly reduces the background from fluorophores in solution, and the technique has been widely applied for single-molecule microscopy[19]. A comprehensive guide for the construction of a TIRF microscope for single-molecule fluorescence imaging has been described previously[17,21]. Multicolor imaging is achieved by using different and spectrally separated fluorophores in conjunction with the corresponding excitation sources and emission filters. We describe the assay as using a prism-type TIRF microscope, though in principle it should be possible to obtain similar results with an objective-type TIRF setup.

Control experiments. The quality of passivation has an important role in determining the specificity of pull-down. It is important to check the quality of PEG passivation for each preparation of slides, as described below (Steps 14 and 15 of the PROCEDURE). Although the PEG surfaces substantially reduce the nonspecific binding, it will occur at protein concentrations >100 nM. Also, antibodies may bind nonspecifically to pulled-down proteins or may cross-react, leading to false positives. Control experiments with suitable control antibodies and control lysates are essential for verifying that the detected fluorescence arises from the anticipated protein complexes. We typically perform control experiments by replacing each of the capture or detection antibodies with the corresponding control antibodies and by using control lysates without bait or prey protein expression. As in conventional pull-down assays, additional controls (for example, mutations that prohibit binding between the bait and the prey proteins) may be performed, depending on the context.

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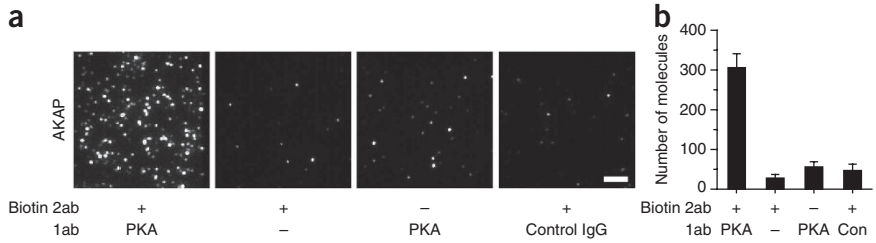

Figure 5 | PKA-AKAP pull-down. The PKA antibody is immobilized through a biotinylated secondary antibody. A tenfold dilution of mouse brain extract is added. The pulled-down AKAP protein is probed by using an orthogonal antibody and the corresponding secondary antibody is labeled with Alexa Fluor 647 (**a**, left). Control experiments show a fivefold reduced secondary antibody binding. Scale bar, 5 µm. (**b**) Bar graph with average number of Alexa Fluor 647 spots per image (2,500 µm^2). Error bars represent s.d. of the mean across >20 images. 2ab, secondary antibody; 1ab, primary antibody.

MATERIALS

REAGENTS

- Methanol (Fisher Scientific, cat. no. A412-4) **! CAUTION** Methanol is flammable; liquid and vapors are toxic. Wear a mask, gloves and chemical safety goggles while handling.
- Acetone (Fisher Scientific, cat. no. A18-4) **! CAUTION** Acetone is flammable, and is a skin and eye irritant. Wear gloves and chemical safety goggles while handling.
- KOH pellets (Fisher Scientific, cat. no. P250-3) **! CAUTION** KOH is corrosive, and is a skin and eye irritant. Use under a chemical fume hood.
- Glacial acetic acid (Fisher Scientific, cat. no. A38-500) **! CAUTION** Acetic acid causes severe eye and skin burns. Handle with a glass pipette. Wear gloves and chemical safety goggles while handling.
- Aminosilane (*N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane) (United Chemical Technologies, cat. no. A0700) **! CAUTION** Aminosilane is potentially toxic, and is a skin and eye irritant. Wear gloves and chemical safety goggles while handling.
- mPEG (Laysan Bio., cat. no. mPEG-succinimidyl valerate, MW 5,000)
- Biotin-PEG (Laysan Bio., cat. no. Biotin-PEG-SVA, MW 5,000)
- Sodium bicarbonate (Fisher Scientific, cat. no. S233)
- Double-sided tape (3M)
- Epoxy (Devcon, cat. no. 14250)
- Tris-HCl (Fisher Scientific, cat. no. BP153-1)
- NaCl (Fisher Scientific, cat. no. S641-212)
- EDTA (Sigma, cat. no E9884-1KG)
- Bovine serum albumin (BSA; New England Biolabs, cat. no. B9001S)
- NeutrAvidin (Pierce, cat. no. 31000)
- Fluorophore-labeled protein for testing quality of PEG passivation
- We have successfully used the following antibodies: biotinylated anti-Flag (Sigma, cat. no. F9291), biotinylated anti-rabbit (Santa Cruz Biotechnology, cat. no. SC2089), biotinylated anti-GFP (Rockland Immunochemicals, cat. no. 600-106-215), anti-goat (Rockland Immunochemicals, cat. no. 605-701-125), rabbit anti-PKARII (Santa Cruz Biotechnology, cat. no. SC909), anti-AKAP150 (Santa Cruz Biotechnology, cat. no. SC6445), and rabbit pre-immune IgG (Cell Sciences, cat. no. NRI01)
- Cell culture medium (Dulbecco's modified Eagle's medium, Hyclone, cat. no. SH30284 or equivalent, supplemented with serum if appropriate)
- Transfection reagent (Lipofectamine 2000, Invitrogen, cat. no. 11668019)
- Nonidet P-40 (NP-40; United States Biological, cat. no. N3500)
- Ultrapurified water (e.g., Milli-Q water (Millipore))

EQUIPMENT

- Rotary drill (Dremel, model no. 395)
- Drill bits (0.75 mm; Kingsley North, cat. no. 1-0500-100)
- Quartz slides (G. Finkenbeiner Inc., 1 inch × 3 inch × 1 mm thick)
- Coverslips (24 × 40 mm; VWR International, cat. no. 48393230)
- Slide holders (Fisher Scientific, cat. no. 08-817)
- Bath sonicator (Bransonic tabletop ultrasonic cleaner)
- Propane torch

Single-molecule TIRF microscope

- Inverted fluorescence microscope (Olympus, cat. no. IX70),
- 1.2 Numerical objective (NA) water-immersion objective (Olympus, cat. no. UPLAPO60XW)
- Pellin-Broca prism (EKSPLA, cat. no. 325-3206)
- EM-CCD detector (Andor Technologies, cat. no. iXon DV 887-BI)
- Laser shutter (Uniblitz electronic shutter, Vincent Associates, cat. no. LS6T2)
- Shutter driver (Vincent Associates, cat. no. VMM-D1)
- Mirrors to align lasers (Thorlabs, cat. no. BB1-E02)
- Polarizing beam splitter (Thorlabs, cat. no. PBS3)
- *XYZ* micrometer translation stage (Newport, cat. no. 462-XYZ)
- Excitation and emission focusing lenses

Excitation lasers

- 488 nm (Coherent, cat. no. Sapphire 488 LP-050)
- 568 nm (Coherent, cat. no. Sapphire 568-50)
- 633 nm HeNe laser (Newport, cat. no. R-30995)

Emission filters

- YFP (Chroma Technology, cat. no. HQ 535/30m)
- mCherry (Semrock, FF01-607/36-25)
- Alexa Fluor 647 (Chroma Technology, cat. no. 640DCLP)

REAGENT SETUP

T50 T50 is prepared by mixing 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl; it can be stored at room temperature (22–25 °C) for up to 1 month.

T50-BSA To prepare T50-BSA, mix 10 mM Tris-HCl (pH 8.0), 50 mM NaCl and 0.1 mg ml^{-1} BSA. This can be stored at 4 °C for up to 1 month.

Lysis buffer Lysis buffer is prepared by mixing 10 mM Tris (pH 7.5), 1% (vol/vol) NP-40, 150 mM NaCl and 1 mM EDTA with protease inhibitors). ▲ **CRITICAL** Lysis buffer should be freshly prepared before each use.

Sample preparation A method for lysate preparation from tissues is provided in the **Supplementary Methods**. Here we describe the method used for lysate preparation from transfected cells. The method can be tailored for different lysis conditions or cell types. Human embryonic kidney 293 cells, for example, are cultured and maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% (vol/vol) fetal bovine serum. Transfect the cells with a suitable transfection reagent according to the manufacturer's protocol. For lysis, wash the cells twice with PBS and add the lysis buffer. Incubate at 4 °C for 30 min. Preclean the lysate by centrifuging it at 14,000*g* for 20 min at 4 °C. We recommend using fresh lysates (i.e., use within ~12 h after preparation).

EQUIPMENT SETUP

TIRF microscope We use a custom-built prism-type TIRF microscope for single-molecule imaging. An inverted microscope is adapted to hold a trapezoidal fused-silica prism on top of the flow chamber. The excitation laser beam is directed towards the objective through the prism at an incidence angle greater than the critical angle (68°). The prism is index matched with the flow chamber by using immersion oil, such that the evanescent excitation field is created at the quartz-aqueous buffer interface. An ×60 water-immersion objective (NA = 1.2) is used to collect the fluorescence signal. The scattered light is rejected by using suitable filters/dichroic mirrors. The emitted light is imaged onto a 512 × 512 pixel EM-CCD. The final pixel size in the imaging plane is ~146 nm. The excitation laser intensity is modulated using a half-wave plate and a polarizing beam-splitting cube to achieve an intensity of 0.5–3 µW µm^{-2} at the quartz-aqueous buffer interface, so as to obtain a five- to tenfold signal above noise.

Software Fluorescence signal is recorded using custom software written in Visual C++. The software acquires movies as a series of frames at a specified time resolution, depending on the camera used. Single-molecule time trajectories are extracted from the movies using scripts written in interactive data language (IDL). The program creates an averaged image of the first ten frames and identifies single molecules as intensity maxima greater than a predetermined threshold. The algorithm also fits the point spread function to a Gaussian to

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avoid including multiple molecules or aggregations into the analysis. Next, the local background is subtracted and intensities from a region of 7 × 7 pixels surrounding the peak are added to obtain the intensities of single molecules. The single-molecule time trajectories and mean fluorophore count per movie are analyzed using MATLAB codes. All programs are available from the authors upon request.

PROCEDURE

PEG passivation of microscope slides ● TIMING 6–8 h

1| Drill two holes in a quartz slide, about 0.75 mm in diameter, 3–4 mm away from the edge (**Fig. 3a**). This is to create an inlet and an outlet for the flow channel. Rinse the slide and place the slide and a coverslip in the slide holder. We typically make 4–5 flow channels per slide.

2| Rinse twice and bath-sonicate the slide and the coverslip in Milli-Q water for 10 min. To remove any organic residue from the surfaces, repeat the process with methanol followed by acetone.
! CAUTION Methanol is flammable; liquid and vapors are toxic. Acetone is flammable and is a skin and eye irritant. Wear a mask, gloves and chemical safety goggles while handling it.

3| Sonicate in 1 M KOH for 20 min and then rinse with Milli-Q water. KOH treatment activates the surface for silane functionalization (Steps 5–7).
! CAUTION KOH is corrosive, and is a skin and eye irritant. Use it under a chemical fume hood.

4| Burn the slide for about 1 min and the coverslip for 1–2 s with a propane torch to dry off any surface moisture. Place the slide and coverslip in a dry slide holder.

5| Mix 95 ml of methanol with 5 ml of acetic acid in a conical flask. Add 1 ml aminosilane, mix and immediately pour this solution into the slide holder with the slide and coverslip. Incubate in dark for 10 min at room temperature.
! CAUTION Aminosilane is potentially toxic, and is a skin and eye irritant. Acetic acid causes severe eye and skin burns. Wear appropriate safety equipment while handling it.
▲ CRITICAL STEP Aminosilane is photosensitive and hydrolyzes rapidly in water. Store it under nitrogen in the dark.

6| Bath-sonicate the slide and coverslip for 1 min, and then incubate for another 10 min at room temperature.

7| Wash the slide and coverslip first with methanol and then with water for 1–2 min per wash. Dry and place them in a humidified box.

8| Weigh 16 mg of mPEG with 0.3 mg of biotin-PEG per slide/coverslip pair. Dissolve in 70 µl freshly prepared sodium bicarbonate buffer (10 mM sodium bicarbonate, pH 8.5). Mix well and spin down for 30 s at 10,000*g* at room temperature to remove bubbles.
▲ CRITICAL STEP The passivation on the coverslip surface is not required when using a prism-type TIRF microscope, but it is recommended to prevent sample loss.
▲ CRITICAL STEP The half-life of succinimidyl valerate PEG in pH 8.5 buffer is only ~10 min. After adding the buffer to PEG, proceed to Step 9 as soon as possible.

9| Apply this solution to the slide surface and sandwich it immediately with the coverslip. Store the slide in the dark for 3–4 h in humidified boxes at room temperature.

10| Wash the slide with copious amount of water, blow it dry with nitrogen, and store the slide and coverslip under vacuum at −20 °C in the dark. We use a food-grade vacuum sealer for sealing the slides.
■ PAUSE POINT The slides can be stored for up to 2 weeks under these conditions.

Construction of flow chambers ● TIMING ~30 min

11| Thaw the slide and coverslip at room temperature for 10 min.

12| Sandwich a piece of double-sided tape between the slide and coverslip, excluding an ~5-mm channel where the inlet/outlet holes are located (**Fig. 3a**). Ensure that the tape sticks to both surfaces.

13| Seal the edges with epoxy and allow it to dry for 10 min. The volume of the flow channel is ~20 µl. Prepare additional chambers for the control experiments as required.

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Testing the quality of PEG slides ● TIMING ~30 min

14| Flow 100 µl of T50 buffer into the flow channel (**Fig. 3b**) and image it under the TIRF microscope. Acquire 10 short movies, at suitable time resolution, and determine the average number of fluorescent molecules per unit imaging area. This is the background fluorescence, which is likely to arise from impurities during surface preparation. Typically, the observed background fluorescence is <0.02 molecules per μm^2 under our experimental conditions.

? TROUBLESHOOTING

15| Test the slides for the quality of passivation. Flow 100 µl of a 10 nM fluorophore-labeled protein through the flow chamber; incubate it for 10 min and wash it by flowing 200 µl T50 twice to remove the unbound protein. Image it under the TIRF microscope and determine the average number of nonspecifically bound molecules. A good passivation should yield <0.01 molecules per μm^2 nonspecifically adsorbed molecules above the background spot count (as determined in Step 14).

? TROUBLESHOOTING

Immobilizing the antibody against the bait protein ● TIMING 30 min–1 h

16| Prepare a 0.2 mg ml^{-1} solution of NeutrAvidin in T50 buffer. Add 70 µl of this solution to the flow chamber. Incubate for 5 min. All incubations are performed at room temperature, unless otherwise specified.

17| Wash excess NeutrAvidin by flowing 200 µl of T50 twice.

18| To immobilize the biotinylated primary antibody against the bait protein, follow option (A). To immobilize the unlabeled bait antibody via a biotinylated secondary antibody, follow option (B).

▲ **CRITICAL STEP** The sensitivity and performance of the assay depends on the affinity of the antibody and accessibility of the epitope. Like any antibody-based assay, it may be necessary to test different antibodies for this application.

(A) Immobilizing the biotinylated primary antibody against the bait protein

(i) Dilute the biotinylated antibody to a working concentration of ~10–20 nM in T50-BSA.
(ii) Add 100 µl of this solution to the chamber and incubate for 10 min.
(iii) Rinse twice with 200 µl of T50-BSA.

(B) Using the biotinylated secondary antibody to immobilize the primary antibody against the bait protein

(i) Flow 100 µl of 20–40 nM of biotin-labeled appropriate secondary antibody and incubate for 10 min.
(ii) Wash twice with T50-BSA.
(iii) Flow 100 µl of 10–20 nM unlabeled primary antibody against the bait protein. Incubate for 10–20 min.
(iv) Wash twice with T50-BSA.

Pull-down of proteins from cell lysates ● TIMING ~30 min

19| Flow 100 µl of an appropriate dilution of cell or tissue lysate on the antibody-coated chambers. Dilutions are typically made in the lysis buffer without detergent, or in T50-BSA. Incubate for 10–20 min and then flush out the unbound extract. If the prey protein bears a fluorescent protein tag, proceed directly to Step 22 for imaging. Otherwise, proceed to Step 20 to fluorescently label the prey protein with antibodies.

▲ **CRITICAL STEP** Expression levels of proteins vary considerably depending on the protein being studied and across preparations. Hence, this dilution factor needs to be determined for each experiment (or sample preparation). As the concentration of the protein of interest in the crude cell extracts is difficult to estimate, we typically start with a 2,000-fold dilution of cell extract for overexpressed proteins or a 20-fold dilution of cell extract for endogenous proteins, where the lysate is prepared from 10^3 to 10^4 cells per µl. The protein concentration is titrated to obtain 0.1–0.2 fluorophores per μm^2, as determined in Step 22.

Immunofluorescence labeling of the pulled-down protein ● TIMING ~30 min

20| Incubate the pulled-down protein with 100 µl of 5–10 nM antibody against the prey protein for 10–20 min. Wash twice with T50-BSA.

▲ **CRITICAL STEP** When you use a secondary antibody for detection, the primary antibodies against bait and prey proteins must be from different organisms.

21| Add 100 µl of 1–2 nM fluorophore-labeled secondary antibody against the prey protein. Incubate for 10 min and flush out the unbound antibody.

▲ **CRITICAL STEP** The secondary antibody against the prey protein can potentially bind to the antibodies against the bait protein. Appropriate controls are recommended to rule out false positives.

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Single-molecule imaging and spot counting ● TIMING 10 min–1 h

22| Image the slide under a prism-type TIRF microscope. Acquire 20 or more short movies (~20 frames each), depending on the statistics desired. Analyze the movies to determine the mean number of fluorophores per unit imaging area.

▲ CRITICAL STEP Fluorescent proteins have a short fluorescence-photobleaching lifetime, typically a few seconds under our excitation scheme. Turn off the excitation shutter when you are not imaging. When imaging organic dyes, through the immunofluorescence labeling scheme, you can use appropriate oxygen-scavenging systems to prolong the fluorescence lifetime of the dyes.

? TROUBLESHOOTING

23| Titrate the concentration of cell lysate depending on the observed surface density of prey molecules to obtain 0.1–0.2 molecules per μm^2; repeat Steps 16–22 as necessary.

▲ CRITICAL STEP Typically, there is some fluorescent background from the surface arising as a result of impurities. The concentration of immobilized molecules due to specific binding should be kept at least five- to tenfold above this background. Under our imaging conditions, the background is ~0.01 molecules per μm^2. For applications involving colocalization of multiple fluorophores and for stoichiometry determination by photobleaching analysis, the density of molecules should be kept between 0.05–0.15 molecules per μm^2.

? TROUBLESHOOTING

24| Once the optimal concentration of the lysate is determined, perform appropriate control experiments by repeating Steps 16–22 with a suitable control antibody (for example, control IgG) and with control lysates lacking the bait-prey interaction.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
14	High background fluorescence without protein immobilization	Surface impurities during slide preparation (for example, aminosilane or PEG may be contaminated)	Make new PEG slides, using fresh reagents
15	Nonspecific binding of proteins	The PEG passivation is not good or has deteriorated	Make new PEG slides
22	No or few bait proteins detected	Low sample concentration in the lysate	Increase the concentration of the lysate added to the flow chamber
	No bait protein detected	Antibodies do not recognize the protein	Try a different antibody against a different epitope on the bait protein
23	No expected prey protein detected	Antibodies do not recognize the protein	Try a different antibody against a different epitope on the prey protein
24	The specific binding yields only two- to threefold higher binding as compared with the control channel	High nonspecific binding of antibodies against prey	Optimize the concentration of cell lysate and/or antibodies Wash with more stringent buffer conditions (high salt or a buffer with detergent) to remove nonspecifically adsorbed proteins
	The control channel has the same number of fluorophores as the sample channel	Surface passivation is not good	Make new PEG slides

● TIMING

Steps 1–10, PEG passivation of microscope slides: 6–8 h
Steps 11–13, construction of flow chambers: ~30 min
Steps 14 and 15, testing the quality of PEG slides: ~30 min
Steps 16–18, immobilizing the antibody against the bait protein: 30 min–1 h

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Step 19, pull-down of proteins from cell lysates: ~30 min
Steps 20 and 21, immunofluorescence labeling of the pulled-down protein: ~30 min
Steps 22–24, single-molecule imaging and spot counting: 10 min–1 h

ANTICIPATED RESULTS

The PEG-passivated surfaces resist nonspecific protein adsorption. **Figure 2** depicts a typical TIRF image of surface-immobilized YFP molecules, pulled down from the cell lysate. When the same amount of lysate was added to a PEG-passivated surface without antibodies, it yielded only ~5 additional fluorescent spots, on average, above the background in an imaging area of 2,500 μm^2. On the other hand, a surface passivated with BSA yielded substantial nonspecific protein binding (**Fig. 2**). Before the experiments, the slides should be checked for quality of passivation by flowing in ~10 nM of the labeled protein.

The lysate concentration is titrated to obtain an optimal fluorescent spot count above the background. Protein kinase A (PKA) tagged with YFP and hemagglutinin (HA) tags is pulled down with antibodies against the HA tag and visualized by using YFP fluorescence (**Fig. 4**). As we increased the lysate concentration, the number of YFP molecules observed also increased. The optimal density for single-molecule imaging is 0.1–0.2 fluorophores per μm^2 imaging area, as depicted in **Figure 4**. At higher concentrations, it is not possible to resolve single molecules.

We demonstrated the immunofluorescence-labeling strategy through pull-down of endogenous PKA-AKAP complexes from mouse brain extracts. We immobilized 20 nM biotinylated donkey-anti-rabbit antibody followed by 10 nM rabbit-anti-PKA antibody. To these surfaces, we then added a tenfold dilution of whole-brain extract. A detailed protocol for preparation of tissue samples is included as the **Supplementary Methods**. The pulled-down AKAPs are labeled with goat-anti-AKAP antibody (10 nM) followed by 2 nM secondary antibody against goat labeled with Alexa Fluor 647. Control experiments excluding the anti-PKA antibody (**Fig. 5a**, second from left), excluding biotinylated secondary antibody against PKA (**Fig. 5a**, second from right) and including a control rabbit IgG (**Fig. 5a**, right) are performed to verify that the observed fluorescence arises from specific binding of prey antibodies to AKAP. Additional controls, by knocking out PKA or AKAP, may be performed to confirm the interaction.

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Note: Supplementary information is available via the HTML version of this article.

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AUTHOR CONTRIBUTIONS A.J., Y.K.X. and T.H. designed the research. R.L. prepared the cell extracts. A.J. conducted single-molecule experiments and analyzed the data. A.J., Y.K.X. and T.H. wrote the paper.

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1. Alberts, B. The cell as a collection of protein machines: preparing the next generation of molecular biologists. *Cell* **92**, 291–294 (1998).
2. Barrios-Rodiles, M. *et al.* High-throughput mapping of a dynamic signaling network in mammalian cells. *Science* **307**, 1621–1625 (2005).
3. Yamada, T. & Bork, P. Evolution of biomolecular networks: lessons from metabolic and protein interactions. *Nat. Rev. Mol. Cell Biol.* **10**, 791–803 (2009).
4. Puig, O. *et al.* The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24**, 218–229 (2001).
5. Gingras, A.C., Gstaiger, M., Raught, B. & Aebersold, R. Analysis of protein complexes using mass spectrometry. *Nat. Rev. Mol. Cell Biol.* **8**, 645–654 (2007).
6. Fields, S. & Song, O. A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245–246 (1989).
7. Kerppola, T.K. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. *Annu. Rev. Biophys.* **37**, 465–487 (2008).
8. Reyes-Lamothe, R., Sherratt, D.J. & Leake, M.C. Stoichiometry and architecture of active DNA replication machinery in *Escherichia coli*. *Science* **328**, 498–501 (2010).
9. Li, H., Zhou, D., Browne, H., Balasubramanian, S. & Klenerman, D. Molecule by molecule direct and quantitative counting of antibody-protein complexes in solution. *Anal. Chem.* **76**, 4446–4451 (2004).
10. Taguchi, H., Ueno, T., Tadakuma, H., Yoshida, M. & Funatsu, T. Single-molecule observation of protein-protein interactions in the chaperonin system. *Nat. Biotechnol.* **19**, 861–865 (2001).
11. Kapanidis, A.N. *et al.* Fluorescence-aided molecule sorting: analysis of structure and interactions by alternating-laser excitation of single molecules. *Proc. Natl. Acad. Sci. USA* **101**, 8936–8941 (2004).
12. Blosser, T.R., Yang, J.G., Stone, M.D., Narlikar, G.J. & Zhuang, X. Dynamics of nucleosome remodelling by individual ACF complexes. *Nature* **462**, 1022–1027 (2009).
13. Jain, A. *et al.* Probing cellular protein complexes using single-molecule pull-down. *Nature* **473**, 484–488 (2011).
14. Ulbrich, M.H. & Isacoff, E.Y. Subunit counting in membrane-bound proteins. *Nat. Methods* **4**, 319–321 (2007).
15. Hoskins, A.A. *et al.* Ordered and dynamic assembly of single spliceosomes. *Science* **331**, 1289–1295 (2011).
16. Yeom, K.H. *et al.* Single-molecule approach to immunoprecipitated protein complexes: insights into miRNA uridylation. *EMBO Rep.* **12**, 690–696 (2011).
17. Roy, R., Hohng, S. & Ha, T. A practical guide to single-molecule FRET. *Nat. Methods* **5**, 507–516 (2008).
18. Fleminger, G., Solomon, B., Wolf, T. & Hadas, E. Effect of polyethylene glycol on the non-specific adsorption of proteins to Eupergit C and agarose. *J. Chromatogr.* **510**, 271–279 (1990).
19. Joo, C., Balci, H., Ishitsuka, Y., Buranachai, C. & Ha, T. Advances in single-molecule fluorescence methods for molecular biology. *Annu. Rev. Biochem.* **77**, 51–76 (2008).
20. Rothenberg, E. & Ha, T. Single-molecule FRET analysis of helicase functions. *Methods Mol. Biol.* **587**, 29–43 (2010).
21. Selvin, P.R. & Ha, T. *Single-Molecule Techniques: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 2008).