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Probing cellular protein complexes using single-molecule pull-down

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Proteins perform most cellular functions in macromolecular complexes. The same protein often participates in different complexes to exhibit diverse functionality. Current ensemble approaches of identifying cellular protein interactions cannot reveal physiological permutations of these interactions. Here we describe a single-molecule pull-down (SiMPull) assay that combines the principles of a conventional pull-down assay with single-molecule fluorescence microscopy and enables direct visualization of individual cellular protein complexes. SiMPull can reveal how many proteins and of which kinds are present in the *in vivo* complex, as we show using protein kinase A. We then demonstrate a wide applicability to various signalling proteins found in the cytosol, membrane and cellular organelles, and to endogenous protein complexes from animal tissue extracts. The pulled-down proteins are functional and are used, without further processing, for single-molecule biochemical studies. SiMPull should provide a rapid, sensitive and robust platform for analysing protein assemblies in biological pathways.

Dynamic interactions between proteins guide almost every aspect of cellular function[1]. Understanding how the macromolecules in living cells interact holds the key to deciphering their roles in cellular function and regulation[2,3]. Individual proteins are also part of diverse sets of protein networks, making it challenging to tease apart various permutations of protein–protein interactions occurring in the cellular context[4]. Currently, the gold standard for determining interactions between proteins is the co-immunoprecipitation assay[5–7], which relies on affinity-based co-purification of interacting proteins, followed by identification via western blot or mass spectrometry. It is, however, difficult to determine how many copies of which proteins are present in the physiological complex using conventional immunoprecipitation. In addition, the many hours and multiple steps that often exist between sample preparation and measurements present uncertainties over the extent to which *in vivo* interactions are preserved before analysis.

In situ imaging methods based on resonance energy transfer[8,9], fluorescence correlation spectroscopy[10,11], two-hybrid methods[12,13] and the bimolecular fluorescence complementation assay[14] are other popular tools for studying pair-wise protein interactions. However, these methods cannot be applied to endogenous proteins and are, in general, blind to heterogeneous interactions between proteins and their stoichiometry.

Here we present a simple, direct and sensitive method to study cellular protein complexes with single-complex resolution. We call this method single-molecule pull-down or SiMPull because physiological macromolecular complexes are pulled down from cell or tissue extracts directly to the imaging surface of single-molecule fluorescence microscopy.

Experimental strategy and YFP pull-down

The key requirement for pull-down assays is the selective capture of a protein of interest (bait), which will bring along its binding partners (prey). We constructed a flow chamber using a microscope slide and a cover slip, passivated with methoxy polyethylene glycol (mPEG)[15] to prevent non-specific adsorption of cell extracts and antibodies, which should minimize false positives[7]. The imaging surface was also doped with biotinylated PEG and streptavidin, followed by biotinylated antibodies against the bait protein (Fig. 1a–d and Supplementary Fig. 1). When cell extracts are infused in the flow chamber, the surface-tethered antibody captures the bait protein together with any interacting partners. After washing away the unbound cell extract, co-immunoprecipitated prey proteins are visualized either through immunofluorescence labelling (Fig. 1a) or using genetically encoded fluorescent protein tags (Fig. 1b). This approach is extendable to multi-protein complexes via multi-colour labelling and has the potential to differentiate between multiple sub-complexes and configurations (Fig. 1c). When proteins are fluorescently labelled with a fixed ratio, photobleaching events yield stoichiometric information[16,17] (Fig. 1d).

We first validated the SiMPull assay for specific pull-down of yellow fluorescent protein (YFP) from cell extracts. When the crude lysate from cells overexpressing His_6-tagged YFP was infused into the flow chamber coated with anti-His antibody, we observed single YFP molecules (Fig. 1e, f), similar to the analysis performed using purified protein[18] (Supplementary Fig. 2). Binding of YFP to the antibody was stable over two hours (Supplementary Fig. 3). The blank slide surface showed ~30 fluorescent spots per imaging area, 2,500 μm^2, possibly due to surface impurities. The number of fluorescent spots per imaging area, N_f, due to specifically pulled-down proteins was 10–20 fold over the background; we could maintain a >10 fold signal-to-noise ratio by controlling the lysate dilution factor. Control experiments with YFP lysate on non-specific antibody and lysate without YFP expression showed the same N_f as blank. Even lysate with a tenfold higher concentration of YFP yielded only ~30 additional spots relative to the blank, implying a less than 0.5% contribution from non-specifically adsorbed proteins (Fig. 1e, f). N_f increased monotonically as the cell lysate concentration increased over three orders

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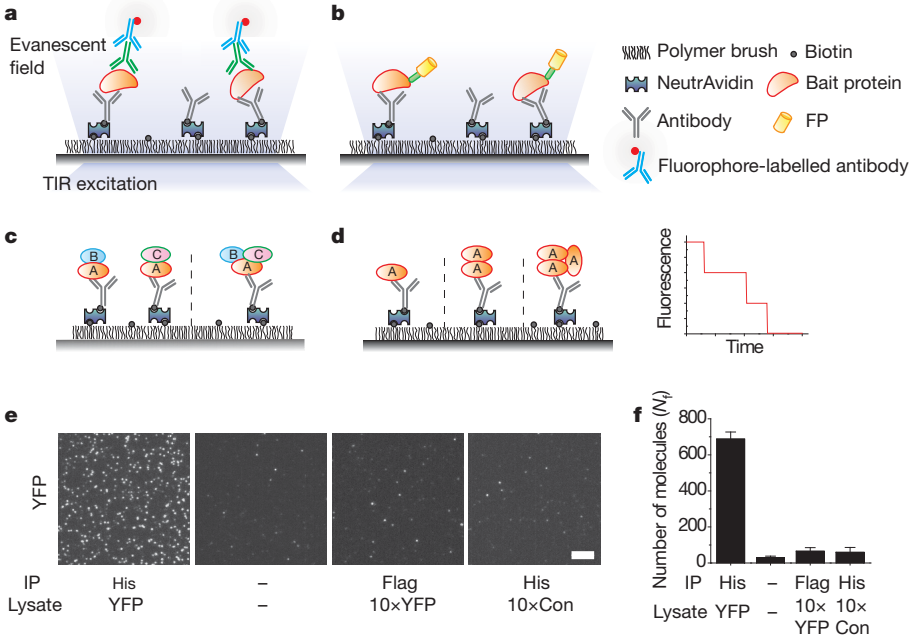

Figure 1 | Schematic for SiMPull assay. **a**, **b**, Immunoprecipitated protein complexes are visualized using TIRF microscopy using fluorophore-labelled antibody (**a**) or fluorescent protein (FP) tags (**b**). TIR, total internal reflection. **c**, Multi-colour colocalization can distinguish between subcomplexes (for example, AB + AC versus ABC). **d**, Photobleaching analysis can provide stoichiometric information. A simulated photobleaching trajectory for a trimeric protein. **e**, TIRF images for YFP pulled down from cells expressing His_6–YFP (YFP) and control cells (Con) using His-tag or a control (Flag-tag) antibody. Minus sign indicates no antibody or sample. IP, immunoprecipitate. Scale bar, 5 μm. **f**, Average number of fluorescent molecules per imaging area, N_f. Error bars denote standard deviation (s.d.) ($n > 20$).

of magnitude (Supplementary Fig. 4), showing that SiMPull can provide a quantitative estimate of protein concentration in cell lysate.

Single-molecule photobleaching analysis performed using the monomeric YFP and tandem dimeric YFP constructs (Supplementary Figs 5, 6) showed that accurate stoichiometric information can be obtained from the pulled-down proteins when we account for the fact that about 75% of YFP is fluorescently active[16].

Two-colour SiMPull of PKA complex

Next, we demonstrate the ability to pull-down single protein complexes from cell extracts using cyclic adenosine monophosphate (cAMP)-dependent protein kinase, protein kinase A (PKA), as our model system. PKA is a ubiquitous serine/threonine kinase that acts downstream of the G-protein coupled receptor (GPCR) pathway[19]. In the inactive state, PKA is a tetrameric complex consisting of two regulatory (R) and two catalytic (C) subunits. In the presence of cAMP, the complex dissociates, thereby activating the enzyme. We prepared C–HA–YFP and R–Flag–mCherry constructs (Fig. 2a).

When only C–HA–YFP was expressed in HEK293 cells, we could specifically pull-down the protein from the cell lysate using surface-immobilized antibodies against the HA or YFP epitope (Fig. 2b). As expected, these samples did not show any detectable fluorescence above background in the mCherry detection channel (Supplementary Fig. 7).

When the two subunits, R and C, were co-expressed, western blot showed that R and C co-immunoprecipitate[20] and they dissociate when cAMP is added to the lysate, confirming that the modified constructs retain the known properties of PKA (Fig. 2a). In a similar fashion, when we pulled down R–Flag–mCherry with anti-Flag antibody, we could detect both YFP and mCherry fluorescence spots. The number of fluorescent spots in mCherry and YFP channels was similar, indicating, on average, a one-to-one association (Fig. 2c, d). Fifty-seven per cent of YFP spots colocalized with a corresponding mCherry, as shown by individual images and their overlay (Fig. 2d). Incomplete colocalization may arise from basal tonic activation of PKA, inactive chromophores[16,21] or unbalanced expression of two proteins in individual cells. Adding cAMP analogue to the flow channel or pre-incubating the lysate with cAMP resulted in a greatly reduced number of C subunit (YFP spots) without a significant change in R subunit (mCherry spots) (Fig. 2c, e). After the reaction, only 4% of remaining YFP molecules colocalized with a corresponding mCherry.

Intracellular levels of cAMP can be increased by stimulating GPCRs for activation of adenylyl cyclases. When the cells overexpressing the PKA complex were stimulated with forskolin, an agonist of

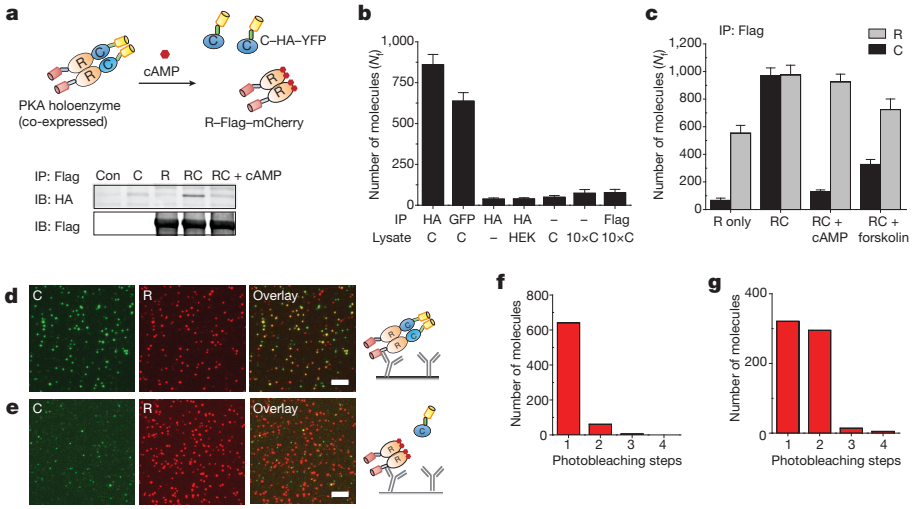

Figure 2 | PKA pull-down. **a**, Schematic of PKA construct. In western blot, C–HA–YFP is pulled down via R–Flag–mCherry; on adding cAMP, PKA dissociates. IB, immunoblot. **b**, N_f for C–HA–YFP (C) as a function of lysates and antibodies demonstrate the specificity of pull-down. **c**–**e**, PKA complex pull-down. **c**, N_f for YFP (C) and mCherry (R) spots. **d**, Images of single PKA complexes, YFP (left), mCherry (centre) and overlay (right). **e**, On adding cAMP, YFP spots decrease significantly. Scale bars, 5 μm. **f**, **g**, Photobleaching step distribution (**f**) for C–HA–YFP-only lysate and (**g**) for C–HA–YFP pulled down via R–Flag–mCherry. Error bars denote s.d. ($n > 20$).

adenylyl cyclase for cAMP production, the amount of active PKA in cells increased, as evidenced by dissociation of PKA in a western blot (Supplementary Fig. 8). Similarly, in our SiMPull assay, the number of C subunits (YFP molecules) pulled down through R subunits decreased significantly (Fig. 2c).

We explored the stoichiometry of PKA using photobleaching analysis. When C–HA–YFP was expressed alone and pulled down using HA antibody, 91% of YFP traces exhibited one-step photobleaching, indicating a monomeric population (Fig. 2f). When C–HA–YFP and R–Flag–mCherry were co-expressed and pulled down using anti-Flag antibody, 47% of YFP traces showed two photobleaching steps (Fig. 2g) and 51% of molecules bleached in one step. Assuming a 75% active fraction of YFP[16], this is consistent with the known stoichiometry of two catalytic subunits in each PKA. We did not perform photobleaching-based stoichiometry analysis for mCherry owing to its inferior photophysical properties[22].

Applications of SiMPull

Next, we examined the applicability of SiMPull to protein complexes from various cellular environments using different capture and detection configurations (Fig. 3).

Receptor pull-down

Membrane protein complexes are particularly difficult to analyse using conventional methods, thus motivating new approaches[23,24]. Their stoichiometry cannot be determined using photobleaching in the cell unless the areal density of the protein complex is low enough for single-molecule detection[16,25,26]. SiMPull should be able to detect individual complexes if membrane patches containing one complex can be isolated. As a test, we applied SiMPull to the β_2-adrenergic receptor (β_2AR), a prototypical GPCR. HEK293 cells were transfected with Flag–YFP–β_2AR and membrane proteins were solubilized. We could specifically pull-down the receptor using antibodies against YFP or Flag (Fig. 3a–c). Twenty-nine per cent of the traces showed two distinct bleaching steps (Supplementary Fig. 9a), indicating a ~51% dimer population, assuming 75% of active fluorophores. Less than 3% of the traces showed three or more photobleaching steps. Our observation of β_2AR homodimerization is consistent with previous studies[27,28]. To test if β_1ARs might form hetero-oligomers with β_2ARs[28], we co-expressed mCherry–β_1AR and YFP–β_2AR. Using antibodies against mCherry–β_1AR, we could pull-down YFP–β_2AR and vice versa (Supplementary Fig. 9b–e). The two fluorophores colocalized with ~42% overlap, consistent with hetero-oligomer formation.

Mitochondrial protein pull-down

Mitochondrial antiviral signalling (MAVS) is a mitochondrial outer membrane protein involved in the innate immune response[29]. When isolated mitochondrial fractions from cells overexpressing YFP–MAVS[30] were applied to a surface with anti-YFP, we observed bright fluorescent structures (Fig. 3e, left), indicating the presence of several YFP molecules. On pre-solubilizing the mitochondrial preparation using mild detergent, we observed isolated single YFP spots (Fig. 3e, centre), 86% of which showed single photobleaching steps (Supplementary Fig. 10), supporting the monomeric state of solubilized MAVS. This observation indicates that the bright fluorescent structures observed in unsolubilized preparations are due to immunoprecipitated mitochondrial membrane patches or whole mitochondria. It may be possible to specifically immobilize cellular organelles or their components using antibodies against suitable marker proteins and perform single-molecule measurements in a physiologically relevant context.

Immunofluorescence detection of single complexes

We extended the assay to detection via antibodies using mammalian target of rapamycin complex 1 (mTORC1) as a model system. mTORC1 is a key signalling complex that regulates cell growth and

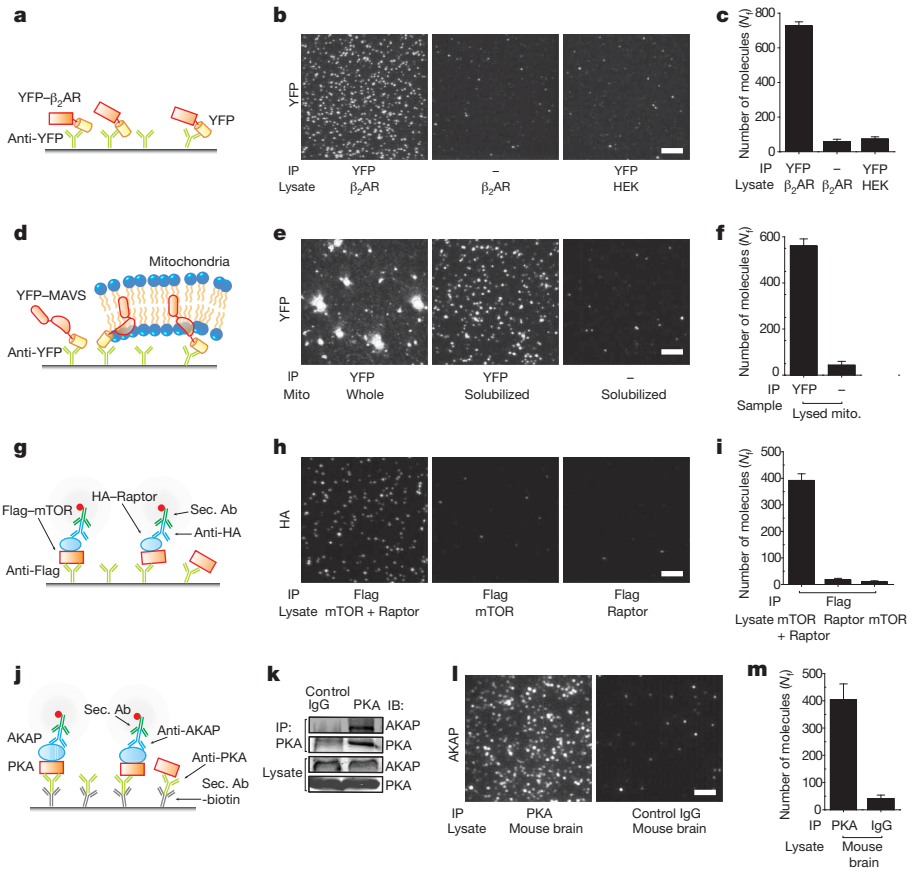

Figure 3 | Applications of SiMPull assay. **a–c**, β_2AR–YFP pull-down. **d–f**, MAVS pull-down. Mitochondrial fraction (mito.) from cells overexpressing YFP–MAVS was added either directly or after detergent solubilization. **g–i**, mTORC1 pull-down. Lysate from cells expressing Flag–mTOR, HA–Raptor or both was applied on chambers with Flag antibody, and probed through primary antibody against HA and labelled secondary antibody (Sec. Ab). **j–m**, Endogenous PKA–AKAP complex pull-down from mouse brain extract. **k**, Western blot shows AKAP immunoprecipitation with PKA antibody. **l**, Immunofluorescence images of AKAP150 pulled down through PKA antibody. **c**, **f**, **i**, **m** show N_f. Scale bars, 5 μm. Error bars denote s.d. ($n > 20$).

metabolism in response to nutrient availability[31,32]. In addition to mammalian target of rapamycin (mTOR), a defining component of mTORC1 is regulatory associated protein of mTOR (Raptor; also known as RPTOR), which associates with mTOR at an equimolar ratio[31,33]. We expressed Flag–mTOR and HA–Raptor in HEK293 cells. Flag–mTOR was pulled down using biotinylated Flag antibody; Raptor was detected using HA antibody followed by fluorescently labelled secondary antibody (Fig. 3g). When both Flag–mTOR and HA–Raptor were co-expressed, we observed the detection antibody binding as fluorescent spots whereas background level of fluorescence was detected when only one of the two proteins was expressed (Fig. 3h, i), demonstrating antibody-based detection in SiMPull.

Pull-down of endogenous complexes in native tissues

Exogenous expression may lead to non-physiological associations between proteins. Pull-down of endogenously expressed proteins, although desirable, is challenging owing to low abundance, high background interaction with other cellular proteins, and general lack of high-affinity antibodies. We tested if SiMPull can be used to detect interactions between endogenous proteins. A kinase anchoring proteins (AKAPs) bind to PKA and confine it to discrete locations in the cell[34]. Figure 3k shows AKAP150 can be co-immunoprecipitated with PKA from mouse brain extract.

Primary antibodies against proteins are often expensive and difficult to label with biotin or fluorophores. Thus, to keep our approach general, we used biotin-labelled secondary antibody to immobilize the antibody against the bait (PKA), and applied mouse brain extract. On probing for the prey protein (AKAP150) using its primary antibody and fluorescently labelled secondary antibody, we observed tenfold more fluorescent spots in the channel with PKA antibody as compared to the control channel (Fig. 3l, m). SiMPull required a 20-fold lower sample volume as compared to the corresponding western blot. This sensitivity allowed detection of PKA–AKAP binding from mouse heart tissue, which was below the detection limit of the conventional western blot under the same conditions (Supplementary Fig. 11).

SiMPull as a preparatory tool

A key advantage of SiMPull is that protein complexes can be directly observed from a fresh cell lysate, bypassing purification procedures. We tested if SiMPull can be used for functional analysis of pulled-down proteins. PcrA, a superfamily 1 helicase, is an ATP-driven motor protein that binds and translocates on single-stranded DNA (ssDNA)[35]. His_6-tagged PcrA was pulled down from bacterial lysate using anti-His antibody and fluorescently labelled DNA molecules were added to the flow channel (Fig. 4a). Fluorescent spots due to labelled DNA binding appeared in the flow channel with pulled-down PcrA, whereas the control channel showed minimal DNA binding (Fig. 4b, c).

When PcrA binds to a partial duplex DNA with a 5′ overhang, it anchors itself to the junction and repetitively reels in the ssDNA[35]. By labelling the DNA with a donor at the tail end and an acceptor at the junction (Fig. 4a), we could observe the reeling-in activity as a gradual increase in fluorescence resonance energy transfer (FRET) (Fig. 4d). Once PcrA reaches the end of the ssDNA, it runs off the ssDNA track and repeats the process from the junction over and over, resulting in cyclic increases and decreases in FRET. Eighty-six per cent (161 of 188) of bound FRET-labelled DNA molecules exhibited repetitive cycling. On increasing the ATP concentration, translocation became faster (Supplementary Fig. 12a), and in the absence of ATP, DNA remained bound but no reeling-in activity was observed (Supplementary Fig. 12b). The mean translocation time matched well with the data obtained with purified protein (Fig. 4e, f). Thus, SiMPull can pull-down functional macromolecules directly from cell extracts for subsequent single-molecule biochemistry *in situ*.

Discussion

We have established a single-molecule platform for analysing the cellular association of macromolecules. SiMPull can be used as an extension of commonly used western blot analysis without requiring additional sample preparation (Supplementary Fig. 13) and confers several key advantages. First, it can provide quantitative data on sub-populations of different association states. Second, it provides information on complex stoichiometry if the proteins can be stoichiometrically labelled. Third, the high sensitivity allows the study of complexes of low abundance, and a suitable calibration (Supplementary Fig. 4) should make it possible to determine the expression level of protein complexes in cell lysate. Fourth, the whole assay took about 30 min, considerably shorter than conventional western blot. In a pilot experiment, we could dilute the cell lysate, pull-down and quantify YFP in 2.5 min (Supplementary Fig. 14). Therefore, it should be possible to analyse even relatively weak protein complexes as long as the dissociation rate constant k_{off} is equal to or smaller than 0.01 s^{-1}. Combining this with microfluidics platform, cross-linking methods or zero mode waveguide[36] may extend the method to complexes with even higher k_{off}.

Cellular processes are under tight spatiotemporal regulation. To overcome ensemble averaging over heterogeneous cell populations, SiMPull may be combined with fluorescence-aided cell sorting to selectively analyse a subpopulation or may even be pushed to the single-cell level. In a preliminary experiment, we could pull-down and quantify proteins from 10 cells obtained through cell sorting (Supplementary Fig. 15) compared to the ~5,000 cells usually required for a western blot[37]. Single-cell SiMPull may enhance the recently demonstrated ability to quantify proteins and RNA numbers in single cells[38]. As in conventional western blot, the sensitivity and specificity of SiMPull are determined by the quality of capture and detection antibodies. The assay may be combined with recent developments in labelling

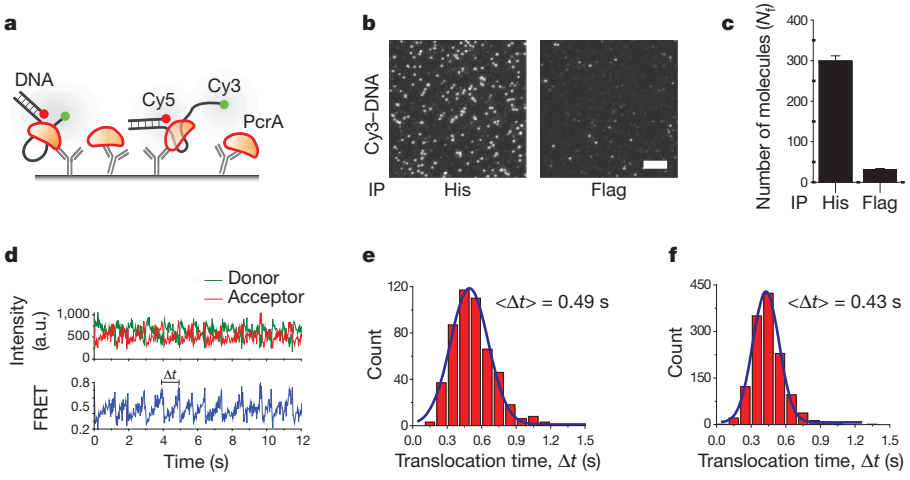

Figure 4 | PcrA pull-down and activity. **a**, Schematic. **b**, **c**, Labelled DNA binding to immunoprecipitated PcrA. Scale bar, 5 μm. Error bars represent s.d. ($n > 20$). **d**, A typical time trace of repetitive reeling-in activity of PcrA monitored by FRET. a.u., arbitrary units. **e**, **f**, The distribution of translocation times, Δt, and its mean, $<\Delta t>$, for purified PcrA (**e**) and for PcrA pulled down from cell extracts (**f**), at 1 mM ATP concentration.

strategies[39] for further improvement in sensitivity and labelling efficiency.

Post-translational modifications have an important role in cellular processes but are difficult to reproduce in recombinant proteins. In addition, the necessary co-factors or ligands for a protein of interest are often unknown. SiMPull as a preparatory tool provides a possibility to study these modified proteins or protein complexes that cannot be purified using conventional methods.

METHODS SUMMARY

Flow chambers were prepared on mPEG passivated quartz slides doped with biotin PEG[15]. Biotinylated antibodies were immobilized by incubating ~10 nM of antibody for 10 min on NeutrAvidin (Thermo) coated flow chambers. A prism type total internal reflection fluorescence (TIRF) microscope was used to acquire the single-molecule data[40]. Samples with fluorescent protein tag were serially diluted to obtain well-isolated spots on the surface upon 20 min of incubation over immobilized antibody surface. All dilutions were made immediately before addition to the flow chamber in 10 mM Tris-HCl pH 8.0, 50 mM NaCl buffer with 0.1 mg ml^{-1} bovine serum albumin (New England Biolabs), unless specified. Unbound antibodies and sample were removed from the channel by washing with buffer twice between successive additions. For immunofluorescence detection, immunoprecipitated complexes were incubated with a different antibody against prey protein (~10 nM) for 20 min and fluorescent-dye-labelled secondary antibody (2–5 nM) for 5 min before imaging. Single-molecule analysis was performed using scripts written in Matlab.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Overview. Flow chambers were prepared on mPEG passivated quartz slides doped with biotin PEG[15]. Biotinylated antibodies were immobilized by incubating ~10 nM of antibody for 10 min on NeutrAvidin (Thermo) coated flow chambers. A prism type total internal reflection fluorescence (TIRF) microscope was used to acquire the single-molecule data[40]. Samples with fluorescent protein tag were serially diluted to obtain well-isolated spots on the surface upon 20 min of incubation over immobilized antibody surface. All dilutions were made immediately before addition to the flow chamber in 10 mM Tris-HCl pH 8.0, 50 mM NaCl buffer with 0.1 mg ml^{-1} bovine serum albumin (New England Biolabs), unless specified. Unbound antibodies and sample were removed from the channel by washing with buffer twice between successive additions. For immunofluorescence detection, immunoprecipitated complexes were incubated with a different antibody against prey protein (~10 nM) for 20 min and fluorescent-dye-labelled secondary antibody (2–5 nM) for 5 min before imaging. Single-molecule analysis was performed using scripts written in Matlab.

Single-molecule imaging and spot counting. A prism type TIRF microscope was used to acquire single-molecule data[40]. YFP was excited at 488 nm; mCherry was excited at 532 or 568 nm. Narrow band-pass filters were used to avoid cross-talk between channels (HQ 535/30 from Chroma Technology for YFP and BL 607/36 from Semrock for mCherry). All experiments were performed at room temperature (22–25 °C) unless specified. Single-molecule analysis was performed as described earlier[15]. Mean spot count per image (imaging area 2,500 μm^2) and standard deviation were calculated from images taken from 20 or more different regions.

Photobleaching analysis. Single-molecule fluorescence time traces of surface immobilized YFP-tagged proteins were manually scored for the number of bleaching steps[16]. To avoid false colocalization, samples were immobilized at an optimal surface density (~300 molecules in 2,500 μm^2 imaging area). The number of photobleaching steps (single frame intensity drops of equal size) in each trace was manually determined, following published procedures[16]. The fluorescence trace of each molecule was classified as having 1–4 bleaching steps or was discarded if no clean bleaching steps could be identified (Supplementary Fig. 5). Some fluorescent protein molecules exhibited blinking, but under most circumstances distinct fluorescence intensity levels could be readily determined despite this blinking behaviour (Supplementary Fig. 5a, b). Separate counts were maintained for each case. At least 500 molecules were analysed for each sample. The probability of missed bleaching events due to simultaneous bleaching of both fluorescent proteins within the same imaging window is ~5%. The population distribution of observed bleaching events and discarded traces is reported in Supplementary Table 1. For future extensions to complexes with many more copies of the same protein, automated algorithms for scoring photobleaching steps would be required[41].

Single-molecule colocalization. Colocalization between YFP and mCherry was performed using a method similar to that described previously[42]. Briefly, we took two separate movies of the same region using YFP and mCherry excitation. The fluorescent spots in both images were fit with Gaussian profiles to determine the centre positions of the molecules to half-pixel accuracy. Next, for each molecule in the YFP image, we determined the mCherry molecules with their centre within a 2-pixel (~300 nm) distance. The number of molecules where this colocalization occurred divided by the total number of YFP molecules was presented as overlap percentage.

YFP constructs and pull-down. As YFP has been shown to dimerize, monomeric YFP was generated through site-directed mutagenesis of alanine 207 to lysine using pEYFP-C1 as the DNA template (Clontech). For bacterial expression, monomeric YFP was cloned into the SalI and XhoI sites of the pET-28b$^+$ vector. BL21 DE3 cells were transformed with the YFP construct and induced by 0.2 mM IPTG for protein expression. Cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH 8.0) and sonicated. The lysate was centrifuged at 15,000*g* for 20 min to collect supernatant used for SiMPull.

For expression in mammalian cells, YFP–His_6 was generated through addition of a 6×His-tag to the carboxy-terminal of YFP and subcloned into the XhoI and XbaI sites of pCDNA3.1$^+$. A second YFP was subcloned into the HindIII and EcoRI sites of pCDNA3.1-YFP-His_6 to make a tandem dimeric YFP construct. Monomeric and dimeric YFP constructs were transiently expressed in HEK293 cells and purified using standard Ni-NTA chromatography. Proteins were detected by western blot using GFP antibody (Clontech) or Penta-His antibody (Qiagen). For single-molecule analysis, samples were immobilized on biotinylated anti-Penta-His antibody (Qiagen) or on biotinylated polyclonal anti-GFP antibody (Rockland Immunochemicals).

PKA constructs and pull-down. HEK293 cells were transfected with R–Flag–mCherry and C–HA–YFP constructs. The regulatory subunit used was PKA RIIβ, and the catalytic subunit is the Cα isoform. After 24 h expression, cells were harvested into lysis buffer (10 mM Tris pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM benzamidine, 10 μg ml^{-1} leupeptin, 1 mM NaF, 1 mM Na_3VO_4). This lysate was centrifuged at 14,000*g* for 20 min and used for SiMPull. For bulk immunoprecipitation, anti-Flag M2 beads were added to the lysate for 3 h at 4 °C. Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes for blot with anti-HA antibody and anti-mCherry antibody.

For cAMP treatment, a non-hydrolysable analogue (8-Br-cAMP; Sigma) was used to activate PKA. For *in vivo* stimulation, R–Flag–mCherry and C–HA–YFP were transiently expressed in HEK293 cells for 24 h. Cells were pre-treated for 10 min with 10 μM 3-isobutyl-1-methlxanthine (IBMX; Sigma) followed by 5 min stimulation with 10 μM forskolin (Sigma). Cells were immediately washed with cold PBS and lysed as described earlier.

Adrenergic receptor constructs and pull-down. Flag–YFP–β_2AR, HA–YFP–β_2AR, Flag–mCherry–β_1AR and HA–mCherry–β_1AR were transiently expressed in HEK293 cells for 24 h. Cells were harvested into hypotonic lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM benzamidine, 10 μg ml^{-1} leupeptin, 0.3% n-dodecyl-B-D-maltoside (DDM), and incubated for 30 min before centrifugation at 600*g* for 10 min. Supernatants were collected and used for SiMPull with antibodies as indicated.

Mitochondria preparation and MAVS pull-down. HEK293 cells were transiently transfected with YFP–MAVS[30]. Intact mitochondria were isolated using MITOISO2 kit (Sigma) and diluted in the storage buffer supplied with the kit. Mitochondrial preparation was immobilized on slides either directly or after solubilization by adding 1% DDM to the storage buffer. We obtained similar results using whole-cell lysates prepared using several different lysing solutions. YFP–MAVS or mitochondria were immunoprecipitated using biotinylated antibody against GFP.

mTORC1 construct and pull-down. Flag–mTOR was stably transfected in HEK293 cells to obtain near endogenous expression levels of mTOR. For mTORC1 pull-down, Flag–mTOR stable cell lines were transiently transfected with HA–Raptor. HA–Raptor-only lysate was obtained by transiently transfecting HEK293 cells with HA–Raptor. Cells were lysed using CHAPS detergent buffer (40 mM HEPES pH 7.5, 0.3% CHAPS, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM EDTA), with protease inhibitor cocktail. Lysate was diluted in the buffer without CHAPS for SiMPull with biotinylated anti-Flag antibody (Sigma). Co-immunoprecipitated HA–Raptor was detected using goat anti-HA antibody (Genscript) and donkey anti-goat secondary antibody (Rockland Immunochemicals) labelled with Cy3.

Endogenous protein pull-down. Mouse brain and heart extracts were prepared from 2-week-old FVB mice after anaesthetization. Both samples were homogenized in lysis buffer (20 mM HEPES pH 7.4, 0.5% Triton X-100, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 5 μg ml^{-1} pepstatin, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4), incubated at 4 °C for 1 h followed by centrifugation at 16,000*g* for 10 min to collect the supernatant. These samples were directly used for SiMPull. For bulk immunoprecipitation, protein-A beads were added to pre-clean the lysate (2 h incubation at 4 °C). PKARII antibody was then added to the lysate for overnight immunoprecipitation followed by 1 h incubation with protein-A beads. Control rabbit IgG was added at a final concentration of 2 μg ml^{-1}. The immunoprecipitated proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes for immunoblot analysis with AKAP150 and PKARII antibodies.

PcrA pull-down and functional assay. His_6-tagged PcrA purified protein and cell lysate were prepared as previously described[35]. The protein was immobilized on slides using antibodies against the polyhistidine tag. A Cy3 and Cy5 dual-labelled partial duplex DNA (Integrated DNA Technologies) with a 5′ tail was added to the immobilized protein. The sequence of DNA used was: 5′ Cy3-$(dT)_{40}$-GCCTCGCTGCCGTCGCCA-3′ + 5′-TGGCGACGGCAGCGAGGC-3′-Cy5.

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