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Single-molecule fluorescence microscopy of native macromolecular complexes

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Macromolecular complexes consisting of proteins, lipids, and/or nucleic acids are ubiquitous in biological processes. Their composition, stoichiometry, order of assembly, and conformations can be heterogeneous or can change dynamically, making single-molecule studies best suited to measure these properties accurately. Recent single-molecule pull-down and other related approaches have combined the principles of conventional co-immunoprecipitation assay with single-molecule fluorescence microscopy to probe native macromolecular complexes. In this review, we present the advances in single-molecule pull-down methods and biological systems that have been investigated in such semi vivo manner.

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Introduction

Single-molecule methods have provided a wealth of unique quantitative information on the functioning of proteins by making it possible to image and mechanically perturb biomolecules with down to subnanometer spatial resolution and submillisecond timescale [1–3]. In single molecule fluorescence imaging, observables such as fluorescence intensity, efficiency of fluorescence resonance energy transfer (FRET) between a donor and acceptor fluorophore or colocalization between multi-fluorophores can yield detailed mechanistic information [4,5]. By monitoring the time dependent changes in fluorescence or FRET signal, one can measure association–dissociation kinetics, enzymatic activity, stoichiometry, local environmental fluctuations and other dynamical effects [6••,7••,8•,9,10••,11••,12•]. Single-molecule fluorescence studies in most cases are performed with a total internal reflection (TIR) scheme where hundreds of surface-immobilized single fluorescent molecules are imaged simultaneously in real-time [13,14•]. TIR configuration permits the excitation of only surface tethered molecules by an evanescent field penetrating 50–200 nm into the solution, reducing background fluorescence and improving the signal-to-background ratio.

Most single-molecule fluorescence studies to date have been performed with molecules in isolation or interaction with one partner molecule. But *in vivo*, these molecules would often assemble into multisubunit complexes, consisting of proteins, lipids and/or nucleic acids, whose activity and composition are under dynamic spatial and temporal regulation [15–17]. The order in which subunits associate is important for the formation and biological function of the complex, making it critical to understand their assembly pathway [18]. Indeed, several research groups recently utilized single-molecule cell-extract based approaches to enable quantitative investigation of macromolecular complexes in physiological environment [6••,7••,8•,19,20] (Table 1). In this review, we highlight a variety of experimental methodologies used for single-molecule pull-down as they are applied to multisubunit complexes in lipid membranes, cytoplasm and in the nucleus (Table 1).

Single-molecule fluorescence studies using cell extracts

Co-immunoprecipitation (co-IP) is a gold-standard for studying protein-protein interactions. However, the information obtained either lacks dynamic and quantitative readouts or is unable to provide stoichiometry of the complex. Performing co-IP experiments at single-molecule resolution reveal static and dynamic molecular properties that are masked by the averaging effects of a conventional co-IP [6••]. To this end, cell extracts, prepared from cultured cell lines or native tissues, is introduced directly onto microscopic slides pre-immobilized with capturing molecules such as antibodies, lipids or nucleic-acids. Surface immobilization of the 'captors' is commonly achieved by tagging molecules with biotin and using avidin to bind them to a biotin-PEG (polyethyleneglycol) surface. The immobilized molecules selectively capture the protein of interest (bait), which brings along its interacting partners (prey) (Figure 1a,b). After washing away unbound lysate, co-immunoprecipitated prey molecules are visualized either using genetically encoded fluorescent tags or through immunofluorescence labeling (Figure 1b). Adequate passivation of microscope slides

Table 1

List of current single-molecule pull-down approaches

Approach	Acronym
Single-Molecule Pull-down [6••]	SiMPull
Colocalization Single-Molecule Spectroscopy [7••]	CoSMoS
Single-Molecule approach to IMmunoprecipitated Protein complexes [8•]	SiMPlex
Single-molecule CoImmunoPrecipitation [33]	SiCoIP
Single-molecule Chromatin ImmunoPrecipitation imaging [44]	Sm-ChIPi
Single Cell Pull-down [49•]	SiCPull

with PEG is required to prevent false positives resulting from non-specific adsorption from extracts [Protocol details: 6••,11••,13,14•]. Choosing appropriate biotinylated molecules, fluorescently-tagged bait and prey molecules, the type of fluorescent tags, and imaging condition enables the exploration of a multitude of single-molecule analysis summarized in Figure 1c and Table 2 [11••]. All the approaches listed in Table 1 utilize similar principles to perform pull-down and vary in their specific biological application, which motivated researchers to name them differently.

Multiprotein assemblies on the lipid membrane

Proteins associated with the membrane — integral or lipid-anchored — are commonly found in multimeric forms obtained as a result of self-assembly (homomeric) or co-assembly (heteromeric) [21]. The number of subunits in membrane proteins, or the stoichiometry, is precisely regulated for their function. Mutations in the oligomerization domains of integral membrane proteins such as ion channels and transporter disrupt their assembly with correct stoichiometry and can cause various pathologies [22]. Yet, it is difficult to determine stoichiometry without disrupting the lipid environment using traditional biochemical techniques. To overcome this challenge, researchers adopted single-molecule total internal reflection fluorescence (TIRF) imaging in live cell membranes of *Xenopus* oocytes to determine subunit stoichiometry of NMDA receptors [23•,24]. Here, NR1 and NR3B subunits were genetically fused to GFP individually, and photobleaching step counting analysis showed that each assembles as a dimer, indicating that they assemble with 2:2 stoichiometry. Live TIRF imaging restricts stoichiometry analysis to plasma membranes. The single molecule pull-down (SiMPull) approach allows stoichiometry determination irrespective of the cellular origin of membrane proteins by immobilizing them directly from cell extracts for TIRF imaging [6••,25–27,28••,29,30]. By capturing membrane patches from the plasma membrane or intracellular organelles the stoichiometry of Best (chloride channels), TREK (potassium channels), MEC (sodium channels) and GluR2 (Glutamate receptors) has been determined through SiMPull (Table 3a). A mitochondrial membrane protein called MAVS was also used as a protein marker to pull down mitochondrial membrane patches from cell extracts [6••]. Similarly, vesicles derived from mouse neuroblastoma cells were pulled down via α3β4 nicotine receptors to determine the receptor stoichiometry [31].

Membrane proteins from the same family with high sequence and structural homology can coassemble into heteromeric complexes with novel functional properties [25,26]. Single-molecule colocalization analysis using multi-color labeling is suitable to probe the coassembly mechanisms. Subunits of Best and TREK channels were tagged by GFP and mCherry, expressed together, and pulled-down to determine the colocalization efficiency between the different subunits [25,26]. Similarly, Vafabakhsh *et al.* used dual labeling for both colocalization and single-molecule FRET measurements between the GluR2 receptor subunits immunoprecipitated from HEK293T cells [32••].

Lipid-anchored proteins that are found in the cytoplasm were also studied using SiMPull. These proteins contain lipid-binding domains (LBDs) that help them anchor to phospholipids in the plasma and intracellular membranes. Arauz *et al.* surface-immobilized lipid vesicles doped with signaling phospholipids and pulled down LBDs and LBD-containing proteins onto these vesicles [12•]. Lipid–protein interactions were transient and by performing real-time TIRF imaging in the presence of cell extracts they could determine the binding and dissociation kinetics and show that different LBDs (FYVE, Spo20-PABD, PLCδ-PH, Akt-PH) and full-length Akt protein have distinct assembly and lipid binding modes [12•].

Table 2

List of quantitative analysis performed on single complexes

Parameter measured	Property determined
Number of single fluorescent molecules	Specificity of pull-down, expression level of proteins
Colocalization percentage between multi-color labeled molecules	Interacting partners and assembly of the complex
Photobleaching step-counting	Stoichiometry
Changes in FRET efficiency	Intra- and intermolecular conformational dynamics
Duration of bound and unbound state, dwell-time distribution	Association and dissociation kinetics of transiently interacting molecules