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# Single-molecule pull-down for investigating protein–nucleic acid interactions

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ABSTRACT

The genome and transcriptome are constantly modified by proteins in the cell. Recent advances in single-molecule techniques allow for high spatial and temporal observations of these interactions between proteins and nucleic acids. However, due to the difficulty of obtaining functional protein complexes, it remains challenging to study the interactions between macromolecular protein complexes and nucleic acids. Here, we combined single-molecule fluorescence with various protein complex pull-down techniques to determine the function and stoichiometry of ribonucleoprotein complexes. Through the use of three examples of protein complexes from eukaryotic cells (Drosha, Dicer, and TUT4 protein complexes), we provide step-by-step guidance for using novel single-molecule techniques. Our single-molecule methods provide sub-second and nanometer resolution and can be applied to other nucleoprotein complexes that are essential for cellular processes.

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## 1. Introduction

Interactions between protein assemblies and nucleic acids are essential elements of cellular processes, such as transcription, translation, and chromatin remodeling. A well-known example of such a protein assembly is the spliceosome, a multi-megadalton ribonucleoprotein complex that uses numerous cofactors to catalyze the splicing of precursor messenger RNA [1,2]. The ribonucleoprotein complex called RISC (RNA-induced silencing complex) is a key player in RNA interference—a cellular process of translational repression [3]. The biogenesis and regulation of microRNA (non-coding RNA that mediates RNA interference) involves several protein complexes such as Drosha-DGCR8 [4,5], Dicer-TRBP [6,7], Dicer-Loqs [8,9] and TUTase-Trim25 [10].

A comprehensive analysis of nucleoprotein complexes is a stepping stone to understanding cellular processes. Recent advances in analytical and biochemical methods have led to numerous breakthroughs in the characterization of multicomponent protein assemblies in complexes with nucleic acids. High-throughput approaches, including large-scale tandem affinity purification, the yeast two-hybrid system, and mass spectrometry analysis, have been used to identify thousands of new protein complexes in yeast [11–15], *Drosophila melanogaster* [16,17] and *Caenorhabditis elegans* [18]. In parallel, advanced computational methods have emerged during the past decade, which made it possible to predict the formation of protein complexes [19]. Major advances in sample preparation and detection techniques have also enabled crystallographers and electron microscopists to determine the structure of large protein complexes interacting with nucleic acid substrates at an atomic resolution [20,21].

Despite the wealth of information acquired from these analytical and biochemical methods, there is a need for complementary techniques that allow for real-time observations of the assembly and function of nucleoprotein complexes. Recently, we and other groups developed such single-molecule fluorescence methods. Hoskins et al. revealed the order of spliceosome assembly during pre-mRNA maturation in cell extract via single-molecule multicolor fluorescence [22,23]. Single-molecule pull-down FRET allowed Nils et al. to visualize in real time the splicing of pre-mRNA by the spliceosome [24,25]. Lee et al. used a single-molecule co-immunoprecipitation approach to investigate weak interactions between different proteins [26,27]. Jain et al. developed single-molecule pull-down techniques to determine the stoichiometry of protein complexes [28–33]. We developed a single-molecule pull-down method to gain insight into the molecular mechanism of large nucleoprotein complexes involved in microRNA uridylation [34].

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Here, we describe various single-molecule pull-down approaches and provide protocols for the purification and immobilization of ribonucleoprotein complexes associated with their native cofactors. Our pull-down methods in combination with single-molecule fluorescence allow for real-time visualization of protein complexes and RNA interactions. We describe several different strategies used in our laboratory and list the challenges that we encountered during the development of these techniques. As a proof-of-concept, we show three examples of protein complexes involved in small RNA biogenesis (human Drosha-DGCR8, human Dicer-TRBP, Drosophila Dicer 2-Loqs-PD, and human TUT4 complex) and illustrate how we elucidate the molecular bases of their functions. With this protocol, single-molecule fluorescence can be widely used to study nucleoprotein complexes.

## 2. Materials and methods

### 2.1. Cell culture and transfection

#### 2.1.1. HEK-293T cells

Human embryonic kidney cells (HEK-293T) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, 31885023, Gibco®) supplemented with 10% fetal bovine serum (FBS, heat-inactivated, Greiner Bio-One) at 37 °C and 5% $CO_2$. Before transfection, cells were split into 10 cm cell culture dishes to a confluence of 25%. After 24 h of growth, plasmids of interest were transfected using a $CaPO_4$ transfection method (*Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, 2001). For the *in vivo* biotinylation of human Dicer and Drosha proteins, an additional plasmid coding for the BirA enzyme was co-transfected. After 5 h, the medium was exchanged with fresh DMEM containing 1 μg/ml biotin (B4639, Sigma), and the transfected cells were incubated for another 48 h to enable protein expression and *in vivo* biotinylation.

#### 2.1.2. SL2 cells

Schneider's Drosophila Line 2 (SL2, CRL-1963™, ATCC®) was maintained in HyClone SFX-Insect Cell Culture medium (SH30278.LS, GE Healthcare HYCLONE) supplemented with 10% FBS (heat-inactivated, Greiner Bio-One) at 25 °C. When the culture reached a density of $0.5 \times 10^6$ cells/mL, the cells were transfected using the FuGENE® HD transfection method (E2311, Promega). After 24 h of incubation, 1 mM $CuSO_4$ was added to the medium, and the cells were incubated for additional 48 h.

### 2.2. Cell harvest and lysis

Before the transfected cells were harvested with scrapers, DMEM was removed and the cells were washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS, 14200 Gibco®). Subsequently, the cells were transferred to 15 mL tubes and centrifuged at 276×*g* and 4 °C for 5 min to form cell pellets. After the removal of the supernatant, the cell pellets were frozen and stored at −80 °C until further processing. Before lysis, the cells were thawed on ice for 30 min. Subsequently, HEK-293T and SL2 cells were resuspended in buffer D (20 mM Tris–HCl [pH 8.0], 200 mM KCl and 0.2 mM EDTA) and lysis buffer SL2 (30 mM HEPES-KOH [pH 7.4], 100 mM KOAc, 10% glycerol, 0.1% Triton X-100), respectively. Lysis was carried out by carefully passing the cells 10 times through a needle (30½ gauge, BD), while avoiding the formation of air bubbles. Afterwards, the lysate was centrifuged twice (16,100×*g* at 4 °C, for 20 min) to remove cell debris (pellet). The recovered cell extract (supernatant) was either directly used for single-molecule experiments (Drosha-DGCR8), or alternatively, tandem purification steps were carried out to obtain higher purity samples (dmDicer-2, hDicer and TUT4). To prevent disturbing the protein complexes, it is important to perform the cell lysis and immunoprecipitation in a gentle manner and in a physiologically relevant buffer. We do not recommend the use of sonication as a cell lysis method because this may cause protein complexes to disassemble and form aggregates [35].

### 2.3. Immunoprecipitation and elution

For immunoprecipitation of 1xFLAG-tagged proteins (dmDicer-2, hDicer, and TUT4), 1 mg of total protein in the cell extract was incubated with 2.5 μL of anti-FLAG antibody-conjugated agarose beads (50% slurry, anti-FLAG® M2 affinity gel, A2220, Sigma) under gentle agitation at 4 °C for 30–60 min. It is noted that a longer incubation time may increase the number of non-specific interactions and result in the pull-down of contaminant proteins. After incubation, the beads were gently washed five times with buffer D or buffer SL2 and resuspended in 10 μL of buffer D or buffer SL2, resulting in 100 μg/μL of total protein concentration. hDicer was eluted from the beads by site-specific cleavage using *Tobacco Etch Virus* TEV protease (0.05 U/μL) (V6101, Promega) at 30 °C for 90 min. Alternatively, the proteins of interest (dmDicer-2 and TUT4) were eluted from the beads using 2 mM 3xFLAG® peptide (F4799, Sigma). The eluted proteins were supplemented with glycerol to a final concentration of 10%, aliquoted and snap-frozen with liquid nitrogen for long-term storage at −80 °C. The immunoprecipitates (IPs) were tested for the enrichment of the proteins of interest using western blot analysis, while the catalytic activities of the IPs were tested with bulk assays (data not shown).

### 2.4. Single-molecule pull-down

To increase purity of the IPs, an additional purification step was carried out directly on the surface of the imaging chamber using streptavidin or specific antibodies targeting the proteins of interest with nanomolar affinity range. This allowed for an efficient immobilization of the protein of interest, while discarding unwanted contaminant proteins (Fig. 1). Single-molecule pull-down procedures are described case by case in the Results and Discussion sections.

### 2.5. Nucleic acids preparation and labeling

#### 2.5.1. Stem-loop RNA

All of the RNA constructs used in this study were synthesized by ST Pharm Co., Ltd., South Korea. Precursor-microRNA (pre-miRNA) molecules were constructed by ligating two synthetic RNAs. First, a single-stranded RNA containing a 5′ phosphate and a half of the terminal loop of pre-miRNA (100 pmol, strand J in Table 1) was mixed with the 5′ strand that contained the other half of the terminal loop (200 pmol, strand K in Table 1). The mixture (20 μL) in TE buffer supplemented with 100 mM NaCl was annealed by heating it to 80 °C, followed by a slow cooling down to 4 °C (−1 °C/4 min in a thermal cycler). The annealed substrate was ligated using 3 μL of T4 RNA ligase (5 U/μL, AM2140, Invitrogen), 3 μL of 0.1% BSA (AM2616, Ambion), 5 μL of the 10× ligation buffer provided, and 19 μL of $H_2O$ at 16 °C for 24 h. After acid phenol-chloroform extraction and ethanol precipitation, the RNA was purified with 12.5% urea polyacrylamide gel.

The primary miRNA (pri-miRNA) substrate was constructed using the method described above. However, due to its length of 116 nucleotides (nt), pri-miRNA had to be ligated in two ligation steps. In the first ligation, a stem-loop structure was constructed (strands A and B in Table 1), followed by an additional ligation with a supplementary single-stranded RNA tail (strand C in Table 1) to obtain the full-length construct.

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