Single Molecule Pulldown with nAb

Background

Intracellular macromolecules very rarely exist independently, their interactions with other macromolecules are vital for cell function. Abnormal protein-protein interaction is the underlying cause for a number of diseases emphasizing the need to study native protein formations. One of the most common methods employed by researchers to assay protein coupling is coimmunoprecipitation (CoIP). Immobilized antibodies pulldown proteins of interest and their interacting partners enabling quantification and downstream analysis.

Classic pulldown assays do not provide any information regarding protein-protein conformation, aggregation stoichiometry or other *in situ* binding properties. Single Molecule Pulldown (SiMPull) is an assay that combines fluorescent imaging with coimmunoprecipitation to examine the intricacies of macromolecule interactions.

nAbs are highly specific antibodies that enable complete sample pulldown. This high specificity makes them especially well-suited for SiMPull applications. They display a high degree of stability, permitting their use in a variety of cell lysates and buffer conditions.

SiMPull reactions involve 5 key steps.

1. Activating a slide for bait antibody attachment
2. Attaching the biotinylated bait antibody to your slide
3. Attracting the bait protein and its interacting partners to the antibodies on your slide
4. Washing any unbound proteins away
5. Single molecule fluorescent imaging of the complexes attached to the slide

Materials

- nAb Antibody (Purified nAb protein, e.g. GFP-nAb™, RFP-nAb™, mNeonGreen-nAb™ or other nAb™)
- Methanol
- Acetone
- KOH pellets
- Glacial acetic acid
- Aminosilane (N-(2-aminoethyl)-3-aminopropyltrimethoxysilane)
- mPEG
- Biotin-PEG
- Sodium bicarbonate
- Double-sided tape (3M)
- Epoxy
- Tris-HCl
- NaCl
- EDTA
- NeutrAvidin
- Lipofectamine 2000
- Nonidet P-40
- Ultrapurified water

- NHS-Biotin (Thermo)
- Alexa Fluor® 647 NHS Ester (Life Technologies) or other fluorescent dye (NHS ester) as appropriate
- DMSO (Anhydrous)
- Zeba Spin Desalting Columns (7K MWCO, 2 ml) (Thermo)

Equipment

- Rotary drill (Dremel, model no. 395)
- Drill bits (0.75 mm)
- Quartz slides (1 inch × 3 inch × 1 mm thick)
- Coverslips (24 × 40 mm)
- Slide holders
- Bath sonicator
- Propane torch
- Single-molecule TIRF microscope
- Inverted fluorescence microscope
- 1.2 Numerical objective (NA) water-immersion objective
- Pellin-Broca prism
- EM-CCD detector
- Laser shutter
- Shutter driver
- Mirrors to align lasers
- Polarizing beam splitter
- XYZ micrometer translation stage
- Excitation and emission focusing lenses
- Excitation lasers
    - 488 nm
    - 568 nm
    - 633 nm HeNe laser
- Emission filters
    - YFP
    - mCherry
    - Alexa Fluor 647

- Solutions
    - T50 T50 [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 [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 [10 mM Tris (pH 7.5), 1% (vol/vol) NP-40, 150 mM NaCl and 1 mM EDTA with protease inhibitors); Lysis buffer should be freshly prepared before each use]

Procedure

Labeling nAb™ with Biotin or Fluorescent Dye

1. Transfer a 250 µg aliquot of nAb™ protein (1 mg/ml) into a fresh microcentrifuge tube. nAb™ proteins are supplied in an amine-free buffer suitable for NHS ester-based labeling of protein amines. 250 µg of nAb™ protein corresponds to 16– 20 nmol of protein, or a concentration of ~70 µM.
2. Dissolve NHS-biotin or fluorescent dye NHS ester in anhydrous DMSO according to the manufacturer's protocol (typically 10 mg/ml for fluorescent dyes and ~10 mM for NHS-biotin).
3. While stirring or gently vortexing, add approximately 20-fold molar excess of NHS-biotin or fluorescent dye NHS ester.
4. Incubate the labeling reaction at room temperature for 1 hour on a rocker.
5. Quench the labeling reaction by adding 25 µl of 1M lysine.
6. Equilibrate a Zeba Spin Desalting column with T50 buffer.
7. Desalt the labeling reaction by passing through the desalting column according to the manufacturer's instructions.
8. Add sodium azide to a final concentration of 0.05% and store the labeled nAb™ protein at 4ºC.

SiMPull Procedure

1. Drill two holes in a quartz slide, about 0.75 mm in diameter, 3–4 mm away from the edge. 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. 4–5 flow channels per slide is a typical number.
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.
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).
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.
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.
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. A food-grade vacuum sealer works well for sealing the slides. [Slides can be stored for up to 2 weeks under these conditions]
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 a ~5-mm channel where the inlet/outlet holes are located. 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.
14. Flow 100 µl of T50 buffer into the flow channel 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 µm2 under our experimental conditions.

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).
16. Prepare a 0.2 mg/ml 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. Immobilizing the biotinylated primary antibody against the bait protein
    a. Dilute the biotinylated antibody to a working concentration of ~10–20 nM in T50-BSA.
    b. Add 100 µl of this solution to the chamber and incubate for 10 min.
    c. Rinse twice with 200 µl of T50-BSA.
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.
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.
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.
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.
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.
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, RFP-nAb™ if using GFP-labeled prey with GFP-nAb™ as the capture antibody) and with control lysates lacking the bait-prey interaction.

Adapted from *Single-molecule pull-down for studying protein interactions*, Ankur Jain, Ruijie Liu,Yang K Xiang & Taekjip Ha. Nature Protocols 7, 445–452 (2012)