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Isolation and characterization of endogenous RNPs from brain tissues

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Abstract

Identification of physiological target RNAs and protein interactors bound to RNA-binding proteins is a key prerequisite to understand the underlying mechanisms of posttranscriptional expression control and RNA granule assembly. Here, we describe a multi-step biochemical approach to isolate endogenous ribonucleoprotein particles from brain tissues by exploiting differential centrifugation and gradient fractionation followed by immunoprecipitation with monospecific, affinity-purified antibodies directed against selected RNA-binding proteins. This protocol results in highly enriched endogenous ribonucleoprotein particles that then can be analyzed by mass spectrometry (for proteins composition) and microarray or RNA sequencing technologies (for target mRNAs).

Keywords

Neuronal RNA granules; Staufen2; Barentsz; differential centrifugation; gradient fractionation; immunoprecipitation

1 Introduction

Ribonucleoprotein particles (RNPs) are highly diverse multi-molecular complexes consisting of RNA binding proteins (RNPs), RNA and other protein interactors[1]. Those RNA granules are essential to transport selected transcripts to specific compartments in the cell, thereby controlling local protein expression in mammalian neurons[2]. Research in the last decades has revealed their importance for regulating neuronal signaling cascades necessary for excitability and the establishment of neuronal circuits[2]. In the last years, a serious effort was taken by several groups to unravel the composition of these granules[3, 4].

To identify the protein interactors and the RNA targets of RBPs, immunoprecipitation is the method of choice. By definition, immunoprecipitation is the isolation of protein-protein or protein-RNA complexes from lysates using antibodies directed against either an endogenous protein or peptide. Alternatively, exogenous RNPs containing tagged-RBPs can be immunoprecipitated using antibodies directed against selected protein tags. Brain tissues are highly heterogeneous and complex tissues challenging immunoprecipitation from crude lysates. Therefore, we developed a combinatorial approach to (i) enrich for RNPs and then (ii) to perform immunoprecipitation using monospecific, affinity-purified antibodies[5, 6].

2 Materials

An essential prerequisite for immunoprecipitation is the availability of highly specific and purified antibodies. Therefore, we purified overexpressed full-length Stauf2 (Stau2) and Barentsz (Btz) from *E.coli* cells and injected them into rabbits to generate polyclonal antibodies. For immunoprecipitation, we exclusively used affinity-purified antibodies. As a control for immunoprecipitation, we routinely chose the pre-immune sera of the same rabbit.

To isolate RNA granules, we prepared all solutions with RNase-free DEPC water and worked under RNase-free conditions.

2.1 Antibody coupling and crosslinking to protein A sepharose beads

1. Phosphate buffered saline (PBS)
2. Antibody (in PBS, affinity-purified)
3. Preimmune sera (PIS)
4. Protein A sepharose
5. RNase inhibitor (e.g Thermo Fisher Scientific Ribolock)
6. Ab and PIS solutions: 100 µg protein (antibody or or PIS) in 250 µl PBS.
7. 4x brain extraction buffer (BEB): 100 mM HEPES pH 7.3, 600 mM KCl, 32% glycerol, 0.4% NP-40
8. Full BEB: 1x BEB supplemented with 1 mM DTT, protease inhibitor (e.g. Roche complete)and RNase inhibitor
9. 0.2 M triethanolamine in PBS, pH 9.5 (TEA). Titrate with NaOH or KOH.
10. 40 mM dimethylpimelimidate dihydrochloride (DMP) in TEA (prepare always freshly for every single reaction step)
11. 200 mM ethanolamine in water pH 8
12. 0.02 % NaN₃ in PBS
13. Motor-driven dounce homogenizer or a hand-driven douncer

2.2 Optiprep gradient fractionation

1. Gradient mixer
2. Polyallomer tubes (e.g. Beckmann 14x89mm, for 11-12 mL)
3. Ultracentrifuge
4. Swing-out rotor (e.g. Beckmann SW41)
5. Optiprep: 60 w/v % solution of iodixanol in dH₂O (sterile) (e.g. Sigma Aldrich)
6. Optiprep gradient solution: 15% and 30% Optiprep in 1x BEB, 1 mM DTT, protease inhibitor

2.3 Immunoprecipitation

1. Blocking solution: 1.25 mg/mL BSA in 1 x BEB
2. 10 mg/ml yeast tRNA
3. 1 mM MgCl₂ in PBS
4. RNase A+T1 mix
5. 0.2 M glycine pH 2.5
6. 1 M Tris-HCl pH 8.5
7. Wash solutions: 1x BEB
8. RNase elution buffer: 200 µg/mL RNase A/T1, 1 mM MgCl₂ in PBS, pH 7.1

3 Methods

All steps for immunoprecipitation should be carried out on ice. Proteins and RNAs are eluted at room temperature (RT).

3.1 Antibody crosslinking to protein A sepharose beads

1. Hydrate beads in 10 mL PBS at RT.
2. Incubate them on a shaker for 30 min.
3. Spin beads at 500x *g* for 2 min.
4. Remove supernatant and resuspend in equal volume PBS (= 1:1 slurry).
5. Beads can be stored hydrated at 4°C in PBS supplemented with 0.02% NaN₃.
6. Prepare Ab and PIS solution by diluting 100 µg protein in 250 µL PBS, respectively.
7. Pipette 100 µL 1:1 slurry protein A sepharose beads (*see* Note 1) into 1.5 mL tube (= 50 µL protein A sepharose beads), spin at 500x *g* for 1 min, wash shortly 3 times with PBS.
8. Add Ab and PIS dilutions to the beads.
9. Incubate at 4°C for 1 h constantly rotating.
10. Store supernatant for coupling test using a Coomassie gel (*see* Note 2).
11. Wash beads at 4 °C by centrifuging at 500x *g* for 1 min, 3 times with PBS, 2 times with TEA.
12. Add 1 mL 40 mM DMP (*see* Note 3) in TEA to the beads (crosslinking step).
13. Incubate at RT for 30 min, constantly rotating.
14. Wash beads 1x with TEA.
15. Repeat steps 12, 13 and 14 three times.

16. Wash with 200 mM ethanolamine.
17. Incubate 2x with 200 mM ethanolamine for 10 min at RT.
18. Wash 2x with PBS.
19. Store beads in 1 mL PBS supplemented with 0.02 % NaN₃.

3.2 Brain Homogenisation and differential centrifugation

1. Brains are homogenized on ice in 5 mL full BEB using a motor-driven dounce homogenizer or using a hand-driven douncer (*see Note 4*).
2. Centrifuge at 20.000x *g* for 15 min at 4°C (resulting supernatant S20).
3. Chill the S20 supernatant (lysate) on ice.

3.3 Optiprep gradient fractionation

1. To prepare Optiprep gradients (Fig. 1), we are using a gradient mixer. Prepare gradient solutions always freshly.
2. Rinse polyallomer tubes and gradient mixer with RNase-free water, fill the 15 % chamber with 5 mL of 15 % Optiprep solution. Then open the valve to remove air bubbles from the chamber connection and pipet the solution from the 30% chamber (front chamber) back. Remove the bubbles from the outlet tubing of 30% chamber as well by filling with 30 % Optiprep solution and sucking the air from the outlet by a 100 µL pipette. Make sure to have an equal volume (5 mL) of corresponding Optiprep solutions in both chambers (*see Note 5*).
3. Turn on the magnetic stirrer and open the two taps carefully, so that the gradient solutions can mix and flow into the tube.
4. Upon pouring the gradients, turn off the magnetic stirrer and put the tube on ice.
5. Wash the gradient maker once with RNase-free water and repeat the whole procedure for the second gradient.
6. Then, carefully add ~1.7 mL of the soluble brain lysate (S20) to each gradient and centrifuge at 280.000x *g* (40,000 rpm in a SW41 swing-out rotor) for 2.5 h at 4 °C (max. acceleration, slow deceleration).
7. Collect 11 x 0.9 mL fractions from the top to the bottom of the gradient. Check every fraction on a Western Blot (*see Note 6*). Fractions can be deep frozen in liquid nitrogen and stored at -80 °C until further use.

3.4 Immunoprecipitation of RNPs

1. Pool OptiPrep fractions, which show an enrichment of the protein of interest (in our case Stau2 and Btz) on a Western blot. Add 2.22 µL Ribolock/mL of pooled fractions (*see Note 7*).
2. Wash 77 µL protein A sepharose beads in PBS, then wash them twice in 1x BEB

3. Add pooled fractions to the washed beads and incubate at 4°C for 1 h constantly rotating (*preclearing step*).
4. In the meantime, wash Ab- and PIS-coupled beads 1x in PBS and 2x in 1x BEB
5. To block the beads, add 400 µL blocking solution to 50 µL Ab- or PIS-coupled beads and incubate at 4°C for 1 h constantly rotating.
6. Wash Ab- and PIS beads once in 1x BEB.
7. For immunoprecipitation, add 1.3 mL of precleared fractions to 50 µL Ab- or PIS-beads, respectively. Add 5 µL of tRNA (10 mg/mL) and incubate for 1.5 h at 4°C while constantly rotating the samples.
8. Centrifuge samples at 500x g at 4°C for 5 min and save unbound fraction.
9. Wash beads 4x in 1 x BEB, 2x with 1 mM MgCl₂ in PBS at 4°C (*see Note 8*).

3.5 Elution of the beads

Depending on the downstream analysis, the protein A beads can be eluted with RNase digestion (protein analysis) or by reversible protein denaturation with glycine (combined protein and RNA analysis)

3.5.1 RNase elution

1. Add 600 µL of elution buffer containing 200 µg/mL of RNase A +T1 to the beads and incubate for 45 min at RT.
2. Centrifuge the beads at 500x g and store the supernatant.
3. Wash beads once with 1x BEB and 2x with ice-cold water (*see Note 9*).

3.5.2 Glycine elution

1. Add 120 µL 0.2 M glycine pH 2.5 to the beads. Incubate at RT for 15 min under constant rotation.
2. Spin beads at 500x g for 5 min, collect supernatant and mix immediately with 30 µL 1 M Tris-HCl pH 8.5.
3. Wash beads 2x with PBS.
4. Store beads in PBS supplemented with 0.02% NaN₃ at 4 °C (*see Note 10*).

3.5 Analysis of RNPs

To identify protein interactors of RBPs, elution fractions were processed for mass spectrometry (*see Note 11*).

4 Notes

1. Depending on the antibody, sepharose A or G show different crosslinking efficiencies. Beads for crosslinking have to be tested.

2. Successful coupling of antibodies to protein A or G beads results in much reduced or almost absent IgG bands around 55 and 25 kDa on a Coomassie gel.
3. DMP is unstable in aqueous solutions. Prepare DMP solutions always freshly before using.
4. Different RBPs behave differently during or upon lysis. For every protein, lysis conditions have to be optimized. Importantly, Mili and Steitz raised the important experimental issue that there might be reassociation of molecules, especially for RBPs, after cell lysis[7].
5. OptiPrep gradients have to be optimized for each RNP of choice. The migration of the respective RBP into the gradient strongly depends on the granule density that is influenced by the number of proteins and/or RNAs embedded in those particles.
6. Check gradient fractions on Western Blot. Generally, Stau2 containing RNA granules are expected to accumulate in fraction 4 to 6 while Btz granules are predominately found in fraction 5 to 7 [6].
7. We recommend to compare Western blots with Coomassie gels of the respective fractions. Pool those fractions that show a signal in the Western blot for your protein of interest and less staining intensity of the total protein.
8. RNase A+T1 requires Mg^{2+} to work. For RNA extraction, add TRIzol to the beads after washing.
9. The efficiency of RNA digestion strongly depends on the chosen RNase. This step has to be optimized. Keep in mind, some RNases cut only single stranded or double stranded RNA while others cut both.
10. Crosslinked beads can be reused several times. Importantly, the yield of proteins in the elution fraction is decreasing with frequency of usage.
11. For analysis of protein interactors or RNA targets, we used the PIS from the same rabbit as negative control. For analytical immunoprecipitation, proteins eluted from antibody and PIS beads are precipitated by trichloroacetic acid or methanol chloroform extraction[8].

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Figure 1. Workflow for neuronal RNP isolation and subsequent downstream analysis

The S20 of brain homogenates is biochemically separated on an OptiPrep gradient (ranging from 15 % to 30 % of OptiPrep). Fractions enriched for Stau2 or Btz proteins are pooled and used for immunoprecipitation with highly specific and affinity purified antibodies (Scheme modified from [9]).