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ImmuCon: in vitro reconstitution and analysis of protein assemblies

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We use this protocol and it's working

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Abstract

ImmuCon is an assay for *in vitro* reconstitution and analysis of condensates and other protein assemblies, typically formed by phase separation-prone proteins. Assemblies formed by recombinant proteins are immobilised on coated coverslips and visualised by immunofluorescence staining. Assembly properties can be investigated by modifying buffer composition, incubation conditions (e.g., temperature, shaking, duration), protein concentration, or by adding different molecules, such as RNA or small molecule chaperones. Moreover, complex protein mixtures can be used – allowing co-condensation or co-aggregation analysis. The technique only requires basic materials and laboratory skills and can be completed within one day.

Materials

Equipment

- Charles Austen Pump Dymax 14 Diaphram
- HulaMixer Sample Mixer (Thermo Scientific, cat. 15920D)
- Pipettes
- Upright fluorescent microscope or confocal microscope (preferably equipped with a 100x objective)

Materials

- 10mm coverslips (VWR, Cover Glasses, Round, cat. ECN 631-1576)
- 24 well plate (Greiner, CELLSTAR, cat. 662 160)

Reagents and buffers

- recombinant protein(s) of interest
- poly-L-Lysine (Sigma Aldrich, cat. P8920-100ML)
- 25% glutaraldehyde (Bangs Labs, EM Grade, cat. AA012)
- BSA (Sigma Aldrich, cat. A3294-50G)
- Primary antibody, e.g.,
  TDP-43 (R&D Biosystems, mouse monoclonal, cat. MAB77781)
  FUS (Proteintech, rabbit polyclonal, 11570-1-AP)
- Secondary fluorescently labelled antibody, e.g.,
  AlexaFluor Secondary Antibodies (ThermoFisher, cat. A11010, A11008, A11003, A11001)
- ImmuMount (Epredia, cat. 9990412)
- 1XPBS
- dH2O

Safety warnings

Glutaraldehyde is used only under a chemical fume hood, and the Safety Data Sheet should be followed for instructions.

Before start

0.02% poly-L-Lysine and 2% glutaraldehyde are made in dH2O, and both keep on ice.

Coverslip coating

2h

1 Place 10mm coverslips to a 24 well plate, wash with water one time and then 70% EtOH one time. 20m

2 Add 300ul of 0.02% poly-L-Lysine to each well and incubate at room temperature for 30min. 30m

3 Remove poly-L-Lysine, wash with water one time. Leave the plate on a bench without a lid to dry the coverslips (usually take 10min around). 10m

4 Add 300ul of 2% glutaraldehyde to each well and incubate at room temperature for 40min. 40m

5 Remove glutaraldehyde, wash with water one time, and dry the plate. 10m

6 After the plate is completely dried, it can be stored at 4°C for up to one month. 10m

Formation of protein assemblies

10m

7 Incubate proteins (e.g., TDP-43, FUS) in an assay buffer at room temperature for 10 min with gentle shaking. 10m
Note:
1) Assay buffer composition, incubation condition, protein concentration for condensation/aggregation has to be optimised for each specific protein.
2) Standard assay buffer has the following composition: 20 mM Tris-HCl pH7.5, 20 mM KCl, 2 mM MgCl2 and 1mM DTT.
3) Using low protein-binding microcentrifuge tubes for sample preparation is recommended.

Assembly fixation

1h 1m

8 After incubation, place a droplet (5ul) of protein sample in the center of the coverslip and incubate at room temperature for 15 min (or an optimised amount of time). 15m

9 Add 5ul of 4% glutaraldehyde (final 2%) to the above sample, gently mix by pipetting and incubate at room temperature for 30min. 30m

10 Wash with 1XPBS three times. 3m

11 The plate can be stored at 4°C for 1-2 days for immunostaining.

Immunostaining 4h 11m

12 Perform blocking step with 1% BSA in 1XPBS/0.25% Triton X-100 at room temperature for 1h. Discard blocking buffer. 1h

13 Add 300ul/well of primary antibody (1: 5000) and incubate for 2h at room temperature. Note: primary and secondary antibodies are prepared in blocking buffer. Dilution and cross-activity need to be tested. 2h

14 Wash with 1XPBS three times. 3m

15 Add 300ul/well of fluorescently labelled secondary antibody (for example, AlexaFluor, 1:1000) and incubate for 1hr at room temperature. 1h

16 Wash with 1XPBS three times and leave in water. 3m

17 Coverslips were mounted on a glass slide using ImmuMount (ThermoFisher). 5m

18 Imaging can be performed on any suitable fluorescent microscope.