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Protocol status: Working
We use this protocol and it's working

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Single-molecule pulldown for immunodetection of protein aggregates

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ABSTRACT

This protocol describe the procedures used to clean and passivate a coverglass surface, functionalise it with biotin and specifically immobilise proteins of interest for detection with single channel imaging (SiMPull), two-colour coincidence aggregate detection (STAPull) or ThT detection (SAVE imaging) on a TIRF microscope. This protocol supports published work (Saleeb et al., 2023).

MATERIALS

Equipment:

- Diener Electronic ZeptoOne Plasma Cleaner
- Oxford Nanoimager

Consumbles:

- Coverslips (Merck, C9056-1CS)
- Ibidi 18-well gasket (Ibidi, 81818)
- 0.02-micron syringe filters (Merck, WHA68092002)

Reagents:

- Potassium hydroxide (Merck, 221473-500G)
- Acetic acid (Merck, 33209-2.5L-M)
- APTMS (Merck, 281778-5ML)
- Methanol (Merck, 32213-2.5l-M)
- mPEG-SVA (5000 Da, Laysan Bio)
- Biotinylated mPEG-SVA (5000 Da, Laysan Bio)
- Sodium hydrogen carbonate (Fisher Scientific, 10020510)
- Streptavidin (ThermoFisher Scientific, 21122)
- Capture antibody, PS129 anti-aSynuclein (Abcam, ab209422) biotinylated by kit (Pierce, 90407)
- Detection antibody, 1:1 mix of PS129 anti-aSynuclein labelled with AF488 or labelled with AF647 by kit (ThermoFisher, A20181/6)
- Thioflavin T (Merck, 596200-500MG)

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Coverslip passivation and functionalisation

1 Treat coverslips with argon plasma for 45 mins

2 Immerse coverslips in 0.22 micron-filtered 1M potassium hydroxide for 20mins

3 Rinse coverslips in ultrapure deionised water and immerse in APTMS solution (prepared as 3.75 mL glacial acetic acid, 1 mL APTMS, 75 mL methanol) and incubate covered for 20 mins at RT.

4 Rinse coverslips sequentially in methanol and ultrapure deionised water and use fast flowing argon to dry slides rapidly. Inspect slide to ensure visually clean.

5 Attach 18-well Ibidi sticky-slide chamber to coverslip, apply pressure to ensure fully adhered.

6 Dispense 50 uL mPEG solution per well (95mg/mL mPEG-SVA, 5 mg/mL biotin-PEG, 0.1M sodium hydrogen carbonate), parafilm seal chamber and incubate covered overnight at RT.

Sample pull-down and labelling

7 Empty PEG solution by inversion and wash wells with ultrapure deionised water x3

8 Dry wells thoroughly using fast-flowing argon gas

9 Apply 50 uL 0.2 mg/mL streptavidin prepared in 0.02-micron filtered T50 buffer (10 mM Tris pH 8.0 supplemented with 50 mM sodium chloride) per well and incubate covered for 10mins at RT

10 Wash wells with 0.02-micron filtered T50 buffer x3

11 Apply 50 uL of 100 nM biotinylated capture antibody and incubate covered for 20 mins at RT

12 Wash wells with 0.02-micron filtered PBS x3

13 Apply 50 uL neat CSF per well and incubate covered for 24 hours at RT

14 Wash wells with 0.02-micron filtered PBS x3

NB. CSF is biohazardous, aspirate to remove liquid waste instead of slide inversion

15 Apply 50 uL 2nM fluorophore-labelled detection antibody and incubate covered for 3 hours at RT

16 Wash wells with 0.02-micron filtered PBS x3

17 Apply 50 uL PBS per well and image immediately

Thioflavin T preparation

18 Prepare approx. 4 mM stock of ThT in 100% ethanol and vortex extensively (approx. 1 hour)

19 Prepare approx. 200 uM ThT dilution in PBS, vortex thoroughly (approx. 20 mins) and filter through 0.02-micron filter.

20 Measure concentration of ThT preparation using a DeNovix spectrophotometer (extinction coefficient 36,000 M^{-1} cm^{-1} at 412 nm)

21 Prepare a 10 uM working stock in 0.02-micron filtered PBS

Imaging

22 Acquire an 8 x 8 grid of 200-micron spaced fields of view per well by total internal reflection fluorescence microscopy using the ONI Nanoimager with 100x/1.4 oil immersion objective lens. Samples are sequentially excited at 638nm and 488 nm, 20 frames captured per field in each channel at 20 frames s^{-1}.

23 On completion of imaging one well, return to position 0 (based on metadata values), cautiously apply 50 uL 10 uM ThT and re-image all fields with 405nm excitation.