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Version 2

Free-floating Mouse Brain Immunohistochemistry V.2

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Jonathan Breiter¹

¹University of Cambridge



Jonathan Breiter

University of Cambridge

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We use this protocol and it's working

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Abstract

This protocol enables immunohistochemical staining of murine tissue with superior penetration of the tissue by the reagents due to the free-floating approach.

In this new version, the **last step** contains a supplemental video with extra context and tips, as part of the ASAP Protocol Particulars, featuring conversations with protocol authors

Materials

10x PBS pH 7.4, Gibco, Cat. No: 70011-036

Triton X-100, Merck, Cat. No: 648466-50ML

Bovine Serum Albumin heat shock fraction, Sigma-Aldrich, Cat. No: A9647 - 100G

Normal Goat Serum, Fisher Scientific, Cat. No: 11819220

Vectashield Antifade Mounting Medium PLUS, Vector Labs, Cat. No: H-1900

Mouse mAb to Alpha-synuclein pS129 (81A), Abcam, Cat. No: ab184674, RRID:AB_2819037

Donkey anti-Mouse IgG (H+L) Alexa Fluor 568, Thermo Fisher, Cat. No: A10037, RRID: AB_2534013

Millex Filter Unit 0.22 um, Merck, Cat. No: SLGP033RS

12 Well Cell Culture Plate, Corning, Cat. No: 3513

24 Well Cell Culture Plate, Thermo Scientific, Cat. No: 144530

Netwell Permeable Supports 15mm Diameter Insert 74 um Polyester Mesh, Costar, Cat. No: 3477

Micro Slides Single Frosted 75 × 25 mm, Corning, Cat. No: 2948-75X25

Cover Glass 22 × 50 mm Thickness No. 1, VWR, Cat. No: 631-0137



Tissue Preparation

- 1 Remove PFA-fixed tissue from storage solution and add to mesh bottom netwell insert inside of 12 well plate that is filled with 0.22 μ m filtered 1x PBS.
- 2 Place the 12 well plate with the tissue on a horizontal shaker and wash tissue 3 $\times$ 5 min at approx. 150 rpm, moving the netwell insert to the next well down after each 5 min period to immerse the tissue in new 1x PBS. 🌡 Room temperature

Buffer Preparation

- 3 Per well of tissue make a minimum of 3.5 mL of blocking solution, consider this is needed for blocking, primary and secondary antibody incubation. All reagents should be 0.22 μ m filtered:

1x PBS

5% Normal Goat Serum

2.5% Bovine Serum Albumin

0.2% Triton-X

Make in excess and keep on ice. Keep at 4 degrees celcius overnight. 🌡 4 °C

Primary Antibody Incubation

- 4 In a new 12 well plate, add 2-3 mL per well of blocking solution and transfer the washed tissue sections inside their netwell inserts into these wells. Incubate for 1h - 2.5 h on horizontal shaker at approx 150 rpm. 🌡 Room temperature
- 5 Meanwhile, dilute primary antibodies in blocking solution to appropriate concentrations and keep 🌡 On ice .

E.g.
- Mouse monoclonal anti pS129 (81A) (ab184674) @ 1:750
- 6 Add approx 250 ul of primary antibody solution to the appropriate number of wells of a 24 well plate for the number of brain sections.



- 7 When blocking is finished, move brain tissue sections from the netwell inserts to their appropriate primary antibody well using a fine paintbrush, being careful not to destroy the tissue.
- 8 Incubate on horizontal shaker at approx. 150 rpm overnight.  4 °C

Secondary Antibody Incubation

- 9 Prepare a new 12 well plate with clean netwell inserts and fill all wells with 0.22 µm filtered 1x PBS.
- 10 Transfer brain tissue sections from 24 well plate into netwell inserts inside 12 well plate and wash 4× 10 minutes on a horizontal shaker at approx. 150 rpm.
 Room temperature
- 11 Meanwhile, make appropriate dilutions of secondary antibody in blocking solution. Keep away from light and keep  On ice .

E.g.
- Donkey anti-Mouse Alexa Fluor 568 (A10037) @ 1:500
- 12 Add approx 250 ul of secondary antibody solution to the appropriate number of wells of a 24 well plate for the number of brain sections.
- 13 When washing step is finished, move brain tissue sections from the netwell inserts to their appropriate secondary antibody well using a fine paintbrush, being careful not to destroy the tissue.

Keep the well plates covered from now to avoid bleaching of fluorophores.
- 14 Incubate 24 well plate on a horizontal shaker at room temperature at approx. 150 rpm for 1 h - 2.5 h.  Room temperature
- 15 Transfer brain tissue sections from 24 well plate into netwell inserts inside 12 well plate and wash 4× 10 minutes on a horizontal shaker at room temperature at approx. 150 rpm.
 Room temperature

Microscope slide preparation & Imaging



- 16 Plasma-clean microscope cover slips in Argon plasma for 15 minutes.
- 17 Using a fine paintbrush, transfer brain tissue sections from 1x PBS onto a microscope slide, using excess 1x PBS to mount multiple sections next to each other and making sure they are not folded over.
- 18 Leave sections on microscope slides to dry out in the dark (they will turn white).
- 19 Add approx. 150 uL of mounting media on top of each tissue section and apply the plasma-cleaned cover slip on top of the tissue, closing the slide.
- 20 Leave the mounting media to dry in the dark.
- 21 Seal the edges of the coverslip with nail varnish and let dry for approx. 30 minutes in the dark.
- 22 Immediately take finished microscope slides to imaging, avoiding unnecessary light exposure.

ASAP Protocol Particulars: context and tips

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<https://www.youtube.com/embed/c7DCf4tjq3k?si=8yEBcWQGgSciqJLK>