Immunofluorescence staining protocol for co-staining of fetuin-A and CD68 in older human autopsy tissue

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Miriam Heinen[1]

[1]RWTH Aachen University

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Miriam Heinen

ABSTRACT

This staining was performed to detect fetuin-A in activated microglia. CD68 is a marker, that stains activated microglia, macrophages and monocytes. Fetuin-A and microglia were detected in paraffin sections (1 µm thickness) of formalin-fixed human brain tissue. CD68 was stained using a monoclonal mouse-anti-human antibody (Dako Cat# M0814, RRID:AB_2314148, clone KP1, dilution 1:50) and a polyclonal goat-anti-mouse Alexa Fluor® 488 conjugated secondary antibody (Thermo Fisher Scientific Cat# A-11029, RRID:AB_2534088, dilution 1:300). Fetuin-A was detected by using a monoclonal IgG2a mouse-anti-human antibody (clone MAHS-1, dilution 1.0 µg/mL), raised against purified human fetuin-A in our laboratories. Antibody binding was detected by tyramide signal amplification using a secondary biotinylated polyclonal goat-anti-mouse antibody (Dako Cat# E0433, RRID:AB_2687905, dilution 1:300) and a Tyramide Signal Amplification Kit (Life Technologies, Carlsbad, USA, T-20933). To minimize lipofuscin autofluorescence, sections were counterstained with Sudan Black (Sigma-Aldrich, Munich, Germany, 199664, dilution 0.3% in 70% ethanol, 5 minutes). Nuclei were stained with DAPI (Sigma-Aldrich, Munich, Germany D9542, dilution 0.25 µg/ml, 5 minutes). Sections were mounted with Immumount (Thermo Scientific, Waltham, USA, 9990402) and stored at 8°C in the dark.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0206597

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Heinen MC, Babler A, Weis J, Elsas J, Nolte K, Kipp M, Jahnen-Dechent W, Häusler M (2018) Fetuin-A protein distribution in mature inflamed and ischemic brain tissue. PLoS ONE 13(11): e0206597. doi: 10.1371/journal.pone.0206597

PROTOCOL STATUS

Working

GUIDELINES

- use with Tyramide Signal Amplification Kit (life technologies, T-20933)
- for this type of tissue fluorescence staining of fetuin-A was only possible by using tyramide signal amplification. Thus co-staining was possible using two mouse-anti-human antibodies one after the other

BEFORE STARTING

Prepare the following components of the Tyramide Signal Amplification Kit according to the manufacturers protocol:

1. Prepare tyramide stock solution by dissolving Component A in 150 µL of Component B (DMSO). (Store unused portions of this stock solution in small aliquots at ≤−20°C, protected from light).
2. Prepare a 1% (10 mg/mL) solution of Component D (BSA) in phosphate-buffered saline (PBS).
3. Prepare the horse radish peroxidase (HRP) conjugate stock solution by reconstituting Component C in 200 µl PBS. (This solution may be stored at 2–8°C for up to 3 months).
4. Prepare amplification buffer/0.0015% H2O2 by adding 30% hydrogen peroxide (Component F) to amplification buffer (Component E) to obtain a final concentration of 0.0015% H2O2. For example, add 1 µL of 30% H2O2 to 200 µL of amplification buffer and then add 1 µL of this intermediate dilution (0.15% H2O2) to a further 100 µL of amplification buffer.

See Guidelines

1 Check tissue requirements and prepare the components of the Tyramide Signal Amplification Kit

Clear slides

2 Place slides in hybridization oven: 37°C overnight, then 1 hour at 65°C

3 Deparaffination in xylene 3x20 minutes (different containers) on a shaker

4 Rehydration in graded ethanol: 3x2 minutes in 100% ethanol, followed by 2x2 minutes in 96% ethanol and at last 2 minutes in 70% ethanol

5 Wash in PBS for 5 minutes

Antigen retrieval

6 Antigen retrieval in Tris-EDTA buffer (pH 9) in a heat steamer for 30 minutes. Afterwards let the slides cool down in PBS for 30 minutes

7 Wash in PBS for 5 minutes

Immunostaining

8 Encircle tissue section with wax pen

9 To block non-specific binding, apply a solution of 10% goat serum in PBS, incubation for 1 hour in a moisture chamber

10 Tip off excess solution and apply the primary anti-fetuin-A-antibody (MAHS-1, dilution 1 µg/ml in 1% BSA in PBS) 100 µl per section, (negative controls: only 1% dilution), incubation overnight in a moisture chamber

11 Tip off excess solution and rinse in demineralized water

12 Wash in PBS for 3x5 minutes

13 Apply the secondary biotinylated goat-anti-mouse antibody (dilution 1:300 in 1% BSA in PBS), 100 µl per section, incubation for 1 hour at room temperature in a moisture chamber

14 Tip off excess solution and rinse in demineralized water

15 Wash in PBS for 3x5 minutes

16 Apply the Tyramide Signal Amplification Kit as follows: Dilute the prior prepared HRP solution 1:100 in 1% BSA in PBS, apply 100 µl per section, incubation for 1 hour at room temperature in a moisture chamber.

17 Tip off excess solution and rinse in demineralized water

18 Wash in PBS for 3x5 minutes

19 Prepare a tyramide working solution following the manufacturer's protocol: dilute the prior prepared tyramide stock solution 1:100 in the prepared 0.0015% hydrogen peroxid solution

20 Apply 100 µl per section, incubation for 10 minutes

keep the tissue samples from now on light protected!

21 Tip off excess solution and rinse in demineralized water

22 Wash in PBS for 3x5 minutes

23 Apply the primary anti-CD68 antibody (dilution 1:50 in 1% BSA in PBS), 100 µl per section (negative controls: only 1% dilution), incubation overnight in a moisture chamber

24 Apply the polyclonal goat-anti-mouse Alexa Fluor® 488 conjugated secondary antibody (dilution 1:300 in 1% BSA in PBS), 100 µl per section, incubation for 90 minutes at room temperature in a moisture chamber

25 Tip off excess solution and rinse in demineralized water

26 Wash in PBS for 3x5 minutes

27 To minimize lipofuscin autofluorescence, perform counterstaining with Sudan Black (dilution 0.3% in 70% ethanol) for 5 minutes

28 Rinse the container with demineralized water until the solution becomes clear and colorless

29 Wash in demineralized water for 3x5 minutes

30 Counterstain with DAPI (dilution 0.25 µg/ml) for 5 minutes

31 Wash in PBS for 3x5 minutes

32 Coverslip using mounting medium

33 Store at 8°C in the dark