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

PLOS One

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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.

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

Protocol status: Working

BEFORE START INSTRUCTIONS

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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 μ L of Component B (DMSO). (Store unused portions of this stock solution in small aliquots at $\leq -20^{\circ}\text{C}$, 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 μ L PBS. (This solution may be stored at $2-8^{\circ}\text{C}$ for up to 3 months).
4. Prepare amplification buffer/0.0015% H_2O_2 by adding 30% hydrogen peroxide (Component F) to amplification buffer (Component E) to obtain a final concentration of 0.0015% H_2O_2 . For example, add 1 μ L of 30% H_2O_2 to 200 μ L of amplification buffer and then add 1 μ L of this intermediate dilution (0.15% H_2O_2) to a further 100 μ L 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