Detection of LC-3 and p62/SQSTM1 in paraffin-embedded mouse tissues by immunohistofluorescence analysis

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METHOD

Fixation of mouse tissues

1. Anesthetize mice first with ether, and then pentobarbital (25 mg/kg intraperitoneally).

NOTE Follow the guidelines for animal experiments formulated in each University or Institute.

2. Perfuse via the heart first with ~30 ml of Ringerõs solution containing 10 U/ml heparin, and then with 50 ml of 4% paraformaldehyde-4% sucrose / 0.1 M phosphate buffer (PB; pH7.4).
3. Excise tissues of interest and cut them into small pieces (e.g. 3-5 mm square).
4. Place them in embedding cassettes or bags, and immerse in the same fixative for 2 h to overnight at 4ęC

Dehydration and paraffin embedding

5. Immerse the tissues sequentially in the following solutions.
   1) Distilled water, 3 changes, 30 min each, room temperature (RT)
   2) 50 % ethanol, 30 min, RT
   3) 70 % ethanol, 30 min, RT
   4) 80 % ethanol, overnight, RT
   5) 90 % ethanol, 30 min, RT
   6) 100 % ethanol, 2 changes, 1 h each, RT
   7) 100 % ethanol that has been treated with Molecular sieves 3A, 1h, RT
   8) Xylene, 3 changes, 20 min each, RT
   9) Paraffin, 3 changes, at 60ęC

NOTE Check the clearness of the tissues at the step 8).

6. Embed into paraffin block.

NOTE Interval time and number of change for each procedure are variable, depending on the block size and laboratory.

NOTE Epitopes can be damaged during these procedures especially at 9),

which influences the final immunohistological results.

Sectioning

7. Prepare 3~5 μm sections using a microtome.
8. Place them on the surface of hot water (45-50ęC) to reduce wrinkles.
9. Mount on glass slides.
10. Dry the sections at 42ęC for 2-3 h.

Deparaffinisation and rehydration

11. Immerse sections sequentially in the following solutions.
    1) Xylene, 10 min
    2) Xylene, 5 min
    3) 100% Ethanol, 5 min
    4) 100% Ethanol, 3 min
    5) 95% Ethanol, 3 min
    6) 90% Ethanol, 3 min
    7) 80% Ethanol, 3 min
    8) 70% Ethanol, 3 min
    9) Distilled water, 5-10 min

Immunostaining

12. Rinse in phosphate buffered saline (PBS) containing 0.1 % tween-20 (TPBS) thrice for 5 min each.
13. (Option) Incubate at 98ęC for 20 min in Immunosaver, a solution for antigen retrieval, using a microwave processor (MI-77).

NOTE Whether this step is included or not should be determined for each antibody. In our laboratory, antigen retrieval is required for anti-p62/SQSTM1 antibody (Progen), but not for anti-MAP1LC3A antibody (Epitomics).

14. Incubate in PBS containing 0.1% Triton X-100 for 15 min at RT for better permeabilization.
15. Rinse with TPBS thrice for 5 min each.
16. Encircle each section with liquid blocker.
17. Incubate with TPBS containing 5% normal goat or donkey serum for 20 min

at RT for blocking.

NOTE Species of the blocking serum is preferably the same one of secondary antibody.

18. Dilute the anti-MAP1LC3A antibody or anti-p62/SQSTM1 antibody with the above blocking solution at 1:100-200 and 1:400-800, respectively. Centrifuge at 15,000 rpm for 5 min, and then apply the supernatant on the sections.
19. Incubate for 1-3 days at 4ęC or 1h at RT in a humidified chamber.
20. Rinse with TPBS thrice for 5 min each.
21. Incubate with goat anti-rabbit or guinea pig IgG conjugated with appropriate fluorescent dye, such as Alexa Fluor® series.

NOTE In the case of double immunolabelling, cross reactivity of secondary antibody should be checked for each combination.

22. Rinse with TPBS thrice for 5 min each.
23. Mount with Fluoromount^{TM}, an antifade mountant. Remove extra mountant and seal with manicure.
24. View with an epifluorescence microscope or a confocal laser scanning microscope.

MATERIALS

REAGENTS

- pentobarbital (Somnopentyl, Kyoritsuseiyaku Corporation)
- Ringerôs solution (Otsuka pharmaceutical)
- Heparin sodium Salt (Nacalai tesque, 17513-54)
- Paraformaldehyde (Merck KGaA, 30525-89-4)
- Ethanol (Wako Pure Chemical Industries, Ltd.057-00451)
- Molecular sieves (Nacalai tesque, 23356-05)
- Xylene (Wako Pure Chemical Industries, Ltd.244-00081)
- Paraffin (Sakura, Finetechnical, Co., Ltd, 7810. Paraffin Wax II60)
- Immunosaver (Nisshin EM Corporation)
- liquid blocker (Dako, s2002)
- Normal goat serum (SIGMA, G 9023)
- Normal Donkey serum (SIGMA, G 9663)

・Rabbit anti-MAP1LC3A antibody (Epitomics, clone ID-EP1983Y)
・Guinea pig anti-p62/SQSTM1 antibody (c-terminal specific, Progen)
・Alexa Fluor® 488 (or 594) Goat Anti-Rabbit IgG (H+L) Antibody (Abcam)
・Alexa Fluor® 488 (or 594) Goat Anti-Guinea pig IgG (H+L) Antibody (Abcam)
・Fluoromount^{TM} (Diagnostic BioSystems, K024)
・Manicure

EQUIPMENT

・Peristaltic pump (ATTO corporation, Perista mini SJ1211)
・Microtome for paraffin (Yamato Kohki Industrial, REM-710)
・Embedding cassettes or bags (Sakura, Finetechnical Co., Ltd, Mega-Cassette, 4173)
・Microwave processor (Azumaya Corporation, MI-77)

REAGENT PREPARATION

0.2 M phosphate buffer (pH 7.4) [stock solution]

400 ml of 0.2 M $Na_2HPO_4 \cdot 12H_2O$ is mixed with 100 ml of 0.2 M $NaH_2PO_4 \cdot 2H_2O$.

10% PFA [stock solution]

10 g of paraformaldehyde is mixed with 80 ml of Milli-Q water that has been heated to 70°C. Add 8 drops of 1 N NaOH to the suspension for complete dissolution, then dilute to 100 ml with Milli-Q water. The solution is filtrated with a filter paper and stored at 4°C. This solution should be used within a few days.

10N NaOH

Dissolve 8 g of sodium hydroxide in 20 ml of distilled water. This reagent can be stored at room temperature.

7.5% sucrose-0.1 M phosphate buffer (pH 7.4) [stock solution]

0.2 M phosphate buffer (pH 7.4)	50 ml
Sucrose	7.5 g
Dilute to 100 ml with Milli-Q water	

10x PBS

Dissolve 80 g of sodium chloride, 29 g of disodium hydrogenphosphate 12-water, 2 g of potassium chloride and 24 g of potassium dihydrogen phosphate in 1 L of distilled water. This reagent can be stored at room temperature.

TPBS 100ml

10×PBS	10 ml
10% Tween20	1 ml
Distilled water	89 ml

TROUBLESHOOTING TIPS

1. Positive controls

It is important to monitor that all processes are properly carried out. For example, we use anti-GM130 antibody, a Golgi marker, which is expressed almost all types of cells, and is detectable when sections are processed for antigen retrieval. For LC3 staining, mouse livers under 24 h-starvation or intraperitoneal injection of leupeptin for 1h could be good models. For detecting p62-aggregates, we use Atg7 knockout livers. Mouse embryonic fibroblasts treated with an oxidative stressor, sodium arsenite, can also be used to detect p62-aggregates. In this case, cells should be collected, embedded in 10% gelatin, and processed as above.

2. Antigen retrieval procedures

Check if the antibodies can be applicable for paraffin embedded tissues, and further if antigen retrieval procedures are required or not. From the preliminary data you can determine the combination of antibodies and method for double immunostaining. It would be worth using other solutions for antigen retrieval, such as 10 mM citrate buffer (pH6.0) or 10 mM Tris-HCl buffer (pH 9.0), Change the incubation time for antigen retrieval may improve the detection of signals.

3. High autofluorescence signal

Tissue samples contain more or less autofluorescent materials, such as lipofuscin in lysosomes and collagen. If such signal is too high to be negligible,

consider using immunohistochemistry using diaminobenzidine (DAB) reaction. Also, please refer to methods for reducing autofluorescence (http://www.uhnres.utoronto.ca/facilities/wcif/PDF/Autofluorescence.pdf,).