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Fixation and Immunostaining V.1

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We use this protocol and it's working

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Abstract

This protocol is based on standard methods for formaldehyde fixation and immunostaining for fluorescence microscopy. However, it contains tested working reagents, advice, and tips.

Guidelines

Read all reagent SDS and follow all safety measures.
Handle, store, and discard Paraformaldehyde according to your institution's guidelines.

Materials

1. PFA - Electron Microscopy Sciences 32% Paraformaldehyde (formaldehyde) aqueous solution, 10x10 mL - Catalog No.50-980-494 – Thermofisher Scientfic
2. PBS (1x)- DPBS, calcium, magnesium - Catalog number: 14040182 - Thermofisher Scientfic
3. DPBS (10X), calcium, magnesium - Catalog number: 14080055 - Thermofisher Scientfic
4. UltraPure™ Glycine - Catalog number: 15527013 - Thermofisher Scientfic
5. 10% Sodium Azide. 100mL, Sterile. 6.1/UN3287/II(L) -10 bottle limited - S0209 - teknova
6. Normal Donkey Serum - Code: 017-000-121 – Jackson Immunoresearch Laboratories
7. Triton™ X-100 solution – 93443 – Milipore Sigma
8. DAPI and Hoechst Nucleic Acid Stains - Catalog number: D21490 - Thermofisher Scientfic
9. Fluoromount-G™ Mounting Medium-Catalog number: 00-4958-02 – Thermofisher Scientific
10. Sucrose - S0389 – Millipore Sigma

BUFFER PREPARATION

1 **2 x Fixation Buffer (8% Paraformaldehyde in 2x PBS):** Use a 32% paraformaldehyde (PFA) in sealed EMS ampule + 30 mL PBS (+/+), pH 7.4 + 8 mL 10x PBS + 22 mL ddH2O
OBS: You can use either PBS (+/+) (with Ca+2 and Mg+2) or (-/-). Some might prefer HBSS instead.

2 **1 x Fixation Buffer (4% PFA in PBS):** Prepare fresh on the day of use. Dilute 2 x Fixation Buffer in ddH2O (1:1 dilution)

3 **Wash Buffer:** 1x PBS + 0.1% Triton-X 100

4 **Quench Buffer:** Wash Buffer + 100 mM Glycine or Lysine + 0.1% Sodium Azide

5 **Blocking Buffer:** Wash Buffer + 10% normal donkey serum + 0.1% Sodium Azide.
OBS: Make sure to filter the Blocking Buffer before adding primaries to remove any precipitates/particles that did not dissolve.

6 **Storage solution:** 1x PBS + 0.1% Triton-X 100 + 0.1% Sodium Azide

PROCEDURES

7 Check all buffers before use - check for precipitates and contamination.

8 Prepare 1x Fix by dilution from 2x Fix.

9 Gently aspirate culture supernatant and replace with cold Fixation Buffer.
OBS: For low-density cultures, check the notes below.

10 Incubate for 30 min at 4° C or on ice.

11 Aspirate Fixation Buffer and wash gently 3x with PBS pH7.4 (wash for 1-2 min each)

12 Aspirate and add Quench Buffer to permeabilize cells/tissue, at least 15 min at room temperature (RT).

OBS: If needed, you can pause now and resume on the next day.

13 Aspirate and add Blocking buffer. Incubate for 30 min at RT.

14 Aspirate and incubate with primary antibodies cocktail prepared in Blocking Buffer (1-2 h at RT or at 4 °C overnight).

15 Wash 3x for 5 min each with Wash Buffer.

16 Prepare secondary antibodies cocktail in Blocking buffer or Wash Buffer (1:500-1:1000), filter, and keep the vial light-protected.

17 Add secondary antibodies cocktail and incubate for 1-2 h at RT, light-protected, or 4 °C overnight.

18 Wash 1x for 5 min (use Wash Buffer).

19 Incubate with 1x DAPI in Wash buffer for 5-10 min.

20 Wash 1x with Wash Buffer.

21 Mount or store humidified Storage Buffer (keep wet, do not let it dry)

NOTES

22 Filter all PBS buffers before use

23 Check buffers for contamination or precipitates weekly, re-filter monthly

24 For thick samples, increase fixation time (1-2 h for thick, 80-800 µm) antibody incubation times. (*e.g.,* at least 4 h at RT for 80 µm thick embryos or embryoid bodies)

25 For low-density and or long-term cultures or cultures where extremely high retention of cells is required for sensitive cells (crucial for oligodendrocytes), it is advisable to use one of two gentle-fixation methods to preserve as many cells as possible with intact morphology.

Option 1 (Pre-Fixation) is usually sufficient.

25.1 Pre-fixation. Before removing the culture supernatant, add a matching volume of 1x Fixation Buffer and incubate at RT for 2 min, then proceed to step #9 above. This 2% pre-fix helps make sure cells are not lost.

25.2 1 volume of 2x fix may also be added to fix-in-place. Note that more cell debris may be retained with this method.

25.3 Sucrose fixation: dissolve sucrose in 1xFix, at 10% (g/100ml). Place on ice when dissolved. Add 0.5-1x culture volume of Fixation buffer + sucrose to culture well and incubate on ice or 4 degrees for 5 minutes. Then aspirate and proceed with the normal fixation as in step #9.

26 Fixation is proportional to time, temperature, and PFA concentration. Incubation of 30 min at 0-4 °C is excellent for most antigens, including some nuclear transcription factors sensitive to overfixation (*e.g.*, HB9). Incubation of 15 min at RT is equivalent. Some antigens may benefit from epitope retrieval. HEIR with buffered EDTA and mild heat (50-60 °C, 2-12 h humidified) is mild and effective for most fixation-sensitive antigens.

27 You can make 100 ml of Blocking Buffer and freeze it in 5-10 mL aliquots for convenience.

28 It is recommended to use number 1.5 coverslips or equivalent optical plastic.

29 Some secondaries aggregate in Wash Buffer, leading to bright dye aggregates, but solubility can be rescued by preparing secondaries in Blocking Buffer.

30 Never use mounting media with DAPI in it: it leads to background fluorescence (*e.g.*, Fluoromount-G, Prolong Gold, or Diamond).