Miltenyi Biotec

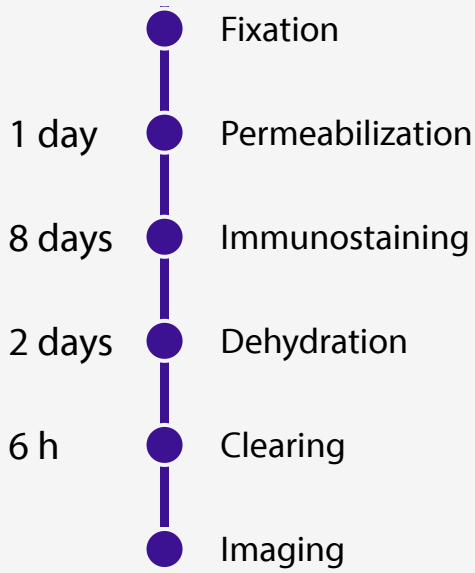
Immunostaining and clearing of mouse brain hemispheres for 3D imaging analysis

This application protocol describes step-by-step the immunostaining and clearing of mouse brain hemispheres using the MACS® Clearing Kit for downstream 3D imaging analysis. The MACS® Clearing Kit enables the immunostaining and clearing of up to 20 brain hemispheres from mice of any age.

Print protocol (javascript:window.print();)

Protocol

Timeline



1. Reagent preparation

- Always freshly prepare reagents. Failure to do so may lead to suboptimal results.

Preparation of PFA buffer

- All steps that include paraformaldehyde (PFA) must be performed under a fume hood.
- The protocol describes the preparation of 500 mL of 4% PFA buffer. For higher or lower volumes scale the amounts of the components accordingly.
- Do not use ready-to-use PFA solutions.

1. Add 20 g of PFA powder to 400 mL of PBS and stir the mixture at 60 °C under a fume hood until it is dissolved.
   ▲**Note:** Do not boil!
   ▲**Note:** PFA powder does not dissolve instantly.
2. Add 0.2-0.4 g of NaOH and continue stirring at 60 °C until the solution clears up. This may take 2–3 hours.
3. Adjust the pH to 7.2–7.4 with HCl and NaOH.
4. Adjust the volume to 500 mL with PBS.
5. Filter the solution using a 0.22 µm filter.

6. PFA buffer can be stored at 4 °C for one week. For indefinite storage prepare aliquots and store at –20 °C. Avoid repeated freeze/thaw cycles.

Preparation of 1× Antibody Staining Solution of the MACS® Clearing Kit

- Before starting make sure that the solution does not contain any precipitates.
- 10× Antibody Staining Solution needs to be diluted 1:10 with sterile distilled water before use, e.g., by diluting 1 mL of 10× Antibody Staining Solution with 9 mL of sterile distilled water. Adjust the volume of 1× Antibody Staining Solution according to the number of samples to be cleared. Always use freshly prepared 1× Antibody Staining Solution.

Preparation of ethanol/Tween® 20 dilutions for dehydration

- Prepare ethanol dilutions of 30%, 50%, 70%, and 90% ethanol containing 2% Tween® 20. Use absolute ethanol (≥99.8%) and sterile distilled water to prepare the dilutions.
  ▲**Note:** Adjust pH values of 30%, 50%, 70%, and 90% ethanol dilutions to pH 9 when working with tissues expressing endogenous fluorescent proteins.

Preparation of ethanol/Tween® 20 (100% ethanol) for dehydration

- Prepare absolute ethanol (≥99.8%) containing 2% Tween® 20 using activated molecular sieves (3Å).
- Prepare within one or two days before use to ensure the absence of residual water.

1. Cover the bottom of a heat-resistant glass petri dish (Ø 14 cm) with molecular sieves.
2. Heat molecular sieves in microwave at 750 W for 2 minutes.
3. Mix the sieves before heating again at 750 W for 2 minutes.
4. Repeat heating until no liquid vaporizes anymore and sieves are completely dry.
5. Let sieves cool down.
6. Transfer sieves into a bottle and add absolute ethanol.
7. Incubate the sieves for 1–2 days to evaporate residual water.
8. Filter the needed volume of ethanol from the previous step using a 0.22 µm filter.
9. Add Tween® 20 to a final concentration of 2% (v/v).

Preparation of Clearing Solution

- Clearing Solution is ready-to-use, but may freeze when stored at 2–8 °C. Therefore, it is recommended to prepare aliquots and store at 2–8 °C. Take out aliquots to room temperature (20–25 °C) before proceeding to clearing of tissues.

2. Protocol

- For details on the reagents of the MACS® Clearing Kit refer to the respective data sheet.
- Use tweezers to handle the tissue.

2.1 Fixation

2.1.1 Fixation of neonatal mouse brain hemispheres (from mice of age ≤P7)

1. Extract mouse brain and collect it in a tube containing PBS/EDTA buffer.
2. Transfer brain into a new tube containing PFA buffer.
   ▲ **Note:** It is possible to fix more than one mouse brain per tube. Make sure brains are fully immersed in PFA buffer.
3. Incubate overnight at 2–8 °C.
   ▲ **Note:** In order to avoid autofluorescence, do not extend the incubation time.
4. Wash tissue 3× in PBS.
   ▲ **Note:** If not immediately processed for clearing, tissues can be stored in PBS/azide buffer at 2–8 °C for several weeks.

2.1.2 Fixation of adult mouse brain hemispheres (from mice of age >P7)

1. For subsequent clearing of adult mouse brain hemispheres, it is highly recommended to perfuse mice transcardially. Use PFA buffer for transcardial perfusion.
2. Extract mouse brain after perfusion and collect it in a tube containing PBS/EDTA buffer.
3. Transfer brain into a new tube containing PFA buffer.
   ▲ **Note:** It is possible to fix more than one mouse brain per tube. Make sure brains are fully immersed in PFA buffer.
4. Incubate for 2 hours at 2–8 °C.
   ▲ **Note:** In order to avoid autofluorescence, do not extend the incubation time.
5. Wash tissue 3× in PBS.
   ▲ **Note:** If not immediately processed for clearing, tissues can be stored in PBS/azide buffer at 2–8 °C for several weeks.

2.2 Permeabilization

1. Cut the brain into two hemispheres using a scalpel.
2. Add 5 mL of Permeabilization Solution of the MACS® Clearing Kit into a 5 mL tube.
3. Transfer one brain hemisphere into the tube.
4. Incubate for 24 hours at room temperature (20–25 °C) under slow continuous rotation using the MACSmix™ Tube Rotator.

5. After incubation discard Permeabilization Solution and immediately proceed to section 2.3 Immunostaining. For analysis of endogenous fluorescence without immunostaining proceed to section 2.4 Dehydration.

2.3 Immunostaining

- Use the 1× Antibody Staining Solution in the following steps (for details of preparation refer to section 1. Reagent preparation).
- The following protocol has been optimized for directly-conjugated antibodies from Miltenyi Biotec. Refer to the respective Miltenyi Biotec antibody conjugate data sheet or product page for indicated dilution. When using other antibodies refer to manufacturer´s recommendations for antibody dilution, incubation temperature and duration of incubation.

1. Dilute primary antibody in a final volume of 1.5 mL of 1× Antibody Staining Solution. Use a 24-well plate to prepare the dilution.
2. Transfer brain hemisphere into the 24-well plate containing the antibody dilution.
3. Carefully seal the plate with parafilm to prevent evaporation.
4. Incubate brain hemisphere with gentle shaking using a horizontal shaker. When using Miltenyi Biotec antibodies, incubate brain hemispheres for 7 days at 37 °C.
5. Add 5 mL of 1× Antibody Staining Solution into a 5 mL tube.
6. Transfer brain hemisphere into this tube.
7. To remove unbound antibody, incubate brain hemisphere under slow, continuous rotation for 4 hours at room temperature (20–25 °C) using the MACSmix™ Tube Rotator.
8. Discard 1x Antibody Staining Solution and repeat steps 5 and 7.
9. Perform one additional washing step with 5 mL of 1x Antibody Staining Solution. Incubate overnight at room temperature (20–25 °C) under slow, continuous rotation using the MACSmix™ Tube Rotator.
10. If working with directly-conjugated antibodies, discard 1x Antibody Staining Solution and continue with section 2.4 Dehydration.
11. (Optional) If performing indirect staining, dilute secondary antibody in a final volume of 1.5 mL of 1× Antibody Staining Solution. Use a new 24-well plate to prepare the dilution.
    ▲**Note:** Refer to manufacturer´s recommendations for optimal antibody dilution.
12. (Optional) Incubate brain hemispheres in secondary antibody dilution with gentle shaking using a horizontal shaker.
    ▲**Note:** Refer to manufacturer´s recommendations for incubation temperature and duration of incubation.
13. (Optional) Proceed with steps 5–9 to wash tissue and remove unbound antibody.

2.4 Dehydration

- For preparation of ethanol series refer to section 1. Reagent preparation.

- When working with mouse brain derived from ≤P7 mice all following incubation steps are performed at room temperature (20–25 °C). When working with mouse brain tissue derived from >P7 mice it is recommended to adapt the temperature during dehydration to 28 °C by using an Eppendorf ThermoMixer® C with Eppendorf SmartBlock™ 50 mL at a rotational speed of 500 rpm or a shaking water bath.

2.4.1 Dehydration of mouse brain hemispheres from ≤P7 mice

- 15 mL tubes must be completely filled up with corresponding ethanol buffer.
- Always use a new tube for each dehydration step. Transfer brain hemispheres into the tube using tweezers.
- Adjustment of ethanol dilutions to pH 9 is important for preservation of endogenous fluorescence (except for 100% ethanol).
- Dehydrate neonatal brain hemispheres by incubating the tissue in a series of ethanol dilutions in 15 mL tubes (one hemisphere per tube) at room temperature (20–25 °C) under slow, continuous rotation using the MACSmix™ Tube Rotator.

1. Incubation in 30% ethanol for 4 hours
2. Incubation in 50% ethanol for 4 hours
3. Incubation in 70% ethanol overnight
4. Incubation in 90% ethanol for 4 hours
5. Incubation in 100% ethanol for 4 hours
6. Incubation in 100% ethanol overnight
   ▲ **Note:** Replace 100% ethanol from previous step with fresh 100% ethanol and incubate overnight.

2.4.2 Dehydration of mouse brain hemispheres from >P7 mice

- 50 mL tubes must be completely filled up with corresponding ethanol buffer.
- Always use a new tube for each dehydration step. Transfer brain hemispheres into the tube using tweezers.
- Adjustment of ethanol dilutions to pH 9 is important for preservation of endogenous fluorescence (except for 100% ethanol).
- Dehydrate mouse brain hemispheres with a series of ethanol dilutions in 50 mL tubes (one hemisphere per tube) at 28 °C using the Eppendorf ThermoMixer® C with Eppendorf SmartBlock™ 50 mL at a rotational speed of 500 rpm.
  ▲ **Note:** The Eppendorf ThermoMixer® C with Eppendorf SmartBlock™ 50 mL is recommended for dehydration. However, if this instrument is not available, a shaking water bath can be used at 28 °C.

1. Incubation in 30% ethanol for 4 hours
2. Incubation in 50% ethanol for 4 hours
3. Incubation in 70% ethanol overnight
4. Incubation in 90% ethanol for 4 hours
5. Incubation in 100% ethanol for 4 hours

6. Incubation in 100% ethanol overnight
   ▲**Note:** Replace 100% ethanol from previous step with fresh 100% ethanol and incubate overnight.

2.5 Clearing

1. Add 5 mL of Clearing Solution of the MACS® Clearing Kit into a 5 mL amber polypropylene tube.
   ▲**Note:** Always use polypropylene tubes to incubate tissue with the Clearing Solution of the MACS® Clearing Kit.
2. Transfer one brain hemisphere into this tube.
   ▲**Note:** Make sure the tube is completely filled with Clearing Solution. Add additional Clearing Solution if necessary to fill up the tube completely.
3. Incubate at room temperature (20–25 °C) under slow continuous rotation for 6 hours using the MACSmix™ Tube Rotator.
4. Discard Clearing Solution and continue with downstream imaging analysis.
   ▲**Note:** Cleared tissues can be stored in 5 mL polypropylene or glass tubes filled with MACS® Imaging Solution at 2–8 °C for several months.
5. (Optional) If imaging analysis is performed immediately after clearing, transfer cleared tissue into the imaging chamber of a light sheet microscope (e.g. Ultramicroscope II or Ultramicroscope Blaze). Fill imaging chamber with MACS® Imaging Solution and proceed with analysis.

Materials

Reagent and instrument requirements

- MACS® Clearing Kit (# 130-126-719)
- MACSmix™ Tube Rotator (# 130-090-753)
- MACS® Imaging Solution (# 130-126-335)
- Sterile distilled water
- Tween® 20
- Absolute ethanol (≥99.8%)
- Phosphate-buffered saline (PBS), pH 7.4
- (Optional) PBS/azide buffer (PBS containing 0.02% sodium azide)
- PBS/EDTA buffer (PBS containing 5 mM EDTA)
- Paraformaldehyde (PFA) powder
- 5 mL, 15 mL, and 50 mL tubes with lid
- Surgical material, such as tweezers, scalpel, and scissors
- 5 mL amber polypropylene tubes with lid

- 24-well plate
- Molecular sieves, 3Å
- Sterivex-GV filter, 0.22 µm
- Heat-resistant glass petri dish, 14 cm in diameter
- Horizontal shaker
- Eppendorf ThermoMixer® C with Eppendorf SmartBlock™ 50 mL
- Parafilm
- (Optional) Shaking water bath

For more information about light sheet fluorescence microscopy refer to Light sheet microscopy [https://www.miltenyibiotec.com/GB-en/products/macs-imaging-and-microscopy/light-sheet-microscopy.html].

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