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# 🌐 Analysis of Primary Cilia in Rodent Brain By Immunofluorescence Microscopy

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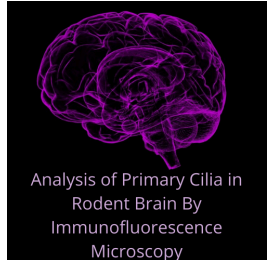
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**We use this protocol and it's working**

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## Abstract

We describe here our method for immunostaining of primary cilia in brain sections from wild type and LRRK2 or other mutant mice. Included are procedures for perfusion, tissue harvesting, sectioning and staining, image acquisition and analysis.

## Guidelines

Pathogenic LRRK2 kinase has been shown to influence ciliogenesis in certain cell types in mouse brain (Dhekne et al., 2018). In this protocol, we describe how to visualize primary cilia in various brain regions to analyze the effect of LRRK2 on ciliogenesis. Critical for these analyses are uniform section thickness and cell type specific cell and ciliary markers to ensure that the correct cell assignment is made for each cilium scored.

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# Materials

**MATERIALS**

**1. Mouse brain processing**
1. Euthatal
2. Surgical tools for incision and brain removal
3. PBS
4. 4% Paraformaldehyde (Sigma; Cat#158127) (w/v), prepared fresh in PBS pH 7.4 4. 30% Sucrose (w/v in water)
5. Optimal Cutting Temperature (O.C.T). Compound (Tissue-Tek, USA; Cat# 4583)
6. 22×22×20mm Embedding Molds (Thermo Scientific; Cat# 1220)
7. Cryotome (Leica CM3050S, Germany)
8. SuperFrost Plus Tissue Slides (VWR; Cat# 48311-703)

**2. Histology**
1. PBS
2. 0.1% Triton X-100
3. 2% BSA
4. DMSO
5. Chicken anti-Glial Fibrillar Associated Protein (EnCOR; Cat# CPCA-GFAP)
6. Guinea pig anti-S100β (Synaptic Systems; Cat# 287-004)
7. Mouse anti-ADP-ribosylation factor-like protein 13B (Arl13b) (NeuroMab; Cat# 75-287)
8. Goat anti-Cholinergic acetyltransferase (CHAT) (Millipore Sigma; Cat# AB144P)
9. Chicken anti-NeuN (Millipore Sigma; Cat# ABN91)
10. Rabbit anti-Adenylate Cyclase 3 (AC3) antibody (EnCOR; Cat# RPCA-ACIII)
11. Rabbit anti-somatostatin receptor 3 (SSTR3) antibody (Sigma; Cat; SAB2900522)
12. Donkey highly cross absorbed H + L secondary antibodies conjugated to Alexa 488, Alexa 568 or Alexa 647 (Life Technologies)
13. DAPI (Invitrogen, D9542)
14. Mowiol 4-88 (Calbiochem; Cat# 405904)
15. DABCO ((1,4-diazabicyclo[2.2.2]octane), Millipore Sigma; Cat#D27802)

**3. Image acquisition and analysis software**
1. Confocal microscope
2. Fiji (https:// fiji.sc)
3. Software for statistical analysis (Graphpad Prism)

# Troubleshooting

# Safety warnings

⚠ Please follow Safety Data Sheets (SDS) for health and environmental hazards.

## Perfusion                                                                 `2d`

1  Anesthetize the mouse using 0.1 mL/10 g of Euthatal **using a 1/5 dilution** of the commercial solution (200 mg/mL), in PBS, per body weight.

2  Soak the hair surrounding the ventral thorax of the mouse with [M] 70 % (v/v) ethanol .

3  Before making the incision, determine anesthetic depth by toe-pinch to verify absence of a withdrawal reflex.

4  Make a midline incision through the skin over the proximal abdomen and thorax.

5  Dissect the skin to expose all underlying muscle. Cut the abdomen, puncture the diaphragm, and perform a thoracotomy by making bilateral paramidline incisions through the ribs toward the thoracic inlet to expose the thoracic viscera.

6  Make an incision to the right atrium to exsanguinate the mouse and allow for drainage of the perfusate.

7  Place a catheter into the left ventricle or aorta to saline, followed by [M] 4 % (v/v) PFA ; perfusion can be carried out by gravity or pump.

8  Cut the skull using sharp scissors and remove the brain.

9  Immediately submerge the whole brain in [M] 4 % (v/v) PFA  ⊙ Overnight in a 15ml plastic conical tube at 🌡 4 °C with gentle agitation.

10  Wash three times with PBS:

10.1  Wash brains with PBS (Wash 1/3).

10.2  Wash brains with PBS (Wash 2/3).

**10.3**     Wash brains with PBS (Wash 3/3). 🖐💧

**11**     Transfer brains into a [M] 30 % (w/v) sucrose solution at 🌡 4 °C with gentle agitation until the brains settle to the bottle of the conical tube (approximately ⏱ 48:00:00 ).   `2d`

## Brain Sectioning   `1h 10m`

**12**     After incubation in 30% sucrose solution, rinse whole brains with PBS and submerge in a 22×22×20mm embedding mold containing O.C.T. compound.

**13**     Note the rostral and caudal positioning of each brain within the embedding mold.

**14**     Place O.C.T. blocks on dry ice to solidify. Frozen blocks can then be stored for long-term storage at 🌡 -80 °C . ⏸

**15**     Remove blocks from -80°C storage and place at 🌡 -20 °C for at least ⏱ 00:10:00 to soften.   `10m`

**16**     Set the Cryostat to 🌡 -20 °C and set cutting thickness at ⊣⊢ 16 μm .

**17**     Cut brain sections either sagitally or coronally, depending on brain region of interest.

**18**     Carefully place sections on SuperFrost microscope slides and allow to dry at 🌡 Room temperature for at least ⏱ 01:00:00 . Slides can be transferred to 🌡 -80 °C for long-term storage.   `1h` ⏸

## Immunohistochemistry of Brain Sections   `7h`

**19**     Take brain sections out of 🌡 -80 °C and place into 🌡 Room temperature PBS for rehydration (~ ⏱ 00:15:00 ).   `15m`

**20** Use a hydrophobic pen (RPI, #19505) to create a barrier around each individual brain section to decrease total volume of reagent needed for subsequent steps.

**21** Remove excess O.C.T. compound with three ~50µl PBS washes (enough to fully immerse tissue), ⏱ 00:05:00 each

5m

**21.1** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 1/3)

5m

**21.2** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 2/3)

5m

**21.3** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 3/3)

5m

**22** Permeabilize brain sections with [M] 0.1 % (v/v) Triton X-100 in PBS for ⏱ 00:15:00 .

15m

> **Note**
>
> Keep permeabilization time consistent across experiments.

**23** Perform three washes with ~50µL PBS for ⏱ 00:05:00 each to remove excess detergent:

5m

**23.1** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 1/3)

5m

**23.2** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 2/3)

5m

**23.3** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 3/3)

5m

**24**  Block brain sections by incubation with PBS containing [M] 1 % (w/v) BSA and [M] 2 % (v/v) fetal bovine serum for ⏱ 02:00:00 at 🌡 Room temperature .

4h

Alternatively, blocking can be performed at 🌡 4 °C ⏱ Overnight .

**25**  Incubate brain sections ⏱ Overnight at 🌡 4 °C with 🧪 50 μL primary antibody in dilution buffer (PBS containing 1% BSA and 1% DMSO) with gentle rocking.

2h

> **Note**
>
> 1. Neuronal primary cilia are visualized with rabbit anti-AC3 at 1:10,000 dilution.
> 2. Astrocyte primary cilia are visualized with mouse anti-Arl13b at 1:500 dilution.
> 3. Rabbit anti-SSTR3 can be used to visualize primary cilia in the cortex and hippocampus but not in the striatal region of the brain

**26**  Perform three washes with (~50μL) PBS for ⏱ 00:05:00 to remove excess primary antibody:

5m

**26.1**  Add 🧪 50 μL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 1/3)

5m

**26.2**  Add 🧪 50 μL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 2/3)

5m

**26.3**  Add 🧪 50 μL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 3/3)

5m

**27**  Incubate with 🧪 50 μL secondary antibody* in dilution buffer for ⏱ 02:00:00 at 🌡 Room temperature . Include DAPI in the dilution mixture ( [M] 0.1 microgram per milliliter (μg/mL) ) at this step if desired.

2h

> **Note**
>
> Donkey highly cross absorbed H + L secondary antibodies (Life Technologies) conjugated to Alexa 488, Alexa 568 or Alexa 647 are used at a 1:1,000 dilution.

**28** Perform three washes with (~50µL) PBS for ⏱ 00:05:00 each to remove excess secondary antibodies:

5m

**28.1** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 1/3)

5m

**28.2** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 2/3)

5m

**28.3** Add 🧪 50 µL PBS to wash (enough to fully immerse tissue) for ⏱ 00:05:00 . (Wash 3/3)

5m

**29** Perform a quick wash in H2O to remove excess buffer. Apply Mowiol mounting medium ( 🧪 25 µL Mowiol 4-88 with 2.5% DABCO ) onto the stained tissue, followed by a 22×50mm coverglass, and gently tap to remove bubbles. Excess mowiol solution can be aspirated from the sides of the cover glass.

> **Note**
>
> Mowiol is prepared as described in Cold Spring Harbor protocol (doi:10.1101/pdb.rec10255) and kept frozen at -20˚C.

**30** Allow slides to dry ⏱ Overnight **in the dark** before image acquisition.

2h

## Image Acquisition and Analysis

**31** Choose multiple brain areas to image – The somatosensory area of the cerebral cortex, pyramidal layer of the hippocampus and the striated area of the caudoputamen next to the lateral ventricle. Image four to five regions within each brain area when investigating primary ciliation in various regions of the brain.

> **Note**
>
> 1. For dorso-striatal Cholinergic interneurons: Cells are triple-labeled with Goat anti-CHAT (Millipore Sigma), Chicken anti-NeuN (Millipore Sigma), and DAPI. Primary cilia are detected with Rabbit anti-AC3 antibody (EnCOR). Cholinergic interneurons are rare, and only comprise 1-3% of the striatal cell population. They are also large compared to the surrounding glia and medium spiny neurons. 15-20 Cholinergic interneurons per brain section are typically scored.
> 2. For dorso-striatal astrocytes: Cells are triple-labeled with Chicken anti-GFAP (EnCOR), Guinea pig anti-S100β (Synaptic Systems), and DAPI. Mouse anti-Arl13b (NeuroMab) is used to label glia primary cilia. Like cholinergic interneurons, GFAP+ astrocytes are also rare in the dorsal striatum (Chai et al., 2017), however, most striatal astrocytes are S100β positive. We typically count 15-20 GFAP+ striatal astrocytes per brain section.

**32** Acquire images using a 63X 1.4NA or 100× 1.4NA objective mounted on a SP8 laser scanning confocal or Yokogawa spinning disk confocal with an emCCD 512 × 512 camera for fast acquisition with high resolution.

**33** Acquire images at 0.5-0.75 μm Z-sampling for each mouse brain. Sizing for Z-sampling depends on cell-type.

> **Note**
>
> Z-steps should be kept uniform across all analyses.

> **Note**
>
> Use larger z-steps of 0.75μm for interneurons.

> **Note**
>
> Use smaller z-steps of 0.5μm for astrocytes.

**34** Select the bottom and top of the Z-series such that the entire area of interest is captured.

> **Note**
>
> For striatal cholinergic interneurons and striatal astrocytes: Capture Z-series from top to bottom of the cell of interest.

35    Using FIJI, open the Z-series image and track Arl13b/AC3 structures by eye.

> **Note**
>
> The cilium of interest should make direct contact with the cell marker (e.g. CHAT for cholinergic interneurons, GFAP for astrocytes). If there is a cilium making direct contact with the cell body, mark the cell as ciliated. If you cannot assign the cilium to a specific cell because it makes contact with two cells, exclude it from your analysis.

36    To manually measure primary cilia length, make a maximum Z projection. Then, select **Analyze > Set Scale**.

> **Note**
>
> Values inserted are based on your microscope calibrations. Make settings global to streamline analysis.

37    In the FIJI toolbar select the "segment line" feature. The segmented line feature can be used to trace the length of the cilium. The shorthand "m" is used to measure ciliary length in μm. Record the results.

38    To automatically score cilia number and length, median filter the maximum intensity projections of Z-stacks, threshold and subject the resulting mask to a skeletonize function.
Then count primary cilia structures in these masks using Analyze Particles. To measure length of primary cilia, the output image from the previous step can be subsequently analyzed using "Longest shortest path function".

39    Count the number of nuclei in the cerebral cortex and striatal regions of the brain by analyzing maximum intensity projections of the DAPI channel. Then median filter images, subject to "Fill Holes", "Watershed" followed by Analyze Particles.

> **Note**
>
> Count number of nuclei in the pyramidal layer of the hippocampus manually by differential pseudo-coloring of Z-stacks to overcome the counting errors caused by tight packing of the nuclei and bright nucleoli of neurons in this region.

40    Pool all data from cells that are analyzed from an individual mouse brain for statistical analysis.

For example, if there are 15 cells counted per brain section, and three brain sections total, a single data point is obtained and graphed in comparison with data from other mouse brains to avoid sampling error. Increase statistical power by increasing total number of cells counted and/or by increasing number of brains per genotype.

41 Choose the statistical analysis measurement based on the number of groups in the experiment (Students t-test for only two groups, ANOVA for three or more).