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## IHC analysis of brain regions from PCB exposed rats V.1

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### ABSTRACT

This protocol describes a method to analyze the effect of PCB52 inhalation on brain cells in adolescent rats. PCB52 (2,2',5,5'-tetrachlorobiphenyl) was administered to adolescent rats via a nose-only apparatus with PCB52-laden air. The exposure was 4 hours a day for 28 consecutive days. Brains were collected, the cerebellum region was frozen, and tissue sections were cut and stained to label apoptotic cells using PSVue, microglia using anti-IBA1, and astrocytes using anti-GFAP. Using a Leica TCS SP8 confocal microscope, Z-Stack images were taken and analyzed for differences in cell density and apoptosis within the distinct cerebellar layers.

**Protocol status:** Working  
We use this protocol and it's working

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## MATERIALS

### Chemicals:

- DPBS (10x) - Thermo Fisher Scientific
- O.C.T. compound - Tissue-Tek
- Ammonium Chloride - Research Products International
- Sodium azide - Sigma Aldrich
- Triton X-100 - Thermo Fisher Scientific
- Formalin

### Antibodies:

- Alexa Fluor 488 Goat anti-rabbit IgG (#A11034, RRID: AB\_2576217) - Thermo Fisher Scientific
- Alexa Fluor 647 Goat anti-mouse (#A28181, RRID: AB\_2536165) - Thermo Fisher Scientific
- Anti IBA-1 polyclonal rabbit anti mouse (cat#100369-764) - Fujifilm
- Anti GFAP mouse anti rat (cat#14-9892-82, RRID: AB\_10598206) - Thermo Fisher Scientific
- PSVue 550 fluorescent probe (cat #P-1005) - Molecular Targeting Technologies, inc.
- DAPI Nuclear counterstain (cat #62248) - Thermo Fisher Scientific

### Materials:

- brain matrix
- parafilm
- small paint brush
- 3 in X 1 in X 1 mm slides - Thermo Fisher Scientific
- 22 X 22 mm #1.5 coverslip - Leica
- 25 X 20 X 5 mm disposable cryomolds - Tissue-Tek
- Costar 12 or 24 well plates - Sigma Aldrich

### Equipment:

- Leica CM3050 S Cryostat
- Leica TCS SP8 Confocal Microscope

### Solutions:

#### Extraction Solution (1% Triton X-100)

(can be stored at 4 °C for several weeks)

50 mL PBS

0.5 mL Triton X-100

#### Ammonium Chloride, NH4Cl (50 mM)

*(can be stored at 4 °C for several weeks)*

50 mL PBS

0.134 g NH<sub>4</sub>Cl

**Blocking Solution (20% serum)**

*(can be stored at 4 °C for 3 weeks)*

5 mL 2x PBS

3 mL dd H<sub>2</sub>O water

2 mL heat inactivated horse serum

**Antibody incubation solution (1% serum)**

*(can be stored at 4 °C for 3 weeks)*

49.5 mL PBS

0.5 mL heat inactivated horse serum

## SAFETY WARNINGS

**Polychlorinated biphenyl (PCB):**

May cause damage to organs through prolonged or repeated exposure. Very toxic to aquatic life with long-lasting effects. Do not breathe dust/fume/gas/mist/vapors/spray. Avoid release to the environment. This statement does not apply where this is the intended use. Get medical advice/attention if you feel unwell. Collect spillage. Dispose of contents/containers in accordance with relevant regulations.

**Formalin:**

Work under hood. Do not inhale substance/mixture. Avoid generation of vapors/aerosols. Keep away from open flames, hot surfaces and sources of ignition. Take precautionary measures against static discharge. After inhalation: fresh air. Immediately call in physician. If breathing stops: immediately apply artificial respiration, if necessary also oxygen. In case of skin contact: Take off immediately all contaminated clothing. Rinse skin with water/ shower. Consult a physician. After eye contact: rinse out with plenty of water. Call in ophthalmologist. Remove contact lenses. After swallowing: immediately make victim drink water (two glasses at most). Consult a physician.

**Dry ice:**

Remove contaminated clothing and protective equipment before entering eating areas. Store in original container protected from direct sunlight in a dry, cool and well-ventilated area, away from incompatible materials and food and drink. Keep container tightly closed and sealed until ready for use. Containers that have been opened must be carefully resealed and kept upright to prevent leakage. Do not store in unlabeled containers. Use appropriate containment to avoid environmental contamination. Immediately flush eyes with plenty of water, occasionally lifting the upper and lower eyelids. Check for and remove any contact lenses. Get medical attention if irritation occurs. Remove victim to fresh air and keep at rest in a position comfortable for breathing. Wash out mouth with water. Do not induce vomiting unless directed to do so by medical personnel.

## ETHICS STATEMENT

The animal protocol used to generate tissue samples was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Iowa. All animals were utilized in accordance with all Public Health Service (PHS) policies and the Guide for the Care and Use of Laboratory Animals, National Institutes of Health (NIH) Publication No. 85-23, revised 2010.

## BEFORE START INSTRUCTIONS

Note that this protocol involves work with experimental animals and requires prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

### Extraction and preparation of cerebellum brain region

- 1 Using a rat brain matrix, the brain is cut into roughly 12 sections, each 2 mm thick
- 2 Drop fix the individual sections in 2 mL of 4% formalin per well for 24 hours at 4°C
- 3 Remove the formaldehyde and add 2 mL of 30% sucrose to each well for 24 hours at 4°C
- 4 Place a drop of O.C.T. compound in a disposable plastic cryomold (or any flat surface specimen block) and place the tissue flat on top of the O.C.T. compound
- 5 Fill the rest of the cryomold with O.C.T. compound so it completely covers the tissue

6 Place the mold on dry ice to freeze the O.C.T. compound

7 Store molds in a -80°C degree freezer

## Cutting and staining

8 Set the temperature of the Leica CM3050 S Cryostat to -18°C and set to a thickness to 100 µm

9 Place O.C.T. tissue molds in the cryostat and wait at least 30 minutes for the blocks to acclimate to the new temperature

10 Preload 12-well plate with 1-2 mL of PBS solution (*optional: prevent contamination by adding sodium azide*)

11 Cut tissues using the cryostat and transfer slices to the 12-well plate, one slice per well (*dispose of excess O.C.T. block as hazardous waste*)

12 Wash with 2 mL of PBS solution 3 times for 15 min at room temp

*(Washing includes switching the current solution for fresh PBS solution 3 times, waiting 15 mins in between, and being careful not to disturb the tissues in the wells. During each of the 15-minute intervals, place the well plate on a rotator at around 100 rpm)*

13 In a new 12-well plate, add 0.5 mL of extraction solution to each labeled well

- 14 Transfer slices to the new 12-well plate containing extraction solution using a very fine paintbrush to pick up the tissue slices
- 15 Leave the tissue slices in the extraction solution for 1 hour on the rotator at around 100 rpm at room temp
- 16 Replace the extraction solution with 0.5 mL fresh extraction solution in each well
- 17 Incubate tissue slices overnight (approximately 16 hours) in a fridge at 4°C
- 18 Remove extraction solution by washing slices with 0.5 mL ammonium chloride solution for 30 min at room temp
- 19 Replace ammonium chloride solution with 0.5 mL of blocking solution for 30 min at room temp
- 20 Replace blocking solution with 0.5 mL antibody incubation solution (1% serum) for 5 min

- 21 Switch the 1% serum solution for ~300 µL of the primary antibody solution (*enough to submerge the tissue; make fresh with each batch*)  
**Primary antibody solution (1 mL total)**  
- 1 µL anti-IBA1 (1:1000)  
- 1 µL anti-GFAP (1:1000)  
- 9993 µL antibody incubation solution (1% serum)
- 22 Wrap the plate with parafilm
- 23 Incubate the 12-well plates with the tissue slices for 3 nights in a fridge at 4°C
- 24 Wash with antibody incubation solution (1% serum) 3 times for 15 min at room temp  
(*Washing includes switching the current solution for fresh antibody incubation solution 3 times, waiting 15 mins in between, and being careful not to disturb the tissues in the wells. During each of the 15-minute intervals, place the well plate on a rotator at around 100 rpm*)
- 25 Switch the 1% serum solution for ~300 µL secondary antibody solution (*enough to submerge tissue; make fresh with each batch*)  
**Secondary antibody solution (1 mL total)**  
- 2 µL Alexa 488 (Goat anti Rabbit) (1:500)  
- 2 µL Alexa 647 (Mouse anti Rat) (1:500)  
- 9996 µL antibody incubation solution (1% serum)
- 26 Wrap the 12-well plate with parafilm, followed by aluminum foil to avoid exposure of the fluorescent probe to light
- 27 Incubate tissue slices overnight (approximately 16 hours) in a fridge at 4°C
- 28 Wash with 1% serum 3 times for 15 min at room temp

*(Washing includes switching the current solution for fresh 1% serum solution 3 times, waiting 15 mins in between, and being careful not to disturb the tissues in the wells. During each of the 15-minute intervals, place the well plate on a rotator at around 100 rpm)*

29 Switch the 1% serum solution for 300 µL of DAPI solution (*make fresh with each batch*)

**DAPI solution (5 mL total)**

- 1 µL DAPI (1:5000)
- 4999 µL antibody incubation solution (1% serum)

30 Incubate tissue slices on a rotator for 30 min at room temp

31 Wash with 1% serum 3 times for 15 min at room temp

*(Washing includes switching the current solution for fresh 1% serum solution 3 times, waiting 15 mins in between, and being careful not to disturb the tissues in the wells. During each of the 15-minute intervals, place the well plate on a rotator at around 100 rpm)*

32 Switch the 1% serum solution for 300 µL of PSVue solution (*make fresh with each batch*)

**PSVue solution (1 mL total)**

- 1 µL PSVue550(1:1000)
- 9999 µL antibody incubation solution (1% serum)

33 Incubate tissue slices for at least 2 hours in a fridge at 4°C

34 Mount on microscope slide with 1-2 drops of PBS with 0.02% sodium azide, flattening the tissue as much as possible before applying the coverslip

35 Secure the coverslip with 2 layers of clear nail polish

36

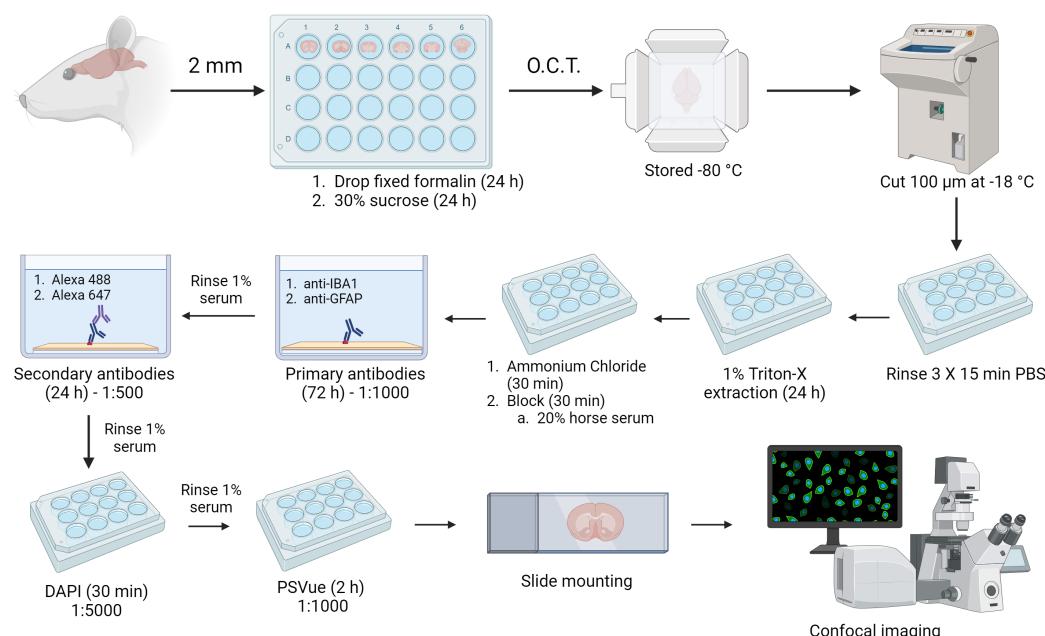


Fig 1. Step-by-step summary of prepping, cutting, and staining brain tissue. Image generated using Biorender.com.

## Imaging and analyzing

**37** Place the microscope slide face down on the inverted Leica TCS SP8 Confocal Microscope

**38** Using the bright field setting, manually focus the microscope

- 39** Adjust the settings, which include: 20x scanning objective, line averaging set to 16 (or as needed), and 1024 x 1024 scan format
- 40** Once focused, take a full overview image to find the area of interest
- 41** Create a sequential setting that includes channels corresponding to the stains used
- 42** Pan through each channel and adjust the lasers and detectors so the cells are not fluorescently oversaturated but just bright enough for analysis
- 43** Once each channel is adjusted, take a Z stack 70  $\mu\text{m}$  thick, with a Z step size of 2  $\mu\text{m}$ :

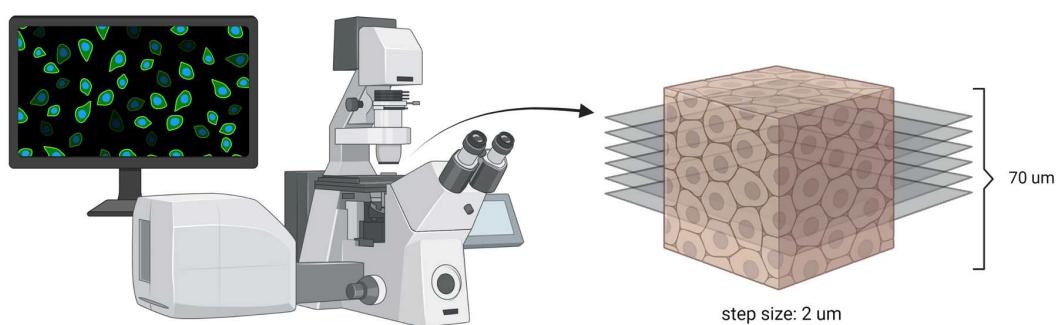


Fig 2. Visual representation of Z stack confocal image methods. With a total of 70  $\mu\text{m}$  imaged at 2  $\mu\text{m}$  intervals. Figure made using Biorender.com

- 44** Once the Z stacks are saved, open them in ImageJ

- 45** Looking at each channel individually, analyze cell density and % area counts for each stain. The following criteria are to be followed for each stain:

- IBA1: particle size of  $15 \mu\text{m}^2$ -infinity, threshold set from 50-60 to 255
- GFAP: particle size of  $10 \mu\text{m}^2$ -infinity, threshold set from 50 to 255
- DAPI: particle size of  $5 \mu\text{m}^2$ -infinity, threshold set from 50 to 255
- PSVue: particle size of  $2 \mu\text{m}^2$ -infinity, threshold set from 100 to 255

*(If desired, these parameters can be analyzed for both the molecular and granular layers within the cerebellum)*

- 46** Record the measurements and transfer data to an Excel spreadsheet for analysis and data visualization

- 47** Channels can be combined to create an overlapping image showing all cell types. Brightness and contrast may need to be adjusted to properly see all of the stains. Representative images are shown below:

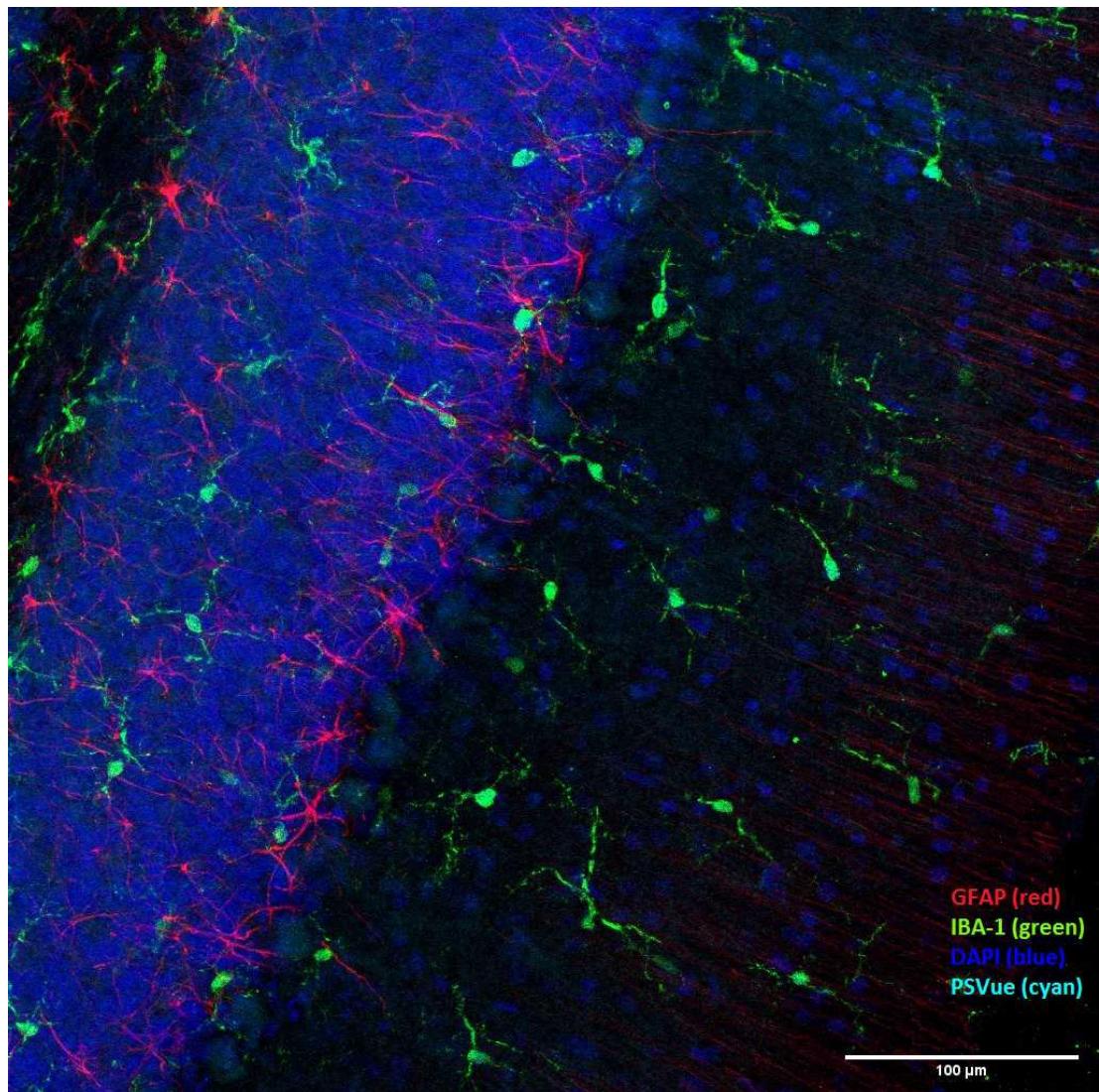


Fig 3. Example of overlapping image (of a male sham specimen) containing all stains used in protocol.  
(note: no PSVue positive cells depicted)

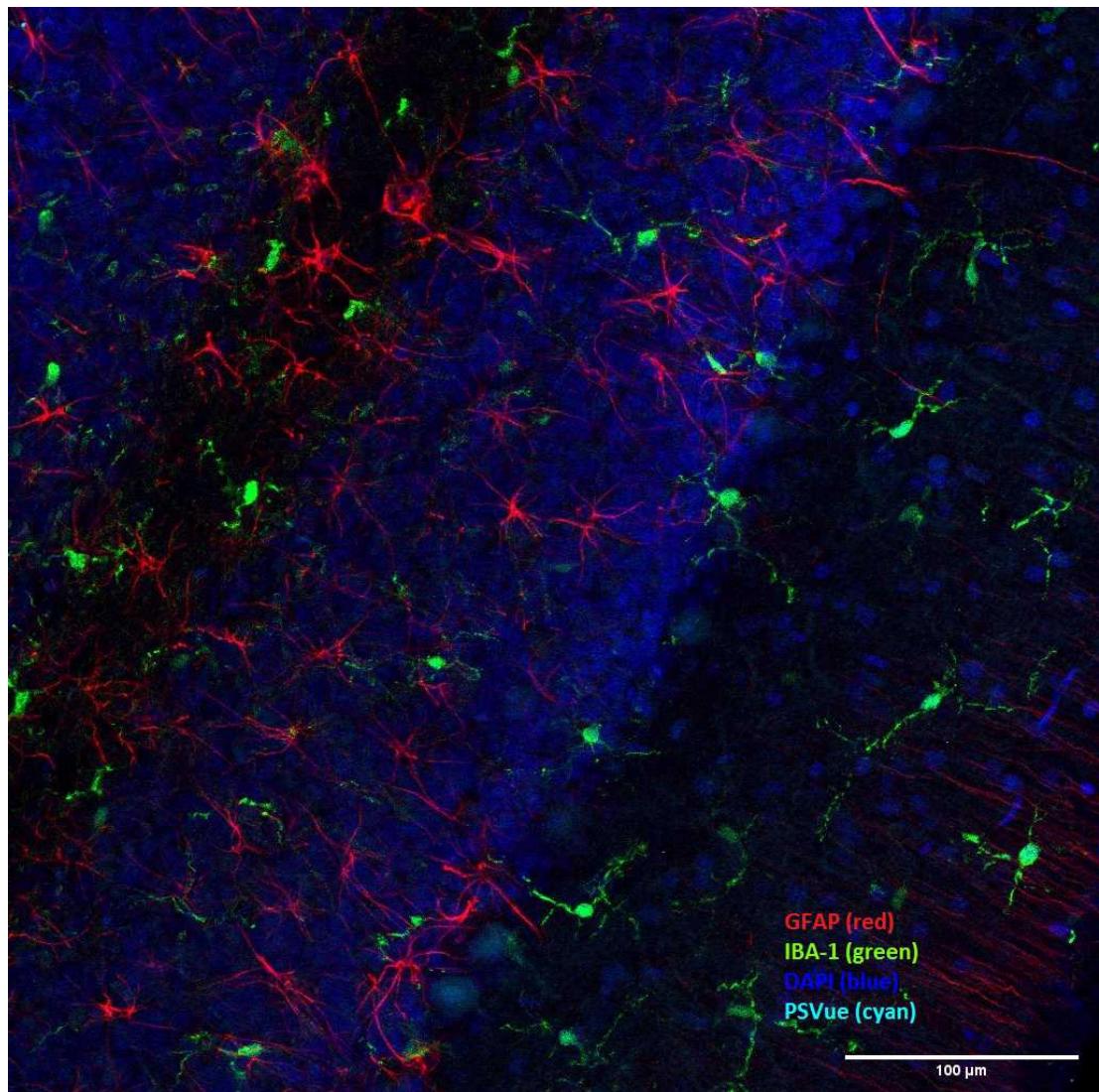


Fig 4. Example of overlapping image (of an experimental male specimen) containing all stains used in protocol. (note: no PSVue positive cells depicted)