Aug 08, 2025

(P34) immunostaining of free floating sections

DOI
https://dx.doi.org/10.17504/protocols.io.261gek32og47/v1

Yunming Wu[1]

[1]Stanford University

Y Yunming Wu
Stanford University

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.261gek32og47/v1

Protocol Citation: Yunming Wu 2025. (P34) immunostaining of free floating sections. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261gek32og47/v1

Protocol status: Working
We use this protocol and it's working. But there is alway room for improvement.

Created: August 08, 2025

Last Modified: August 13, 2025

Protocol Integer ID: 224354

Keywords: performing routine immunostaining, routine immunostaining, floating section, p34, protocol, optional section

Disclaimer

The method used here is widely used in many labs and is not invented in this protocol. This protocol is a brief and concise version of the the commonly used immunostaining protocol for brain slices for use as a routine procedure.

Abstract

This is a protocol for performing routine immunostainings of free floating sections. It includes 2 optional sections for enhancing weak signals.

Materials

- 1X PBS
Dilute 10X PBS 1:10 with ddH2O.
Filter with 0.24um Vaccum filter.
This solution can be stored under room temperature for 1 year.

- PBST
Add 1 mL TritonX100, 100mL 10X PBS, to 500mL ddH2O.
Stir mix with a stir bar until tritonX is fully dissolved.
Add water to 1L.

- 1M Citric acid
dissolve 192.1g citric acid in 1L ddH2O.

- 1M Sodium citrate
dissolve 25.1g sodium dihydrogen citrate in 1L.

- 10X Citrate buffer
In a beaker, add 82 mL 1M sodium citrate
add 18 mL 1M Citric acid
add ddH2O to 1L.

- 1X Citrate buffer
dilute 10X citrate buffer to 1L with ddH2O.

- ABC amplification kit: VECTASTAIN™ Elite ABC-HRP Kit, Peroxidase (Standard), PK-6100

- TSA amplification kit: Alexa Fluor™ 488 Tyramide Reagent, Catalog number: B40953

- HRP Stop buffer:
Make 5M sodium azide by dissolving 3g sodium azide (Sigma S2002) into 10mL ddH2O.
Make 0.2M Trolox by dissolving 1g Trolox (Sigma 238813) into 20mL H2O.
Make 1M sodium ascorbate by dissolving 1g sodium ascorbate (Sigma 11140) into 5mL ddH2O.
Store the stock solution at -20C.
At the day of experiment:
In 10mL PBST, add 10uL 1M sodium ascorbate, 100uL 0.2M Trolox, 10uL 5M sodium azide.

Transcardial perfusion surgery

16h

1

Note

All experiment involving the use of live animals must be reviewed and approved by the IACUC, and comply with NIH and institutional guideline.

2 Weight the animal.

3 Anesthetize animals 0.1 mL 2.5% Avertin per 10 g bodyweight i.p. using a sterile tuberculin syringe (26g needle).

4 Perfuse with 10 mL ice cold 1X PBS.

5 Perfuse with 10 mL ice cold 4% PFA.

6 Postfix the sample in 4% PFA 4 °C Overnight with gentle agitation. 16h

7 Store the samples at 4 °C in 1X PBS supplied with 1 µL of 4% PFA. The samples can be stored for upto 1 week.

Preparing free floating sections with a vibratome

1h 48m

8 Melt 4% low melting point agarose with a microwave briefly and keep it at 37 °C afterwards. 2m

9 Pour a thin layer of melt agarose to the peel-a-way mold. 1m

10 Keep the mold in 4 °C until it turns solid. 5m

11 Make a mark on one side of the brain. 5m

> Note
>
> This can be done by cutting a small piece off on one side, depending on what brain region will be used.

12 Embed the brain sample in the mold with the melt agarose. The A-P axis should be vertical to one edge of the mold. The brain is placed upside down so that bragma and lambda are in contact of the surface. 5m

13 Section into 50 µm with a vibratome. 1h

14 Place the sections into 24 well plate (for serial sectioning), or 6 well plate (selected sections without serial sectioning). 30m

Antigen retrival (Optional) 16m

15

> Note
>
> This is an optional step to enhance antibody binding. It is recommended for antibodies with weak signals or no signal.

16 Transfer the brain sections into a beaker with enough citrate buffer to cover all sections. e.g., 20 sections in a 500 mL beaker filled with 100 mL citrate buffer. 5m

17 Microwave 00:01:00, or until boiling (depends on the power). 1m

18 Wait until the citrate buffer cools down to Room temperature. 10m

Immunostaining 16h 38m

19 Transfer the brain sections back to the multi-well plate if the antigen retrival step is performed. 5m

20 Dilute Primary antibody in PBST 1:1000. 1m

21 Incubate the sections with the diluted primary antibodies at room temperature Overnight with gentle agitation. Each well of 24 well plate uses 100uL. 16h

22 Wash PBST 5min X3. 15m

23 Dilute Secondary antibody in PBST 1:1000. 1m

24 Incubate the sections with the diluted secondary antibodies at room temperature Overnight with gentle agitation. Each well of 24 well plate uses 100uL. 1m

25 Wash PBST 5min X3. 15m

26 Mount in Ymount and image.

Signal amplification through biotin-avidin-hydrogen peroxidase 2h 23m

27

Note

This step can further enhance weak signals. The secondary antibody must be conjugated with biotin.

28 Deactivate endogenous peroxidase activity with [M] 3 % (v/v) H_2O_2 in PBST 01:00:00 with gentle agitation. 1h

29 Wash with PBST 00:05:00 . 5m

30 Dilute reagent A and reagent B 1:50 in PBST. 1m

31 Mix and RT 00:30:00 . 30m

32 Add A B mix to sample, 00:30:00 (Optional overnight for complete penetration). 30m

33 Wash with PBST 00:05:00 . repeat 3 times. 5m

34 Incubate with 1X fluorescently conjugated TSA reagent with 0.0015% H2O2 in PBST in dark, 00:02:00 . 2m

35 Wash with HRP stop buffer 00:05:00 X3. 5m

36 Wash with PBST 00:05:00 . 5m

37 Mount in Ymount and image.