

Neuronal Morphology Analysis

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[Abstract] This protocol describes how to visualize neuronal morphology and how to determine neuronal complexity of immature and mature hippocampal neurons in the mouse *in vivo* including tissue preparation, staining of brain sections and confocal cell analysis.

Materials and Reagents

1. Mice
2. 0.9% sterile Sodium chloride (NaCl) (Fresenius Kabi)
3. Ketamine hydrochloride (Ketavet, 100 mg/ml) (Pfizer)
4. Xylazine hydrochloride (Rompun, 20 mg/ml Xylazine) (Bayer)
5. 4% Paraformaldehyde in phosphate buffer (4% Roti-Histofix) (Roth, catalog number: P087.1)
6. Hank's balanced salt solution (HBSS) (Life Technologies, Invitrogen™, catalog number: 14170-138)
7. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 31434)
8. Sodium phosphate dibasic heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: S9390)
9. Sodium phosphate monobasic monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) (Roth, catalog number: K300.2)
10. Potassium chloride (KCl) (AppliChem GmbH, catalog number: A3582)
11. Potassium phosphate monobasic (KH_2PO_4) (Gerbu, catalog number: 2018)
12. Sodium azide (Sigma-Aldrich, catalog number : S2002)
13. Hydrochloric acid (HCl, 37%) (Sigma-Aldrich, catalog number: 30721)
14. Trizma base (Sigma-Aldrich, catalog number: T1503)
15. Horse serum (Biochrom, catalog number: S9135)
16. Triton X-100 (Sigma-Aldrich, catalog number: X-100)
17. Chicken anti-GFP antibody (Aves, catalog number: GFP-1020)
18. Goat anti-Doublecortin antibody (DCX, C18) (Santa Cruz, catalog number: sc-8066)
19. Mouse anti-NeuN antibody (EMD Millipore, catalog number: MAB377)
20. Donkey anti-chicken DyLight488 antibody (Dianova, catalog number: 703-485-155)

21. Donkey anti-goat Alexa 647 antibody (Dianova, catalog number: 705-605-147)
22. Donkey anti-mouse Alexa 546 antibody (Life Technologies, Invitrogen™, catalog number: A10036)
23. Hoechst (33342) (Biotrend, catalog number: 40047)
24. Gelatine to coat glass slides (Sigma-Aldrich, catalog number: G7041)
25. Chromium (III) potassium sulfate dodecahydrate (Sigma-Aldrich, catalog number: 60152)
26. Bromothymol Blue sodium salt (Sigma-Aldrich, catalog number: 114421)
27. FD Rapid GolgiStain™ Kit (FD NeuroTechnologies, catalog number: PK401)
28. Millipore water
29. Ethanol (Sigma-Aldrich, catalog number: 459844)
30. Xylene (Sigma-Aldrich, catalog number: 33817)
31. Eukitt (Fluka, catalog number: 03989)
32. Agarose (AppliChem GmbH, catalog number: A8963)
33. Phosphate buffer saline (PBS) (20x) (see Recipes)
34. TBS (10x) (see Recipes)
35. TBS++ (see Recipes)
36. 0.1 M Phosphate buffer (see Recipes)
37. Gelatine to coat glass slides (see Recipes)
38. Mowiol (Merck/Calbiochem, catalog number: 475904) (see Recipes)

Equipment

1. Syringe (1 ml syringe 27 G for *i.p.* injections)
2. 0.5 ml Eppendorf Safelock tubes
3. 15 ml and 50 ml Falcon tubes
4. Micro dissecting scissors
5. Forceps
6. Leica VT1200 Vibratome
7. Brush to transfer slices
8. Netwell carriers and plates (Corning Inc., catalog numbers: 3477 and 3520)
9. Rocking platform
10. Tube roller mixer
11. Hot plate stirrer
12. Staining containers
13. Microscope glass slides
14. Cover slips
15. Confocal microscope

16. Centrifuge

Software

1. Amira Filament Editor Analysis (Visage Imaging) or other neuron morphology analysis software

Procedure

A. Tissue preparation

1. Transcardial perfusion

Animals are anesthetized with an overdose of Rompun (14 mg/kg bodyweight) and Ketavet (100 mg/kg bodyweight) in 0.9% NaCl.

Mice are transcardially perfused with 30 ml HBSS followed by 10 ml of 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer pH 7. For transcardial perfusion the thorax cavity is opened, and the right auricle cut with a scissor to allow bleeding. A butterfly cannula is introduced in the left ventricle and mice are perfused with 30 ml HBSS followed by fixation with 10 ml 4% PFA.

Brains are removed and post-fixed overnight in 10 ml 4% PFA in 0.1 M phosphate buffer pH 7 in a 15 ml Falcon tube on a tube roller mixer at 4 °C.

Tissue is washed twice with PBS and may be stored in PBS with 0.01% sodium azide for up to a year.

B. Neuronal morphology analysis of immature dentate gyrus neurons

1. Vibratome cutting

- a. For coronal vibratome sections (see Figure 1), the cerebellum is cut, removed and the brain glued upright with the cutting site using superglue onto the holder plate of the vibratome. Coronal sections may have a thickness of 50 µm or 100 µm.

Note: The NeuN antibody does not very well penetrate 100 µm thick sections. If you have to use 100 µm thick sections primary antibody incubation should be 72 h.

- b. For sagittal sections (see Figure 1) brains are embedded in 2% agarose in PBS. The brain is then glued in a solid gel block on the lateral side to the holder plate. Sagittal sections are cut 100 µm thick. Agarose can be removed from the slices during cutting or may be kept during the staining process in order to stabilize the tissue (especially olfactory bulbs).
- c. Sections can be stored in PBS with 0.01% sodium azide at 4 °C. Tissue might be used for up to one year after perfusion.

Coronal brain sections



Sagittal brain sections

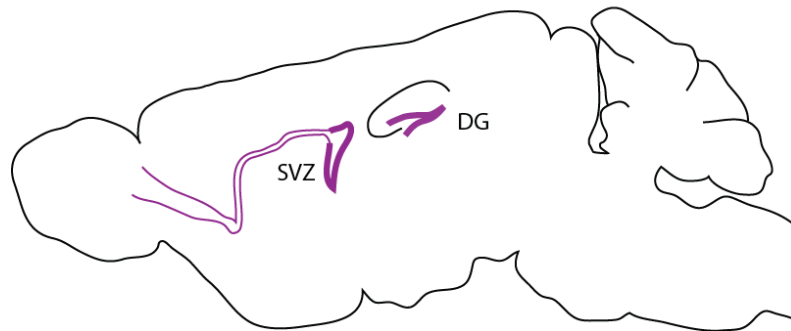


Figure 1. Overview of neurogenic niches in coronal and sagittal brain sections (SVZ: subventricular zone; DG: dentate gyrus)

2. Immunofluorescence staining

- a. For each mouse, 4 brain slices (50 or 100 μm thick, 250 μm or 300 μm apart, respectively) are stained. Brain sections are placed in net carriers in 12 well plates (2 slices per well) filled with 4 ml 0.1 M Tris Buffer pH 7.4 supplemented with 8% NaCl (TBS). The sections are washed three times in TBS each 15 min at RT on a rocking platform (50 rpm).
- b. Blocking of unspecific antibody binding is performed by incubating sections for 1 h in TBS++ at RT.
- c. Sections are transferred to 0.5 ml Eppendorf Safelock tubes (2 sections per tube) containing 200 μl TBS++ and the diluted primary antibodies, and incubated at 4 $^{\circ}\text{C}$ for 24-72 h. For this 12 tubes are put in a 50 ml Falcon and rotated at 4 $^{\circ}\text{C}$ on a tube roller mixer.
 - i. For staining of GFP/YFP of either genetically or retrovirally labeled immature neurons sections are stained with the primary chicken anti-GFP antibody (1:1,000).
 - ii. Otherwise non-labeled immature neurons are stained with the goat anti-Doublecortin antibody (1:200). Mouse anti-NeuN (1:200) might be used as additional marker to determine cell maturity.

- d. After incubation sections are transferred back to net carriers in 12 well plates, washed three times with TBS at RT.
 - e. After blocking in TBS++ for 30 min at RT sections are transferred again into 0.5 ml Eppendorf Safelock tubes containing the diluted secondary antibody mix in TBS++. Sections in Eppendorf tubes in Falcons are incubated in secondary antibodies at 4 °C on a tube roller mixer for 2 h.
 - i. Secondary antibodies are diluted 1:400: Donkey anti-chicken DyLight488, donkey anti-goat Alexa 647 or donkey anti-mouse Alexa 546.
 - ii. Hoechst 33342 (1:10,000) is used to counterstain DNA and added to the secondary antibody mix.
 - f. Finally, sections are placed back into net carriers in 12 well plates, washed three times for 15 min with TBS and additionally 4 times for 1 min in TBS at RT.
 - g. Sections are floated in 0.1 M PB in a Petri dish, mounted on glass slides and embedded with 100 µl Mowiol.
3. Confocal microscope pictures are taken with a 40x objective on a confocal microscope. Branching points and total dendrite length are measured using Amira Filament Editor Analysis (Visage Imaging).

C. Neuronal morphology analysis of mature hippocampal neurons

1. For analysis of neuronal morphology of mature CA or DG neurons PFA fixed brains are cut in two hemispheres and stained with the FD Rapid GolgiStain™ Kit.
 - a. Hemispheres are incubated in impregnation solution (A and B) for 2 weeks at RT in the dark.
 - b. After that tissue is transferred into solution C and stored for five days at 4 °C protected from light.
 - c. The tissue is cut in 100 µm thick coronal sections floating in solution C with a Leica VT1200 vibratome and mounted on gelatine coated glass slides. To coat glass slides 1.5 g gelatine and 0.25 g chromium potassium sulfate are mixed with 500 ml distilled water, a few crystals of bromthymol blue are added as preservative, heated up to 60 °C in order to dissolve gelatine and then glass slides are dipped into the solution and the lower part of the slide is cleaned with a tissue. Coated slides are dried overnight at RT.
 - d. Mounted slides are washed 2x for 2 min in Millipore water.
 - e. Sections are stained for 10 min in staining solution (40 ml solution D, 40 ml solution E and 80 ml Millipore water) at RT.

- f. Sections are washed 2 x 4 min in Millipore water, once for 4 min in 50% ethanol (EtOH), once for 4 min in 75% EtOH, once for 4 min in 95% EtOH and four times for 4 min in 100% EtOH at RT.
 - g. Finally slices are washed three times for 4 min in xylene and embedded with Eukitt.
2. Neurons of the dentate gyrus and CA regions can be analysed with this method. Stacks can be recorded on a confocal microscope with a 40x objective. Branching points and total dendrite length are measured using Amira Filament Editor Analysis (Visage Imaging) (see Figure 2).

Representative data

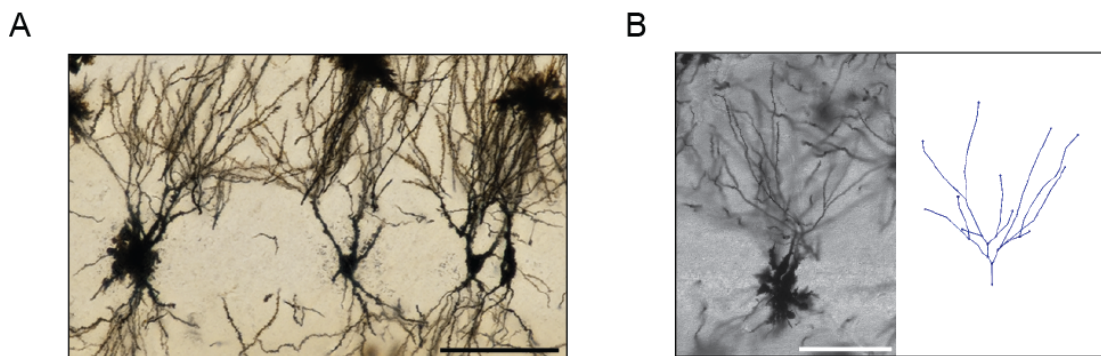


Figure 2. Light microscope (A) and confocal microscope picture (B) of Golgi stained neurons. Picture shown in B (left panel) was used for image analysis with Amira (right panel).

Recipes

1. PBS (20x)

NaCl	160 g/L
Na ₂ HPO ₄	23 g/L
NaH ₂ PO ₄	28.84 g/L
KCl	4 g/L
KH ₂ PO ₄	4 g/L

Adjust pH to 7.4 with HCl and fill volume up to 1 L with dH₂O.

2. TBS (10x)

Trizma base	24.23 g/L
NaCl	80.06 g/L

Mix in 800 ml ultra-pure water, adjust pH to 7.6 with pure HCl and fill up to 1 L.

3. TBS ++

- TBS 100 ml
 Horse serum 3 ml
 Triton X-100 0.25 ml
4. 0.1 M Phosphate buffer
 0.2 M Monobasic Stock
 NaH₂PO₄·H₂O 13.9 g/500 ml
 0.2 M Dibasic Stock
 Na₂HPO₄·7H₂O 53.65 g/L
 Combine indicated amounts of 0.2 M monobasic and 0.2 M dibasic stock solutions and bring volume up to 600 ml.

0.2 M Monobasic Stock	0.2 M Dibasic Stock	pH
57 ml	243 ml	7.4

5. Gelatine to coat glass slides
 Gelatine 1.5 g
 Chromium (III) potassium sulfate dodecahydrate 0.25 g
 Add a few crystals of bromthymol blue as a preservative
 Fill up to 500 ml with H₂O and heat up to 60 °C to dissolve gelatin.
6. Mowiol
 1x PBS 40 ml
 Mowiol 10 g → stir for 24 h
 Add Glycerol 20 ml → stir for 24 h
 Centrifuge 15 min at 5,000 rpm, RT
 Aliquot and store at -20 °C

Acknowledgments

This protocol is adapted from Seib *et al.* (2012).

References

- Seib, D. R., Corsini, N. S., Ellwanger, K., Plaas, C., Mateos, A., Pitzer, C., Niehrs, C., Celikel, T. and Martin-Villalba, A. (2013). [Loss of Dickkopf-1 restores neurogenesis in old age and counteracts cognitive decline](#). *Cell Stem Cell* 12(2): 204-214.