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Whole-brain Staining and Tissue Clearing Protocol of LINCS

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We use this protocol and it's working

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Abstract

Here we present LINCS (*L*abeling *I*ndividual *N*eurons with *C*hemical dyes and controllable *S*parseness), an ultrabright neuronal labeling system that achieves cell-type specific labeling with chemical dyes. We established and optimzed an efficient pipeline for rapid whole-mount staining procedure, integrating LINCS with tissue clearing and commercially available light-sheet microscopy.

Image Attribution

The protocol image was created using **BioRender** (**https://biorender.com/**).
Created in BioRender. LIN, R. (2025) https://BioRender.com/giul03e

Materials

Buffer

PTx.2

0.2% TritonX-100 in PBS

PTx.5

0.5% TritonX-100 in PBS

Reagent

Biotin (300 mM stock in DMSO stored at -20℃; completely dissolved before diluted in water; Sigma-Aldrich, V900418)

Heparin sodium (10 mg/mL stock)

NaN_3 (10% solution)(can be substituted with ProClin)

20%, 40%, 60%, 80%, 100% Methanol (v/v in ddH_2O)

Dichloromethane (DCM, J&K, 908525)

H_2O_2 (30% solution)

Alexa Fluor 647-conjugated monovalent Streptavidin (monovalent SA-AF647, Abcam, ab272190)

Dibenzyl ether (DBE, Sigma, 108014)

Troubleshooting

Safety warnings

Refer to the material safety data sheets (MSDS) for proper handling and usage guidelines for NaN_3, DCM and DBE.

Ethics statement

Animal care and use followed the approval of the Animal Care and Use Committee of the National Institute of Biological Sciences, Beijing (Approval ID: NIBS2023M0013), in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of China.

This protocol needs prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

Before start

- Tissue clearing is processed based on a modified iDISCO+ protocol.
- Steps 5-9,11,14-16 are done with gentle shaking on a rocker (~5 rpm).
- Steps 12-13 are done with gentle shaking on a shaker (~120 rpm).
- Perform all steps in closed tubes (Eppendorf 5 mL tubes), ensuring they are fully filled to prevent oxidation.
- It is recommended to cover marker labels on tube caps with transparent tape to prevent dissolution by organic reagents.
- Keeping the samples in dark after step 11.
- Samples should not be exposed to water after step 16.

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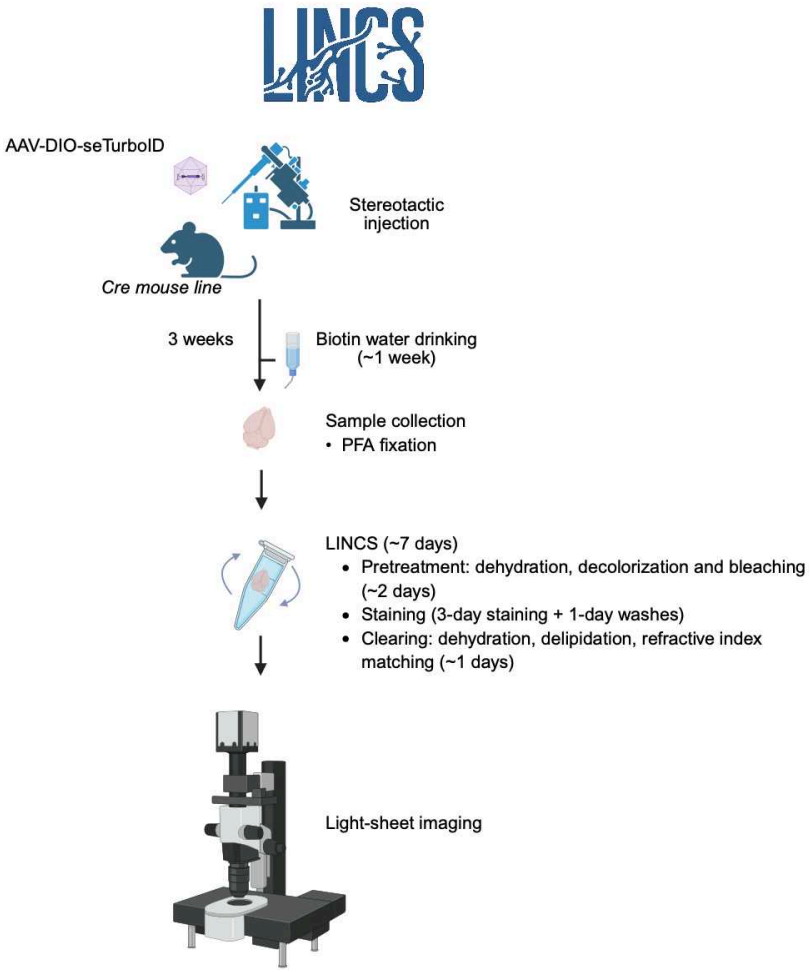
LINCS workflow summary

1



Specific neuronal populations are labeled *in vivo* using seTurboID, delivered via AAV in *Cre* transgenic mice. Biotin is administered through drinking water. Three weeks after viral injection, brains are harvested and fixed in 4% paraformaldehyde (PFA). The tissue then undergoes pretreatment, whole-mount staining, and clearing procedures before light-sheet microscopy imaging.

AAV injection and biotin administration

2 AAVs is delivered to target regions at a rate of 23 or 46 nL/min using a Nanoliter 2020 Injector pump controlled by a Micro4 controller (WPI). Tissue samples are typically harvested 3 weeks post-injection to allow for sufficient transgene expression.

3 One week prior to sacrifice and perfusion, mice are administered biotin (2 mM) in their drinking water *ad libitum*. The biotin-supplemented water should be provided in sterilized bottles and replaced weekly.

Note: Extending the administration period beyond one week does not affect the final labeling efficiency.

Sample collection and fixation

1d

4 Anesthetize the mouse and perform intracardial perfusion, first with ice-cold heparinized PBS (10 U/mL concentration), then with ice-cold 4% PFA, followed by ice-cold heparinized PBS.

5 Harvest the brains and post-fix in 4% PFA o/n at 4℃ and then for 1 h at RT.

18h

Sample Pretreatment

2d

6 Wash post-fixed samples with PBS for 3×30 min at RT.

1h 30m

7 Dehydrate samples at RT by incubating them in a graded methanol series (20%, 40%, 60%, 80%, v/v in ddH_2O, 1h for each), followed by two 1 h incubations in 100% methanol.

6h

8 Delipidate samples in 66% DCM (v/v in methanol) at RT o/n.

18h

9 Wash samples with 100% methanol for 2×2 h at RT.

4h

10 Pre-cool samples at 4℃ and then bleach samples in 5% H_2O_2 (v/v in ice-cold methanol) at 4℃ o/n.

18h

11 Rehydrate samples at RT by incubating them in a graded methanol series (80%, 60%, 40%, 20%, v/v in ddH_2O, 1h for each), followed by two 1h incubations in PTx.2.

6h

Staining

4d

12 Stain samples with monovalent SA-AF647 (1:100 diluted for bulk labeling; 1:250 diluted for sparse labeling) in PTx.5 supplemented with 0.05% NaN_3 for 3 days at 37–42℃ (37℃ is recommended for sparse labeling samples).

3d

13 Wash sampels with PTx.5 first for 2×1 h at RT, and then o/n at 25–37℃ (the lower temperature is recommended for samples with labeled axons enriched in cortical regions; Adding 0.05% NaN_3 to PTx.5 is recommended for o/n washing).

14h

14 Wash samples with PBS for 4×1 h at RT.

4h

Tissue Clearing

1d

15 Dehydrate samples at RT by incubating them in a graded methanol series (20%, 40%, 60%, 80%, v/v in ddH_2O, 1h for each), followed by two 1 h incubations in 100% methanol.

Note: Can stop optionally at this step o/n at RT.

6h

16 Delipidate samples in 66% DCM (v/v in methanol) for 3 h at RT. Wash samples for 2×15 min in 100% DCM.

3h 30m

17 Transfer samples to DBE in a new tube and store at RT until clear. The tube should be fully filled with DBE to prevent oxidation.

18 Before imaging, gently invert the tube to ensure the solution is homogeneous.

Note: It is recommended to perform imaging within one week.

Protocol references

Zhong et al., Ultrabright chemical labeling enables rapid neural connectivity profiling in large tissue samples, Neuron (2025)
https://doi.org/10.1016/j.neuron.2025.08.022

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