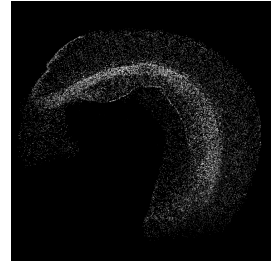


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Click histochemistry for whole-mount staining of brain structures

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We use this protocol and it's working

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Abstract

Labeling of the replicating DNA with synthetic thymidine analogs is commonly used for marking the dividing cells. However, until now this method has only been applied to histological sections. A growing number of current approaches for three-dimensional visualization of large tissue samples requires detection of dividing cells within whole organs. Here we describe a method for labeling dividing cells with 5-ethynyl-2'-deoxyuridine (EdU) and their further detection in whole brain structures (for example, hippocampus) using the Cu (I) -catalyzed [3 + 2] cycloaddition reaction (so-called click-reaction). The presented method can be used for brain neurogenesis studies as well as for whole-mount staining of any preparations in which the terminal ethynyl group has been introduced.

- New click histochemistry method based on Cu (I) -catalyzed [3 + 2] cycloaddition reaction allows whole-mount staining of brain structures and other tissues.
- Our whole-mount click histochemistry method allows to visualize dividing cells in 3D and can be used in neurogenesis studies, i.e. for birthdating dividing early progenitors and further tracking of proliferation, survival, migration, differentiation, and fate of their progeny.
- Our whole-mount click histochemistry staining demonstrates high staining specificity, high signal intensity, and low background levels in young and adult mouse brain tissue.



Attachments



2019_Click histochem...

700KB

Guidelines

Microtubes must be filled fully to prevent the contact of specimens with air and development of autofluorescence on the samples' surface.

All procedures described below must be performed with constant stirring and at  Room temperature , unless otherwise specified.



Materials

MATERIALS

- ☒ Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P6148**
- ☒ Alexa Fluor[®] 555 Azide, Triethylammonium Salt **Thermo Fisher Catalog #A20012**
- ☒ 5-ethynyl-2'-deoxyuridine **Lumiprobe Catalog #40540**
- ☒ PBS Tablets **Merck MilliporeSigma (Sigma-Aldrich) Catalog #524650**
- ☒ Chloral hydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C8383**
- ☒ DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #W387520**
- ☒ Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #34860**
- ☒ Sodium L-ascorbate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4034**
- ☒ Copper(II) sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #61230**
- ☒ Dibenzyl ether **Merck MilliporeSigma (Sigma-Aldrich) Catalog #108014**
- ☒ Hydrogen peroxide 30% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #8.22287**
- ☒ UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #15575020**
- ☒ Saponin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #84510**
- ☒ Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T8787**
- ☒ UltraPure[™] 1M Tris-HCl pH 8.0 **Thermo Fisher Scientific Catalog #15568025**

Step 1: tissue sample preparation

- 5-ethynyl-2'-deoxyuridine, 6.15 mg/ml
- 0.1 M phosphate buffer (PBS) pH 7.4
- 4% paraformaldehyde (PFA) in PBS, pH 7.4 at  4 °C
- 15% chloral hydrate
- 2 ml microtubes
- 1 ml syringes

Step 2: pretreatment and storage

- Eppendorf ThermoMixer Temperature Control Device (Eppendorf[™] 5382000023)
- DMSO
- 100% methanol
- 50% methanol
- 25% methanol
- 12.5% methanol
- 30% H₂O₂ stock solution
- 10% Triton X-100 stock solution in PBS

- 10% saponin stock solution in PBS
- 0.1 M Tris-HCl buffer (pH 8.0)

Step 3: click reaction

- DMSO
- 10% Triton X-100 stock solution in PBS
- 10% saponin stock solution in PBS
- 0.1 M Tris-HCl buffer (pH 8.0)
- 1 M sodium ascorbate stock solution (freshly prepared)
- 100 mM CuSO₄ stock solution
- 1 mM fluorescent azide (e.g. Alexa Fluor 555 Azide, Triethylammonium Salt, A20012, Invitrogen) stock solution in DMSO
- 0.5 M EDTA (pH 8.0) stock solution

Step 4: clearing

- 25% methanol
- 50% methanol
- 75% methanol
- 100% methanol
- Dibenzyl ether

Safety warnings

- ❗ Paraformaldehyde requires careful handling since it is highly toxic and potentially carcinogenic. Work only in a fume hood.

Methanol requires careful handling since it is highly toxic and highly inflammable liquid.

The methanol/DMSO solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

Dibenzyl ether requires careful handling since it is strong non-polar solvent. Use only polyethylene and glass labware.



Before start

A method described here allows staining whole brain structures using the Cu (I) -catalyzed [3 + 2] cycloaddition reaction (so-called click-reaction) between the terminal ethynyl group incorporated into DNA upon 5-ethynyl-2'-deoxyuridine (EdU) injection and fluorescently labeled azide. This method can be used in neurogenesis studies, i.e. for birthdating of dividing early progenitors and further tracking of proliferation, survival, migration, differentiation, and fate of their progeny. This whole-mount click method is based on the original protocol for staining tissue sections developed by Salic and Mitchison (2008). The following procedures were developed and optimized for whole hippocampus preparation staining and imaging.

Citation

Salic A, Mitchison TJ (2008). A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proceedings of the National Academy of Sciences of the United States of America.

<https://doi.org/10.1073/pnas.0712168105>

LINK

All procedures described below must be performed with constant stirring and at  Room temperature , unless otherwise specified.

Tissue sample preparation

1 Inject animal with a synthetic thymidine analogue 5-ethynyl-2'-deoxyuridine at a dose of 40-123 mg/kg. The time to euthanasia may vary depending on the specific research question.

2 Anesthetize animal with chloral hydrate (10 mg/kg) immediately before euthanasia.

 00:10:00

3 Perfuse animal intracardially with 30 ml of PBS and 30 ml of cold 4% paraformaldehyde, pH 7.4.

 01:15:00

Safety information

Paraformaldehyde requires careful handling since it is highly toxic and potentially carcinogenic. Work only in a fume hood.

4 Dissect the brain in 1 h after perfusion.

 00:05:00

5 Postfix the brain samples by overnight (ON) immersion in 4% PFA at  4 °C .

 14:00:00

6 Rinse the brain twice with PBS for 2 h each time.

 04:00:00



7 Remove the cerebral cortex from the fixed hemispheres using a microsurgical spatula, then isolate the hippocampi and place it in 2 ml microtube with cold PBS.

 00:10:00



Pretreatment and storage

8 Incubate the specimen in Dent solution (100% methanol/DMSO, in the ratio of 4:1) for 2 h at  4 °C .

 02:00:00



Safety information

Methanol requires careful handling since it is highly toxic and highly inflammable liquid.

The methanol/DMSO solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

- 9 To destroy endogenous pigments, bleach the specimen in Dent bleach solution (100% methanol/DMSO/30% H₂O₂, in the ratio of 4:1:1) in bright light for 2 h until the samples are completely white.
 02:00:00
- 10 After bleaching, rinse the specimen three times in 100% methanol for 1 h each time.
 03:00:00
- 11 At this stage, specimens can be stored in 100% methanol at  -70 °C . It is important to warm up the preparations gradually after storage to preserve the morphology. For this, transfer the sample first to  -20 °C , then to  4 °C , and only after that brought it to  Room temperature . 
- 12 Rehydrate samples stepwise in 75%, 50%, 25% and PBS for 1 h in each solution.
 04:00:00
- 13 Wash rehydrated sample twice in PBS for 1 h each time. 
 02:00:00
- 14 Permeabilize the preparation in 2% saponin and 5% DMSO solution in PBS for 1 h at  37 °C .
 01:00:00
- 15 After permeabilization, wash the preparation in Tris-HCl buffer (pH 8.0) with 0.2% Triton X-100 for 1 h.
 01:00:00

Click reaction

- 16 Incubate the specimen in click reaction solution (Tris-HCl buffer containing 5% DMSO, 0.2% Triton X-100, 0.2% saponin, 100 mM sodium ascorbate, 1 mM CuSO₄ and 10 mM fluorescent azide) for 2 h in the dark. 
 02:00:00



Note

Click reaction solution must be prepared immediately before staining. 1 M sodium ascorbate stock solution must be prepared from the powder immediately before the click reaction.

- 17 Stop the click reaction by three 1 h incubations in Tris-HCl buffer with 5% DMSO, 0.2% Triton X-100 and 0.1 M EDTA.

03:00:00

- 18 Wash stained preparation twice for 1 h with PBS containing 5% DMSO and 0.2% Triton X-100 and twice with PBS for 1 h each time. Final rinsing with PBS was done overnight at

4 °C .

18:00:00



Clearing

- 19 Dehydrate the preparation in 25%, 50%, and 75% methanol and three times in 100% methanol for 1 h for each incubation.

06:00:00



- 20 Clear the preparation for ON in dibenzyl ether (DBE) in the dark at 4 °C . Samples can be stored in clearing solution.

14:00:00



Safety information

Dibenzyl ether requires careful handling since it is strong non-polar solvent. Use only compatible plastic (polyethylene etc.) and glass labware.

Citations

Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. <https://doi.org/10.1073/pnas.0712168105>