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The Detection of 5-hydroxymethylcytosine in Neural Stem Cells and Brain of Mouse

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TITLE:

The Detection of 5-Hydroxymethylcytosine in Neural Stem Cells and Brains of Mice

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KEYWORDS:

DNA demethylation, 5-hydroxymethylcytosine, immunofluorescence staining, dot-blot, mouse, brain, neural stem cells, neuron

SHORT ABSTRACT:

Here, we present a protocol to detect 5-hydroxymethylcytosine in cells and brain tissues, utilizing immunofluorescence staining and DNA dot-blot methods.

LONG ABSTRACT:

More than 20 DNA modifications have been identified in the mammalian genome. Of that, 5-methylcytosine and 5-hydroxymethylcytosine-mediated epigenetic mechanisms have been intensively studied. 5-hydroxymethylcytosine displays dynamic features during embryonic and postnatal development of the brain, plays a regulatory function in gene expression, and is involved in multiple neurological disorders. Here, we describe the detailed methods including immunofluorescence staining and a DNA dot-blot to detect 5-hydroxymethylcytosine in cultured cells and brain tissues of mouse.

INTRODUCTION:

Epigenetic modifications, including DNA modification, histone modification and RNA modification, have been shown to play essential functions in diverse biological processes and diseases[1-7]. For a long time, DNA methylation (i.e., 5-methylcytosine (5-mC)) was viewed as a highly stable epigenetic marker and could not be further modified in the genome. Recently, it has been found that 5-mC could be oxidized to 5-hydroxymethylcytosine(5-hmC) by TET (Ten-eleven translocations) family proteins including TET1, TET2, and TET3[8,9]. Further studies show that 5-hmC could serve as a stable marker and play biological roles by regulating gene expression[4,10-12].

The present evidence indicates that 5-hmC is highly enriched in neuronal tissues/cells relative to other types of tissues in mammals, and exhibits dynamic features during neuronal development[13,14]. In a neuronal system, 5-hmC mediated epigenetic modifications play an important role in regulating neural stem cells, neuronal activity, learning and memory, and is involved in multiple neurological disorders including Rett syndrome, autism, Alzheimer's disease, Huntington's disease, etc.[2,13,15-20].

There are several approaches for detecting 5-hmC in cells and tissues[14,21-24]. Here, we describe two methods to detect the existence of 5-hmC and quantify the global level of 5-hmC: immunofluorescence staining and DNA dot-blot. These two methods are convenient and sensitive, and have been successfully used in previous studies[25-31]. The key steps of these two methods are DNA denaturation. For immunofluorescence staining of 5-hmC, pre-treatment of samples with 1 M HCl is required. For a 5-hmC dot-blot, a NaOH solution is used to denature DNA. These two methods together with next-generation sequencing are very useful tools for investigating the function of 5-hmC.

PROTOCOL:

All the animal procedures have been approved by the Animal Ethics Committee of Zhejiang University.

1. The culture of adult neural stem cells and neurons

1.1. Isolate adult neural stem cells from the forebrain of an adult (8-10 week old) C57/BL6 male mouse as described previously[32,33].

1.2. Culture adult neural stem cells in DMEM/F-12 medium containing 20 ng/mL FGF-2, 20 ng/mL EGF, 2% B27 supplement, 1% antibiotic-antimycotic, and 2 mM L-Glutamine in a 5% CO_2 incubator at 37 °C. Induce the differentiation of adult neural stem cells with 1 µM retinoic acid and 5 µM forskolin for 48 h as described previously[32,33].

1.3. Isolate neurons from the embryonic day 17 (E17) hippocampi of the mouse and culture with neurobasal medium containing of 0.25% L-Glutamine, 0.125% GlutaMax and 2% B27 supplement in a 5% CO_2 incubator at 37 °C as previously described[34].

2. Transcardial perfusion of the mouse

2.1. Prepare 10% chloral hydrate, 4% paraformaldehyde (PFA) and phosphate buffered saline (PBS) one day before the experiment, and store at 4 °C.

2.2. Anesthetize an adult male or female C57 BL/6J mouse with 10% chloral hydrate (50 mg/kg, i.p.), and ensure that the animal is deeply anesthetized by checking the body reaction. Fix each limb with sticky tape on a plastic board (in a face-up position).

2.3. Cut the skin and then the muscle with surgery scissors. Open the thoracic cavity with a surgical scissor. Expose the heart and cut off a little part of the right atrium with a fine surgical scissor.

2.4. Perfuse the mouse with a 10 mL disposable sterilized syringe from the left ventricle with cold PBS (around 30 mL per adult mouse).

2.5. Perfuse the mouse with 4% PFA (around 30 mL in 10 min per adult mice) until it is stiff.

2.6. Open the skull of the mouse with bone forceps. Remove the brain and put into 5 mL of 4% PFA in a 15 mL centrifuge tube for post-fixation at 4 °C.

NOTE: Clean the surgical area after surgery has finished.

2.7. At least 24 h later, transfer the brain samples into a 30% sucrose solution for complete dehydration at 4 °C.

3. Brain sectioning

3.1. Embed the brain samples in optimal cutting temperature compound (OCT) in a small container, and cool down at -20 °C for at least 1 h.

3.2. Section brain samples at a thickness of 20-40 µm with a cryostat microtome.

3.3. Collect sections into PBS and store at 4 °C.

4. Immunofluorescence staining

4.1. Pick up the sections with the targeted brain regions and put them into a 24-well plate with PBS. For cultured cells on a coverslip, go directly to step 4.2.

4.2. Wash with PBS on the shaker at room temperature for 10 min. Repeat this twice more.

4.3. Remove PBS, and treat with preheated 1 M HCl for 30 min at 37 °C.

NOTE: Prepare 1 M HCl by adding 1 mL of hydrochloric acid (36-38%) into 10 mL of water in a chemical hood. Preheat 1 M HCl in the incubator at 37 °C.

4.4. Wash the samples with PBS for 5 min. Repeat this twice more.

4.5. Block samples with PBS containing 3% normal goat serum and 0.1% Triton X-100 for 1 h on the shaker at room temperature.

4.6. Incubate sections with specific primary antibodies overnight at 4 °C on the shaker.

NOTE: Use the following primary antibodies: polyclonal rabbit antibodies anti-5-hydroxymethylcytosine (1:5,000), mouse monoclonal antibody anti-NeuN (1:500).

4.7. Take out the samples, and further incubate at room temperature for 1 h.

4.8. Wash the samples with PBS for 10 min. Repeat this twice more.

4.9. Incubate with secondary antibodies corresponding to the primary antibodies at room temperature for 1 h on the shaker. Cover the plate with aluminum.

NOTE: Use the following secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG (1:500), Alexa Fluor 568 goat anti-mouse IgG (1:500). Counterstain nuclei with 4'-6-diamidino-2-phenylindole (DAPI).

4.10. Wash the samples with PBS for 10 min on the shaker. Repeat this twice more.

4.11. Mount brain sections onto the slides, add proper amount of antifade mounting medium (around 100-150 µL), and cover with premium cover glass. Seal with nail polish.

4.12. Take images with a regular or confocal fluorescence microscope.

5. Genomic DNA isolation

5.1. Euthanize an adult C57 BL/6J mouse (male or female) by cervical dislocation and remove the brain.

5.2. Dissect the hippocampus, cortex and cerebellum tissues on an ice-cooled dish. Grind tissues with a tissue grinder in 1 mL of DNA lysis buffer and transfer into clean microcentrifuge tube. Add 250 µg of proteinase K per 600 µL of lysis buffer. For cell pellets, directly add lysis buffer, proteinase K, and mix thoroughly.

NOTE: Prepare DNA lysis buffer: 5 mM EDTA, 0.2% SDS, 200 mM NaCl in 100 mM Tris-HCl, pH 8.5. Wash and autoclave the tissue grinder before the experiment.

5.3. The second day, add about 50 µg of RNase A per sample for at least 12 h at 37 °C.

5.4. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and mix completely.

5.5. Centrifuge at 20,817 x *g* for 15 min, and remove the supernatant into a new microcentrifuge tube.

5.6. Add 600 µL of chloroform to the supernatant to precipitate DNA, and mix thoroughly.

5.7. Centrifuge at 20,817 x *g* for 15 min, and remove the supernatant into another new tube.

5.8. Add 500 µL of isopropanol to the supernatant, and mix thoroughly.

5.9. Centrifuge at 20,817 x *g* for 15 min, and remove the supernatant completely.

5.10. Wash the precipitation with 1 mL of 70% ethanol, centrifuge at 20,817 x *g* for 1 min, and remove the supernatant completely. Repeat once.

5.11. Dry the DNA pellet completely.

5.12. Dissolve DNA pellet with Tris-HCl buffer (pH 8.5) to the proper concentration.

6. DNA dot blot

6.1. Prepare the solutions: 2 M NaOH, Tris-HCl buffer (pH 8.5), 6x saline sodium citrate (SSC).

6.2. Make the sample mixture as **Table 1**.

6.3. Denature DNA samples at 100 °C for 10 min, and cool down on ice.

6.4. Cut the proper size of nylon membrane (e.g., Hybond-N+) and rinse with 6x SSC.

6.5. Put the membrane on dot-blot apparatus and connect to the vacuum pump. Spot 6 µL of mixture per dot onto the membrane.

6.6. Hybridize for 30 min at 80 °C, and block the sample membrane with fat-free milk in Tris-buffered saline (TBS) for 1 h.

6.7. Incubate with primary antibody at 4 °C overnight.

NOTE: Use the following primary antibody: polyclonal rabbit anti-5-hydroxymethylcytosine (1:5000).

6.8. On the second day, incubate the sample membranes at room temperature for 1 h. Wash with TBS for 10 min. Repeat this wash twice more.

6.9. Incubate the membrane with anti-rabbit secondary antibody (1:5,000) for 30 min at room temperature.

6.10. Wash with TBS for 10 min. Repeat this wash twice more.

6.11. Visualize the chemiluminescence signals, and quantify signal intensities.

REPRESENTATIVE RESULTS:

To reveal the distribution of 5-hmC in the hippocampus of adult mice, we performed immunofluorescence with antibodies against neuronal cells (NeuN) and 5-hmC. In the hippocampus, 5-hmC co-localized well with neuronal cell marker NeuN (**Figure 1A-H**), suggesting an enrichment of 5-hmC in neurons.

To determine the dynamics of 5-hmC during neuronal development, a dot-blot was first performed with DNA samples isolated from proliferating and differentiated adult neural stem cells (NSCs). Dot-blot results showed that the global level of 5-hmC significantly increased during the differentiation of NSC (**Figure 2A-B**). Further, dot-blot results showed that the level of 5-hmC in neurons was significantly higher than that of NSCs (**Figure 2C-D**), suggesting a dynamic 5-hmC modification during neuronal development.

FIGURE AND TABLE LEGENDS:

Figure 1. Immunofluorescence staining of 5-hmC in hippocampus of adult mice. 5-hmC co-localized well with neuronal cell marker NeuN. Scale bar in **A-D**: 100 μm; Scale bar in **E-F**: 50 μm.

Figure 2. DNA dot-blot detection of 5-hmC in adult neural stem cells and neurons. (**A**) 5-hmC dot-blot of NSCs under proliferation (Proli) and differentiation (Diffe) conditions. (**C**) 5-hmC dot-blot of NSCs and primary neurons. Methylene blue staining (**B, D**) indicating an equal loading of genomic DNA at each concentration in (**A**) and (**C**), respectively.

Table 1. The preparation of samples for dot-blot.

DISCUSSION:

Epigenetic modifications play essential roles during brain development, maturation, and function. As a stable marker for DNA modification, dynamic 5-hmC responds to behavioral adaptation, neuronal activity, and is positively correlated with gene expression; thus, it is involved in the normal function of the brain and neurological disorder[4]. To explore its function in cells and tissues, it is necessary to detect the existence of 5-hmC and compare the level before and after treatment. Here, we demonstrated two convenient methods to detect 5-hmC in cells and tissues, which could be performed with common equipment in the lab.

The key reagent of detecting 5-hmC with immunofluorescence staining and DNA dot-blot is the 5-hmC antibody. The 5-hmC antibody used in the method has been proven to have high sensitivity and is very specific. For 5-hmC staining, it requires DNA denaturation with HCl. The proper treatment of tissues and cells with HCl is critical for complete DNA denaturation and affects the results. The DNA dot-blot is a sensitive method to quantify the amount of 5-hmC, and is much more convenient than mass spectroscopy. For a successful dot-blot, precise spreading of DNA samples onto the membrane is required. Further, methylene blue staining helps determine whether DNA samples were equally loaded. Of note, the methods described here detect the global level of 5-hmC in multiple types of cells and tissues. To measure the amount of 5-hmC relative to other bases and distinguish its distribution feature in genome, it requires LC-MS/MS and next-generation sequencing.

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DISCLOSURES:

No competing financial interests exist.

REFERENCES:

1 Tan, L., Shi, Y. G. Tet family proteins and 5-hydroxymethylcytosine in development and disease. *Development.* **139** (11), 1895-1902 (2012).

2 Yao, B. et al. Epigenetic mechanisms in neurogenesis. *Nature Reviews Neuroscience.* **17** (9), 537-549 (2016).

3 Day, J. J., Sweatt, J. D. DNA methylation and memory formation. *Nature Neuroscience.* **13** (11), 1319-1323 (2010).

4 Wu, X. J., Zhang, Y. TET-mediated active DNA demethylation: mechanism, function and beyond. *Nature Reviews Genetics.* **18** (9), 517-534 (2017).

5 Sun, W. J., Guan, M. X., Li, X. K. 5-Hydroxymethylcytosine-Mediated DNA Demethylation in Stem Cells and Development. *Stem Cells and Development.* **23** (9), 923-930 (2014).

6 Li, S., Mason, C. E. The pivotal regulatory landscape of RNA modifications. *Annual Review of Genomics and Human Genetics.* **15** 127-150 (2014).

7 Hwang, J. Y., Aromolaran, K. A., Zukin, R. S. The emerging field of epigenetics in neurodegeneration and neuroprotection. *Nature Reviews Neuroscience.* **18** (6), 347-361 (2017).

8 Kriaucionis, S., Heintz, N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science.* **324** (5929), 929-930 (2009).

9 Tahiliani, M. et al. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science.* **324** (5929), 930-935 (2009).

10 Guo, J. U. et al. Neuronal activity modifies the DNA methylation landscape in the adult brain. *Nature Neuroscience.* **14** (10), 1345-1351 (2011).

11 Feng, J. et al. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nature Neuroscience.* **13** (4), 423-430 (2010).

12 Jaenisch, R., Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Geneticset.* **33 Suppl** 245-254 (2003).

13 Szulwach, K. E. et al. 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. *Nature Neuroscience.* **14** (12), 1607-U1150 (2011).

14 Song, C. X. et al. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nature Biotechnology.* **29** (1), 68-72 (2011).

15 Shu, L. Q. et al. Genome-wide alteration of 5-hydroxymenthylcytosine in a mouse model of Alzheimer's disease. *BMC Genomics.* **17** (2016).

16 Cruvinel, E. et al. Reactivation of maternal SNORD116 cluster via SETDB1 knockdown in Prader-Willi syndrome iPSCs. *Human molecular genetics.* **23** (17), 4674-4685 (2014).
17 Bernstein, A. I. et al. 5-Hydroxymethylation-associated epigenetic modifiers of Alzheimer's disease modulate Tau-induced neurotoxicity. *Human Molecular Genetics.* **25** (12), 2437-2450 (2016).
18 Wang, F. L. et al. Genome-wide loss of 5-hmC is a novel epigenetic feature of Huntingtons disease. *Human Molecular Genetics.* **22** (18), 3641-3653 (2013).
19 Yu, H. et al. Tet3 regulates synaptic transmission and homeostatic plasticity via DNA oxidation and repair. *Nature Neuroscience.* **18** (6), 836-843 (2015).
20 Wu, H., Zhang, Y. Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell.* **156** (1-2), 45-68 (2014).
21 Lister, R. et al. Global epigenomic reconfiguration during mammalian brain development. *Science.* **341** (6146), 1237905 (2013).
22 Inoue, A., Zhang, Y. Replication-Dependent Loss of 5-Hydroxymethylcytosine in Mouse Preimplantation Embryos. *Science.* **334** (6053), 194-194 (2011).
23 Pastor, W. A. et al. Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. *Nature.* **473** (7347), 394-397 (2011).
24 Ito, S. et al. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature.* **466** (7310), 1129-U1151 (2010).
25 Shu, L. et al. Genome-wide alteration of 5-hydroxymenthylcytosine in a mouse model of Alzheimer's disease. *BMC Genomics.* **17** 381 (2016).
26 Wang, T. et al. Genome-wide DNA hydroxymethylation changes are associated with neurodevelopmental genes in the developing human cerebellum. *Human Molecular Genetics.* **21** (26), 5500-5510 (2012).
27 Song, C. X. et al. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nature Biotechnology.* **29** (1), 68-72 (2011).
28 Wang, T. et al. Subtelomeric hotspots of aberrant 5-hydroxymethylcytosine-mediated epigenetic modifications during reprogramming to pluripotency. *Nature Cell Biology.* **15** (6), 700-711 (2013).
29 Li, X. et al. Ten-eleven translocation 2 interacts with forkhead box O3 and regulates adult neurogenesis. *Nature Communications.* **8**, 15903 (2017).
30 Szulwach, K. E. et al. 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. *Nature Neuroscience.* **14** (12), 1607-1616 (2011).
31 Tao, H. et al. The Dynamic DNA Demethylation during Postnatal Neuronal Development and Neural Stem Cell Differentiation. *Stem Cells International.* **2018**, 2186301 (2018).
32 Li, X. K. et al. Ten-eleven translocation 2 interacts with forkhead box O3 and regulates adult neurogenesis. *Nature Communications.* **8** (2017).
33 Li, X. et al. Epigenetic regulation of the stem cell mitogen Fgf-2 by Mbd1 in adult neural stem/progenitor cells. *Journal of Biological Chemistry.* **283** (41), 27644-27652 (2008).
34 Kaech, S., Banker, G. Culturing hippocampal neurons. *Nature Protocols.* **1** (5), 2406-2415 (2006).

Figure

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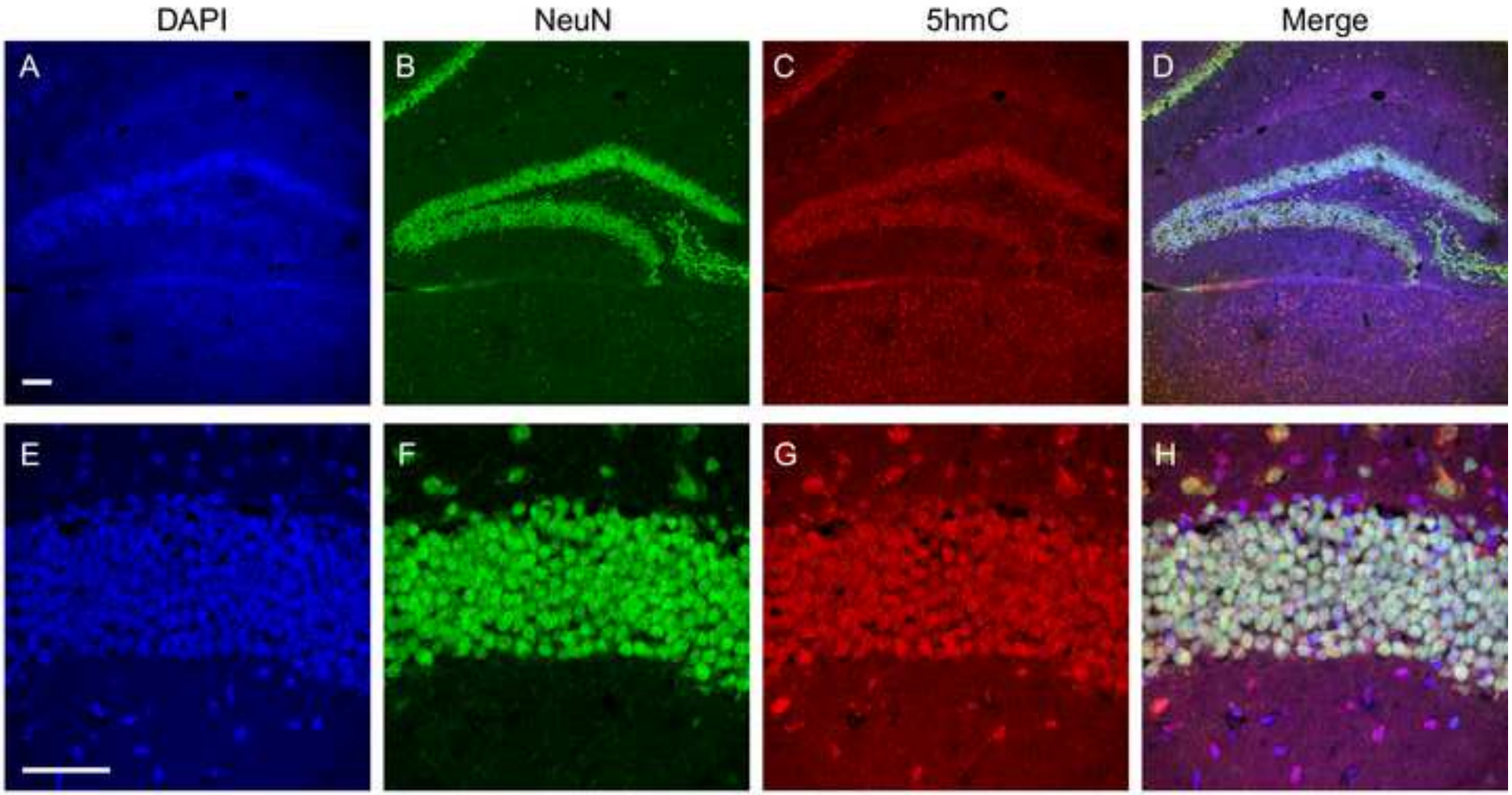

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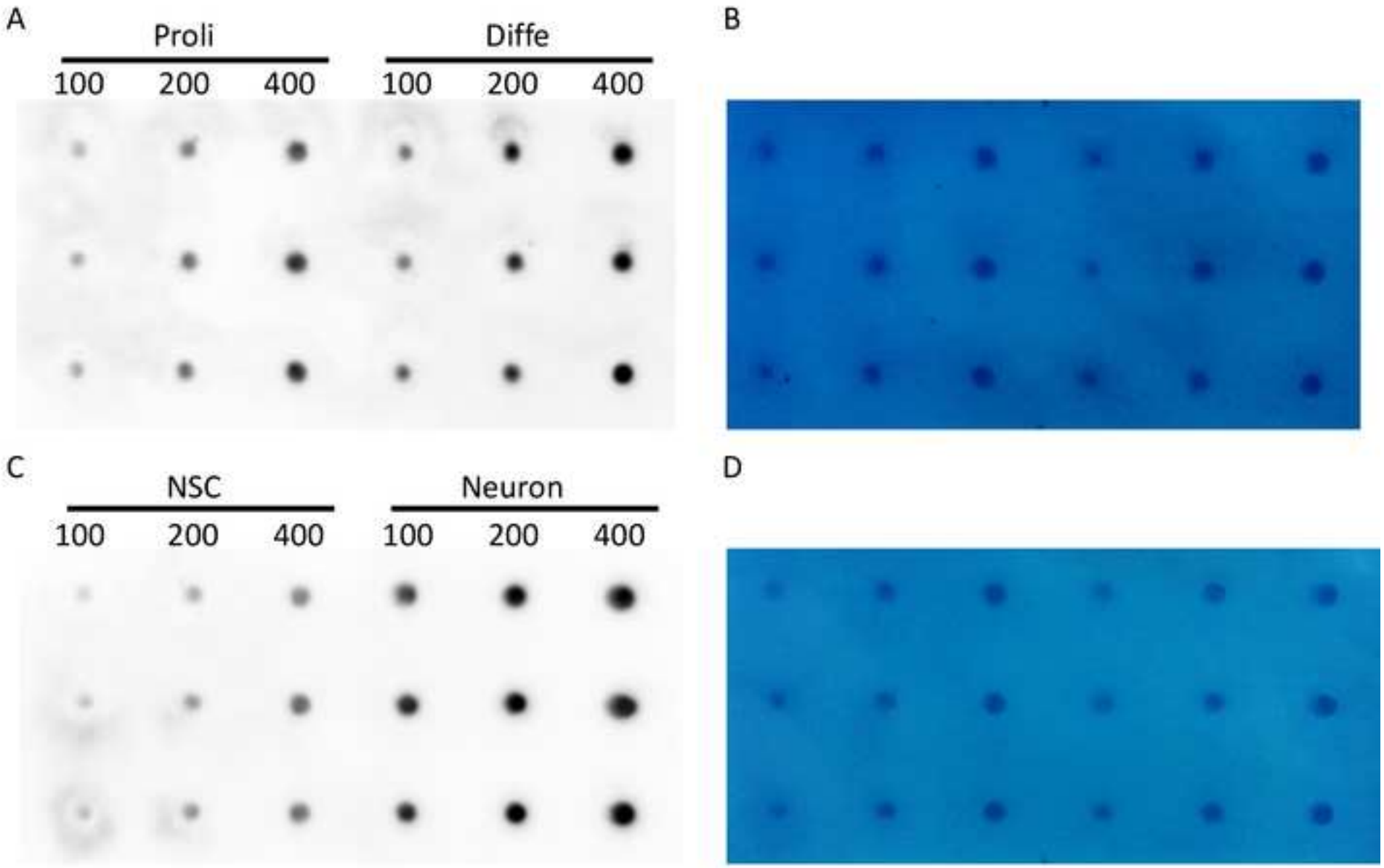

Table

	200 ng/dot	400 ng/dot	1000 ng/dot
DNA	470 ng	940 ng	2350 ng
2 M NaOH	2.81 µL	2.81 µL	2.81 µL
Tris-HCl buffer, pH 7.5	Make the volume up to 14.06 µL		

Name of Material/Equipment	Company
4'-6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich
Adobe Photoshop software	Adobe Inc.
Alexa Fluor 488 goat anti-rabbit IgG	Thermo Fisher
Alexa Fluor 568 goat anti-mouse IgG	Thermo Fisher
anti-5-hydroxymethylcytosine	Active Motif
anti-NeuN	Millipore
B27 supplement	Gibco
B27 supplement	Gibco
B27 supplement	Gibco
Cryostat microtome	Leica
DMEM/F-12 medium	OmegaScientific
epidermal growth factor	PeproTech
Fibroblast growth factor-basic	PeproTech
forskolin	Sigma-Aldrich
GlutaMax	Thermo
L-Glutamine	Gibco
neurobasal medium	Gibco
normal goat serum	Vector Laboratories
nylon membrane (Hybond™-N+)	Amersham Biosciences
OCT	Leica
Pen Strep	Gibco
phenol: chloroform: isoamyl alcohol (25: 24:1)	Sigma-Aldrich
Poly-D-Lysine	Sigma
proteinase K	VVR
retinoic acid	Sigma-Aldrich
Triton X-100	Solarbio

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A11001
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DM25
100-15
100-18B
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Manuscript Summary:
The manuscript describes two antibody-based techniques (immunostaining and dot blot) used for detection of 5-hydroxymethylcytosine in the mouse brain. The protocols and their visualisation should be of interest for researchers working in the fields of epigenetics and neuroscience.
Major Concerns:
Text of the manuscript requires heavy editing. There are numerous issues with grammar and synthaxis that make some parts of the paper difficult to understand. Both Introduction and Discussion are very vague, superficial and sometimes factually questionable. Large part of Discussion reiterates the Introduction without bringing any new information. No information on suitable experimental controls is given. In fact, the text of the manuscript needs to be completely rewritten and edited by a (preferably) native English speaker.
** we have thoroughly edited the manuscript.

Reviewer #2:
Manuscript Summary:
This is a clear protocol describing two different ways to monitor 5-hydroxymethylcytosine in neuronal stem cells and sections from mouse brains. The first involves immunostaining and the second is a dot-blot.
Overall, the details of the protocols are sufficient and clear.
Major Concerns:
The manuscript requires extensive English editing.
** we appreciate the reviewer’s overall positive comments, and formatted the manuscript.

Reviewer #3:
Manuscript Summary:
In this manuscript, Zhuang et al. described a protocol for detecting the 5-

hydroxymethylcytosine in mouse neural stem cells and brain by immunofluorescence staining and DNA dot-blot methods. This protocol is detailed and promising, and I support its publication in Journal of Visualized Experiments.

Major Concerns:

n/a

Minor Concerns:

n/a

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