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Methodology article

Isolation of neuronal chromatin from brain tissue

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Abstract

Background: DNA-protein interactions in mature brain are increasingly recognized as key regulators for behavioral plasticity and neuronal dysfunction in chronic neuropsychiatric disease. However, chromatin assays typically lack single cell resolution, and therefore little is known about chromatin regulation of differentiated neuronal nuclei that reside in brain parenchyma intermingled with various types of non-neuronal cells.

Results: Here, we describe a protocol to selectively tag neuronal nuclei from adult brain – either by (anti-NeuN) immunolabeling or transgene-derived histone H2B-GFP fusion protein – for subsequent fluorescence-activated sorting and chromatin immunoprecipitation (ChIP). To illustrate an example, we compared histone H3 lysine 4 and 9 methylation marks at select gene promoters in neuronal, non-neuronal and unsorted chromatin from mouse forebrain and human cerebral cortex, and provide evidence for neuron-specific histone methylation signatures.

Conclusion: With the modifications detailed in this protocol, the method can be used to collect nuclei from specific subtypes of neurons from any brain region for subsequent ChIP with native/unfixed or crosslinked chromatin preparations. Starting with the harvest of brain tissue, ChIP-ready neuronal nuclei can be obtained within one day.

Background

An increasing number of neurodevelopmental and neuropsychiatric disorders are thought to result from defective DNA:protein interactions specifically in neurons; furthermore, sustained changes in neuronal gene expression and behavior after exposure to certain drugs or stimuli are likely to involve chromatin remodeling, including DNA methylation and histone modification changes [1-5]. However, even the most sensitive chromatin immuno precipitation assays and most other approaches used to study the regulation of DNA and histone modifications, transcription factor binding etc., lack single cell resolution and instead require the preparation of homogenates from at least 10^3 – 10^7 nuclei. Consequently, detailed chromatin analysis was until now not feasible for nuclei of terminally differentiated neurons that typically reside in brain parenchyma intermingled with various types of glia and other cells mostly in a 2:1 – 1:2 ratio, dependent on species and brain regions [6,7].
Recently, immunostaining in conjunction with fluorescence-activated cell sorting (FACS) was used successfully to selectively collect neuronal nuclei from human (postmortem) brain tissue for the purposes of retrospective birth dating [8] or assessment of age-related changes in DNA cytosine methylation [9]. However, these studies utilized the nuclear harvest for highly sensitive radiation and PCR assays, and it remained unclear whether the protocol could be modified for the purposes of chromatin immunoprecipitation and other techniques that require comparatively larger amount of input (for example, \(10^5\) \(10^7\) nuclei). We provide a detailed protocol for selective sorting of neuronal nuclei from mouse and human brain in quantities sufficient for immunoprecipitation with different chromatin preparations (enzyme-based digestion and crosslinking/sonication), followed by microarray or PCR studies. In addition, we introduce a transgenic mouse line for neuron-specific expression of GFP (enhanced green fluorescent protein)-tagged histone H2B. Evidence is presented that even under baseline conditions, promoter-bound histone methylation in neuronal samples is significantly different when compared to unsorted, or non-neuronal nuclei from the same brain region. Therefore, the methods presented here will be important for the study of molecular mechanisms governing epigenetic control of neuronal gene expression and chromatin remodeling specifically in mature brain.

**Results**

**H2B-GFP transgenic mice**

The promoter of the \(\beta\) subunit of the \(\text{Ca}^{2+}/\text{calmodulin}\) dependent protein kinase II gene (CAMKII) was used to drive H2B-EGFP expression; as expected, this transgene labeled most of the neuronal populations in the fore- and midbrain, including cortex, striatum, hippocampus, with the notable exception of the GABAergic interneurons in cerebral cortex and hippocampus (Fig. 3). In contrast, labeling in hindbrain, incl cerebellum, was less consistent (data not shown). The transgene-derived labeling of neuronal nuclei with H2B-EGFP was robust pre- and post-FACS (Fig. 2, panel a-d). To date, our oldest transgenic mice are 5 months of age and so far we did not observe any overt neurological phenotypes, even in animals expressing the fusion protein at comparatively high levels in CNS neurons. Likewise, no adverse effects were reported for transgenic mice expressing high levels of H2B-EGFP in a wide range of tissues, including brain [10].

**Loss of nuclei during the procedure**

Each of the major steps in this protocol – (1) extraction of nuclei from the tissue, (2) ultracentrifugation, (3) immunolabeling and (4) fluorescence-activated sorting – could result in loss of nuclei, together totaling perhaps up to 50% of the neuronal nuclei in the starting material. However, despite of these limitations, the alternative approach, such as nuclei harvesting from primary neuronal culture, is likely not to improve results in a higher yield and would have the additional disadvantage of any \textit{ex vivo} preparation.

**Nucleosomes are preserved in sorted nuclei**

Nucleosomes as the elementary unit of chromatin – are comprised of 146 bp of genomic DNA wrapped around core histones H2A, H2B, H3 and H4. To find out whether
nucleosomal structures remain intact in sorted NeuN+ nuclei, we compared nucleosome occupancy at promoters in forebrain from unsorted and NeuN+ nuclei, using a modification-independent anti-H3 C-terminus antibody for ChIP-DSL in conjunction with M8K promoter array (Fig 4A, B). No significant differences between chip-to-input ratios of sorted NeuN+ and unsorted nuclei were observed. Instead, H3 occupancy at specific promoter sequences showed a strong correlation between the NeuN+ and unsorted samples (Spearman rank, r = 0.651370, p < 2.2e-16) (Fig. 4B). We conclude that promoter-bound nucleosomes are intact after the sorting procedure.

Differential histone H3 methylation in neuronal nuclei
To find out whether or not neuronal chromatin, under baseline conditions, shows a histone methylation signature that is different compared to unsorted nuclei, we conducted ChIP for two histone lysine methylation marks, H3K4me2 and the "repressive" mark, H3K9me3. All studies were conducted on the forebrain of male mice 8–12 weeks of age. Histone methylation levels were at 3 out of 4 – arbitrarily chosen – gene promoters significantly different in neuronal chromatin (Fig. 4C). Furthermore, in 2 out of 4 promoters (B2m, MeCP2), changes in the "open" mark, H3K4me2 and the "repressive" mark, H3K9me3, were in the opposite direction (Fig. 4C). Notably, H3K9me3 levels at the MeCP2 promoter – i.e., the Rett syndrome gene which is robustly and ubiquitously expressed in mature CNS neurons [11-15] – were higher in neuronal vs. unsorted chromatin. This particular finding was unexpected and requires further investigations.

Next, we wanted to (i) find out whether the above findings can be extrapolated to species other than mouse and (ii) further confirm that histone lysine methylation at select gene loci is differentially regulated in neurons as compared to non-neuronal cells residing in the same tissue. To this end, we utilized postmortem tissue from the human prefrontal cortex for ChIP with H3-tri-methyllysine 4 (H3K4me3), a chromatin mark enriched at sites
of active gene expression [16,17]. For each tissue sample, NeuN immunopositive and immunonegative nuclei were sorted separately by FACS, and then processed in parallel for anti-H3K4me3 ChIP followed by qPCR for the following genes: (1) The subunit 2B of the NMDA receptor (GRIN2B) and (2) brain-derived neurotrophic factor (BDNF); both these genes are in postnatal and adult brain predominantly or exclusively expressed in neurons [18,19]. (3) ? 2 microglobulin (B2M), which in CNS is expressed in a mixed population of neuronal and non-neuronal cells and considered as a "housekeeping gene" in postmortem studies [20,21], and (4) the locus control region of the ? globin locus (HBB), which is highly regulated in erythopoetic tissues but silent and inactive in brain [22,23]. Levels of H3K4me3 at promoters of the neuron-specific genes, GRIN2B and BDNF, were consistently higher in chromatin of the NeuN+ nuclei as compared to NeuN- (Fig. 5). These changes were highly specific because H3K4me3 levels in chromatin surrounding B2M were higher in NeuN- nuclei, and furthermore, H3K4me3 at the HBB locus control region were overall very low (Fig. 5). Taken together, our findings in mouse...
forebrain and human prefrontal cortex suggest that – even at baseline – important differences exist between histone methylation signatures of neuronal and non-neuronal chromatin.

Conclusion
The protocol presented here should be particularly useful to investigators who wish to study chromatin regulation, including epigenetic mechanisms of neuronal gene expression, in the mature and the aging brain. The alternative approach, i.e. the study of primary neuronal cultures or neuron-like cell lines, certainly is suited to model developmental mechanisms and neurodevelopmental disease [24-29]. However, neurons and other CNS cells are sustainable in culture, at best, for a few weeks [30,31] and therefore are not ideal from the viewpoint of chronic neuropsychiatric diseases, which often involve a protracted course, late onset and predilection for a specific disease [24-29]. However, neurons and other CNS cells are sustainable in culture, at best, for a few weeks [30,31] and therefore are not ideal from the viewpoint of chronic neuropsychiatric diseases, which often involve a protracted course, late onset and predilection for a specific disease [24-29].

Methods
An overview of the procedure is provided in Fig. 1

Human
Procedures involving human postmortem brain were approved by the institutional review boards of the University of Massachusetts Medical School. Frozen tissues (prefrontal cortex) from 3 specimens (age range: 8–69 years, autolysis interval 5–28 hours) were obtained from the Brain and Tissue Banks for Developmental Disorders, University of Maryland (National Institute of Child Health and Human Development Contract NO1-HD-8-3284) and from a brain bank at the University of California at Davis (Center for Neuroscience, Director Dr. Edward G. Jones).

Mice
A cassette, comprised of 8 kbp of the CamIIK (? subunit) promoter and 1.4 kbp of histone H2b-eGFP cDNA (CaMK II-H2B-EGFP), was used to generate 3 lines of transgenic mice by pronuclear injection. All the experiments were performed in strict accordance with Institutional Guidelines regarding use of experimental animals.

Reagents and reagent setup
Chemicals used include the following: 1 × PBS (Cellgro, 21-040-CV), Triton X-100 (Fluka, 93426), Igepal CA-630 (NP-40) (Sigma, 1–3021), Protease inhibitor cocktail tablets (Roche, 11697498001), Phenylmethanesulfonyl Fluoride (PMSF) (Sigma, P7626), Mouse anti-NeuN (Upstate, MAB377), Goat anti-mouse IgG, Alexa Fluor 488 (Invitrogen, A11029), Bovine serum Albumin (BSA) (Sigma, B-4287), Normal goat serum (Vector, S-1000), Micrococcal nuclease (Sigma, N-3765), Protein G Agarose, (Upstate, 16–266), Rabbit Anti-H3 (Abcam, ab1791), Rabbit Anti-H3K4me2 (Upstate, 07–030), Rabbit Anti-H3K9me3 (Upstate, 07–442), Rabbit Anti-H3K4me3 (Upstate, 07–473), Normal rabbit IgG (Upstate, 120370)

Nuclei Extraction Buffer (NEB): 0.32 M Sucrose, 5 mM CaCl$_2$, 3 mM Mg(AC)$_2$, 0.1 mM EDTA, 10 mM Tris-HCl (pH8), 1 X Protease inhibitor cocktail, 0.1 mM PMSF, w/o 0.1% Triton X-100, w/o 0.1% NP-40. Sucrose Cushion: 1.8 M Sucrose, 3 mM Mg(AC)$_2$, 10 mM Tris-HCl (pH8). Blocking Solution: 0.5%. Bovine serum albumin (BSA), 10% normal goat serum, in 1 × PBS.

Nuclei extraction from brain tissue
Total nuclei were extracted via sucrose gradient ultracentrifugation. In the work presented here (mouse), each sample was derived from a single forebrain (adult males, 8–12 weeks of age); (human) 1000 mg of cerebral cortex. All the reagents used were pre-chilled and the entire procedure was performed on ice. Fresh or frozen samples were homogenized by douncing 50 times in 5 mL NEB.
with 0.1% Triton X-100, or alternatively, 0.1% NP-40. Triton X-100 was preferable if nuclei require immunotagging (with NeuN, for example), while NP-40 as a milder detergent left more nuclei intact and sufficient when working with nuclei expressing GFP. After douncing, brain homogenates were transferred into 14 mL ultracentrifuge tubes (Beckman, 14 × 95 mm, 344061), and 9 mL of Sucrose Cushion was carefully loaded directly to the bottom of the ultracentrifuge tube. Ultracentrifugation was performed at 24400 rpm for 2.5 hrs at 4°C (Beckman, L8-70M, SW28 rotor). After centrifugation, a nuclei pellet – thin and typically with a light yellow taint – was formed on the bottom of the tube. The sticky white tissue debris was restrained in the middle interface of the two sucrose layers. Supernatant, including debris, was carefully removed. 1200 µl of chilled 1 × PBS was then added into the tube and incubated without disturbing the pellet, for 20 min on ice. This incubation is recommended because the nuclei pellet is easier to dissociate and consequently, there will be less damage to the nuclei. After incubation, the nuclei were dissociated by pipetting up and down. FACS required single nuclei in solution but excess pipetting tended to break nuclei, at least in the unfixed preparations. Pipetting required optimization by checking the nuclei “quality” (intact single nuclei, non-adhering, without debris) under the light microscope. The total number of nuclei was counted by using a hemacytometer. One mouse forebrain typically yielded 20–30 × 10⁶ unfixed nuclei after ultracentrifugation.

One alternative option was to fix the nuclei by directly douncing the tissue in NEB containing 1% formalin. Typically, we fixed with formalin at room temperature for 5–10 min, and then added glycerol to a final concentration of 125 mM. The fixed nuclei were pelleted by centrifugation, re-suspended in 5 mL of NEB, and subjected to ultracentrifugation as described above. Nuclei clumping during isolation was – in our experience – usually to blame if the final yield was lower than expected. For unfixed samples, factors that appear to promote clumping are (i) contamination from brain debris, or (ii) DNA leaking from broken nuclei, which appears to be alleviated via careful handling and performing the whole procedure on ice. When a fixation step was included (see above), the only way to significantly alleviate the clumping was to remove Ca²⁺ from the NEB, albeit this increased the proportion of broken and damaged nuclei. Thus, lower post-FACS yield is to be expected when working with fixed samples.

**Immunotagging of neuronal nuclei**

Next, neuronal nuclei were tagged by immunofluorescence staining, using an anti-NeuN antibody (Fig. 2A, panels e-j). Firstly, anti-NeuN (Ms) and anti-Ms IgG (Alexa 488) antibodies were co-incubated at room temperature for 5 min (Table 1), then nuclei solution was added and the mixture incubated in darkness for at least 20 min at 4°C before FACS. The proportion of NeuN positive nuclei in the material recovered after ultracentrifugation was about 50%. The FACS background signal was minimal in these preparations (Fig. 2B, panel a).

**Neuronal nuclei isolation via FACS**

Next, the fluorescent nuclei (H2B-GFP transgene or NeuN labeled) were filtered through a 40 µm Nitex mesh to remove any remaining clumps, and then run through a FACS machine (Vantage SE/Diva, BD Biosciences) with proper gates settings based on the size and density of nuclei, to ensure that only single NeuN+ nuclei were sorted, which typically included 40% of the whole population (Fig. 2B, panel b). After FACS, the NeuN+ nuclei were reanalyzed to confirm the purity. Sample purity typically is > 95%. For selected samples, the purity of NeuN+ nuclei was checked under the microscope (Fig. 2A, panels c, d and g, h). FACS sorting should be performed on the same day as extraction and NeuN labeling of nuclei. For a subset of experiments, including those involving human postmortem brain, immunonegative (NeuN-) nuclei were collected and processed in parallel to the fraction of neuronal (NeuN+) nuclei.

**Expected yield of neuronal nuclei**

We usually obtained – from a single mouse forebrain – 2.5 × 10⁶ NeuN+ nuclei post-FACS if fixative was added to the NEB and up to 8 × 10⁶ nuclei if processed unfixed. We usually obtained from 1000 mg of human (prefrontal) cerebral cortex 5 × 10⁶ NeuN+ nuclei if processed unfixed.

**Post-FACS nuclei pelleting**

After FACS, nuclei were collected in a large volume of 1 × PBS, roughly 4 × 10⁶ nuclei in 10 mL of 1 × PBS. This was not ideal for ChIP because processing such a large volume is associated with very high expense and potential "wast-

### Table 1: Antibodies used for immunotagging of neuronal nuclei.

| Primary/secondary antibody conjugate | anti-NeuN (Ms) | anti-Ms IgG Alexa 488 | Blocking Solution | 1 × PBS | Nuclei solution |
|-------------------------------------|---------------|----------------------|-------------------|---------|----------------|
| NeuN                                | 1.2 µl        | 1 µl                 | 100 µl            | 300 µl  | 1000 µl        |
| Negative control                    | 0 µl          | 0.2 µl               | 20 µl             | 60 µl   | 200 µl         |

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ing" of antibodies and other reagents. Therefore, pelleting was important, but the challenge thereby was that post-FACS nuclei became fragile and most of them were destroyed by centrifugation in 1 × PBS. Therefore, we tested different buffers and noticed that the presence of Ca$^{2+}$ and Mg$^{2+}$ is crucial for integrity of nuclei during pelleting. Hence, the concentration of sucrose, Ca$^{2+}$, and Mg$^{2+}$ in post-FACS nuclei solution was adjusted by adding the following to each 10 mL of post-FACS nuclei: 2 mL of 1.8 M Sucrose, 50 µl of 1 M CaCl$_2$, and 30 µl of 1 M Mg(Ac)$_2$. Samples were mixed gently, incubated on ice for 15 min, and centrifuged at 3000 rpm for 15 min at 4°C.

**Chromatin immunoprecipitation**

The FACS sorted nuclei were prepared either by microccocal nuclease digestion for native chromatin immunoprecipitation (NChIP) as described [37], or in case of fixed nuclei, sheared by sonication (Branson Sonifier 250) for XChIP. (Typically, samples were sonicated at power level 6 (Branson, Danbury CT) in ice water, by applying 10 runs of 30 sec impulse with 1 min resting interval. Again, sonication conditions need to be optimized before each experiment.) In the work presented here, only NChIP was used. In brief, microccocal nuclease (MNase) digestion was performed with a working concentration of 4U/mL at 37°C for 5 min. The resulting mono-nucleosomal prepa-

**Real time PCR**

Quantification of DNA extracted from immunoprecipitates was done by real time PCR using custom-designed primers targeting the promoter regions of the following genes: (mouse) methyl CpG binding protein 2 (MeCP2), glutamate decarboxylase 1 (Gad1), β2-microglobulin (B2m), and Glial fibrillary acidic protein (GFAP); (human) glutamate receptor, ionotropic, N-methyl D-aspartate 2B (GRIN2B), brain derived neurotrophic factor (BDNF), β2-microglobulin (B2M), and the locus control region of the globin genes (HBB). MouseMeCP2 primer sequence (119 bp): forward GCCTCCTTTCTCCGCCTAAA, reverse GCCCTTGTCTTGTGAGAT; Gad1 (98 bp): forward TGTCTCACCACATACTCTAGTCTC, reverse CCCCTGTCTTGGCAGAT; B2m (112 bp): forward GGAAAGTCTGACCCCTAACC, reverse GGCGGCCTTACAGATTGT; GFAP (100 bp): forward TACCAGGAGGCTGCTCCITT, reverse AACTCTCTCTACCCCACTGA; HBB primer sequence (64): forward TCTCTTGCATTGAGTGTTGG, reverse GGCTATACCATTCTGGGACA; BDNF (102): forward AGCCCAACACATTTGGGCGG, reverse GAGACGCCTGGTTACACAGG; B2m (99): forward GGGACACCATTGCAAGTC, reverse GGCCTGTATCTCAGGACTTC; HBB (87): forward CCCAGGTATGTTCCCTTT reverse TCCAGGCTCTTGTTGTGCT. Quantification was done as described [37]. For each of the antibodies used in this study (anti-H3K4me2, -H3K9me3, and -H3K4me3), specific signal was limited to input and chip fractions, and differed from controls by at least 3–4 cycle thresholds [see Additional file 1, panel B, C].

**ChIP-on-Chip**

To study histone occupancy at gene promoters on a larger scale, a mouse promoter array, M8K (Aviva Systems Biology, San Diego, CA), containing 8000 40-mer oligonucleotide probes targeting gene promoters, was used. The DNA samples were amplified and labeled by Aviva’s ChIP-GLAS system. Briefly, both input and immunoprecipitated (ChIP) DNA were biotinylated and annealed to the M8K-Oligo-Mix. The DNA samples were then amplified by ligation mediated PCR with T3 and T7 based primers (25 cycles), labeled with Atto 550 or 647, and hybridized onto the chip. Amplification, labeling, hybridization, and washing was carried out according to the manufacturer’s instruction (Aviva, AK-0524). Slides were scanned with DNA Microarray Scanner BA (Agilent Technologies, Santa Clara, CA, USA) and intensities extracted with Feature Extraction Software Version 9.1 (Agilent Technologies, Santa Clara, CA, USA). Raw data were read into the R statistical computation environment for preprocessing and data analysis (R: Development core team (2004). R: A language end environment for statistical computing. Vienna, Austria.URL found in Availability and requirements section). Local background intensities were subtracted from raw signals and negative values were replaced with small positive ones. Signals were then normalized using a variance stabilization method described in [38,39] and implemented in the vsn Bioconductor package (Gentleman, RC. et. al., Bioconductor: open software development for computational biology and bioinformatics. Genome Biology 5, R80 (2004)).

**Other amplification procedures**

We used the Genomeplex whole genomic amplification kit (WGA2, Sigma) to amplify DNA immunoprecipitates, which resulted in 2 to 5 ug of (amplified) DNA from starting material (10 ng of input DNA; ChIP DNA from 0.5 × 10$^6$ nuclei).

**Availability and requirements**

RA language end environment for statistical computing: http://www.r-project.org
Authors' contributions
YJ, H-SH and SA contributed to conception and study design, YJ, H-SH and AM conducted experiments and data analyses; JS performed microarray data analyses; YJ and SA wrote the paper.

Additional material

Additional file 1
(A) Images from ethidium bromide-stained 1.3% agarose gels showing chromatin DNA from mouse forebrain before (MNase-) and after (MNase+) micrococcal nuclease (MNase) digestion. All samples were treated with RNase A. The DNA ladder is shown on the left side of gel picture. Notice approximately 146 bp DNA fragment only in MNase+ samples. (B), SYBR-green based melting curves from immunoprecipitates with anti-H3K4me2 antibody using primer pairs for mouse B2m and Gad1; notice single peak for specific product. (C) Representative amplification curves of inputs (black circles), immunoprecipitates (red circles) and IgG control (green circles), dotted line indicating cycle threshold. Data shown for Gad1 and B2m separately. Notice samples processed with non-specific IgG show much higher cycle thresholds than input and immunoprecipitates.

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