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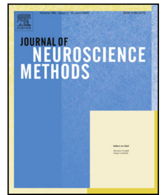

Basic neuroscience

Chromatin immunoprecipitation and gene expression analysis of neuronal subtypes after fluorescence activated cell sorting

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HIGHLIGHTS

- This paper describes a one day chromatin immunoprecipitation protocol for neuronal subtypes isolated using fluorescence activated cell sorting.
- The protocol achieves increased yield of cells per animal relative to previously reported FACS studies.
- ChIP is able to discriminate differences in histone acetylation and methylation at multiple gene promoters that correspond to gene expression.

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ABSTRACT

Background: With advances in cell capture, gene expression can now be studied in neuronal subtypes and single cells; however, studying epigenetic mechanisms that underlie these changes presents challenges. Moreover, chromatin immunoprecipitation (ChIP) protocols optimized for low cell number do not adequately address technical issues and cell loss while preparing tissue for fluorescence activated cell sorting (FACS). Developing a reliable FACS–ChIP protocol without the need for pooling tissue from multiple animals would enable study of epigenetic mechanisms in neuronal subtypes.
Methods: FACS was used to isolate dopamine 1 receptor (D1R) expressing cells from the nucleus accumbens (NAc) of a commercially available BAC transgenic mouse strain. D1R+ cells were used to study gene expression as well as histone modifications at gene promoters using a novel native ChIP protocol.
Results: Isolated cells had enrichment of the dopamine 1 receptor (D1R) mRNA and nearly undetectable levels of GFAP and D2R mRNA. ChIP analysis demonstrated the association of activating or repressive histone modifications with highly expressed or silent gene promoters, respectively.
Comparison with existing methods: The ChIP protocol developed in this paper enables characterization of histone modifications from ~30,000 FAC-sorted neurons.
Conclusions: We describe a one day FACS–ChIP protocol that can be applied to epigenetic studies of neuronal subtypes without pooling tissue.

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1. Introduction

Regulation of gene expression is critical for neuronal adaptation to the environment and is enacted by epigenetic mechanisms. These mechanisms are induced by a range of chromatin modifying enzymes and include covalent modifications to histone subunit N-terminal tails and methylation of cytosine residues on DNA (Kouzarides, 2007). Changes in histone modifications and DNA methylation across the brain have been implicated in regulation of neuropsychiatric and neurological disorders (Robison and Nestler, 2011; Sun et al., 2013; Urdinguio et al., 2009); therefore, characterizing epigenetic processes that control gene expression may reveal new targets for treatment.

While discovery of epigenetic modifications in the brain has greatly accelerated over the past two decades, technical issues remain in studying histone modifications in this complex tissue type. One issue relates to correlating changes in RNA transcript levels to changes in histone modifications at gene promoters using chromatin immunoprecipitation (ChIP). For instance, while a cell may produce hundreds of RNA molecules of the same transcript, it only contains two loci for those RNA sequences in the genome (excluding the potential contribution of copy number variation). Therefore, it may be difficult to detect changes in histone modifications at a specific gene promoter using chromatin made from

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millions of cells if only a few thousand cells are contributing to a detectable increase in RNA transcript number. This issue may be particularly relevant in the brain, which is composed of a plethora of neuronal subtypes, glia, and other cell types with distinct transcriptional profiles (Molyneaux et al., 2007). Additionally, substantial loss of chromatin occurs during formaldehyde cross-linking, multiple cell lysis steps, and sonication that are components of nearly all standard ChIP protocols, so that most protocols recommend using tens of millions of cells as input (Collas and Dahl, 2008; Dahl and Collas, 2008). Some of these limitations are overcome using cell culture systems where histone modifications within a distinct cell type can be studied; however, this lacks complexity relative to whole animal models.

Recent advances in fluorescence activated cell sorting (FACS) and laser capture microdissection using brain tissue have made it possible to isolate large numbers of neuronal subtypes. In particular, FACS allows for automated capture of a large number of cells within a matter of hours based on antibody-mediated or endogenous cell fluorescence. FACS studies also benefited by the completion of the Gene Expression Nervous System Atlas project, which developed transgenic mice with promoter-specific fluorescent proteins integrated into the genome using bacterial artificial chromosomes (BAC) (Gong et al., 2003). Studies characterizing neuronal subtypes using BAC transgenic strains indicate fluorescent reporter expression is highly cell-type specific and these animals have been used in anatomical, electrophysiological, and optogenetic studies (Gong et al., 2003; Valjent et al., 2009; Durieux et al., 2011).

Transcriptional differences between neuronal subtypes are also emerging. An early study utilized BAC transgenic mouse strains and FACS to identify transcriptional differences between dopamine D1 receptor (D1R) and dopamine D2 receptor (D2R) medium spiny neurons (MSN) in the striatum (Lobo et al., 2006). Application of this technique has been particularly important for studying drug-induced regulation of gene expression. Eric Nestler's group identified differences in cocaine-induced gene expression in D1R versus D2R MSNs (Lobo et al., 2010) and showed that epigenetic mechanisms intrinsic to these neuronal subtypes underlie the observed transcriptional changes (Maze et al., 2014). A recent study utilized BAC transgenic strains along with antibody-mediated tagging of histone modifications to examine how cocaine alters histone modifications in D1R and D2R MSNs (Jordi et al., 2013). Other groups have used antibody-mediated FACS to study gene regulation induced by cocaine (Guez-Barber et al., 2011; Ozburn et al., 2015), opioids (Fanous et al., 2013; Schwarz et al., 2013), and methamphetamine (Liu et al., 2014) in cellular subtypes. However, while antibody-based approaches allow for selection of a wider array of cell types compared to BAC transgenic strains, they also require fixing cells and have issues with off-target binding by antibodies that may reduce their specificity.

While the use of FACS to study neuronal subtype specific gene expression is well-established, interrogating neuronal subtype-specific epigenetic mechanisms would benefit by studying histone modifications at gene promoters using ChIP. Several papers have reported methods for ChIP from small numbers of cells (Acevedo et al., 2007; Gilfillan et al., 2012); however, they have not adequately addressed technical issues of handling FAC-sorted neurons. One study reported a ChIP protocol after isolating olfactory epithelial cells using FACS (Magklara et al., 2011); however, they required an average of ten animals per ChIP to achieve reliable results. Additionally, isolation of neuronal nuclei using FACS is associated with increased cell yield and can be used to study epigenetic modifications in neuronal subtypes (Mo et al., 2015; Jiang et al., 2008); however, this technique precludes simultaneous study of cytosolic RNA in these neurons.

This study utilized a commercially available BAC transgenic strain where the dopamine D1 receptor (D1R) promoter drives expression of tdTomato, a variant of the red fluorescent protein. This strain has been well-characterized with tdTomato expression restricted to D1R MSNs and no apparent behavioral differences relative to wild type mice (Ade et al., 2011). We describe a one day protocol to isolate D1R+ medium spiny neurons (MSN) from the nucleus accumbens (NAc) and complete ChIP and RNA-specific studies using FAC-sorted neurons from a single animal.

2. Materials and methods

2.1. Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Two heterozygous transgenic males containing a gene for the tdTomato fluorescent protein driven by the D1R promoter were purchased from the Jackson Laboratory (Stock #016204). These males were bred to isogenic C57BL/6J females and offspring were checked for the presence of the transgene with PCR after weaning. Male offspring that possessed the transgene were housed with their wild type littermates and used for all experiments at 8–16 weeks of age. Mice were housed under 12 h light/dark cycles and had ad libitum access to food and water. Separate animals were used for chromatin and RNA extraction described below.

2.2. PCR genotyping

Tail snips (<0.5 cm) were taken at the time of weaning and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instruction. DNA was used for a PCR assay containing GoTaq HotStart Polymerase (Promega, Madison, WI), primers for the transgene and positive control region (20 μm), dNTPs (2.5 mM), 5× reaction buffer, and $MgCl_2$ (2.5 mM). Primer sequences used were Transgene, F: 5′-CTT CTG AGG CGG AAA GAA CC-3′ and R: 5′-TTT CTG ATT GAG AGC ATT CG-3′, Positive control region, F: 5′-CTA GGC CAC AGA ATT GAA AGA TCT-3′ and 5′-GTA GGT GGA AAT TCT AGC ATC ATC C-3′. PCR conditions were 3 min at 94 °C, then 30 s at 94 °C, 1 min at 59 °C, 1 min at 72 °C repeated 35 times, then 2 min at 72 °C. PCR products were run on a 1.5% agarose gel. Transgenic animals were identified by the presence of two bands: a 750 bp band for the transgene and 324 bp band for the positive control region.

2.3. Dissociation of the nucleus accumbens into a single cell suspension

Tissue dissociation was adapted from a previous report of FACS using BAC transgenic mouse strains (Crook and Housman, 2012). The following reagents were made fresh before the start of each experiment. *HABG:* 200 μl of 50× B27 supplement (Life Technologies) and 25 μl of 100× Glutamax (Life Technologies) into 9.8 ml of Hibernate A cell culture media (Life Technologies). *Papain dissociation buffer:* 14 μl of 100× Glutamax (Life Technologies) into 5.5 ml Hibernate E cell culture media (BrainBits LLC, Springfield, IL). *Papain:* 5 ml of papain dissociation buffer into one vial containing 100 U of papain (Worthington Biochem, Lakewood, NJ). *DNase:* 500 μl of papain dissociation buffer into one vial containing 1000 U of DNase (Worthington Biochem).

Adult animals were sacrificed by cervical dislocation and the brain was extracted and placed into an ice cold adult mouse brain

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