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High content analysis of histone acetylation in human cells and tissues

Pritika J. Narayan[a,b], Michael Dragunow[a,b,*]

[a] National Research Centre for Growth and Development, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand
[b] Centre for Brain Research, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand

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ABSTRACT

There is increasing demand for automated image analysis of cell nuclei to be fast, objective and informative. Here, we have developed a high content analysis method for quantifying histone acetylation within any given population of cells. To demonstrate the utility of this method we quantified the effect of valproic acid (VPA) on histone H3 acetylation levels in SK-N-SH cells, a human neuroblastomal cell line. VPA, commonly used for treatment of bipolar disorder and epilepsy, has also been shown to act as a histone deacetylase inhibitor (HDACi), and to maintain the N-terminals of susceptible histones in an acetylated and transcriptionally active state. The Discovery-1™ (Molecular Devices) platform was used for automated image acquisition of immunolabelled cells. Multiple parameters of labelled nuclei were analysed in 1.82 s per image using the built-in count nuclei assay from MetaMorph™ (Molecular Devices) image analysis software. Data were presented in two forms: summary graphs or heterogeneity profiles using frequency distributions within GraphPad Prism (SmartDrawNet). Results showed that VPA increased histone H3 acetylation in a concentration- and time-dependent manner in SK-N-SH cells. The same analysis was shown to accurately quantify histone acetylation changes in human tissue sections also. Trichostatin A, a known HDACi was used to validate VPA action. Western blotting was used to validate the specificity of the antibodies. Overall these data demonstrate that this novel method for quantifying average treatment effects *and* the heterogeneity within any given population of cells, is fast, reproducible and can be applied to many different cellular contexts (immunocyto- and immunohisto-chemistry).

1. Introduction

With increasing evidence suggesting that epigenetic changes such as histone acetylation play a pivotal role in the biology of human disease (Narayan and Dragunow, 2010), there is increasing need to measure these phenomena with accuracy, objectivity and in a reproducible manner. In light of this we propose a method of high content analysis (aided by high-throughput image acquisition) to extract multiparametric information from immunolabelled nuclei which is fast, information-rich and relatively user friendly.

To date, image based methods used for assessing and quantifying epigenetic changes include electron microscopy (Hendzel et al., 1998; Khorasanizadeh, 2004), image correlation spectroscopy (Fejes Toth et al., 2004; Gorisch et al., 2005), confocal imaging and flow cytometry (Le Beyec et al., 2007), high resolution texture analysis of chromatin phenotype (Mohamed et al., 2007), in situ localisation methods (Tumbar et al., 1999; Verschure et al., 1999), protein chromatin interaction studies (Musri et al., 2006; Padilla-Parra et al., 2008) which in some instances include complex proteomics based analysis (Sjoholt et al., 2005) or can be coupled to nucleosomal arrays (Sous et al., 2004). In light of these methods, which each have their obvious advantages the main benefit of the method proposed in this article, is its simplicity.

We show that images acquired with either high-throughput imaging platforms such as Discovery-1™ or images acquired using basic light microscopy (Nikon E800 equipped with a digital camera) of cells, immunolabelled for histone modifications can be processed (including plating of cells, duration of drug treatments, immunolabelling, imaging and analysis) within 3–4 days (unless antibody validation and optimisation of techniques is required) and yield semi-quantitative measures that accurately represent real drug effects.

To demonstrate the utility of this method we treated SK-N-SH cells with Trichostatin A (TSA), a known histone deacetylase

Abbreviations: VPA, Valproic acid; HDACi, Histone deacetylase inhibitor; TSA, Trichostatin A; FBS, Foetal bovine serum; DMSO, Dimethyl sulfoxide; PBS-T, Phosphate buffered saline and Triton X-100; PVDF, Polyvinylidene difluoride membrane; ECL, Enhanced chemiluminescence; ATCC, American Type Culture Collection; PFA, Paraformaldehyde; DAB, 3,3′-Diaminobenzidine; SDS, Sodium dodecyl sulphate; EDTA, Ethylenediaminetetraacetic acid; CCD, Charge coupled device; dH_2O, deionised water.

* Corresponding author at: Department of Pharmacology and Clinical Pharmacology, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand. Tel.: +64 9 9236403; fax: +64 9 3737556.
E-mail addresses: p.narayan@auckland.ac.nz (P.J. Narayan), m.dragunow@auckland.ac.nz (M. Dragunow).

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inhibitor (HDACi) (Futamura et al., 1995; Hoshikawa et al., 1994; Takahashi et al., 1996; Taunton et al., 1996) and valproic acid, more commonly known for its use in the treatment of epilepsy and bipolar disorder, however more recently also shown to possess HDACi properties (Gottlicher et al., 2001; Phiel et al., 2001). We measured the effect that HDAC inhibition had on the pattern of histone H3 acetylation using image analysis of immunolabelled cells. By profiling the patterns of histone acetylation and how they are altered in different drug conditions one can gain important insights into changes in chromatin conformation and/or transcriptional regulation.

2. Materials and methods

2.1. Materials

All media components (RPMI, Penicillin-Streptomycin-Glutamine, FBS, trypsin) and all nunc plasticware used for cell culture were purchased from Invitrogen, NZ. Valproic acid sodium salt (2-propylpentanoic acid sodium, P4543) and Trichostatin A (T8522) reconstituted in DMSO (D2650) in addition to all PBS reagents and Triton X-100 were purchased from Sigma–Aldrich, NZ. Normal goat serum (16210-072) was obtained from Life Technologies and Merthiolate (Thiomersal, 304164H) from BDH. The rabbit polyclonal anti-acetyl histone H3 antibody (Upstate/Millipore 06-599) raised against acetylated residues lysine 9 and 14, which was used for immunolabelling of cells, tissue sections and Western blots, was purchased from Abacus ALS, NZ. The mouse monoclonal anti-acetyl histone H3 antibody (H0913, used for immunolabelling of cells and Western blots), the goat anti-rabbit (B7389) and goat anti-mouse (B7264) secondary antibodies and the tertiary ExtrAvidin (E2886) were all purchased from Sigma, NZ. For immunohistochemistry, the secondary sheep anti-rabbit (Chemicon AP322B) and StreptAvidin (Chemicon SA202) were purchased from Abacus ALS, NZ. The mouse monoclonal raised against beta actin (Abcam ab6276) used for Westerns was obtained from Sapphire Bioscience, NZ. All Western blot running reagents (NuPage 4–12% Bis-Tris gels, MOPS SDS Running Buffer, Antioxidant, 4× LDS Sample Buffer, 10× reducing agent and Novex® Sharp Protein Standard (LC5800)) except methanol (from Merck, NZ) were purchased from Invitrogen, NZ. Hybond polyvinylidene difluoride membrane (PVDF, Amersham Pharmica Biotech) was obtained from Global Science, NZ. All ECL reagents used for Western blots (ECL Mouse IgG, HRP-Linked Whole Ab from sheep (NA931V), ECL Rabbit IgG, HRP-Linked Whole Ab from donkey (NA934V), ECL Western Blotting Detection Reagents) and hyperfilm were purchased from GE healthcare, NZ. The Bio-Rad protein assay reagents and Dual Color precision plus protein standard (161-0374) were obtained from Bio-Rad Laboratories, Inc., Hercules, CA.

2.2. Cell culture

The SK-N-SH human neuroblastoma cell line was obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 supplemented with 10% FBS (v/v), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 37 °C in a humidified atmosphere (5% CO_2 and 95% air). For passaging, cells were trypsinised for 5 min at 37 °C with 2 mL 0.25% trypsin (v/v) in PBS, centrifuged for 5 min at 200 rcf and reseeded in fresh supplemented media. Cells were passaged every 3–4 days or when 80–90% confluent. Viable cells (which exclude Trypan blue dye) were counted on a haemocytometer. For experiments, cells were plated 24-h prior to drug addition. For concentration response experiments SK-N-SH cells were plated at 4×10^4 cells/well plating density, and treated the following day with 1 mM VPA for 6 h. Experiments were stopped by fixation with 8% PFA (w/v) on top of an equal volume of media. For timecourse experiments SK-N-SH cells were plated at 3×10^4 cells/well, and a staggered reverse timecourse was started the following day (i.e. 1 mM VPA was added to appropriate wells within one 96-well microplate in the following order 24 h, 12 h, 8 h, 6 h, 4 h, 2 h, 1 h). This enabled all time points to be fixed at the same time (at time = 0 h) with 8% PFA on top of equal volume of media. To obtain Western blot protein lysates, cells were plated at 5×10^6 cells/dish in a 30 mm diameter petri dish.

2.3. Immunocytochemistry

All antibodies were diluted in immunobuffer (PBS, 1% normal goat serum (v/v) and 0.4 mg/mL merthiolate). All washes were carried out 3 times for 5 min with PBS containing 0.2% Triton X-100 (v/v, PBS-T). Cells plated in 96 well microplates were washed then incubated with 50 μL/well of primary acetyl histone H3 antibody (Sigma), diluted 1:1000 (v/v), overnight at 4 °C with shaking. Cells were washed and incubated with 50 μL/well secondary anti-rabbit (Sigma) antibody diluted 1:500 (v/v) overnight at 4 °C with shaking. Cells were washed again and incubated with 50 μL/well ExtrAvidin (Sigma) diluted 1:500 (v/v) for 3–4 h at RT with shaking. After washing, cells were incubated with 50 μL/well of freshly prepared DAB solution (0.5 mg/mL, in 0.1 M Phosphate buffer, with 0.1% hydrogen peroxide (v/v); BDH, added immediately before use) for 5 min (or until sufficient colour change could be visualized), washed and stored long term in 50 μL/well PBS-T with 0.4 mg/mL merthiolate.

2.4. Protein lysates, biorad protein assay, Western blotting

Protein lysates from SK-N-SH cells plated in petri dishes were prepared using a novel lysis buffer recipe adapted from histone extraction protocols used by Dunn et al. (2006); 5% NP40 (v/v), 10% SDS (w/v), 10% glycerol (v/v), 50 mM Tris, 0.6 M NaCl, 8.3 mM EDTA, 0.25% Na deoxycholate (w/v), with 1 Complete mini tab; Roche, per 10 mL buffer, pH 10; 200 μL of buffer was added per petri dish, and surface of the dish scratched with a cut 1 mL pipette tip to lyse adherent cells. The properties of each ingredient was considered carefully to ensure penetration of cell membrane and nuclear membrane. In addition, pH was a critical factor, demonstrated previously by Dr Steve Hodgkinson (personal communication), more alkaline pH improved the signal to noise ratio of histone molecules against total protein and thus significantly improved the specificity of antibody binding in Western blots. If lysates were too viscous to pipette accurately, they were triturated with a range of 21–27 in. syringe needles to shear the DNA. Homogenates were kept on ice at all times during preparation and at −80 °C for long term storage.

Protein concentrations were determined using the Bio-Rad DC Protein assay protocol according to manufacturers instructions. All samples were diluted to the same concentration using lysis buffer, NuPAGE® LDS sample buffer 4× and NuPAGE® Reducing agent 10×. Diluted samples were boiled at 70 °C for 10 min, immediately before loading onto gels, centrifuged and 30 μg of each sample were loaded onto NuPAGE® Novex 4–12% Bis-Tris gradient gels. Samples were run alongside protein ladders (6 μL of Dual Color precision plus protein standard and 6 μL of Novex® Sharp Standard). Proteins were electrophoresed at 100 V for 60–90 min using standard NuPAGE® procedures. Briefly, running buffer (1×) was prepared by diluting 20× NuPAGE® MOPS running buffer in deionised water (dH_2O) and for reduced samples, 500 μL of NuPAGE® antioxidant was added to the buffer used to fill the upper buffer chamber. Proteins were electrophoretically transferred to PVDF using a Bio-Rad Mini PROTEAN system according to manufacturers' instructions. Membranes were blocked for 1 h at RT with 5% non-fat milk powder (w/v, Anchor, NZ) made up fresh in TBS-T