Cover Page

DNA Labeling *in Vivo*: Quantification of Epidermal Stem Cell Chromatin Content in Whole Mouse Hair Follicles Using Fiji Image Processing Software

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Short title: BrdU Label Retaining Quantification in the Hair Follicle Using Fiji

Key words: DNA labeling, Bromo-deoxyuridine (BrdU), Label retaining cells, Epidermal stem cells, Hair follicle, Fiji software

Number of figures: 2.

Fig. 2 must be printed in color (paper edition).

Chapter …

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Summary

DNA labeling *in vivo* using nucleoside analogues is a current experimental approach to determine cell proliferation rates in cell cultures and tissues. It has also been successfully used to localize adult stem cell niches through the identification of nucleoside label retaining cells (LRC) in long term experiments. A major hindrance of this methodology relies on the selection of adequate procedures to quantify the nucleoside analogue content from image data files. Here we propose a simple procedure using Fiji image processing software to accurately calculate nucleoside analogue retaining chromatin/total chromatin (LRC/DAPI) signal ratios in the well-known mouse hair follicle stem cell niche.

1. Introduction

Metabolic labeling of the DNA molecule *in vivo* is a powerful methodology to study cell proliferation and differentiation dynamics in culture cells and whole tissues or organisms ***(1).*** The basis of this methodology is the addition of chemically modified

nucleoside analogues to target samples. After a short labeling pulse, passive or active cellular uptake mechanisms and subsequent nuclear translocation result in the accumulation of phosphorylated modified nucleosides in the nuclear compartment, where they are incorporated into the DNA molecule mainly through successive rounds of conservative replication (**Fig. 1A**). Cells showing high DNA replication rates are thus expected to show a faster and greater accumulation of modified nucleosides.

Interestingly, an immediate and fruitful application of DNA labeling in living cells and tissues has been the identification of putative stem cell niches in adult eukaryotic organisms ***(2-9)***. Delivering huge nucleoside analogue amounts *in vivo* through consecutive labeling pulses results in a widespread nuclear accumulation and subsequent massive incorporation of these compounds into the DNA molecule through different mechanisms, including DNA replication, excision repair mechanisms and reverse transcription in retrotransposon genomic regions. This process ultimately results in a homogenous modified nucleoside labeling of most nuclei in the target organism (**Fig. 1B**). A defining hallmark of mammalian adult stem cells, along with the capacity of differentiation in one or more different cell types and the potential to self-renew, is the active maintenance of a quiescent, low-proliferating state. In this context, an extensive and homogenous DNA nucleoside analogue label is expected to be progressively lost in a long term time period in most proliferating cells in a tissue after successive DNA replication rounds, but retained in the quiescent, reported adult stem cell population (**Fig. 1C**). The existence of this Label Retaining Cells (LRC) population has been nicely demonstrated in the mouse hair follicle stem cell niche model and this experimental approach has enormously contributed to our knowledge on adult stem cell biology ***(4, 9)***.

Historically, metabolic DNA labeling has been performed by using thymidine derivatives including tritiated thymidine ***(10)*** or halogenated analogues like bromo-deoxyuridine (BrdU), chloro-deoxyuridine (CldU) or iodo-deoxyuridine (IdU) ***(11-14)***. Detection of

incorporated marks is subsequently performed by autoradiography, in the case of radioactive derivatives, or by using specific antibodies, in the case of halogenated nucleosides. Due to its radioactive nature, tritiated thymidine is at present virtually out of experimental use. For its part, major drawbacks of experimental protocols using halogenated nucleosides are the requirement of specific treatments to unmask analogue epitopes on fixed chromatin, typically by mild acid hydrolysis or nuclease digestion, and the overall constraining of detection efficiency by the permeability of target samples to antibody diffusion ***(11-14)***. As an additional hindrance, some degree of cell and tissue toxicity, including mutagenic and teratogenic effects ***(15-20)***, has also been reported for both radioactive or halogenated nucleoside analogues.

In the last decade, bioorthogonal chemical reporter approaches ***(21, 22)*** have been developed trying to override the experimental limitations of halogenated nucleosides. Bioorthogonal labeling *in vivo* of DNA with the “clickable” 2´-deoxy-5-ethynyluridine (EdU) nucleoside has been reported in different biological samples ***(23, 24)***. This deoxythymidine analogue is highly diffusible, its detection with fluorescent azides after copper(I)-catalyzed azide-alkaline cycloaddition (CuAAC) does not require sample fixation or DNA denaturation and the staining procedure is not constrained by the limitations of immunological procedures. However, EdU is significantly more toxic than BrdU ***(25)***. More recently, less toxic variations on the same theme have been developed by using “clickable” arabinosyl nucleosides such as (2´S)-2´-deoxy-2´-fluoro-5-ethynyluridine (F-ara-EdU) ***(25)***. Despite this technical progress, BrdU is still at present the most widely used nucleoside analogue in DNA labeling experiments.

A major theoretical hindrance of DNA labeling experiments is the methodology of choice to quantify the incorporated nucleoside label. In most experimental models, the lack of enough physiological and/or biochemical parameters defining cell and/or chromatin turnover rates in each biological system constrains the establishment of an adequate mathematical framework, forcing the use of qualitative instead of quantitative

strategies to measure nucleoside analogue incorporation dynamics ***(14, 26, 27)***. This is particularly true for procedures to quantify nucleoside analogue content from image data files. In most cases, the measure of choice is the number of cells showing positive nucleoside labeling in the nucleus ***(28)***, being this signal a small spot or a large nuclear region. Such measure guideline has several inherent pitfalls ranging from the distortion introduced by the subjective human factor to the actual biological significance, in terms of cell function, of the label amount in each particular nucleus. These pitfalls are specially noticed in the case of LRC quantification in animal models. Trying to circumvent these inconveniencies, here we propose a simple procedure that uses Fiji image processing software ***(29)*** to calculate the ratio of BrdU label retained in long term experiments with respect to the total chromatin content in the well-known hair follicle stem cell niche ***(30, 31)***.

2. Materials

2.1. BrdU DNA labelling in new born mice.

1. Ten-day-old C57BL/6j mouse pups.

2. Distilled water.

3. PBS, 5mM EDTA in PBS, 0.02 % sodium azide in PBS.

4. 1N HCl (Merck).

5. Tris-borate-EDTA (TBE) buffer.

6. 5-bromo-2’-deoxyuridine (BrdU; Sigma) (*see* **Note 1**).

7. 12-O-tetradecanoil-phorbol-13-acetate (TPA; Sigma). Prepare a 20 nM stock in acetone and store at -20 ^{o}C.

8. Fixation solution: 3.7 % formaldehyde (Merck) in PBS (*see* **Note 2**).

9. Permeabilization and blocking (PTG) buffer solution: 0.5 % Triton X-100 (Sigma), 0.2% gelatin (Merck) in PBS (*see* **Note 3**).

10. Mouse FITC-conjugated monoclonal antibody against BrdU (Roche).

11. Mounting medium: Vectashield (Vector Labs)-DAPI (Sigma) (*see* **Note 4**).

12. Insulin type syringes, needles, sterile surgical scalpel blades, scissors and tweezers.

13. Heater (at 37 ºC).

2.2. Confocal microscopy and image processing software (*see* **Note 5**).

1. Leica TCS SP2 or SP5 AOBS Laser Scanning Spectral Confocal Microscopes.

2. Image acquisition software: Leica Confocal Software (LCS) Lite and Leica Application Suite Advanced Fluorescence (LAS AF) Suite 2.6.1 (Leica). http://www.csc.mrc.ac.uk/microscopy/links.html

3. Image processing and quantification software: Fiji. http://fiji.sc/Fiji

3. Methods

3.1. DNA labelling with BrdU and production of long term Label Retaining Cells (LRC).

1. Inject ten-day-old mice intraperitoneally (i.p.) once a day during 4 consecutive days with 12 mg/ml BrdU in PBS (50 mg/kg body weight) (*see* **Note 6**).

2. Let the mice grow for a minimum of 50 days in standard housing conditions.

3. For TPA stimulation of cell growth and differentiation ***(9)***, topically treat 50-day-old, BrdU-injected mice on tail skin with 20 nm TPA in acetone 3 times in 48 hours (*see* **Note 7**).

3.2. Preparation of hair follicle whole mounts and immunodetection of LRC.

This procedure essentially follows the guidelines stated by Braun et al. ***(9)***.

1. Sacrifice the mice following the guidelines of the corresponding housing animal facility, cut the tail and peel the skin in a whole piece from the tail backbone.

2. Incubate the peeled tail skin in PBS with 5 mM EDTA at 37 ºC for 4 h.

3. Separate the epidermis from the dermis using tweezers and fix the intact sheet of epidermal tissue with 3.7 % formaldehyde in PBS at 4 ºC for at least 72 h.

4. Thoroughly wash the tissue with PBS and store at 4 ºC in PBS containing 0.02% sodium azide.

5. Cut fixed epidermal sheets into 0.5 cm x 0.5 cm pieces for further processing.

6. Incubate the epidermal pieces with 1 N HCl for 45 min at 37 ºC to hydrolyze chromatin and expose incorporated BrdU epitopes (*see* **Note 8**). Wash before and after the incubation with distilled water.

7. Incubate with Tris-borate-EDTA 5 min at room temperature (RT) to neutralize acid hydrolysis and thoroughly wash with distilled water.

8. Permeabilize and block tissue pieces by incubating with PTG buffer for 30 min at RT.

9. Incubate tissue samples with FITC-conjugated mouse monoclonal antibody against BrdU (Roche) diluted 1:50 in PBS for 8-12 h (overnight) at 37 ºC.

10. Thoroughly wash samples at least three times in PBS and mount in Vectashield-DAPI on slides facing upwards the inner side of the epidermis. Keep samples in the dark at 4 ^{o}C until confocal microscopy analysis.

3.3. Image capture and digital quantification of BrdU LRC area fraction with respect to total chromatin area fraction in the hair follicle (*see* **Fig. 2**).

1. Use the image acquisition software coupled to the confocal microscopy device to obtain stacks of images of LRC-FITC or DAPI distribution covering the whole thickness of the hair follicles between selected boundaries. Obtain the maximum projection of each image stack and save as a single snapshot.

2. Open snapshot files with Fiji software.

3. Use the *Freehand selection* tool to delimit the bulge area on each hair follicle, based on the DAPI image of global chromatin distribution in the whole mount. Perform this operation on each LRC-FITC and correlative DAPI image pairs.

4. Duplicate selected images and turn into 8 bit type (*Image* → *Type* → *8 bit*). Invert the image (*Edit* → *Invert*) and set an adequate threshold (*Image* → *Adjust* → *Threshold*) to have a sharp image of LRC-FITC or DAPI stained nuclei. Select the *Area fraction* option for measurement (*Analyze* → *Set measurements*) and make the corresponding measure for each selected area (*Analyze* → *Measure*).

5. Calculate the ratio between *Area fraction* measurements from each BrdU LRC-FITC and DAPI image pair in each particular hair follicle, obtaining the relative BrdU label retaining chromatin/total chromatin value.

6. Repeat steps 4 and 5 for all the hair follicles to be included in the study and perform the statistical analysis of choice to compare means of relative BrdU label retaining chromatin/total chromatin values between different experimental groups.

4. Notes

1. Prepare a 12 mg/ml BrdU stock solution in PBS by stirring 2-3 h at RT in the dark. If necessary, heat slightly at 40-50 ºC to ensure complete dissolving. Sterilize using a 0.2 µm pore size syringe filter. Store at -20 ºC.

2. The fixation solution should be kept in a neutral pH range (6.8-7.5). Acidification of the solution, which progressively takes place at RT, results in a drastic reduction of formaldehyde fixative properties.

3. For PTG buffer preparation, weight 100 mg of gelatin powder, heat 20 ml of PBS at 60 ºC in a glass beaker, slowly pour the gelatin powder into the PBS volume and thoroughly stir until the solution reach RT. At this point, complete the PBS volume up to 50 ml, add 250 µl of Triton X-100 and stir for additional 20 min.

4. Prepare a DAPI stock solution, 10 mg/ml in distilled water and store at -20 ºC. Combine 5 µl of concentrated DAPI stock with distilled water to a final volume of 1 ml, obtaining a 50 ng/ml intermediate stock. Add 100 µl of DAPI intermediate stock solution to 900 µl of Vectashield, to obtain 1 ml of mounting medium containing DAPI at 5 ng/ml. Store at 4 ºC in the dark. Samples should be kept in mounting medium a minimum of 30 min before microscopy analysis to ensure that minor groove insertion of DAPI in the DNA molecule reaches a suitable chemical equilibrium for optimal detection. Mounted samples can be stored at -20 ºC in the dark 1-2 weeks showing good emission and resolution efficiencies.

5. Confocal microscopy devices and coupled image acquisition software are circumstantial and equivalent equipment can be used to obtain raw image data. Fiji image software is strongly recommended for image analysis and quantification.

6. BrdU can be stored for years at -20 ºC as powder or diluted stock. However, after prolonged storage it is strongly recommended to check BrdU viability in cell cultures before performing experiments in animal models.

7. For each TPA treatment, homogeneously distribute 100 µl of 20 nM TPA in acetone on the whole surface of tail skin and let evaporate for at least 5 min. Animals can be anesthetized at this step. TPA is extremely hazardous and mutagenic. Handle with extreme care.

8. Mild acid hydrolysis of chromatin is a critical step for proper detection of incorporated BrdU. A short hydrolysis time results in a defective BrdU epitope unmasking, while excessively longer hydrolysis results in extensive chromatin degradation.

Acknowledgements

This work was supported by grants of the Spanish Ministerio de Economía y Competitividad (SAF 11-23493) and the Comunidad Autónoma de Madrid (SkinModel, CAM S10/BMD-2359) to J.E.; E.C and M.I.C. are supported by PhD fellowship grants of the Spanish Ministerio de Educación and Universidad Autónoma de Madrid, respectively.

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Figure legends

Fig. 1. DNA labeling strategies in the mouse hair follicle using BrdU. Upper panels outline different pulse-chase DNA labeling strategies based on BrdU incorporation in cells going through the S phase of the cell cycle. Lower panels show representative confocal microscopy images (maximum projection of image stacks) of BrdU distribution in mouse telogen hair follicles in tail epidermis whole-mounts after different DNA labeling strategies. **A**: 24 hours after a single intraperitoneal injection of BrdU in adult mice, extensive DNA labeling is preferentially detected in proliferating cells. In the telogen hair follicle, BrdU labeling is observed in the interfollicular epithelium,

infundibulum and dermal papilla, but not in the bulge region, a major niche of quiescent, epidermal stem cells. **B**: Serial injections of large BrdU amounts in neonatal mice result in an extensive and homogeneous DNA labeling of most cells in the hair follicle. Developing neonatal mice present active proliferation and differentiation in most cell types and lineages, facilitating the incorporation of BrdU into the DNA molecule. **C**: In adult mice, only low-proliferating, quiescent cells are expected to retain DNA labeling after serial BrdU injections in the neonatal state. In telogen tail skin hair follicles of 50 days old mice, these label retaining cells (LRC) (arrow) are mainly localized in the bulge area, showing different degrees of nuclear staining. Inf: infundibulum; SG: sebaceous gland; IFE: interfollicular epithelium; Bg: bulge; DP: dermal papilla Scale bar: 100 μm.

Fig. 2. Quantification of the BrdU retaining chromatin/total chromatin ratio of long term label retaining cells (LRC) in the mouse hair follicle using Fiji image processing software. A: Confocal microscopy images (maximum projection of image stacks) of telogen hair follicles in whole mounts of 50 days old mouse tail epidermis showing total chromatin DAPI staining (left) and BrdU LRCs in the bulge region (right). Bg: bulge; SG: sebaceous gland; DP: dermal papilla; Inf: infundibulum; IFE: interfollicular epithelium; LRC: label retaining cells. Scale bar: 100 μm. **B**: Selected areas of images in **A** containing one hair follicle for the quantification of the BrdU retaining chromatin/total chromatin ratio. **C**: Bulge area marked off with the *Freehand selection* tool in 8 bit-type inverted images. **D**: Once the threshold has been established, positive nuclei appear as black regions over the white background. AF: *Area fraction* value in the selected region. **E**: Left panels show representative confocal microscopy images (maximum projection of image stacks) showing a strong increase of LRC in the bulge area after treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). Right panel represent the quantification of the BrdU retaining chromatin/total chromatin ratio, expressed as the AF ratio of BrdU staining with respect to DAPI

staining in the bulge region. AF values were obtained as described above. The mean ± SD of values calculated in thirty hair follicles in three different animals for each treatment are represented (**, $p < 0.01$).

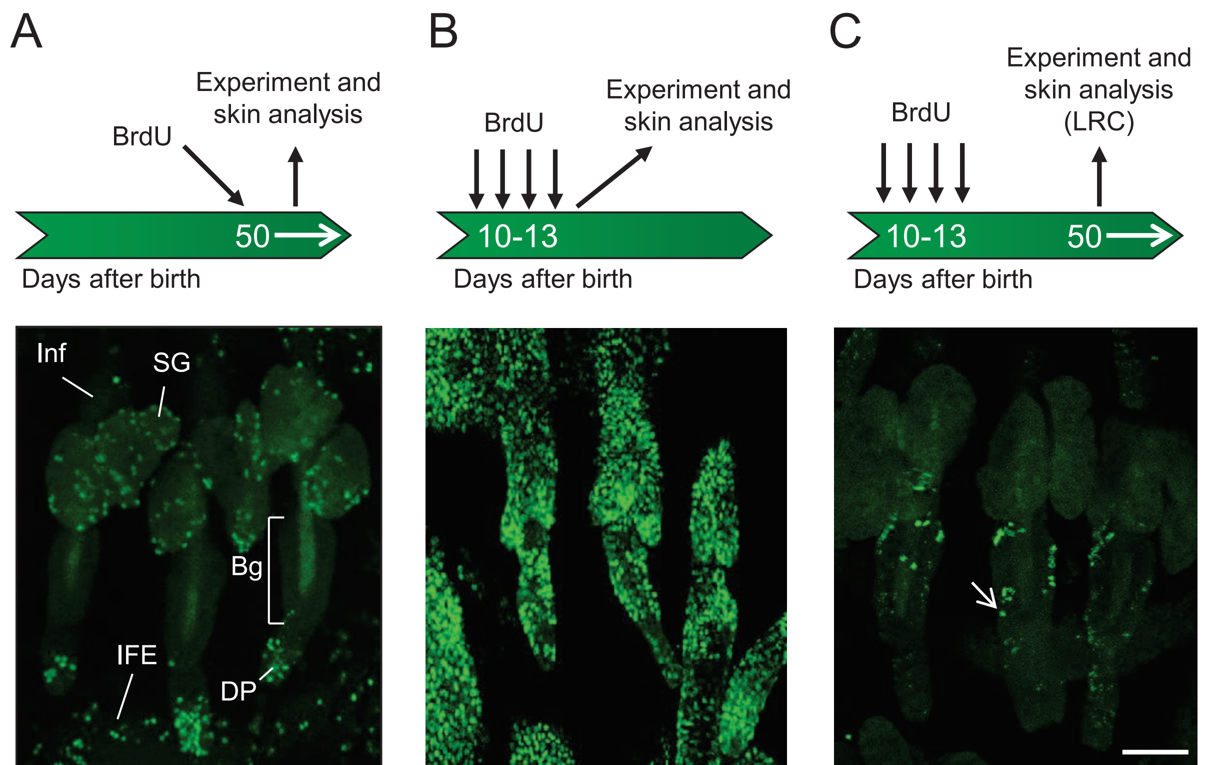

Figure 1. Carrasco et al. 2013

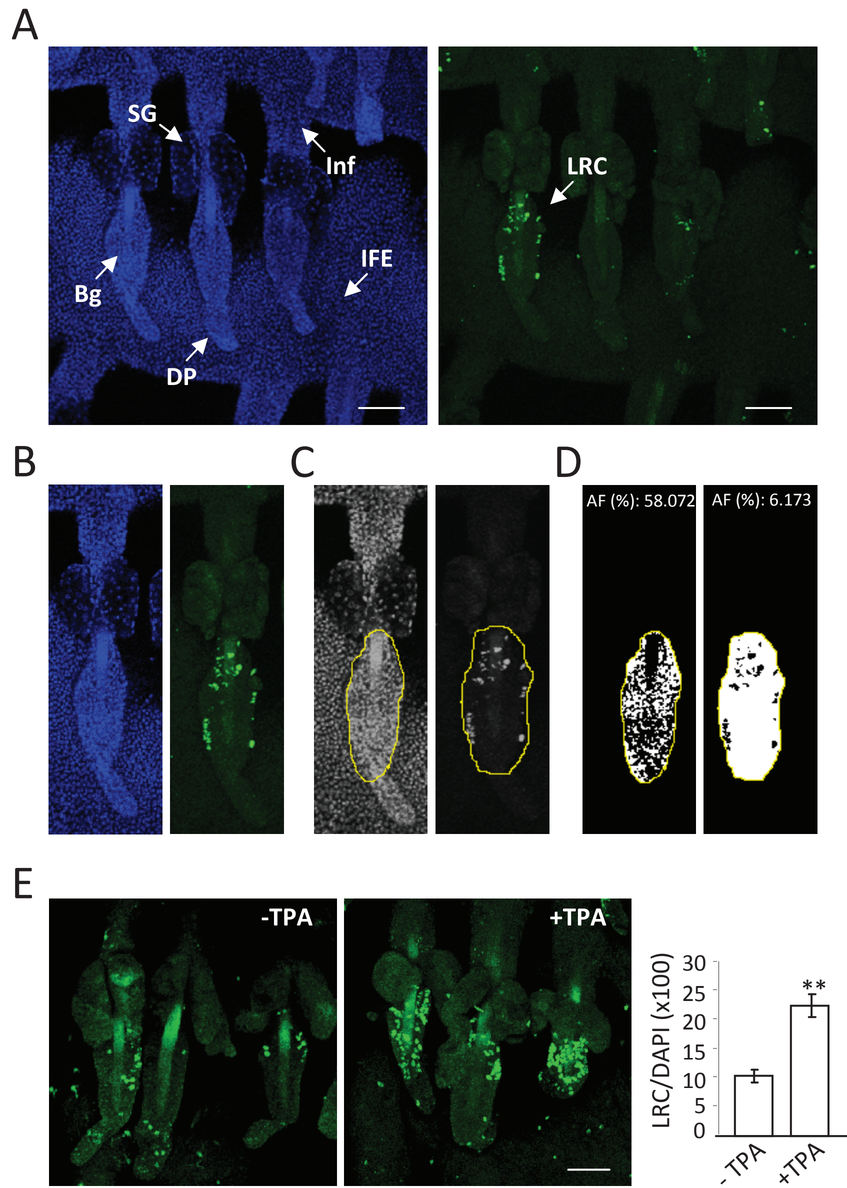

Figure 2. Carrasco et al. 2013