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Observing S-Phase Dynamics of Histone Modifications With Fluorescently Labeled Antibodies

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Summary

Histone modifications are central to epigenetic regulation and must be reestablished with each round of DNA replication. Here we describe methods to localize these modifications within mammalian nuclei and to relate them to specific spatiotemporal patterns of DNA replication.

Key Words: Nuclear structure; histone modification; histone methylation; immunofluorescence microscopy.

1. Introduction

The postgenomic era has brought with it an appreciation of the limitations of DNA sequence information and the importance of epigenetic regulation through nuclear structure/chromatin dynamics. Histone proteins, the major components of chromatin, are subject to dynamic covalent modifications, mostly at their N-terminal tails, and different combinations of these modifications are thought to represent different "histone codes" that regulate processes such as transcription, replication, recombination, and repair by forming myriad interactive surfaces for regulatory proteins ***(1,2)***. Layered upon this histone modification code are various isoforms of the core histones, known as histone variants, as well as many linker histones, all of which appear to have specialized roles in modulating chromosomal functions ***(3)***. One tool to probe the functional roles of these various epigenetic regulators is to determine their subcellular localization and how this localization changes during the cell cycle and development. An important opportunity for plasticity in epigenomic structure occurs during DNA replication, when histones are disrupted and then reassembled ***(4)***. Here, we describe methods to examine histone modifications and their relationship with DNA replication during S-phase.

From: *Methods in Molecular Biology, vol. 325: Nuclear Reprogramming: Methods and Protocols*
Edited by: S. Pells © Humana Press Inc., Totowa, NJ

A typical immunofluorescence protocol includes the following steps: (1) cellular fixation to immobilize the proteins of interest, (2) cellular permeabilization to allow antibodies to access antigens, (3) blocking nonspecific immunoglobulin binding, (4) incubation with antibody to recognize proteins of interest, and (5) visualization by microscopy. Visualization is achieved either by conjugating the specific antibody itself with a fluorochrome (direct immunofluorescence) or by including a subsequent incubation with a fluorescently tagged anti-immunoglobin secondary antibody whose specificity matches the primary antibody isotype (indirect immunofluorescence). Indirect immunofluorescence is used more commonly because the same fluorescently conjugated secondary antibody can be used for many different primary antibodies, avoiding the need to fluorescently conjugate each new antibody and providing continuity in experiments through the use of the same reagent for many different visualizations. In addition, because the secondary antibody recognizes multiple epitopes on the primary antibody, an amplification of signal is achieved over direct immunofluorescence. The primary drawback of indirect immunofluorescence is the added risk of crossreactivity and increased background resulting from the use of two antibodies rather than one.

As with all methods, certain limitations should be considered when interpreting the results of immunofluorescence experiments. These limitations and potential approaches to overcome them have been discussed extensively elsewhere ***(5,6)***. However, two of the most important issues related to immunofluorescence microscopy of histone modification are stressed here. First, antibody specificity is the single most important determinant for successful immunofluorescence microscopy, yet this determinant is often ignored as investigators all too frequently assume that their reagents are highly specific. Importantly for our discussion here, some sites of histone modification (for example, H3-K9 and H3-K27) have similar neighboring amino acid sequences that can easily cause crossreactivity between respective antibodies. Thus, quality control for antibody specificity, for example, by using histone modification-peptide arrays ***(7)*** to spot potential crossreactivity, is an invaluable asset for interpretation of results. Second, it is important to remember that histone modifications are embedded inside nuclei and that epitopes may not be presented in the same way as they are in Western blots or even peptide arrays. Hence, the fixation and permeabilization steps should be controlled carefully to preserve in vivo distribution of histone modification and allow for antibody access as well. If the same pattern is obtained with multiple fixation and permeabilization methods, one can feel more comfortable with the results. If not, more complicated methods, such as mass spectrometry and mutational analyses ***(8,9,16)*** may be needed to aid in the interpretation of results.

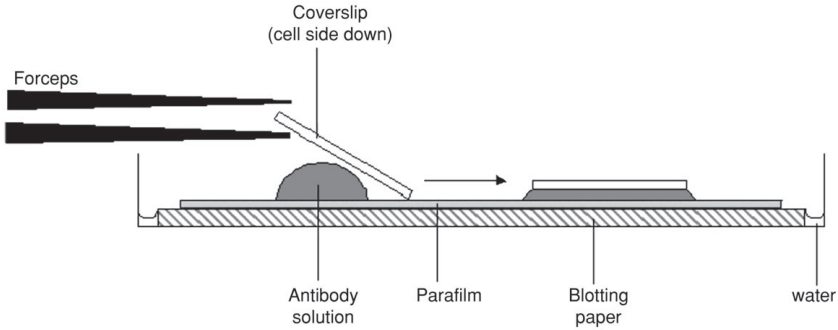

Fig. 1. Summary of antibody reaction in humidified chamber. A piece of filter paper slightly smaller than the box size is placed in the box and moistened with ddH_2O. After draining off excess ddH_2O, a piece of parafilm is placed on top of the filter paper. For antibody incubation, a drop of antibody solution is pipetted onto the parafilm and the cover slip is placed onto it, cell side facing down, by gently lowering one side of the cover slip.

2. Materials

2.1. Supplies and Equipment

1. Microscope cover glasses (12CIR; cat. no. 12-545-80; Fisher Scientific): wrapped in aluminum foil, autoclaved and transferred to sterile dishes.
2. Sterile culture dishes and 24-well plates.
3. Microscope slides.
4. 24-well cell culture plates (e.g., Costar brand, cat no. 07-200-84; Fisher Scientific).
5. Forceps with fine tips (e.g., Jewelers Microforceps from Fisher Scientific, cat. no. 08-953E straight end or 08-953F curved end).
6. Humidified chamber (*see* **Fig. 1**): any small box with a lid can be made into a humidified chamber. For example, we use the shipping box (~6.5 × 9.0 cm) for Vectashield mounting medium from Vector laboratories, Inc. Alternatively, plastic microscope slide storage boxes make another suitable chamber. A piece of blotting paper slightly smaller than the box size is placed in the box and moistened with ddH_2O. After draining off excess ddH_2O, a piece of parafilm is placed on top of the blotting paper and the chamber is ready for use.
7. Conventional or confocal-equipped epifluorescence microscope with appropriate filter set and high power, high numerical aperture lenses.

2.2. Reagents

1. 5-Bromo-2'-deoxyuridine (BrdU; Sigma Chemical, St. Louis, MO): make a 10 mg/mL stock solution in ddH_2O, filter sterilize (0.2 μm; cat. no. 21062-25; Corning), aliquot, and store at –20°C. After thawing the stock solution, the white precipitate should be redissolved in solution by vortexing and/or heating to 37°C.

2. 2% and 4% Paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4. It is optimal to prepare the fixative freshly. However, this is very time-consuming. For most applications, we make an 8% paraformaldehyde in PBS stock and keep it frozen at –20°C. When needed, the stock is thawed at 60°C and diluted with PBS to the desired concentration.
3. 8% (w/v) PFA in PBS, pH 7.4. Add 8 g of solid paraformaldehyde to approx 80 mL of ddH_2O. Add a few drops of 1 *N* NaOH to the solution and heat to 60°C while stirring. Cool the solution to room temperature, add 10 mL of 10X PBS, adjust pH to 7.4, and bring the total volume to 100 mL. Aliquot and freeze at –20°C in the dark for up to several months.
4. 1X PBS.
5. 70% EtOH (ice cold).
6. PBS/T: 0.5% Tween-20 in PBS.
7. 0.2% Triton X-100 in PBS.
8. Blocking buffer (*see* **Note 1**): 3% bovine serum albumin\PBS\0.5% Tween-20.
9. Primary antibody solution: dilute antibody with blocking buffer. For different batches of antibodies, titration is needed to determine the optimal dilution. In this protocol, we describe the use of a rabbit polyclonal antibody raised against histone H3 tri-methylated at lysine 9 (rabbit αMe_3K9H3 ***[10]***).
10. Secondary antibody solution: choose fluorescence as required by experiments (*see* **Note 2**). For example, here we use Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen, cat. no. A-11034) that is diluted 1/400 in blocking buffer.
11. 0.3 *M* Glycine in PBS.
12. 0.5% NP40 (a nonionic detergent) in PBS.
13. 1.5 *N* Hydrochloric acid.
14. Mouse anti-BrdU antibody (Becton Dickinson, cat. no. 347580): diluted 1/10 to 1/20 with blocking buffer.
15. Secondary antibody for BrdU: Alexa 592-conjugated goat anti-mouse IgG diluted 1/400 with blocking buffer.
16. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; cat. no. D9540; Sigma Co.): make a 10 mg/mL stock by dissolving the powder in ddH_2O, aliquot, and store at –20°C in the dark. (It is stable for more than 1 yr.) Also, make a 0.1 mg/mL solution and keep at 4°C for routine use. (This solution also is very stable.)
17. Vectashield (cat. no. H-1000; Vector Laboratory) or other antifading reagent.

3. Methods

3.1. Preparation of Cells Attached to Cover Slips (see Note 3)

1. Place several sterile cover slips in the culture dish either with sterile tweezers or by glass pipet aspiration (the vacuum is sufficient to hold the cover slip).
2. Seed the cells on the dish and let the cells grow to the desired cell density. Usually 50 to 70% cell confluency is desirable. High cell densities will facilitate microscopic analysis, but too high a cell density will inhibit BrdU incorporation and introduce staining background.

3. Label the cells by adding BrdU (from stock solution) to a final concentration 10 to 30 μg/mL. Mix well and return to the incubator for 10 min. The minimum labeling time is 2 min at higher concentrations ***(11)***, but do not exceed 30 min because each replication site takes 45 to 60 min to complete its replication ***(12)***.

*3.2. Fixation and Permeabilization of Cells (***see *Notes 4 and 5)***

Cells on cover slips can either be fixed directly in dishes or the cover slips can be removed one at a time and fixed in 24-well plates.

3.2.1. Paraformaldehyde Fixation and Triton Permeabiliztion

1. Remove the medium from the dish and quickly rinse the cells with PBS twice to remove cell debris.
2. Incubate with 2% PFA in PBS at room temperature for 10 min (*see* **Note 4**).
3. Wash the cells twice for 5 min each in PBS. Cover slips can be stored at 4°C for up to a week.
4. Incubate for 5 min with 0.2% Triton X-100 in PBS to permeabilize the cell membrane (*see* **Note 6**).
5. Wash the cells twice for 5 min each in PBS.

3.2.2. Ethanol Fixation

1. Remove the medium from the dish and quickly rinse the cells with PBS twice to remove cell debris.
2. Incubate with ice-cold 70% ethanol at 4°C for 10 min.
3. Wash the cells twice for 5 min each in PBS. Cover slips can be stored at 4°C for up to a week.

*3.3. Immunostaining of Cells for Histone Modification (***see *Notes 7 and 8)***

Do not allow cover slips to dry at any step to avoid high background staining.

1. Incubate the cells with blocking buffer for 20 min (*see* **Note 1**).
2. Rinse the cells with PBS/T.
3. Incubate the cells with primary antibody for 1 h.

All antibody incubations should be conducted in a humidified chamber, usually at room temperature (*see* **Note 9**). As shown in **Fig. 1**, pipet drops of antibody solution (as little as 4 μL for each drop) onto the parafilm, being careful to avoid air bubbles. One cover slip should be placed on top of each drop with the cell side facing down.

4. Add PBS/T to a 24-well cell culture plate and transfer the cover slips to the 24-well plate with the cell side facing up. Wash with PBS/T three times for 5 min each.
5. Incubate with Alexa 488-conjugated secondary antibody solution for 1 h (*see* **Note 2**).

OPTIONAL: if staining is to be done only for protein and not for BrdU:

6. Transfer the cover slips back to a 24-well plate and incubate with 0.01 μg/mL DAPI in PBS/T for 5 min.
7. Wash with PBS/T three times for 5 min each.
8. Pipet approx 2 to 3 μL of Vectashield onto slides and place the cover slips on top of the Vectashield with the cell side facing down. Seal the cover slips with nail varnish and store the specimens in a sealed dark box at 4°C or –20°C. Significant loss of fluorescence signal may be experienced after 1 wk of storage at 4°C or longer than 1 mo at –20°C.

3.4. Postfixation and Stain for BrdU Staining

If colocalization with sites of DNA replication is desired, the initial antibodies must be covalently fixed with formaldehyde to preserve their localization throughout the denaturation step that is necessary to visualize sites of BrdU incorporation. In this case, cover slips after **step 5** (in **Subheading 3.3.**) should be treated as follows:

1. Wash with PBS/T twice for 5 min each.
2. Wash with PBS once for 5 min.
3. Incubate with 4% PFA in PBS at room temperature for 15 min.
4. Wash in PBS once for 5 min.
5. Incubate with 0.3 *M* glycine in PBS for 5 min to quench unreacted formaldehyde.
6. Wash in PBS once for 5 min.
7. Incubate with 0.5% NP40 in PBS for 15 min.
8. Wash in PBS twice for 5 min each.
9. Denature DNA in 1.5 *N* hydrochloric acid at room temperature for 30 min.
10. Wash in PBS twice for 5 min each.
11. Wash in PBS/T once for 5 min.
12. Incubate with mouse anti-BrdU diluted 1/10 to 1/20 in blocking buffer for 1 h.
13. Wash in PBS/T three times for 5 min each.
14. Incubate with Alexa 592-conjugated goat anti-mouse IgG diluted 1/400 in blocking buffer for 1 h.
15. Proceed to **step 6** in **Subheading 3.3. Figure 2** shows an example of the kinds of results obtained with this protocol.

4. Notes

1. Bovine serum albumin is a practical and economical blocking agent that is usually sufficient. Ideally, however, to eliminate background staining, commercially available nonimmune serum from the species of the secondary antibody (e.g., rabbit, sheep, horse, donkey) should be used to block nonspecific antibody binding. We use 10% serum in PBS\Tween-20.
2. For the secondary antibody, you can choose the specific fluorochrome according to your experimental requirement. Fluorescein and Texas Red are used commonly. However, they are prone to photobleaching and are not the best choice for

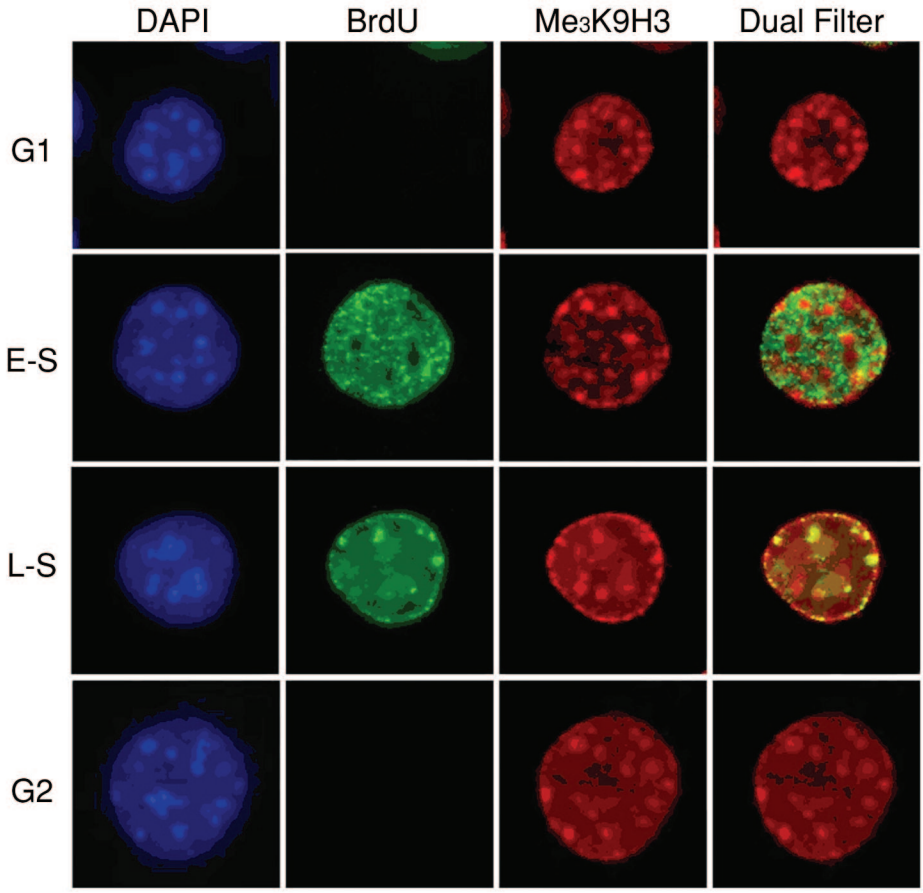

Fig. 2. Localization of Me_3K9H3 during cell cycle. Exponentially growing mouse C127 cells were pulse-labeled with BrdU and then costained for anti-BrdU and anti-methyl K9 H3 ***(10,16)***, as described herein. Although we recommend cell synchronization to verify the spatiotemporal order of BrdU staining patterns, once established, one can easily distinguish cells in different phases of the cell-cycle based upon these patterns. Shown are examples of G1-phase (small BrdU-negative nuclei), early and late-S-phase (distinguished by the spatial pattern of BrdU labeling), and G2-phase (large BrdU-negative nuclei).

image acquisition requiring long exposure times, such as Z- stack acquisition by confocal (or deconvolution) microscopy. Companies are now introducing more photostable fluorochromes. Among them, our favorite is the Alexa dye series (Invitrogen, Inc).

3. Adherent cells can be grown on slides or cover slips before fixation. In our hands, growing cells directly on 12-mm round glass cover slips is easier and more convenient for subsequent immunostaining manipulation. For those cells that adhere

less strongly, cover slips should be pretreated with poly-(L-lysine) or with calgon metasilicate to facilitate cell attachment ***(6)***. Suspension cells can be stained in suspension, as is done for flow cytometry. However, for convenience, suspension cells can be adhered to clean microscope slides using a cytocentrifuge (e.g., cytospin2 or 3; Shandon) and the same procedures described here for adherent cells can be followed. Cytocentrifuges are typically designed for slides. However, a round cover slip can be placed atop the slide to spin cells onto a cover slip. One drawback of adhering cells with a cytocentrifuge is the apparent change in cell morphology (flattening) that occurs as a result of the cytospin method, which needs to be taken into account when interpreting results.

4. Sometimes, triton extraction can be performed before cell fixation to remove any soluble pool of proteins that are present in excess within nuclei and mask the fluorescence pattern of the chromatin-bound fraction ***(13)***. This is not necessary for most histones because there is very little soluble histone pool.
5. Choosing a suitable fixative may be the most important and the most challenging task during the development of a protocol for a new antigen because the choice of fixative will directly affect the accuracy and interpretation of the final result. Very different localization patterns can be obtained with different fixatives and confirming the true in vivo localization of a protein can be difficult, for example, by comparison with the localization of a GFP-tagged version of the same protein (e.g., *see* **Fig. 2** in **ref. *14***). For the localization of histone modifications, such confirmation is not available, and many of the existing results will need to be confirmed by microdissection or other purification methods coupled with modern mass spectrometry. There are two classes of fixative: crosslinking reagents and organic solvents. Crosslinking reagents, including formaldehyde and glutaraldehyde, covalently crosslink molecules and generally are believed to more accurately preserve cell morphology. Glutaraldehyde fixation produces poor accessibility of antibodies to nuclear antigen and is rarely used to study nuclear antigen. Formaldehyde (~1–4%) fixation works relatively well to preserve cell morphology and antigen accessibility and can be chosen as a starting fixation protocol for new antibodies. Longer fixation times can introduce artifacts and therefore it is recommended to use the shortest fixation time that maintains antigen localization. Obviously, if the epitope of the antigen is crosslinked by formaldehyde or if it is masked within the structure of the protein or within protein complexes, the antigenicity of that protein will be reduced. Hence, in cases of poor antigenicity, organic solvent fixation can be tried. The most commonly used organic solvents are cold methanol (100%) and ethanol (70%), which maintain insoluble components and large soluble components (>100 kDa) in place while washing away small soluble components ***(15)***. Although organic solvents cause cell shrinkage and disrupt cytoplasmic structure dramatically, they can produce very good results with insoluble nuclear proteins**.** Unfortunately, there is no perfect fixation method and the best results must be determined empirically for each new antibody.

6. After formaldehyde fixation, the cell and nuclear membrane should be permeabilized to allow antibodies to penetrate the nuclei. The most commonly used permeabilization agents for nuclear antigens are the nonionic detergents Triton X-100, NP40, Bij35, and Tween-20. In our hands, Triton X-100 and NP40 are more efficient whereas Tween-20 is less efficient for permeabilizing nuclear membrane. Conditions presented here are very robust for most nuclear antigens but permeabilization conditions should be optimized for each new antibody.
7. Several experiments should be included to control for the specificity of the antibody reaction, e.g., (1) compete the primary antibody with the antigen peptide to check the specificity of the primary antibody; (2) omit the secondary antibody to examine the autofluorescence from the specimen; (3) omit the primary antibody to check the specificity of the secondary antibody.
8. Here, we describe the protocol for one protein antigen, co-localized with BrdU. Quite often, two protein antigens need to be stained simultaneously to evaluate co-localization. Often, one can mix two antibodies together and follow the same protocol. However, sometimes the presence of one antibody can inhibit another antigen's antibody reaction, particularly when they co-localize very closely. In this case (i.e., if one antigen is not observed when simultaneously stained with another), sequential staining with each antibody added in both orders should be tried ***(11)***. When co-localizing with BrdU, the harsh denaturation steps required to visualize BrdU necessitate staining for protein first.
9. The length and temperature of incubation can be varied. In many cases, reactions can be accelerated (15–30 min) at 37°C, but we routinely incubate for 1 h at room temperature. However, if poor signals are obtained it is recommended that antibody incubations be carried out overnight at 4°C. With either of these deviations, the risk of sample dehydration is increased; hence, it is advised to add more antibody solution.

Acknowledgments

We thank D. Cameron for critical evaluation of the manuscript. Research in the Gilbert lab is supported by NIH grant GM-57233-01, NSF grant MCB-0077507, and American Cancer Society grant RPG-97-098-04-CCG.

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