How to stain nucleic acids and proteins in Miller spreads

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ABSTRACT

The spreading technique proposed by Miller and Beatty in 1969 allowed for the first time the visualization at transmission electron microscopy of nucleic acids and chromatin in an isolated and distended conformation. This approach is beneficial since it can reveal many aspects of chromatin organization and function that otherwise can only be indirectly inferred by biochemical methods. The final step of staining chromatin spreads is critical because it can strongly influence the interpretation of the results. We evaluated different staining techniques, and almost all provided a good result. Specifically, well-contrasted micrographs were obtained when staining with $H_3PW_{12}O_{40}$ (phosphotungstic acid, PTA), as originally proposed by Miller and Beatty, and with two alternatives proposed here: uranyl acetate or terbium citrate. Quite a good contrast of the spread DNA could also be achieved using osmium ammine; while no or little contrast of nucleic acids was observed by staining with $KMnO_4$ (potassium permanganate) and $H_3PM_{o12}O_{40}$ (phosphomolybdic acid, PMA) respectively.

Key words: Chromatin spread; transmission electron microscopy; staining techniques.

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Introduction

Several methodologies have emerged in recent years to study higher-order chromatin structures and the interactions between specific nucleic acid sequences and proteins, like chromatin immunoprecipitation (ChIP), RNA immunoprecipitation (RIP), and the chromosomes conformation capture techniques,[1] associated with high-throughput genome sequencing strategies and bioinformatics. Thanks to these, it is possible to make inferences on genome architecture and its functional output. Optical microscopy represents a complementary approach that allows non-invasive direct visualization of cellular and subcellular structures, in different colors, in 3D (for instance, in 3D immunoFISH experiments[2]). Moreover, super-resolution microscopy techniques allow studying chromatin organization at a nanoscale.[3,4]

The spreading technique proposed previously by Miller and Beatty, as early as 1969, provided a revolutionary morphological approach that allowed the direct visualization of chromatin at the ultrastructural level in a distended conformation after lysis of nuclei. Using the spreading technique, the authors were able to image the ongoing transcription of the ribosomal genes, which forms a Christmas-tree-like structure, where the stem corresponds to the transcribed rDNA genes, while the branches stand for the nascent rRNAs.[5] This technique was subsequently coupled with immunocytochemistry to identify and localize the presence of specific proteins on distended DNA and RNA.[6] For example, the proteinic complexes visible at the end of the Christmas tree branches were shown to be 5'ETS rRNA processing complexes.[7]

Here we propose different contrast protocols to visualize the nucleic acids and the associated proteins in chromatin spread as alternatives to the first phosphotungstic acid (PTA) staining method presented by Miller and Beatty.[5] The staining methodology greatly impacts the final result of this experiment. When one observes a tissue section at the transmission electron microscopy (TEM), biological structures, such as membranes and organelles, provide spatial coordinates and show some weak intrinsic contrast even when the staining procedure has not worked well. On the other hand, the chromatin spread images completely lack these points of reference, so image contrast strongly influences the information gathered from the micrograph. As a result, the possibility of false-negative or false-positive signal interpretation is definitely increased. To improve this aspect, we made different staining attempts in order to try to improve contrast intensity or specificity.

Materials and Methods

Solutions for chromatin spread

The solutions necessary for the spread must be prepared in advance. First, a 0.2 mM EDTA was prepared from a 0.1 M stock solution, and the pH was adjusted to 7.5 with 0.1 N NaOH. The solution must be kept 1 week at 4°C to stabilize, and then the pH checked again.

Solution A was prepared by dissolving 1.71 g of sucrose in 40 mL of distilled water (dH_2O) and adding 1 mL of 10 mM NaH_2PO_4 $2H_2O$. The pH was then adjusted using 0.1 M KOH solution till reaching 7.5. dH_2O was added to reach the final volume of 50 mL.

Solution F/S was prepared by dissolving 1.71 g of sucrose in 40 mL of dH_2O. 5.4 mL of 37% paraformaldehyde were then added, and finally, the pH was adjusted using boric buffer until reaching a value of 9-10.

Solution B: 250 µL of Nonidet P40 or Tween20 were dissolved in 40 mL of 0.2 mM EDTA solution. Boric buffer was added dropwise to reach a final pH value of 9-10.

Solution C: 0.2 mL of Photo-Flo 200 Solution (Eastman Kodak Co., Rochester, NY, USA) or AGEPON (AGFA) were dissolved in 40 mL of dH_2O. The pH could be adjusted, if necessary, using boric buffer by adding it dropwise till reaching a pH value of 7.6-7.9.

The following day, the pH of each solution was measured again to ensure that it was correct and stable.

Spreading procedure

We used HeLa cells in the experiments described here, but any transcriptionally active cells may be used. Hela cells were grown in Eagle's Minimum Essential Medium (ATCC, Manassas, VA, USA) added with Gibco fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) to a final concentration of 10% and 1x Penicillin Streptomycin Solution (Sigma-Aldrich, St. Louis, MO, USA).

2 to 5 x 10^6 Hela cells were harvested for each experiment and centrifuged for 5 min at 1500 g to remove the supernatant medium. The cell pellet was suspended with 2 mL of phosphate-buffered saline (PBS) to wash away the medium residues, then this centrifugation step was repeated and the supernatant removed. Next, 1 mLof filtered solution A was added and vortexed carefully at medium speed (excessive speed could cause dissociation of proteins from nucleic acids). Then, 1 mL of filtered solution B was added dropwise while vortexing at medium speed. Then the suspended cells were transferred into 20 mL EDTA solution (0.2 mM) and shortly vortexed. At this step, the immunocytochemical analysis could be run to individuate a specific protein in the spread lysate, as follows. First, the primary antibody against the protein of interest must be diluted in the cells lysate EDTA solution (dilution fold depends on the antibody properties and efficiency), and incubation must last 30 min at 4°C. A final dilution 1:20 for 30 min at 4°C is recommended for the secondary gold-conjugated antibody. To localize the large ribosome subunits, we used an antibody against the P1/P2 proteins as components of this ribonucleoprotein complex (mouse monoclonal antibody, courtesy of Dr. J. Gordon). The primary antibody dilution was 1:100.

Aliquots of the final incubation mixture were then, directly or after immunocytochemical reaction, layered onto formaldehyde 4% sucrose (0.1 M, RNase free) cushions (F/S solution) in special plastic centrifugation chambers,[8] on the bottom of which glow-discharged,[9] formvar- and carbon-coated electron microscope gold grids were previously placed. Spreading was performed by centrifugation at 2200 g for 10 min at 4°C. The grids were then removed and, without drying, immediately immersed in 0.4% Photo-Flo 200 (Eastman Kodak Co., Rochester, NY, USA) (solution C) (pH 7.6-7.9) for 30-45 s, blotted on filter paper to remove the excess liquid, and air-dried.

Solutions for staining

0.1% $H_3PW_{12}O_{40}$ (PTA) in 50% ethanol.
4% uranyl acetate aqueous solution.
0.2 M terbium citrate, prepared according to Biggiogera and Fakan.[8]
0.1% $KMnO_4$ (potassium permanganate) in dH_2O.
Osmium ammine solution, prepared according to Vázquez-Nin *et al.*[10]
0.1% $H_3PMo_{12}O_{40}$ (phosphomolybdic acid, PMA) in 50% ethanol.

Staining procedures

Staining with 0.1% PTA: The grid was carefully put, section side down, on a drop surface of 0.1% PTA solution, and rotated slowly for 1 min. Then the grid was transferred to a drop of 95% ethanol with the same orientation and rotated slowly for

20 s. Finally, it was dried by moving it slowly in the air for a few min and stored in a box with the section side facing upwards.

Staining with 0.1% PMA: The same procedure as for the 0.1% PTA staining was followed, using 0.1 % PMA in place of PTA.

Staining with uranyl acetate: A small drop of uranyl acetate aqueous solution and several dH_2O drops were made on a parafilm sheet, using a glass Pasteur pipette. The grid was lain on the uranyl acetate drop for 2 min and then on the dH_2O drops maintaining it for a few seconds on each drop in order to wash away the uranyl acetate excess and avoid precipitates.

Staining with terbium citrate: The grid put on the terbium citrate drop with the section side facing the drop for 10 min. Then, two fast washes were performed by putting the grid on a dH_2O drop for 10 s, and then on another dH_2O drop for 5 s.

Staining with 0.1% $KMnO_4$: The grid put on a 0.1% $KMnO_4$ drop for 1 min with the section side facing the drop and then washed by putting it on several dH_2O drops maintaining it for a few seconds on each drop in order to remove the $KMnO_4$ excess and reduce the formation of precipitates.

Staining with osmium ammine preceded by HCl hydrolysis: The grid was floated on a drop of 0.1 N HCl for 30 min in a well. Then, the subsequent washes in dH_2O in a multiwell were performed: 7 quick rinses (soaking, making quick movements in water, blotting on an absorbent paper dish) and 3 rinses of 2 min each in dH_2O. The grid was blotted on an absorbent paper dish at the end of each wash. Afterward, the grid put on osmium ammine solution in a well for 60 min, followed by quick rinses in dH_2O as above to reduce precipitates formation and then 3 rinses of 5 min each and 1 rinse of 20 min in dH_2O.

Staining with osmium ammine without HCl hydrolysis: The same procedure reported above was followed, without the step of 0.1 M HCl hydrolysis.

At the end of each staining procedure, the grids were stored in a box with the section side facing upwards to let them dry.

Results and Discussion

In this study, we evaluated different techniques to stain spread nucleic acids and the associated proteins at TEM. The majority of the contrasting agents tested here provided good staining results

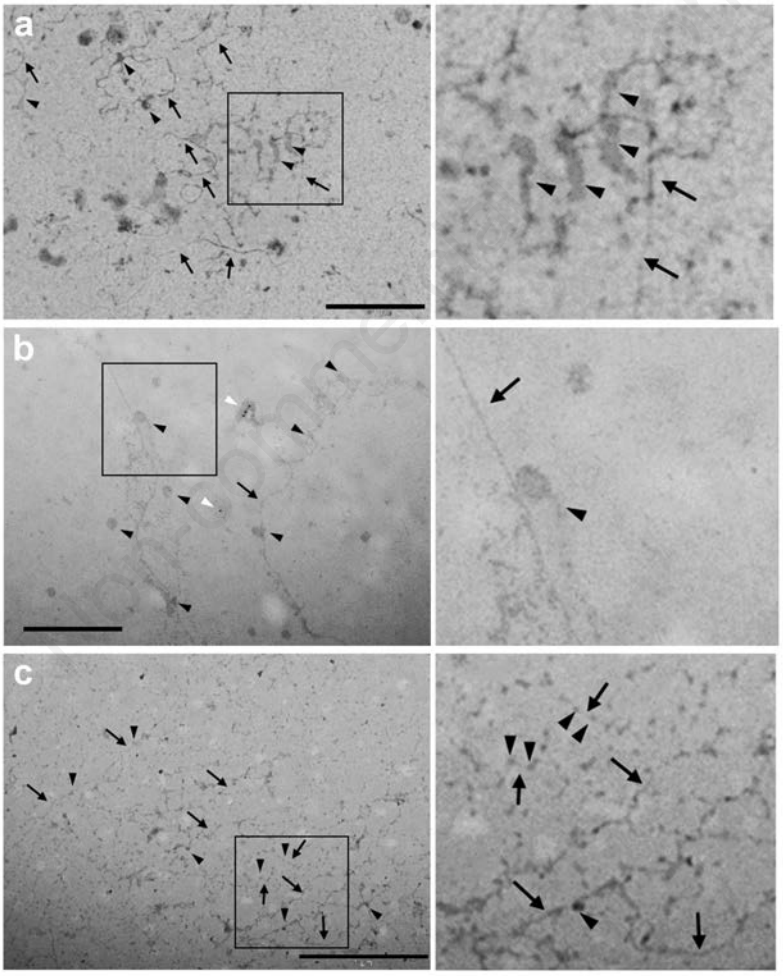

Figure 1. The micrographs show chromatin spread from Hela cell lysates, stained by different techniques. a) PTA staining. This staining, traditionally used, binds to both nucleic acids (arrows) and proteins (arrowheads), which appear well contrasted. b) Uranyl acetate staining. It is possible to appreciate well-defined and contrasted nucleic acids (arrows) with some associated proteins (arrowheads). The image is free from precipitates. Example of immunocytochemistry on chromatin spread: protein complexes labeled with 12 nm gold grain conjugated with a secondary antibody indicating the presence of P1/P2 proteins, components of large ribosome subunit (white arrowheads). c) Terbium citrate staining. Terbium provides a marked contrast to nucleic acids (arrows) and proteins (arrowheads). Areas delimited by the squares are reported on the right of each image at higher magnification to show in detail the contrast provided to the spread chromatin by each staining technique. Scale bars: 1 µm.

and are shown in Figures 1 and 2.

PTA has been used at TEM as an anionic stain for the positively charged groups of proteins (lysine and arginine residues)[11,12] in tissue sections. This property has allowed studying the ultrastructure of nuclear DNA, which is associated with histones, and therefore is well contrasted by ethanolic PTA; nucleoli and mitochondrial matrix are also intensely stained.[13] The PTA staining technique to contrast the Miller spreads is still among the best because, as expected, it ensures a good contrast of both nucleic acids and proteins (Figure 1a). However, some of the staining techniques proposed here could be a valid alternative, and one could choose among these staining procedures depending on what is desired to observe and on the experimental goal.

Uranyl acetate is commonly used for TEM because it contrasts many biological structures, including nucleic acids and basic proteins in tissue sections, by reacting with the phosphate groups present in the backbone of nucleic acids and with the amino groups of proteins. Uranyl acetate staining, therefore, contributes to the stabilization of nucleic acids and confers electron density to them and to proteins.[14] A similar result is observed in spread chromatin cell lysate: it provides a contrast very similar to that of PTA staining solution, but the results are free from precipitates (Figure 1b). An additional advantage is that the formvar membrane on which the cell lysate is spread is more resistant to the uranyl acetate staining than the alcoholic PTA staining solution. However, the ethanol content constitutes the major risk of membrane detachment from the grid, leading to experimental failure.

Terbium citrate was proven to contrast RNA fibrils at TEM preferentially. In their oxidation form 3+, Lanthanide elements can interact with single-stranded nucleic acids,[15] especially with guanosine monophosphate in RNA.[16] Indeed, it was demonstrated that this staining is abolished by previous treatment with RNase or nuclease S1, but not with DNase or pronase, and moreover, terbium citrate does not react with single-stranded DNA in adenovirus 5-infected HeLa cells. The contrast provided by terbium is quite weak.[8] When staining spread cell lysates, terbium citrate shows different behavior because it seems to contrast both DNA, RNA, and the associated proteins markedly. This technique provides good results, with a high contrast of all the cell lysate components (Figure 1c). The absence of the embedding resin and the different sample preparation may create a different chemical environment around the chromatin components that could modify terbium behavior and specificity. It may also stain DNA and protein in this particular condition. A similar example of loss of staining specificity by changing sample preparation procedure was reported by Derenzini and Farabegoli for the osmium ammine staining.[17]

Staining with osmium ammine specifically contrasts DNA on thin sections at TEM so that nuclear domains characterized by a condensed chromatin conformation, as heterochromatin, appear dark on a lighter background.[18] The Schiff reagent is widely used in light microscopy because it binds aldehyde groups on DNA molecules previously subjected to HCl hydrolysis, resuming a red fuchsia color. More in general, all reagents, which specifically react with aldehyde groups in the presence of H_2SO_3, are defined as Schiff-like reagents. They demonstrated that if acid hydrolysis is not performed, the Osmium ammine stains both RNA- and DNA-containing structures. Differently from tissue sections, in the spread experiment, nucleic acids are directly exposed to staining reagents because they are distended and because of the loss of some associated proteins. Considering this, we decided to perform very mild acid hydrolysis (with 0.1 N HCl instead of the routinely applied 5 N HCl) or to avoid this step in order to prevent the com-

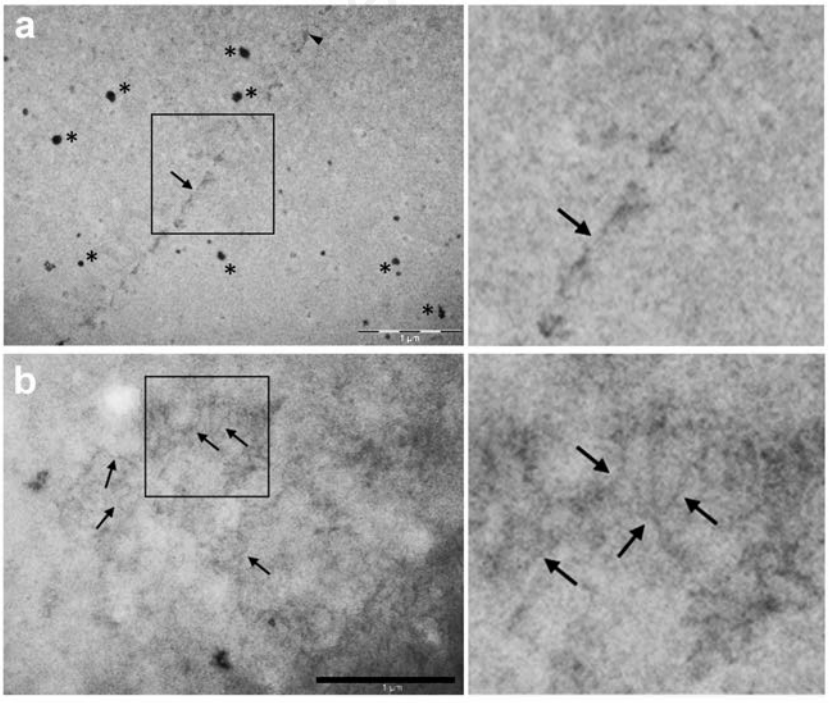

Figure 2. a) Osmium ammine staining. Feebly contrasted nucleic acids can be appreciated (arrow), but evident precipitates appear (asterisks). Proteins are not contrasted. b) Staining with Osmium Ammine preceded by acid hydrolysis (HCl 0.1N). As in d, the acid hydrolysis additional step does not improve the staining result. Note the weakly contrasted nucleic acids (arrows). Bars: 1 µm. a.,b. Areas delimited by the squares are reported on the right of each image at higher magnification to show in detail the contrast provided to the spread chromatin by each staining technique.

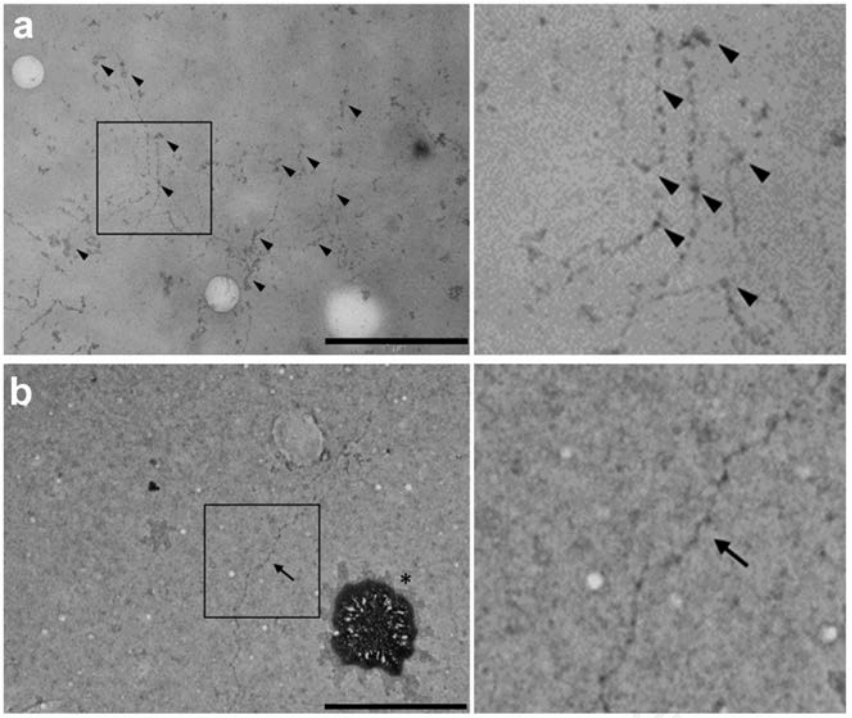

Figure 3. The micrographs show spread cell lysate from Hela cells. Examples of inefficient contrast attempts. a) $KMnO_4$ staining. The image appears free from precipitates, but no contrast of nucleic acids can be appreciated; only protein components are visible (arrowheads). b) PMA staining. The image shows large precipitates (asterisks), which severely disturbs the observation of the feebly contrasted nucleic acids (arrows). Areas delimited by the squares are reported on the right of each image at higher magnification to show in detail the contrast provided to the spread chromatin by each staining technique. Scale bars: 1 µm.

plete nucleic acid degradation. In both cases, the osmium ammine technique still contrasts the isolated nucleic acid, even though the contrast is weak. Indeed, also in tissue sections, the areas characterized by loose chromatin show feeble contrast compared with chromatin domains characterized by a condensed conformation. As expected, proteins are not contrasted, so this biological component is not visible if this technique is chosen (Figure 2 a,b).

Finally, here are a few words to comment on the staining attempt with $KMnO_4$ and PMA (Figure 3). We discourage the use of these techniques.

$KMnO_4$ has been employed in electron microscopy both as a fixative and as a stain. It is considered a good fixative for cytological membranes.[19] An example of cleanly stained tissue ultrathin sections using $KMnO_4$ was reported by Sutton,[20] who stained Araldite- and Maraglas-embedded tissue sections using 0.1 % aqueous solution of $KMnO_4$ for 30 min. Today, this method is seldom used because some researchers complained of the granularity of the sections and their fragility under the electron beam.[21] Instead, we stained chromatin spreads with 0.1% $KMnO_4$ solution and reduced the incubation time to 1 min. This staining modality is rapid, and the result is clean with very few precipitates, but it stains only proteins and not nucleic acids, which are not contrasted (Figure 3a).

PMA, like PTA, is a heteropolyacid and has been reported to behave similarly to PTA in electron microscopy when used to stain thin sections of glutaraldehyde-fixed tissues. In this case, both staining solutions mainly contrast tissue structures containing a significant amount of carbohydrate components.[22] Unfortunately, our protocol for PMA staining of Miller spreads gives only weak contrast and many precipitates (Figure 3b).

In conclusion, some of the staining techniques proposed here, especially the uranyl acetate and terbium citrate staining, could be as valid as the standard PTA staining, allowing even cleaner and stronger signals in certain circumstances. Furthermore, comparing images stained by alternative techniques may allow one to visualize different details and obtain complementary information on chromatin organization, helping to shed light on different aspects of chromatin function.

Moreover, uranyl acetate, which was proven to contrast chromatin spread markedly without making precipitates, is standard staining routinely used in TEM laboratories; this avoids the need to be dependent on the less used PTA solution.

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