The Feulgen reaction at the electron microscopy level

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ABSTRACT

The Feulgen reaction has been the first specific method for detecting DNA available at light microscopy since 1924. However, a similar specific method was proposed for electron microscopy only 50 years later. Here, we discuss the problems encountered in finding the electrondense reagent capable of taking advantage of the extremely high resolution offered by electron microscopy as well as some applications of the method.

Key words: Feulgen reaction; osmium ammine complex; transmission electron microscopy.

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The story so far

In the 1960s there was an explosion of new cytochemical techniques for the high-resolution detection of molecules at electron microscopy (EM).[1] In parallel with new methods for detecting proteins, polysaccharides and lipids, the search begun for finding a reliable way to stain (and consequently localize with unsurpassed resolution) the DNA and only the DNA.

Indeed, a reliable and specific method to selectively stain DNA existed already for light microscopy[2] and had been utilized also for quantifying the DNA content[3] of single cells.

Several researchers tried to translate the Feulgen reaction from light microscopy to EM, but the results were not satisfactory.[4-6] The main problem was related to incomplete staining specificity.[4] The source of the problem lies in the second part of the staining reaction. The Feulgen technique, in fact, is composed of two steps. In the first, the tissue section is subjected to acid hydrolysis, usually by utilizing 5N HCl for about 45-60 min. HCl selectively removes the purine bases from deoxyribose thus engendering the formation of aldehyde groups. It must be noted that the hydrolytic process starts two different processes at the same time: apurination and DNA depolymerization. This latter tends to accelerate with a prolonged treatment time and ends, in due course, with the complete dissolution of DNA as a polymer.[7] Moreover, HCl cannot engender free aldehyde groups on the ribose of RNA and, actually, depolymerizes RNA very fast. Consequently, the reaction concerns only DNA.

The free aldehyde groups on DNA then react in a stoichiometric way with the Schiff reagent.[7] This latter is a transparent solution which resumes its magenta colour in presence of aldehydes. The final result at light microscopy is a nucleus (or chromosomes) specifically stained. However, the Schiff reagent is very weakly electrondense and any attempt to increase its electrondensity *via* heavy metals resulted in a more or less severe loss of specificity.[4,5] Once again part of the solution came from light microscopy observations. Feulgen and Voit[8] demonstrated that it was possible to reveal aldehyde groups with ammoniacal silver. Thus, the very first Feulgen-like staining at EM was due to Breitschneider[9] who stained a whole sperm head (not a section). Peters[10] then produced a silver methenamine staining, revealing, beside cell nuclei, viral particles.

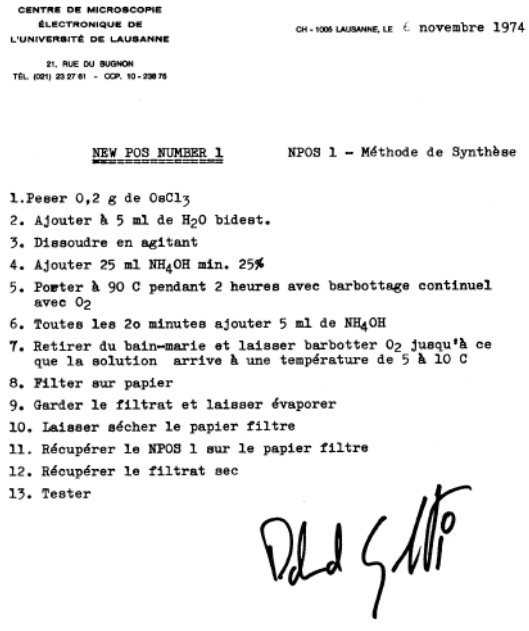
During the search for a reliable staining, Gautier[4] tested several compounds on thin sections, among which ruthenium red, ammonium hexachloroosmate, Bismarck brown and others. The best results were obtained by Moyne,[11] with a reaction involving thallium ions; the method was difficult to carry out, and (due also to the poisoning effects of thallium) not much utilized. Consequently, the search for an EM-dedicated Schiff reagent started.

The ideal reagent would display amino/ammino groups as well as an electrondense core. After several years of attempts Cogliati and Gautier[12] succeeded in synthesizing an osmium-based polyammine which behaved like a true Schiff reagent and was perfect both for Feulgen-type and PAS-type reaction at EM. The reagent was called osmium ammine complex (OA). In their first paper as well as in the following ones[11,12] all the tests of specificity were satisfied. The real problem, however, was the reproducibility of the synthesis procedure. The slow chemical synthesis (more or less one week) although detailed in the protocols I could examine in Gautier's lab at the Centre of Electron Microscopy in Lausanne, was not so precise. At a given point, for instance the solution was ready when turning burgundy in colour, or needed an extra oxygen bubbling if the colour was like brandy. In Figure 1, a protocol signed by Roland Cogliati is shown. It is a more stringent procedure, but also in this case it worked in 50% of the trials. Very often, indeed, the final black powder could stain both DNA and RNA (and probably proteins). Eventually, the only lab which was successful in the synthesis was Derenzini's lab in Bologna. To solve the situation, Olins and coworkers[14] proposed a standardized procedure for the synthesis of osmium ammine B (OA-B). The synthesis gave a brownish powder, very fine, which yielded an almost grainless end-product and was, most importantly, reproducible. The reagent was then commercialized, and it is still available.

An important modification of the technique was proposed.[15-17] So far, the active reagent was prepared by dissolving the OA powder in H_2O and then bubbling with SO_2. In these papers, the use of acid and metabisulfite added to the solution avoided the use of gaseous and potentially harmful SO_2. In our lab, we have constantly used this last modification since it has an additional advantage. OA-B treated with metabisulphite can be used for a couple of weeks, while the SO_2-bubbled solution must be used within a day.

Another, although often forgotten, problem is related to the embedding medium. The first attempts of acid hydrolysis were carried out on glycolmethacrylate or Vestopal embedded tissues, with varying and, in some cases, destructive results. Then came the epoxies, Epon and Araldite, which offered a much higher stability of the section to the harsh treatments. In this context one must consider that SO_2-bubbled OA has a pH of about 0.8. Epoxy resins proved to be the best in term of resolution, whereas the overall contrast was not comparable to acrylic resin embedded specimens.

A particular property of OA-B was shown by Derenzini and Farabegoli[18] on methacrylate sections: the reagent, in the absence of any hydrolysis, could bind to both DNA and RNA due to electrostatic interactions. The use of DNase or RNase could then help in detecting either nucleic acid. This behaviour of OA-B was exploited by Olins and coworkers on Balbiani ring granules.[19, 20]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CH - 1005 LAUSANNE, LE 6 novembre 1974

NEW POS NUMBER 1 NPOS 1 – Méthode de Synthèse

1. Peser 0,2 g de $OsCl_3$
2. Ajouter à 5 ml de H_2O bidest.
3. Dissoudre en agitant
4. Ajouter 25 ml NH_4OH min. 25%
5. Porter à 90 C pendant 2 heures avec barbottage continuel avec O_2
6. Toutes les 20 minutes ajouter 5 ml de NH_4OH
7. Retirer du bain-marie et laisser barbotter O_2 jusqu'à ce que la solution arrive à une température de 5 à 10 C
8. Filter sur papier
9. Garder le filtrat et laisser évaporer
10. Laisser sécher le papier filtre
11. Récupérer le NPOS 1 sur le papier filtre
12. Récupérer le filtrat sec
13. Tester

Figure 1. Reproduction of a protocol, signed by Roland Cogliati, reporting a modification in the synthetic procedure of osmium ammine, posterior to the original published paper. The reagent is named New POS (POS stands for Préparation d'Osmium – in French).

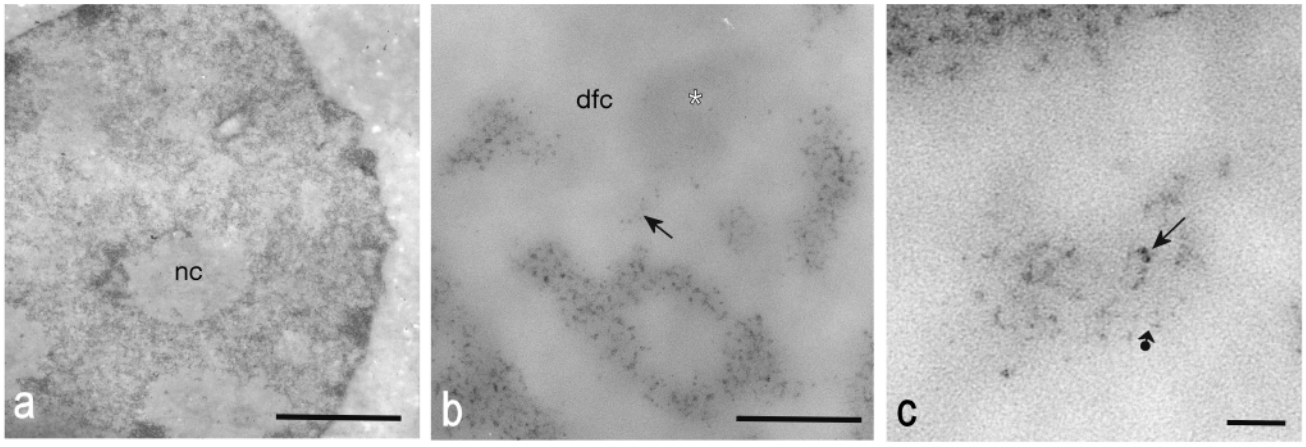

Figure 2. OA staining of a P815 cell nucleus. **a**) Only DNA is stained, the contrast in the cytoplasm is due to acrylate embedding; note the nucleoli (nc) which appear clearer than the surrounding nucleoplasm; scale bar: 2.5 µm. **b**) High magnification of the nucleolar chromatin; dfc, dense fibrillar component; asterisk, fibrillar centre; arrow, thin fibres of DNA; scale bar: 500 nm. **c**) In the inner part of the nucleolus, fibrils of naked DNA (arrowhead) as well as in the nucleosomal configuration (arrow) are present; scale bar: 100 nm.

Applications of osmium ammine

In the very first papers, OA was shown not only to be specific but also capable of giving a high resolution end product, *i.e.*, a very fine-grained staining.[4,12] In Figure 2, an example of DNA staining and its resolution is shown. The first top level results were published by the group of Derenzini[21,22] with the high resolution visualization of nuclear distribution of DNA in fibrils *in situ* in the nucleus, including the picture of DNA forming nucleosomes and leaving the unstained histone core. Moreover, the same group showed the presence of DNA in discrete foci inside the nucleolus, including areas in which the DNA was naked, not in a nucleosomal configuration.[23,24] Several other papers utilized OA on different tissue and cell models and even on ultrathin cryosections.[25] An interesting application of the reaction was published in 1985 on mouse sperm cells[26] showing a leopard-skin like pattern of the DNA distribution. This strange appearance was also obtained by Courtens and colleagues[27-29] in other species, leading to the conclusion that microheterogeneities in DNA compaction were present in the apparently homogeneous sperm cell nucleus. Fakan and Odartchenko[30] showed the presence of a DNA rim around the mouse embryo in 2-cell and 4-cell embryos and followed the movement of DNA inside the reactivating nucleolus. A review of several applications of OA[31] showed the potential of this technique on thin sections. Other applications were, for instance, made to the *in situ* configuration of viral genomes in virus-infected cells.[32] The authors studied adenovirus, poxvirus, herpes virus and SV40. By far, chromatin structure was the prime target for DNA staining by OA. In a review, Derenzini and coworkers[33] extended the approach to the nuclear chromatin compartmentalization and explored the unravelling of chromatin during lymphocyte activation by phytohemagglutinin. On the medical side, megakaryoblastic leukaemia was also studied.[4] el-Alfy and colleagues[35] studied DNA changes involved in the formation of metaphase chromosomes in mouse duodenal crypt cells stained by OAC. They described new structures appearing during the S phase and condensing at prophase into "chromomeres" which fuse at prometaphase into mitotic chromosomes. Liu *et al.*[36] in the same tissue model followed by OA the nascent DNA labelled by bromodeoxyuridine into discrete structures arising in S phase. Finally, a very interesting point is the possibility to combine OAC staining with immunocytochemistry: as an example, Biggiogera *et al.*[37] demonstrated the presence of DNA-related phosphorous by EFTEM as well as OA staining in the dense fibrillar component of the nucleolus. A few words must be added to the story of OA. From the very beginning, Gautier proved the true Schiff-like nature of this reagent also by PAS reaction for polysaccharides. Von Schack and Fakan[38] demonstrated the retention of glycogen in high-pressure frozen, cryosubstituted mouse liver, testing the technique for the first time on a chemically-unfixed specimen.

Future perspectives

EM is a "stable" technique, in the sense that the basics of the preparation of the specimens have been established some 60 years ago, with a few exceptions including the use of low temperature embedding. Almost everything is standardised and there is only need for the curiosity of young researchers to continue exploiting its unsurpassed high resolution. Osmium ammine is then qualified as an established reagent ready to be used for detecting DNA even in extremely small amount.

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