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IN SITU HYBRIDIZATION AT THE ELECTRON MICROSCOPE LEVEL

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Abstract

This tutorial paper will review radioactive and non-radioactive in situ hybridization (ISH) techniques at the electron microscope (EM) level; describe our efforts in comparison to those of other groups who recently have published in this field and discuss some potential future applications. Our contribution is the development of a non-radioactive, postembedding technique for the detection of transcripts on thin sections of Lowicryl K4M-embedded cells or tissues. Biotinylated probes were prepared by nick-translation. Signal detection was accomplished with anti-biotin antibody and protein A-gold complexes. Specific labeling was obtained over structures known to be the site of expression. Compared to EM autoradiography this technique offers advantages: (a) rapid signal detection; (b) superior morphological preservation and (c) superior spatial resolution.

KEY WORDS: Biotinylated probes, protein A-gold complexes, Lowicryl K4M-embedding, nick-translation, mitochondrial large rRNA and U1 small nuclear RNA expression, Drosophila ovaries.

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Introduction

The technique of ISH at the light microscope (LM) level was introduced in 1969 by Gall and Pardue (16) and John et al. (25). With its potential to visualize specific DNA and RNA sequences it had a continuing impact on diagnostic pathology, molecular genetics and developmental biology.

For obvious reasons - namely correlation of DNA replication and transcription phenomena with ultrastructural details - it was attempted to extend ISH to the EM level soon after its development. Two lines of development were pursued: one was EM visualization of nucleic acid hybrids by means of spreading techniques (not dealt with in this paper; for review see Brack (8)); the other was ISH on thin sections of resin embedded tissue. Initially, approaches to ISH on thin sections were adaptations of LM level procedures of ISH for hybridization and signal detection (i.e. radiolabeled probes and autoradiography). Recently, however, with the advent of non-radioactive probes (6, 9, 13, 20, 21, 26, 28, 36, 40, 43, 45) in consort with sensitive immuno- or affinity-cytochemical signal detection (27, 29, 31, 33, 36, 41), new aspects for improvement of ISH at the EM level emerged. This in turn made the prospects of alternative approaches worth exploration.

History of EM level ISH

The first paper on this subject was published in 1971 by Jacob et al. (23). They succeeded in hybridizing tritiated rRNA to DNA in Xenopus oocytes at the EM level. This was a postembedding ISH technique where necessary pretreatments (treatment with ribonuclease, denaturation) as well as hybridization were carried out directly on thin sections of glycol methacrylate embedded oocytes. Croissant et al. (11), and Geuskens et al. (17) used preembedding

EM level ISH, i.e., embedding after pretreatments and hybridization.

With these early attempts only highly reiterated DNA sequences could be visualized. The probes used in these experiments were isolated and purified by standard physical methods. Radiolabeling of probes was performed either in vivo in the presence of ^{3}H-Uridine prior to isolation or after purification by in vitro transcription. Signal detection at the EM level was accomplished by autoradiography.

The above methods were subject to several limitations: long exposure times, limited resolution, low sensitivity and low efficiency. Thus, it came as no surprise that these somewhat cumbersome techniques had not gained much attraction. A number of developments over the past years have improved the utility of LM ISH techniques (i.e. the availability of pure probes in sufficient quantity by recombinant DNA technology, non-radioactive probes and non-radioactive signal detection). The basic principle of non-radioactive probes is the chemical or enzymatic incorporation of so called "reporter molecules" into DNA or RNA sequences which can then be detected immuno- or affinity-cytochemically. The obvious advantages of non-radioactive over radioactive probes are (a) stability, (b) high resolving power, (c) no hazards, (d) no disposal problems, (e) rapid signal detection and (f) modest requirements of laboratory facilities. The main disadvantage was claimed to be their relatively low sensitivity, although detection levels comparable to those of radioactive probes have been reached on blot hybridization (9). Sensitivity enhancement was improved at various levels of the ISH procedure, for example by optimizing the hybridization efficiency, by increasing the amount of label at the hybridization site (30) or by physical means with reflection-contrast microscopy (27).

These developments at the LM level had little effect on EM level ISH. Although Manning et al. were the first to exploit the biotin-avidin interaction for ISH in 1975 (33), it was not until 1982 that biotinylated probes were successfully applied for ISH to whole mount chromosomes at the ultrastructural level (22, 34). Hutchison et al. (22) hybridized biotinylated mouse satellite DNA and cRNA to whole mount chromosomes using anti-biotin antibody and a secondary antibody-gold complex for detection. Manuelidis et al. (34) also hybridized mouse satellite DNA to chromosomes. For signal detection, however, they used anti-biotin antibody and peroxidase labeled anti-rabbit IgG. With this technique major tRNA gene cluster of Xenopus laevis (14) and a human interspersed repetitive DNA (35) were localized on chromosomes and - very recently - the relationship between mouse satellite DNA, sister chromatid pairing and centromere function was studied (32).

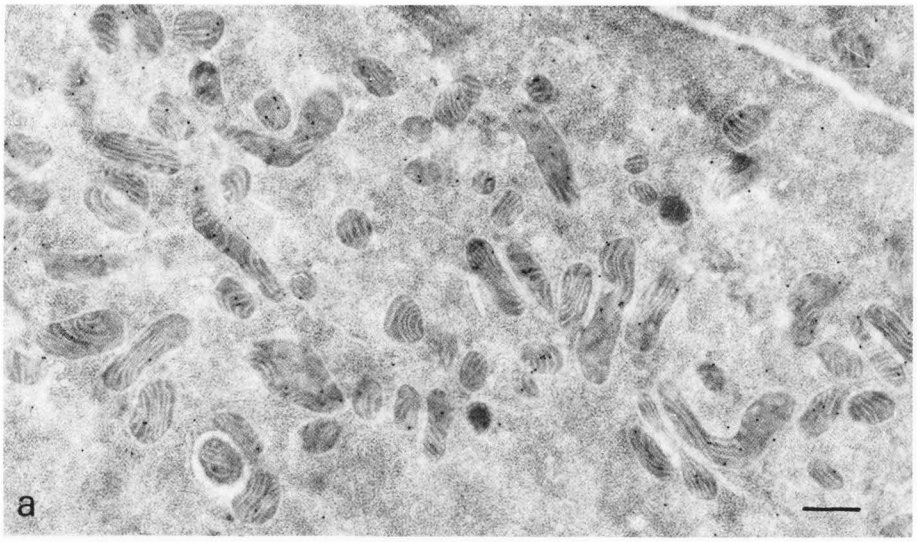
For non-radioactive localization of transcripts on thin sections at the EM level, however, no comparable method was available. Prompted by the progress in the field of developmental biology (1, 2, 4, 5, 10, 18, 19, 24, 44) which was brought about by LM level ISH in order to answer questions concerning gene expression and its modulation during developmental transitions, we set out to establish an appropriate technique at the EM level.

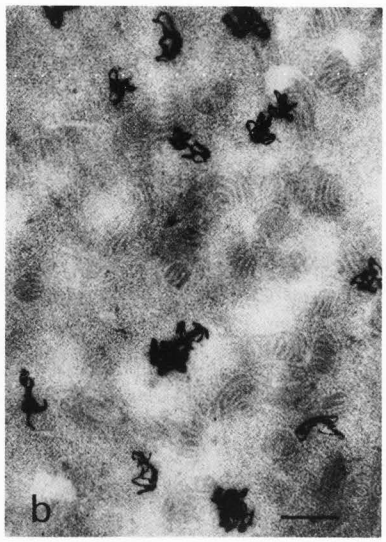
Conceptual approach

The establishment of a new technique is a multistep task since this entails that each parameter has to be appropriately controlled and optimized. In our approach we (a) tested the suitability of the low temperature embedding resin Lowicryl K4M by checking RNA retention and accessibility by acridine orange staining on semi-thin sections; (b) tested the sensitivity of the detection system - namely - anti-biotin antibody in consort with protein A-gold complexes with dot blots; and (c) developed hybridization and signal detection protocols for ISH to semi-thin and ultrathin sections of Lowicryl K4M embedded tissue for radiolabeled and biotinylated probes. We chose mitochondrial large rRNA and small nuclear RNA expression in Drosophila ovaries as our model systems since the transcripts are assumed to be confined within well-defined structures (i.e., mitochondria and nuclei).

Chronologically the first question to be answered was whether RNA was retained in the embedding resin and moreover, whether it was accessible to the hybridization probe on the section profile in our postembedding approach. As mentioned above, this was checked by acridine orange staining. The positive results obtained with this staining technique on semi-thin sections strongly favored the use of Lowicryl K4M. Additional aspects in favor of this resin are as follows: (a) methacrylate embedding has already been successfully used for ISH techniques (24, 42); (b) this highly crosslinked acrylate- and methacrylate-based hydrophilic resin has shown to provide superior preservation of structure together with significantly lower background labeling compared to other resins and (c) the low temperature embedding protocol may prevent enzymatic degradation of RNA.

The next logical step was to check signal generation with conventional LM





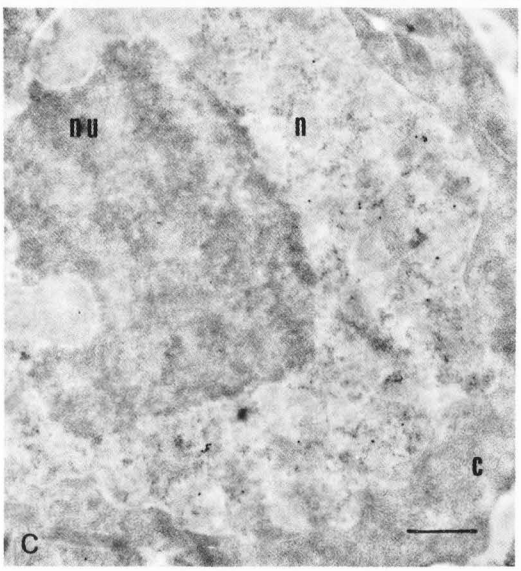


Fig.1. EM level ISH.
(a) Follicle cell hybridized with biotinylated mitochondrial rDNA, signal detection with anti-biotin antibody in consort with protein A-gold. The gold particles can be observed over mitochondria. The labeling pattern is heterogeneous. Morphometric analysis showed that approximately one-third of the mitochondria is unlabeled, nearly one-third is labeled with one gold particle, and the residual third is labeled with more than one gold particle with a number being reciprocal to the frequency of occurrence. With serial sectioning we found that the labeling pattern of individual mitochondria changed on consecutive sections.
(b) Follicle cell hybridized with ^{3}H-labeled mitochondrial rDNA; signal detection with autoradiography (exposure 2 months). The labeling pattern obtained with the tritiated rDNA probe is similar compared to that of Fig.1a. It is evident that the autoradiographic detection system is inferior to the biotin system in terms of spatial resolution and structural preservation (morphological details of mitochondria are somewhat obscured).
(c) Nurse cell; hybridization with biotinylated U1 probe. Signal detection with anti-biotin antibody and protein A-gold. The gold particles are observed over the nucleus. Note that the nucleolus is free of label. n = nucleus, nu = nucleolus, c = cytoplasm. Bar = 0.5 µm.

ISH on semi-thin sections (i.e., nick-translated, radioactive probes and autoradiography). Since label was found at the expected sites (cytoplasmic labeling with the mitochondrial rDNA probe, nuclear labeling with the small nuclear U1 probe), the influence of various fixation protocols and pretreatments on labeling intensity were tested. Common pretreatments for hybridization to cellular RNA at the LM level are protease treatment in order to improve access of probe to RNA, and acetylation for reduction of non-specific binding. In agreement with Steinert et al. (42) the same results were obtained whether or not pretreatments were performed before hybridization.

The sensitivity test of the intended EM detection system (anti-biotin antibody-protein A-gold system) with dot blots of the biotinylated probes was then carried out. A sensitivity of 1-5pg could be obtained with visual dot blots on nitrocellulose. However, one should keep in mind that magnification with the EM, per se, enhances sensitivity. For example, the accumulation of a few gold particles over a specific binding site - never visible with the naked eye or under LM - can be detected under the EM.

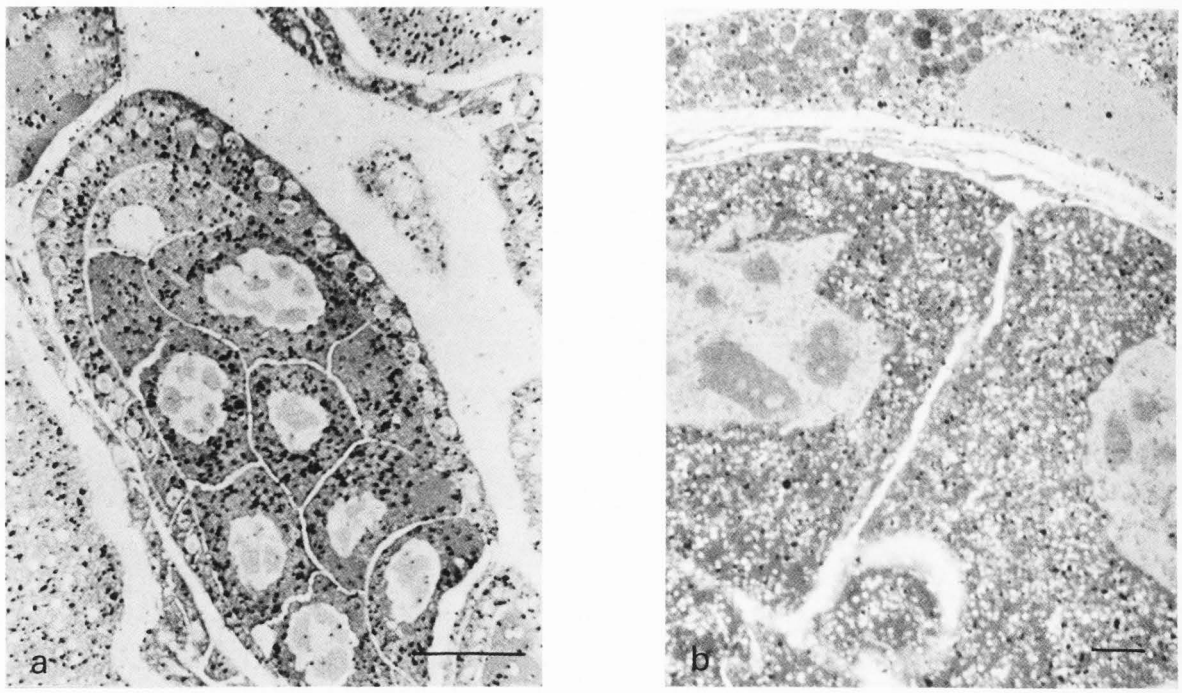


Fig.2. Comparison of hybridization using either tritiated or biotinylated probes on Lowicryl K4M semi-thin sections.
(a) LM autoradiograph of a late stage ovarian chamber hybridized with tritiated mitochondrial rDNA. Note that nuclei of nurse cells and nuclei of follicle cells are free of label.
(b) Late stage ovarian chamber. Hybridization with a biotinylated mitochondrial rDNA probe. Non-radioactive signal detection with anti-biotin antibody -protein A-gold - anti-protein A antibody -protein A-gold - silver enhancement. The labeling pattern resembles that of LM autoradiography (Fig.2a). The only difference appears to be the heterogeneous grain size compared to autoradiography. Bar = (a) 50 µm; (b) 10 µm. With the permission of the publisher, The Rockefeller University Press.

Taking all the foregoing into account, we arrived at the crucial point - that is to say, the localization of hybridization sites on thin sections with biotinylated probes (prepared by nick-translation using biotinylated dUTP). It turned out that some procedures common in conventional ISH cannot be used with the biotinylated probes; at least not at the EM level. Addition of formamide to the post-hybridization washing buffer as well as acetylation for reduction of non-specific binding was deleterious at the EM level.

After having succeeded with the EM demonstration of the two RNA species on thin sections (Fig.1, a, b and c), we asked ourselves if it were possible to adapt this technique for LM level ISH. In the EM version (hybridization with biotinylated probes, anti-biotin antibody, protein A-gold) no signal could be detected on semi-thin sections under the LM. However, after substantial amplification (hybridization with biotinylated probes, anti-biotin antibody, protein A-gold, antiprotein A antibody, protein A-gold and silver enhancement) a labeling pattern could be obtained which closely resembled that obtained with radioactive probes (signal detection with autoradiography) (Fig. 2, a and b). Controls such as hybridization with the nick-translated plasmid (without insert) or omission of anti-biotin antibody, yielded no labeling above background. Furthermore, morphometric analyses at the EM level was done on 608 different mitochondrial profiles. This convincingly demonstrated labeling densities significantly above background over the respective structures (mitochondria, nuclei).

Signal-to-noise ratios (S/N) of the radioactive and the non-radioactive techniques at both levels (LM and EM)

compared well. They ranged from 5.4 - 10.2. The S/N ratio of 7 obtained with hybridization of biotinylated rDNA probes to thin sections also compares well with that obtained by Hutchison et al. (22) with hybridization of a biotinylated probe to whole mount chromosomes at the EM level. This is the only data available in literature which has been obtained using a technique similar to ours.

The usefulness of an ISH technique depends on the sensitivity achievable with this method and the precision with which target molecules can be localized. In our case a sensitivity check for the signal generating system is feasible (with dot blots as mentioned) whereas an accurate overall estimate of sensitivity is not possible due to several yet unknown parameters on which sensitivity depends. To obtain a rough estimate of the order of magnitude of sensitivity of hybridization plus signal detection we extrapolated from morphometric and biochemical data. This calculation yields the number of approximately 10 RNA molecules equalling one gold particle of specific label. Precision of localization with an EM technique is superior compared to an LM one and moreover, a non-radioactive EM technique is superior compared to a radioactive one. In conclusion with the described technique it should be possible to visualize moderately abundant RNAs on thin sections.

Methods

Since the original protocol is described fully elsewhere (7) the procedures will be only briefly listed in this paper.

Tissue processing

Drosophila ovaries were fixed in 4% formaldehyde containing 0.1% glutaraldehyde. Dehydration and infiltration at low temperatures with Lowicryl K4M were performed as previously described (39).

Preparation of protein A-gold complex

Colloidal gold (14 nm) was prepared according to the method of Frens (15). Protein A was complexed to colloidal gold as previously described (38).

Probes and nick-translation with ^{3}H-labeled and biotinylated probes

Two different Drosophila DNA probes have been used in this study: a plasmid containing a 1.05-kb EcoRI fragment of the mitochondrial large rRNA gene cloned into pBR 322, and a second plasmid containing 131 nucleotides from the U1 snRNA gene cloned at the Hinc II site of pUC8 (3). ^{3}H-labeled hybridization probes were prepared by nick-translation according to the protocol of Hafen et al. (18).

Biotinylated probes were prepared essentially according to the above protocol for ^{3}H-labeled probes with the following modifications: instead of the tritiated nucleotides a mixture of dATP, dCTP and dGTP, biotinylated dUTP (Bio-11- or Bio-16-dUTP; Enzo Biochem. Inc. New York) and ^{32}P-dCTP were added to the reaction mixture. The preparations were checked for probe length (35 - 120 nucleotides) by acrylamide gel electrophoresis (5%) and incorporation of label by trichloroacetic acid precipitation.

LM in situ hybridization on semi-thin sections

Before hybridization with tritiated probes, semi-thin sections were subjected to acetylation with acetic anhydride in order to diminish background. Then they were washed in PBS and finally the sections were dehydrated in a graded series of ethanol. Hybridization, washing in formamide buffer, dipping slides in emulsion, exposure and development were carried out as described by Hafen et al. (18). Biotinylated probes were denatured prior to hybridization. Hybridization was performed at 37°C for 5 - 10 h. Subsequently sections were washed five times for 10 min in PBS and then incubated with rabbit anti-biotin antibody for 2 h at room temperature. After two washes with PBS the sections were incubated with protein A-gold (1 h) followed by anti-protein A antibody (2 h), and again protein A-gold (1 h) with washes between incubations. Finally, sections were washed with distilled water and air dried. Silver enhancement of the gold signal was carried out as described by Danscher and Rytter Nørgaard (12).

EM in situ hybridization on thin sections

^{3}H-labeled probes were denatured by boiling. Hybridization was performed by floating the grids on drops of the hybridization mixture in a moist chamber for 24 h at 37°C. After hybridization, the grids were placed on drops of formamide buffer at 37°C for approximately 20 h with several buffer changes. The grids were then washed with PBS, distilled water and air dried. L4 Ilford emulsion was applied to the grids with the loop technique. Exposure was carried out at 4°C. After exposure autoradiograms were developed, fixed, rinsed with water, air dried and stained with uranyl- and lead acetate.

After denaturation, the biotinylated probes were hybridized as afore mentioned from 1 - 24 h at 37°C. Subsequently the grids were rinsed five times for 10 min each by floating them on drops of PBS. After incubation with the antibody (2 h at room temperature), grids were washed twice with PBS. Then they were incubated with the protein A-gold complex for 1 h at room temperature as described above. Finally the grids were washed in PBS and distilled water. The air dried sections were stained with uranyl- and lead acetate.

Potential future applications

The ability to identify RNA molecules on thin sections may be the starting-point for the study of all aspects of RNA processing at an ultrastructural level. One could ask whether defined spatial domains are involved within the nucleus or whether three dimensional topography is important in higher order regulation of gene expression. Furthermore, one could simultaneously localize a species of mRNA molecules and the corresponding protein on the same section using double labeling techniques with gold markers of different size (37). Such an approach would assist our understanding of regulatory systems for tissue differentiation.

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Discussion with Reviewers

R. Albrecht: Have different size gold beads been used with this procedure; if so is there a particular size which is superior?
Author: We used 8 nm and 14 nm gold particles; the former prepared by tannic acid - the latter by citrate reduction. Both sizes performed equally well in our procedure. Eventually, we decided to use 14 nm gold particles because of their better visibility on the micrographs at the magnification used.

R. Albrecht: What is the size, roughly, of the enhanced gold labels compared to the silver grains in the radiographic emulsion?
Author: The size of the developed silver grains of the Kodak NTB-2 emulsion which we used for radioactive LM ISH is approximately 0.4 µm with size variations depending on conditions of development.

The particle size of the silver enhanced gold signal detected with our non-radioactive LM ISH is roughly within the

same size range. However, as can be seen from Fig. 2 b there is a considerably greater variation in grain size compared to that of autoradiography.

E. Carlemalm: Can you explain how the magnification enhances the sensitivity of the labeling?
Author: Whenever one can observe a gold label consisting of a few gold particles over a particular cellular structure under the EM (verified as specific label with the appropriate control experiments) the same signal will not be detectable without substantial amplification under LM conditions on semi-thin sections (in our case with anti-protein A antibody, protein A gold and silver enhancement). Therefore it makes sense to say that magnification with the EM per se enhances sensitivity of ISH.

E. Carlemalm: Why have you not used acridine-gold complexes?
Author: Colloidal gold in water carries a negative net surface charge and its stability is maintained by electrostatic repulsion. The addition of electrolytes causes flocculation of the colloidal gold particles whereas they are stabilized against flocculation by electrophilic macromolecules such as proteins or glycoproteins. Acridine orange is a dye salt; that means it is an electrolyte which probably rather causes flocculation than stabilization of the colloidal gold. However, acridine compounds could be covalently attached to a protein (e.g. bovine serum albumin) and used for complex formation in the conjugated form. Then it could be used as reagent for DNA or RNA detection on sections at the EM level. It was not our intention to do this; we only wanted to check the accessibility of RNA molecules on semi-thin sections of K4M embedded tissue with acridine orange staining.

Notes added in Proof

During the preparation of this manuscript Harris and Croy (20) published a non-radioactive preembedding method for the localization of mRNA for pea legumin on thin sections of cotyledon storage parenchyma tissue of developing pea seeds embedded in Spurr resin. The biotinylated cDNA probe was hybridized to fixed tissue slices. For signal detection at the ultrastructural level they used avidin-peroxidase and avidin-ferritin.