NEWSLETTER

an immunogold users exchange platform for practical problem solving

nr. 3

In Situ Hybridization
for
Light and Electron Microscopy
using
Ultra Small Immunogold Detection
and
Silver Enhancement

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AIM of this NEWSLETTER

Whereas immunocytochemistry (IC) mainly deals with the localization of specific proteins, in situ hybridization (ISH) aims at the localization of specific DNA or RNA sequences. Both approaches require the preservation and exposure of target molecules in biological specimens as well as the preservation of ultrastructural details to allow high resolution detection. Since between the two approaches target molecule chemical characteristics differ significantly, specimen preparation conditions as well as specific detection procedures will have to be adapted accordingly.
In this Newsletter procedures are presented that allow the sensitive intranuclear and cytoplasmic detection of 28S ribosomal RNA (rRNA) by ISH as a model system. To this end a specimen preparation technique was adapted to allow preservation and exposure of RNA. Secondly, a pre-embedding immunogold detection procedure is presented which warrants a high degree of penetration and detection efficiency. As a derivative a post-embedding procedure is presented. Apprehensive schedules and extensive procedures illustrate the specimen preparation techniques used, as well as the in situ hybridization and the pre and post-embedding labeling protocols.

Although 28S rRNA is quite abundant the high label density and structural integrity provide a firm basis for the sensitive detection of low copy numbers of both RNA and DNA sequences.

INTRODUCTION

An extensive number of biochemical studies deal with nuclear functions like replication of DNA, transcription and metabolism of mRNA. However relatively little is known about the localization of these nuclear functions. In situ hybridization, a detection technique which enables the localization of specific nucleic acid sequences, will be of help to place the nuclear functions in a morphological context. Especially when applied at the electron microscopical level, in situ hybridization enables the correlation of for instance specific RNA sequences with cytoplasmic and nuclear structures like the cytoskeleton, interchromatin granules, nucleolar sub-structures, lamines and the nuclear matrix.
The nuclear matrix is defined as the structure which remains after removal of most of the DNA with DNAse and high salt. The structure is thought to play an essential role in nuclear functions (reviewed in Verheyen et al 1988) and is a topic of research at the Molecular Cell Biology Department. Localization of specific RNA sequences in such preparations may elucidate the involvement of the nuclear matrix in nuclear functions.

Gold particles are the marker of choice for the electron microscopical detection of target molecules. The use of ultra small gold particles, the smallest available, in combination with silver enhancement results in maximized labeling efficiency (De Graaf 1991; Humbel and Biegelmann 1992) and the possibility to evaluate the results both at the light and electron microscopical level.
The combined in situ hybridization and immunogold detection procedures can be performed in the pre and post-embedding mode.
Post-embedding in situ hybridization/immunogold labeling (ISH/IG) is performed on sections. The specimen preparation steps (fixation, dehydration and embedding) are critical for the adequate preservation of the target sequences. As opposed to pre-embedding approaches it enables the localization of different nucleic acid sequences in serial sections.
Pre-embedding ISH/IG is performed on fixed but unembedded cells or tissue. It is only feasible when the target nucleic acid sequences are within reach of both the nucleic acid probe and the immunogold reagent. Limited accessibility makes it necessary to apply detergents (see for an extensive description of pre-embedding immunocytochemical localization using ultra small gold conjugates the AURION Newsletter nr. 2).

It has already been shown that nuclear proteins can be successfully localized in nuclear matrix preparations, using pre-embedding immunolabeling with ultra small gold conjugates and silver enhancement (de Graaf 1992). We have combined this pre-embedding labeling technique with in situ hybridization experiments for the localization of specific RNA sequences in the nuclear matrix. The pre-embedding in situ hybridization approach was developed since pre-embedding labeling of permeabilized specimens results in in-depth labeling throughout the whole cell or nuclear matrix, whereas with post-embedding labeling only those targets can be reached which are exposed at the surface of resin sections. Using this approach EGF-receptor transcripts have been successfully localized in nuclear matrices (submitted for publication).

With the pre-embedding approach we localized 28S rRNA using a digoxigeninylated DNA probe specific for human 28S rRNA and a F(ab) anti-digoxigenin Ultra Small ImmunoGold conjugate. The 28S rRNA was detected in the nucleolus, a dense and relatively difficultly accessible nuclear structure.

In this Newsletter a detailed protocol is presented for the use of Aurion ultra small gold conjugates in the pre-embedding in situ hybridization study as mentioned above. It is demonstrated that with this labeling method, hybridization and detection methods can be optimized first at the light microscopical level before proceeding with electron microscopy.
The light microscopical detection obtained with the immunogold conjugate is related to the detection using alkaline phosphatase as a marker.

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MATERIALS

Unless otherwise indicated all chemicals were obtained from MERCK and were of pro analysi grade.

Triton-X-100, Dextrane sulphate (D-6001), Bovine Serum Albumin (A-4503), Polyvinylpyrrolidone (PVP-10) and Tween-20® were obtained from Sigma,
Vanadyl ribonucleoside complex from Gibco,
Formamide (deionized) from Fluka,
Ficoll 400 from Pharmacia (17-0400-01),
Herring sperm DNA, Yeast tRNA and F(ab)-anti-Digoxigenin-alkaline phosphatase conjugate from Boehringer Mannheim,
Cold water fish skin gelatine and F(ab)-anti-Digoxigenin Ultra Small Gold conjugate from AURION

RNAse inactivation of Buffers:

All buffers that were used before fixation were made RNAse-free by addition of diethylpyrocarbonate (0.05%). Buffers were left for at least 16 hrs while stirring and autoclaved before use.

SOLUTIONS

CSK-buffer, pH 6.8:

(cytoskeleton stabilizing buffer)
100 mM NaCl
2 mM $MgCl_2$
0.5 mM $CaCl_2$
1% Triton-X-100
10 mM Pipes
300 mM Sucrose*
4 mM Vanadyl ribonucleoside

*Sucrose (RNAse-free) was added after autoclaving

2xSSC , pH 7.3:

30 mM Sodium citrate
300 mM NaCl

Hybridization mix:

50% Formamide
5% Dextrane sulphate
0.3% Bovine Serum Albumin
0.3% Ficoll ®
0.3% Polyvinylpyrrolidone
0.2 mg/ml Herring sperm DNA
0.2 mg/ml Yeast tRNA
in 2xSSC
1 ng/μl Digoxigenin labeled 28S DNA probe (average length 200-300 base pairs)

Plasmid specific for 28S human rRNA containing a 2.1 kb insert (bauman and Bentvelzen, 1988) was labeled by random priming with digoxigenin according to the applications manual of Boehringer Mannheim.
In control experiments a plasmid without the 28S DNA insert was used.

PBS-buffer, pH 7.4:

10 mM phosphate buffer (Na_2HPO_4, KH_2PO_4)
150 mM NaCl

PBG-buffer, pH 7.4:

PBS buffer with
0.5% Bovine Serum Albumin
0.1% Cold Water Fish Skin Gelatine

AP-incubation buffer, pH 7.4:

100 mM Tris/HCl
150 mM NaCl
1% Bovine Serum Albumin
0.3% Tween-20®

AP-wash buffer, pH 7.4:

100 mM Tris/HCl
150 mM NaCl

Silver enhancement (Danscher, 1981)

Solution A: Protective colloid

100 g Gum Arabic
in distilled water, final volume 200 ml.

Solution B: Buffer

2.55 g citric acid (1.H_2O)
2.35 g sodium citrate (2.H_2O)
in distilled water, final volume 10 ml.

Solution C: Developer

0.57 g Hydroquinone
in distilled water, final volume 10 ml
(use immediately!)

Solution D: Silver ion supply

0.073 g Silver lactate (Fluka)
in distilled water, final volume 10 ml
(use immediately, protect from light!)

Working Solution:

6 ml Solution A
1 ml Solution B
1.5 ml Solution C
Mix on Vortex
1.5 ml Solution D
Mix on Vortex
(use immediately, protect from light!)

AP-staining buffer, pH 9.5

100 mM Tris/HCl
100 mM NaCl
50 mM $MgCl_2$
immediately before use add:
0.33 mg/ml nitro blue tetrazolium and
0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate

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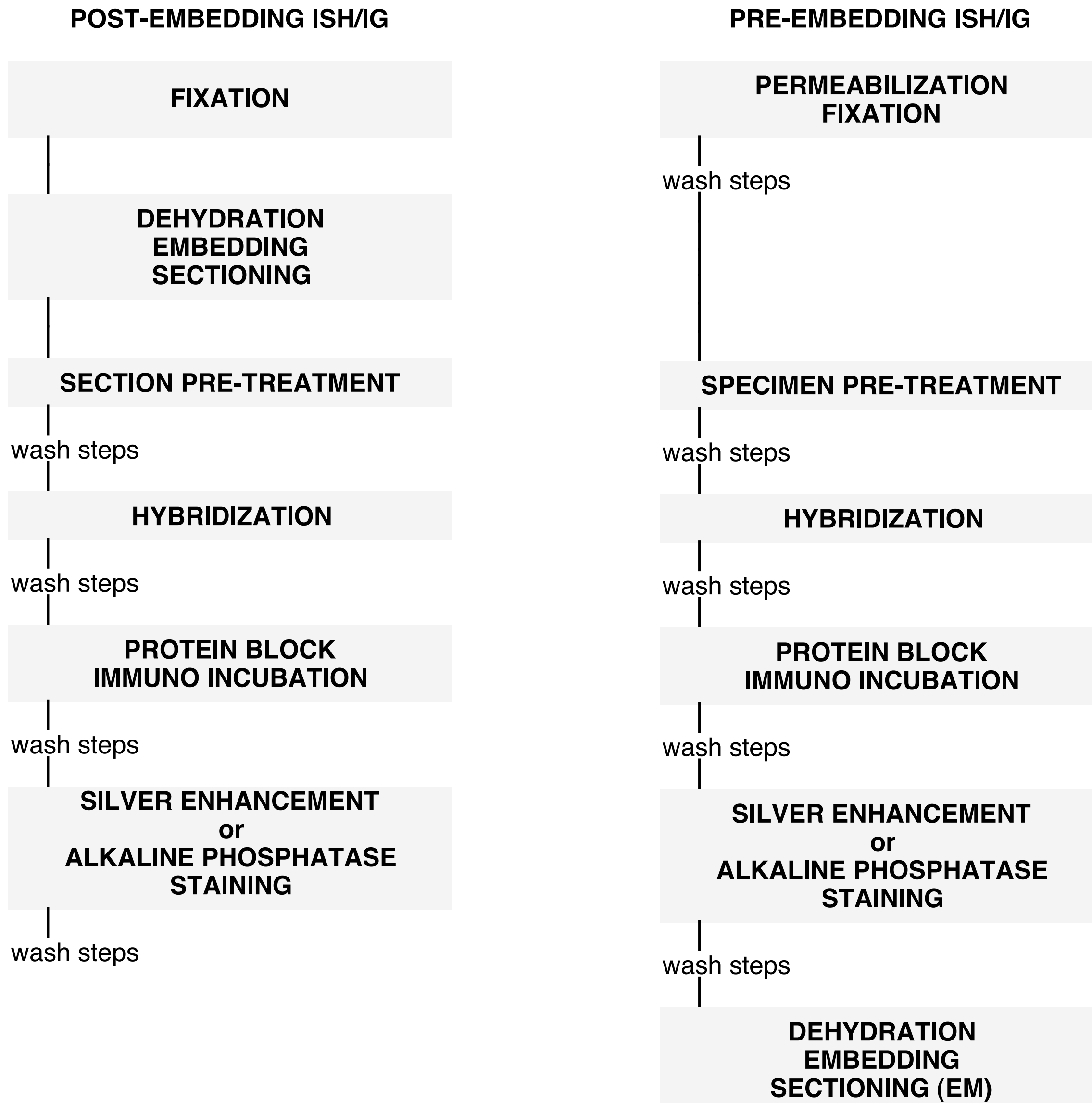
SCHEMATIC OVERVIEW OF CRITICAL STEPS



Whereas most steps are explained at length in the "DETAILED PROCEDURE" on page 5, the missing steps are explained below.

PRE-TREATMENT

Pre-treatment steps can be used in both pre and post-embedding in situ hybridization methods to improve the signal by digesting proteins surrounding the target nucleic acid sequences. These steps involve an incubation with e.g. proteinase K (up to 500 μg/ml in 200 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4 for 5-30 minutes at 37°C) or with pepsin (500 μg/ml in 0.2 M HCl at 37°C for 5-30 minutes) prior to the hybridization. However, since these treatments affect the ultrastructure, a compromise has to be found between optimal signal and acceptable morphological preservation.

PRE-HYBRIDIZATION

To improve the hybridization signal/noise ratio a pre-hybridization step can be introduced using the hybridization mixture with omission of the nucleic acid probe. The presumed action is two-fold:
(i) by melting out the hairpins present in the RNA sequence under investigation at higher temperatures (50-80°C) more target sequences are exposed,
(ii) background on accord of aspecific nucleic acid interactions is blocked by the herring sperm DNA and yeast tRNA present in the hybridization mix.
In the present study the application of pre-treatment or pre-hybridization steps did not result in signal improvement. Therefore these steps were no longer included in the presented protocol.

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DETAILED PROCEDURE for PRE-EMBEDDING ISH/IG

PERMEABILIZATION and FIXATION

A431 cells were grown on coverslips for light microscopy
and on thermanox for electron microscopy.
Nuclear matrix preparations were made according to Fey et al (1986) as follows:
All steps are at room temperature, unless indicated otherwise.
Remove specimens from the growth medium, transfer to wells filled with RNAse free PBS.
Remove PBS immediately and extract the cells on ice for 3 minutes in CSK-buffer.
Digest the chromatin with 1000 U/ml DNase I in CSK buffer for 30 minutes,
Wash in 250 mM ammonium sulphate (RNAse-free) to remove DNA digests.
Fix in 0.25 % glutaraldehyde in CSK-buffer for 10 minutes.

wash steps
rinse 2x5 minutes in PBS
rinse 2x5 minutes in PBS, supplemented with 50 mM glycin

HYBRIDIZATION

Hybridization to detect the 28S rRNA was performed for at least 3 hrs at 42°C.
Hybridization time may be extended overnight at 42 ˚C.

wash steps
2x15 minutes washing in 50 % formamide, 2xSSC at the hybridization temperature,
2x10 minutes 2xSSC, 2x10 minutes 1xSSC, 2x10 minutes 0.1xSSC

PROTEIN BLOCK and IMMUNO INCUBATION

using Gold labeling:

Blocking: 30 minutes in PBG
Incubation: overnight with
anti-digoxigenin F(ab) fragments,
coupled to ultra small gold particles,
diluted 1/20 in PBG.

wash steps
two hours washing in PBG
(change every 15 minutes),
10 minutes in PBS,
post-fixation with 1% glutaraldehyde in PBS
for 10 minutes.
wash in deionized water for 3x3 minutes

SILVER ENHANCEMENT

Silver enhancement was performed in the dark,
40 minutes for light microscopical preparations,
20 minutes for electron microscopical preparations.

wash steps
rinse with distilled water

DEHYDRATION, EMBEDDING, SECTIONING

Light microscopical preparations were embedded in mowiol,
Electron microscopical preparations were bloc-stained with 0.5 % uranyl acetate,
dehydrated and embedded in EPON.
0.2 μm sections were examined at 120 KV.

using Alkaline Phosphatase labeling

Blocking: 30 minutes in AP-incubation buffer
Incubation: 2 hours
with anti-digoxigenin F(ab) fragments,
coupled to alkaline phosphatase,
diluted 1/500 in AP-incubation buffer.

wash steps
4x10 minutes in AP-wash buffer

COLOUR DEVELOPMENT

10-15 minutes in AP-staining buffer.

wash steps
rinse with distilled water

DEHYDRATION and MOUNTING

Light microscopical preparations were embedded in mowiol.

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RESULTS

Figure 1
Light microscopy detection of 28S rRNA in A431 cells using a digoxigenin-labeled 28S DNA probe.
1a: Alkaline phosphatase detection system.
1b: ImmunoGold Silver detection system.
1c: As 1b with control probe.
A clear signal was visible in the nucleoli, parts of the cytoskeleton were also labeled. With the control probe no signal was observed (Figure 1c). Bar = 25 µm

Figure 2
Electron microscopy detection of 28S rRNA in A431 cells using a digoxigenin-labeled 28S DNA probe.
The nucleoli (Nu) show a dense labeling (Figure 2a). Control experiments were negative (data not shown).
Bar = 0.3

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DISCUSSION / EVALUATION

In this newsletter a detailed protocol for the light and electron microscopical detection of nucleic acid sequences is presented. The use of the immunogold silver detection system offers the possibility to work out protocols at the LM level and to extend the approach to EM specimens.

Ultra small gold particles are shown to be suited for the sensitive pre-embedding in situ hybridization at both the light and electron microscopical level.

Pre-embedding ISH

The pre-embedding in situ hybridization method as presented here was used to localize a specific RNA sequence in the nuclear matrix. The nuclear matrix is defined as the structure in the nucleus that resists treatment with strong detergents as present in the CSK-buffer (Fey et al., 1986). For this approach the use of CSK-buffer was therefore mandatory. For general applications where the localization of nuclear or cytoplasmic nucleic acid sequences is the objective, it may not always be required to use such extreme conditions. Much of the cytoplasmic and membranous structure is removed as a consequence of this treatment. This may not only hamper interpretation of the ultrastructure but may also lead to removal of target molecules. A limited degree of permeabilization will however always be necessary with the pre-embedding approach, if only to render the plasma membranes permeable. Alternatives may be found in the use of saponin (Willingham, 1983) which must be present during all of the steps from fixation to the wash steps after the immunogold incubation. For the immunocytochemical localization of cytoskeletal components it was found that even a sodium borohydride treatment may be sufficient to allow ultra small immunogold conjugates to pass the plasma membrane of paraformaldehyde/glutaraldehyde fixed neurones (van Lookeren Campagne, 1993) and in 0.5% glutaraldehyde fixed epithelial cells (Leunissen and van de Plas, personal communication). Finally, a dehydration/rehydration sequence with graded ethanol and acetone, as used in standard Epon-812 embedding, may prove useful.

Post-embedding ISH

Similarity with pre-embedding

From the schedules presented on page 4 it follows that the sequence of hybridization, protein block/immunoincubation and enhancement/staining steps in post-embedding in situ hybridization is not different from the one used in pre-embedding. The same holds for the pre-treatment and pre-hybridization steps (if applied or necessary).

Differences with pre-embedding

Permeabilization vs. sectioning

The major difference is found in the omission of a physicochemical permeabilization step based on the use of detergents. Instead accessibility of the target is achieved by making sections of the specimen.

Sections which can be fully rehydrated (paraffin, cryostat, ultrathin cryosections) before proceeding with the specific localization steps may have advantages over sections of plastic embedded material on the following points:

1. More targets are preserved;
2. The section surface is not as smooth as found with plastic sections. This leads to higher detection efficiencies at the section surface;
3. In the depth of the hydrated section more targets may be exposed and available for binding with the nucleic acid probe and the immunoconjugate.

Resin embedding generally leads to improved ultrastructural preservation. Resins commonly used are Lowicryl K4M, HM20, LR-white and LR-gold.

The wash steps after the immunogold incubation may be limited to a total of 30 minutes instead of 2 hours since a limited reactive area is available.

DNA-ISH

With the following minor adaptations the presented approach should also be useful to localize DNA sequences:

1. An RNAse step has to be performed instead of the DNAse step (temperature preferentially 37°C);
2. Furthermore the DNA present in the specimen has to be denatured before hybridization with a nucleic acid probe can take place. This can be achieved in two ways:

-- either through a pre-hybridization step using the hybridization mixture without probe for 10 minutes at 80°C prior to the actual hybridization or

-- by incubating at 80°C with the complete hybridization mixture for 10 minutes after which the temperature is lowered to the hybridization temperature.

Detection systems

The results obtained with the alkaline phosphatase colour reaction and the detection with gold conjugates are highly comparable. The detection method with the gold conjugates as described here involves a one-step-detection method i.e. the hybrids are detected with an anti-digoxigenin F(ab)-fragment directly coupled to ultra small gold particles. The signal can be improved with a two-step detection system using secondary antibody gold conjugates. For instance the digoxigeninylated nucleic acid can be detected with a mouse monoclonal antibody which in turn is detected with a goat-anti-mouse IgG ultra small gold conjugate.

For starters

When immunogold detection of nucleic acids is aimed at, the following approach is recommended:

Firstly optimize the hybridization procedure in combination with alkaline phosphatase detection at the light microscopical level, since the detection with alkaline phosphatase is easy and sensitive. The hybridization conditions found to be optimal in this system are also valid for the immunogold detection system. After establishing a specific hybridization signal with immunogold detection at the light microscopical level the samples can be prepared for examination at the electron microscopical level.

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AURION PRODUCTS for in situ hybridization

A. direct detection of labeled nucleic acid probes.

Ultra Small Conjugates
for LM and EM applications

100.199 Sheep-Anti-Digoxigenin
100.211 F(ab) of Sheep-Anti-Digoxigenin
100.088 Goat-Anti-Biotin

EM-applications
The conjugates described are also available as EM-grade conjugates with particle sizes of 6, 10, 15 and 25 nm. However, sensitivity is optimal with the ultra small conjugates.

B. two-step detection of labeled nucleic acid probes

Ultra Small Conjugates
for LM and EM applications

100.155 Rabbit-Anti-Sheep IgG (H&L)
100.077 Rabbit-Anti-Goat IgG (H&L)
100.011 Goat-Anti-Rabbit IgG (H&L)
100.166 F(ab')$_2$ of Goat-Anti-Rabbit IgG (H&L)
100.022 Goat-Anti-Mouse IgG (H&L)
100.177 F(ab')$_2$ of Goat-Anti-Mouse IgG (H&L)
100.033 Goat-Anti-Mouse IgM (μ-chain)
100.044 Goat-Anti-Mouse IgG/IgM
100.188 F(ab')$_2$ of Goat-Anti-Mouse IgG/IgM (μ)
100.055 Goat-Anti-Rat IgG (H&L)
100.066 Goat-Anti-Human IgG (H&L)
100.099 Streptavidin
100.122 Biotinylated Albumin
100.144 Goat-Anti-Guinea-Pig IgG (H&L)
100.222 Rabbit-Anti-Chicken IgG (H&L)

EM-applications
The conjugates described are also available as EM-grade conjugates with particle sizes of 6, 10, 15 and 25 nm.

AURION SILVER ENHANCEMENT REAGENTS

500.011 AURION R-GENT 2x30 ml
500.022 AURION R-GENT 2x250 ml

AURION BSA-C BACKGROUND SUPPRESSION REAGENT

900.099 AURION BSA-C (10%) 30 ml
900.022 AURION BSA-C (10%) 100 ml

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