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−Review Article−

In Situ Hybridization Techniques for Electron Microscopy

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In order for messenger RNA (mRNA) to be localized in cells and tissues by *in situ* hybridization at the electron microscopic level, the following conditions must be accomplished. The target mRNA must be retained in the specimen with well-preserved morphology. The labeled-nucleic acid probes must be accessible to the mRNA and make hybrids with the mRNA efficiently. The labels should be detectable by either transmission or scanning electron microscopes. To achieve these, appropriate techniques for the electron microscopic *in situ* hybridization (EMISH) for the target mRNA were developed and have been improved. This review will describe representative procedures of EMISH of mRNA and will make some comments on the procedures.

Keywords : *in situ* hybridization, electron microscopy, backscattered electron imaging, RNA

Introduction

In situ hybridization (ISH) is a method that demonstrates localization of specific nucleic acids at the cell and tissue levels. Recently the method was adapted for electron microscopy to define the sites of specific nucleic acids at the level of cellular organelles.

Electron microscopic *in situ* hybridization (EMISH) is currently used to investigate the intracellular distribution of messenger RNA (mRNA)[1-6], viral nucleic acids[7] and genomic DNA[8,9]. EMISH of mRNA has shown to be particularly useful for demonstration of the synthesis of proteins in cytosol[10,11]. This is because immunoelectron microscopy provides evidence only for the synthesis of proteins that are secreted or associated with the extracellular domain of plasma membrane by virtue of their association with rough endoplasmic reticulum. EMISH is used to detect RNA-viral replication complexes[12], to analyze DNA-virus life cycles within cells[13] and to detect viral nucleic acid in virus infected cells in the latency period[14] in which the viral RNA is not translated and cannot be detected by immunoelectron microscopy. EMISH is also useful for the precise localization of specific genomic DNA in interphase cell nuclei or in chromosomes[8,9] with high resolution. This review will be centered on the technical aspects of EMISH of mRNA and will include procedures for EMISH of mRNA.

Principal of EMISH

A single stranded-nucleic acid has a disposition to anneal with another single stranded nucleic acid with a complementary nucleotide sequence and forms a double stranded nucleic acids, i. e., DNA : DNA, DNA : RNA, or RNA : RNA. The annealing between two single stranded nucleic acids in vitro is called "hybridization" and the newly formed double stranded nucleic acid is often referred as a "hybrid". The stability of a hybrid correlates with "Tm", i. e., the temperature that half of the hybrid reanneal to single stranded nucleic acid. Tm is influenced by salt concentration, GC content of the nucleic acid, length of the nucleic acid and the frequency of mismatches between the single stranded nucleic acids. The formation of the hybrid is usually recognized by labeling one of the single stranded nucleic acid either with haptens or radioisotopes. The labeled nucleic acid is called a "probe" or "labeled probe". A hybrid is first formed between a labeled probe and single stranded nucleic acid in cells and tissues using the ISH method. Then the site of the hybrid is recognized using a localization of marker hapten or radioisotope by immunohistochemistry or radioautography, respectively. When the site is defined at the ultrastructural level, it is called EMISH.

Preparation of cells and tissues for EMISH of mRNA.

To preserve ultrastructural morphology and retain mRNA intact, all cells and tissues require fixation. It has been our experience as well as others that solutions of formaldehyde are best suited for EMISH[15-17].

Target mRNA in Cells and EMISH :

To accomplish EMISH, 1) target mRNA must be retained in specimens of which ultrastructure is well preserved, 2) probes must be accessible to the target mRNA to form hybrids, and 3) the amount of the target mRNA must be sufficient for detection by electron microscopy.

1. Retention of RNA in Cells :

The retention of nucleic acids in cells and tissues should be confirmed before starting EMISH, since mRNA is easily degraded by endogenous RNase. For assessment of the RNA retention, cells and tissues are specifically stained for RNA. A preferred method for the staining in our laboratory is methylgreen-pyronin staining[18]. This is because ready made methylgreen-pyronin staining solution is commercially available, methylgreen intercalates larger-sized nucleic acid, e. g., DNA, and stains cell nuclei green, pyronin intercalates smaller-sized nucleic acid, e. g., RNA, and stains cytoplasm and nucleoli red. The stained preparations are permanent and can be observed with an ordinal light microscope. Those preparations in which cytoplasm is not stained reddish should not be processed for EMISH of mRNA.

2. Accessibility of probe to RNA :

mRNA in sections of fixed tissues is usually mantled by fixed proteins and embedded in embedding medium and labeled probes are not readily accessible to the mRNA. So, the proteins and embedding medium should be partially removed before EMISH. The removal of proteinaceous matrixes is usually accomplished by either washing in acid[19,20] or digestion by protease[21,22]. Embedding medium may be removed by organic solvents. The efficiency of the removal often depends upon the fixative and embedding media employed[22] and the accessibility of probes to target mRNA should be assessed independently.

3. Frequency of target mRNA in cytosol :

The intracellular density of target mRNA nucleotide sequence is much lower than that of most antigens detected by immuno-electron microscopy. The average thickness of polyribosome being about 20 nm and a diameter of about 200 nm and one strand of mRNA is translated by a polyribosome. Thus mRNA is dispersed in cytosol minimum of 200 nm apart from each other. As the fact that only about 0.1% percent of a polyribosome is estimated to be with a given specific mRNA makes the intracytosolic density of specific mRNA extremely low[11]. In addition, one expects that the entire length of mRNA is not exposed even after acid and proteinase treatments. In the chance that a given specific mRNA is at or near the surface of ultra-thin section is astronomically low. For this reason, little or no meaningful results are acquired by post-embedding EMISH and we opted to utilize either pre-embedding or thick-section EMISH.

Procedures of EMISH

Reported EMISH procedures include five major steps as list below.

Step 1 : Fixation ;
a. Perfusion-fixation with aldehyde solution.
b. Immersion-fixation in aldehyde solution.

Step 2 : Starting Materials ;
a. Thick sections of plastic embedded tissues. The sections were mounted on glass slides.
b. Frozen sections of fixed tissues. The sections which were mounted on glass slides.
c. Vibratome or chopper sections of fixed tissues. The sections were suspended in solution.
d. Ultrathin frozen sections of fixed tissues. The sections were mounted on grids.
e. Ultrathin sections of plastic embedded tissues. The sections were mounted on grids.

Step 3 : Hybridization With ;
a. Thymine-thymine (T-T) dimerized DNA probe[23].
b. Biotinylated DNA or RNA probe.
c. Digoxigenin labeled DNA or RNA probe.
d. Isotope (^{35}S, ^{32}P, ^{3}H) labeled DNA probe.

Step 4 : Detection of the Hybridized Probe by ;
a. Rabbit anti-T-T and horseradish peroxidase (HRP) labeled anti-rabbit IgG, followed by incubation in a solution of 3,3'-diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2)[24], then osmication.
b. Avidin-biotin-HRP complex (ABC), followed by incubation in a solution DAB+H_2O_2, then osmication.
c. Colloidal gold-rabbit anti-biotin.
d. Colloidal gold-streptavidin.
e. Goat anti-biotin, colloidal gold-anti-goat IgG.
f. Ferritin-avidin.
g. Mouse anti-digoxigenin, colloidal gold-protein A.
h. Sheep anti-digoxigenin, colloidal gold-anti-sheep IgG.
i. Silver grain by autoradiography.

Step 5 : Electron Microscopic Observation by ;
a. Backscattered electron imaging (BEI) of scanning electron microscope (SEM).
b. Transmission electron imaging of transmission electron microscope (TEM).

By utilizing a combination of the above five steps, EMISH is performed. Representative procedures are briefly described.

I. Step 1a→Step 2a→Step 3a→Step 4a→Step 5a.

Izumi et al.[16,25], have developed and performed EMISH with following steps.

1. Fixation : Fix tissues by perfusion with 4% paraform-

aldehyde in 0.01M phosphate buffered saline (PBS), pH 7.2, for 5 min (Step 1a).

2. Fix tissue blocks with 4% paraformaldehyde in PBS for 4 hours at 4℃.

3. Washing : Wash with PBS overnight at 4℃.

4. Embedding : Embed in JB-4 resin[26] (Step 2a).

5. Sectioning : Mount 2-3 μm tissue section on gelatin-coated glass slide.

6. Removal of embedding medium : Treat with a mixture of chloroform and isoamyl alcohol (= 19 : 1, v/v) for 30 min at room temperature (RT).

7. Protein removal : Treat with 0.2N HCl in distilled water for 20 min at RT.

8. Washing : Wash with PBS for 15 min (5 min each, three changes) at RT.

9. Protein digestion : Treat with proteinase K (15 units/ml) in PBS for 15 min at 37℃.

10. Washing : Wash with PBS for 15 min (5 min each, three changes) at RT.

11. Hybridization : Hybridize with hybridization solution containing probe overnight at 37℃ (Step 3a).

Use T-T dimerized synthetic antisense-oligodeoxynucleotide for the probe, and T-T dimerized synthetic sense-oligodeoxynucleotide for the control probe.

12. Washing : Wash with 50% formamide in 2X SSC for 60 min at RT.

13. Washing : Wash with 2X SSC for 60 min at RT.

14. Wash with PBS for 15 min (5 min each, three changes) at RT.

15. First antibody : React the tissue section with rabbit anti-T-T dimer for 60 min at RT (Step 4a).

16. Washing : Wash with PBS for 15 min (5 min each, three changes) at RT.

17. Second antibody : React the tissue section with HRP-goat anti-rabbit IgG for 60 min at RT (Step 4a).

18. Washing : Wash with PBS for 15 min (5 min each, three changes) at RT.

19. Visualization : Immerse the tissue section in DAB+ H_2O_2 solution for 10 min at RT.

20. Washing : Wash with distilled water for 15 min (5 min each, three changes) at RT.

21. Osmication : React with 2% OsO_4 for 2 min (Step 4a).

22. Washing : Wash with distilled water for 15 min (5 min each, three changes) at RT.

23. React with 0.1% thiocarbohydrazide for 1 minute.

24. Washing : Wash with distilled water for 15 min (5 min each, three changes) at RT.

25. Osmication : React with 2% OsO_4 for 2 min.

26. Washing : Wash with distilled water for 15 min (5 min

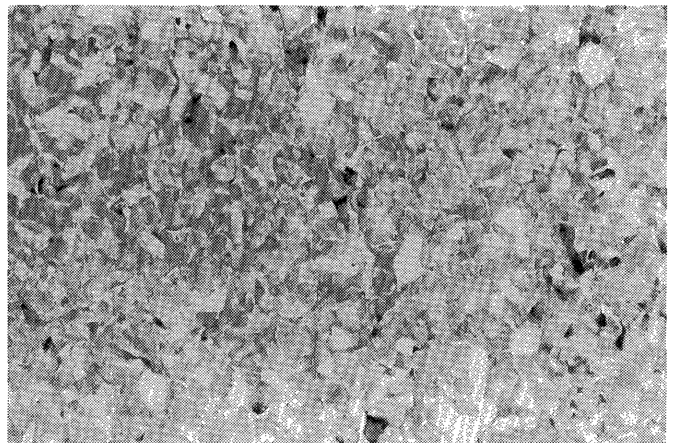
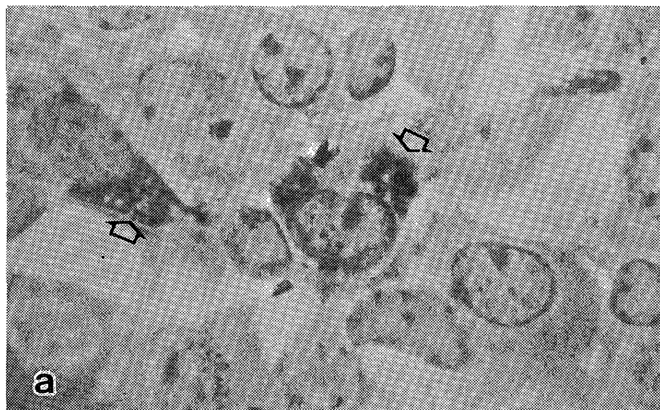


Fig. 1 Light Microscopic *In Situ* Hybridization for Prolactin mRNA in Cells of Frozen Section.

The frozen tissue section was treated with 0.2N HCl followed with proteinase K (0.02 units/ml) before hybridization using a rat prolactin anti-sense oligodeoxynucleotide T-T-dimerized probe. Positive staining is seen in some of the rat anterior pituitary cells. X224



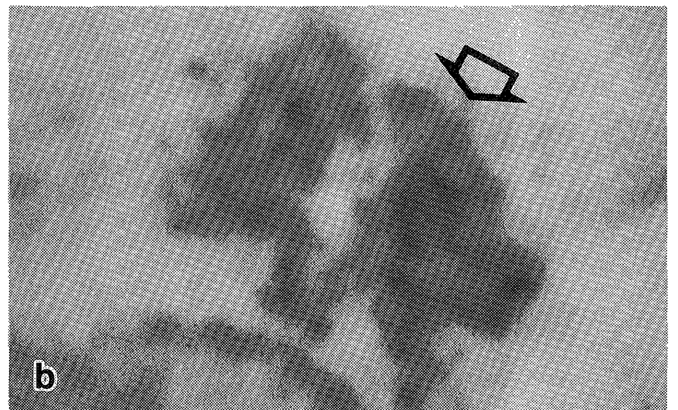


Fig. 2. Backscattered Electron Microscopic *In Situ* Hybridization for Prolactin mRNA in Cells of JB-4 Section.

The JB-4 section was treated with chloroform plus isoamyl alcohol and with 0.2N HCl followed with proteinase K (15 units/ml) before hybridization using a rat prolactin anti-sense oligodeoxynucleotide T-T-dimerized probe.

a) Backscattered electron image of prolactin mRNA is formed in confined area of the cytoplasm of some anterior pituitary cells (arrows). X1683.

b) A higher magnified micrograph of "a". Backscattered electron image of prolactin mRNA is recognized as a fine granular network of the reaction products in the cytoplasm. X11217.

each, three changes) at RT.

27. Evaporation : Dry and then evaporate carbon film on the tissue section.

28. Observation : Observe the tissue section with BEI of SEM with accelerating voltage 15-25 kV (Step 5a).

In this procedure water soluble JB-4 plastic resin was used as an embedding medium since it withstands the electron bombardment[27]. Osmium per oxidized DAB polymer was increased by successive reaction with osmium, thiocarbohydrazide and osmium for better detection by BEI[26,28]. This method of EMISH is easy, rapid and useful, although the resolution of a backscattered electron image is less than that of a transmission electron image. Using JB-4-embedded light microscopic "thick" sections, backscattered electron microscopic location of prolactin mRNA is shown in the Figure 2. Light microscopic location of prolactin mRNA in frozen section using the same probe is shown in Figure 1.

II. Step 1a→Step 2b→Step 3a→Step 4a→Step 5b.

Matsumura et al[29]., have performed EMISH using the following procedure.

1. Fixation : Fix tissue blocks with 4% paraformaldehyde in PBS for 4 hours at 4℃ (Step 1a).
2. Washing : Wash with 30% sucrose in PBS overnight at 4℃.
3. Embedding : Embed in OCT-compound.
4. Quick freeze the embedded tissues in a mixture of ethanol and dry ice.
5. Frozen sectioning : Section the tissues at 8 μm thickness (Step 2b). Mount the frozen tissue section on a gelatin-coated glass slide and air dry.
6. Washing : Wash with PBS for 15 min (5 min each, three changes) at RT.
7. Protein removal : Treat with 0.2N HCl in distilled water for 20 min at RT.
8. Washing : Wash with PBS for 15 min (5 min each, three changes) at RT.
9. Protein digestion : Treat with proteinase K (0.02 units/ml) in PBS for 15 min at 37℃.
10. Washing : Wash with PBS for 15 min (5 min each, three changes) at RT.
11. Hybridization : Hybridize with hybridization solution containing probe overnight at 37℃ (Step 3a).

Use T-T dimerized synthetic antisense-oligodeoxynucleotide for the probe and T-T dimerized synthetic sense-oligodeoxynucleotide as the control probe.

12. Washing : Wash with 50% formamide in 2X SSC for 60 min at RT.
13. Washing : Wash with 2X SSC for 60 min at RT.
14. Wash with PBS for 15 min (5 min each, three changes) at RT.
15. First antibody : React the tissue section with rabbit anti-T-T dimer for 2 hours at RT (Step 4a).
16. Washing : Wash with PBS for 30 min (10 min each, three changes) at RT.
17. Second antibody : React the tissue section with HRP-goat anti-rabbit IgG for 2 hours at RT (Step 4a).
18. Washing : Wash with PBS for 30 min (10 min each, three changes) at RT.
19. Visualization : Immerse the tissue section in DAB+H_2O_2 solution for 10 min at RT.
20. Washing : Wash with distilled water for 15 min (5 min each, three changes) at RT.
21. Osmication : React with 2% OsO_4 for 1 hour.
22. Dehydrate with ethanol, embed the tissue section by inverting the gelatin capsule filled with Epoxy resin, and polymerize the resin at 60℃.
23. Ultramicrotomy : Section the tissues by an ultramicrotome for TEM.
24. Mount ultrathin sections on grids with supporting film.
25. Counterstain : Counterstain slightly with uranyl and lead salts.
26. Observation : Observe the tissue section with a transmission electron image of TEM with accelerating voltage 80 kV (Step 5b).

III. Step 1a→Step 2c→Step 3b→Step 4b→Step 5b.

Mitchell et al[4]., performed EMISH using the following steps : The specimen was perfusion-fixed with a solution of 4% paraformaldehyde and 0.1% glutaraldehyde (Step 1a). The vibratome sections of fixed tissues were suspended in solution (Step 2c) and were hybridized with biotinylated probe (Step 3b), and thereafter the hybridized probes on the sections were detected immunohistochemically using avidin-biotin-HRP complex (ABC). The sections were incubated in DAB+H_2O_2 solution and osmicated (Step 4b). The sections were embedded in plastic and thin-sectioned. Ultrathin sections were mounted on a grid and observed by transmission electron imaging of TEM (Step 5b).

IV. Step 1b→Step 2b→Step 3b→Step 4e→Step 5b.

Mitsui et al[5]., performed EMISH using the following steps : The specimen was immersion-fixed with a solution of 4% paraformaldehyde and 0.5% glutaraldehyde (Step 1b). The frozen tissue sections were mounted on glass slides (Step 2b) and were hybridized with biotinylated probe (Step 3b), and thereafter the hybridized probes on the sections were detected immunohistochemically using goat anti-biotin followed with colloidal gold-anti-goat IgG (Step 4e). The sections on the slide were embedded in plastic, thin-sectioned on a grid and finally observed by transmission electron imaging of TEM (Step 5b).

V. Step 1b→Step 2c→Step 3b→Step 4b→Step 5b.

Le Guellec et al[30]., performed EMISH using the following steps : The specimen was immersion-fixed in 4% paraformaldehyde solution (Step 1b). Vibratome tissue sections was made (Step 2c). The sections were hybridized with biotinylated probe (Step 3b). The hybridized probes on the sections were detected immunohistochemically with rabbit anti-biotin followed with ABC (Step 4b). The sections were embedded in plastic, thin-sections were made, and observed by transmission electron imaging of TEM (Step 5b).

VI. Step 1b→Step 2d→Step 3b→Step 4d→Step 5b.

Le Guellec et al[30]., performed EMISH using the following steps : The specimen was immersion-fixed in 4% paraformaldehyde solution (Step 1b). Ultrathin frozen tissue sections were made. The sections were mounted on grids (Step 2d) and were hybridized with biotinylated probe (Step 3b). The hybridized probes on the sections were detected immunohistochemically with rabbit anti-biotin followed with colloidal gold-streptavidin (Step 4d). The sections were observed by transmission electron imaging of TEM (Step 5b).

VII. Step 1b→Step 2d→Step 3b→Step 4e→Step 5b.

Wenderoth et al[2]., performed EMISH using the following steps : The specimen was immersion-fixed in 4% paraformaldehyde solution (Step 1b). Ultrathin frozen tissue sections were made. The sections were mounted on grids (Step 2d) and were hybridized with biotinylated probe (Step 3b). The hybridized probes on the sections were detected immunohistochemically with rabbit anti-biotin followed with colloidal gold-anti-rabbit IgG (Step 4e). The sections were observed by transmission electron imaging of TEM (Step 5b).

VIII. Step 1b→Step 2d→Step 3c→Step 4g→Step 5b.

Dirks et al[3]., performed EMISH using the following steps : The specimen was immersion-fixed in 1% paraformaldehyde solution (Step 1b). Ultrathin frozen tissue sections were made. The sections were mounted on grids (Step 2d) and were hybridized with digoxigenin labeled-probe (Step 3c). The hybridized probes on the sections were detected immunohistochemically with mouse anti-digoxigenin followed with colloidal gold-protein A (Step 4g). The sections were observed by transmission electron imaging of TEM (Step 5b).

IX. Step 1b→Step 2e→Step 3b→Step 4c→Step 5b.

Le Guellec et al[30]., performed EMISH using the following steps : The specimen was immersion-fixed in 4% paraformaldehyde solution (Step 1b). The tissues were embedded in Lowicryl and ultrathin sections of the embedded-tissues were mounted on grids (Step 2e). The sections were hybridized with biotin labeled-DNA probe (Step 3b). The sections were reacted with colloidal gold rabbit anti-biotin (Step 4c) and were observed by transmission electron imaging of TEM (Step 5b).

X. Step 1b→Step 2e→Step 3c→Step 4h→Step 5b.

Lin et al[31]., performed EMISH using the following steps : The specimen was immersion-fixed in 1% glutaraldehyde solution (Step 1b). The tissues were embedded in Lowicryl and ultrathin sections of the embedded-tissues were mounted on grids (Step 2e). The sections were hybridized with digoxigenin-RNA probe (Step 3c). The sections were reacted with sheep anti-digoxigenin folloewed with colloidal gold anti-sheep IgG (Step 4h) and were observed by transmission electron imaging of TEM (Step 5b).

XI. Step 1b→Step 2e→Step 3d→Step 4i→Step 5b.

Le Guellec et al[1]., performed EMISH using the following steps : The specimen was immersion-fixed in 2% paraformaldehyde solution (Step 1b). The tissues were embedded in Lowicryl and ultrathin sections of the embedded-tissues were mounted on grids (Step 2e). The sections were hybridized with ^{35}S labeled-cDNA probe (Step 3d) and were processed for autoradiography (Step 4i). The silver grains were observed by transmission electron imaging of TEM (Step 5b).

General comments of procedures for EMISH

1. Fixation

As fixatives, paraformaldehyde (4%, 2%, 1%)[3,15,17,32], glutaraldehyde (1%) and paraformaldehyde/glutaraldehyde mixture (4%/3~0.08%)[33,34] were used mainly. The addition of glutaraldehyde to formaldehyde fixative improves morphology, but results in a decrease of signal intensity for EMISH of mRNA[3,35]. Perfusion fixation with 4% paraformaldehyde in PBS is recommended to retain target nucleic acids in tissues with satisfactory morphology.

2. Embedding and Sectioning

Embedding conditions affect the hybridization efficiency of target mRNA. OCT-compound-embedded fixed-frozen Cryostat sections yield good hybridization, although the morphology is somewhat deteriorated[5]. Ultrathin frozen ultracryotomy[2,32,36] yields a higher signal

from EMISH of mRNA, but the sections were morphologically fragmented and disrupted during the EMISH processing. Plastic (Glycolmethacrylate[33], JB-4[16], Lowicryl K4M or K11M[1,10,37,38], LR White[2])-embedded tissue sections were used by many because of better hybridizability and morphology, but not much mRNA is recognized because of it low frequency. Vibratome sections[34,39] and Agar-embedded Tissue Chopper sections are relatively thick, so that the probes may not penetrate into the target RNA, resulting weak signal of EMISH.

3. Treatment

Basic protein surrounding nucleic acids may be partially removed by immersing in 0.2N HCl[19,20]. The concentration of proteinase K for the digestion of protein is different with the fixation conditions, the embedding medium[14], and tissues. In our hands, the proteinase K concentration of 0.02 units/ml for OCT-compound embedded frozen tissue sections, 2 units/ml for paraffin embedded tissue sections, and 15 units/ml for JB-4 embedded tissue sections were best when assessed by either the RNA immuno-staining intensity[16] or 28S ribosomal RNA hybridization[40]. Some investigators used TritonX-100 (0.2%~0.03%)[37] for EMISH. Saponin, which leaves holes large enough for penetration of nucleic acids through membranes, is effective for pre-embedding EMISH of mRNA in cultured cells[35]. But we found no appreciable benefit on fixed tissue sections with these detergents.

4. Probe Nucleic Acid

cDNA[1,16,41,42], cRNA[2,43], and synthetic oligo-probes (oligo-DNA[34,36,44,45,46] and oligo-RNA[47]) are used as probes for EMISH of mRNA. We recommend the use of oligodeoxynucleotide since it can be easily synthesized with desired nucleotide sequences and length. The relatively small-sized oligo-DNA probes are expected to penetrate into target mRNA in cells more easily than large-sized cDNA, however, the signals using oligo-DNA are weaker than that of using cDNA[48]. To avoid the problem, the use of a mixture of multiple non-overlapping oligonucleotides is recommended[49]. In case of EMISH for rat prolactin mRNA in pituitary gland tissues embedded in JB-4, the best hybridization was obtained when 45 base oligonucleotide with five ATT repeats of the synthetic prolactin oligo-DNA probes was used.

5. Labeling of Probe and Electron Imaging

For EMISH many authors use non-radioactive rather than radioactive (^{3}H[38,45], ^{32}P[1], ^{35}S[4,50]) procedures because of superiority of high resolution, an ease of handling and of less time consumption[4]. Recently, T-T-, biotin-, or digoxigenin-labeled oligonucleotides with a desired sequence can be ordered commercially. As for the electron dense label, osmium[16,35,51], colloidal gold particles[2,3], and ferritin[41] are used and the choice depends on the procedure of EMISH.

Summary

When subcellular localization of a particular nucleotide sequence is essential for an investigation, EMISH may probe to be a useful technique. Based on our experience, we recommend following the procedures for EMISH of mRNA : Step 1a→Step 2a→Step 3a→Step 4a→Step 5a, Step 1a→Step 2b→Step 3a→Step 4a→Step 5b, or Step 1b →Step 2b→Step 3b→Step 4e→Step 5b.

References

1) Le Guellec D, Frappart L, Desprez PY : Ultrastructural localization of mRNA encoding for the EGF receptor in human breast cancer line BT20 by in situ hybridization. J Histochem Cytochem 39 : 1-6, 1991.
2) Wenderoth MP, Eisenberg BR : Ultrastructural distribution of myosin heavy chain mRNA in cardiac tissue : A comparison of frozen and LR White embedment. J Histochem Cytochem 39 : 1025-1033, 1991.
3) Dirks RW, Van Dorp AGM, Van Minnen J, Fransen JAM, Van Der Ploeg M, Raap AK : Electron microscopic detection of RNA sequences by non-radioactive in situ hybridization in the Mollusk Lymnaea stagnalis. J Histochem Cytochem 40 : 1647-1657, 1992.
4) Mitchell V, Gambiez A, Beauvillain JC : Fine-structural localization of proenkephalin mRNAs in the hypothalamic magnocellular dorsal nucleus of the guinea pig : a comparison of radioisotopic and enzymatic in situ hybridization methods at the light- and electron-microscopic levels. Cell Tissue Res 274 : 219-228, 1993.
5) Mitsui T, Kawai H, Saito S : Ultrastructural localization of myoglobin mRNA in human skeletal muscle. Histochemistry 101 : 99-104, 1994.
6) Matsuno A, Teramoto A, Takekoshi S, Utsunomiya H, Ohsugi Y, Kishikawa S, Osamura RY, Kirino T, Lloid RV : Application of bitinylated oligonucleotide probes to the detection of pituitary hormone mRNA using Northern blot analysis, in situ hybridization at the light- and electron-microscope levels. Histochem J 26 : 771-777, 1994.
7) Wolber RA, Beals TF, Maassab HF : Ultrastructural localization of Herpes simplex virus RNA by in situ hybridization. J Histochem Cytochem 37 : 97-104, 1989.
8) Cremers, AFM., Jansen in de Wal N, Wiegant J, Dirks RW, Weisbeek P, van der Ploeg M, Landegent JE : Non-radioactive in situ hybridization. A comparison of several immunocytochemical detection systems using reflection-contrast and electron microscopy. Histochemistry 86 : 609-615, 1987.
9) Fetni R, Lemieux N, Malfoy B, Dutrillaux B, Messier P-E, Richer C-L : Detection of small, single-copy genes on protein-G-banded chromosomes by electron microscopy. Cytogenet Cell Genet 60 : 187-189, 1992.
10) Webster H deF, Lamperth L, Favilla J T, Lemke G, Tesin D, Manuelidis L : Use of a biotinylated probe and in situ hybridization for light and electron microscopic localization of P0 mRNA in myelin-forming Schwann cells. Histochemistry 86 : 441-444, 1987.
11) Singer RH, Langevin GL, Lawrence JB : Ultrastructural visualization of cytoskeletal mRNA and their associated proteins using double-label in situ hybridization. J Cell Biol 108 : 2343-2353, 1989.
12) Egger D, Troxler M, Bienz K : Light and electron microscopic in situ hybridization : non-radioactive labeling and detection, double hybridization, and combined hybridization-immunocytochemistry. J Histochem Cytochem 42 : 815-822, 1994.
13) Morey AL, Ferguson DJP, Leslie KO, Taatjes DL, Fleming KA : Intracellular localization of parvovirus B19 nucleic acid at the

ultrastructural level by in situ hybridization with digoxigenin-labelled probes. Histochem J 25 : 421-429, 1993.

14) Mandry P, Murray AB, Rieke L, Becke H, Hofler H: Postembedding ultrastructural in situ hybridization on ultrathin cryosections and LR White resin sections. Ultrastructural Pathology 17 : 185-194, 1993.

15) Feldman M Ya : Reactions of nucleic acids and nucleoproteins with formaldehyde. Prog Nucl Acid Res Mol Biol 13 : 1-49, 1973.

16) Nakane P K, Koji T, Terasaki T, Izumi S : In situ localization of mRNA for peptide hormones. In : Prolactin gene family and its receptor, ed. by K. Hoshino, Elsevier Science Publishers B. V. (Biomedical Division), Amsterdam, 1988, p. 299-305.

17) Weiss LM, Chen Y-Y : Effects of different fixatives on detection of nucleic acids from paraffin-embedded tissues by in situ hybridization using oligonucleotide probes. J Histochem Cytochem 39 : 1237-1242, 1991.

18) Shulte EKW, Lyon HO, Hoyer PE : Simultaneous quantification of DNA and RNA in tissue sections. A comparative analysis of the methyl green-pyronin technique with the gallocyanin chromalum and Feulgen procedures using image cytometry. Histochem J 24 : 305-310, 1992.

19) Capco D G, Jeffery W R : Differential distribution of poly (A)-containing RNA in the embryonic cells of oncopeltus fasciatus. Analysis by in situ hybridization with a [3H] poly (U) probe. Development Biol 67 : 137-151, 1978.

20) Godard C M, Jones K W : Improved method for detection of cellular transcripts by in situ hybridization : Detection of poly (A) sequences in individual cells. Histochemistry 65 : 291-300, 1980.

21) Angerer LA, Angerer RC : Detection of poly A^{+}RNA in sea urchin eggs and embryos by quantitative in situ hybridization. Nucl Acid Res 9 : 2819-2840, 1981.

22) Koji T, Moriuchi T, Nakane P K : Improved tissue preparation for in situ localization of specific mRNA using non-radioactive DNAprobes : Effects of proteinase digestion and probe size on signal detection in frozen and paraffin sections of rat pituitary glands. Acta Histochem. Cytochem. 21 : 187-200, 1988.

23) Nakane P K, Moriuchi T, Koji T, Tanno M, Abe K : In situ localization of mRNA using thymine-thymine dimerized cDNA. Acta Histochem Cytochem 20 : 229-243, 1987.

24) Nakane PK, Pearce GBJr : Enzyme-labeled antibody : preparation and application for the localization of antigens. J Histochem Cytochem 14 : 929-931, 1966.

25) Izumi S, Koji T, Nakane P K : Backscattered electron imaging (BEI) for non-radioactive in situ hybridization. J Electron Microsc 39 : 304, 1990.

26) Izumi S, Nakane P K : Immunohistochemical localization of antigens in tissue embedded in JB-4 by backscattered electron imaging utilizing colloidal gold-labeled and peroxidase-labeled antibodies. Acta Histochem Cytochem 21 : 237-251, 1988.

27) Nakane P K, Izumi S, Hartman A L : Application of backscattered electron imaging for vascular research. In : Glomerular Dysfunction and Biopathology of Vascular Wall, ed. by S. Seno, A. Copley, M. A. Venkatachalam, Y. Hashimoto & T. Tsuji, Academic Press, Tokyo, p. 43-50, 1985.

28) Nakane P K, Izumi S : Histochemical and immunohistochemical observations with back scattered electron imaging. Proc XIth Int Cong on Electron Microscopy, Kyoto, 2013-2016, 1986.

29) Nakane PK., Matsumura H, Koji T : *In situ* hybridization on using T-T dimerized non-radioactive probes. In : Tabuchi K, ed. Biological Aspects of Brain Tumors. Tokyo : Springer-Verlag, p. 52-62, 1990.

30) Le Guellec D, Trembleau A, Oechoux C, Gossard F, Morel G :Ultrastructural non-radioactive in situ hybridization of GH mRNA in rat pituitary gland : Pre-embedding vs ultra-thin frozen sections vs Post-embedding. J Histochem Cytochem 40 : 979-986, 1992.

31) Lin N-S, Chen C-C, Hsu Y-H : Post-embedding in situ hybridization for localization of viral nucleic acid in ultrathin sections. J Histochem Cytochem 41 : 1513-1519, 1993.

32) Morel G, Dihl F, Gossard F : Ultrastructural distribution of growth hormone (GH) mRNA and GH intron I sequences in rat pituitary gland : effects of GH releasing factor and somatostatin. Mol Cellul Endocrinol 65 : 81-90, 1989.

33) Steinert G, Felsani A, Kettmann R, Brachet J : Presence of rRNA in the heavy bodies of sea urchin eggs. An in situ hybridization study with electron microscope. Experi Cell Res 154 : 203-212, 1984.

34) Penchow JD, Haralambidis J, Coghlan JP : Localization of glandular kallikrein mRNA in mouse submandibular gland at the cellular and ultrastructural level by hybridization histochemistry using 32P- and 3H- labeled oligodeoxyribonucleotied probes. J Histochem Cytochem 39 : 835-842, 1991.

35) Macville MVE, Wiesmeijer KC, Dirks RW, Fransen JAM, Raap AK : Saponin pre-treatment in pre-embedding electron microscopic in situ hybridization for detection of specific RNA sequences in culture cells : A methodological study. J Histochem Cytochem 43 : 1005-1018, 1995.

36) Morel G, Chabot J-G, Gossard F, Heisler S : Is atrial natriuretic peptide synthesized and internalized by gonadotrophs? Endocrinology 124 : 1703-1710, 1989.

37) Binder M, Tourmente S, Roth J, Renaud M, Gehring WJ : In situ hybridization at the electron microscope level : Localization of transcripts on ultrathin sections of Lowicryl K4M-embedded tissue using biotinylated probes and protein A-gold complexes. J Cell Biol 102 : 1646-1653, 1986.

38) Binder M : In situ hybridization at the electron microscope level. Scanning Microscopy 1 : 331-338, 1987.

39) Tong Y, Zhao HF, Simard J, Labrie F, Pelletier G : Electron microscopic autoradiographic localization of prolactin mRNA in rat pituitary. J Histochem Cytochem 37 : 567-571, 1989.

40) Yoshii A, Koji T, Ohsawa N and Nakane, P. K.: In situ localization of ribosomal RNAs is a reliable reference for hybridizationable RNA in tissue sections. J. Histochem Cytochem 43 : 321-327, 1995.

41) Brangeon J, Prioul JL, Forcioni A : Localization of mRNAs for the small and large subunits of Ribisco using electron microscopic in situ hybridization. Plant Physiol 86 : 990-992, 1988.

42) Yi J, Michel O, Sassy-Prigent C, Chevalier J : Electron microscopic localization of mRNA in the rat kidney : improved post-embedding in situ hybridization. J Histochem Cytochem 43 : 801-809, 1995.

43) Dorries U, Bartsch U, Notle Ch, Roth J, Schachner M : Adaptation of a non-radioactive in situ hybridization method to electron microscopy : detection of tenascin mRNAs in mouse cerebrellum with digoxigenin-labelled probes and gold-labelled antibodies. Histochemistry 99 : 251-262, 1993.

44) Larsson L -I, Christensen T, Dalboge H : Detection of proopiomelanocortin mRNA by in situ hybridization, using a biotinylated oligodeoxynucleotide probe and avidin-alkaline phosphatase histochemistry. Histochemistry 89 : 109-116, 1988.

45) Trembleau A, Favre-Mongtange M, Calas A : Ultrastructural visualization of oxitocin mRNA by in situ hybridization. A high resolution radioautographic study using a tritiated oligonucleotide probe. C. R. Acad Sci Paris, t. 307, Serie III, p. 869-874, 1988.

46) Koji T, Nakane PK : Recent advances in molecular histochemical techniques : in situ localization and southwestern histochemistry. J Electron Microsc 45 : 119-127, 1996.

47) Denny P, Hamid Q, Krause J E, Polak J M, Legon S : Oligoriboprobes. Tools for in situ hybridisation. Histochemistry 89 : 481-483, 1988.

48) Priestry J V, Hynes M A, Han V K M, Rethelyi M, Perl E R, Lund P K : In situ hybridization using 32P labelled oligodeoxynucleotides for the cellular localisation of mRNA in neuronal and endocrine tissue. An analysis of procedural variables. Histochemistry 89 : 467-479, 1988.

49) Trembleau A, Bloom FE : Enhanced sensitivity for light and electron microscopic in situ hybridization with multiple simultaneous non-radioactive oligodeoxynucleotide probes. J Histochem Cytochem 43 : 829-841, 1995.

50) Guitteny A-F, Bloch B : Ultrastructural detection of vasopressin messenger RNA in the normal and Brattleboro rat. Histochemistry 92 : 277-281, 1989.

51) Matsuno A, Ohsugi Y, Utsunomiya H, Takekoshi S, Osamura RY, Watanabe K, Teramoto A, Kirino T : Ultrastructural distribution of growth hormone and prolactin mRNAs in normal rat pituitary cells : a comparison between preembedding and postembedding methods. Histochemistry 102 : 265-270, 1994.