

***In situ* reverse transcription: the magic of strength and anonymity**

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ABSTRACT

In this study, we describe an approach that enables a highly specific, effective and fast detection of polyadenylated RNA sequences *in situ* at the light and electron microscopy levels. The method developed is based on the incorporation of 5-bromo-2'-deoxyuridine into the growing cDNA strand by means of the reverse transcriptase. We have shown that unlike the previously used deoxyuridine tagged with biotin or digoxigenin, 5-bromo-2'-deoxyuridine is 'invisible' in the DNA–DNA duplex but easily detectable in the DNA–RNA duplex. This feature is an important pre-requisite for the correct interpretation of the data obtained, as our results strongly indicate that reverse transcriptase uses DNA breaks as primers efficiently. We have also shown that the replacement of deoxythymidine by 5-bromo-2'-deoxyuridine considerably stabilizes the growing DNA–RNA duplex, thus enabling the one-step detection of polyadenylated RNA in structurally well-preserved cells. The method developed provides a highly specific signal with the signal/noise ratio higher than 130 for permeabilized cells and 25 for conventional acrylic resin sections under the conditions used. When the high pressure freezing technique followed by the freeze substitution is employed for the cell's preparation, the ratio is higher than 80.

INTRODUCTION

The use of reverse transcription for the detection of RNA sequences has been described in several earlier studies. Besides isotopic markers (1–5), deoxyuridine tagged with biotin or digoxigenin has been used in some studies as well (6–9). The detection of RNA by means of reverse transcription is sometimes designated as IST—*in situ* transcription (1), or PRINS—primed *in situ* labelling (7), and is

typically performed in two consecutive steps. First, the short DNA sequence is hybridized with the target sequence in living or already fixed cells. During this step, the use of formamide is usually needed to optimize the hybridization conditions. The short hybridized DNA sequence serves in the next step as a primer and is elongated by reverse transcriptase. The marker nucleotides incorporated in the cDNA strand are detected in the subsequent steps. This procedure enables a higher labelling intensity than the *in situ* hybridization with the labelled probe alone (6). The majority of the mentioned studies used the approach described above only at the light microscopy (LM) level. Although Bassell *et al.* (6) have used this method for the detection of polyadenylated RNA (polyA RNA) also at the electron microscopy (EM) level, it is not clear whether this method is applicable for the localization of polyA RNA on the resin sections, since they performed the reverse transcription and the detection of the signal before embedding the permeabilized cells into the resin. However, the detection of target RNA after embedding and sectioning is an important pre-requisite for the good preservation of cell structure, as any permeabilization step before embedding results in its damage.

In this study, we have developed an approach based on a reverse transcription enabling a highly specific, effective and fast detection of polyA RNA sequences *in situ*. The approach developed does not require an individual hybridization step. Therefore, it allows the simultaneous detection of protein epitopes sensitive to formamide. The omission of formamide during detection is also an important pre-requisite for the efficient detection of polyA RNA in the cell cytoplasm. This method is suitable not only for localization studies in permeabilized cells but also for acrylic resin sections, including samples prepared by the techniques of high-pressure freezing (HPF) and freeze substitution (FS).

MATERIALS AND METHODS

Cell culture

Human HeLa cells were incubated in culture flasks or on coverslips in Dulbecco's modified Eagle's medium

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with L-glutamine (DMEM, Gibco) supplemented with 10% foetal calf serum (PAA Laboratories), 1% gentamicin and 0.85 g/l of NaHCO₃ at 37°C in a humidified atmosphere containing 5% CO₂.

Fixation/permeabilization protocols

In most experiments, the cells were fixed with formaldehyde and permeabilized with Triton X-100. The cells were mostly fixed with 2% formaldehyde for 5 min, washed in 1× PBS, permeabilized in 0.2% Triton X-100 for 10 min and washed in 1× PBS (low-extraction conditions).

In some experiments, this protocol was modified: the cells were fixed first with 2% formaldehyde for 20 s, then washed in 1× PBS, permeabilized with 1% Triton X-100 for 10 min, then washed in 1× PBS, fixed again with 2% formaldehyde for 10 min and, finally, washed in 1× PBS (high-extraction conditions).

In other experiments, the cells were fixed and permeabilized with methanol/acetone. In this case, the cells were first incubated in ice-cold 100% methanol for 10 min at −20°C. After that, the cells were incubated at room temperature in pure acetone for 30 s. The cells on the coverslips were then dried and subsequently washed in distilled water.

Those cells conventionally embedded in resins were fixed in 8% formaldehyde in 0.2 M PIPES (pH 6.95; Serva) for 2 h at room temperature. After the exchange of formaldehyde, the cells were fixed for 12 h at 4°C and then washed in 1× PBS.

Embedding of cells in resins

After fixation, the cells were scraped off and centrifuged at 500g, at 37°C for 5 min. The 1× PBS was replaced by 10% gelatine (~200 µl, 37°C; Sigma Aldrich), and 1 µl Cibatron Blue (Sigma Aldrich) was added. The cells were centrifuged at 500g, at 37°C for 5 min again. The excess of gelatine was removed; the cells in gelatine were incubated on ice for 10 min and post-fixed in 8% formaldehyde in 0.2 M PIPES for 20 min. After washing in a 1× PBS buffer, the small gelatine pieces were cut up (to < 1 mm³). The samples were dehydrated in methanol or ethanol and embedded in a Lowicryl K4M (Polysciences) or LR White (Sigma Aldrich) or Epon (Spi-Chem) resin.

HPF and FS

In this case, the culture medium was removed from the cells cultivated in Petri dishes and replaced by a CO₂-independent medium (GIBCO) supplemented with 20% bovine serum albumin (Sigma Aldrich). Most of the CO₂-independent medium was removed, only 200–300 µl were left in the Petri dish and the cells were scraped off. One microlitre of Cibatron Blue was added to the cell suspension. About 1 µl of such a treated cell suspension was transferred to the membrane carriers (Leica, 1.5 mm cavity diameter), and the cells were frozen in the Leica EM PACT high-pressure freezer. The time interval between the removal of the cells from the incubator and the freezing was kept at a minimum (always for < 10 min).

The frozen samples in the carriers were transferred under the liquid nitrogen to the Leica AFS machine and placed in the substitution solution pre-cooled to −90°C. The FS solutions were as follows:

(i) acetone; (ii) acetone + 0.25% glutaraldehyde + 0.1% uranyl acetate and (iii) acetone + 0.5% uranyl acetate. The uranyl acetate was dissolved in acetone from a 20% methanol stock solution. The cells were freeze-substituted in substitution solutions at −90°C for 2 days. The acetone was dehydrated using dried molecular sieves (J.T. Baker). Then, the temperature was being raised at a rate of 10°C per hour to −40°C. After 12 h, the samples were transferred into the special plastic capsules (Leica Reagent bath and Leica Flow-through rings) with cold acetone to wash out the residues of the substitution solutions. The samples were infiltrated at −40°C in a cold mixture of acetone and Lowicryl HM20 using the following mixtures (acetone:Lowicryl HM20): (i) 2:1 for 30 min; (ii) 1:1 for 1 h and (iii) 1:2 for 2 h. The samples were then incubated in the Lowicryl HM20 for 2 h at −40°C. Lowicryl HM20 was exchanged three times to remove the residue of acetone. After 2 h, Lowicryl HM20 was removed, the freshly prepared Lowicryl HM20 was added to the samples in the special plastic capsules and the samples were polymerized. The polymerization was done by UV light. It was performed first at −40°C for 24 h. Then, the temperature was raised at a rate of 10°C/h to 20°C and the samples were polymerized by UV for 2 days.

Reverse transcription

One-step reaction. The cells/sections on coverslips or the sections on grids were washed with distilled water before reverse transcription. The following components were used in the reaction mixture (RM) during testing: 1× AMV reverse transcriptase buffer (Promega, 5× AMV reverse transcriptase buffer: 250 mM Tris-HCl, 250 mM KCl, 50 mM MgCl₂, 2.5 mM spermidine, 50 mM DTT), 0.2 U/µl AMV reverse transcriptase (Promega), 0.4 U/µl RNasin (Promega), 0.25 mM dATP, dGTP, dCTP and dTTP (Promega), 0.25 mM 5-bromo-2'-deoxyuridine triphosphate (BrdUTP, Sigma Aldrich), 0.05 mM biotin-16-2'-deoxyuridine-5'-triphosphate (biotin-dUTP, Roche), 0.05 mM digoxigenin-11-2'-deoxyuridine-5'-triphosphate (digoxigenin-dUTP, Roche), 0.05 mM Alexa Fluor[®] 555-aha-2'-deoxyuridine-5'-triphosphate (alexa-dUTP, Invitrogen), 0.05 mM ChromaTide[®] fluorescein-12-2'-deoxyuridine-5'-triphosphate (fluorescein-dUTP, Invitrogen) and 0.01 µg/µl oligonucleotides. The following oligonucleotides were used: oligonucleotides consisting of 15, 20 or 25 deoxythymidines (oligo dT15, Promega; oligo dT20, oligo dT25, Generi Biotech), oligonucleotides consisting of 15 deoxyadenines (oligo dA15, Generi Biotech) or a random hexanucleotide (Promega). The coverslips/grids were incubated on the 20 µl/10 µl drops of the RM in the moisture chamber for 1 h at 42°C and washed in 1× PBS. Coverslips with cells labelled with alexa-dUTP or fluorescein-dUTP exclusively were washed in 1× PBS and distilled water and mounted in Mowiol. The coverslips/grids with fluorescently non-labelled nucleotide analogues

were incubated for 1 h with the primary antibody and after washing in PBS they were incubated for 1 h with the secondary antibody. The following primary antibodies were used in the comparative studies focused on the detection of cDNA tagged by bromodeoxyuridine (BrdU) or biotin-dUTP: a rabbit anti-biotin antibody (Enzo Lifesciences) diluted 1:100 in 1×PBS and a mouse anti-BrdU antibody (Roche) diluted 1:20 in 1×PBS. Digoxigenin-dUTP was detected by a mouse anti-digoxigenin antibody (Roche) diluted 1:100 in 1×PBS. For a comparison of the different antibodies against the BrdU, a rat anti-BrdU antibody (Abcam) diluted 1:100 in 1×PBS and a mouse anti-BrdU antibody (Becton-Dickinson) diluted 1:4 in 1×PBS were used in addition to the mouse anti-BrdU antibody purchased from Roche. For the detection of the splicing factor SC35, the mouse anti-SC35 antibody (Abcam) diluted 1:500 in 1×PBS was used. For LM, we used the anti-rabbit, anti-rat or anti-mouse secondary antibody conjugated with Cy3 or FITC fluorochrome. All of the secondary antibodies were diluted 1:100 in 1×PBS and were purchased from Jackson ImmunoResearch. In the comparative studies focused on the detection of cDNA tagged by BrdU or biotin-dUTP and the comparison of different antibodies against BrdU, secondary antibodies conjugated with Cy3 were used exclusively. The chosen secondary antibodies (cat. number 115-165-146 and 111-165-144) provided a similar strength of the replication signal after the hypotonic introduction (10) of biotin-dUTP and BrdUTP into cells. For EM, we used an anti-mouse antibody conjugated with 10 nm of gold adduct diluted 1:100 in 1×PBS (Aurion). After incubation with the secondary antibody, the cells on coverslips were washed on drops of 1×PBS, then on drops of distilled water and then mounted in Mowiol. The sections on coverslips were washed on drops of 1×PBS, drops of distilled water, stained with DAPI for 20 min, washed in distilled water and mounted in Mowiol. The sections on grids were washed in 1×PBS, then in distilled water and subsequently incubated on drops of 3% uranyl acetate for 45 min. After that, the grids were washed in distilled water and dried.

The following components were used in the RM during the control experiments with DNA polymerase I, *E. coli* (Fermentas): 1×DNA polymerase I, *E. coli* buffer (Fermentas, 10x polymerase I buffer: 500 mM Tris-HCl, 100 mM MgCl₂, 10 mM DTT), 0.2 U/μl DNA polymerase I, *E. coli*, 0.25 mM dATP, dGTP, dCTP and dTTP (Promega), 0.25 mM BrdUTP and 0.05 mM biotin-dUTP.

In the other control experiments, the cells/sections were treated by DNase I or RNase A. We used DNase I (RNase-free, Fermentas, 0.1 U/μl in 10 mM Tris-Cl, pH 7.5, 2.5 mM MgCl₂ and 0.1 mM CaCl₂) or RNase A (DNase and protease free, Fermentas, 5 μg/ml in 10 mM Tris-Cl, pH 7.4).

Two-step reaction. The cells fixed with 2% formaldehyde and permeabilized in 0.2% Triton X-100 were washed in 1×PBS, in 2×SSC and then incubated on the hybridization mixture consisting of 25% deionized formamide, 4×SSC, 1 mM EDTA, 0.5 mg/ml Salmon sperm DNA

and 200 ng/ml oligonucleotide consisting of 37 deoxythymidines labelled by fluorescein at the 5'-end (oligo dT37, Generi Biotech) at room temperature overnight. After hybridization, the cells were washed two times in 2×SSC for 30 min, two times in 1×SSC for 30 min, two times in 0.1×SSC for 15 min and, finally, three times in distilled water for 5 min. Reverse transcription was performed in the RM without oligonucleotide as described earlier. Biotin-dUTP or BrdUTP were present as the marker nucleotides in this case. After a wash in 1×PBS, BrdU or biotin was detected.

Sectioning and microscopy

Ultra-thin sections (70 and 200 nm thick) were cut on a Leica UltraCut S microtome (Leica Microsystems) with a diamond knife (Diatome Ltd). Seventy-nanometre thick sections were put on nickel grids coated with formvar and carbon, whereas 200-nm thick sections were placed on the coverslips coated with poly-D-lysine (Sigma Aldrich).

The permeabilized cells and 200 nm sections were observed with an Olympus IX81 microscope equipped with a Hamamatsu ORCA II camera (resolution 1344×1024 pixels). The 70 nm sections were viewed with a Morgagni 268 (FEI Company) electron transmission microscope equipped with a Megaview II camera (resolution 1280×1024 pixels).

RESULTS

Localization of polyA RNA in permeabilized cells

We detected the polyA RNA sequences in permeabilized HeLa cells. The cells were fixed with formaldehyde and permeabilized with 0.2% Triton X-100 if not stated otherwise.

In the first set of experiments, we used biotin-dUTP as a marker nucleotide for the localization of polyA RNA. After fixation and permeabilization, the cells were incubated for 1 h at 42°C in a RM consisting of oligo dT15, a nucleotide mixture including biotin-dUTP, RNase inhibitor and AMV reverse transcriptase (Table 1). As the presence of biotin-dUTP in DNA strands decreases the melting temperature (T_m), we used biotin-dUTP in the mixture with deoxythymidine triphosphate (dTTP). The ratio between biotin-dUTP and dTTP

Table 1. The constitution of the RM, the time and the temperature for reverse transcription

dATP	0.25 mM
dGTP	0.25 mM
dCTP	0.25 mM
dTTP	0.25 mM
Biotin-dUTP	0.05 mM
AMV reverse transcriptase	0.2 U/μl
RNasin	0.4 U/μl
Oligo dT15	0.01 μg/μl
AMV RT buffer	1×
Temperature	42°C
Time	1 h

was 1:5 (compare e.g. with 6). The incorporated biotin was detected by means of a rabbit anti-biotin antibody and an anti-rabbit antibody conjugated with fluorochrome. We observed a signal both in the cytoplasm and in the nucleus (Figure 1A). In contrast to the previously

published hybridization results (11,12), we did not observe the localization of the signal into nuclear speckles. The same pattern of labelling was also observed when oligo dT20 or oligo dT25 was used instead of oligo dT15. When we completely replaced

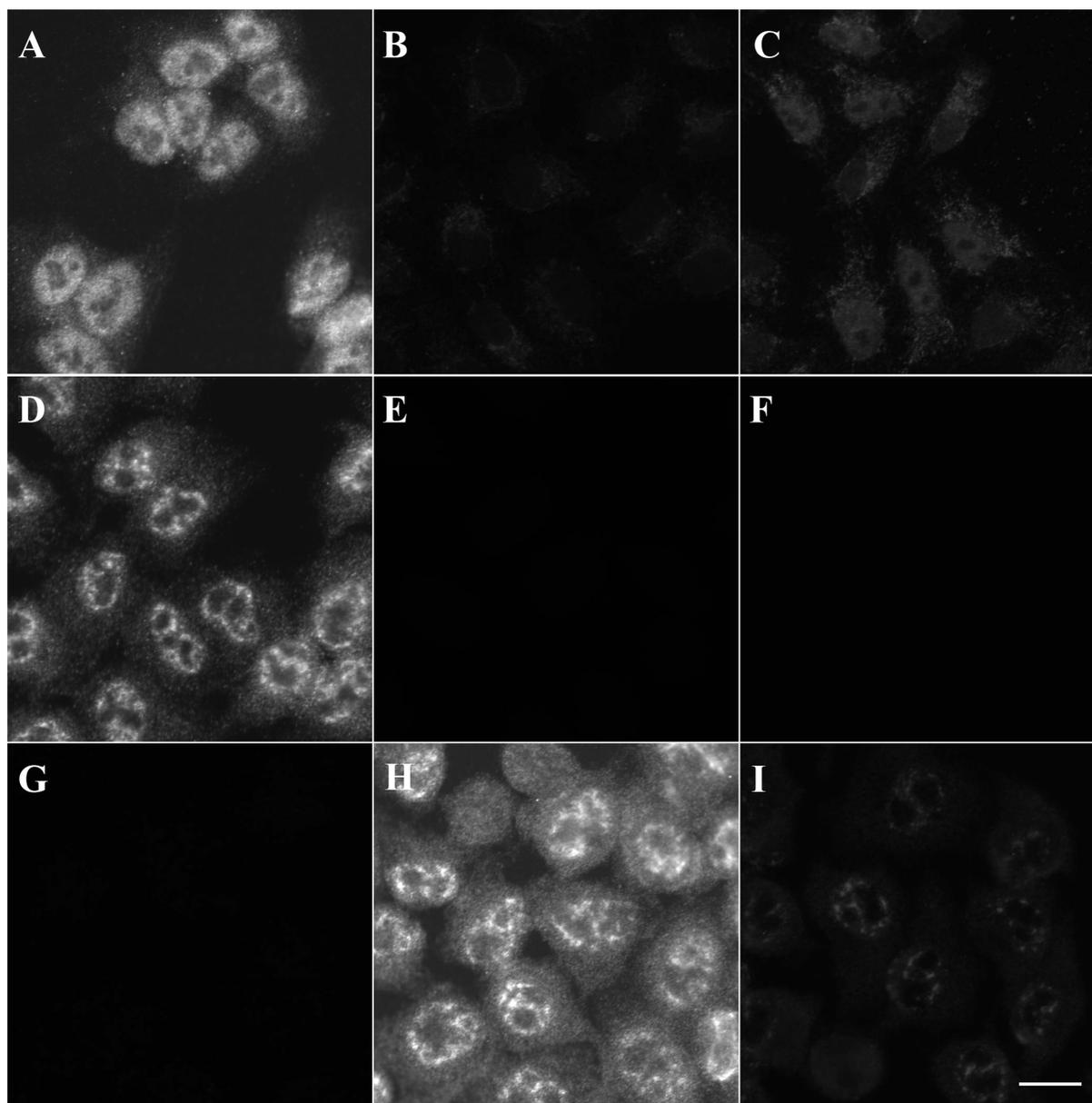


Figure 1. The detection of polyA RNA in permeabilized HeLa cells. The cells were fixed with formaldehyde and permeabilized with 0.2% Triton X-100 (A–H) or fixed and permeabilized in methanol/acetone (I). Cy3-labelled secondary antibodies were used in all of the experiments. (A) The detection of polyA RNA after the incubation of the cells in RM containing biotin-dUTP, dTTP, dATP, dCTP, dGTP and oligo dT15 by means of an immunolocalization of the incorporated biotin. (B) The immunolocalization of biotin in the cells without incubation in RM. (C) The immunolocalization of biotin after the incubation of the cells in RM containing biotin-dUTP, dTTP, dATP, dCTP and dGTP (without oligo dT15). (D) The detection of polyA RNA after the incubation of the cells in RM containing BrdUTP, dATP, dCTP, dGTP and oligo dT15 by means of an immunolocalization of the incorporated BrdU. (E) The immunolocalization of BrdU after the incubation of the cells in RM containing BrdUTP, dATP, dCTP and dGTP (without oligo dT15). (F) The immunolocalization of BrdU after the incubation of the cells in RM containing BrdUTP, dATP, dCTP, dGTP and oligo dA15. (G) The immunolocalization of BrdU after the incubation of the cells in RM containing BrdUTP, dATP, dCTP, dGTP and random hexanucleotide. (H) The detection of polyA RNA after the incubation of the cells in RM containing biotin-dUTP, BrdUTP, dATP, dCTP, dGTP and oligo dT15 by means of an immunolocalization of the incorporated biotin. (I) The detection of polyA RNA after the incubation of the methanol/acetone-fixed cells in RM containing BrdUTP, dATP, dCTP, dGTP and oligo dT15 by means of an immunolocalization of BrdU. The images of the biotin immunolocalization were acquired at 2000 ms (A–C, H), whereas the images of the BrdU immunolocalization at 70 ms (D–G, I). Bar: 20 μ m.

dTTP with biotin-dUTP in RM, a decrease of the signal was found. This finding is in accordance with the destabilization effect of biotin-dUTP mentioned earlier.

To exclude the possibility of the non-specific labelling, we omitted the incubation of cells in RM (Figure 1B) or we incubated the cells in RM without oligonucleotides (Figure 1C). In the first case, only the cytoplasmic signal was observed (Figure 1B), probably as a consequence of the presence of endogenous biotin. In the second case, a relatively weak signal was detected in many cell nuclei (Figures 1C and 2A). This signal was nearly eliminated by a pre-incubation of the cells in RM without oligo dT15 but with dTTP or BrdUTP instead of biotin-dUTP. On the other hand, the biotin signal was not suppressed in those cells pre-incubated in RM without oligo dT15 and without BrdUTP or dTTP. These results indicate that the observed signal may

correspond to the biotin-dUTP incorporated into the oligo dT15-independent sites, probably in one-strand DNA breaks. This supposition was supported by our experiments with DNA polymerase I. We found that the 10-min incubation of cells in RM containing DNA polymerase I, dATP, dGTP, dCTP, dTTP and biotin-dUTP (Figure 2B) provided a signal similar to the previous experiments with the incubation of the cells in RM with AMV reverse transcriptase without oligo dT15 (compare Figure 2A and B). In addition, the oligo dT15-independent signal produced by AMV reverse transcriptase was completely suppressed by the pre-incubation of the cells in RM containing DNA polymerase I, dATP, dGTP, dCTP and dTTP (Figure 2C). In some experiments, we used DNase I or RNase A digestion. In this case, the fixed and permeabilized cells were first incubated for 1 h with DNase I at 37°C or with RNase A at room

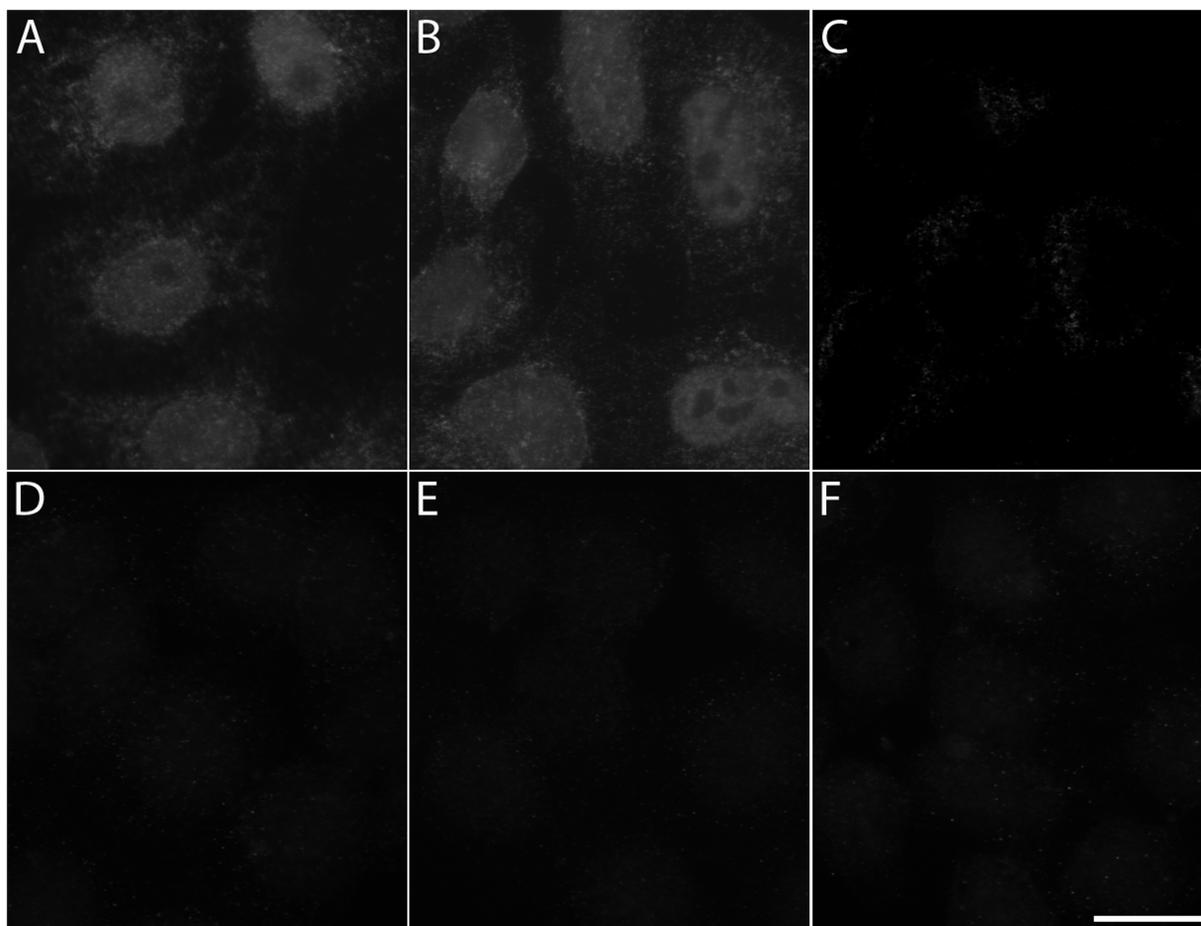


Figure 2. The comparison of DNA polymerase I and AMV reverse transcriptase activity *in situ*. The HeLa cells were fixed with formaldehyde and permeabilized with 0.2% Triton X-100. Cy3-labelled secondary antibodies were used in all of the experiments. (A) The immunolocalization of biotin after a 1-h incubation of the cells in RM containing biotin-dUTP, dTTP, dATP, dCTP and dGTP and AMV reverse transcriptase. (B) The immunolocalization of biotin after a 10-min incubation of the cells in RM containing DNA polymerase I, biotin-dUTP, dTTP, dATP, dCTP and dGTP. (C) The immunolocalization of biotin after a 10-min pre-incubation of the cells in RM containing DNA polymerase I, dTTP, dATP, dCTP and dGTP and a subsequent incubation in RM containing AMV reverse transcriptase, biotin-dUTP, dTTP, dATP, dCTP and dGTP. (D) The immunolocalization of BrdU after an incubation of the cells in RM containing AMV reverse transcriptase, BrdUTP, dATP, dCTP and dGTP. (E) The immunolocalization of BrdU after a 10-min incubation of the cells in RM containing DNA polymerase I, BrdUTP, dATP, dCTP and dGTP. (F) The immunolocalization of BrdU after a 10-min pre-incubation of the cells in RM containing DNA polymerase I, dTTP, dATP, dCTP and dGTP and a subsequent incubation in RM containing AMV reverse transcriptase, BrdUTP, dATP, dCTP and dGTP. All of the images were acquired at 2000 ms. Bar: 20 μ m.

temperature. The cells treated in this way were further incubated either in RM containing DNA polymerase I or AMV reverse transcriptase and dATP, dGTP, dCTP, dTTP and biotin-dUTP. While the pre-incubation with DNase I completely removed the signal produced by DNA polymerase I or AMV reverse transcriptase, no effect was observed after the pre-incubation of cells with RNase A.

We obtained similar results with digoxigenin-dUTP. The only substantial difference was the absence of the cytoplasmic signal in the experiment when the incubation of the cells in RM was omitted. This is in agreement with the supposition that the cytoplasmic signal in the biotin-labelled cells is a consequence of the presence of endogenous biotin.

Further, we tested BrdUTP instead of biotin-dUTP or digoxigenin-dUTP as a marker nucleotide for the labelling of cDNA. We completely omitted dTTP in RM, and the concentration of BrdUTP was the same as the concentration of all of the other nucleotides (0.25 mM). BrdU was detected by a mouse anti-BrdU antibody and an anti-mouse antibody conjugated with fluorochrome. In contrast to the biotin or digoxigenin signal, in the BrdU-labelled cells the signal was intensively accumulated into the nuclear speckles (Figure 1D, compare with Figure 1A). This accumulation was completely independent of the primary antibody used, as similar results provided three different antibodies against BrdU (see 'Material and Methods' for the details about the antibodies used). For the further experiments, we used the mouse anti-BrdU antibody (Roche) if not stated otherwise. The same labelling pattern provided experiments with oligo dT15, oligo dT20 or oligo dT25. As the signal was insensitive to oligonucleotide length, we used oligo dT15 in the next experiments exclusively.

No significant signal was observed in any cellular compartment in the control experiment without the incubation of the cells in RM. A similar result was also obtained in the experiments with the incubation of cells in RM without oligo dT15 (Figures 1E and 2D) or in RM containing DNA polymerase I, dATP, dGTP, dCTP and BrdUTP or by the pre-incubation of the cells in RM containing DNA polymerase I, dATP, dGTP, dCTP and dTTP followed by the incubation of the cells in RM with AMV reverse transcriptase, dATP, dGTP, dCTP and BrdUTP (Compare Figure 2D, E and F). Furthermore, the experiments with the pre-incubation of the cells with DNase I or RNase A followed by an incubation in RM containing dATP, dGTP, dCTP and BrdUTP and either DNA polymerase I or AMV reverse transcriptase do not provide any significant signal. In contrast to biotin or digoxigenin, BrdU is apparently not accessible for the antibody at the oligo dT15-independent sites, and this inaccessibility is independent of the primary antibody used, as all of the anti-BrdU antibodies generated similar results. This phenomenon resembles the detection of BrdU incorporated into DNA by cellular DNA polymerases. In this case, additional steps, such as the incubation of the cells in an acid environment, are commonly used for the visualization of BrdU incorporated during DNA replication (13).

Although we have treated the permeabilized cells after their incubation in RM without oligo dT15 and with BrdUTP for 20 min in 4N hydrochloric acid (HCl), we did not observe any signal. We can only speculate that such treatment may result in the partial degradation of BrdU-labelled DNA and/or in the release of BrdU-labelled DNA from the cells. As the visualization of BrdU in these sites was beyond the scope of this study, we did not continue in additional experiments.

To estimate the portion of the signal corresponding to the DNA synthesis from non-specifically paired primer, oligo dT15 was replaced in RM by oligo dA15. In the case of the use of oligo dA15, no signal was found in any cell (Figure 1F). If a random hexanucleotide primer was a component of RM instead of oligo dT15, only a very weak diffused signal was observed in the cells (Figure 1G). All of the above-mentioned results strongly indicate that cDNA synthesis *in situ* by means of AMV reverse transcriptase, oligo dT15 and BrdUTP represents a highly specific system for the detection of polyA RNA molecules in cells.

In the next experiment, we used RM containing oligo dT15, dATP, dGTP, dCTP, biotin-dUTP and BrdUTP. We found that the replacement of dTTP by BrdUTP results in a change of the localization of biotinylated cDNA (Figure 1H). The signal increased both in the cytoplasm and nucleoplasm; however, the most apparent change was the appearance of an intense signal in the nuclear speckles (compare Figure 1H and A). This result indicates that BrdU strongly stabilizes a growing cDNA strand and/or reverse transcriptase during cDNA synthesis. As the increase of the signal was attributed especially to the nuclear speckles, it seems that polyA RNA in the nuclear speckles exhibited a different organization with respect to the other cellular compartments.

In some experiments, we used methanol-acetone fixation/permeabilization of the cells to compare the effect of the fixation/permeabilization protocol on the detection of polyA RNA. We found the speckled pattern of the nuclear signal in these cells as well, but the overall intensity of the signal was lower as compared to the formaldehyde-fixed cells (Figure 1I, compare with 1D). The decrease was most apparent in the cytoplasm.

To confirm that the change in the localization of polyA RNA after BrdUTP addition is independent of the detection system, we used RM containing AMV reverse transcriptase, oligo dT15, dATP, dGTP, dCTP, alexa-dUTP or fluorescein-dUTP and BrdUTP or dTTP. We observed a clear difference between the localization of fluorochrome-tagged cDNA in those cells incubated in RM containing BrdUTP (Figure 3A) or dTTP (Figure 3B). The replacement of dTTP by BrdUTP resulted in the speckled localization of the originally diffused nuclear signal and a progressive increase of the cytoplasmic signal (Figure 3). This result clearly documented the stabilization effect of BrdU.

The results of the simultaneous localization of BrdU-labelled cDNA and splicing factor SC35, which is highly concentrated in the nuclear speckles (14), confirmed that the BrdU-containing cDNA is specifically enriched in this nuclear compartment (Figure 4).

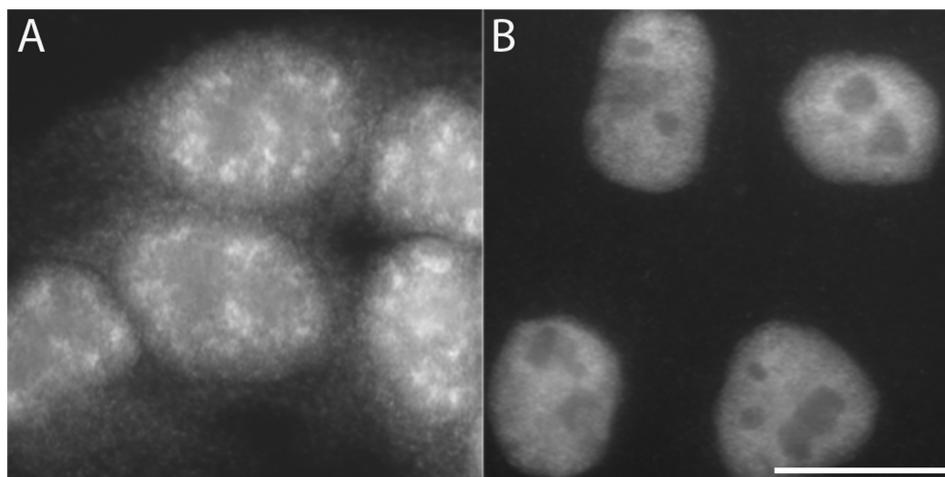


Figure 3. The detection of polyA RNA by means of Alexa-dUTP in permeabilized HeLa cells. The cells were fixed with formaldehyde and permeabilized with 0.2% Triton X-100. (A) The detection of polyA RNA after an incubation of the cells in RM containing AMV reverse transcriptase, Alexa-dUTP, BrdUTP, dATP, dCTP, dGTP and oligo dT15. (B) The detection of polyA RNA after an incubation of the cells in RM containing AMV reverse transcriptase, Alexa-dUTP, dTTP, dATP, dCTP, dGTP and oligo dT15. All of the images were acquired at 2000 ms. Bar: 10 μ m.

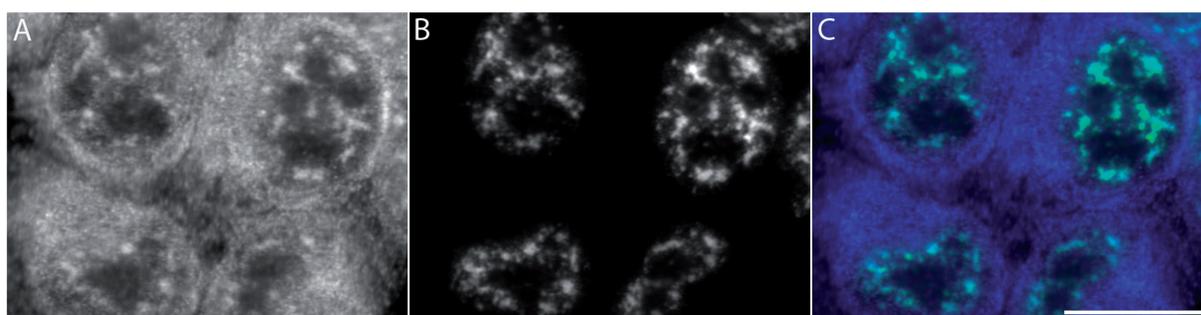


Figure 4. The co-localization of polyA RNA and splicing factor SC35 in permeabilized HeLa cells. The cells were fixed with formaldehyde and permeabilized with 0.2% Triton X-100. (A) The detection of polyA RNA by means of immunolocalization of incorporated BrdU. (B) The immunolocalization of SC35. (C) Merge image of BrdU (blue) and SC35 (green). Bar: 20 μ m.

The comparison of the signal from selected experiments is provided in Table 2. We used the minimal times necessary for the saturation of the signal in any region of the acquired image to compare signals from individual experiments. These measurements indicated that the best result was provided by the one-step protocol based on the incubation of the cells in RM containing BrdUTP, dATP, dGTP, dCTP and alternatively oligo dT15/dT20/dT25. When the experiment with oligo dA15 was taken as a negative control, the signal/noise ratio was around 130.

Next, we performed a two-step procedure to discover whether there is any difference in the signal strength and distribution as compared to the one-step protocol. We used a longer oligonucleotide, because more stringent conditions were used during the hybridization step. The oligo dT37 was labelled by fluorescein at the 5'-end to verify the efficiency of the hybridization step. The oligonucleotide was first hybridized with polyA RNA in the presence of formamide. In a separate step, the hybridized oligonucleotides were then elongated by reverse transcriptase in RM containing dATP, dGTP, dCTP and

alternatively BrdUTP, a combination of BrdUTP and biotin-dUTP or dTTP and biotin-dUTP at 42°C for 1 h. In all three cases, the signal corresponding to the detection of marker nucleotides was localized mainly in the nuclear speckles, which indicated that no stabilization effect of BrdUTP is necessary (Figure 5A). Interestingly, the hybridization signal from the fluorescein-labelled probe was much more diffused than the signal provided by the detection of the marker nucleotides after the reverse transcription step (Figure 5B). The hybridization step apparently resulted in two groups of annealed primers. Only one of these groups could efficiently serve as primers for cDNA synthesis. The other clearly represented non-specifically hybridized oligonucleotides or oligonucleotides whose 3'-end could not be elongated. The overall level of the BrdU signal was much lower when compared to the one-step protocol. The appearance of the first saturation in the image required a 10- to 20-fold longer time as compared to the one-step procedure. The next apparent difference when compared to the one-step procedure was an extremely low signal in the cytoplasm (compare Figure

Table 2. A comparison of the minimal times necessary for signal saturation in the permeabilized cells

Experiment	EX/+BrdUTP	EX/+ biotin-dUTP, + dTTP
+ BrdUTP	1	1
+ biotin-dUTP, + dTTP		
+ biotin-dUTP, + BrdUTP	1.3 ± 19	0.6 ± 16.6
- oligo, + BrdUTP	131.4 ± 22.6	
- oligo, + biotin-dUTP, + dTTP		1.8 ± 15.7
- oligo, + hexanucleotide, + BrdUTP	28.8 ± 18.4	
- all	134.8 ± 19.9	6.9 ± 18.5
MA, + BrdUTP	3.9 ± 19.6	
- oligo, + BrdUTP → - oligo, + biotin-dUTP, + BrdUTP	126.7 ± 22.6	6 ± 21.6
- oligo → - oligo, + biotin-dUTP, + BrdUTP	132.9 ± 18.8	2.1 ± 21.4
+ BrdUTP, + oligo dA15	130.6 ± 22.6	

The table compares the minimal times necessary for the saturation of signal in any region of the acquired image. In the column 'Experiment', the changes in the composition of the RM are indicated for the individual experiments. '+' means an addition of the mentioned component, whereas '-' means an omission of the mentioned component with respect to the RM shown in Table 1 without dTTP and biotin-dUTP. An arrow indicates two consecutive experiments. MA indicates an experiment with cells after methanol/acetone fixation/permeabilization. In all of the other experiments, the cells were fixed with formaldehyde and permeabilized with Triton X-100. In the second column, the ratio between the minimal time necessary for the signal saturation after BrdU detection in that experiment and in the experiment designated as '+BrdUTP' is indicated. The mean value and standard deviation (%) are provided. A similar value is shown in the third column for the biotin signal. In this case, the signal after biotin detection in the experiment was related to the biotin signal from the experiment designated as '+biotin-dUTP, + dTTP'.

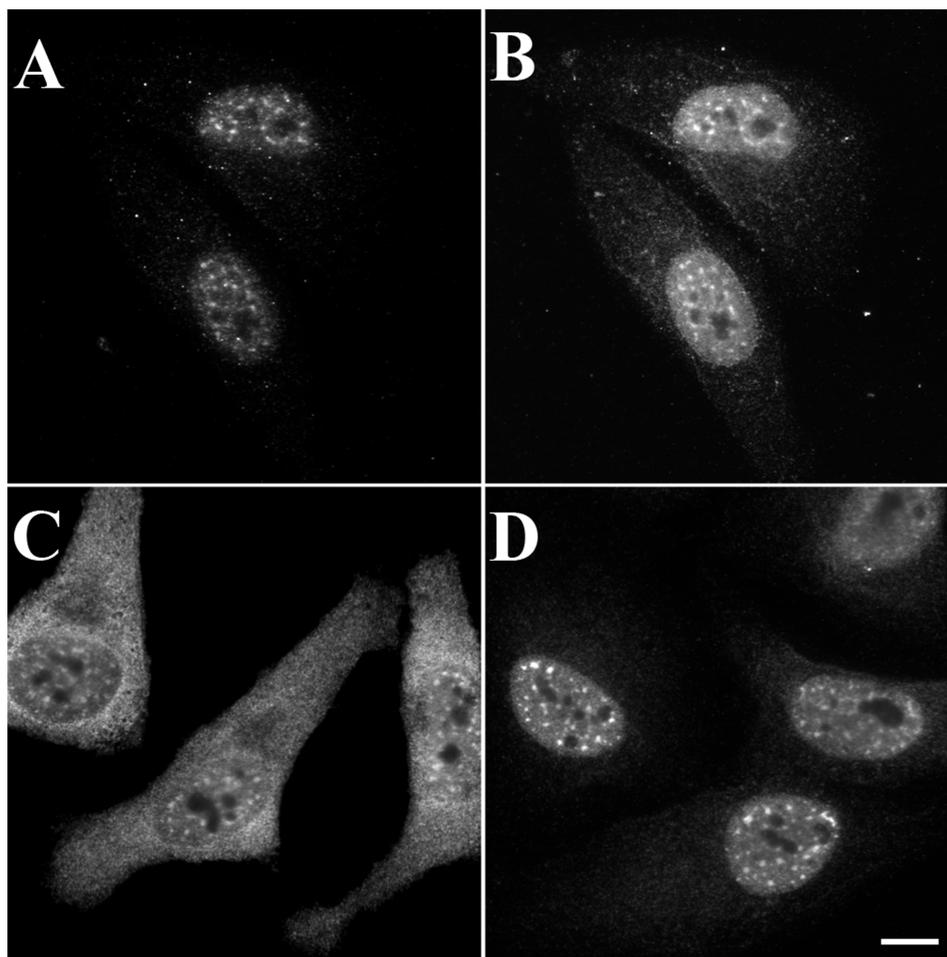


Figure 5. A comparison of the two-step and one-step procedure after using low-extraction or high-extraction conditions. A comparison of the two-step (A and B) and one-step (C and D) procedures after using low-extraction (C) or high-extraction conditions (D) is shown on the pictures. The signal corresponding to the localization of oligo dT37 is shown in (B). The signal corresponding to the sites of the incorporated BrdU is shown in (A, C and D). The images were acquired at 1000 ms (A), 6000 ms (B), 60 ms (C) and 45 ms (D). Bar: 10 μ m.

5A and C). As the hybridization signal in the cytoplasm was very weak as well, the use of formamide probably resulted in the extraction of RNA from the cytoplasm. This supposition was verified by the experiment when weakly formaldehyde-fixed cells were permeabilized in 1% Triton X-100 and a one-step protocol was used. Even though the signal in the nuclear speckles was higher as compared to the signal from the cells permeabilized with 0.2% Triton X-100, the signal in the cytoplasm decreased substantially (Figure 5D).

Localization of polyA RNA sequences on the sections of the cells embedded in resin

We tested the developed protocol for the detection of polyA RNA sequences on the sections of the cells fixed in 8% formaldehyde and embedded in the following resins: Epon (epoxide resin), Lowicryl K4M (acrylic resin) and LR White (acrylic resin). The resin sections were incubated in RM containing BrdUTP as the marker nucleotide. No signal was found on the Epon sections. The Lowicryl K4M and LR White (Figure 6A and B) sections provided a strong signal in the form of discrete tiny spots both in the cytoplasm and cell nuclei. As both acrylic resins provided a similar signal, we used only sections of cells embedded in LR White in all of the further experiments.

When we omitted the incubation of the sections in RM, no signal was detected in cells. The same result was obtained when sections were incubated in RM without oligo dT15 (Figure 6C) or with oligo dA15 (Figure 6D). Similar results were provided by the experiments with the incubation of the sections in RM containing DNA polymerase I, dATP, dCTP, dGTP and BrdUTP or experiments with the pre-incubation of the sections in RM with DNA polymerase I, dATP, dCTP, dGTP and dTTP followed by incubation of the sections in RM with AMV reverse transcriptase, dATP, dCTP, dGTP and BrdUTP or incubation in DNase I or RNase A prior to the incubation of the sections in RM with AMV reverse transcriptase or DNA polymerase I and dATP, dGTP, dCTP and BrdUTP. If we used a random hexanucleotide primer instead of oligo dT15, two tiny areas per cell were observed on average (Figure 6E and F).

Experiments similar to those with BrdUTP were also performed with biotin-dUTP as the marker nucleotide. In this case, the sections were incubated in RM containing dATP, dGTP, dCTP, BrdUTP and biotin-dUTP (BrdUTP: biotin-dUTP, 5:1). A strong signal of biotin was found in all these experiments except for the experiment when we omitted the incubation of the sections in RM. The very high signal obtained after the omission of oligo dT15 in RM (Figure 6G and H) apparently resulted from the high number of DNA breaks caused by the sectioning of the samples, as similar results were provided by the experiments with the incubation of the sections in RM containing DNA polymerase I, biotin-dUTP, dATP, dCTP, dGTP and BrdUTP. Moreover, if DNase I digestion or the incubation of the cells in RM containing DNA polymerase I, dATP, dGTP, dCTP and dTTP was used

prior to the incubation of the sections in RM with AMV reverse transcriptase the signal was completely removed. In contrast, RNA digestion by RNase A has no effect on the signal strength. In this respect, we observed especially heavily labelled mitotic chromosomes. This signal could not be resolved from the signal of the labelled cDNA. On the other hand, this signal could be, like in permeabilized cells, nearly removed by the pre-incubation of the sections in RM without oligo dT15 and containing dATP, dGTP, dCTP and BrdUTP or dTTP. When dTTP was used in RM containing oligo dT15 instead of BrdUTP, the biotin signal in most areas decreased, but the signal emanating from mitotic chromosomes was unchanged. Similar results were also obtained with digoxigenin-dUTP. These findings clearly indicated that BrdU contributed to the cDNA stabilization also on the acrylic sections. Our experiments also strongly indicated that BrdUTP is efficiently masked in double stranded DNA but not in the RNA-DNA complex. This observation is in complete agreement with our results from a similar experiment in permeabilized cells (Figure 1C).

In the last experiment, we used the sections of the cells processed by means of HPF followed by FS in three different substitution solutions: (i) acetone, (ii) acetone and 0.5% uranylacetate, (iii) acetone, 0.1% uranylacetate and 0.25% glutaraldehyde, and embedded in Lowicryl HM20. For the reverse transcription, RM with oligo dT15, BrdUTP, dATP, dGTP and dCTP was used. The highest number of labelled areas was found when only acetone was used as the substitution solution. The second and third solutions provided a slightly lower number of labelled domains. On the other hand, the number of domains and the intensity of the signal were still much higher as compared to the conventional embedded cells in Lowicryl K4M or LR White. The localization of polyA RNA sequences was performed at the LM (Figure 7A and B) and EM levels (Figure 7C and D). The EM data showed that the BrdU signal is localized particularly in the region of interchromatin granules, perichromatin fibrils and around the chromatin areas. In the cytoplasm, the signal was mainly in the ribosome-enriched regions. Labelled mitochondria were also found regularly. The experiments without oligo dT15 in RM or with oligo dA15 instead of oligo dT15 provided results very similar to the same experiments on the sections of the cells embedded in LR White (see above).

The comparison of the signal from selected experiments is provided in Table 3. We have compared the minimal acquisition times necessary for the saturation of signal in any region of the image like in the experiments with permeabilized cells. It is clear that the approach developed provides a highly specific signal not only in a permeabilized cell but also on the sections of the cells embedded in acrylic resins. The signal/noise ratio was around 25 for the conventional fixation/embedding protocol. In the case of HPF followed by FS, the ratio was even higher, around 80 ($\approx 26.9/0.3$). The time from the experiment with oligo dA15 was taken as a negative control.

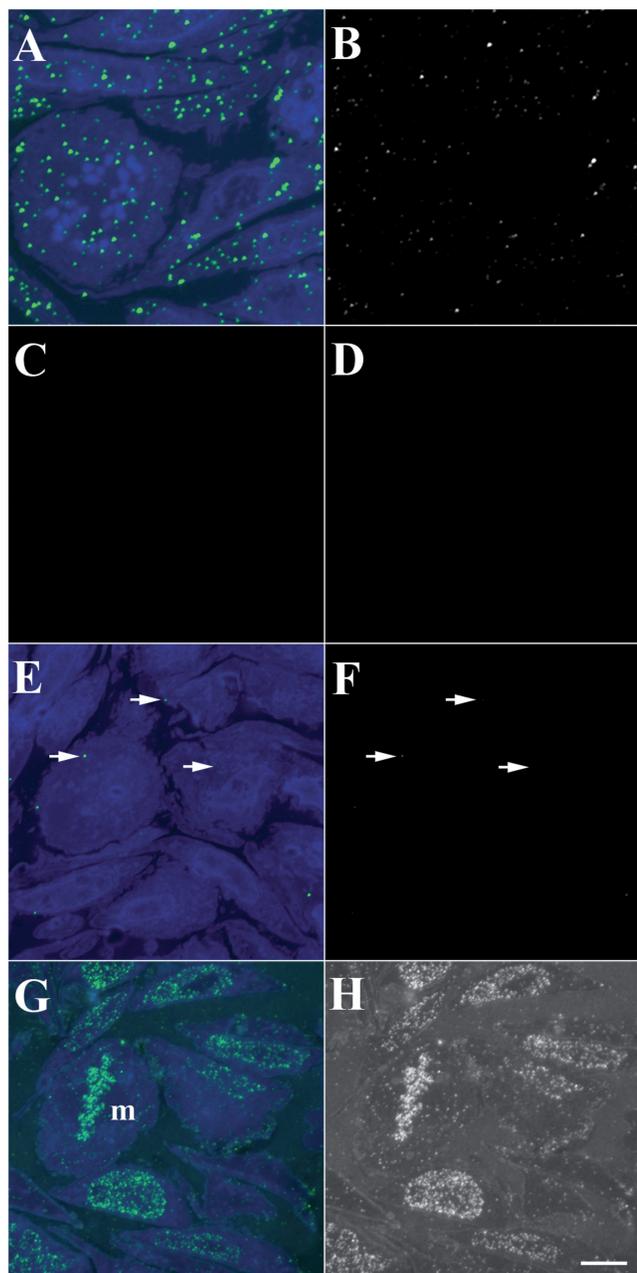


Figure 6. The detection of polyA RNA on the 200nm sections of the HeLa cells embedded in LR White. The cells were fixed with 8% formaldehyde. (A and B) The detection of polyA RNA after the incubation of the sections in RM containing BrdUTP, dATP, dCTP, dGTP and oligo dT15. BrdU was localized by means of primary and secondary antibodies (green). The green signal was processed in order to enhance the visualization of the labelled domains. The non-processed BrdU signal is shown in (B). The colour blue corresponds to the DAPI staining. (C) The immunolocalization of BrdU after the incubation of the sections in RM containing BrdUTP, dATP, dCTP and dGTP (without oligo dT15). The non-processed signal is shown. (D) The immunolocalization of BrdU after the incubation of the sections in RM containing BrdUTP, dATP, dCTP, dGTP and oligo dA15. The non-processed signal is shown. (E and F) The immunolocalization of BrdU (green) after the incubation of sections in RM containing BrdUTP, dATP, dCTP, dGTP and hexanucleotide. The green signal was processed in order to enhance the visualization of the labelled domains. The non-processed BrdU signal is shown in (F). The colour blue corresponds to the DAPI staining. (G and H) The immunolocalization of biotin (green) after the incubation of the

DISCUSSION

We have described a new method for the fast and highly effective detection of polyA RNA both in permeabilized cells and on the sections of the cells embedded in acrylic resins. We showed here that AMV reverse transcriptase effectively uses oligonucleotide-independent sites, most probably the one-strand DNA breaks, for priming the DNA strand synthesis. As a large number of DNA breaks is produced during the sectioning of the samples embedded in resin, this problem especially concerns the detection of polyA RNA on the acrylic resin sections. In this respect, the use of biotin- or digoxigenin-labelled nucleotides for the labelling of cDNA results in a high signal emanating from the labelled chromatin. Although the signal can be substantially decreased by the incorporation of non-labelled nucleotides at these sites before the detection of polyA RNA, a more convenient way is the replacement of biotin- or digoxigenin-labelled nucleotides by BrdUTP. We have shown that BrdU added to these sites by the AMV reverse transcriptase is inaccessible for the anti-BrdU antibody. In contrast, the BrdU in cDNA strands is easily detected by the same antibody. Moreover, BrdUTP can be used without any addition of dTTP. Therefore, the received signal is much more intense than the signal of biotin or digoxigenin. Our results also showed that the incorporation of BrdU into the cDNA results in the stabilization of the cDNA–RNA complex. This phenomenon enabled us to use the one-step procedure instead of the protocol with a separate hybridization step. Although the high stability of the long, double-stranded DNA containing BrdU had been described earlier (15,16), it was not clear whether this effect appeared during reverse transcription. It is likely that at many molecules only short parts of cDNA are synthesized in the formaldehyde-fixed and permeabilized cells because of the low accessibility of the target RNA. Undoubtedly, even much shorter segments are synthesized on the sections. Nevertheless, the higher stability of BrdU-enriched regions in the case of oligonucleotides has not been described (17). Another advantage of the described method is the omission of the hybridization step including the omission of the use of formamide. This is an important pre-requisite for the simultaneous detection of protein epitopes sensitive to its presence. Moreover, the omission of the hybridization step progressively shortens the whole procedure. In agreement with previously published studies dealing with the localization of polyA RNA sequences (11,12), we have shown a high accumulation of polyA RNA in nuclear speckles. However, in contrast to the commonly used methods or two-step method, our method provided a much higher signal in the cytoplasm and other nuclear areas in the permeabilized cells. This can be attributed to the massive

sections in RM containing biotin-dUTP, BrdUTP, dATP, dCTP and dGTP (without oligo dT15). The non-processed signals are shown. The colour blue corresponds to the DAPI staining. Only the biotin signal is shown in (H). m: mitotic cell. The images of BrdU immunolocalization were acquired at 600ms (A–F), the images of biotin immunolocalization at 8000ms (G and H). Bar: 10 μm.

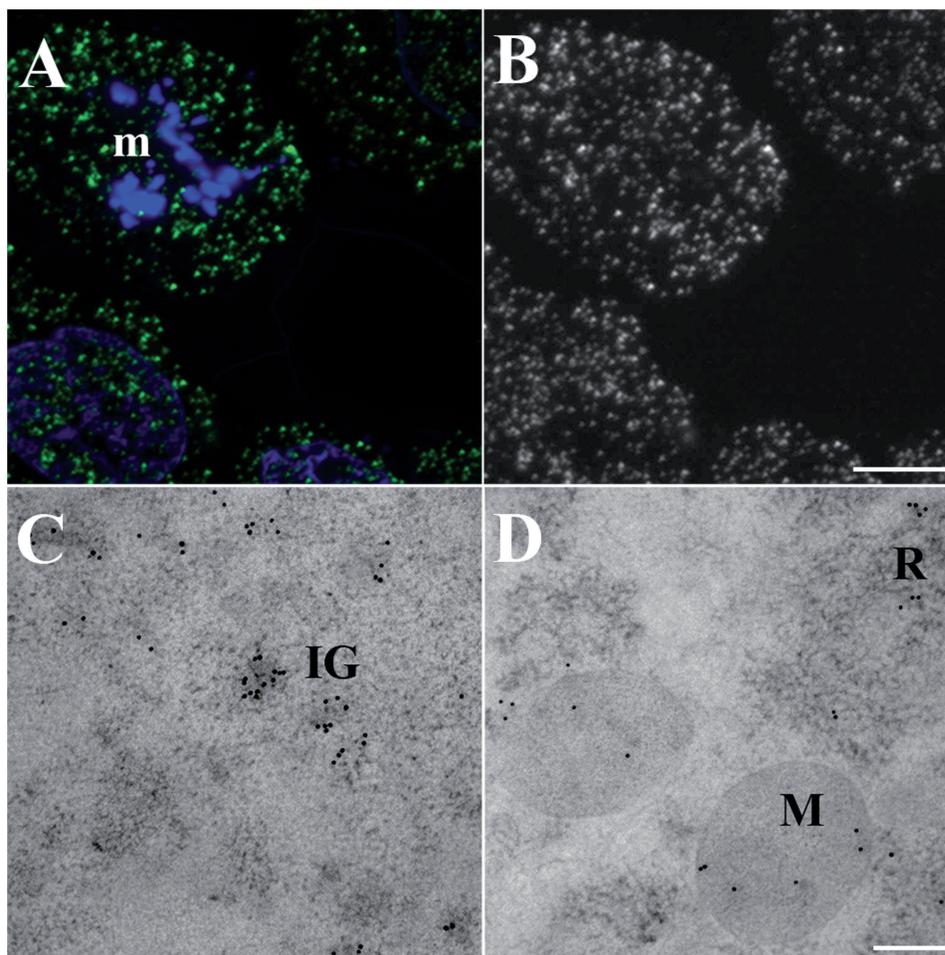


Figure 7. The detection of polyA RNA on the 200-nm and 70-nm sections of the HeLa cells processed by HPF followed by FS. The detection of polyA RNA on the 200-nm (A and B) and 70-nm (C and D) sections of the HeLa cells processed by HPF followed by FS is shown in the pictures. The substitution medium used contained a mixture of glutaraldehyde, uranylacetate and acetone. The cells were embedded in Lowicryl HM20. PolyA RNA was detected by fluorescence (A and B, green) or EM (C and D, gold particles). The localization of BrdU was performed after incubation in RM containing BrdUTP, dATP, dCTP, dGTP and oligo dT15. The green signal was processed in order to enhance the visualization of the labelled domains. The non-processed BrdU signal is shown in (B). The colour blue corresponds to the DAPI staining. m: mitotic cell, IG: interchromatin granules, M: mitochondrion, R: ribosome-enriched region. Bar: 10 μ m (A, B), 200 nm (C, D).

Table 3. A comparison of the minimal times necessary for signal saturation from the experiments with acrylic resin embedded and sectioned cells

Experiment	EX/+ BrdUTP	EX/+ biotin-dUTP, + BrdUTP
LR, + BrdUTP	1	
LR, + biotin-dUTP, + BrdUTP	1.1 \pm 21.3	1
LR, - all	26.3 \pm 22.3	3.4 \pm 19.8
LR, - oligo, + BrdUTP	27.6 \pm 25.3	
LR, - oligo, + biotin-dUTP, + BrdUTP	25.6 \pm 21.3	1.6 \pm 21.9
LR, + biotin-dUTP, + dTTP		1.5 \pm 19.6
LR, - oligo, + BrdUTP \rightarrow - oligo, + biotin-dUTP, + BrdUTP	25.9 \pm 21.1	3 \pm 22.2
LR, - oligo \rightarrow - oligo, + biotin-dUTP, + BrdUTP	29 \pm 24.2	1.8 \pm 17.9
LR, - oligo, + hexanucleotide, + BrdUTP	3.6 \pm 21.8	
LR, + BrdUTP, + oligo dA15	27.9 \pm 18.6	
HPF	0.3 \pm 20.6	
HPF, + BrdUTP, + oligo dA15	26.9 \pm 16.4	

The table compares the minimal times necessary for the saturation of signal in any region of the acquired image. In the column 'Experiment', the changes in the composition of the RM are indicated for the individual experiments. '+' means an addition of the mentioned component, whereas '-' means an omission of the mentioned component with respect to the RM shown in Table 1 without dTTP and biotin-dUTP. An arrow indicates two consecutive experiments. The cells were fixed in 8% formaldehyde and embedded in LR white (designated as 'LR') or processed by HPF followed by FS and embedded in Lowicryl HM20 (designated as 'HPF'). In the second column, the ratio between the minimal time necessary for signal saturation after BrdU detection in that experiment and in the experiment designated as 'LR, +BrdUTP' is indicated. The mean value and standard deviation (%) are provided. A similar value is shown in the third column for the biotin signal. In this case, we have related the signal after biotin detection in the experiment to the biotin signal from the experiment designated as 'LR, + biotin-dUTP + BrdUTP'.

extraction of RNA when formamide is used in the hybridization mixture. The higher signal observed in the cytoplasm can also be partially explained by the stabilization effect of BrdUTP in the one-step procedure. The stabilization effect probably enables the unveiling of polyadenylated mRNA even with very short polyA sequences. In this respect, we found that mainly the sequences in the speckles are required for the effective visualization of the presence of BrdUTP in the RM. This observation indicates that the tracked polyA sequences in nuclear speckles may exhibit a different organization than the polyadenylated sequences in most of the other cellular regions. They can be e.g. shorter than the others or much more occupied by proteins, and therefore short segments that require stabilization by BrdU are synthesized during reverse transcription. According to our results the polyA RNA is rather extensively occupied by proteins as the signal increased progressively in highly extracted cells.

The method developed enabled an effective and fast detection of polyA RNA on acrylic resin sections including the sections of the cells prepared by HPF followed by FS. The EM localization performed showed a relatively strong signal in the regions of the interchromatin granules, perichromatin fibrils and around the chromatin areas. In the cytoplasm, we observed the most intense signal in the ribosome-enriched regions. A signal was also detected in the mitochondria. These results were in complete agreement with the data from the common hybridization techniques (12). All of these results show that the technique employing the stabilization effect of BrdU provides a very useful tool for the trustworthy localization of polyA sequences. Under the conditions used, the method developed provides a highly specific signal with a signal/noise ratio of more than 130 for the permeabilized cells and 25 for the acrylic resin sections of the cells embedded by conventional procedure. HPF followed by FS increased this ratio to more than 80.

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Conflict of interest statement. None declared.

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