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Combined Detection of Newly Synthesized RNA and Nuclear Proteins at the Ultrastructural Level: A Modification of the Protocol for Immunoelectron Microscopy[1]

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Abstract—In the present work, we propose a novel variant of a protocol that allows combined revealing of nascent RNA transcripts and representative proteins at the ultrastructural level using immunoelectron microscopy. Early mouse embryos injected with BrUTP were used as a test system. The standard procedure for processing of ultrathin sections with a mixture of the appropriate primary antibodies in this case gives unsatisfactory results, perhaps due to a mismatch of blocking buffers and diluents. We used a new variant of the technique, which contains two steps: a complete cycle of processing the sections on grids with an antibody to specific nuclear protein and a complete cycle of processing the same sections with an antibody that recognizes BrUTP. In the interval between the steps, the sections are allowed to dry, thereby eliminating the mixing of the buffer solutions used in the first and second cycles of immunolabeling. Here, we demonstrate the efficiency of this protocol and the specificity of the observed immunolabeling using examples of simultaneous detection of nascent RNA, as well as ATRX protein and a functional histone modification, H4K5ac.

Keywords: immunoelectron microscopy, double labeling, BrUTP, mouse embryos

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INTRODUCTION

During the past 25 years or so, halogen-containing nucleotide analogues, including 5-bromouridine 5'-triphosphate sodium salt (BrUTP), have been widely used to localize transcription sites and nascent RNA as RNA synthesis precursors (Jackson et al., 1993; Wansink et al., 1993, 1994). BrUTP assay has, in various modifications, almost completely replaced the classical method of autoradiography, because it does not require the use of a radioactive precursor (H^3-uridine) and expensive photoemulsion, availability of a special darkroom, and long exposure time, especially in the case of research on the ultrastructural level. The use of BrUTP makes it easy to localize the precursor incorporation by a simple immunodetection procedure with appropriate commercially available antibodies not only at the light-optical level, but also at the ultrastructural level. BrUTP assay remains a widespread method of detecting nascent transcripts to characterize the efficiency of RNA synthetic activity in the cell, although other precursors of RNA synthesis, e.g., flurorouridine (FlU), have been proposed in a number of other studies (Hendzel et al., 1998; Boisvert et al., 2000; Kießlich et al., 2002). A problem is posed by the low permeability of cellular membranes to BrUTP, but this is solved by adding Triton X-100 detergent to the medium in the case of somatic cells cultured in vitro (Cmarko et al., 1999). In the case of significantly larger cells, including invertebrate and vertebrate oocytes, zygotes, or two- to four-cell mammalian embryos, the microinjection technique is an efficient method for solving this problem (Bogolyubov, 2007; Pochukalina and Parfenov, 2008; Bogolyubova, 2011).

Long-term personal experience in the field of immunodetection of various nuclear antigens has shown that, in some cases, a certain modification of the traditionally used protocols is required for simultaneous detection of specific nuclear proteins and transcription sites using BrUTP injections. This primarily concerns immunoelectron microscopy, which, on the one hand, makes it possible to investigate the intranuclear distribution of BrUTP incorporation sites at high resolution, but, on the other hand, imposes increased requirements on the affinity and specificity of the antibodies used, especially when applied to ultrathin sections (the postembedding technique), as well as on the selection of buffer solutions.

[1] The article was translated by the authors.
Abbreviations: ATRX—α-thalassemia/mental retardation syndrome X-linked, BrdU—5-bromodeoxyuridine, BrUTP—5-bromouridine 5'-triphosphate, H4K5ac—histone H4 acetylated at lysine 5.

Despite the fact that the simultaneous detection of BrUTP incorporation and certain nuclear antigens has occasionally been successful with somatic cells in the practice of ultrastructural studies (Cmarko et al., 1999, 2000), we found that the use of the sample preparation protocols described in these articles, which have become traditional by now, may be inapplicable to the embryonic material injected with BrUTP. With the use of a standard sample preparation procedure, which includes fixation with formaldehyde, embedding of the material in an acrylic resin LR white, and the application of gelatin-containing buffer solutions and demonstrates high efficiency in detecting a wide range of nuclear proteins at the ultrastructural level (Bogolyubova and Bogolyubov, 2017), we often observed a pronounced nonspecific reaction using a wide palette of antibody dilution (from 1 : 50 to 1 : 10000) when BrUTP antibodies are used, including in control experiments with noninjected material. In turn, the use of the buffer solutions recommended by the manufacturer for BrUTP antibodies significantly reduces the efficiency of immunoelectron detection of other nuclear antigens. Taking into account these problems of method, we attempted to modify the protocol of immunoelectron labeling for simultaneous detection the sites of BrUTP incorporation and some endogenous nuclear proteins. The chromatin-remodeling protein ATRX and one of the modified forms of histone (H4K5ac) have been chosen as representative proteins.

The proposed variant of the protocol for immunocytochemical reactions at the ultrastructural level involves sequential treatment of the material using buffer solutions most suitable for each of the antibodies: to BrUTP according to the recommendations of the manufacturer (Sigma-Aldrich) or to specific nuclear protein, a "standard procedure" (Bogolyubova and Bogolyubov, 2017). As compared to the conventional use of a mixture of different antibodies (Cmarko et al., 1999), this approach somewhat prolongs the duration of the experiment but demonstrates reliable and specific results. Here, we provide a detailed description of the modified protocol and illustrate its efficiency by the example of simultaneous detection of BrUTP and some nuclear proteins (ATRX, H4K5ac) in blastomeres of mouse two-cell embryos at the ultrastructural level.

MATERIAL AND METHODS

Embryo collection. Two-cell embryos of BALB/c mice obtained from the Rappolovo nursery (Leningrad region, Russia) were the test object for the study. To synchronize the ovary cycle and stimulate ovulation, females were injected with 10 IU serum gonadotropin (Folligon, Intervet, Netherlands) and chorionic gonadotropin (Chorulon, Intervet, Netherlands) at an interval of 44–48 h. After 46 h, embryos were collected in an F10 medium (Sigma-Aldrich) containing 25 mM HEPES.

BrUTP microinjections into the cytoplasm of embryos were performed using an Eppendorf 5242 microinjector (Germany) and a Narishige MMO-202ND micromanipulator (Japan) secured on a Leica DM IRB inverted microscope (Germany). Femtotips® II capillaries (Eppendorf) and Cook® K-HPIP-1035 holding pipettes were used. During microinjections, embryos were placed in a drop of medium of approximately 50 μL. An amount of 100 mM BrUTP sodium salt (Sigma-Aldrich) diluted with injection buffer containing 140 mM KCl and 2 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), pH 7.4, was used for microinjections (Wansink et al., 1994). Approximately 5% of each blastomere volume was injected (Cmarko et al., 1999). The material was fixed 15 min after the injection.

Confocal microscopy. The material was fixed with 4% formaldehyde prepared from paraformaldehyde (Ted Pella, United States) in PBS for 40–60 min at room temperature, then postfixed with 2% formaldehyde for at least 12 h at 4°C. After rinsing in PBS, the material was treated with 0.5% Triton X-100 in PBS for 10 min and then incubated in blocking buffer containing 5% bovine fetal serum (Gibco, United States) in special diluent for BrUTP antibodies for 10 min. The diluent is PBS containing 1% BSA, 0.5% Tween-20, and 0.1% sodium azide, pH 7.4. Incubation in a solution of primary antibodies (see below) was carried out overnight at 4°C in a moist chamber. Then, preparations were treated with FITC- or Alexa-568-conjugated secondary antibodies (Molecular Probes) diluted 1 : 128 or 1 : 200, respectively, for 1.5 h at room temperature and mounted in Vectashield® medium (Vector Laboratories, United States) containing 0.25 μg/mL 4',6-diamidino 2-phenylindole (DAPI) to reveal DNA simultaneously. Preparations were analyzed in a TCS SP5 laser scanning confocal microscope (Leica, Germany) equipped with appropriate lasers and a 40×/1.25 objective. Digital images were merged using ImageJ 1.37a.

Primary antibodies. Mouse monoclonal antibody against BrdU (clone BU-33, Sigma-Aldrich), which recognizes BrUTP with high specificity and affinity (Vanderlaan and Thomas, 1985), was used to detect the sites of BrUTP incorporation. The antibody was diluted in the above-described diluent at a working dilution of 1 : 1000. The adequate working concentration of antibodies was determined in control experiments with noninjected material.

Rabbit polyclonal antibodies against histone H4 acetylated at lysine 5 (H4K5ac) (ab124636, Abcam), dilution 1 : 500, and against the chromatin-remodeling protein ATRX (H-300, Santa Cruz Biotechnology), dilution 1 : 100, were used for the combined detection of representative nuclear proteins.

Immunoelectron microscopy. The material was fixed in PBS containing 4% formaldehyde prepared

from paraformaldehyde and 0.5% glutaraldehyde (Polyscience, United States) for 1.5 h at room temperature, and then in 2% formaldehyde in PBS without glutaraldehyde overnight at 4°C. After fixation, the material was rinsed in PBS, then in PBS containing 0.05 M ammonium chloride (Sigma-Aldrich), dehydrated in an ascending ethanol series (up to 85%), and embedded in acrylic resin LR white (Sigma-Aldrich). Ultrathin sections 70–80 nm in thickness were prepared using a Reichert Ultracut E ultra microtome (Austria) and mounted on nickel grids.

Simultaneous detection of the sites of BrUTP incorporation and representative nuclear proteins (H4K5ac and ATRX) was performed according to the following procedure.

1. Immunocytochemical detection of endogenous proteins was performed according to the protocol described previously (Bogolyubova and Bogolyubov, 2017). Grids were incubated in blocking buffer containing 0.5% gelatin from cold water fish skin (Sigma-Aldrich) and 0.02% Tween-20 in PBS, pH 7.4, for 10 min, and then in solution of appropriate rabbit antibodies overnight at 4°C in a moist chamber.

2. After rinsing in PBS containing 0.1% gelatin and 0.02% Tween-20 (5 × 2 min), grids were treated with secondary goat anti-rabbit antibodies conjugated with 15 nm colloidal gold particles (Aurion, Netherlands) for 1.5 h at room temperature in a moist chamber, then rinsed in the same buffer (2 × 2 min), rinsed briefly in distilled water, and allowed to dry.

3. The same grids with sections were incubated in blocking buffer (5% bovine fetal serum in diluent for BrUTP antibodies, see above) for 10 min in a moist chamber and then in primary antibody to BrUTP overnight at 4°C.

4. After fivefold rinsing in diluent (2 min in each portion), grids were treated with secondary goat anti-mouse antibodies conjugated with 10-nm colloidal gold particles (Aurion, Netherlands), dilution 1 : 10, for 1.5 h at room temperature. Then, grids were rinsed in diluent (2 × 2 min), rinsed briefly in distilled water, and allowed to dry entirely on a piece of 4/N (80 g/m^2) filter paper for at least 4–6 h at room temperature.

5. Grids were contrasted with a saturated solution of uranyl acetate (Electron Microscopy Sciences, United States) in 70% ethanol for 10 min and analyzed in a Libra 120 electron microscope (Carl Zeiss, Germany) at 80 kV.

The control material was (1) embryos injected with BrUTP, whose ultrathin sections were treated as described above but without the use of primary antibodies, incubating grids in steps 1 and 3 in an appropriate buffer solution containing no antibodies, and (2) noninjected embryos, whose ultrathin sections were processed according to the described procedure.

To evaluate the differences in the intensity of labeling, the number of colloidal gold particles was counted on randomly chosen squares of 9 μm^2. A comparison of the mean values obtained was carried out using the Mann–Whitney U-test.

RESULTS AND DISCUSSION

In the late two-cell stage of mouse development, which corresponds to the age of the embryos 46 h after administration of chorionic gonadotropin to the female, the blastomere nuclei are transcriptionally active, since the main events of zygotic genome activation are already being completed by then (Li et al., 2014). The ultrastructure of the nucleus of mouse blastomeres during zygotic genome activation is characterized by a number of specific features (Fakan and Odartchenko, 1980) related with the special functional status of embryos at the initial stages of development. Therefore, preimplantation mouse embryos are an interesting experimental model for comparative studies of the functional morphology of the cell nucleus.

As an analog of uridine, it is convenient to use BrUTP for the localization of transcription sites and detection of nascent RNA in the nucleus of mammalian embryos, including at the ultrastructural level. The incorporated precursor is traditionally detected with the use of available commercial antibodies to BrdU. These antibodies reveal bromouridine with high efficiency, since they cannot recognize the antigens by their sugar residues, ribose or deoxyribose (Vanderlaan and Thomas, 1985). The technique of microinjection into two-cell mouse embryos is not a problem if suitable certified equipment (microinjector and micromanipulator) and consumable materials (capillaries and holding micropipettes) used in the practice of in vitro fertilization are available. In our research practice, problems arose when an attempt was made to detect simultaneously the sites of BrUTP incorporation and some endogenous nuclear proteins at the ultrastructural level. In double immunostaining experiments, we used a mixture of appropriate antibodies as is traditional (Cmarko et al., 1999). This accelerates the process significantly and usually provides reliable and reproducible results. In such experiments, we use well-proven gelatin-containing buffer solutions in PBS (Bogolyubova and Bogolyubov, 2017). Unexpectedly, this turned out to be impossible in our ultrastructural experiments due to a nonspecific or weak reaction for no apparent reason in the case of the combined use of antibodies against BrUTP and against representative nuclear proteins H4K5ac and ATRX. With regard to the localization of endogenous proteins, we decided not to choose buffer solutions instead of those traditionally used in our laboratory, but performed the treatment with antibodies to these proteins and to BrUTP in different time. In the case of BrUTP, we used buffer solutions recommended by the manufacturer (Sigma-Aldrich) for fluorescent microscopy and applied them in an electron microscopic study for the first time. Our variant of the pro-

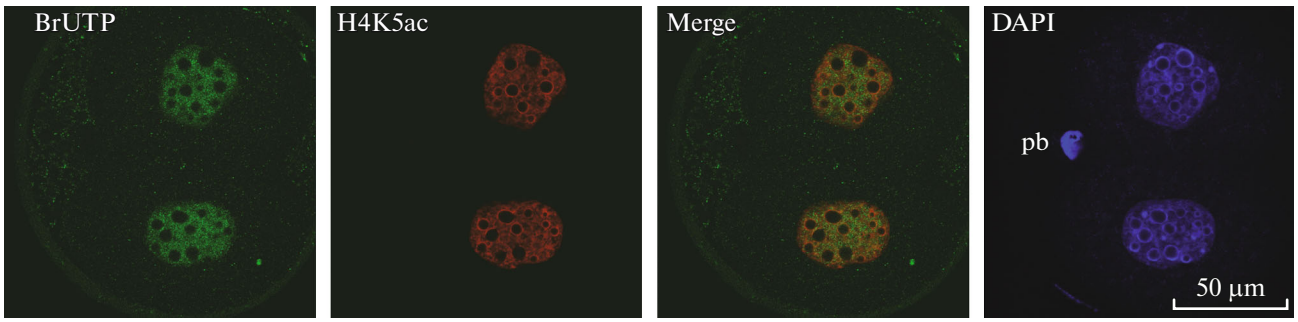

Fig. 1. The results of double immunostaining of BrUTP-injected two-cell mouse embryos at the light-optical level after treatment with antibodies to BrdU (green signal) and histone H4 acetylated at lysine 5 (H4K5ac, red signal); confocal microscopy; merge, the image combined at two channels; DNA is stained with DAPI (blue signal); pb, polar body.

tocol for immunoelectron microscopy makes it possible to use optimally suitable buffer solutions for each type of antibody, which increases the number of colloidal gold particles in sections without reducing the specificity of immunocytochemical reactions, although the procedure increases duration of sample processing.

Figure 1 illustrates the results of simultaneous detection of BrUTP incorporation sites and histone H4 acetylated at lysine 5 (H4K5ac) in transcriptionally active two-cell mouse embryos at the light-optical level with the use of an antibody mixture and buffers recommended by the manufacturer of BrUTP antibodies (for details, see MATERIALS AND METHODS). Numerous small fluorescence foci distributed throughout the nucleus with the exception of the nucleolus precursor bodies (the specific nuclear structures of preimplantation mammalian embryos; for details, see Fléchon and Kopečný, 1998) are detected in the nucleoplasm (Fig. 1, green signal). This pattern of labeling is stably reproduced, and the specificity of antibody binding was confirmed in additional experiments with the use of both noninjected embryos and injected embryos treated with RNase (not shown). This approach does not prevent the successful simultaneous detection of endogenous nuclear proteins, e.g., H4K5ac, at the light-optical level (Fig. 1, red signal).

At the same time, this technique proved inefficiency to detect BrUTP simultaneously with other nuclear antigens at the ultrastructural level as noted above, and only a few colloidal gold particles are detected in this case. Figure 2 illustrates the results obtained according to our modified protocol. The images demonstrate both the colloidal gold particles (10 nm, black arrows) corresponding to the sites of BrUTP incorporation and the particles (15 nm, white arrows) that correspond the localization of H4K5ac (Fig. 2a) or ATRX (Fig. 2b). Without going into details of the intranuclear distribution of H4K5ac and ATRX, which have been chosen only as major and representative antigens in this study, we note that the distribution patterns of BrUTP, H4K5ac and ATRX revealed at the ultrastructural level are in accordance with the data of immunofluorescent staining, both single and double. For example, we never observed the colocalization of fluorescent signals in the pairs BrUTP-ATRX and BrUTP-H4K5ac at the light-optical level, which is confirmed by immunoelectron microscopy (Fig. 2).

To test the specificity of the labeling observed as a result of the use of our modified protocol for double immunoelectron labeling, we performed a number of control experiments, the results of which are shown in Fig. 3. In the case of the standard version of testing the specificity of immunolabeling, we used only secondary antibodies conjugated with colloidal gold particles and replaced the incubation of sections in primary antibody solution with incubation in the appropriate buffer solution. As expected, preparations remained almost completely unlabeled except for single gold particles in the field of view at the background level (Fig. 3a).

To confirm the visual data, we estimated the number of gold particles in several randomly chosen squares of 9 μm^2. The intensity of labeling was significantly different ($P \leq 0.01$) between the control and experimental material both for 10-nm colloidal gold particles corresponding to the sites of BrUTP incorporation (1.0 ± 0.8 and 11.5 ± 5.2, respectively) and for 15-nm particles that correspond to the places of H4K5ac localization (1.25 ± 0.5 and 11.1 ± 3.9, respectively).

In another variant of control experiments, we completely reproduced our protocol of immunolabeling in ultrathin sections of noninjected embryos. In this case, the pictures of the distribution of labels, characteristic for specific endogenous protein, in particular, H4K5ac (Fig. 3b), remained unchanged. The difference in the number of gold particles marking the potential sites of BrUTP incorporation in control and experimental preparations (3.0 ± 1.1 and 11.5 ± 5.27, respectively) was also confirmed with the use of the Mann–Whitney U-test ($P \leq 0.01$).

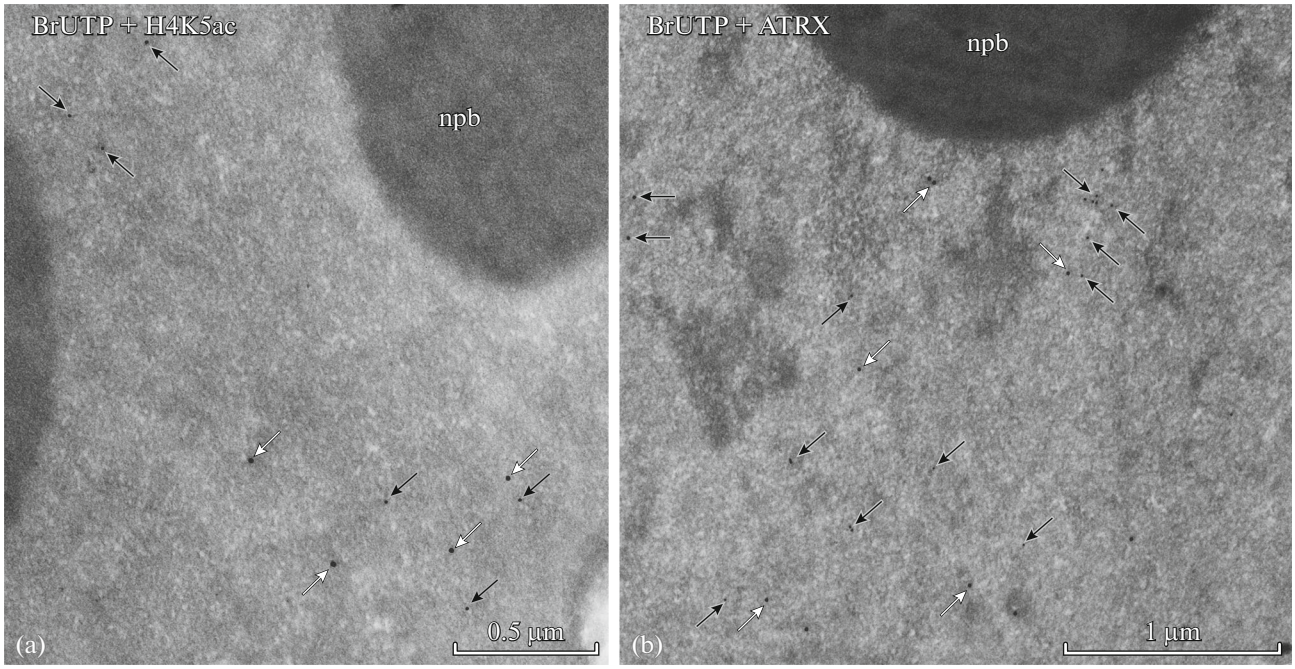

Fig. 2. The results of double immunostaining of BrUTP-injected two-cell mouse embryos at the ultrastructural level after treatment with antibodies to BrdU (10-nm gold particles, black arrows) and two endogenous nuclear proteins (15-nm gold particles, white arrows): (a) H4K5ac and (b) ATRX; fragments of the nuclei of two-cell embryos are presented; immunoelectron microscopy; the procedure of combining detection of BrUTP incorporation sites and nuclear proteins is described in MATERIALS AND METHODS; npb, nucleolus precursor body.

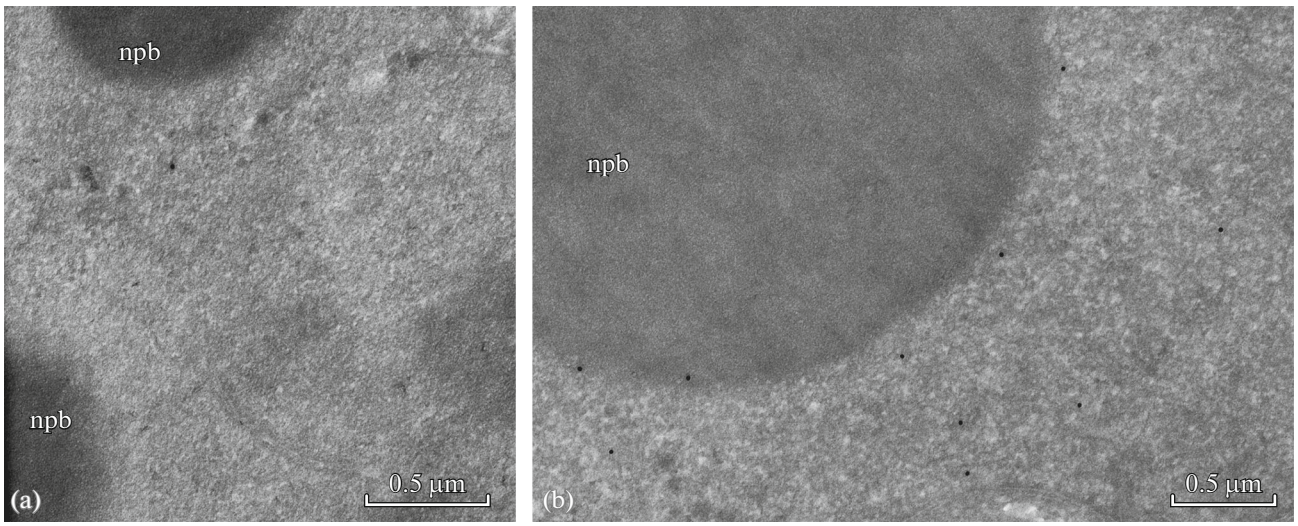

Fig. 3. Ultrastructural micrographs of control samples of the nuclei of two-cell mouse embryos; immunoelectron microscopy. (a) A fragment of the nucleus of blastomere injected with BrUTP, but treated with only secondary antibodies conjugated with colloidal gold particles of 10 and 15 nm. (b) A fragment of the nucleus of noninjected blastomere after treatment with antibodies to BrdU and histone H4 acetylated at lysine 5 (H4K5ac); only endogenous H4K5ac (15-nm gold particles) is detected; for more information on control experiments, see the MATERIALS AND METHODS; npb, nucleolus precursor body.

Thus, the variant of the processing protocol for ultrathin sections proposed by us allows successfully combining the use of BrdU antibodies to localize BrUTP incorporation sites and antibodies to representative endogenous proteins (H4K5ac and ATRX). This allows identifying nuclear proteins simultaneously with nascent RNA transcripts at the ultrastructural level. Nonspecific binding of antibodies is minimal in these conditions, and the distribution pattern of antigens studied is in agreement with the results obtained using confocal microscopy.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. The study was approved by the Animal Ethics Committee of the Institute of Cytology of the Russian Academy of Sciences (Assurance Identification number F18-00380).

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