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METHODS

Extra Perspectives of 5-Ethynyl-2'-Deoxyuridine Click Reaction with Fluorochrome Azides to Study Cell Cycle and Deoxyribonucleoside Metabolism

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Abstract—Beginning with the pioneering work of Salic and Mitchison (2008), the application of thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) for the detection of cells replicating DNA is actively expanding. Being incorporated into DNA, this nucleoside after click reaction of azide–alkyne cycloaddition with azides of fluorochromes can be easily detected by fluorescence. Recently, protocols of EdU application in combination with click reaction adapted for plant cells appeared, and they are help for a monitoring S-period of the cell cycle in the root meristems and in vitro cultured cells with the help of a microscope and flow cytometer. In this work, we focused some details of developed methods and their modifications and also recommended new protocols. In particular, we suggested combining EdU incorporation into the cells replicating DNA with subsequent isolation of protoplasts from them and their preparation for the microscopic analysis and flow cytometry. In addition, the method of determination of EdU phosphorylation dynamics in the cells in vivo is suggested.

Keywords: Arabidopsis thaliana, *Chlamydomonas reinhardtii*, *Synechocystis*, *Vigna radiata*, cell cycle, cell culture, nucleotides, S-period, protoplasts, flow cytometry, thymidine kinase, fluorescence microscopy

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INTRODUCTION

In the cell cycle (CC) there are two main stages. One of them—mitosis, during which substantial structural changes occur caused by an equal distribution between daughter cells of nuclear DNA packaged into chromosomes. The name "chromosome" is associated with the ability of these structures to be intensively colored by cytological dyes, which, coupled with their rather large sizes, determined for a long time their fruitful study, primarily by various microscopic techniques. The second stage is DNA replication occurring without obvious structural changes. It is this stage binds together CC periods and determines the success of continuous transmitting of identical hereditary information through a number of generations.

Certainly, the possibility of rapid visualization of cells in the period of nuclear DNA replication (S-period/S-phase of CC) is important for various areas of cell biology and physiology, including the studies of the CC regulation, cell responses to stressors, in screening for inhibitors of DNA replication, etc.

It is known that the cell requires a balanced supply of all four deoxyribonucleoside triphosphates—the immediate precursors of DNA synthesis [1]. Thymine is a nitrogenous base unique for DNA; therefore, its nucleotides are involved only in the synthesis of this macromolecule, but the usage of somehow labeled dTTP for the identification of DNA synthesis in living cells, in plant cells in particular, is difficult because of plasmalemma poor permeability for nucleoside triphosphates. However, nucleosides, including thymidine (dT) penetrate the cell easily with the help of transporters [2]. Within the cell, dT is phosphorylated by specific thymidine kinase (TK; EC 2.7.1.21) or deoxyribonucleoside kinase (EC 2.7.1.145) to produce dTMP and further, in the process of successive kinase reactions, a dTTP is produced—a substrate for a DNA polymerase reaction.

The classic method for detecting cells in S-period is associated with the use of tritium-labeled thymidine followed by radioautographic detection the precursor incorporated into DNA [3]. This method was practised widely for half a century, and basic discoveries in cell cycle kinetics and in the study of CC are on its

Abbreviations: 7-AAD—7-aminoactinomycin D; BrdU—5-bromo-2'-deoxyuridine; CC—cell cycle; DAPI—4',6-diamidino-2-phenylindol; DMSO—dimethylsulfoxide; dT—thymidine; EdU—5-ethynyl-2'-deoxyuridine; MMC—mithramycin A; PBS—phosphate-buffered saline; PI—propidium iodide; TK—thymidine kinase; TPFC—two-parameter flow cytometry.

account. Nevertheless, the radioautographic detection of precursors incorporated into DNA is quite laborious and takes a long time (up to several weeks of preparation exposure to the photographic emulsion). In addition, there was a desire to replace the radioactive nucleoside by safer compounds. Halogen-substituted dT analogues, such as 5-bromo-2'-deoxyuridine (BrdU) and 5-iodo-2'-deoxyuridine, came to the aid. As a rule, dT analogues are easily transported into the cell and involved in the metabolism by deoxyribonucleoside kinases and/or TK. After their incorporation into DNA, they are detected using specific monoclonal antibodies. The method takes much less time than radioautography, but has the following disadvantages: (1) DNA should be denatured (HCl, formamide, heating, etc.) for the interaction between epitops of 5-halogen-substituted dT analogues and antibodies, and this disturbs DNA structure and antigen properties of the nucleus and cell; (2) antibodies poorly penetrate in bulk samples; plant cell walls create a special obstacle for antibodies, so, cell walls must be broken [4–6].

In recent years, it was suggested [4] and actively expanding the application of dT analogue, 5-ethynyl-2'-deoxyuridine (EdU), for the detection of cells replicating DNA. After incorporation into DNA this precursor is easily detected in the click reaction (the reaction destined for rapid and reliable obtaining of novel molecules by combining of individual elements) of azide -alkyne cycloaddition catalyzed by Cu(I); this reaction was firstly described in 2002 independently by two groups of researchers [7, 8]. The terminal alkyne group of EdU reacts with fluorochrome azides (for example, with the azide of Alexa Fluor 488), producing stable covalent bonds. This method does not require strong DNA denaturation, cell wall breakage, the additional permeabilization of the plasmalemma and nuclear membrane, and allows a reduction of the time to detect cells replicating DNA at least three times as compared with the protocol using BrdU and immunodetection [4–6].

After the work of Vanstraelen et al. [9], who applied the 24-h exposure of *Arabidopsis* roots to EdU to elucidate the role of *CCS52A2* gene in the functioning of the root quiescent center, the first detailed paper of Kotogány et al. [5] appeared in 2010 about the methods of EdU usage in combination with click reaction for the monitoring of CC S-period in meristems of the roots and in the in vitro cultured cells with the application of microscopy and flow cytometry. In the review of Bass et al. [10] appeared in 2014, twenty presently available publications, where DNA replication in plant tissues was detected using EdU with subsequent click reaction with azides of fluorochromes, were briefly analyzed. Most works were destined to the analysis of the proportion of S-phase cells in different tissues, changes of DNA replication in mutant and transgenic plants, characterization of replication "pattern" on the levels of chromosome and chromatin.

Certainly, EdU application in plant experimental biology will be expanded. In this connection, we focused on the methodological details of available and modified protocols, and we also tried to complement the possible EdU applications in the click reaction with fluorochrome azides for the physiology and biochemistry of plant cells.

MATERIALS AND METHODS

Materials used. *Arabidopsis thaliana* (L.) Heynh. cell suspension cultures of two genotypes: wild type Col-0 (ecotype Columbia) and *ein2-1* mutant (EIN2 is one of the components of ethylene signaling), were produced from calli of leaf origin. Seeds of Col-0 [N1092] and *ein2-1* [N3071] were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC, Great Britain). Cell cultures were grown in Schenk and Hildebrandt (SH) medium supplemented with 3% sucrose, 1 mg/L 2,4-D, and 0.1 mg/L kinetin in glass flasks in darkness at 26°C and constant stirring (120 rpm). Subculturing was performed every 10 days.

The *Synechocystis* sp. PCC 6803 GT strain was kindly presented by Dr. N. Murata (National Institute for Basic Biology, Okazaki, Japan). It was grown on the BG11 medium [12] supplemented with needed quantity of 20 mM Hepes–NaOH to adjust pH to 7.6. The *Chlamydomonas reinhardtii* 137C+ IPPAS L-1014 strain was grown on TAP medium [13]. Algae were cultivated in 300-mL flasks in 80 mL of the medium at 25°C, constant stirring (70 rpm), and continuous illumination with luminescence lamps (35 μmol photons/(m^2 s).

The seeds of *Vigna radiata* L. cv. Berken were washed, soaked in warm water for 1 h, the seed coat was removed, and seeds germinated for 24 h in darkness at 25°C in the wells of 12-well plate in a small volume of water covering not more than the half of the seed.

Incubation with EdU and fixation of cells and tissues. Aliquots of the suspension cultures of *Arabidopsis* cells were sampled at the logarithmic growth phase (3rd–5th day), and 10–20 μM EdU (Invitrogen, cat. no. A10044) was added from its 10 mM stock solution in dimethylsulfoxide (DMSO). The cells were incubated at 26°C on the shaker (120 rpm): for detection of S-phase cells – for 30–120 min, for the determinations of cells capable of DNA replication – for 2–3 days. Algal and cyanobacterial cells were sampled on the 3rd day of culturing and incubated with 20 μM EdU for 2 h. Germinating seeds of *Vigna radiata* were treated with 10 μM EdU for a day immediately after seed coat removal.

When incubation with EdU was short, its incorporation in to the synthesizing DNA was stopped by the addition of 200 μM thymidine (dT, Sigma-Aldrich, cat. no. T9250) from the 10 mM aqueous stock solution. The cells were kept with dT for 5 min and fixed or used for protoplast preparation (see below). *Arabidopsis* cells were fixed in 4% formalin (prepared from 10% buffered solution from Sigma-Aldrich, cat. no. HT50-1-1) in PBS (2.7 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4, pH 7.4) with 0.1% Triton X-100. Algae were fixed in 4% formalin in PBS without Triton X-100 or in 70% cold methanol. *Vigna* roots were kept in 0.05% aqueous solution of colchicine (Sigma-Aldrich, cat. no. C3915) for 1 h, then in ice for 1 h, and fixed in the mixture of methanol and propionic acid (3 : 1).

Isolation and fixation of protoplasts. Equal volumes of cell suspension (usually 5 mL) and the heated to 28°C solution containing macrosalts of SH medium, 0.8 M sorbitol, 8 mM $CaCl_2$, 25 mM Mes-KOH (pH 5.7), 2% cellulase Onozuka R10 (Kinki Yakult, Japan), 0.3% pectinase Macerozyme R10 (Kinki Yakult), and 0.8% hemicellulase Driselase (Fluka) were mixed in 100-mL glass beakers. Enzyme solution was prepared beforehand, clarified by centrifugation, and frozen. Protoplasts were isolated at 26°C on the shaker (120 rpm) for 1.0–1.5 h.

The protoplast suspension was passed through a nylon sieve (40 μm mesh) and transferred to 10-mL plastic tubes. Protoplasts were sedimented by centrifugation at 100 *g* for 5 min in the bucket rotor at room temperature; the pellet of protoplasts was resuspended in 10 mL of 0.5 M sorbitol with 2.5 mM $CaCl_2$ (Sorb-Ca), kept for 5 min, and centrifuged (100 *g* for 5 min); the procedure was repeated; Sorb-Ca was added to the pellet up to 1.5 mL; suspension was dropwise added to 3.5 mL of cold (4°C) methanol in 5-mL tubes and kept for 20–30 min.

Protoplasts fixed in 70% methanol can be stored at 4°C during 2–3 weeks. Protoplasts can be fixed in 3–4% formaldehyde. To this end, 2 mL of 2× fixative was added to 2 mL of the protoplast suspension; this fixative was prepared as follows: 1.82 g of sorbitol was dissolved in 5 mL of distilled water, 2 mL of Mops (0.5 M, pH 7.0), 4 mL of 36.5% formaldehyde (Sigma-Aldrich, cat. no. F8775), and 100 μL of 1 M $MgCl_2$ were added, and the volume was adjusted to 20 mL. Protoplasts were fixed for no less than 15 min. In this fixative protoplasts can be stored at 4°C for a week; for long-term storage they should be transferred into 70% .

The basic protocol for detection of EdU incorporated into DNA using click reaction with the azide of Alexa Fluor 488. EdU incorporation was detected by the reaction with the azide of Alexa Fluor 488, although azides of other fluorochromes are now commercially available spanning the entire visible spectrum and suitable for multicolor cell labeling.

In the standard protocol for EdU detection, we used Click-iT EdU Alexa Fluor 488 HCS assay (Invitrogen, cat. no. C10350). Fixed *Arabidopsis* cells were washed in PBS three times and placed 20–30 μL of the packed cell volumes in the Eppendorf tubes. In each tube, 150 μL of the reaction mixture was added; this mixture was prepared in the following sequence recommended by the manufacturer: 113.3 μL of H_2O + 15 μL of the 10X reaction buffer (component C) + 6 μL of $CuSO_4$ (component D) + 0.7 μL of the azide of Alexa Fluor 488 (component B) + 15 μL of 1X buffer additive (component E; frozen 10X solution was diluted with water immediately before staining). The reaction mixture cannot be stored and must be used immediately after preparation. It should be noted that firstly we doubled the amount of fluorochrome (optimization is possible) and secondly many researchers refer to the protocol of Kotogány et al. [5]; but in their text [5] the names and letter designations of components are mixed up, whereas the sequence of their adding is very important.

After the click reaction (exposure for 30 min at room temperature in darkness), the cells were washed with 200 μL of component F or PBS with 2 mM sodium azide, then with 1 mL of PBS and, for nuclei staining, with PBS containing 100 ng/mL of DAPI (4',6–diamidino-2-phenylindole, Sigma-Aldrich, cat no. D9542) from the stock solution (250 μg/mL in water). Stained cells can be stored at 4°C for several days.

The click reaction is catalyzed by the Cu(I) ions, which can be easily produced when $CuSO_4$ is used in the presence of reducing agents, such as sodium ascorbate. Therefore, in addition to the reaction cocktail recommended by Invitrogen, we applied two compositions as media for click reaction.

Medium 1. Modified reaction medium of Salic and Mitchison [4]: water (up to the required final volume) + 100 mM Tris–HCl (pH 8.0) + 2 mM $CuSO_4$ + 5 μM azide of Alexa Fluor 488 (Invitrogen, cat. no. A10266) from 1 mM stock solution in DMSO + 100 mM sodium ascorbate (Sigma-Aldrich, cat. no. A7631) from 0.5 M freshly prepared solution.

Medium 2. PBS (up to the required final volume) + 2 mM $CuSO_4$ + 5 μM azide of Alexa Fluor 488 + 100 mM sodium ascorbate.

Let us note that the correct sequence of component addition and the usage of freshly prepared solutions are important factors for the successful reaction. We did not observe any substantial differences between the application of commercial set from Invitrogen and suggested reaction media 1 and 2. The choice depends on the compatibility of the subsequent after-staining procedures with the used buffer system.

Protocol for protoplasts. Protoplasts fixed in methanol or formalin were washed twice in 0.5 M sorbitol without calcium with centrifugation for 7–10 min at 100 *g*; then they were washed with PBS with 0.1% Triton X-100 and PBS (with 10-min incubation between centrifugations); 1 mL of PBS was added to the pellet; the suspension was transferred into 1.5-mL Eppendorf tubes, centrifuged, and 150 μL of the click reaction medium was added to 20–30 μL of the pellet. In 30 min, protoplasts were centrifuged again, washed with 200 μL of the component F or PBS with 2 mM sodium azide, then with the excessive PBS and 1 mL of PBS with one of fluorochromes staining DNA: 100 ng/mL of DAPI; 5 μg/mL of PI (propidium iodid, Fluka, cat. no. 81845) from the stock solution of 1 mg/mL in water with 0.1% sodium azide, 25 μg/mL of MMC (mithramycin A, Serva, cat. no. 29803.01) from the stock solution of 2.5 mg/mL in water with 10 mM $MgCl_2$ and 0.1% sodium azide; 15 μg/mL of 7-AAD (7-aminoactinomycin D, Molecular Probes, cat. no. A1310) from the stock solution of 500 μg/mL in DMSO. To stain nuclei, 10–20 min is sufficient, but they may be left at 4°C for 2–3 days.

Protocol for protoplasts with RNase treatment. The concentrated solution of RNase was prepared as follows: RNase A (Sigma-Aldrich, cat. no. R5500) at the concentration of 10 mg/mL was dissolved in 10 mM Tris–HCL (pH 7.6) with 15 mM NaCl and heated at 95°C for 20 min. Then, the solution was slowly cooled to room temperature, centrifuged at 10000 *g*, poured in 0.5-mL Eppendorf tubes (200 μL in each), and frozen.

Fixed protoplasts were centrifuged at 100 *g* for 10 min, fixative was removed, 5 mL of 0.5 M sorbitol without calcium was added to the pellet, incubated for 20 min (protoplast suspension was periodically stirred carefully), centrifuged at 120 *g* for 10 min, and washing with 0.5 M sorbitol was repeated. Thereafter, sorbitol was removed, 4 mL of PBS with 0.5% Triton X-100 was added to the pellet of protoplasts, and they were incubated for 30 min. Protoplasts were centrifuged at 200 *g* for 10 min and washed with PBS; 0.5 mL of RNase solution (the required amount of the stock solution was added to the warm PBS (37°C) with 2.5 mM Na-EDTA up to the final concentration of 100 μg/mL) and incubated at 37°C for 1 h. After treatment with RNase, protoplasts were sedimented (at 200 *g* for 10 min), washed with PBS twice, and transferred into 1.5-mL tubes (30–50 μL of packed protoplast volume in each). Then, 150 μL of the click reaction mixture was added. In 30 min protoplasts were centrifuged, washed with 200 μL of component F or PBS with 2 mM sodium azide, then with PBS and 1 mL of PBS with fluorochromes: 100 ng/mL DAPI or 5 μg/mL of PI (10 μg/mL for flow cytometry).

Detection of EdU into DNA of algal cells and *Vigna* roots. Fixed algal cells were sedimented (at 2000 *g* for 3 min), incubated in 70% methanol for 10 min, centrifuged, washed twice with 70% methanol (10 min each) and twice with PBS; then the cells were incubated in PBS with 0.1% Triton X-100, washed with PBS, and transferred into 1.5-mL tubes. 200 μL of the reaction mixture (**medium 1** in Tris–buffer) was added to 30–50 μL of the pellet, the cells were incubated for 30 min in darkness. Thereafter, the cells were washed with PBS with 2 mM sodium azide and PBS; then cold 80% acetone was added to the pellet and kept for 10 min, centrifuged, acetone was removed, and the procedure was repeated still twice. After washing with PBS, the cells were stained with DAPI (100 ng/mL in PBS).

Fixed *Vigna* roots were twice washed with 70% methanol (10 min each), then with water, and incubated in 45% acetic acid for 1.5 h. Then, the material was twice washed with water, PBS, kept for 20 min in PBS with 0.2% Triton X-100, washed twice with PBS, and click reaction with the azide of Alexa Fluor 488 was performed for 1 h in the reaction mixture with PBS (**medium 2**). After washing with PBS with 2 mM sodium azide and PBS, roots were stained with DAPI and squashed preparations were made.

Fluorescence microscopy and flow cytometry. Immediately before microscopic analysis, protoplasts, cells, and tissues were washed free of the excess of fluorochromes (DAPI, PI, MMC, and 7-AAD) with PBS. The analysis was performed with the Univar microscope (Reichert-Jung, Austria) with the Canon PowerShot G6 digital camera and filter blocks: 42 (Ex 18 × 2 UG1; reflection up to 420 nm, absorption 18 × 3 KV 418/VG) for DAPI; 45 (Ex 18 × 2 BG3; reflection up to 450, absorption 18 × 3 KV 418/VG) for MMC; 51 (Ex 18 × 4 VG9; reflection up to 510 nm, absorption 18 × 3 RG645/VG) for Alexa Fluor 488, PI, and 7-AAD. The Axio Imager Z2 microscope (Carl Zeiss, Germany) was equipped with the digital camera AxioCam MR and filter blocks: 44 (Ex BP 475/40; Em BP 530/50) for Alexa Fluor 488; 02 (Ex G365; Em LP420) for DAPI; 43 (Ex BP 550/25 HE; Em BP 605/70 HE) for PI. Images were analyzed with the AxioVision 4.8 program.

For two-parameter fluorescence analysis of protoplasts stained with PI and Alexa Fluor 488, we used the Guava easyCyte 6HT flow cytometer (Merck Millipore, Germany) equipped with the capillar of the inner diameter of 100 μm, exciting argon-ion laser 488, two detectors (525/30 nm for Alexa Fluor 488 and 680/30 nm for PI), and the InCyte software.

Determination of EdU Phosphorylation Dynamics in Arabidopsis Cell Culture

This protocol is based on the method of Wolcott and Colacino [14], who used the property of La ions to sediment phosphates, including nucleoside phosphates; it

was used for the determination of in vitro thymidine kinase activity with radioactive substrates. Before experiment, the *Arabidopsis* Col-0 cell suspension at the logarithmic growth phase was passed through the sieve with 1–2-mm mesh size into wide beaker and placed on the magnetic stirred.

Cell sampling and fixation. Cell suspension (2 mL) was filtered through the glass microfiber GF/C filter (Whatman, cat. no. 1822-021) fixed in the dismountable. Teflon filter holder on the Bunsen flask. Cell fresh mass formed on the filter as a "tablet" was quickly placed on a brass table cooled with liquid nitrogen. Frozen cell mass was transferred into the polypropylene tube with the filtering insert with pores of 10 μm (VectaSpin Micro, Whatman, cat. no. 6838-0002); then the tube was placed in the freezer (–20°C). Several filtered but not frozen specimens (before and after the end of experiment) were weighed for the evaluation of the uniformity of sampling.

Experiment. EdU (25 μM) was added to the beaker with stirred cell suspension. In 2, 4, 6, and 8 min, the aliquots (2 mL) of suspension were taken, filtered as described above, and before a dense pellet of cells was formed on the filter, the cells were washed with 2 mL of 1 mM dT. Further we followed the protocol of cell sampling and fixation (see above). The experiment was repeated in 40 min, using cell suspension cultivated in parallel in another flask.

The VectaSpin tubes with frozen cell mass were thawed at 4°C, centrifuged at 1500 *g* for 30 min (at 4°C), and filtered cell sap was transferred into preliminary weighed 1.5-mL Eppendorf tubes. After measuring the volume of the cell sap, its volume was equalized by cold deionized water; then equal amount of 200 mM Tris–HCl (pH 8.0) with 10 mM NaF and 200 μL of the reaction **medium I** (in Tris buffer) was added. In 30 min the required volume of 100 mM dTMP (as a ballast nucleoside phosphate) was added up to the concentration of 5 mM; thereafter, 1 mL of 100 mM $LaCl_3$ solution with 5 mM triethanolamine was added. Samples were thoroughly stirred and centrifuged at 1500 *g* for 10 min. The pellets were washed with 1 mL of $LaCl_3$, dissolved in 200 μL of 0.05 M HCl, and the solution was transferred in the wells of the 96-well flat-bottomed plate. The fluorescence intensity of Alexa Fluor 488 covalently bound to the EdU phosphates was determined using Typhoon Trio$^+$ Imager (GE Healthcare Life Sciences, Sweden) at Ex 480 and Em BP 520/40. Click reaction with the cell sap without cell incubation with EdU was used as a control.

RESULTS AND DISCUSSION

The Presence of Thymidine Kinase Determines EdU Phosphorylation and Incorporation into Replicating DNA

As it was noted, the presence of enzymes phosphorylating EdU, TK or deoxyribonucleoside kinase, is necessary for EdU incorporation into replicating DNA molecule; these enzymes are absent from many marine and fresh-water cyaonobacteria [15, 16]. For example, to reveal asynchrony in the replication of multicopy chromosomes of *Synechococcus elongatus* PCC 7942, Watanabe et al. [16] developed the S. 7942^{TK} strain expressing the gene encoding TK of herpes virus. The cells of this strain were capable of BrdU (dT analogue) incorporation into replicating DNA molecules; thereafter, BrdU incorporation was detected by specific antibody. Revealing and subcellular localization of DNA replication, but already with the help of EdU, in the *Schizosaccharomyces pombe* were also coupled with a necessity to construct the strain expressing *TK* gene of the herpes virus and *hENT1* gene encoding the nucleoside transporter. For hyperthermophilic archaea *Sulfolobus* sp., the application of EdU for the monitoring of DNA replication became possible after the construction of strains expressing *TK* from another species of hyperthermophilic archaea [17, 18].

In our work, we studied a possibility of EdU incorporation into DNA of well known model strain *Synechocystis* sp. PCC 6803 GT, which genome does not contain *TK* gene. After 2 h of incubation with 20 μM EdU, fixation (in methanol or formalin), click reaction with the azide of Alexa Fluor 488, and the additional removal of chlorophyll and other pigments with acetone, we did not detect any "EdU-positive" *Synechocystis* cells (Figs. 1a–1c).

As distinct from cyanobacteria, many unicellular green algae are armed with TK, *Chlamydomonas reinhardtii* is among them, and this enzyme was isolated from its cells [19].

Using fluorescent microscopy, we revealed 5–6% of "EdU-positive" *C. reinhardtii* cells (Fig. 1e) after 2-h incubation with 20 μM EdU and subsequent click reaction with the azide of Alexa Fluor 488. It should be noted that the culture was not synchronized and it was cultivated under low light conditions. Because of a great amount of green pigments, fixation in methanol and washing with methanol are insufficient for the detection of EdU incorporation. The signal of Alexa Fluor 488 was detected in the cells of *C. reinhardtii* only after three additional washes with cold acetone.

Thus, the incubation of cyanobacterium and green algal cells with EdU with subsequent click reaction with azides of fluorochromes may be used not only for revealing cells replicating DNA but also as the express test on the presence of TK and/or deoxyribonucleo-

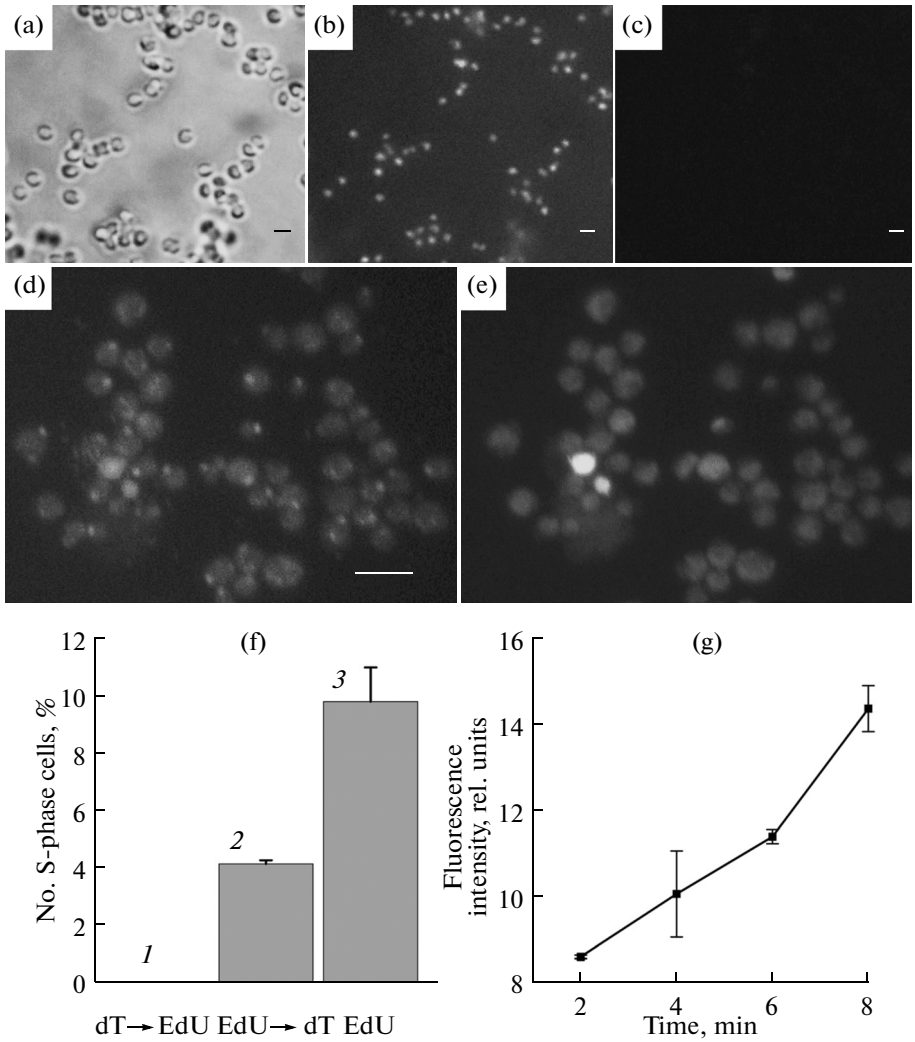

Fig. 1. Detection of EdU incorporated into DNA in dependence on the presence of TK, competitive inhibition of EdU incorporation by dT, and the analysis of EdU phosphorylation in the cells.

The cells of *Synechocystis* sp. (a–c) and *Chlamydomonas reinhardtii* (d, e) were incubated with 20 μM EdU, and precursor incorporated into DNA was detected in the click reaction with the azide of Alexa Fluor 488. (a) Transmitted light; (b, d) staining with DAPI; (c, e) Alexa Fluor 488. The cells of *Arabidopsis* Col-0 (f) before isolation of protoplasts were incubated with dT followed by EdU (*1*), or with EdU followed by dT (*2*), or with EdU alone (*3*). EdU incorporation was detected in the click reaction with the azide of Alexa Fluor 488; (g) dynamics of EdU phosphorylation in the *Arabidopsis* Col-0 cells in vivo (see Materials and Methods section). Scale bar: 2 μm (a–c) and 30 μm (d, e).

side kinase in tested organisms. This can be a beneficial primary trait in their systematics. In addition, it should be noted that DNA labeling with Alexa Fluor 488 is tolerant to acetone treatment.

Successful half-century application of dT and its analogues in the studies of CC and DNA replication in higher plants indicates the indubitable presence in these organisms of enzymes phosphorylating dT. Nevertheless, plant TK was not recognized immediately. Early studies of Hotta and Stern [20] on microspores of *Lilium longiflorum* showed that TK activity appeared shortly before DNA synthesis, coinciding with the accumulation of deoxyribonucleoside phosphates, and was induced by dT. Then, TK functioning in plant cells was questioned [21]; it was assumed that dT phosphorylation was catalyzed by nucleoside phosphotransferase (EC 2.7.1.77). Later TK was isolated and purified from broad beans [22], and quite recently two genes encoding TK were identified in the *Arabidopsis* genome [23].

When the quantity of S-phase cells was determined in experiments with the great number of treatment (for example, at the study of the action of many effectors on CC) and short exposures to EdU, its incorporation into DNA should be stopped by cell and tissue incubation with the excess of dT. We demonstrated the competitive inhibition of EdU incorporation into DNA by dT on cultured cells of *Arabidopsis* Col-0 (Fig. 1f).

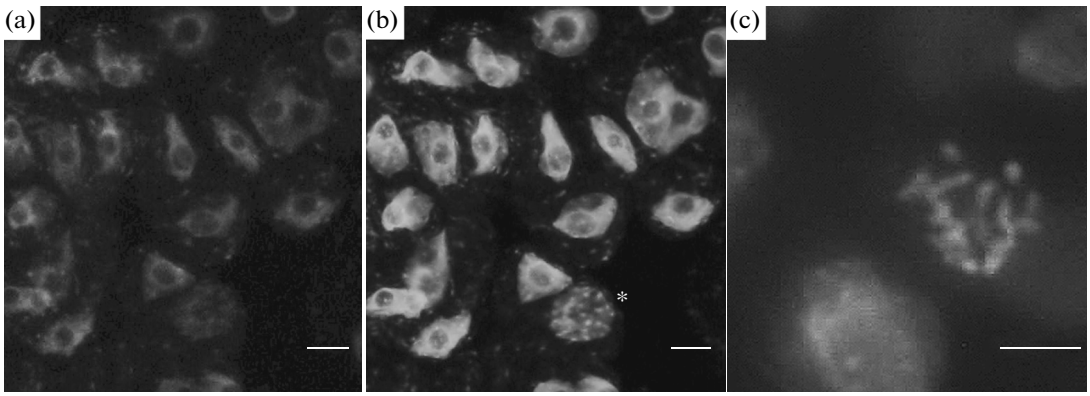

Fig. 2. Detection of EdU incorporated into DNA after long-term incubation with nucleoside.
Arabidopsis Col-0 cells were incubated for 48 h with 10 μM EdU (a, b) and germinating seeds of *Vigna radiata* were incubated for 24 h with 10 μM EdU (c). Then the precursor incorporated into DNA was revealed in the click reaction with the azide of Alexa Fluor 488. (a) Staining with DAPI; (b, c) staining with Alexa Fluor 488. Scale bar is 10 μm for all photos. The nucleus transited to the S-phase shortly before cell fixation is marked with the snowflake.

When the cells were first exposed to 400 μM dT for 10 min and then to 20 μM EdU for 30 min with subsequent protoplast isolation (1.5 h), S-phase cells were not detected. When dT was added to the cells after 30-min incubation with EdU, 4% of S-phase cells was detected, whereas without dT addition their number increased to 10%, i.e., the cells continues the DNA synthesis DNA during protoplast isolation.

Since EdU phosphorylation is necessary for its incorporation into DNA, we suggested to use this dT analogue for the analysis of the dynamics of deoxyribonucleoside phosphorylation in the cells. The essence of the method is cell incubation with EdU, rapid cell sampling and their fixation by freezing, isolation of the cell sap containing EdU and its phosphorylated derivatives, click reaction EdU and its phosphates with the azide of Alexa Fluor 488, selective precipitation of phosphorylated complex EdU–Alexa Fluor 4888 by $LaCl_3$, and measuring the fluorescence signal. Cultured cells of *Arabidopsis* Col-0 phosphorylated EdU rapidly (Fig. 1g). In this case, the intensity of fluorescence was measured using the Typhoon Trio$^+$ Imager and obtained reproducible results of two independent experiments. Obviously, EdU can be applied instead of tritium-labeled dT for the analysis of TK activity in vitro, whereas the combination of EdU with dT and other dT analogues – in the investigations of substrate specificity of TKs and deoxyribonicleoside kinases.

Choice of EdU Concentration and Incubation Duration

Kotogány et al. [5] analyzed in detail a dependence of detected S-phase cells on the EdU concentration and incubation duration. It was shown that 15-min incubation with 10 or 50 μM EdU was sufficient for the detection of culturing *Arabidopsis* cells replicating DNA, whereas at the concentration of 1 μM 30-min exposure was required. For slowly growing cultures, it was recommended to apply at least 10 μM EdU for 30–60 min.

In our experiments, for the determination of the S-phase index, we incubated cells with 10–20 μM EdU for 30–120 min with the obligatory stoppage of nucleoside incorporation with the dT excess. To determine the proliferative pool of cultured cells (cell fraction involved in division), they are usually incubated with the precursor of DNA synthesis (in classical methods with tritium-labeled dT) for the period slightly exceeding the CC duration, adding the precursor immediately or in small doses with intervals shorter than the duration of S-period. Thus, after the incubation of cultured *Arabidopsis* Col-0 cells with 10 μM EdU for 48 h, essentially all nuclei were stained with Alexa Fluor 488 after click reaction with the azide of the fluorochrome (Figs. 2a, 2b). Let us note that even after 48 h we could detect nuclei transited to the S-period of the CC shortly before cell fixation, which was evident from the pattern of label incorporation as separate focuses (Fig. 2b, marked by the snowflake).

In several works performed on mammal cells, cytotoxic EdU effects were observed, mainly at long-term (longer than 24 h) exposures, which were manifested in the CC arrest, the arising of double-stranded breaks in DNA, and cell death [24–26]. EdU effects depended on the cell genotype, in particular on the expression of p53 protein. The fact of negative EdU influence on plants was noted only in one study, when in *Vicia faba* roots about 3% of metaphase chromosome aberrations were seen after meristematic cell synchroni zation with hydroxyurea in the S-period of the CC and subsequent short-term incubation with EdU [27].

Three-day-long incubation of cultured *Arabidopsis* cells with 10 μM EdU did not affect their viability determined by the absence of staining with Erythrosin B (there was about 84% of alive cells in both control culture and that treated with EdU). The exposure of germinating *V. radiata* seeds with 10 μM EdU did not lead to CC arrest at early stages during first 24 h after soaking, because we observed prophases and metaphases colored with Alexa Fluor 488 (Fig. 2c). Nevertheless, at long-term incubation, a possibility of cytotoxic and genotoxic EdU action should be taken into account, and applying low EdU concentrations is preferable.

Using the Protoplasts for Detection of Cells in the S-phase of the Cell Cycle by Microscopy and Flow Cytometry

During most developmental stages, the organism of higher plants comprises tissues where individual cells are mechanically connected through a common cell wall. It is a difficult task to analyze individual cells within the tissue. More convenient material for studying some issues, including CC regulation, is cell suspension culture consisting of small cell aggregates. However, even in this case, due to the superposition of cell layers in squashed cytological preparations of cell aggregates, some difficulties arise in the calculating of S-phase cells, identified after EdU incorporation into DNA and subsequent click reaction with azides of fluorochromes. Protoplast isolation is an efficient approach for the conversion of cell aggregates into the suspension of single protoplasts, which is much more convenient for experiments, in particular for the calculation of S-phase cells.

Some important points of the procedure should be noted. Firstly, it is necessary to stop EdU incorporation into replicating DNA by the addition of dT to the cell suspension at the concentration 10–20 times higher than EdU concentration, because EdU continues to incorporate into DNA even in the process of protoplast isolation (Fig. 1f). Secondly, it is necessary to shorten the time of protoplast isolation. Using our proposed enzyme mixture, protoplasts can be easily isolated from cultured cells of many plant species for 1.0–1.5 h; however, it is important to constantly monitor the process of protoplast isolation and not to keep the protoplasts in the enzyme solution too long (better a little shorter) to avoid their possible disturbance. Thirdly, the protoplast suspension should be filtered through the nylon sieve with pores 1.5-fold higher than the average diameter of protoplasts of a given culture to lessen the losses of large protoplasts and the analysis of single cells, protoplasts of which are not still isolated.

We isolated protoplasts from the cultured *Arabidopsis* cells after their incubation with EdU. Protoplasts fixed in methanol or formalin retained well their shape (Figs. 3a, 3d, 3g, 3h). It seems likely that the formalin fixation would be preferable if you want to keep the structure and antigenic properties of the cell cytoplasm. On preparations of protoplasts after click reaction with the azide of Alexa Fluor 488 and DAPI staining of nuclei, we can easily differentiate and count the number of S-phase cells after short or prolonged incubation with EdU (Figs. 3a–3f).

Certainly, flow cytometry is a more powerful tool for the analysis of the CC events, especially in the two-parameter case, where the fraction of nuclei replicating DNA can be determined in parallel with their distribution after ploidy [5, 10, 28]. For two-parameter flow cytometry (TPFC), as a rule, the suspension of nuclei isolated from fresh or fixed tissues and cells preliminary incubated with BrdU is used [28]. To determine the amount of DNA in nuclei, the intercalating dye PI (as distinct from DAPI, PI does not manifest any selective affinity for the composition of bases in DNA) is usually applied, whereas nuclei in the S-period of CC are detected by antibody specific toward BrdU and secondary antibody labeled with some fluorochromes. Recently, the TPFC with the replacement of BrdU with Edu was suggested for mammal cells; in this case the laborious procedure of immunodetection is replaced by click reaction of EdU with azides of fluorochromes [24]. First works appeared with the application of EdU for TPFC of plant cells and tissues and nuclei isolation from fresh or fixed material [5, 10].

Many researchers are skeptical relative to the use of protoplasts for CC analysis by TPFC [5, 29], indicating that protoplast isolation is a long-term process and the quality of histograms of protoplast distribution by the amount of DNA is bad. As was described above, the time of protoplast isolation can be shortened substantially by the optimization of enzyme mixture; however, histogram quality of protoplast distribution by the amount of DNA is affected by PI capacity to stain RNA. Independently of the way of fixation of protoplasts isolated from the cultured *Arabidopsis* cells after incubation with EdU, click reaction with the azide of Alexa Fluor 488 clearly reveals S-phase nuclei, which green fluorescence is surrounded by red fluorescence of the cytoplasm intensively stained by PI (Figs. 3g, 3h). In protoplasts pretreated with RNase (before click reaction and DNA staining), cytoplasm staining disappears almost completely (Fig. 3i); on these protoplast preparations S-phase nuclei are clearly seen (Figs. 3k, 3l). Thus prepared protoplasts stained with PI or 7-AAD or protoplasts without RNase treatment and stained with DAPI or MMC (Figs. 3b, 3c, 3e, 3f, 3j) are suitable for TPFC. On the one-parameter histogram of the distribution of protoplasts by the amount of DNA (staining with PI)

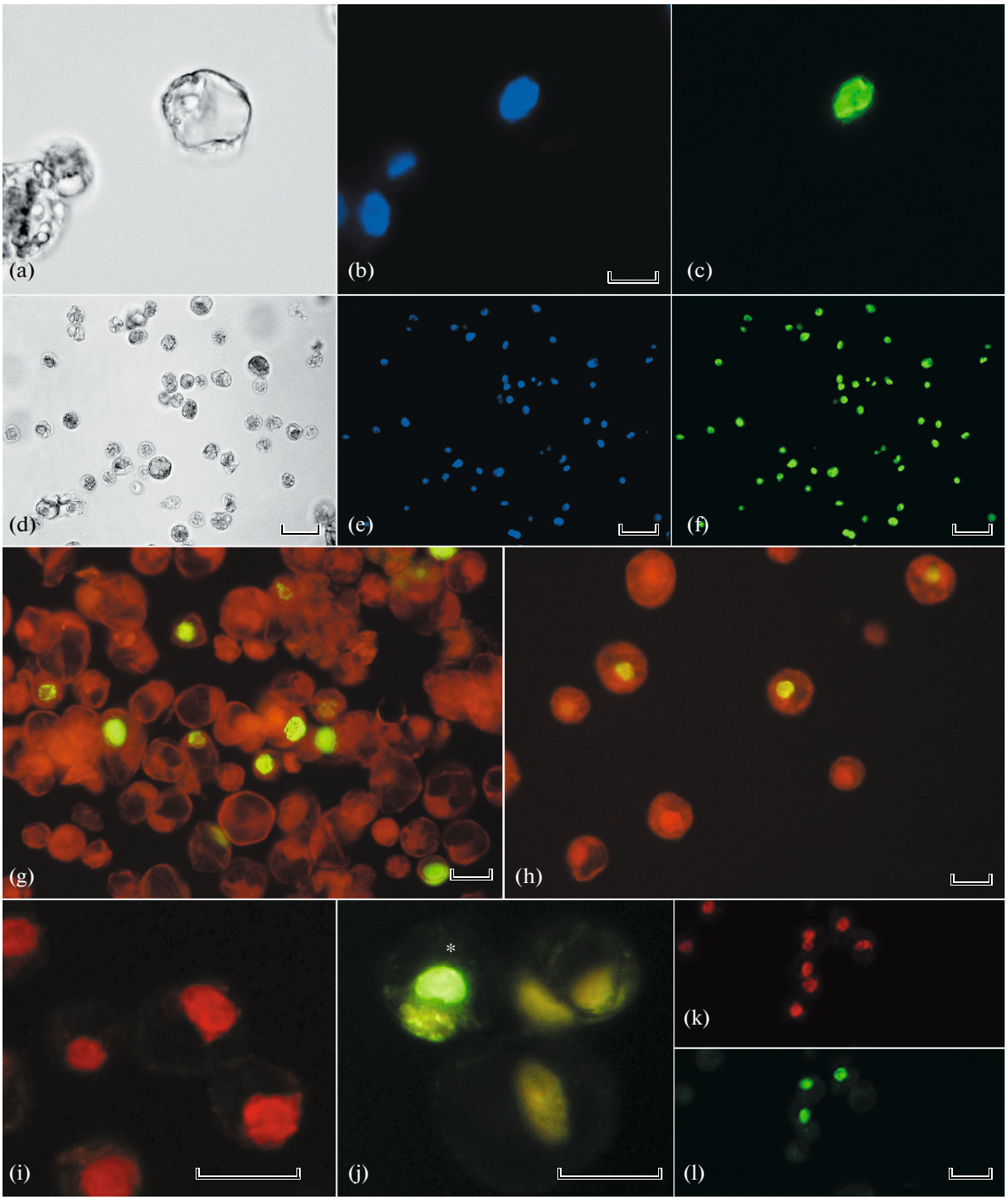

Fig. 3. Protoplast using for revealing the cells in the S-phase of the CC.

Protoplasts were isolated from the *Arabidopsis* Col-0 cells (a–c, g, i, k, l) and *Arabidopsis ein2-1* cells (d–f, h, j) after their incubation with EdU in the concentration of 20 μM (a–c, g–l) or 10 μM (d–f) for 1 h ((a–c, g–j), 3 h (k, l), or 48 h (d–f). Protoplasts were fixed with 70% methanol (a–f, h–l) or with formalin (g); the incorporated EdU was revealed in the click reaction with the azide of Alexa Fluor 488 without preliminary protoplast treatment with RNase (c, f–h, j) or after treatment with RNase (i, k, l). DNA was additionally stained with DAPI (a–f), PI (g–i, k, l), or MMC (j). (a, d) Transmitted light; (b, e, g–k) DNA staining; (c, f, g, h, j, l) staining with Alexa Fluor 488. Scale bar: 10 μm (a–c), 50 μm (d–f),and 20 μm (g–l). The nucleus with green fluorescence of Alexa Fluor 488 surrounded by nuclei with yellow fluorescence of MMC is marked with the snowflake.

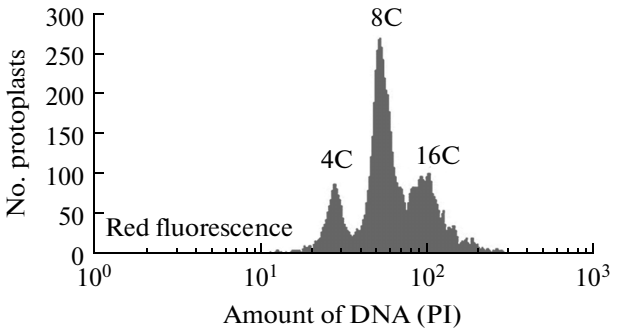

Fig. 4. Histograms of distribution of protoplasts from cultured *A. thaliana ein2-1* cells by the amount of nuclear DNA after one-parameter flow cytometry.

Protoplasts were stained with PI after preliminary treatment with RNase.

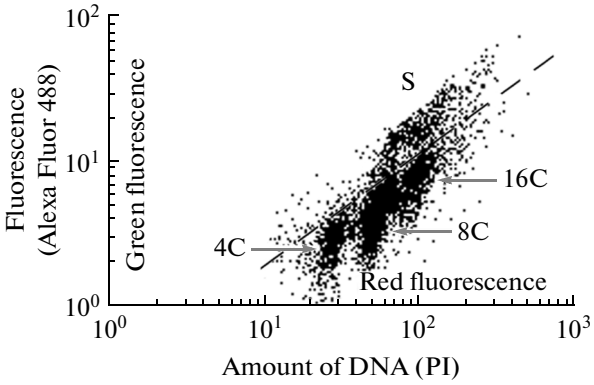

Fig. 5. The results of TPFC of protoplasts of cultured *A. thaliana ein2-1* cells after revealing EdU incorporated into DNA in click reaction with the azide of Alexa Fluor 488 and nuclei staining with PI.

Above the dashed line is a cluster of protoplasts (S), having tag EdU–Alexa Fluor 488, the intensity of which green fluorescence is substantially different from fluorescence of closely spaced dots below of the dashed line.

(Fig. 4), peaks corresponding to 4C, 8C, and 16C are well seen. The analyzed population of cultured *Arabidopsis ein2-1* cells is mixoploid, and the data obtained by flow cytometry coincided with the results of two wave length cytophotometry of these cells after staining after Feulgen (data not shown). The results of TPFC presented as a point diagram (Fig. 5) demonstrate clearly the presence of protoplast cluster with the label EdU–Alexa Fluor 488 (S, protoplasts synthesizing DNA), which green fluorescence intensity differed substantially from fluorescence of closely positioned points (Fig. 5, above and below of the dotted line, respectively).

In general, protoplasts isolated from the cells after incubation with EdU and click reaction with azides of fluorochromes can be successfully used for revealing S-phase cells by microscopic analysis; they can serve by an alternative of isolated nuclei in TPFC even when working with mixoploid cell population.

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