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New method for cell cycle synchronization in *Amoeba proteus* culture

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Summary

A method to synchronize cell cycle in the culture of *Amoeba proteus* is proposed. *Amoeba* cells were kept in quail egg albumen and thus having been fed by the pinocytosis, then thoroughly washed from the protein and examined at stained slides in different periods of time after washing. It appeared that practically all the cells on the slide corresponding to certain time after feeding/washing underwent the same stage of cell cycle – either interphase or one of the phases of mitosis depending on time. The method of amoebae feeding by the pinocytosis also allows avoiding negative effect of food remains in the cell cytoplasm, thus providing the higher quality of stained slides.

Key words: *Amoeba proteus*, cell cycle, mitosis, pinocytosis, synchronization

Introduction

Large free-living freshwater amoebae, such as *Amoeba proteus* (Pallas, 1766) Leidy, 1878, are traditionally used as model objects while studying the biology of eukaryotic cell (Ord, 1971; Yudin, 1990; Jeon, 1995). Large (about 500 μm) amoeba cells with well pronounced ovular nucleus 30-40 μm in size (Page, 1986) are rather resistant to damaging factors and unpretentious in laboratory culturing. These protists which bear simultaneously the features of a cell and an organism are attractive when study physiology, biochemistry and mainly adaptations by watching some marker characters in the course of environmental alterations. Being obligatory agamic organism amoebae represent a number of strains (clones), and thus they might be extremely suitable for researches in genetics, or epigenetics (Yudin, 1982).

Although the representatives of well-known species *Amoeba proteus* are under study for about 200 years their main enigmas – the number of chromosomes and the ploidy – are still unsolved. It was usually considered that the chromosomes are very small and their number is about 500 or even much more (Ord, 1973; Marahova et al., 1993), although Liesche (1938) inclined to believe that their number is less than 100. It is impossible to use DNA content as a measure of ploidy due to some peculiarities of the amoebae cell cycle – the G_1 phase is absent there, and the S-phase begins directly after the karyokinesis; the disproportionate DNA replication occurs during S-phase (Ord, 1979; Yudin, 1982; Afon'kin, 1989; Makhlin, 1993). Earlier it was presumed that numerous (several hundreds, see: Bogdanowicz, 1930; Gromov, 1985) nucleoli which are located at the nucleus periphery mainly contribute to this DNA replication, but

more recently some data were obtained that made this presumption rather doubtful (Borisenko et al., 2010).

According to traditionally accepted view, in self-running culture one division (mitosis) was found per approximately 1000 cells. Thus, the important requirement to study various stages of mitosis in amoebae, the ploidy of the amoeba cells, the number and morphology of their chromosomes, is to use the culture synchronized by its cell cycle. It was shown that the generation time and the amount of DNA synthesized in amoeba nucleus depend upon the nutritional regime of the amoebae culture (Rogerson, 1980; Makhlin, 1993). Feeding by ciliates (*Tetrahymena pyriformis* GL) alternated with medium replacement was used to regulate multiplication rate of the culture, and usually after applying such a technique the cells start to divide more or less simultaneously (Yudin, 1990), but rather significant dispersion of this character also took place (Makhlin, 1993). To determine generation time of the strain and to study amoeba's mitosis, dividing amoebae were selected from the culture (Gromov, 1985) as they easily can be distinguished from the interphase cells by their appearance (Chalkley and Daniel, 1933). Unfortunately, such a method does not allow to obtain mass material of this or that stage of mitosis.

More or less satisfactory results were obtained when after 3 days of starvation amoebae were fed by tetrahymenas; then followed double cleaning, second feeding and next 3 days of starvation (A.V. Goodkov, unpublished data). In this case the ciliate remains are kept in digestive vacuoles of amoeba up to 72 h after feeding and form "background noise" on the stained slides. It is even impossible to measure DNA content by cytofluorimetry during mitosis because of abundance of tetrahymenas in the amoeba cell, and it results in necessity to isolate the nucleus to carry out the measurements (Afon'kin, 1989; Borisenko et al., 2010).

Thus, taking into account all these side effects, the necessity of looking for more reliable method to synchronize the culture of *Amoeba proteus* becomes evident.

Material and methods

Amoebae *Amoeba proteus* of strain B from the Collection of amoeba cultures in the Laboratory of unicellular organisms of the Institute of Cytology RAS (Yudin, 1990; Podlipaeva and Farka, 1995; Podlipaeva and Goodkov, 2009) were used in experiments. The culture was intensively fed by tetrahymenas in Prescott-Carrier's (PC) solution (Prescott and Carrier, 1964) in glass vessel during 2 weeks to reach sufficient density – approximately 1000 cells per 1 cm^2. Then the cells starved for 3 days to achieve the elimination of the tetrahymena remains from food vacuoles.

To avoid the "negative effect" of small particles on the stained slides which is inevitable in the process of nutrition by phagocytosis, we decided to stimulate amoebae to feed by pinocytosis. Quail egg albumen was used instead of tetrahymenas. Portion of egg-white was obtained by pipette from the quail egg, placed into eppendorf tubes and centrifuged at 10000 rpm during 20 min to unload solid and mucous contaminations. Transparent supernatant was used for amoebae feeding.

The fixed material was studied on the total preparations stained with modified traditional ethanol-orcein technique (Dyomin and Schobanov, 1990). The light microscope Leika DM2500 with component ingredients for differential-interference contrasting method (DIC) was used; the microscope was equipped by photo-camera Leika DFC420 with software for registration of images and their treatment.

Results and discussion

PC medium was removed from the culture vessel and replaced by 1 ml of albumen to cover totally the amoebae, attached to the bottom of the vessel. Amoebae have immediately stopped locomotion, synchronously started taking spherical shape (Fig. 1a), and begun the process of pinocytosis (Figs 1b and 1c). After 1 h of such feeding all the cells in the culture were perfectly spherical and were lying at the vessel bottom. One huge vacuole is formed in the very middle of each cell.

Amoebae were washed during 1 h, the medium was changed every 10–12 min, as cells were sinking and attaching to the bottom. First two times we used NaCl solution instead of PC to avoid the forming of phosphate and sulphate sediments, and then the culture was several times washed with PC. Then the amoeba culture was placed into dark chamber at room temperature (20–22 °C).

The stained slides were prepared starting from 30 hrs after washing and then every 2 hrs (32, 34, 36 and 38 hrs) to find definite stage of mitosis and to check the extent of culture synchronization. In other experiments we also tested the cells at 39, 40, 41 and 42 hrs after washing. All these points were

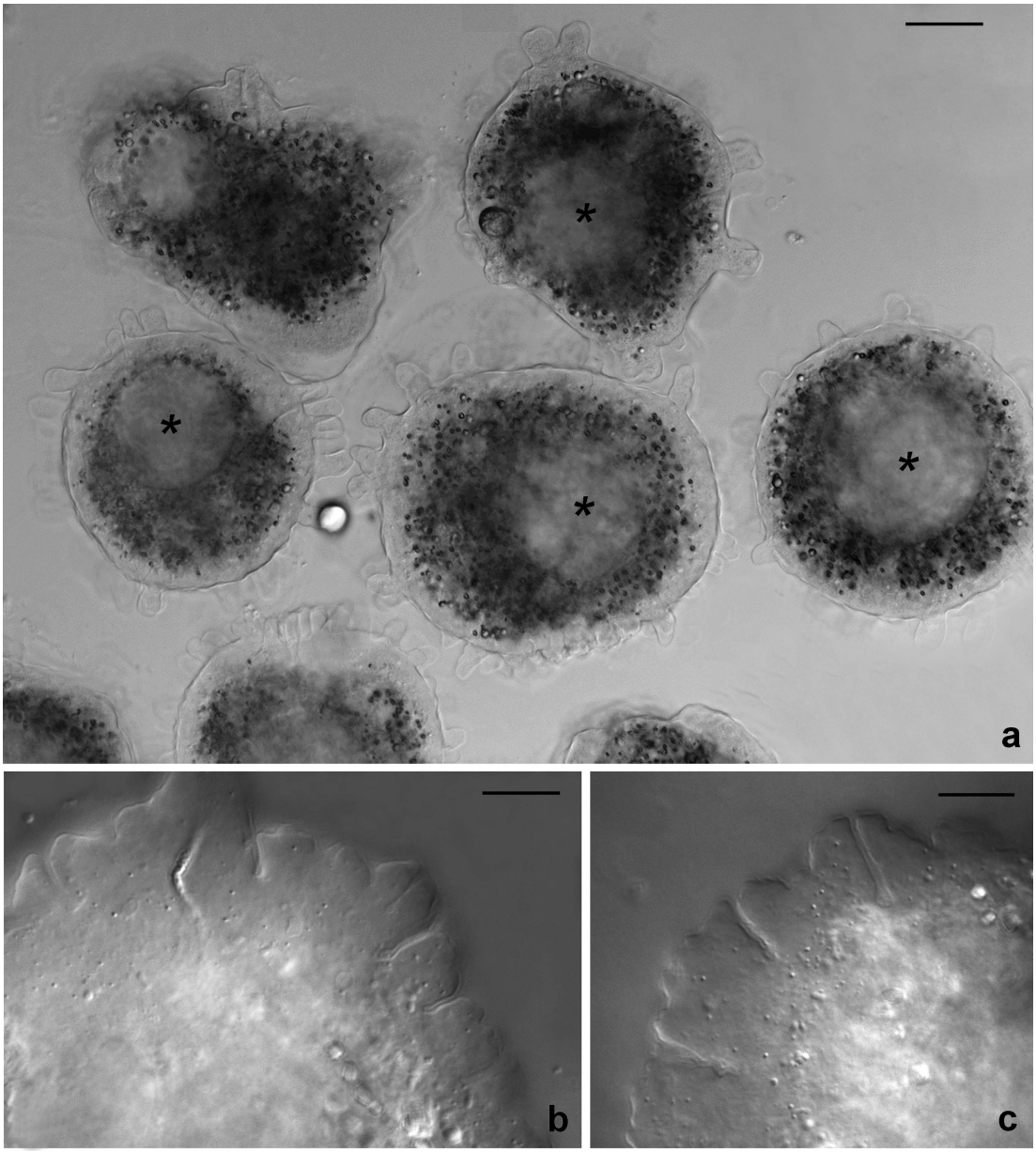

Fig 1. Photomicrographs of alive *Amoeba proteus* in experimental culture (DIC). a – Rounded amoebae in the process of pinocytosis after adding the quail protein solution; b, c – numerous pinocytic canaliculi piercing the layer of peripheral hyaloplasm. Growing central vacuole in amoebae cells is marked by *asterisk*. Scale bars: a – 50 μm; b, c – 25 μm.

chosen and checked according to cell cycle timing of *Amoeba proteus* at different temperatures (Ord, 1968; Prescott, 1973; Yudin, 1990; Jeon, 1995).

Totally 9 experiments were carried out with different periods passed after quail egg albumen pinocytosis feeding, followed by washing with PC. In the experiments, the amoeba nuclei in 98.7% of the organisms, examined after the same period after washing, had the identical nuclear morphological features (Fig. 2). These results witness for the fact that those amoebae were passing the same phase of the cell cycle synchronously.

Interphase nuclei (Figs 2a and 2b) were observed on the slides examined 30–38 h after washing. Prophase of mitosis was found after 39 h (Fig. 2c), metaphase – 41 h (Fig. 2d), and anaphase pictures appeared 41.5 h after washing cells from the quail egg protein (Fig. 2e). Starting from 42 h, only interphase nuclei were found, thus mitosis being over.

To sum up, we may recommend feeding amo-

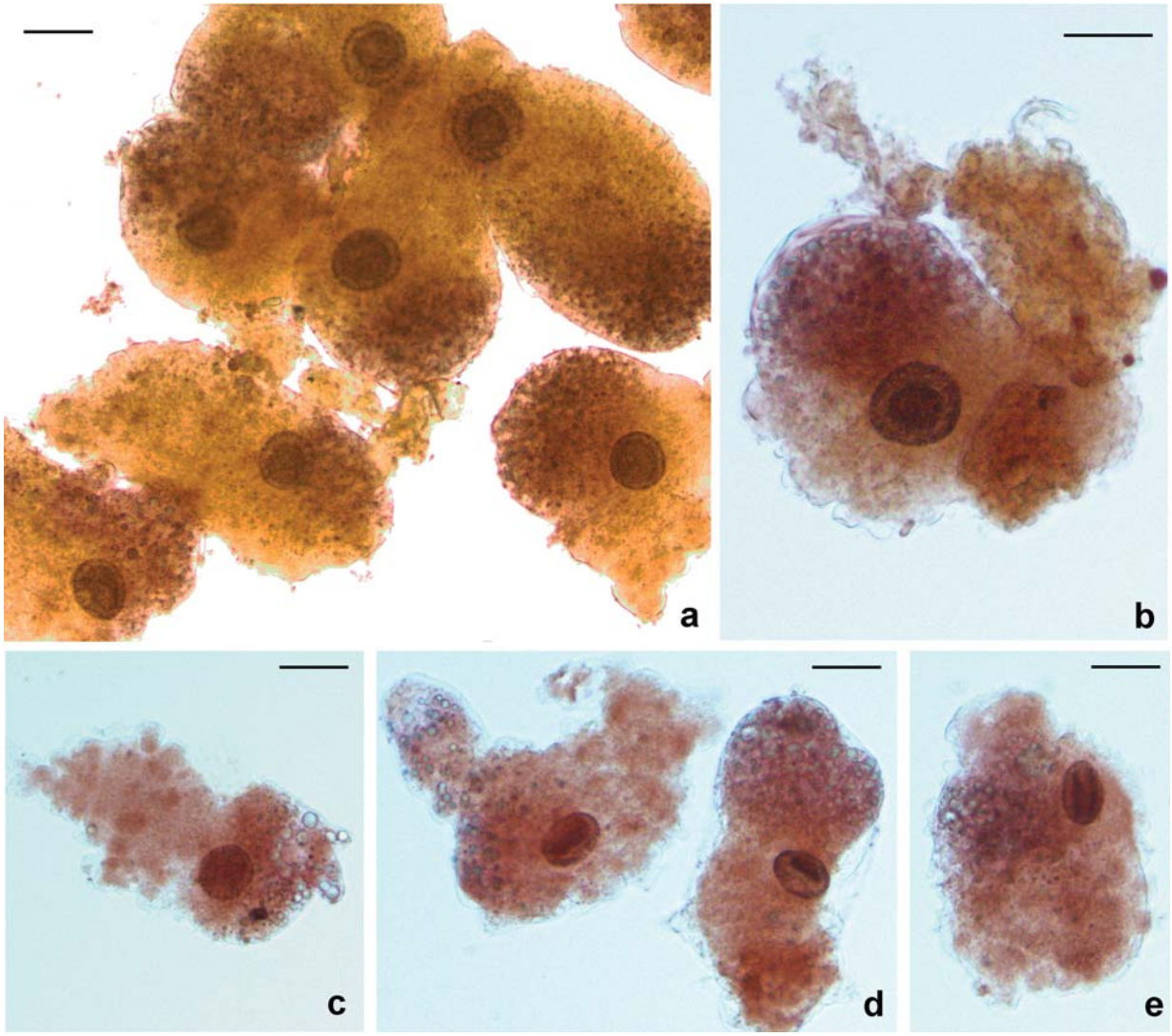

Fig 2. Photomicrographs of stained permanent preparations of Amoeba proteus at different stages of cell cycle. a, b – Interphase nuclei; c – prophase of mitosis; d – metaphase, and e – anaphase. Scale bars: 30 μm.

ebae with quail protein by pinocytosis in order to:

(a) achieve the higher extent of synchronization of cell culture examining at the same period of time after washing from the protein,

(b) obtain the cells on the certain stage of mitosis in a quantity enough for studying,

(c) avoid contaminations caused by particles of food remains.

In particular, such a method gives an opportunity to get mass material of metaphase nuclei and thus an approach for studying amoeba chromosomes. We are looking forward to solving some of the amoebae puzzles using this methodological approach in near future.

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