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ИнБЮМ – IBSS

Морской биологический журнал
Marine Biological Journal
2022, vol. 7, no. 3, pp. 60–79
https://marine-biology.ru

UDC 593.1:576.3 DOI: 10.21072/mbj.2022.07.3.06

TRICHOPLAX SP. H2 CULTIVATION AND REGENERATION FROM BODY FRAGMENTS AND DISSOCIATED CELL AGGREGATES: OUTLOOK FOR GENETIC MODIFICATION

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Received by the Editor 31.07.2020; after reviewing 26.08.2020;
accepted for publication 19.08.2022; published online 13.09.2022.

Trichoplax sp. H2, a simple multicellular animal cultivated in the laboratory, was studied with the aim of its further genetic modification. The idea here is to introduce genetic information into a cell suspension after dissociation of the *Trichoplax* body into single cells, followed by their aggregation and regeneration of the resulting agglomerates into a viable animal. 1. We analyzed the dynamics of the *Trichoplax* growth in Petri dishes on *Tetraselmis marina* algal mats. Specimens were uniform on the exponential growth stage. 2. Trichoplaxes were cut radially in a post-traumatic regeneration research, and the regeneration of the obtained parts was investigated under a microscope. Growth and reproduction rate of animals on nutrient mats were determined that decreased as the animals had been cut. The missing part of the *Trichoplax* body was replaced by remodeling of remaining cells. 3. The animals after a vital staining were dissociated into single cells in a medium with no divalent cations. Pear-shaped or rounded cells were identified, as well as epithelial cells with flagella maintaining motion activity for more than 12 hours. 4. *Trichoplax* plates were disintegrated in the presence of 10 μM amlodipine to quantify a cell population using flow cytometry. As estimated, *Trichoplax* (0.5–1 mm in size) consists of approximately 10,000 cells. 5. Treatment of animals with 10 % BSA (Bovine Serum Albumin) during various exposure intervals suggests a hypothesis on the existence of totipotent cells at the periphery of the *Trichoplax* body, probably in the rim. 6. In the course of reparative regeneration experiments, we achieved *Trichoplax* dissociation into single cells with 0.1 % BSA treatment and the following recreation of the viable organisms by centrifugation of a cell suspension and subsequent dispersion of a large pellet into fragments up to 0.1 mm *prior* to plating multicellular aggregates on nutrient mats. 7. The development of the aggregates was accompanied by active motion of cells and epithelialization of the surface, which resulted in cell growth, formation of a plate, and further vegetative division of *Trichoplax*. As assumed, the artificial stage of a single cell in a line of asexual reproductions allows to introduce foreign genetic information into *Trichoplax*, for example, in order to study the signal processing, organization, and functioning of this multicellular organism. Transgenesis, which is based on the dissociation of an animal body into single cells, could be applied to other organisms with high regenerative potential.

Keywords: *Trichoplax*, Placozoa, post-traumatic and reparative regeneration, cell dissociation and aggregation, cellular engineering, methods of transgenesis

Trichoplax belongs to the Placozoa phylum and is considered one of the simplest multicellular animals (Seravin & Gudkov, 2005 ; Schulze, 1883, 1891). It is a flat invertebrate with an asymmetric body 0.2–2.0 mm in diameter and 25 μm thick; it is found throughout tropical seas (Eitel & Schierwater, 2010 ; Pearse & Voigt, 2007). Trichoplax has no muscle cells and neurons; it consists of three cell layers. The animal adheres to a substrate with the help of microvilli and slides over the surface due to beating of the ventral epithelium cilia and observed rhythmic contractions of the dorsal epithelium under the effect of stellate fibre cells of the middle layer, performing amoeboid movement (Armon et al., 2018 ; Eitel et al., 2013 ; Pearse & Voigt, 2007 ; Schierwater et al., 2009 ; Smith et al., 2019). Two possible feeding types are described for trichoplax – external and internal. With external feeding, the animal crawls onto large prey (*e. g.*, an algal agglomerate) and adheres closely to a substrate forming a kind of food cavity, into which it releases secretions dissolving the food. Then, this food is absorbed by trichoplax during the clathrin-mediated endocytosis (Smith et al., 2015, 2019). Also, trichoplax is capable of picking up small prey (single algae) with cilia and moving it to the dorsal side; there, it is phagocytosed by cells of the middle layer temporarily exposed on the surface (Wenderoth, 1986).

Interestingly, the animal with such a primitive body structure contains genes that are responsible for functions of the immune and nervous systems in highly organized animals (Kamm et al., 2019 ; Varoqueaux et al., 2018). *Trichoplax* sp. H2 has a 94.88-Mb genome with 12,225 genes identified (Kamm et al., 2018). The mitochondrial genome of trichoplax is the largest among all Metazoa; it is a 43-Kb circular DNA (Dellaporta et al., 2006). Six main cell types were described for *Trichoplax adhaerens* H1, using electron microscopy (Table 1). Additional cell types were characterized in *Hoilungia* sp. H4 based on confocal microscopy and differential staining of H4 strain, and the membrane potential of mitochondria was measured (Romanova, 2019).

Table 1. *Trichoplax* prevailing cells types identified by electron and confocal microscopy using specific antibodies (Smith et al., 2014)

Position	Type	Description	Function	Content
Lower layer	Ventral epithelial cells	Small cells with a cilium and microvilli, ventrodorsally elongated; numerous inclusions and vesicles	Active sliding on the surface and adherence to a substrate, absorption, pinocytosis	72 % of all trichoplax cells
	Lipophilic cells	Large cells without cilia; those form contacts with fibre cells and are absent in the edge 20-μm area; one large inclusion with lipid content	Secretion of digestive enzymes	11 % of cells
	Gland cells	Medium-sized, located in the edge area, with a cilium and microvilli; granules of various shapes and colors	Secretion of neurotransmitters	3 % of cells
Middle layer	Fibre cells	Tetraploid cells with outgrowths; various inclusions, intracellular symbiotic bacteria and food odds	Mechanical changes in the body shape, phagocytosis, digestion	4 % of cells
	Crystalline cells	Cells contain crystals ~2 μm in size, contact with fibre cells, do not come to the surface	Possibly, acting as statocysts	< 0.2 % of cells in an animal body
Upper layer	Dorsal epithelial cells	Small T-shaped cells with sensory cilium; intracellular granules	Protective, sensory, contractile	9 % of cells

The most representative cells in the trichoplax body are ventral epithelial cells; those provide adherence to the surface *via* microvilli, sliding with the help of cilia, and absorption of digested food (Table 1). Lipophilic cells are also located in the lower epithelium; those contain a large lipid inclusion. The outer edge of animal body, or the peripheral belt (~20 μm), consists of two types of epithelial cells – dorsal and ventral ones; there are no fibre cells here. There are gland cells on the periphery of the trichoplax plate. Those express such neurospecific proteins, as syntaxin 1, synaptobrevin, and SNAP25. Moreover, neurosecretory cells contain FMRF-amide and other neuropeptides (Mayorova et al., 2019 ; Senatore et al., 2017 ; Smith et al., 2014). Various types of peptidergic cells are recorded not on the periphery alone: those are also located concentrically towards the center in both epithelia (Varoqueaux et al., 2018). Fibre cells are tetraploid, contain symbionts, and are responsible for body shape change, phagocytosis, and digestion (Gruber-Vodicka et al., 2019). Some cells have crystalline inclusions of aragonite. Apparently, they play the role of a vestibular apparatus allowing trichoplax to navigate through environment, turn over, and adhere to a substrate directly on the ventral side (Mayorova et al., 2018 ; Smith et al., 2014). Dorsal epithelial cells are located in the upper layer and have one sensory cilium. Assumedly, there are other, less representative or yet unidentified cell types (Sebé-Pedrós et al., 2018). For example, unusual cells – looking like shiny balls – were described in individuals from natural environment; there are no such cells in cultivated animal lines (Grell & Ruthmann, 1991 ; Syed & Schierwater, 2002). Trichoplaxes are known to deter potential predators, possibly *via* the release of toxins by specialized cells (Jackson & Buss, 2009 ; Pearse & Voigt, 2007). As assumed, stem cells can be located in the peripheral belt, which ensure the growth of an animal on its outer edge (Albertini et al., 2019).

Trichoplaxes reproduce mainly by body binary fission or budding with the participation of “spherical buds” (Kamm et al., 2018 ; Thiemann & Ruthmann, 1991, 1988). In Placozoa, the formation of gonocytes is usually observed in aging cultures; oocytes are extremely difficult to detect (Grell, 1972, 1971 ; Grell & Benwitz, 1974). Spermatozoa have not yet been described (Grell & Benwitz, 1981). Embryos degrade after cleavage for unknown reasons under laboratory conditions; the development of embryos is studied up to the stage of 64–128 cells (Eitel et al., 2011). The life span of individual cells in the animal body is also unknown. Stem cells and their niches have not been found. As assumed, all trichoplax cells are capable of reverse differentiation because an animal can regenerate after dissection into small fragments and even from individual cells (Ruthmann & Terwelp, 1979). However, regenerative morphogenesis experiments showed partial cell differentiation. Specifically, fragments of the middle of the plate do not regenerate. When connecting the belt and the central part of the animal body, redundant material is rejected (Schwartz, 1984). As shown, there are small cells along the edge of the trichoplax body in which the *Trox-2* gene is expressed. Apparently, those are multipotent stem cells since the suppression of the *Trox-2* expression by antisense oligonucleotides or by RNA interference stops trichoplax growth and regeneration (Jakob et al., 2004).

Since genome sequences for several trichoplax strains became available (Dellaporta et al., 2006 ; Kamm et al., 2018 ; Signorovitch et al., 2007 ; Srivastava et al., 2008), it is possible now to manipulate them (for example, by turning off one or another gene) and analyze what changes this would lead to (Hardy et al., 2010). On the other hand, reporter genes encoding fluorescent proteins are often used to identify individual cells and their descendants (Currie et al., 2016). This approach allows to label cells by transgenic mRNA and to study the spatiotemporal distribution of cells in population under a fluorescence microscope, separate them applying fluorescence-activated cell sorting (FACS), and investigate

transcriptomes of individual cells by scRNA-seq analysis (Lush et al., 2019). The molecular genetic study of communication between cells is of great interest in case of trichoplax: it is the cause of the formation of multicellular ensembles and systemic behavior (Kuznetsov et al., 2020b).

Unfortunately, limited data on the fine structure, poor knowledge of the reproductive cycle of trichoplax, and lack of comprehensive information on the dynamics of animal growth and physiology during cultivation hinder work on its genetic modification and reverse genetics. Due to a lack of methods for genetic modification of trichoplax organism, researchers are limited to the analysis of genetically unmarked cells (Moroz et al., 2020 ; Romanova et al., 2020 ; Sebé-Pedrós et al., 2018) and heterologous gene expression (Elkhatib et al., 2019 ; Smith et al., 2017). It should be noted, methods for the introduction of genetic information, such as electroporation and lipofection (except for ballistic transfection) (Sambrook & Russell, 2001), are focused on the manipulation of cells in culture, whether they are prokaryotes or eukaryotes. In case of multicellular organisms with a sexual reproduction, transgenesis occurs at the single cell stage, for example, by injecting DNA into the zygote (Transgenesis Techniques, 2009). Therefore, it is necessary to develop a special method of transgenesis for trichoplaxes, which are not capable of sexual reproduction under laboratory conditions but reproduce vegetatively.

Our key aim was to study the regenerative abilities of *Trichoplax* sp. H2 for the cellular and genetic engineering of this animal.

MATERIAL AND METHODS

Cultivation. *Trichoplax* sp. H2 strain was used for experiments. Every time, 15 animals were placed with a micropipette in a Petri dish 90 mm in diameter and cultivated at a temperature of +25 °C and pH 7.8–8.0. Unicellular green alga *Tetraselmis marina* (Cienkowski) R. E. Norris, Hori & Chihara, 1980 was used as a food source (Kuznetsov et al., 2020b). Artificial seawater (hereinafter ASW) with a salinity of 35 ‰ was changed every 5–7 days. Trichoplaxes were transferred onto a fresh algal mat every 3–5 weeks. Animals were kept in the “starvation” mode the day before the beginning of the experiment: we placed them in a dish with ASW without alga.

Microscopy and image analysis. Animals were investigated under Zeiss Primo Star or Zeiss Stemi 305 microscope with a built-in camera at 8× and 40× magnification. The images were analyzed applying the ImageJ package (https://imagej.nih.gov/ij/). The contrast threshold was selected to separate the images from the background noise. The areas of trichoplax plates were measured. Individual cells and their agglomerates were studied under an inverted Nikon Eclipse Ts2R microscope with DIC optics at up to 600× magnification.

Vital staining. The samples of individual animals or their parts were sequentially rinsed in two drops of ASW. To the second drop, 20 μL of 0.01 % neutral red solution (Sigma-Aldrich, USA) was added; it was exposed for 10 min at room temperature and then rinsed again in two drops of ASW for 30 min.

Microsurgery. We took large animals for the experiments, at least 1 mm in diameter, which changed the behavioral strategy to “waiting” and flattened on a substrate after the active seek for food. For this purpose, trichoplaxes were placed into a Petri dish with a plastic substrate and ASW for about 60 min – until their transition to a resting state occurred. Individual animals in this state were dissected into radial parts. Medial incisions were made with a scalpel under a Zeiss Stemi 305 microscope at 8× magnification. To investigate the ability of trichoplax parts to grow and reproduce, the animals were dissected into 2, 4, or 8 radial lobes; 10 parts were sown on *T. marina* algal mats – with the condition of 1 fragment from an individual. The post-traumatic regeneration was directly observed for 3–4 hours

under a Nikon Eclipse Ts2R microscope. Photo and video recording was carried out at various time intervals.

Dissociation into individual cells. From 50 to 150 individuals, 0.5–1.0 mm in size and of the same age, were taken *prior* to the stationary phase; this corresponded to 2–3 weeks after inoculation. Those were twice rinsed in ASW for 30 min and transferred into 300-μL wells. For dissociation of trichoplax plates into separate cells, bovine serum albumin (hereinafter BSA) (Sigma, USA) at concentrations of 10 and 0.1 %, 10 μM amlodipine (Teva, Russia), or 3.5 % NaCl was used with an exposure of 15 to 90 min. Trichoplaxes were kept in ASW with 0.1 % BSA for 15 min at room temperature, and the medium was intensively stirred for the last 5 min – until a homogeneous cell suspension was obtained.

Flow cytometry. Cytomics™ FC 500 flow cytometer (Beckman Coulter, USA) equipped with an argon laser with a wavelength of 488 nm was used to study the efficiency of trichoplax dissociation into individual cells and to assess animals' abundance and size. Cytometric data were processed applying Flowing Software v2.5.0 (www.flowingsoftware.com, Perttu Terho, University of Turku, Finland). Total abundance of individual cells and their aggregates was determined in unstained samples by gating a cell population on 2-parameter light scattering cytograms (forward scatter, FS, and side scatter, SS), as well as after staining the samples with SYBR Green I (Molecular Probes, USA). The final dilution was 10^{-4} in each sample. Staining was carried out in the dark for 30 min just before cytometry. The stained samples were analyzed using FS and SYBR Green I fluorescence in the green area of the spectrum (FL1 channel, 525 nm). Cell concentration was calculated from a sample flow rate (60 μL·min^{-1}), time, and abundance of cells registered during this interval (60 s). Measurements were controlled and calibrated using fluorescent microspheres (Beckman Coulter, USA) with a size of 1.0, 4.2, and 10.7 μm. Cell sizes (L, μm) were determined based on the FS channel data as an equivalent spherical diameter (ESD), the volume of which is equal to a cell volume regardless of its morphology.

Cell reaggregation and reparative regeneration. A resulting cell suspension was immediately placed on nutrient mats in the first series of experiments. In the second series, the homogenate was transferred into a 1.5-mL microtube, and cells were rinsed three times with 1 mL of ASW by sedimentation on an FVL-2400 Combi-Spin microcentrifuge (BioSan, Latvia) for 2 min. At the final stage, centrifugation lasted for 5 min and was followed by a pellet dispersion on a vortex for 2 sec. Mini-aggregates of cells up to 100 μm in size were sown on algal mats for further trichoplax regeneration, growth and reproduction.

The research was carried out during the year. In total, 14 series of experiments were performed with BSA and amlodipine in various modifications; control tests with 3–5 dishes in a separate experiment were carried out. The data in the article are given as "mean ± standard deviation."

RESULTS

Animal growth. *Trichoplax* sp. H2 were in a state of adaptation to new conditions for a week after sowing on algal mats in Petri dishes. After a lag phase (up to 7–10 days), the animals began to grow and then began to divide, with the formation of an area of stretching and thinning between the cells, which was followed by rupture. The exponential phase occurred, characterized by uniform morphology of the animals. The subsequent logarithmic phase of the trichoplax culture was accompanied by slight changes in the population size (Fig. 1a), and the biomass gain occurred stepwise (Fig. 1b). Deceleration of the culture growth with the transition to the stationary phase and subsequent shrinking of some animals was recorded on the 20th day; the beginning of the terminal phase was registered on the 25th day.

For further cultivation, 10 intact animals were placed into dishes with fresh algal mats. As before, there was an exponential increase in abundance after a 7-day pause (Fig. 1c), with a certain slowdown in the biomass gain at the late stage (Fig. 1d) accompanied by a temporary decrease in animals' size.

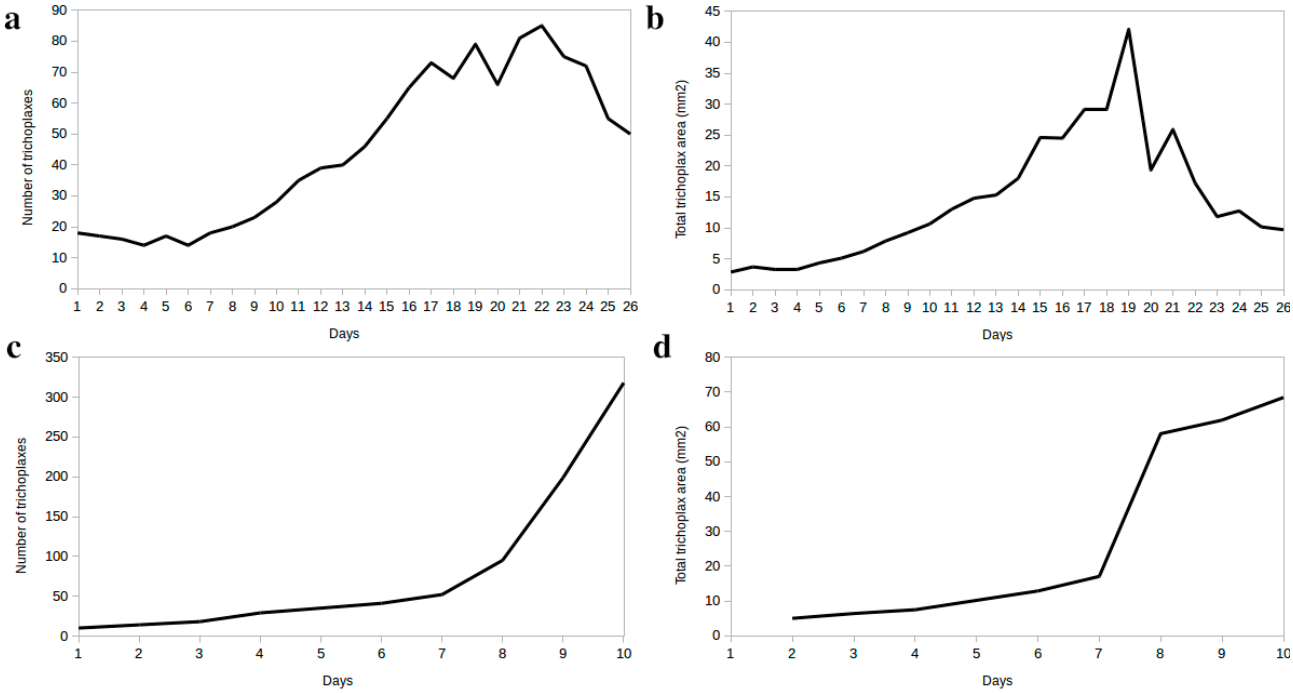

Fig. 1. *Trichoplax* sp. H2 culture growth: a, b, the long-term cultivation; c, d, the following short-term cultivation. Total surface area of animals is in mm^2 (b, d)

Feeding behavior of trichoplaxes. With the excess of food, the animals rested on an algal mat (Fig. 2a). As a mat was consumed, trichoplaxes slowly moved to a new spot abounding in food. In case the animals were sown in ASW without alga, they began to move actively seeking for food (Fig. 2b). After several tens of minutes of unsuccessful seek, the animals calmed down and switched to the "waiting" mode (Fig. 2c); a thin peripheral rim was formed, and trichoplaxes were adhered closely to a substrate as the movement of cells inside their bodies slowed down. The addition of microalga next to an animal stimulated its positive taxis in the direction of food and its subsequent consumption. The addition of a small volume of medium from the old culture or the placement of dead alga near trichoplax, on the contrary, caused an escape reaction.

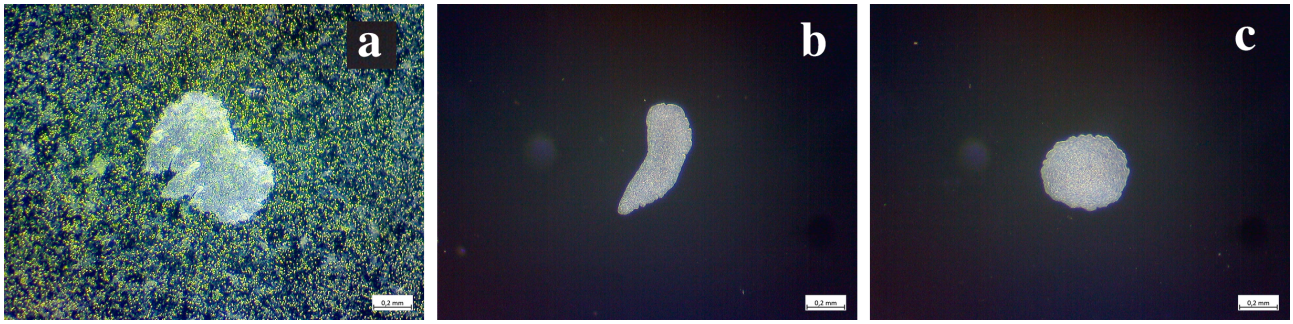

Fig. 2. *Trichoplax* in various physiological states: a, animal resting on an algal mat; b, *Trichoplax* in motion; c, motionless animal. Magnification 40 times

Microdissection of animals. After dissecting trichoplax into two parts (Fig. 3a), the halves began to regenerate into an intact individual and healed the wound in 1 hour (Fig. 3b, c). Dissected animal stopped moving, remained flattened on a substrate, and curled up isolating the damaged area from the environment. The injured part of the plate is characterized by the absence of cilia and belt while its native edge forms a belt consisting of small dark cells with clearly visible mobile cilia. The internal content of the plate was very dynamic, in contrast to the wound edge. Trichoplax permanently changed its shape but did not rise above the surface – just remained adhered closely to a substrate. The belt on the side opposite to the wound somewhat thickened in height, became darker, and adhered closely to a substrate fixing the animal in such a way that it performed amoeboid movement but could not tear itself away from the adherence site (see Supplementary 1: https://marine-biology.ru/mbj/article/view/353). The wound healing lasted about 30 min (Fig. 3a) and ended with deep invagination of the belt towards the central part of the plate in 60 min after bisection (Fig. 3b) (see Supplementary 2: https://marine-biology.ru/mbj/article/view/353). It took about an hour for further repair leading to an alignment of the edge of the plate (Fig. 3c, d). Animals no longer adhered to the surface with the opposite side; trichoplaxes began to rotate. After 3 hours or more, the edge of the plate in the damaged area was rounded, an intact belt was formed, and the scar inside the plate was resorbed. All cells inside the trichoplax plate began to move in a coordinated manner, the plate acquired the initial plasticity, and the animal got the ability to move progressively.

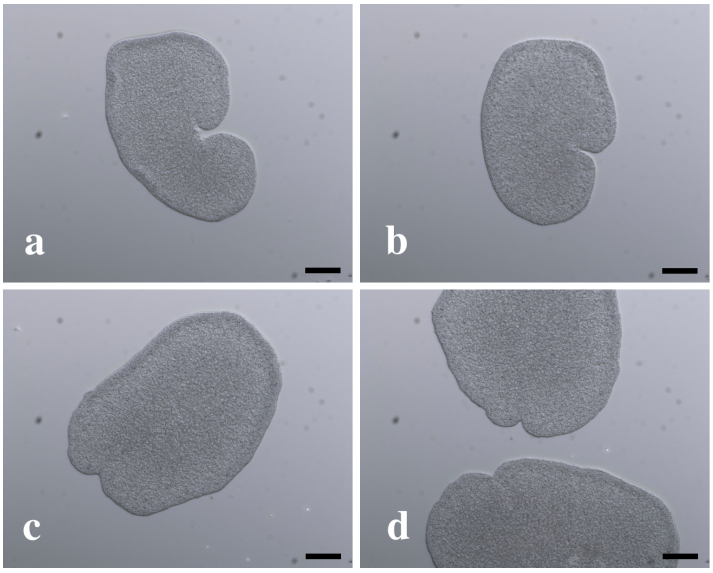

Fig. 3. Repair of a wound area in half of *Trichoplax* sp. H2 within 30 min (a), 60 min (b), and 120 min (c, d). Magnification 400 times. Scale bars are 50 μm

When trichoplax was cut into 4 parts and, especially, into 8 parts, the initial recovery proceeded longer (up to 60–120 min) because the wound area exceeded the undamaged one. Interestingly, to restore the wound area with the help of remaining small cells of the belt of the plate, the animal had to thicken (Fig. 4b), form folds, and even reject some large cells from the central part of the plate. A dissection of the animal resulted in the immobilization of the plate in the damaged area: trichoplax lost its ability to move progressively, performed rhythmic movements in one plane accompanied by cell restructuring and leading to a decrease in the wound area, and later began to rotate. As the wound was healed, the edge of the plate flattened out, and the animal regained its mobility.

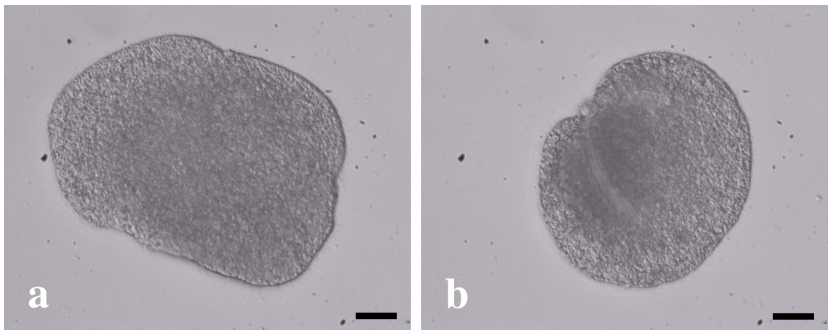

Fig. 4. Repair of a wound area in 1/8 of *Trichoplax* sp. H2 specimen: a, small intact animal as a positive control; b, healing of 1/8 of a large animal within 1–2 hours. Magnification 400 times. Scale bars are 50 μm

In two weeks after sowing 10 halves and 10 quarters of trichoplax on an algal mat, 40 intact individuals were found in each of two dishes (Table 2). Animals were located separately on and under alga, adhered closely to a substrate, were inactive, and had a typical morphology with smooth edges and corresponding plate sizes (about 1 mm). This result indicates that trichoplax halves and quarters have approximately the same potential for recovery over a long period of time in the presence of a food source. Fewer individuals regenerated out of 1/8 of trichoplax, since only some of them were capable of giving rise to full-fledged animals. Nevertheless, all recovered individuals had a reproductive potential: a 4–5-fold increase in the population size *per* week was recorded.

Table 2. *Trichoplax* sp. H2 regeneration after microdissection of the plate

Weeks	Abundance of regenerating animals			
	Whole part	1/2 part	1/4 part	1/8 part
2	42	40	40	10
3	> 200	> 200	> 200	51
4	> 200	> 200	> 200	> 200

Dissociation of animals into individual cells. As a result of placing trichoplax in a medium without divalent cations (3.5 % NaCl), animals' bodies gradually dissociated into individual cells (Fig. 5b). There were mostly rounded cells in 60 min; those performed erratic movements due to cilia beating even the next day. Immobile pear-shaped cells were revealed as well but in a much smaller abundance, because the pear-shaped cells turned into spherical ones with prolonged incubation.

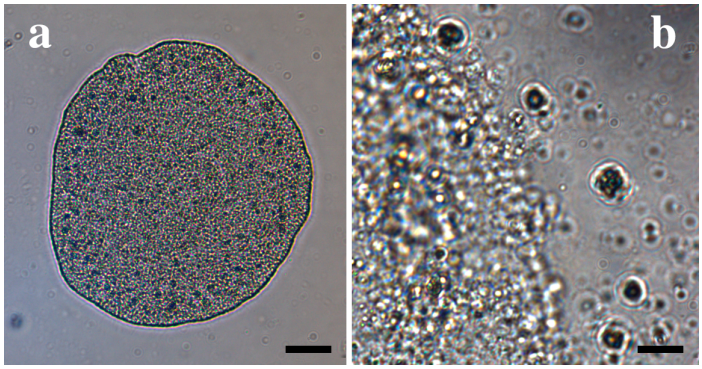

Fig. 5. *Trichoplax* plate stained with neutral red: a, intact animal, magnification 200 times, scale bar is 75 μm; b, animal during dissociation with 3.5 % NaCl solution, magnification 600 times, scale bar is 25 μm

Evaluation of individual cells and their aggregates using a flow cytometer. The analysis of trichoplax cells in unstained samples (Fig. 6a, d) was restricted by the presence in the medium of suspended particles of a comparable size. This problem was especially felt when samples were treated with 3.5 % NaCl; to a lesser extent, with 0.1 % BSA (Fig. 6a). The formation of different-sized cell aggregates after dissociation of the trichoplax body did not allow obtaining a compact cluster on cytograms: it had a core of individual cells (IC) and a plume of cell aggregates (CC) (see Fig. 6a, d).

The efficiency of cell dissociation was estimated by the ratio of individual cells in the total number of recorded objects. The value varied within 60–76 % [(68 ± 8) %]. In terms of this indicator, there was no statistically significant difference between the treatment with 0.1 % BSA and 10 μM amlodipine. Cell abundance in one organism ranged 7,000 to 12,000; the value may be underestimated since the efficiency of tissue dissociation did not exceed 80 %.

Staining of nucleic acids with SYBR Green I fluorochrome facilitated the identification of trichoplax cells on cytograms (TR gates in Fig. 6b, e) and gave more accurate estimations of cell abundance and size based on the gating of their populations (Fig. 6c, f). According to calibration measurements, mean size (ESD) of individual trichoplax cells was of (3.5 ± 0.4) μm; there was no significant difference between the samples with BSA and amlodipine.

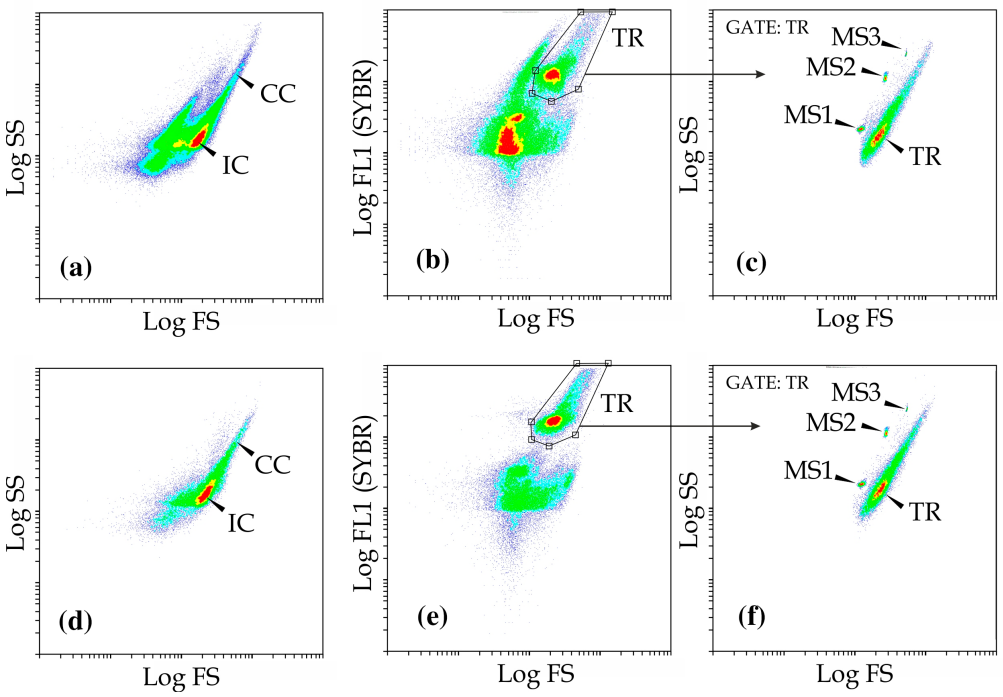

Fig. 6. Flow cytometry of dissociated *Trichoplax* cells with no staining (a, d) and after their staining with SYBR Green I fluorochrome (b, c, e, f). Treatment with 0.1 % BSA (a, b, c) and 10 μM amlodipine (d, e, f). Single *Trichoplax* cells (IC), their aggregates (CC), gating of *Trichoplax* cells in stained samples (TR), and calibration microspheres of 1.0 μm (MS1), 4.2 μm (MS2), and 10.7 μm (MS3) are marked. Data from the TR gate are given on two graphs (c, f)

Regeneration of animals after treatment with 10 % BSA. It was necessary to find out whether trichoplaxes are able to regenerate from fragments of less than 1/8 of the body. To obtain such small fragments, we applied trichoplax plate dissociation technique with BSA. In total, 50 individuals were involved in the experiment, and BSA was added in a concentration up to 10 %. The animals were kept for 15, 45, and 90 min at room temperature; the suspension of animal fragments was removed and sown

on nutrient mats for growth. The use of BSA instead of a scalpel did not allow to obtain decreasing fragments with saving of axes – such as sectors of 1/16, 1/32, *etc.* Instead, random fragments of animals' bodies were obtained. Thus, the viable trichoplaxes were found on mats after only 3 weeks of cultivation. Interestingly, the animals were of different size – from very small to large, about 1 mm. Some individuals remained in close contact with each other after division.

The sizes of trichoplax plate fragments after 90 min of treatment were significantly smaller than after 15 min of incubation. The longer the exposure to 10 % BSA, the lower the ability of trichoplax to reparative regeneration, growth, and reproduction was observed. Specifically, 83, 38, and 1 animal were found in dishes in 3 weeks of cultivation; 333, 220, and 4 intact organisms in 4 weeks of cultivation (after exposure to BSA for 15, 45, and 90 min, respectively) (Table 3). In the latter case, three out of four regenerating animals were registered only in 4 weeks of cultivation, and the reproduction of animals was slow.

The obtained result shows that the lasting of trichoplax disassembling into fragments with 10 % BSA negatively affects the repair of experimental animals. Despite the fact of body fragments differ significantly in structure after microsurgery and BSA treatment, the efficiency of trichoplax recovery after 15 min of incubation with 10 % BSA is comparable to animal regeneration from 1/8 parts (Table 2). However, reparative regeneration of animals and further reproduction required almost one month of cultivation on algal mats after 90 min of treatment with 10 % BSA. Interestingly, the recovered individuals gave rise to a new population which reached a maximum of 182 animals in the second month of culture growth and was characterized with the gradual death of trichoplax by the third month (Table 3).

Table 3. *Trichoplax* sp. H2 regeneration after 10 % BSA treatment depending on incubation period

Weeks	Abundance of regenerating animals		
	15 min	45 min	90 min
3	83	38	1
4	333	220	4
5	> 400	> 400	8
6	> 400	> 400	18
7	> 400	> 400	47
8	> 400	> 400	182

Restoration of animals from an aglomerate of individual cells. Are trichoplaxes capable of recovering after complete dissociation into individual cells? The animals did not grow in case of sowing a suspension of such cells on a nutrient mat. However, we achieved the formation of viable animals after centrifuging a cell suspension, dispersing a pellet, and sowing cell aggregates on mats. Specifically, 74 individuals were recorded in 1 week of cultivation on an algal mat in case of 0.1 % BSA for dissociation, and 2 animals were registered in case of 10 μM amlodipine; then these animals successfully reproduced reaching the value of 380 in 4 weeks (Table 4). In contrast, the treatment of trichoplaxes with 3.5 % NaCl negatively affected their subsequent regeneration.

The experiments were consistently reproduced, and abundance of regenerating trichoplaxes directly depended on abundance of animals taken. Specifically, 5, 74, and 93 individuals grew when we involved 50, 100, and 150 trichoplaxes, respectively, in case of 0.1 % BSA. So, a gain in trichoplax quantity increased the individual cell concentration in suspension, and this positively affected the number of fragments after pellet's disaggregation and, ultimately, the ratio of recovered animals.

Table 4. *Trichoplax* sp. H2 regeneration from aggregates of single cells after dissociation with various reagents

Weeks	Abundance of regenerating animals		
	0.1 % BSA	10 μM amlodipine	3.5 % NaCl
1	74	2	0
2	> 400	25	0
3	> 400	236	0
4	> 400	380	0
5	> 400	> 400	0

Over time, new cell aggregations appeared on algal mats; those grew on a substrate, acquired a typical trichoplax morphology and abilities to move, external feeding, and division (Fig. 7a, b). Trichoplaxes regenerated from cell aggregates asynchronously and formed different-sized colonies with uneven edges (interestingly, initially large aggregates developed faster than small ones). Then these swarms rounded turning into mobile animals, grew up to their usual size (about 1 mm), and began to divide by thinning and stretching – like intact individuals. If animals regenerated from the cell aggregates after centrifugation of a cell suspension and subsequent pellet dispersion without food in ASW, then, initially random, shapeless, and chaotic trichoplax cell aggregates were rearranged due to regenerative morphogenesis into rounded structures similar to “spherical buds”: in those, small epithelial cells with mobile cilia are settled on the surface while large cells are located inside (Fig. 7c).

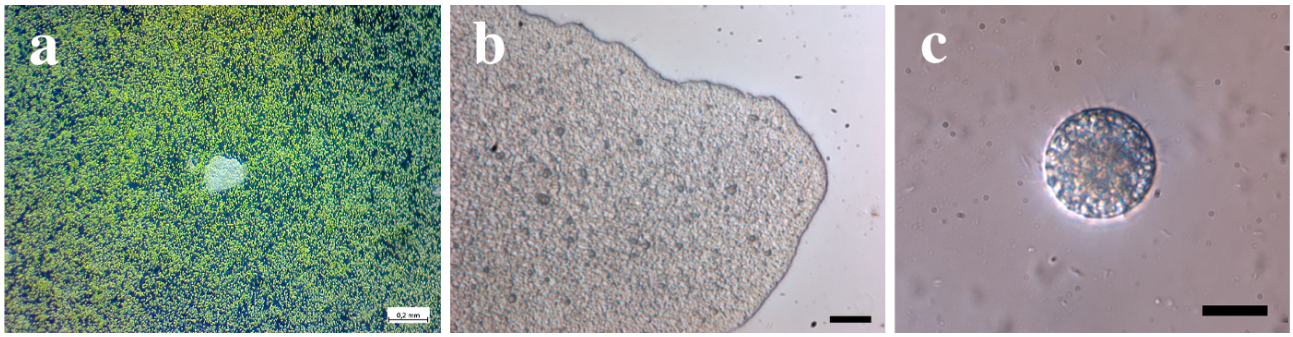

Fig. 7. Regenerative morphogenesis of *Trichoplax* sp. H2: a, 7-day animal formed on an algal mat, magnification 40 times, scale bar is 0.2 mm; b, *Trichoplax* from a 6-week culture, with small cells in the rim and cilia on the periphery, magnification 400 times, scale bar is 50 μm; c, 7-day animal regenerated in ASW with no food source, magnification 2×400 times, scale bar is 100 μm

The dynamics of recovery, subsequent growth, and reproduction of trichoplaxes on nutrient mats varied in experiments and depended on age and state of the selected animals (Fig. 8). The best results were obtained on trichoplaxes taken at the exponential phase. In contrast to intact animals with a lag phase duration of about 1 week (Fig. 1c, d), experimental animals were characterized by the fact that either agglomerates did not regenerate or this stage was delayed up to two or more weeks. However, this was followed by the exponential and logarithmic phases; then, there were the stationary phase (in the second month of the culture existence) and slow death (starting from the sixth week after sowing). The death of the culture was stated in a decrease in the total biomass including a drop in trichoplax abundance in Petri dishes and animals’ shrinking.

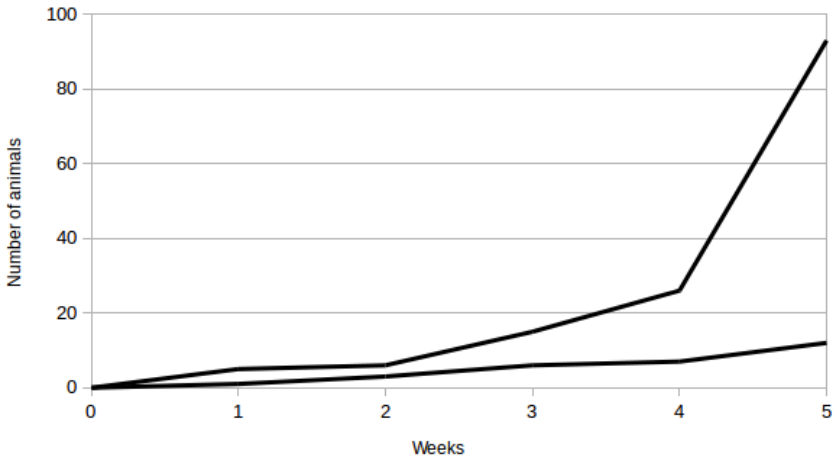

Fig. 8. Dynamics of *Trichoplax* sp. H2 regeneration after dissociation with 0.1 % BSA. Two independent experiments with animal growth in separate Petri dishes

DISCUSSION

The employment of a limited number of model organisms, such as *Escherichia coli* (Migula, 1895), *Caenorhabditis elegans* (Maupas, 1900), *Drosophila melanogaster* Meigen, 1830, *Mus musculus* Linnaeus, 1758, *etc.*, representing different taxonomic groups, allowed biologists to focus their attention on studying the mechanisms of life and led to an understanding of biological processes at the molecular level; moreover, this allows to modify living objects (Sommer, 2009). Out of hydrobionts, in addition to *Danio rerio* (Hamilton, 1822), researchers are interested in new model organisms – *Hydra vulgaris* Pallas, 1766 and *Nematostella vectensis* Stephenson, 1935 (Layden et al., 2016), *Ciona intestinalis* (Linnaeus, 1767) (Liu et al., 2006), *Paracentrotus lividus* (Lamarck, 1816) (Gildor et al., 2016), *etc.* One of them is *Trichoplax* sp. This unique multicellular animal without a nervous system belongs to basal Metazoa (Heyland et al., 2014). Thereby, it is interesting to know the way its functioning is coordinated. At the same time, this organism remains difficult for both developmental biology and molecular genetics study due to poor knowledge of its life cycle and regenerative abilities (Eitel et al., 2011 ; Kamm et al., 2018), implicit symmetry, uncertainty of the body plan, and expression of the corresponding genes (DuBuc et al., 2019 ; Schwartz, 1984 ; Zuccolotto-Arellano & Cuervo-González, 2020), as well as due to a supposed absence of stem cells because of predominant vegetative reproduction (Ruthmann, 1977).

The work with individual trichoplax cells is the basis for studies by methods of molecular genetics. As known, the ability for genetic transformation in bacteria and for genetic transfection in eukaryotic cells in culture is associated with the exponential growth phase characterized by a maximum number of mitoses (Sambrook & Russell, 2001). For this reason, we paid considerable attention to obtaining individual trichoplax cells and their further aggregation to restore reproductive animals. Interestingly, trichoplax growth in Petri dishes differs much from its cultivation in an aquarium (Pearse, 1989).

Under our conditions, the crucial factors were pH stability in the medium and periodic change of ASW allowing to remove trichoplax waste products. Acidification of the medium and water change affected the shape of a growth curve which was manifested in appearance of steps (Fig. 1); this hinders identification of the beginning of the transition to the stationary phase. Consumption of alga by animals

eventually led to a culture death. Such behavior of trichoplaxes, as seeking for food and fixing and flattening on a substrate, as well as feeding behavior, served as a criterion for the physiological state of animals. The most uniform individuals were registered at the exponential phase of growth.

To remember, the ability to manipulate individual cells and regenerate after dissociation was first demonstrated in the early XX century for sponges (Galtsoff, 1925 ; Wilson, 1910, 1907). Hydra regeneration was discovered by A. Trembley even earlier – in the middle of the XVIII century (Lenhoff & Lenhoff, 1986). The regenerative abilities of *Trichoplax adhaerens* are widely studied since the second half of the XX century (Kuhl & Kuhl, 1963, 1966). *Trichoplax* sp. H2 showed high regenerative potential and tissue plasticity in our experiments when it was dissected radially into several parts. The dissected animals remained adhered to a substrate but curled up to minimize the damaged area and surround the wound with healthy tissue. Trichoplaxes recovered much more slowly from 1/4 and, especially, from 1/8 part, than from 1/2 part due to lower ratio of the intact surface area to the wound area. Epithelial cells with a cilium maintaining motor activity for at least 12 hours prevailed among the individual cells obtained after the dissociation of the trichoplax plate. However, their rounded shape did not correspond to that of cells that make up the animal body (Smith et al., 2014) which may result from the absence of neighbors or the effect of the osmotic pressure of the environment. Immobile pear-shaped cells, presumably derivatives of lipophilic cells, were found as well. However, it was impossible to detect less representative cell types under a light microscope. Therefore, the entire pool of available cells was used in the course of reparative regeneration experiments in hope of natural selection of poorly differentiated cells during regenerative morphogenesis. This is confirmed by the rejection of some material which, apparently, contained differentiated epithelial cells and cells of the middle of the plate (Schwartz, 1984). The total abundance of cells in 0.5–1.0-mm animal was rounded up to 10,000 according to flow cytometry data; the value is in agreement with the results of electron microscopy where cell abundance in 1-mm trichoplax was 50,000 (Smith et al., 2014).

Experiments on trichoplax dissociation with 10 % BSA for different time intervals showed that animals lose their ability to recover in accordance to the duration of exposure; apparently, this occurs because of the predominant loss of peripheral cells and is consistent with the results of trichoplax incubation in a calcium-free medium. This evidences in favor of the existence of totipotent cells in the belt of the trichoplax plate that disagrees with the data on sponges where most cells are capable of movement and transdifferentiation (Bond, 1992 ; Harris, 1987). Dissociation of the trichoplax plate into individual cells with 10 μM amlodipine is more effective than with 0.1 % BSA but has the opposite effect on the restoration of animals after centrifugation of a cell suspension. The difference found may be due to different mechanisms of trichoplax tissue dissociation. Specifically, BSA is capable of binding calcium ions and blocking receptors on a cell surface (Kuznetsov et al., 2020b) while amlodipine disrupts calcium channels (Kuznetsov et al., 2020a). The use of a calcium-free medium did not result in a restoration of animals, as in other experiments (Ruthmann & Terwelp, 1979).

The results of our experiments suggest that hypothetical stem cells of trichoplax are located on the periphery in the edge belt of the plate but are not able to proliferate and differentiate independently, without contact with neighboring cells and without active morphogenetic movements that is consistent with the assumption in (Albertini et al., 2019). This is confirmed by cell reconfiguration within several days in the absence of food from shapeless cell aggregates into pronounced spherical bodies with a flat epithelium and large internal cells (Thiemann & Ruthmann, 1991, 1988) but is not supported by self-assembly from individual cells, as described in (Ruthmann & Terwelp, 1979).

On the other hand, the development of multicellular aggregates on algal mats, their subsequent regeneration, and growth of experimental trichoplaxes recapitulated the development of intact animals in culture with a 1–2-week delay.

Conclusion. The widely represented cell types of trichoplax disguise the possible existence of totipotent cells and obstruct their search. However, the system of stem cell selection used by us during the assembling of dissociated cells – centrifugation and further pellet dispersion followed by germination of the obtained aggregates – can be useful and/or critical when working with competent cells for genetic transfection. This assumption requires additional research. The future work on trichoplax cell suspension can involve existing methods of transfection – lipofection and electroporation – using species-specific DNA constructs: this will allow studying and modifying the mechanisms of cell signaling, functioning, and organization of this ancient multicellular organism. In a broader aspect, the transgenesis and genome editing method based on the dissociation of tissue into individual cells can be applied to other hydrobionts with a high regenerative potential – sponges, cnidarias, and planarians.

The work was supported by a grant from the Government of the Russian Federation under Order No. 220 (agreement No. 14.W03.31.0015 dated 28.02. 2017).

Acknowledgement. We thank A. Soldatov for support; A. Ponomareva, A. Doroshkov, O. Krivenko, and E. Sakhon for their assistance in carrying out the work; A. Pirkova and I. Dovgal for critical reading of the manuscript; and anonymous reviewers for their useful comments and advices.

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КУЛЬТИВИРОВАНИЕ И РЕГЕНЕРАЦИЯ ТРИХОПЛАКСА *TRICHOPLAX* SP. H2 ИЗ ФРАГМЕНТОВ ТЕЛА И АГРЕГАТОВ ДИССОЦИИРОВАННЫХ КЛЕТОК: ПЕРСПЕКТИВЫ ГЕНЕТИЧЕСКОЙ МОДИФИКАЦИИ

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Выполнены исследования на культивируемом в лаборатории простейшем многоклеточном животном *Trichoplax* sp. H2 с целью дальнейшей генетической модификации этого организма. Предлагается вводить генетическую информацию в суспензию клеток после диссоциации тела трихоплакса на отдельные клетки с последующей их агрегацией и регенерацией полученных агломератов в жизнеспособное животное. С этой целью мы исследовали динамику роста трихоплаксов в чашках Петри на матах из одноклеточной водоросли *Tetraselmis marina*. Особи были однородны на стадии экспоненциального роста. В экспериментах по посттравматической регенерации разрезали подопытных животных радиально и исследовали восстановление полученных частей под микроскопом. Оценивали интенсивность роста и размножения трихоплаксов на водорослевых матах — показатели, ухудшавшиеся по мере измельчения животных. Обнаружено, что утраченная часть тела трихоплакса замещается за счёт ремоделинга оставшихся клеток. После витальной окраски животных подвергали диссоциации на отдельные клетки в среде, лишённой двухвалентных катионов. Идентифицированы клетки грушевидной или округлой формы и клетки эпителия со жгутиками, которые сохраняли двигательную активность более 12 ч. Для количественной оценки популяции клеток с помощью проточной цитометрии пластинки трихоплаксов дезинтегрировали при добавлении 10 мкМ амлодипина. Показано, что трихоплакс размером 0,5–1,0 мм состоит примерно из 10 000 клеток. Обработка животных 10%-ным бычьим сывороточным альбумином (БСА) в течение различных промежутков

времени свидетельствует в пользу существования тотипотентных клеток на периферии трихоплакса, вероятно в пояске пластинки. В экспериментах по репаративной регенерации удалось добиться диссоциации трихоплаксов на отдельные клетки при обработке 0,1%-ным БСА, а затем воссоздать живые организмы путём центрифугирования суспензии клеток и последующего диспергирования крупного осадка на фрагменты до 0,1 мм перед высевом многоклеточных агрегатов на питательные маты. Развитие этих агрегатов сопровождалось активными движениями клеток и эпителизацией поверхности, что приводило к увеличению клеточной массы, формированию пластинки, росту и дальнейшему вегетативному делению трихоплаксов. Предполагается, что пребывание экспериментальных животных на искусственной стадии одиночной клетки в ряду бесполых размножений позволит интродуцировать в трихоплакса чужеродную генетическую информацию, например с целью изучения сигнальных систем, организации и функционирования этого многоклеточного организма. Трансгенез, основанный на диссоциации тела животного на отдельные клетки, возможно, будет применим и к другим организмам, обладающим высоким регенеративным потенциалом.

Ключевые слова: трихоплакс, пластинчатые, посттравматическая и репаративная регенерация, диссоциация и агрегация клеток, клеточная инженерия, методы трансгенеза