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Current state and perspectives of single-cell studies in ecophysiology of protists

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Summary

Protists (eukaryotic microbes) play a tremendous role in aquatic and terrestrial ecosystems worldwide, because their physiological activity is important for biogeochemical cycling of elements. For a long time studies of microbial physiology have been carried out with the focus on populations. However, nowadays development and application of new techniques lead to rapid accumulation of data obtained at the single-cell level. Single-cell approaches have already brought a lot of invaluable information about prokaryotic microbes, whereas protists are still less studied in this respect. In this paper, we review the range of single-cell studies in the field of ecophysiology of eukaryotic microbes published to the date and discuss future prospects of individual-based protist research.

Key words: ecophysiology, protists, single-cell techniques

Introduction

Ecophysiology of protists is a research area of high importance, since these eukaryotic microorganisms represent key members of natural communities and shape many crucial processes in ecosystems (Caron et al., 2009; Matantseva and Skarlato, 2013). For many years physiology of protists in the context of changing environmental conditions and their ecological relevance have been studied at the level of populations. However, this approach does not allow estimation of individual performance of distinct cells within a population, thus leaving a significant aspect of protist physiology out of consideration. Moreover, population-based approaches cannot be applied in case of uncultured organisms and in the investigations of most symbiotic relationships. Recent implementation of various single-cell techniques in microbiological studies has provided new possibilities to investigate microbial ecophysiology (Brehm-Stecher and Johnson, 2004). Indeed, application of these methods has allowed obtaining very intriguing and promising results in ecophysiology of prokaryotes (Musat et al., 2012; Blainey, 2013). Nevertheless, until now only a limited number of studies have used modern analytical achievements to investigate protists at the level of single cells. In this paper, we provide an overview of such works where single-cell sequencing and direct observations of cellular activities were applied in order to answer important questions of protist ecophysiology. Furthermore, we discuss what can be expected to come in the nearest future.

The power of single-cell sequencing

Single-cell sequencing became feasible less than 10 years ago, but nowadays it is a wide-spread and fruitful approach to study microorganisms (Lasken, 2007, 2012). One of the major problems of microbiology is the inability to culture organisms from most of existing taxa. This applies not only to prokaryotes, but also to eukaryotic microorganisms. Often uncultured protist species are rare and small in size (pico- and nanoeukaryotes), hence it is particularly difficult to isolate them and, consequently, to sequence their genomes by conventional approaches requiring significant DNA amounts. Development of methods to isolate single protist cells and amplify their DNA by multiple displacement amplification (MDA; Spits et al., 2006) has allowed researchers to overcome this hindrance and decipher genomic information of uncultivated species. The methods to capture individual cells of protists for subsequent DNA amplification and sequencing are well described and reviewed elsewhere (Ishoey et al., 2008; Ashida et al., 2010; Bouchillon et al., 2014; Kodzius and Gojobori, 2016).

Sequencing of DNA from single cells of protists provides invaluable information about biodiversity, functional roles and metabolic potential of uncultured but relevant species (Heywood et al., 2011). Such information is a solid basis for testing the existing viewpoints and generating new sound hypotheses; it provides a good start to further experimental work on protist ecophysiology. The recently discovered group of uncultivated picoeukaryotes named picobiliphytes (Not et al., 2007; Cuvelier et al., 2008) had been thought to possess plastids and perform photosynthesis until Yoon and co-workers (2011) sequenced a single-cell genome of these organisms and did not find plastid-related genes within it. Later the absence of plastids and heterotrophic lifestyle of 'picobiliphytes' were confirmed when the member of this enigmatic group was isolated and investigated by classical methods (Seenivasan et al., 2013; Moreira and López-García, 2014). As a result, picobiliphytes have been renamed to Picozoa.

Recently Roy and co-authors (2014) produced a draft genome of a stramenopile belonging to the lineage MAST-4 (Massana et al., 2004) and identified nearly 7000 protein-encoding genes. This allowed researchers to place the species onto the eukaryotic tree of life and to get some insights regarding physiology of these organisms. It was shown that MAST-4 eukaryotes possess most of the pathways required for the basic metabolism of sugars, lipids, and amino acids, as well as TCA (tricarboxylic acid) cycle and oxidative phosphorylation chain. However, it was also demonstrated that genomes of these organisms lack urea cycle and photosynthetic machinery typical for many other stramenopiles.

Apart from prediction of metabolic properties of uncultivated and rare protists, single-cell sequencing can also provide ecologically relevant information on their interactions with other organisms and environment. This approach offers a good way to investigate so far mysterious prey preferences and symbiotic associations in protists. For example, amplification of DNA originating from individual cells of heterotrophic and mixotrophic planktonic protists can be followed by 18S and 16S rRNA sequencing, which allows identification of all pro- and eukaryotic organisms associated with a certain cell. If compared to the single-cell data of free planktonic cells, the conclusions can be made whether organisms found inside the target protistan cell represent free-living prey or symbionts. This approach allowed Yoon and co-workers (2011) to propose the spectrum of prey bacteria typical for 'picobiliphytes'.

Moreover, single-cell sequencing data can be used to identify protistan cells experiencing viral infections. In the previously mentioned work by Yoon et al. (2011), it was shown that one of the investigated protistan cells contained many viral sequences. Phylogenetic analysis and database search revealed that they belonged to a previously unknown virus widely distributed in marine ecosystems (Dhillon and Lee, 2015).

Physiology and metabolic interactions at the single-cell level

Although single-cell sequencing helps to get an idea about physiology and functions of protists in ecosystems, activities of every species have to be demonstrated and measured experimentally. Development of the nanoscale secondary ion mass spectrometry (nanoSIMS) has marked a new era in this field. In combination with the use of stable isotope tracers and various techniques for species identification (e.g. FISH) this approach allows to link taxonomic identity of uncultivated species to their physiology (Kuypers and Jørgensen, 2007; Musat et al., 2012) and to study metabolic interactions in symbiotic systems (Behrens et al., 2012).

For example, many diatoms inhabiting oligotrophic regions of the ocean possess symbiotic N_2-fixing cyanobacteria to withstand nitrogen limitation. NanoSIMS measurements showed that nitrogen fixation rates by cyanobacteria *Richelia* are 171-420 times higher in cells living in association with diatoms, as compared to free-living *Richelia* cells. Remarkably, host diatom cells receive over 97% of nitrogen fixed by their symbionts in the process of rapid and effective N transfer (Foster et al., 2011).

A similar partnership was found between uncultured cyanobacterium of the group UCYN-A and unicellular prymnesiophytes. UCYN-A cells possess substantially reduced genomes lacking many important enzymes of carbon metabolism. NanoSIMS analysis demonstrated that these cyanobacteria obtain organic carbon from their eukaryotic hosts, whereas prymnesiophytes benefit from this association due to the input of nitrogen fixed by symbiotic partners (Thompson et al., 2012).

Symbiosis between scleractinian corals and dinoflagellates of the genus *Symbiodinium* has been receiving much attention due to its relevance for reef-associated ecosystems. Nevertheless, metabolic interactions between hosts and their endosymbionts were poorly understood for a long time because of methodological limitations. It was known that both corals and their symbionts possess enzymatic pathways for ammonium fixation, but relative contribution of each partner within a native coral-dinoflagellate symbiotic system remained uncertain until recently. Assimilation of isotopically labeled inorganic ammonium by coral cells and *Symbiodinium* sp. cells was investigated using nanoSIMS (Pernice et al., 2012). In that study, the authors found out that dinoflagellates have a much higher capacity for rapid ammonium fixation than host cells, although both symbiosis members contribute to the process. Further studies on this symbiotic system showed that distinct genetic clades of symbiotic dinoflagellates *Symbiodinium* differ in physiological ability to fix inorganic carbon and ammonium to their hosts with one clade performing this processes more effectively than the other (Pernice et al., 2014).

An important advantage of the nanoSIMS approach is the possibility to study uptake of various compounds by cells inhabiting intact structured environment. For example, significant light gradients exist within host tissues in the previously discussed coral-dinoflagellate symbiotic system (Wangpraseurt et al., 2014). NanoSIMS analysis of carbon fixation rates by individual symbiont cells demonstrated that dinoflagellates inhabiting the aboral ('dark') coral parts fixed carbon only 6-fold slower than cells in the oral ('light') parts of a coral body, although the former had 15-fold less light available. This finding indicates that enhanced light-harvesting efficiency may be characteristic of the *Symbiodinimum* cells inhabiting aboral tissues (Wangpraseurt et al., 2015).

Intrapopulation heterogeneity

A relatively new and promising direction of single-cell biology is the investigation of cell-to-cell heterogeneity or variability within populations of microorganisms (Kreft et al., 2013). Differences in morphology and physiology of distinct cells of the same population may arise due to external factors, such as patchiness of microenvironment, or due to differences in physiological state and cell cycle stage, or even due to stochastic events in the course of gene expression.

The phenomenon of cell-to-cell variability in bacterial populations has received a lot of attention, since it seems to be very important for population dynamics, especially under changing environmental conditions (Booth, 2002; Elowitz et al., 2002; Kussel and Lebler, 2005; Acar et al., 2008; Lidstrom and Konopka, 2010; Martins and Locke, 2015). Less is known about variability in natural and laboratory populations of protists. A large proportion of works in this field focuses on variation in nutrient content of microalgae. In the study on dinoflagellates *Dinophysis norvegica* researchers used a nuclear microprobe to measure particulate C, N, and P content of cells within a population growing under the same environmental conditions and found out that cellular nutrient content may considerably vary, which was especially prominent in case of cellular N (Gisselson et al., 2001). Synchrotron X-ray fluorescence was used to confirm similar inter-cell variability in P content (between 3.8- and 5-fold) for a population of diatoms *Thalassiosira pseudonana* representing another group of marine protists (Núnez-Milland et al., 2010). Later Bucci et al. (2012) not only demonstrated P-content variability in populations of diatoms *Cyclotella meneghiniana,* but also unveiled the mechanisms generating such variability. By means of *in silico* simulations they found out that P content heterogeneity in diatoms was mostly (85%) due to microscale patchiness of nutrient content in the environment. This finding is ecologically important, since micropatches of

high nutrient content typically exist around lysing cells and/or sinking particles and can support some members of microbial community in natural ecosystems. In the same work, Bucci and co-authors (2012) showed that conventional modeling based on population-average cellular nutrient content gives higher population growth rates than modeling based on nutrient content of individual cells.

Thus, accounting for intrapopulation heterogeneity and using individual-based modeling may influence our comprehension of protist community dynamics, and data on heterogeneity in various physiological parameters are necessary (Kreft et al., 2013). One of the rare studies to provide such data focused on the diatom species *Cyclotella cryptica* and used high-throughput imaging flow cytometry to observe cell-to-cell variability in triacylglycerol (potential lipid fuel) and chlorophyll content of distinct algal cells under stress conditions, i.e. silicon and nitrogen limitation (Traller and Hildebrand, 2013).

Although currently data on heterogeneity in protists are extremely limited, not to say nearly lacking, new methods and protocols appear to investigate this phenomenon. For example, in recently published works, researchers provided a protocol to estimate DNA, chlorophyll and lipid content in microalgae by confocal laser scanning microscopy (Chansawang et al., 2015) and reported the use of aerosol time-of-flight mass spectrometry to obtain metabolomes of thousands of single protistan cells (Cahill et al., 2015). This fact gives a hope that much more data on cell-to-cell variability obtained with the range of different methods will be available in the nearest future.

Future prospects

Ecophysiology of protists is a quickly developing area of research that is strongly influenced by advances in analytical techniques. Despite significant progress in single-cell methods, they have not been widely applied to explore eukaryotic microorganisms. However, the situation will likely change in the coming years. Single-cell sequencing becomes more and more common (Stepanauskas, 2015), and the number of various approaches to investigate physiology of individual cells increases at a fast pace (Vasdekis and Stephanopoulos, 2015). For example, a possibility to measure formation of small metabolites in single *Chlamydomonas reinhardtii* cells by means of synchrotron Fourier-Transform Infrared spectromicroscopy was reported several years ago (Goff et al., 2009), and now we can expect its effective use in protist ecophysiology.

Moreover, nowadays relatively old techniques to study physiology at the level of single cells expand to cover previously inaccessible groups of organisms as research targets. One of such cases is the application of electrophysiological techniques to study ion channels and signaling in some protists (Martinac et al., 2008), including such ecologically relevant groups as dinoflagellates (Pozdnyakov and Skarlato, 2012, 2015; Pozdnyakov et al., 2014).

Heterogeneity within populations of protists is the least studied phenomenon, but it is likely to receive much more attention in the coming years due to several reasons. First, previously mentioned achievements in high-throughput single-cell techniques will promote heterogeneity research. Second, the need to incorporate cell-to-cell variability into ecological models becomes evident, since it should facilitate development of individual-based ecology (Hellweger and Bucci, 2009; Kreft et al., 2013; Stilmann et al., 2015). Taken together, the spread of modern analytical approaches, modeling and, most importantly, growing interest in the processes going on at the level of single cells and their dependence on the environmental alterations represent the most significant factors that will probably change our perspective on ecophysiology of protists very soon.

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