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A microfluidic chip for studying the reproduction of *Enteromorpha prolifera*

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ABSTRACT

In recent years, green tides caused by water eutrophication, has brought serious environmental problems. *Enteromorpha prolifera* (*E. prolifera*), an opportunistic macroalgae, is one of the main source contributing to the formation of green tides. It has been estimated that the excessive growth of *E. prolifera* is closely related to various reproductive ways of germ cells which are at the micrometer scale. Here we report a microfluidic device named Germ Cell Capture Chip (GCChip) to investigate the *E. prolifera* reproductive mechanism. GCChip integrates the functions of algal growing, and the release, capture and selective culture of germ cells. Automatic separation and capture of germ cells on the chip allows to study germ cells' response to different stimuli. The novel device greatly facilitates long-term live-cell imaging at cellular resolution and implements the rapid and accurate exchange of growth medium without manual intervention. Results showed that the starting time of germ cell releases were earlier on the chip than that of traditional experiments with more concentrated breakout. Moreover, GCChip can be widely applied on the study of other algae. The study of algae growth process, including the elongation of somatic cell, the generation, and the release of reproductive cells, can all be improved by using this microfluidic platform.

1. Introduction

The situation of water eutrophication has become deteriorate in the past decades, which is caused by the pollutant rich in nitrogen and phosphorus discharged from human industry, agriculture, and daily life [1–4]. The polluted water contributes to the continuous colonization and flourishing of the benthic algae, and was termed "green tide" [5–7]. The green tide was known as mass-stranding events of macroalgae which usually occurs in spring and summer [8,9]. Dense drifting macroalgae can excessively consume dissolved oxygen in water, seriously inhibit phytoplankton from producing oxygen, and turn into a stinking morass [10,11]. They can also produce toxic hydrogen sulfide (H_2S), which have noxious effects on coastal ecosystems. On the other side, tons of algae smothering the shoreline, deterring shore-based activity, and depressing tourism in the coastal regions diminish the aesthetic appeal and hinder the development of voyage [8,12–14].

In recent years, the number of worldwide reports on the outbreaks of benthic algae has been increasing [9,15]. In China, from 2007, unprecedented summer green tides had broken out many times in the coastal area of the Yellow Sea [16]. In 2008, without warning, the coast region of Qingdao, where was going to hold the Olympic sailing events, had experienced a large-scale green tide caused by free-floating green alga [7,17]. The green tide, had lasted two months, involved an area of 1.29×10^4 km^2 approximately, and covered 32.04% of sailing venues at the peak period of the bloom [5,18]. The expenditure of the removal was more than $30 million, a total loss of more than $100 million to aquaculture (abalone, clam and sea cucumbers) [8,19]. Another large-scale green tide disaster assaulted coastal regions of Qingdao in 2013 and caused more serious damages than ever. Similarly in the Europe and the United States, the regions affected by green tides suffered severe losses of the local economy, e.g. H_2S gas produced

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from *Ulva* rotting on a beach of Brittany, and even caused the death of a horse [20]. In fact, Green tides have become a serious common problem annoying many countries [14,19,21].

Macroalgae play a significant role in the nutrient enrichment and transfer of the ecosystem. They work as filters for nutrients and eventually exported them to the ocean [22]. *Enteromorpha prolifera*, as an opportunistic macroalgae with tenacious vitality, which contributes mostly to the formation of green tides, is characterized by rapid nutrient uptakes and high growth rates [23,24]. It is essential to study explosive growth of *E. prolifera* for the solution of green tides. *E. prolifera* itself owns several pathways of reproduction including sexual, asexual and vegetative reproduction [25]. Previous studies have revealed that the excessive reproduction of *E. prolifera* mainly rely on the asexual reproduction based on the differentiation and clone of germ cells (spores and gametes) [26]. Most cells of *E. prolifera* thalli can develop into reproductive cells (zoosporangia or gametangia) [25]. The division situation of each reproductive cell varies from different environments. It was estimated that 1 cm^2 blade (single layer) of *E. prolifera* can release 10^6 spores or 10^7 gametes, and most of them can germinated into younger seedlings. This means that 1 g (fresh weight) algae can produce, approximately, 10^8 to 10^9 new younger seedlings in theory [19]. This gives *E. prolifera* the ability of massive and explosive propagation [27]. In addition, compared to adult thalli, germ cell is more sensitive to the environmental change [28,29]. Above all, it is crucial to study the development process of germ cells (the germination, release, and differentiation, etc.) to reveal the reproduction mechanism of *E. prolifera*.

While many experiments concerning the growth of *E. prolifera* have showed that some external factors, such as salinity, temperature, light intensity, and pH, could affect the proliferation of *E. prolifera* [22,28,30], unfortunately, the real mechanism of excessive growth of *E. prolifera* has not yet been uncovered.

The traditional methods for the study of *E. prolifera* using multiwell plates or other vessels as platforms are usually associated with the following shortcomings: (1) They lack local control to change culture medium in situ, so it is inflexible to change algal growing medium accurately. (2) The spatial resolution cannot meet the technical requirements of morphological measurements for cellular component on the micrometer scale [31]. It is challenging to achieve real-time observation of cellular behaviors (such as the division and elongation of *E. prolifera* cells, release and clone of germ cell, band together of two gametes). (3) It lacks the approach to locally and accurately manipulate the position of *E. prolifera* in the medium [32]. The released germ cells cannot be separated for further studying without manual intervention. (4) Traditional static culture exist the problems of substrate limitation and accumulation of toxic metabolites. All of the disadvantages may lead to information missing and mistaking during experimentation. Accordingly, there is an urgent demand of microsystem that can be applied to study cellular processes on micrometer scale.

Microfluidics, which refers to the study and control of fluidic property is characterized by its structures of micrometer dimensions [33]. Technical innovations in microfluidics have realized miniaturization for handling fluid on nanoliter or microliter scale, greatly increasing the accuracy of experiments and facilitating high-throughput studies on the cellular level. Moreover, rapid and accurate exchange of culture medium offers an unparalleled advantage over traditional culture techniques, which can decrease manual intervention during culture and imaging [34]. Microfluidic chip also makes it practicable for long-time and non-invasive monitoring by using various chambers and controlling experiment conditions [32,35,36]. So far, microfluidic chip has been widely applied on the animal cell culture and pathogen detection but quite few on studying the plant cells [37,38].

Here we introduced a powerful microfluidic chip for the study of algae, which overcomes the disadvantages of traditional methods. To the best of our knowledge, it is the first try to study the growth and reproduction of *E. prolifera* on the chip. The microfluidic device, Germ Capture Chip (GCChip), has integrated the function of algal growing, the germ cells release, capture, and its selective culture. It realizes the automatic separation of *E. prolifera* thallus and its germ cells, cell culture in situ, and the real-time observation. The perfusion culture on the microfluidic chip provides a flow condition that similar to the actual current, and facilitates nutrient delivery and toxic metabolite removal [39]. In conclusion, GCChip provides a flexible and feasible approach to explore the intrinsic reasons of the reproductive strategies of *E. Prolifera*.

2. Materials and methods

2.1. Reagents and materials

Sea salt was purchased from Dongshan county salt company Fujian, China, $NaH_2PO_4 \cdot 2H_2O$, $NaNO_3$, HCl, NaOH, and all the other reagents used for f/2 medium preparation was purchased from Sigma (China). For the experiments with microfluidic chip, 10 mL syringe was purchased from Becton Dickinson, NJ, USA, and microbore tubings were purchased from Cole-Parmer, IL, USA.

F/2 medium was prepared as previous study [40], which is used as the basal culture medium for *E. prolifera* culture. To prepared the nutrient medium, $NaNO_3$ and $NaH_2PO_4 \cdot 2H_2O$ was added in f/2 medium to the final concentration of 420 mg/L and 60 mg/L, respectively. The f/2 medium and nutrient medium were diluted to different concentrations of salinity and nutrient as needed. HCl (1 mol/L) and NaOH (5 mol/L) were used to regulate the pH of medium. Ultrapure water was used to prepare all the medium. All kinds of medium were sterilized at 121 °C for 30 min and stored at 4 °C for further use [41].

The *E. prolifera* was obtained from Shanghai Ocean University as a gift. It had been experimented and could regular grow by its' own microflora. It was cultivated in a 250 mL conical flask with 150 mL f/2 medium under a light: dark photoperiod of 12:12 h using a white light source with the photon flux density of 20–75 mmol/m^2 per s at 20 °C for further experiments [25]. The medium was replaced weekly under sterile conditions.

2.2. Loading of chopped thalli of the E. Prolifera

The experimental thalli of the *E. prolifera* were checked carefully to make sure that there was no germ cell formation. The distal end of thalli from which germ cells mainly derived were chopped into pieces of 350 ± 50 μm long , loaded into a syringe with a 500 μm inner diameter needle, then injected into the chip by a syringe pump (KDS200, KDScientific, MA, USA) at an infusion flow rate of 20 μL/s. The other chopped thalli were cultured in the 96-well plates as a control.

2.3. Culture of the E. prolifera on the chip

After the thalli were trapped in the culture chamber, the device was placed at room temperature of 20 °C, relative humidity of ~40%, and a light: dark photoperiod of 12: 12 h with the white light source. The device was automatically perfused with slowly flowing medium (30 μL/h) by the syringe pump. The shear force of current at this flow rate could exactly carry away released spores or gametes.

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