Establishment of primary cell culture from the temperate symbiotic cnidarian, *Anemonia viridis*

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Abstract The temperate symbiotic sea anemone *Anemonia viridis*, a member of the Cnidaria phylum, is a relevant experimental model to investigate the molecular and cellular events involved in the preservation or in the rupture of the symbiosis between the animal cells and their symbiotic microalgae, commonly named zooxanthellae. In order to increase research tools for this model, we developed a primary culture from *A. viridis* animal cells. By adapting enzymatic dissociation protocols, we isolated animal host cells from a whole tentacle in regeneration state. Each plating resulted in a heterogeneous primary culture consisted of free zooxanthellae and many regular, small rounded and adherent cells (of 3–5 µm diameter). Molecular analyses conducted on primary cultures, maintained for 2 weeks, confirmed a specific signature of *A. viridis* cells. Further serial dilutions and

micromanipulation allowed us to obtain homogenous primary cultures of the small rounded cells, corresponding to *A. viridis* “epithelial-like cells”. The maintenance and the propagation over a 4 weeks period of primary cells provide, for in vitro cnidarian studies, a preliminary step for further investigations on cnidarian cellular pathways notably in regard to symbiosis interactions.

Keywords Cnidarian · Primary cell culture · Symbiosis

Introduction

Primary cell culture of marine invertebrates has been sporadically developed since the late 1960s. However despite the research efforts and the development of attractive short-term in vitro systems, no established cell line is yet available (reviewed by Rinkevich 2005, 2011). Major limitations are the large contaminations of primary cultures within 7 days of establishment and the slow rate of cell proliferation in vitro. The main challenge to resolve these problems is to understand the in vitro needs of marine invertebrate cells. In order to improve our knowledge in the discipline of marine invertebrate cell culture and to attempt the formulation of appropriate culture media, the developments of novel approaches or the study of novel species are crucial requirements. Short-term culture experiments with cnidarian species, during periods ranging from a

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few days to several weeks, Frank et al. (1994), Apte et al. (1996), Kopecky and Olander (1999), Frank and Rinkevich (1999), and Domart-Coulon et al. (2001, 2004) reported encouraging results and provided guidelines for further primary cell culture investigations. Moreover, despite the recent genomics and transcriptomics efforts contributing to increase our understanding of the molecular pathways existing in cnidarians, few insights are available on cnidarian cell physiology. The maintenance and propagation of cnidarian primary cell cultures will then provide a valuable tool with which to explore functional genomic and cellular pathways.

For the past decade, the Mediterranean sea anemone, *Anemonia viridis*, has proven to be a suitable model to investigate the physiological, biochemical and genomics aspects of cnidarian adaptations to symbiotic lifestyle but also to stress response. The reasons for the choice of this cnidarian model are the possibility to separate each tissue cell layers (gastroderm and epiderm) from the large polyps of *A. viridis*, to maintain symbiotic and aposymbiotic specimens and to cultivate separately the dinoflagellate symbionts (commonly named zooxanthellae and belonging to *Symbiodinium* temperate A clade (Savage et al. 2002). Additionally for the development of cell cultures, *A. viridis* also presents many advantages: (1) it is a non-calcifying cnidarian, without bulky mineralized skeleton, (2) tentacles are long and not retractable; (3) its tentacles possess a high regenerative capacity. Finally, an increasing amount of studies have accumulated a wealth of data from this species, using physiological, biochemical and functional genomics approaches (Furla et al. 1998, 2000; Roberts et al. 1999; Richier et al. 2003, 2006; Merle et al. 2007; Sabourault et al. 2009; Ganot et al. 2011; Moya et al. 2012), highlighting the need of a derived animal cell culture to start to investigate cnidarian cellular physiology.

In this study, to obtain a homogenous primary cell culture of animal host cells, we adapted enzymatic dissociation method from published protocols developed for cnidarians (Frank et al. 1994; Domart-Coulon et al. 2004). As observed previously two major difficulties for the establishment of such primary cell cultures have been highlighted: the occurrence of contaminations during cultivation and the limited number of viable cells of interest. In order to resolve these challenges, we investigated the suitability of new decontamination and dissection protocols to decrease

susceptibility to contamination and to improve cell type homogeneity and maintenance of cell cultures. Using a collagenase tissue dissociation method, reducing the contaminants in the first days of culture and enhancing cell homogeneity by serial dilutions and micromanipulation, we successfully obtained primary cultures of viable sea anemone animal cell types, which could be passaged and maintained over a 4 weeks period.

Materials and methods

Anemonia viridis specimens

Specimens of the Mediterranean sea anemone, *A. viridis* (Forskål 1775), were collected and maintained in closed-circuit seawater aquaria at 18.0 ± 0.5 °C with 50 % renewal every week. A metal halide lamp (HQI-TS OSRAM) provided light at a constant saturating irradiance of $250 \mu\text{mol}/\text{m}^2$ per s (measured using a special sensor QSL-100, Biospherical Instruments Inc., San Diego, CA, USA) on a 12/12-h light/dark cycle. Sea anemones were fed twice a week with an equal combination of frozen *Artemia salina* and *Osmerus eperlanus* (total quantity per ten liter tank: 4 g of food).

Cell dissociation protocol

Tentacle dissection

Four to five *A. viridis* large tentacles were collected and incubated in $0.2 \mu\text{m}$ -filtered sterile artificial seawater (SASW) prepared according to Domart-Coulon et al. (2004), and supplemented with 1 % Kanamycin ($100 \mu\text{g}/\text{ml}$; Sigma-Aldrich, St. Louis, MO, USA) and 1 % Amphotericin B ($2.5 \mu\text{g}/\text{ml}$; Interchim, Montluçon, France). Tentacles were then dissected and cut into 4 mm^2 tissue pieces using a scalpel under aseptic conditions in a sterile laminar-flow hood.

For the “post-cutting” protocol, tentacles in regeneration state were cut again 3 days after the first cut and treated as below.

Tissue dissociation

For enzymatic tissue dissociation, pieces of tentacles were first rinsed in calcium-free artificial seawater (CaFSW) (Gates and Muscatine 1992; Frank et al. 1994; Domart-Coulon et al. 2004) and then placed in a

tissue culture dish. The cell dissociation was performed as previously described (Gates and Muscatine 1992; Frank et al. 1994) with the following modifications. Pieces of tentacles were treated with 3 ml of 0.05 % collagenase I (Sigma-Aldrich) in SASW for at least 1 h at room temperature under agitation. The cell pellets were collected after centrifugation at 300 g for 5 min at room temperature, and was then suspended in 1 ml of culture medium and seeded in wells of a 12-well tissue culture plate (Corning, Corning, NY, USA).

For spontaneous dissociation, corresponding to explant culture, 4 pieces of tentacles (4 mm² each) were placed in 30 × 15 mm tissue culture dish and left at least 1 week before any culture medium renewal.

Media and culture conditions

Cells were cultured in the dark at 20 °C in an incubator without CO₂ environment. The *A. viridis* cell culture medium, modified from Frank et al. (1994), was based on commercial vertebrate cell culture medium DMEM (Dubecco's modified Eagle Medium; Invitrogen/Life Technologies, Carlsbad, CA, USA) adjusted to surface seawater ionic ratios (modified DMEM). The final culture medium consisted of 80 % modified DMEM, 5 % foetal bovine serum (PAA/GE Healthcare, Velizy-Villacoublay, France), 1 % Kanamycin (100 µg/ml; Sigma-Aldrich), 1 % Amphotericin B (2.5 µg/ml; Interchim) and SASW.

To notably reduce the contamination, the culture medium was supplemented with MycoKill AB[®] (1× in final medium; PAA) during the first week of cell maintenance, according to the manufacturer. For cultured cells from enzymatic tissue dissociation protocol, the MycoKill AB[®] treatment consisted of 7 days with one replacement of medium after 3 days. For explant cultures, there was no medium replacement during the week of treatment.

Primary cell culture maintenance consisted in weekly medium replacement and cell harvesting was performed by serial dilutions of cell culture suspensions collected during each passage. Cultures were observed under light and phase contrast on an inverted microscope (Nikon, Tokyo, Japan).

Micromanipulation

Cell-handling micromanipulation consisted in detecting morphological cell types of interest under inverted

microscope (20× objective) and picking them with a glass micropipette. Picked cells were then seeded in 24-well culture plate.

Viability assessment

Cell viability was determined by the Evans Blue vital stain method. Cells were rinsed with SASW and then detached with Accutase Solution[®] (Sigma-Aldrich; 200 µl/well in 12-well tissue culture plate), during 15 min at room temperature and suspended in 1 ml of culture medium. Then, 50 µl of suspended cells were incubated at 1:1 ratio with 0.05 % Evans blue (Sigma-Aldrich) solution for 10 min at room temperature. Cells were counted under a regular microscopy using a Neubauer improved hemocytometer. The number of viable cells was expressed as a percentage of the total population.

Molecular characterization

Genomic DNA extraction

Genomic DNA was extracted from primary cell cultures. Cells were rinsed with SASW and then detached with Accutase Solution[®] (Sigma-Aldrich; 200 µl/well in 12-well tissue culture plate), during 15 min at room temperature and suspended in 1 ml of culture medium. Extracts were then harvested by centrifugation at 300 g for 10 min at room temperature. Pellets were kept in lysis buffer (0.02 M EDTA, pH 8.0, 0.01 M Tris-HCl, pH 7.5, 0.4 M NaCl, 6 % sodium dodecyl sulfate, 1 µl/ml RNase and 100 µg of proteinase K) at 56 °C for 2 h. DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. DNA pellets were suspended in 50 µl of TE buffer. DNA was quantified on a 150 ND-1000 Spectrophotometer (NanoDrop/Life Technologies).

PCR experiments

Genomic DNA (50–100 ng) extracted from tissue or primary cell cultures were used. PCR amplifications were performed using primers specifically designed against the *A. viridis npc1* (Niemann Pick type C1) gene (Ganot et al. 2011). PCR products were electrophoretically analyzed on 2 % agarose gel stained with gelRed (Interchim) under UV transillumination.

For PCR directly performed on cultured cells: cultures were rinsed with SASW and then cells were detached with Accutase Solution® (Sigma-Aldrich; 200 µl/well in 12-well tissue culture plate), during 15 min at room temperature and suspended in 1 ml of culture medium. Cells were then harvested by centrifugation at 300 g for 10 min at room temperature. Pellets were kept in 50 µl of TE Buffer and 5 µl of suspended cells were added directly to PCR reagent mix.

DNA fragmentation assay

Genomic DNA (500 ng up to 1 mg) extracted from tissue or primary cultures were loaded on a 1.5 % agarose gel and electrophoretically separated at 80 V for 1 h. The gel was stained with gelRed (Interchim) and revealed under UV transillumination.

Results

Limitation of contamination

Within 7 days after collagenase treatment we observed frequent contamination in the culture medium (Fig. 1a). This contamination is thought to be due to mycoplasma, bacteria and *Thraustochytrid* protists, as previously reported in cnidarian and other marine invertebrate cell cultures (Frank et al. 1994; Rinkevich 1999), contamination against which Kanamycin and Amphotericin B are unfortunately ineffective. The reduction observed in our laboratory of the contamination of cultured zooxanthellae when treated for 7 days with MycoKill AB® without a notable reduction of viability (F. Priouzeau, personal communication) drove us to test this product in animal cell cultures. As recommended by the manufacturers the product must be added from the start of the culture for a maximum of 7 days. Following collagenase cell dissociation from whole tentacle of *A. viridis* (see “Materials and methods” section) we compared the rates of cell culture contamination in the absence or presence of MycoKill AB®. The first observations were made after 3 days of culture, corresponding to the first replacement of medium. At this stage no difference was observed between the two media (data not shown). After 7 days without MycoKill AB® the entire tissue plates were covered by bacteria/

contaminants contrary to the cultures treated with MycoKill AB® (Fig. 1). Furthermore, as the cell viability of the 7-day treated culture was determined as 75.6 % by the Evan’s blue method, we decided to further investigate culture establishment protocols under these conditions.

Optimizing the cell extraction protocol

Classical collagenase tissue dissociation yielded heterogeneous cultures (Frank et al. 1994). A large number of free zooxanthellae were recorded while no zooxanthellae-containing gastrodermal cell has been observed. Free discharged intracellular capsules of cnidocytes (discharged cnidocysts) were also observed in these primary cell cultures, a likely consequence of the tissue manipulation (Fig. 2). Excluding the microalgal cells, the cell population consisted of small adherent and non-adherent rounded cells (Fig. 2). This last group might presumably contain “epithelial-like cells” and thus represented our cells of interest. To increase the number of viable cells of interest we tested a new tentacle dissection protocol. The new protocol, named “post-cutting”, consists of cutting the regenerating tentacles again 3 days after the first cut. Considering the high regenerative capacities of the tentacle, we postulated that proliferative cells could be present in the healing point. Following cultures in the same medium conditions after enzymatic cell dissociation, with 0.05 % collagenase, we compared both dissection protocols. As shown in Fig. 2b, the “post-cutting” protocol increased the number of small rounded cells observed in 14-day culture compared to the “first-cut” classical protocol (Fig. 2a). Because of this successful enrichment of target cell types, this method was considered suitable for use in further experiments.

Temporal follow-up and molecular analysis of *A. viridis* primary cell culture

The combination of MycoKill AB® treatment and “post-cutting” dissection protocol allowed to strongly reduce two major limitations of primary cell culture establishment, i.e. contamination and number of viable cells of interest. Considering our symbiotic cnidarian model another technical challenge was to establish a culture exclusively of animal host cells. In order to address this critical point we compared the

Fig. 1 Observation of cultured cells from whole tentacles following collagenase treatment (7-day culture). **a** With standard culture medium, **b** with culture medium supplemented with MycoKill AB[®]. Phase contrast microscopy, scale bar = 10 μ m

Fig. 2 Whole tentacle primary cell cultures following collagenase treatment (14-day culture). **a** With the “classical” protocol, **b** with the “post-cutting” protocol. Zooxanthellae (z),

discharged cnidocytes (c) and small rounded cells (sr) (2–5 μ m). Phase contrast microscopy, scale bar = 10 μ m

pattern of cultured cells after enzymatic cell dissociation and explant culture. After 0.05 % collagenase treatment and serial dilutions from a 3-day heterogeneous culture (Fig. 3a) we obtained an 18-day homogeneous primary cell culture (Fig. 3b). The culture consisted mostly of small rounded cells clustered in adherent aggregates. The explant technique carried out in parallel led to spontaneous cell migration outside the dissected tentacle. However, the high level of contamination by other cell types never allowed us to obtain homogeneous *A. viridis* cell culture with the explant technique (data not shown). We, therefore, decided to continue with the successful 18-day culture derived from enzymatic cell dissociation.

As the classification of invertebrate cell types observed under in vitro conditions is not yet available, it was necessary to confirm that observed cells in 18-day culture were animal cells from sea anemone indeed. The characterization of these cultured cells required a signature typical of the animal host. We used specific designed primers of *A. viridis npc1* gene (Ganot et al. 2011). Previous transcriptomic analyses we performed on *A. viridis* symbiotic and

aprosymbiotic specimens allowed the identification of genes specifically and exclusively expressed in animal host tissue (Ganot et al. 2011). Among these genes we chose to target molecular analyses with *npc1* (Niemann Pick type C1), which encodes a membrane protein involved in intracellular transport of cholesterol. *Npc1* is exclusively expressed in animal tissue (Ganot et al. 2011) and presents a unique isoform specific to sea anemone (Dani et al. submitted). The DNA amplicon obtained after PCR reaction was previously used as animal reference sequence to exclude *Symbiodinium* DNA contamination (Zamoum and Furla 2012). Thus, a PCR assay with the specific primers of *A. viridis npc1* gene was done on genomic DNA extracted from 18-day cultured cells. Figure 4a showed that culture samples exhibited a DNA amplicon of expected size (lanes 2 and 3) compared to PCR reaction on genomic DNA extracted from *A. viridis* fresh tissue (lane 1). Genomic DNA extracted from cultured zooxanthellae was used as negative control (Fig. 4a, lane 4). No amplification in primary culture samples was observed with specific primers of *Symbiodinium* gene (data not shown). These molecular

Fig. 3 **a** Heterogeneous culture following collagenase treatment (3-day culture). Observation of small numbers of viable cells containing: zooanthellae (z) and *small rounded cells* (sr), and discharged cnidocytes (c). Phase contrast microscopy, scale

bar = 10 µm. **b** Homogeneous culture following serial dilutions (18-day culture). Observation of aggregates of small rounded and adherent cells (arrow). Phase contrast microscopy, scale bar = 10 µm

analyses validated that homogeneous 18-day culture contained host animal cells and seemed devoid of *Symbiodinium* cells. However, without molecular analysis with Thraustochytrid or fungi specific marker we cannot exclude the potential simultaneous presence of these contaminants in these primary short-term cell cultures of *A. viridis*.

Maintenance and propagation of *A. viridis* primary cell culture

Usually, the establishment of in vitro cell cultures is the first step to further studies on cellular pathways. Our cnidarian model of primary cell cultures may be suitable to investigate not only the molecular and cellular pathways in cnidarian cells but also cellular mechanisms involved in maintenance or breakdown of symbiosis. After validation of the protocol for homogeneous 18-day primary cell cultures, we analysed the physiological status of cultured cells. Indeed, a high proportion of apoptotic cells would preclude efficient cell culture maintenance and propagation. As an indicator of apoptosis, analysis of DNA fragmentation showed that three of the four samples (lanes 1, 2 and 4, Fig. 4b) of genomic DNA extracted from 18-day cultures exhibited the same pattern as that obtained from *A. viridis* fresh tissue (lane 5, Fig. 4b). Unfortunately, no DNA could be detected in one of the four samples (lane 3, Fig. 4b). As no typical scale of apoptotic DNA was observed, the selected samples of 18-day cultures were then maintained in culture with weekly changes of medium (see “[Materials and methods](#)” section). In order to propagate the adherent aggregates of small rounded cells, we tested different

Fig. 4 **a** PCR on genomic DNA with specific primers of *A. viridis npc1* gene. Lane 1 extracted from *A. viridis* fresh tissue, lanes 2, 3 extracted from cell culture (18 day-culture), lane 4 extracted from cultured zooxanthellae. **b** Analysis of genomic DNA (1 mg) fragmentation. Lanes 1–4 extracted from cell culture (18 day-culture), lane 5: extracted from *A. viridis* fresh tissue

methods of cell detachment. Classically the propagation of adherent cells is obtained by cell passaging and re-plating after enzymatic treatment, with trypsin–EDTA or accutase. In our experimental temperature conditions (20 °C), the trypsin–EDTA solution was completely unsuccessful. Accutase was only a good alternative for nucleic acid extraction but not for efficient cell detachment. Thus, we decided to move to a mechanical technique of micromanipulation (see

“Materials and methods” section). After a further 10 day-period, i.e. 4 weeks since collagenase tissue dissociation, we isolated adherent cell aggregates by micromanipulation. Picked cells were then seeded in a 24-well culture plate. After 1 week, we observed small adherent rounded and isolated cells, which largely covered the surface of the wells (Fig. 5a). Ten days after picking (38 days after initial collagenase treatment), PCR analysis performed directly on replicate samples of primary cultures of cells obtained by micromanipulation showed that these cells still exhibited a specific molecular signature of *A. viridis* (Fig. 5b).

Discussion and conclusions

This is the first time that a protocol for primary cell culture using *A. viridis* epithelial cells is reported. In this study we developed an appropriate cell isolation method, which enabled the establishment of a long-term primary culture of host animal cells. First, by supplementing the culture medium with MycoKill AB[®] to limit the contamination and optimizing target cell type isolation with the post-cutting tentacle protocol, we strongly reduced two major limitations of cell primary culture establishment. Secondly, we selected an enzymatic tissue dissociation method with collagenase over a classic explant technique to increase cell type homogeneity. Moreover, as our

study model is a symbiotic cnidarian, we overcame another technical challenge by establishing an exclusive animal host cell culture, i.e. without zooxanthellae. Through weekly serial dilutions, we obtained after 3 weeks a homogeneous primary cell culture composed of small rounded cells, putatively enriched in “epithelial-like cells”. Insufficient information for the classification of invertebrate cell types under in vitro conditions required us to specifically distinguish the anemone host cells from zooxanthellae and unicellular eukaryotic contaminants (Rinkevich 1999). Results of PCR analyses using specific primers of an *A. viridis* gene confirmed that small rounded cells, which comprised the 18-day homogenous cultures, are likely the cells of interest. However, potential concomitant presence of protist contaminants cannot be ruled out. Considering the slow rate of in vitro cell proliferation classically observed for a variety of marine invertebrates, the evaluation of physiological state using DNA analyses during cell culture maintenance might be regarded as an important tool. Indeed, it is crucial to discriminate between non-dividing vs apoptotic cells in primary cultures. DNA fragmentation analysis performed on 18-day cultures showed that *A. viridis* cultured cells did not exhibit typical scale of apoptotic DNA. Maintenance of 18-day cultures of cells isolated using micromanipulation allowed us to obtain a long-term (more than 1 month) primary cell culture of *A. viridis* epithelial cells. Future experiments will consist in characterizing these cultured

Fig. 5 **a** Observation of small rounded and adherent cells 7 days after micromanipulation. Phase contrast microscopy, scale bar = 10 μ m. **b** PCR on DNA with specific primers of *A. viridis* *npc1* gene. Lane 1 genomic DNA extracted from *A.*

viridis fresh tissue, lane 2 genomic DNA extracted from cultured zooxanthellae, lanes 3, 4: total DNA extracted from isolated cells (10 days after micromanipulation)

epithelial-like cells, i.e. to determine their differentiation status. Are they totally undifferentiated cells, which could be considered as totipotent cells, or are they specific cells (gastrodermal or epidermal cell type)? To solve this question it would be particularly informative to study the expression of totipotency markers, such as *piwi* or *vasa* genes, whose homologs are present in *A. viridis*. Moreover, measuring the expression level of genes previously identified as being specific of the gastrodermal or the epidermal tissue layer (Ganot et al. 2011) could complement this analysis.

The method presented here may thus be considered as a preliminary step for further investigations on cnidarian cellular pathways. In addition, another perspective of this work could be the analysis of the expression of symbiosis specific genes in animal host primary cell cultures.

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