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RESEARCH ARTICLE

A simple method for cell culture of 'Nemo' ocellaris clownfish (*Amphiprion ocellaris*, Cuvier 1830)

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Abstract

Worldwide, the 'Nemo' ocellaris clownfish (*Amphiprion ocellaris*, Cuvier 1830) is one of the top three most exported ornamental fishes. It also served as a subject in various fields of study except for cell culture. This first report described a simple explant method for culturing cells from the vertebra of ocellaris clownfish. The fish was first anesthetised with iced cold water and decapitated. The body trunk was disinfected in isopropanol and washed in sterile PBS. The vertebra was aseptically excised, washed two times in PBS and minced in the dissection solution (PBS containing 250 IU/mL penicillin, 250 μg/mL streptomycin, 50 μg/mL gentamycin and 2.5 μg/mL amphotericin-B). Then, the vertebral biopsies were washed three more times in PBS before being seeded in 25 cm^2 culture flasks containing 1.5 mL of RPMI-1640 supplemented with 20% FBS. A small amount of CO_2 was injected into the flask before it was tightly capped and incubated at 28°C in the regular incubator. When the monolayer reached 40–50% confluence, the vertebral biopsies were dislodged together with the medium to initiate a new primary culture. The cell monolayer was subcultured with short, cold 0.05% trypsin. The Nemo cell line was grown in the medium containing 15% FBS. The cell line at passage 4 had the population doubling time of 39.6 h and the cell line at passage 5 could be cryopreserved with 80% viability. This simple and reliable explant method has been applied successfully to culture cells of both marine and freshwater fishes for the prometaphase chromosome preparation.

Keywords: *Amphiprion ocellaris* (Cuvier 1830); cell cryopreservation; cell culture; ocellaris clownfish; vertebra

Introduction

The ocellaris clownfish or false percula clownfish (*Amphiprion ocellaris*, Cuvier 1830) is well known worldwide as 'Nemo', the hero from the movie 'Finding Nemo' released by Disney and Pixar 10 years ago. It belongs to the subfamily Amphiprioninae of the family Pomacentridae. In this subfamily, there are 29 species of the clownfish in the genus *Amphiprion* and only one species of the clownfish in the genus *Premnas*, known as the maroon anemonefish, *Premnas biaculeatus* Bloch, 1790 (Allen et al., 2008, 2010). All these described species are monophyletic (Quenouille et al., 2004; Santini and Polacco, 2006; Cooper et al., 2008). The ocellaris clownfish is found in Indo-West Pacific Ocean and Eastern Indian Ocean including Andaman and Nicobar Islands, Thailand, Malaysia, Northwest Australia, Singapore, Indonesia, the Philippines, Taiwan and the Ryukyu Islands. But it is not found in the Atlantic Ocean or Caribbean Sea (Thornhill, 2012).

This charismatic fish is one of the most popular tropical marine ornamental fishes because of its attractive colour pattern, small-sized body, durability and high adaptability to life in captivity. Three species of the clownfishes namely ocellaris clownfish, percula clownfish (*A. percula* Lacépède, 1802) and maroon anemonefish are amongst the top 20 marine aquarium fish imported into the U.S. (Rhyne et al., 2012). Thus, the clownfish is considered the 'goldfish' of marine aquaria (Hoff, 1996).

The population of the wild clownfish declined drastically because of over exploitation in response to its increasing popularity and deterioration of its natural habitats. Concerns such as these have led to efforts of the captive breeding of many ornamental marine fishes for commercial purposes as well as for conservation (Dawes, 2003; Green, 2003). As a

*Corresponding author: e-mail: topas999@gmail.com
Abbreviations: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PBS, phosphate buffer saline

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result, the clownfishes were amongst the first coral reef fishes raised successfully in captivity throughout their entire life and now represent one of the most well-developed captive breeding programs for marine fishes (Dawes, 2003).

Although, many research aspects of the clownfish, including molecular genetics, have been investigated (Nelson et al., 2000; Timm et al., 2012), at present, there is still no report on the cell culture of the fish in the subfamily Amphiprioninae. Actually, the fish cell culture has become an essential tool in various fields and provided the best experimental specimens for studying virology, environmental toxicology, cytobiology, oncology, genetics and genomics (Lee et al., 2009; Taju et al., 2012). Fish cell culture will probably become even more important in the future, when the automated and high-throughput technologies in ecotoxicological hazard assessment are incorporated (Castaño et al., 2003). Furthermore, fish cell lines are most demanded not only to combat fish diseases especially with viral etiology (Perez et al., 2002; Sobhana, 2009), but also to be reserved in a genome bank for the fish cloning (Mauger et al., 2006).

This first report of the cell culture from the ocellaris clownfish describes a simple explant method from the fish vertebra and its growth characteristics.

Materials and methods

Initiation of primary cultures and routine maintenance

Two normal and apparently healthy juvenile ocellaris clownfish were kindly provided by Krabi Coastal Fisheries Research and Development Centre, Thailand and transported live to the laboratory. The preparation of the primary cultures from the vertebra followed the previously modified protocol (Tantithakura, 1989; Tantithakura et al., 1993; Patkeaw and Tantithakura, 2003). Briefly one fish (2–3 cm long) was anesthetised in iced cold water before its head and belly were cut off. Its body trunk was disinfected in isopropyl alcohol for 10–15 s and transferred into a 5-cm sterile Petri dish containing sterile PBS.

The next steps were performed under sterile conditions in a biological safety cabinet. The fish skin and its underneath muscle were removed. The vertebral column was excised and transferred to be washed in fresh PBS. Adherent muscle was taken off as much as possible. The vertebra was washed two more times in sterile PBS. Then, it was cut into small pieces in the dissection solution (PBS containing 250 IU/mL penicillin, 250 μg/mL streptomycin, 50μg/mL gentamycin and 2.5 μg/mL amphotericin-B). After that, the vertebral biopsies were washed two more times in fresh dissection solution and three times in fresh PBS to remove the antibiotic and antimycotic traces from the tissue fragments.

RPMI-1640 (Gibco) supplemented with 20% FBS (Gibco) 1.5 mL was first pipetted into a 25 cm^2 culture flask (SPL Lifesciences). Then, 5–7 pieces of vertebral biopsies were placed in the flask. A small amount of CO_2 was given directly into the flasks before they were tightly capped and incubated at 28°C in a regular incubator (WTB Binder). The primary cultures were monitored for tissue adherence and cell outgrowth every day under the inverted microscope (Nikon TS 100). The culture medium was changed every 6–7 days.

Re-initiation of the new primary culture

When the cell outgrowth from the vertebral biopsies reached 40–50% confluence, the vertebral biopsies were dislodged from the culture flasks. Then, the vertebral biopsies and the medium were transferred to a new culture flask to re-initiate a new primary culture. The cells in the original flask were fed with 2 mL of the medium supplemented with 20% FBS.

Subculturing

When the primary cultures in both original and new flasks reached confluence at 60–70%, they were subcultured with short and cold trypsin (0.05% trypsin for 10–15 s) at a split ratio of 1:2 or 1:3. The cell lines in the later passages, scheduled at a 5-day interval, were fed with the medium containing 15% FBS.

Growth study of the Nemo cell line

For the growth study, the cells at passage 4 were seeded into a 6-well plate at an initial density of 1.2×10^4 cells/mL and incubated at 28°C. Cells from triplicate wells was trypsinised and counted (three counts per well) with a hemocytometer under an inverted light microscope every day. This experiment took 7 days.

Cell cryopreservation and cell recovery

The viability and stability of the Nemo cell line at passage 5 in liquid nitrogen were assessed. The cell cryopreservation method proposed by Wang et al. (2012) was slightly modified. The assessment was performed in triplicate. The Nemo cell line at passage 5 was first subcultured and seeded in 75 cm^2 culture flasks. When the cells were in the exponential phase on day 3 after seeding, the culture medium was changed. On the following day, the cells were harvested, washed in PBS and suspended in the freezing medium: RPMI-1640 medium supplemented with 20% FBS and 5% DMSO (Sigma–Aldrich) at the concentration of 3.5×10^6 cells/mL. Aliquots of 1 mL were transferred to 2-mL cryovials (Nunc) and kept sequentially at −20°C for 4 h, −80°C for 16 h and finally stored in liquid nitrogen −196°C.

The frozen cells were recovered in 1 month by thawing in a water bath at 28°C. They were centrifuged to remove the

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cryopreservative and then suspended in 1.5 mL RPMI-1640 medium supplemented with 15% FBS. The viable cells were counted before they were seeded in a 25 cm^2 culture flask and incubated at 28°C. The cell growth and morphology were observed. The viable attached cells were determined within 24 h after seeding. The quality of cell recovery was assessed by the comparison of the percentage of viable cells recovered after adhesion with the percentage of viable cells after thawing (Mauger et al., 2006).

Results

Cell outgrowth in the original primary culture

In the original primary culture, fibroblastic cells emerged from the vertebral biopsies on day 4 after implantation (Figure 1). Subsequently, they outgrew from the vicinity of the tissues in large numbers and multiplied rapidly. Some epithelial cells could be observed in the primary culture.

Cell outgrowth after re-initiating in the new primary culture

The outgrowth of the fibroblastic cells from the dislodged vertebral biopsies could be observed on the next day after the re-initiation of another primary culture (Figure 2). There were also many liberated cells from the original flask. These cells also adhered to the flask surface and formed individual colonies on the next day (Figure 3). Most of them were fibroblastic cells. They proliferated rapidly and reached 70–80% confluence in ~3–4 days. In comparison with the cell outgrowth in the original primary culture less epithelial cells were observed in the new primary culture.

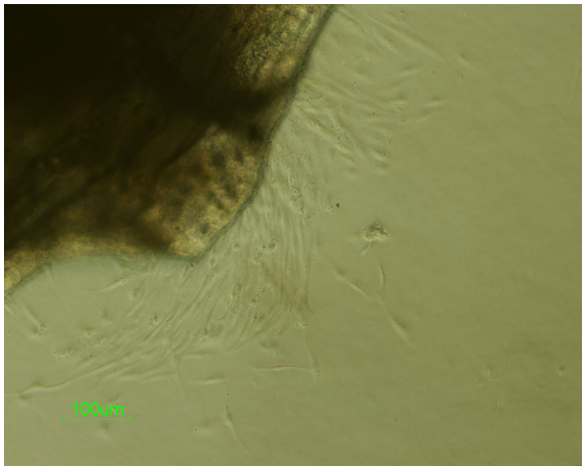

Figure 1 Outgrowth of the fibroblastic cells from the vertebral biopsies of the 'Nemo' ocellaris clownfish on day 4, scale bar = 100 μm.

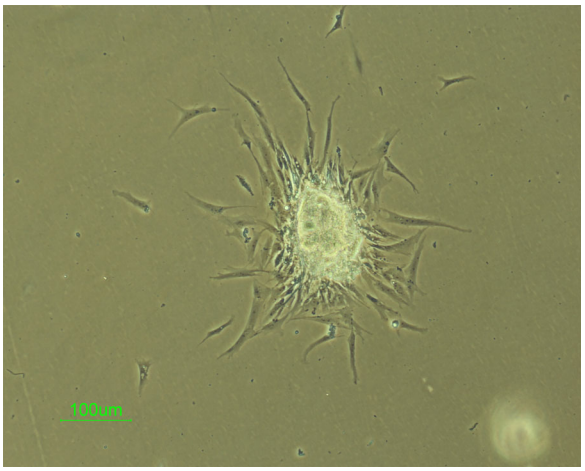

Figure 2 The outgrowth of the fibroblastic cells from the dislodged vertebral biopsies in the new primary culture, scale bar = 100 μm.

The Nemo cell line

The primary cells in both original and new flasks were subcultured when they reached 60–70% confluence. After the subculturing process, the cell growth was accelerated. The cells reached 70–80% confluence again within 4–5 days especially from passage 3 onwards (Figure 4). The epithelial cells which were also found in the primary culture gradually depopulated in the later passages while the fibroblastic cell outnumbered them.

Growth pattern of the Nemo cell lines

The Nemo cell line at passage 4 mostly contained typical long spindle-shaped fibroblasts. The cell line at this passage still

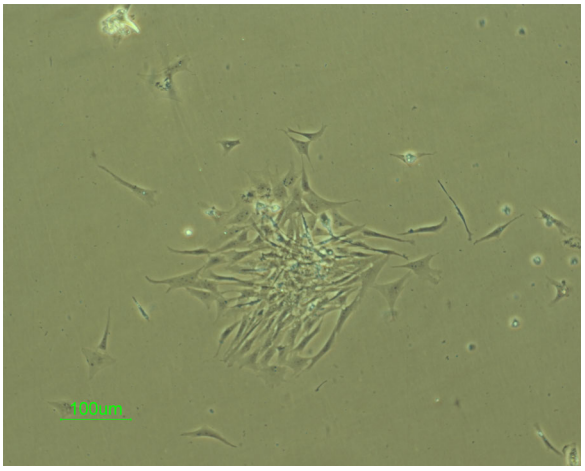

Figure 3 Colonies of the fibroblastic cells from the dislodged vertebral biopsies in the new primary culture, scale bar = 100 μm.

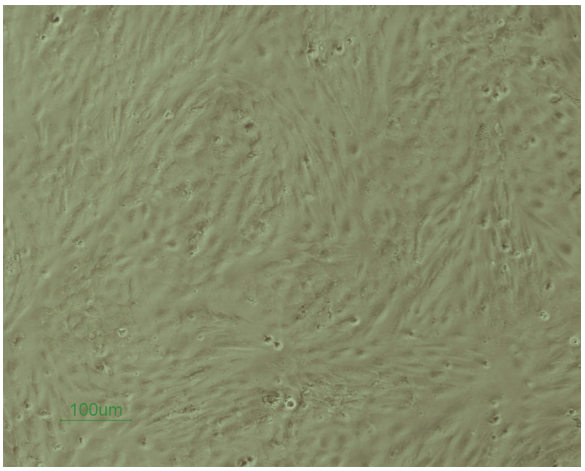

Figure 4 The Nemo cell line at passage 3 on day 3 after passaging, scale bar = 100 μm.

exhibited the typical growth curve of a normal cell (Figure 5). The lag phase and the exponential phase lasted 2 and 4 days respectively. The cells reached the stationary phase on day 7. The population doubling time which was calculated from the exponential phase of the curve data took 39.6 h.

Cell cryopreservation and cell recovery

The Nemo cell line could survive the freezing and thawing process. The mean number of viable cells after being thawed was 2.8×10^6 cells/mL. Then, the average viability was 80%. The mean number of viable attached cells was 1.6×10^6 cells/mL. Therefore, the quality of the cell recovery from 1 month of cryopreservation was approximately 57%. No alterations in the morphology or growth pattern were observed from the cryopreserved Nemo cell lines.

Discussion

Even though fish cell cultures have be practiced for almost 60 years, it seems that the technology of the fish cell culture is still at the beginning of the exponential phase when the number of the published papers and the number of fish cell line discoveries are considered. Up until now there are approximately 300 fish cell lines (Pandey, 2013) from a limited number of fish species.

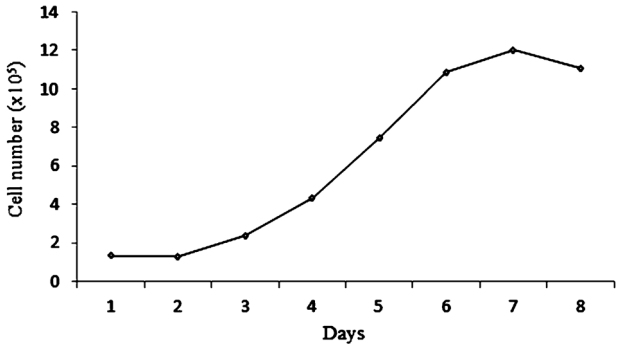

Figure 5 Growth curve of the Nemo cell line at passage 4.

At present, there have been very few reports on cell culture systems from the fish vertebrae (Pombinho et al., 2004; Marques et al., 2007). This group of researchers used collagenase and trypsin respectively to disaggregate calcified tissues of vertebrae and branchial arches for the primary culture establishment. Tantithakura and Patkaew (2004, unpublished data) used only the explant method to culture cells from the vertebrae of both marine and freshwater fishes. Meanwhile, this explant method has been applied successfully with other species of anemonefish, the saddleback anemonefish *Amphiprion polymnus* (Linnaeus, 1758). From our experience, the fish vertebra is a promising tissue especially for the fibroblast cell culture, which is our most needed subject. The uppermost goal of our research is to prepare the chromosomes at prometaphase for a high-resolution karyotype analysis.

The method of the fish cell culture described in this paper was based on the explant method and was simplified without any application of enzymes for the cell liberation (Patkeaw and Tantithakura, 2003). In this way, our success in the primary cell culture from the gills fins, skins and vertebrae of other species of anrmonefishes was more than 90%. There were also some researchers such as Lakra et al. (2006) and Sahul Hameed et al. (2006) who developed their cell culture systems from Asian seabass *Lates calcarifer* (Bloch) without enzymatic digestion.

Some pioneers in fish cell cultures used the enzyme disaggregation method for the establishment of primary culture (Grützner, 1958; Wolf et al., 1960; Clem et al., 1961). Fryer (1964) used many enzymes to liberate cells from the embryonic tissue of the steelhead trout, *Salmo gairdneri gairdneri*. He found that pancrease could not disperse cells as much as collagenase, papain, hyaluronidase, trypsin and *Vibrio comma* receptor destroying enzyme (*N*-acetylneuraminidases). He also found that collagenase, papain and hyaluronidase were more toxic than trypsin. Then, trypsin was the only satisfactory enzyme for his cell dispersion. Currently, trypsin and collagenase, either separately or together, has been applied to isolate fish cells for the fish primary cell culture (Pärt et al., 1993; Ostrander et al., 1995; Chi et al., 1999; Qin et al., 2006; Butler and Nowak, 2008; Grunow et al., 2011). Anyway, the irreversible effects and batch variability of the disaggregated enzymes may result in poor or aggressive dissociation of tissues and lead to low cell viability (Wolf and Quimby, 1969). Furthermore, most enzymes are expensive.

RPMI 1640 supplemented with 20% FBS was the media of choice for our primary cell cultures. For the later passage, the concentration of 15% FBS was proved to be an optimal

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concentration for our cell lines (Patkaew and Tantithakura, in preparation). The medium was buffered with $NaHCO_3$, so a small amount of CO_2 was injected directly into the culture flask before the culture flask was tightly capped. Thus, the cell culture could be incubated in the regular incubator instead of the CO_2 incubator.

It should also be noted that in our cell culture system, antibiotics and antimycotics were used only in the dissection step and two more washes afterwards. Their traces then were removed by three washes in fresh PBS. Antibiotics have a number of significant disadvantages; thus, routine uses of antibiotics in the culture media should be avoided (Freshney, 2005). Furthermore, their stability in the medium at the incubation temperature lasts as much as 5 days. Ryan (2008) suggested using antibiotics and antimycotics only for short-term applications and he also referred to many experienced animal cell culturists' recommendation that antibiotics and antimycotics will prevent the growth of the usually more easily detected contaminants but allow mycoplasma or other cryptic contaminants to grow undetected. Our primary cultures were sometimes contaminated with fungi in some flasks during the first 2–4 days.

The re-initiation of the new primary culture in the original flask was an ingenious way to obtain more cell cultures from the outgrowing tissues. The morphology and growth pattern of cells in the original primary culture and re-initiated primary culture were somewhat different. The cell population in the original primary culture was more heterogeneous, composing of more fibroblastic and some epithelial cells, while the cell population in the new primary cultures was mostly fibroblastic cells. Moreover, the proliferation rate of the cells in the re-initiated primary cultures was remarkably faster than that in the original primary cultures.

The morphology of the cell lines from the Nemo in the third passage onwards was similar to the cell line from the vertebra of the saddleback anemonefish-SAV-1 (Patkaew and Tantithakura, in preparation). The growth curve of the Nemo cell line at passage 4 was a typical growth curve of a normal cell line. The population doubling time which reflected the proliferation kinetics during the log phase was 39.6 h. This population doubling time exhibited the fast growing nature of the cell line. Thus, this cell line has been successfully used for prometaphase chromosome preparation. (Patkaew and Tantithakura, in preparation).

The cryopreserved Nemo cell lines were revived after thawing with 80% viability and the quality of cell recovery after 24 h attachment was 57%. These percentages were not much different from the feasibility studies in other fish species (Wang et al., 2012; Yadav et al., 2012). These percentages also ensured that the cryopreserved Nemo cell line could provide consistent results serving as the seed stock of cells for the genome bank of this fish species.

Conclusions

The method described here is simple and reliable for culturing the 'Nemo' ocellaris clownfish cells. In addition, this method is economical in terms of both chemicals and time. The enzymatic disaggregation of the tissues and the supplement of antibiotics, antimycotics and other additives in the culture medium are not required. With differential trypsinisation, the cell population in the later passages was mostly fibroblasts which were our most needed cells for the prometaphase chromosome preparation. This method has been applied successfully to culture cells of both marine and freshwater fishes. Therefore, this simple method will open new vistas for researches especially in the field of high-resolution karyotype analysis and fish genome cryobanking.

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