Protistology 4 (2), 185-190 (2005)

Protistology

A simple mass culture of the amoeba *Chaos carolinense*: revisit

Olivia Li Ling Tan, Zakaria Ali Moh. Almsherqi and Yuru Deng

Department of Physiology, Faculty of Medicine, National University of Singapore, Singapore

Summary

The free-living amoeba *Chaos carolinense* used to be a very popular laboratory object in the 1950s-60s and is still one of the favorite classroom objects for demonstration of amoeboid movement. Large-scale production of this organism is often needed for various experimental purposes. We have established a reproducible, simple mass culture method specifically suitable for *C. carolinense*, with feeding on only one food organism (*Paramecium multimicronucleatum*), and discuss the problems that have been encountered during establishing and maintaining of this culture.

Key words: amoeba, *Chaos carolinense*, mass culture, *Paramecium*

Introduction

The uniquely large size (1-5 mm) of *Chaos carolinense* amoebae allows easy manipulation in the laboratory, making them an excellent model for certain physiological (Griffin, 1960; Daniels, 1962), biochemical (Bruce and Marshall, 1965; Friz, 1968) and morphological studies (Brandt and Pappas, 1960). These studies often require a large number of cells. However, to date there is no protocol, which allows continuous mass culturing of *C. carolinense*. So far this organism was supported in laboratories in mixed cultures (where many types of organisms grow together). Such cultures are not easy to maintain for a long time in the laboratory, as they have been documented to vanish due to overgrowth of the accompanying organisms or simply for no reason at all (Lorch, 1973). Simple cultures fed on only one food organism, unlike mixed cultures, can provide abundant and cytologically uniform specimens, where unpredictable parameters from food sources are limited to only one food object (Andresen, 1956; Griffin, 1973). So far, continuous culture methods have only been successful on a relatively hardy species, *Amoeba proteus* (Goldstein and Ko, 1976), while the longest-living cultures of *C. carolinense* were maintained by Andresen (1956) for only 3 months.

We revisited *C. carolinense* mass culture methods of growing amoebae in simple inorganic medium (Bruce and Marshall, 1965) with *Paramecium multimicronucleatum*, which is the a more suitable food object

for this species (Kudo, 1946) than Tetrahymena, which is more appropriate for *A. proteus* (Prescott and James, 1954; Griffin, 1960). This method allowed us to obtain a continuous, large-scale culture of this amoeba and was adapted to reach optimal cell growth. The amoebae were observed under various conditions (well-fed, over-fed, and starved) to establish features, allowing easy assessment of the culture status.

Material

Mixed cultures of *C. carolinense* were obtained from Carolina Biological Supply Co (catalog number #13-1324). Living amoebae were picked individually under a stereoscope (Nikon SMZ645) with low light illumination (Fibre-Lite™ MI-150) using a glass Pasteur pipette pulled to obtain a fine tip with a diameter of about 0.5 mm into tissue culture treated dishes (Bector Dickinson) filled with amoeba inorganic medium. The picked amoebae were then left overnight, without food in the medium, so that they got accustomed to the new environment. Healthy looking amoebae were then individually transferred to large, treated Pyrex® baking dishes to start the mass cultures.

To make the surface of culture dishes "wettable", which is crucial for growing *Chaos*, non-wettable dishes were treated overnight with 10% sulphuric acid, rinsed with running tap water and then soaked in MilliQ water before use. Used dishes, which were still wettable, were cleaned using a soft sponge and MilliQ water to remove debris left from the previous culture. All detergents must be strictly avoided in routine cleaning of culture dishes, because *Chaos* amoebae are extremely sensitive to any residual detergents or chemicals.

Method for the mass culture of *Chaos carolinense*

1. Maintenance of cultures

The cultures were maintained in amoeba inorganic medium (Bruce and Marshall, 1965) containing 0.5 mM $CaCl_2$, 0.05 mM $MgSO_4$, 0.16 mM K_2HPO_4, 0.11 mM KH_2PO_4, made up in MilliQ water, at a depth of 7-10 mm and kept in the dark, at 22°C- 24°C on bench top or in a 22°C cool incubator (Sanyo MIR-553). Amoebae were counted using a large magnifying lens and fed once every 2-3 days, with the medium changed before each feeding. The counting is crucial, since the feeding scheme is based on the number of amoebae in the dish. The amoebae were split to a new dish when the cell density in the dish became too high. Culture dishes were changed every 2-3 weeks, when the debris accumulated in the culture was becoming detrimental.

2. Feeding of amoebae

The cultures were fed on 7-day old *Paramecium multimicronucleatum* cultures at late log phase or early stationary phase (Fok and Allen, 1979). The growth medium of *Paramecium* was removed by filtering the cultures through a 3-piece glass filter funnel (Whatman) lined with a layer of GF/A glass microfibre filter (Whatman) and the cells were washed with amoeba medium (Fig. 1). The ciliates were concentrated by allowing the medium to be filtered through; concentrated suspension of *Paramecium* in amoeba inorganic medium was collected into a beaker. The number of *Paramecium* per ml was estimated by taking an average of the number of *Paramecium* individuals in ten 10 µl drops. The amount of food was calculated from the density of paramecium suspension and the abundance of amoebae in each dish so that there were 36 ± 3 *Paramecium* per amoeba cell.

3. Changing amoeba culture medium

The culture dishes were tilted and after the amoebae settled to the bottom of the dish, the old medium was carefully removed by suction. Fresh amoeba medium was gently squirted onto the surface of dish. The process was repeated 2-3 times until the culture was free from the remaining food organisms and cell debris. Fast growing and healthy cultures had little cell debris (dead amoebae, cell fragments) and usually few or no lingering *Paramecium* left from the previous feeding, whereas slow-growing or sick cultures had an increased amount of cell debris and a large number of *Paramecium* left from the previous feeding.

4. Harvesting *Chaos*

The dish was tilted like when changing the medium before feeding. The excess medium was removed and *Chaos* individuals were collected using 3-ml disposable Pasteur pipettes. Multiple dishes of *Chaos* can be collected in a beaker simply by letting the amoeba settle by gravity and removing the excess amoeba medium.

5. Culture and harvest of food organism *Paramecium multimicronucleatum*

The axenic culture of *P. multimicronucleatum*, originally from Dr. Richard D. Allen (University of Hawaii, USA), was grown in modified medium (Table 1) for axenic cultures of *P. aurelia* (Soldo et al., 1966) in Erlenmeyer flasks of various sizes according to our needs. Each flask was filled to only 20% of its total volume to obtain maximum aeration. Only 5-7 days old

Fig. 1. Three-piece Glass Filter setup with the final wash of *Paramecium* in amoeba inorganic medium.

cultures, at log phase and with a minimum density of 4500 *Paramecium*/ml of culture medium, were used for inoculation of new cultures. Using aseptic techniques, 10-15% of the volume of medium to be inoculated was taken from the growing culture and transferred to the flask to be inoculated. The newly inoculated cultures were then grown in the dark at 26°C with no agitation or shaking needed.

Table 1. Growth medium of axenic *P. multimicromucleatum* culture.

L-a-Phosphatidylethanolamine	0.2 g/L
Proteose peptone	10 g/L
Peptone from casein, tryptic digest	0.5 g/L
Ribonucleic acid	1.0 g/L
Magnesium sulfate heptahydrate	4.2 mM
Stigmasterol	0.006 mM
D-Pantothenic acid hemicalcium salt	0.02 mM
Nicotinamide	0.04 mM
Pyridoxal hydrochloride	0.25 mM
Pyridoxamine dihydrochloride	0.01 mM
Riboflavin	0.01 mM
Folic acid	0.06 mM
Thiamine hydrochloride	0.04 mM
Biotin	0.01 µM
Lipoic acid	0.24 µM

Notes on the feeding schemes, amoebae development and recovery of sick cultures

In the process of optimization of the maintenance of the *Chaos* mass culture, different feeding schemes based on the parameters affecting the culture conditions have been employed. Two main feeding schemes were finally selected.

The first scheme was based on the number of *Paramecium* fed per ml of amoeba medium in the dish. Various concentrations have been tried and checked by proportion of the size of *Paramecium multimicronucleatum* (200 µm long) to *Tetrahymena pyriformis* (50 µm long), to keep the density of *Paramecium* in the dish similar to those of *Tetrahymena* used in the earlier attempt at *Chaos* mass cultures (Griffin, 1960). The cultures were observed to have fluctuating growth rates and inconsistent culture conditions. The cultures would sometimes have a high growth rate but drop drastically at the next feeding, even though the feeding was consistent. Most of the time, the drastic drop in growth was accompanied by the presence of sick or unhealthy looking amoebae. This scheme could lead to a frequent occurrence of over-feeding, because the volume of culture medium in the dish usually remains constant, whereas the number of amoeba may differ at each feeding.

The second feeding scheme was based on the number of *Paramecium* fed per *Chaos* cell. This feeding scheme gave more consistent growth rates and improved culture conditions. A decrease in growth rate would not necessarily be accompanied with the deteriorated culture conditions occurring in the first scheme. Less healthy cultures could be brought back to a healthy state easily by either washing away the excess food or reducing the amount of the food unlike the first scheme where a reduction would not necessarily help to improve the culture condition. Culture conditions were not only improved in the sense of the state of the amoebae but also visually, where the culture would be cleaner with less debris and a lesser occurrence of excess food present in the dish, since the amount of food given would specifically cater to the number of amoebae in the dish. Therefore, the second scheme has been employed to maintain the *Chaos* mass culture.

The mass culture has been found to thrive and grow at its fastest rate by balancing the ratio of the number of *Paramecium* fed to the number of *Chaos* in the dish with the optimal range of 35-38 *Paramecium* to one *Chaos* cell. If this amount of food was fed every 2-3 days, the growth rate could increase to 30% at each feeding. Thus, a mass culture starting with a minimum of 20 large Pyrex® baking dishes (33cm × 21cm × 4.5cm) can produce at least 18 000 *Chaos* individuals or 700 µl of

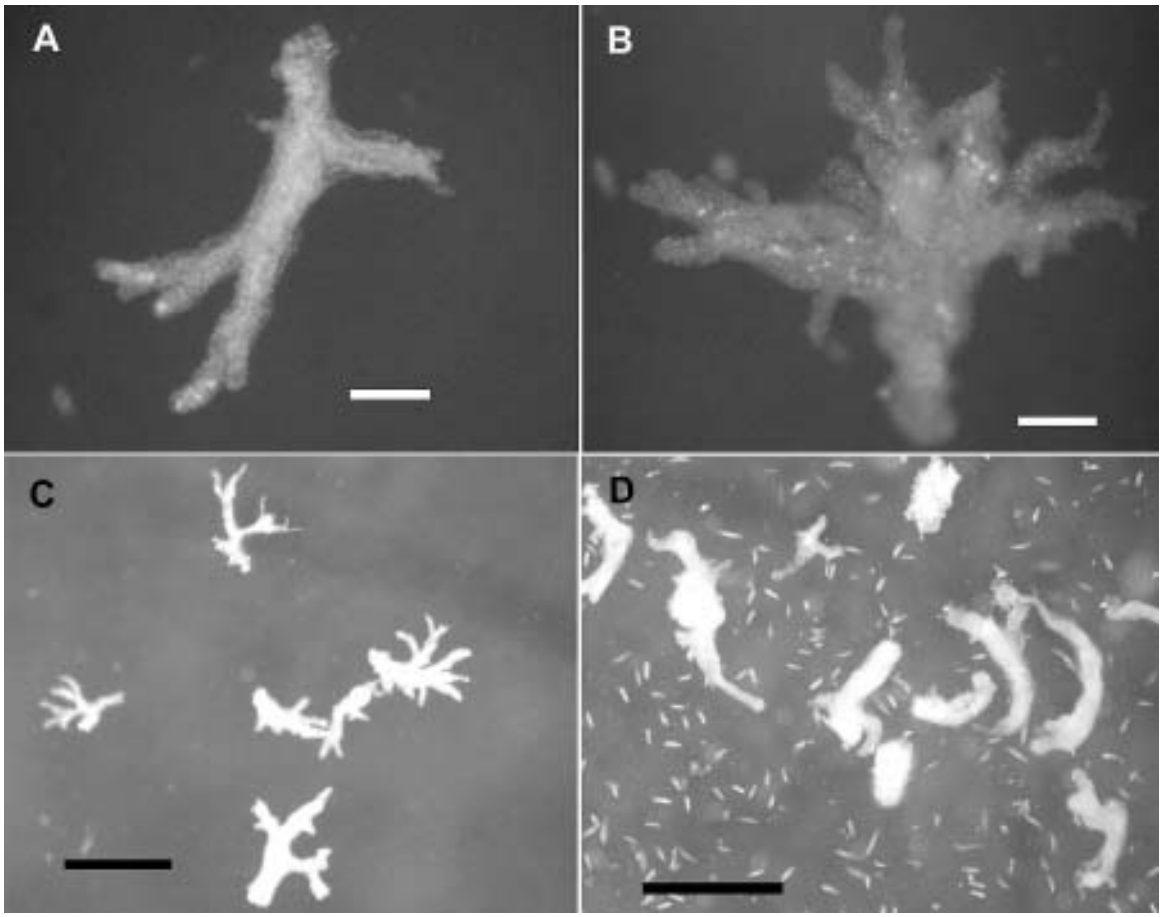

Fig. 2. Characteristic appearance of *Chaos carolinense* amoebae under various mass culture conditions. A - well-fed and healthy *Chaos*; B - well-fed *Chaos* in a recently washed culture; C - *Chaos* culture staved for 2-3 days; D - over-fed *Chaos* culture with amoebae that are rounded and rod-like. Scale bars: A, B - 250 μm; C, D - 1 mm.

packed cells per week. The subtle line between the amount of appropriate feeding and overfeeding must be noted (Table 2). A difference of only 2 *Paramecium* cells fed may seem small, but the outcome can be drastic and extreme. Overfed cultures are clearly unhealthy and do not have significant growth, appropriately fed cultures thrive and the *Chaos* can be morphologically identified to be healthy and well-fed (Fig. 2 A). In addition, well-cleaned *Paramecium*, that is, without any remnants of their own culture medium is essential for healthy *Chaos* cells, since we have observed that proteose peptone, one of the components in the *P. multimicronucleatum* growth medium is toxic to *Chaos*.

Table 2. Growth rates and doubling times of amoeba *Chaos carolinense* under different feeding schemes employed.

Feeding Scheme	Amount Fed	Average Growth Rate (%)	Doubling Time (days)
Paramecium / mL	200 p/mL	10.2	9.8
	250 p/mL	12.6	7.9
	275 p/mL	9.43	10.6
Paramecium/ amoeba	20 p/a	8.5	11.7
	30 p/a	8.9	11.2
	38 p/a	20.6	4.85
	> 40 p/a	7.4	13.5

When the *Chaos* cells exhibit any of the unhealthy characteristics demonstrated in Figures 2 C and 2 D, it is important to take steps to 'rescue' the culture from further deterioration. Dishes with even as little as 10% of the slightly starved amoebae (which have numerous long and thin pseudopods; Fig. 2 C), or severely starved amoebae (that appear as long thin rods), or over-fed amoe-

bae (that become irregular masses with a few small pseudopods; Fig. 2 D) should be washed clean of any excess food organisms and the amoebae should be transferred to a clean dish.

Amoebae that have been under-fed or, in other words, are slightly starved form long, thin pseudopods that increase in number as starvation proceeds (Fig. 2 C). They differ from the amoebae in recently washed cultures that are generally branched but are still 'plump' (Fig. 2 B). On the surface of the culture there might be some patches of oily film, presumably of lipid nature, that is extruded from the starved amoebae. Such dishes should be carefully washed to remove as many of the oily patches as possible, and the amoebae should be fed with a slightly higher amount of food (about 1-2 more *Paramecium* per amoeba). Then the feeding should be further adjusted, if necessary, and the number of amoebae in the dish should be checked to ensure that the feeding amount is appropriate.

Amoebae not fed for over a long time (approximately 7 to 14 days) become thin and rod-like. The surface of the culture would be covered with a thin oily film. Such severely starved cultures should also be washed and fed with a lesser amount of food, about half amount of the normal, with slowly increasing the amount with each consecutive feeding until the *Chaos* return to their normal behavior.

When *Chaos* are fed with an excessive amount of food, they tend to round up and appear as very dense, white balls (Fig. 2 D), some with a few small and thin pseudopods. If allowed to continue in this condition, some of *Chaos* would take on the thin rod-like appearance seen in severely starved cultures while others would round into tight balls and eventually die. The culture surface would sometimes also have similar oily patches observed in starved dished but to a lesser extent. However, the most distinguishing characteristic of an over-fed culture is an unusually large amount of food left unconsumed. The *Chaos* individuals return to the normal branched appearance (Fig. 1 C) when the excess food and debris have been removed by thorough washing of the culture and in severe cases, transfer of a culture into a clean dish. These amoebae can be immediately moved back to a normal feeding scheme (36-38 *Paramecium*/ amoeba) with possibly a slower growth rate observed until the culture stabilizes again.

Acknowledgments

This work is supported in part by research grants from NMRC, Singapore (R-185-000-058-213), and BMRC, Singapore (R-185-000-065-305) to Y. Deng.

We thank Dr Richard D. Allen of University of Hawaii, USA for providing us with starter cultures of *Paramecium multimicronucleatum*.

References

Andresen N. 1956. Cytological investigations on the giant amoeba *Chaos carolinensis*. Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 29, 435-555.

Brandt P.W. and Pappas, G.D. 1960. An electron microscopy study of pinocytosis in amoeba. I. The surface attachment phase. J. Biophys. Biochem. Cytol. 8, 675-687.

Bruce D.L. and Marshall J.M. Jr. 1965. Some ionic and bioelectric properties of the ameba *Chaos chaos*. J. Gen. Physiol. 49, 151-178.

Daniels E.W. 1962. Limits of transplantation tolerance in large amoebae. I. Microfusion studies using *Amoeba proteus, Pelomyxa illinoisensis*, and three strains of *Pelomyxa carolinensis*. J. Protozool. 9, 183-187

Fok A.K. and Allen R.D. 1979. Axenic *Parameciu*m *caudatum*. I. Mass culture and structure. J. Protozool. 26, 463-470.

Friz C.T. 1968. The biochemical composition of the free-living amoebae *Chaos chaos, Amoeba dubia* and *Amoeba proteus*. Comp. Biochem. Physiol. 26, 81-90.

Goldstein L. and Ko C. 1976. A Method for the mass culturing of large free-living amebas. Methods Cell Biol. 13, 239-246.

Griffin J.L. 1960. An improved mass culture method for the large, free living amoebae. Exp. Cell Res. 21, 170-178.

Griffin J.L. 1973. Culture: Maintenance, large yields, and problems of approaching axenic culture. In: The biology of amoeba (Ed. K.W. Jeon). Acad. Press, New York. pp. 83-98.

Kudo R.R. 1946. *Pelomyxa carolinensis* Wilson I. General observation on the Illinois stock. J Morphol. 78, 317-351.

Lorch J. 1973. Historical aspects of amoeba studies. In: The biology of amoeba (Ed. K.W. Jeon). Acad. Press, New York. pp. 1-36.

Pappas G.D. and Brandt P.W. 1959. Mitochondria. I. Fine structure of the complex patterns in the mitochondria of *Pelomyxa carolinensis* Wilson (*Chaos chaos* L.). J. Biophys. Biochem. Cytol. 6, 85-90.

Prescott D.M. 1956. Mass and clone culturing of *Amoeba proteus* and *Chaos chaos*. Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 30, 1-12.

Prescott D.M. and James T.W. 1955. Culturing of *Amoeba proteus* on *Tetrahymena*. Exp. Cell Res. 8, 256-258.

Soldo A.T., Godoy G.A. and Van Wagtendonk W.J. 1966. Growth of particle-bearing and particle-free *Paramecium aurelia* in axenic culture. J. Protozool. 13, 492-497.

Whatley J.M. and Chapman-Andresen C. 1990. Phylum Karyoblastea. In: Handbook of Protoctista. (Eds. L.Margulis, J.O.Corliss, M.Melkonian and D.J.Chapman). pp. 167-185.

Address for correspondence: Yuru Deng. Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, MD9, 2 Medical Drive, 117597 Singapore. E-mail: phsdy@nus.edu.sg

Editorial responsibility: Alexey Smirnov