

Chapter 3

Procedures for Cultivation, Observation, and Conventional Experiments in *Cyanidioschyzon merolae*

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Abstract *Cyanidioschyzon merolae* 10D was originally isolated from a mixture of hot spring water sampled in Naples, Italy. Currently, this strain is available in the Microbial Culture Collection at the National Institute of Environmental Studies in Japan. The strain has been cultured in 2× Allen’s medium or its derivatives. The optimal growth conditions for this strain are as follows: pH 2.5, 42 °C, and ~100 μmol photons m⁻² s⁻¹, allowing the cell density to reach ~5 × 10⁸ cells mL⁻¹. *C. merolae* can also grow slowly at >20 °C. We generally store stock cultures at room temperature or at 30 °C under a low light condition (~20 μmol photons m⁻² s⁻¹) or as frozen stock in liquid nitrogen. Cell cycle progression can be synchronized by subjecting the culture to a 12-h light/12-h dark cycle. In addition, cells can be arrested at the S or M phases by adding relevant inhibitors. The shapes of cells and chloroplasts are clearly observed by phase-contrast or differential interference contrast microscopy. Because *C. merolae* lacks a cell wall, cellular contents (e.g., DNA, RNA, and proteins) are easily extracted.

Keywords Cell cycle • Cell division • Cyanidiales • *Cyanidioschyzon merolae* • Red algae • Synchronous culture

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3.1 Introduction

Unicellular algae are potentially useful for basic biological studies in several different disciplines and also for interdisciplinary studies for the following reasons:

1. The chemical formulas of many kinds of media for algal growth are completely defined.
2. A unicellular algal culture often provides a homogeneous population in terms of cell types and surrounding environment (including pH, temperature, light strength, and inorganic nutrient condition). This contrasts with land plants in which many different types of cells and tissues differentiate and each cell is exposed to a different environment, even in the same organism.
3. Some unicellular algal species exhibit relatively simple cellular and genomic architecture among eukaryotes.
4. Many unicellular algae are relatively easy to cultivate at a low cost and at a shorter generation time than required for land plants. These simple characteristics of unicellular algae potentially offer ideal experimental platforms; however, experimental techniques, particularly those for genetic modification of eukaryotic algae, are unfortunately extremely limited. However, the recent rapid development of genetic modification tools in *C. merolae* shows promise for overcoming this issue.

The methods for several “omics” analyses (Chap. 6), genetic modification (Chap. 7), fluorescence and electron microscopy (Chaps. 8, 9 and 10), and “in silico” analyses in *C. merolae* are described in other chapters. In the present chapter, we briefly summarize procedures for cultivation and synchronization of *C. merolae* in the laboratory and other conventional experimental procedures with relevant references.

3.2 Cultivation of *C. merolae*

3.2.1 Stock Culture

C. merolae 10D was originally isolated by Dr. Toda belonging to Prof. Kuroiwa’s group at the University of Tokyo from a mixture of hot spring water samples collected in Naples, Italy, that was prepared by Prof. Pinto (Toda et al. 1995). Currently, this strain is available from the Microbial Culture Collection at the National Institute of Environmental Studies in Japan (NIES-3377; <http://mcc.nies.go.jp>).

Many laboratories culture *C. merolae* in 2× Allen’s medium (Allen 1959) or its derivatives (e.g., MA medium) (Minoda et al. 2004). Although the optimal temperature for this strain is 42 °C, *C. merolae* can also grow at >20 °C at a reduced rate. Thus, we generally store stock cultures at room temperature or at ~30 °C in

Erlenmeyer flasks or plastic culture flasks which are gently agitated with illumination ($\sim 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Fig. 3.1). Under these conditions, the cell density reaches $\sim 5 \times 10^8 \text{ cells mL}^{-1}$, and the culture can be kept for 2 months until the next subculture.



Fig. 3.1 Stock and asynchronous cultures of *Cyanidioschyzon merolae*. (a) Stock cultures of the wild type and transformants in 50 mL plastic culture flasks. Cultures are stocked at room temperature with illumination ($\sim 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) on a rotator ($\sim 50 \text{ rpm}$). (b) Stock cultures of the wild type and transformants in 500 mL Erlenmeyer flasks. Cultures are stocked in a growth chamber at 30°C with illumination ($\sim 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) on a rotator ($\sim 50 \text{ rpm}$). (c) Asynchronous batch culture of *C. merolae* in an incubator at 40°C . Cultures in 100 mL test tubes (70 mL medium) are aerated with an air pump. The air from the pump is passed through distilled water before being passed into the culture to prevent desiccation of the culture. The test tubes are illuminated by fluorescent lamps ($100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). (d) Asynchronous batch culture of *C. merolae* in a 24-well plate. The plate is agitated using a shaker ($\sim 50 \text{ rpm}$) in a growth chamber at 40°C with illumination ($100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$)

Alternatively, *C. merolae* 10D or its transformants can be stored as frozen stock in liquid nitrogen (Ohnuma et al. 2011); however, long-term storage (e.g., ~1 year) using this method has not yet been tested. In addition, storage in liquid nitrogen is relatively costly. Thus, cultivation under a lower temperature at a slow growth rate is much easier for the storage of many strains.

Cultivation of *C. merolae* is also feasible on a starch bed on a gellan gum plate containing 2× Allen's medium or its derivative. The cultivation of cells on gellan gum plates has been applied only to isolate colonies of transformants and is described elsewhere (Imamura et al. 2010) (Chap. 7).

Cell density in the culture is determined using a Neubauer-improved cell counting chamber or a Coulter counter (Z2, Beckman Coulter) equipped with a 50 µm aperture (Miyagishima et al. 2014). When determining the cell density with a cell counting chamber, the chamber is left for 5 min after the injection of the culture to allow the cells to adhere to the surface of the bottom glass before counting.

3.2.2 Asynchronous Batch Culture

The optimal conditions for *C. merolae* growth are as follows: temperature of 42 °C, pH 1–5, and light illumination of ~100 µmol photons m⁻² s⁻¹ (depending on the thickness of the culture bottle). We generally use 2× Allen's medium at pH 2.5; however, a similar growth is achieved in the medium at pH 4.6 (Nishida et al. 2005). Because the medium is acidic, the solubility of CO₂ is relatively low; therefore, aeration is important to yield higher cellular growth. We usually culture the cells in 100 mL test tubes (~3 cm thick; containing ~70 mL culture) or 700 mL flat bottles (~5 cm thick; containing ~500 mL culture), which are aerated with 1 L min⁻¹ or 3 L min⁻¹ ambient air by pumps (Fig. 3.1). These conditions are usually sufficient to achieve good algal growth (doubling time is ~24 h) (Miyagishima et al. 2014); however, the addition of ~5% CO₂ accelerates cellular growth under certain conditions. The tubes or bottles can be incubated in an air incubator as well as in a transparent water bath that is illuminated from the outside.

When many cultures of small volume are required, for example, to expose cells to a series of concentrations of inhibitors, we usually culture the cells in 24-well plates that are agitated with a shaker under illumination (Fig. 3.1). Although cellular growth under these conditions is somewhat slower than that under the methods described above, the 24-well plate culture is sufficient to test the effects of inhibitors or other chemicals on cellular growth.

3.2.3 Synchronous Culture

Cell cycle progression of *C. merolae* cells can be synchronized by a 12-h light/12-h dark cycle (Suzuki et al. 1994; Fujiwara et al. 2013; Miyagishima et al. 2014). Cells

remain in the G1 phase during the light period and synchronously enter into the S and then M phases during the dark period (Suzuki et al. 1994; Fujiwara et al. 2013; Miyagishima et al. 2014). The details of the cell cycle of *C. merolae* are described elsewhere (Fujiwara et al. 2013) (Chap. 11), and the cell cycle progression is regulated by circadian rhythms (Miyagishima et al. 2014) (Chap. 12). Briefly, during light period, cells grow by photosynthesis, but G1/S transition is inhibited. During the dark period, cell growth ceases and only cells that have grown beyond a certain size threshold during the light period divide during the dark period (Miyagishima et al. 2014). Thus, to increase the mitotic index during the dark period, it is important to achieve good cell growth during the light period. The simplest method to accelerate cellular growth is to increase the rate of aeration.

The cells in a stationary culture ($\sim 5 \times 10^8$ cells mL⁻¹ at 30 °C) are subcultured to $< 1 \times 10^7$ cells mL⁻¹ (OD₇₅₀ = 0.15–0.20) in 500 mL of 2× Allen's medium at pH 2.5 (in a 700 mL flat bottle) and then cultured in the dark for 12 h under aeration with ambient air (3 L min⁻¹) at 42 °C. The culture is then subjected to a 12-h light/12-h dark cycle (100 μmol photons m⁻² s⁻¹) (Fig. 3.2). The cells divide synchronously from the second dark cycle (Fig. 3.3). The temperature should be maintained at 40 °C–50 °C (we usually culture cells at 40 °C–42 °C). The timing of G1/S transition is temperature compensated by circadian rhythms; however, the durations of the S and M phases largely depend on the temperature (Miyagishima et al. 1999b, 2014). Therefore, at a lower temperature (<40 °C), the cell cycle does not complete within the 12 h dark period and no synchrony is evident (Miyagishima et al. 2014).

3.2.4 Procedures to Arrest Synchronized Cells in the S or M Phase

Inducible transgene expression systems are now available in *C. merolae* (Sumiya et al. 2014; Fujiwara et al. 2015) (Chap. 7). Thus, the arrest of synchronized cells at a desired point of the cell cycle by expressing a dominant negative form of a certain cell cycle regulator is likely feasible. However, to date, no example of such experiments has been published.

An alternative method to arrest cells in the S or M phases is the treatment of cells with cell cycle inhibitors. We have arrested cells in the S phase in the past by the addition of ampicillin (Terui et al. 1995; Itoh et al. 1996), 5-fluorodeoxyuridine (Miyagishima et al. 1999a; Nishida et al. 2005), or camptothecin (Nishida et al. 2005, 2007; Miyagishima et al. 2012) to the culture at 10–12 h after the onset of the light period in the synchronous culture (Fig. 3.3). To arrest cells in the M phase, MG132, a proteasome inhibitor, has been added to the synchronous culture at 4 h after the onset of the dark period (Nishida et al. 2005; Miyagishima et al. 2012) (Fig. 3.3). Because chloroplast division occurs in the S phase in eukaryotic algae (Miyagishima et al. 2012), the chloroplast in the S-phase-arrested cell continues to divide, producing abnormal cells that contain four to eight chloroplasts (Itoh et al.

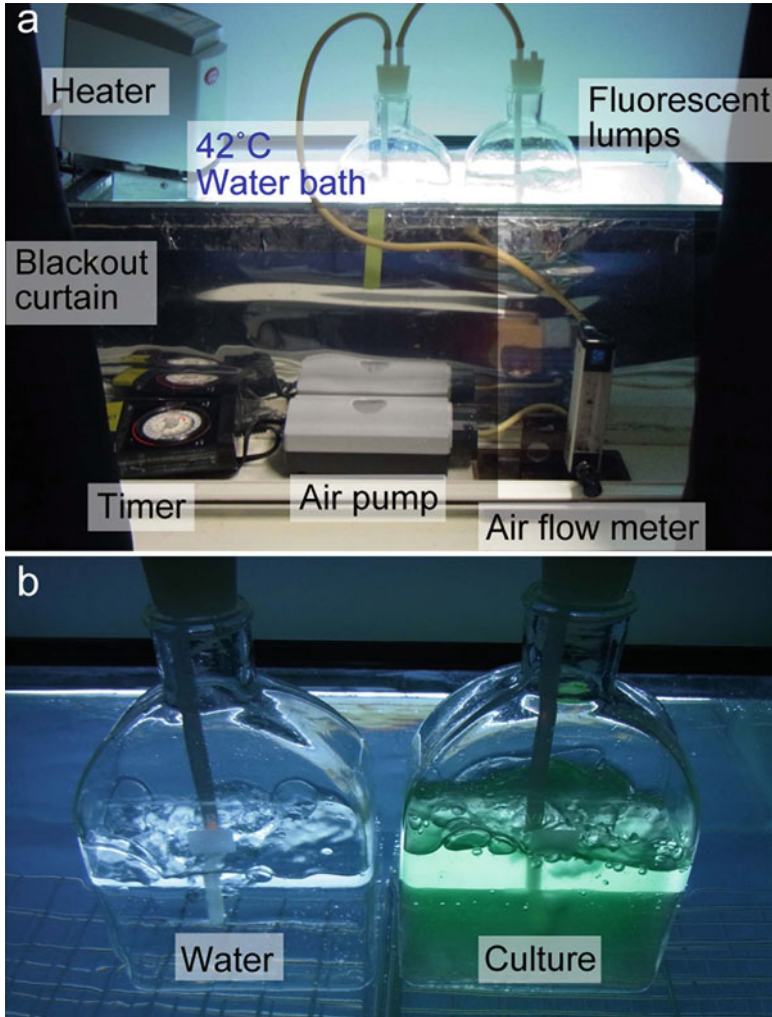


Fig. 3.2 Synchronous culture of *Cyanidioschyzon merolae* in a water bath. A stock culture is diluted to $\sim 1.0 \times 10^7$ cells mL^{-1} ($\text{OD}_{750} = 0.15\text{--}0.2$) in 500 mL fresh $2\times$ Allen's medium in a 700 mL flat bottle. (a) The culture is aerated with ambient air (3 L air min^{-1}) in a water bath at 42°C . The air from the pump is passed through distilled water before being passed into the culture to prevent desiccation of the culture. The culture is incubated in dark for $>12 \text{ h}$ and then subjected to a 12-h light/12-h dark cycle. The bottles are illuminated ($100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) by fluorescent lamps located behind the water bath. The fluorescent lamps are regulated by a timer. (b) A magnified view of the bottles containing distilled water and the culture inside the water bath

1996; Miyagishima et al. 2012) (Fig. 3.3). In contrast, when cells are arrested in the M phase, the chloroplast has completed one round of division but never divides again, resulting in cells containing two chloroplasts (Miyagishima et al. 2012) (Fig. 3.3). Oryzalin, an inhibitor of microtubule polymerization, has also been

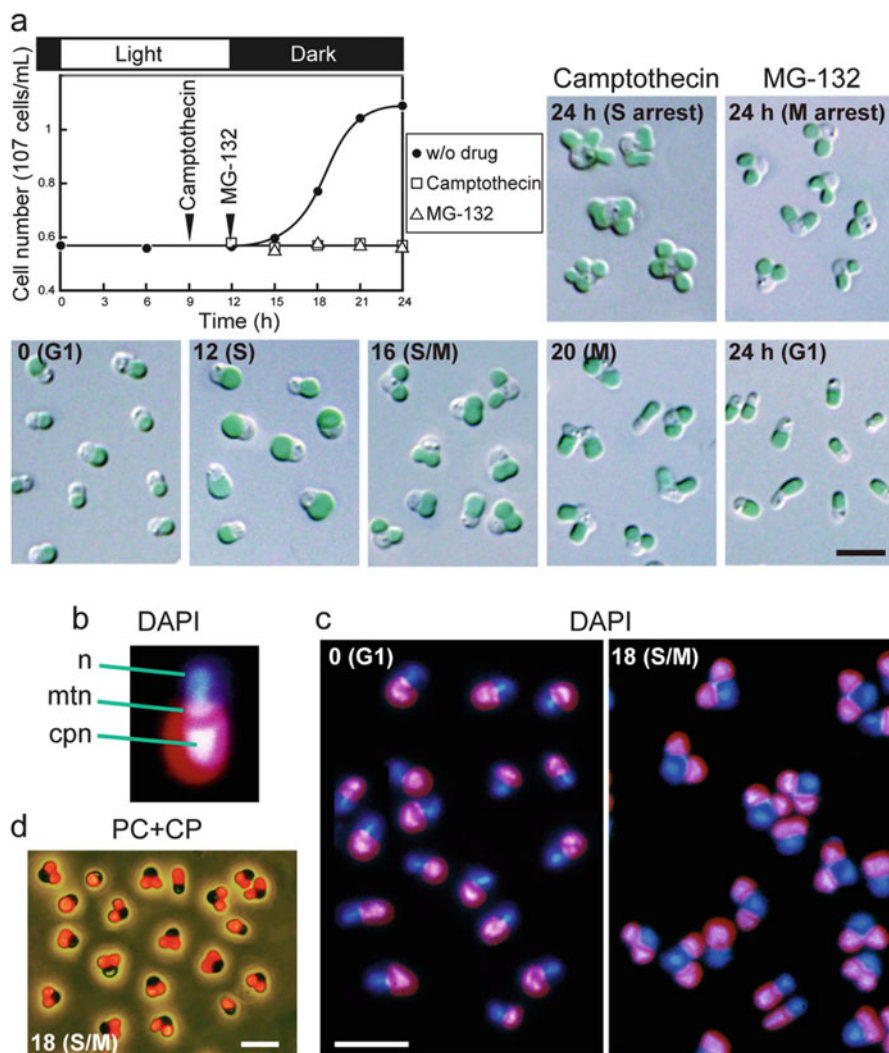


Fig. 3.3 Observation of cell cycle progression in synchronous culture by microscopy. (a) Cells were entrained by a 12-h light and 12-h dark cycle; the cells in the second cycle are shown. To arrest the cells in the S phase, 1/50,000 volume of 10 mg mL⁻¹ camptothecin (an inhibitor of DNA topoisomerase-I) solution in dimethyl sulfoxide (DMSO) was added 8 h after the onset of the second light period. To arrest the cells in the M phase, 1/500 volume of 50 mM MG132 (an inhibitor of proteasomes) solution in DMSO was added at the onset of the second dark period. Cells were observed without fixation by differential interference contrast microscopy with a 60× objective lens. (b) A DAPI-stained fluorescent image of a G1 cell. n, nucleus; mtn, mitochondrial nucleoid; cpn, chloroplast nucleoid. Blue represents DAPI fluorescence of DNA and red represents autofluorescence of chloroplast. (c) DAPI-stained fluorescent images of G1 (at the onset of the light period) and S/M phase (6 h after the onset of the dark period) cells. Cells were fixed by the addition of glutaraldehyde (final concentration of 1%) to the culture. After fixation for 10 min, cells were stained with 1 μg/mL DAPI by gently squashing the fixed cells with the slide and cover glasses. (d) An image of phase-contrast and chloroplast autofluorescence of the S/M phase (6 h after the onset of the dark period) cells. Cells were observed without fixation with a 100× objective lens. Scale bars = 5 μm

applied to arrest synchronized cells in the M phase (Nishida et al. 2005, 2007). However, the effect does not last for a long time, and a halt of pump aeration is required when oryzalin is added to the culture (Fujiwara et al. 2013). Thus, MG132 is more suitable than oryzalin to arrest cells in the M phase.

3.3 Microscopic Observation

Here we briefly describe how we observe *C. merolae* cells by ordinary optical microscopy. To examine shapes of the cell and the chloroplast, we generally use phase-contrast (Miyagishima et al. 2003) or differential interference contrast microscopy (Miyagishima et al. 2012) using 40–100 \times objective lenses (Fig. 3.3). To clearly visualize the chloroplast, red fluorescence by green or UV excitation is useful (Terui et al. 1995; Miyagishima et al. 1999b, 2001) (Fig. 3.3). Cells are either not fixed or fixed with ~1% glutaraldehyde (added to the culture directly); however, without fixation, the cells will be crushed by the slides and cover glasses within a short period. Centrifugation at 800 $\times g$ is conducted when the cells have to be concentrated before observation, following which the cell pellet is resuspended with a small volume of the medium. We often add 0.01–0.001% Tween-20 to the culture before centrifugation, which prevents cells from adhering to the walls of centrifugation tubes.

3.4 Molecular, Genetic, and Biochemical Analyses

C. merolae cell lacks a rigid cell wall in contrast to its relatives, *Cyanidium* spp. and *Galdieria* spp. Therefore, there is no need to break the cells vigorously (with glass beads, ultrasonic treatment, etc.) to extract the cellular contents such as proteins, DNA, and RNA. In most cases, procedures applied for mammalian cultured cell lines are also applicable to *C. merolae*. In addition, there are no specific requirements for PCR or reverse transcription PCR because *C. merolae* nuclear genome has a moderate GC content (55%) (Matsuzaki et al. 2004; Nozaki et al. 2007). Because the *C. merolae* genome contains few introns (0.5% of the total nucleus-encoded genes) (Matsuzaki et al. 2004; Nozaki et al. 2007), genomic DNA is used to clone cDNA instead of reverse transcribing the RNA.

To extract proteins, DNA, or RNA, 0.01–0.001% of Tween-20 is often added to the culture before centrifugation to prevent cells from adhering to the walls of

centrifugation tubes as above. We usually isolate DNA by ordinary phenol-chloroform extraction. For RNA extraction, TRIzol (Thermo Fisher Scientific) is often used. For ordinary PCR amplification of the genomic DNA, we usually add a small aliquot of the culture directly into the reaction mix instead of using purified DNA.

For SDS-PAGE and subsequent immunoblotting of the cellular whole proteins, the cell pellet is directly resuspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 6% 2-mercaptoethanol, 2% SDS, and 10% glycerol). The protein content can be determined with XL-Bradford (APRO Science) even in the presence of SDS and 2-mercaptoethanol or dithiothreitol. When the sample becomes too viscous to handle because of high concentration of DNA, samples are treated by sonication or heat (e.g., 96 °C for 10 min), which reduces the viscosity. To detect the proteins using commercially available antibodies, we have expressed proteins tagged with sfGFP (Sumiya et al. 2014), 3× FLAG (Imamura et al. 2013), or 3× HA (Ohnuma et al. 2008; Fujiwara et al. 2013; Sumiya et al. 2016) in *C. merolae* by transformation. Anti-GFP (JL-8, mouse monoclonal, Clontech), anti-FLAG (M2, mouse monoclonal, Sigma-Aldrich), and anti-HA (HA7, mouse monoclonal, Sigma-Aldrich) have yielded specific detection of the tagged proteins in *C. merolae* total proteins by immunoblotting. The anti-HA antibody has also been used for the detection of proteins by immunofluorescence microscopy (Fujiwara et al. 2013; Sumiya et al. 2016).

To extract native proteins without denaturation, the cell pellet is rapidly frozen in liquid nitrogen and resuspended in adequate buffer. This freezing and thawing step is sufficient to extract soluble proteins. Alternatively, a fresh cell pellet is resuspended in a buffer, following which sonication is applied to break the cells. We usually supplement the buffer with Complete Mini protease inhibitor mixture (Roche) to avoid protein degradation (Imamura et al. 2009; Miyagishima et al. 2014). When required, phosphatase inhibitor mixture (25 mM NaF, 2.5 mM Na₃VO₄, 5 mM Na₄P₂O₇, and 5 mM beta-glycerophosphate) is also added to the buffer (Miyagishima et al. 2014). After extraction of proteins with or without adequate detergents, the cellular lysate is ultracentrifuged to remove insoluble materials. The supernatant fraction is subjected to further analyses. Alternatively, a specific organelle can be isolated before protein extraction using methods for organelle isolation described elsewhere (Miyagishima et al. 1999a; Yagisawa et al. 2009) (Chap. 4).

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