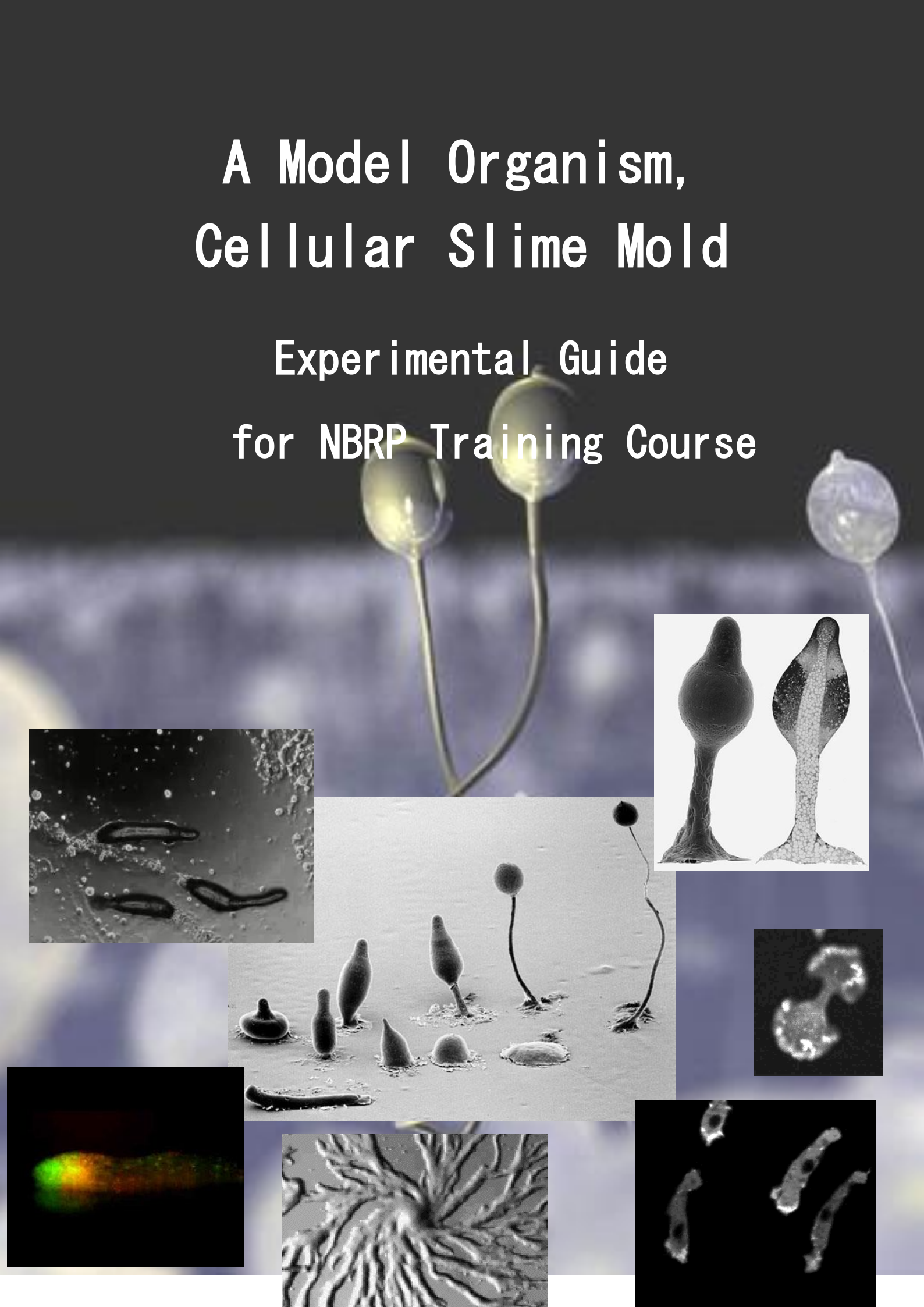
A Model Organism, Cellular Slime Mold

Experimental Guide for NBRP Training Course



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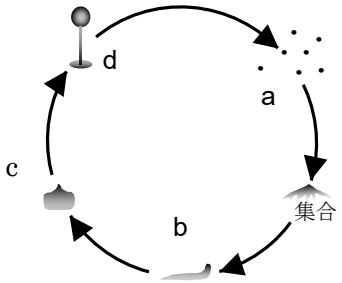
Ⅰ. Introduction

Cellular slime molds are amoeboid eukaryotic microorganisms in the soil that normally divide and multiply while feeding on bacteria. Upon starvation, they undergo a simple developmental process and within 24 hours complete multicellular bodies (zygotes) with only two different cell differentiation patterns (right figure).



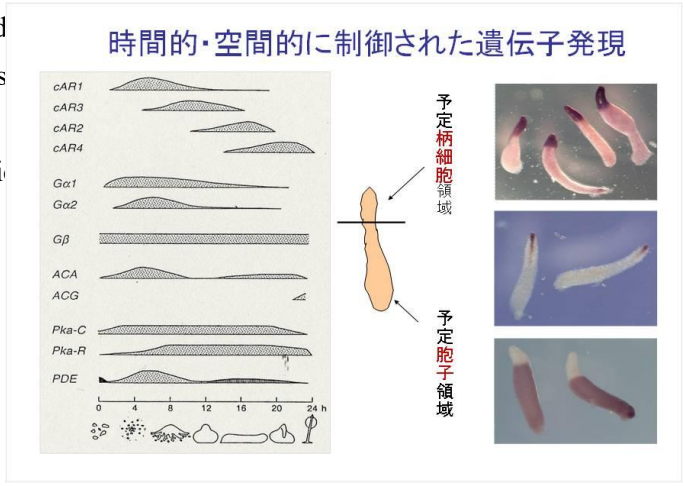
細胞性粘菌の生活史

a: 増殖期（単細胞期）

b-d: 形態形成期（多細胞期）

Multicellularity in cellular slime molds is due to chemotactic movement in response to cAMP secreted by the cells of the assembly center. Subsequently, slug-shaped migratory bodies

The slug-shaped migratory body is then formed. At this stage, the cells that will become spores and the cells that will become their supporting scapes have already been determined (lower right figure).



The following is a list of experimental characteristi of cellular slime molds

-Simple cell division in culture medium
-Bacterial predation and digestion
-Directed amoebic movements such as chemotaxis
-Easy induction of morphogenesis
-Haploid through its life cycle.
-Easy transformation
-Various expression vectors
-Completion of genome sequencing
-Enrichment of gene, strain, and publication databases
-High homologous recombination efficiency
-Ease of storage
-A lot of cell lines etc.

This manual is intended to help newcomers to the field of cellular slime molds to handle *Dictyostelium discoideum* as an experimental standard strain for their research. The contents of the book focus on cellular slime mold culture and the isolation of transformants using molecular biological techniques and include a description of methods for isolating cellular slime molds from soil.

It is my hope that this book will help to utilize the model organism cellular slime molds in many research settings and lead to useful biological discoveries.

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Ⅱ. Culture and preservation methods for cellular slime molds

Wild strains of cellular slime molds are basically grown on agar or in liquid medium, using bacteria as a food source. However, if a sterile culture strain is used, it is possible to culture only pure slime mold cells in a bacteria-free medium.

Methods for culturing cellular slime molds

(1) Co-member culture method with bacteria (observation of developmental process)

(Preparation) Standard strain of *Dictyostelium discoideum* (strain AX2)

Bacteria (food): *Escherichia coli* B/r or Klebsiella aerogenes

5LP agar (lactose 5.0 g, Bacto Peptone (BD REF No.; 211677) 5.0 g, Bacto Agar 15.0 g / 1L Distilled H_20; autoclaved) x 20 mL per 9 cm diameter petri dish,
Incubator (21°C)

1. Incubate the bacteria (food) (*Escherichia coli* B/r or *Klebsiella aerogenes*) in 5LP liquid medium (5LP agar medium without Agar) at 37°C overnight, then drop onto 0.5 mL 5LP agar medium. Spread evenly on the agar and air-dry on a clean bench until the moisture is absorbed.
2. Inoculate a standard strain of *Dictyostelium discoideum* (ex. strain AX2) in the center.
3. Incubate in an incubator maintained at 21 ºC for 4 to 5 days.
4. bacteria feed and form a plaque at the center. Near the center, starvation is in progress and fruiting bodies are observed. Slug bodies, aggregates, and proliferating amoebae are observed as one approaches the periphery.

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(2) Cell preparation by two-member culture method with bacteria on agar medium (Under Water Culture method)

(Preparation) Standard strain of *Dictyostelium discoideum* (AX2 strain)

Bacteria (food): *Escherichia coli B/r* or *Klebsiella aerogenes*

N agar medium (glucose 10.0 g, Bacto Peptone (BD REF No.; 211677) 10.0 g, Na_2HPO_4-$12H_20$ 0.96 g, KH_2PO_4 1.44 g, Bacto Agar 15 g / 1 L Distilled H_20; (Autoclave sterilization)

Sterile phosphate buffer (Na_2HPO_4-$12H_20$ 1.07 g, KH_2PO_4 0.96 g / 1 L; autoclaved)

Sterile distilled water, sterile test tubes, centrifuge tubes

Incubator (21°C)

1. Scrape an appropriate amount of bacteria (*Escherichia coli B/r* or *Klebsiella aerogenes*) cultured on N agar medium and suspend in 3 mL of sterile water in a sterile test tube.
2. Scrape an appropriate amount of *Dictyostelium discoideum* standard strain (strain AX2) amoeba or spores and suspend in sterile water with the bacteria.
3. Transfer the suspension onto N agar.
4. Incubate in an incubator at 21 °C for 4 to 5 days.
5. Transfer the cell suspension to a centrifuge tube by gently scraping the surface with an alcohol-sterilized glass rod (spreader).

   Centrifuge at 300-350 g (1,500 - 2,000 rpm) for 2 min.

   Discard the supernatant and resuspend the cells in cold sterile phosphate buffer.

8. Repeat steps 6 and 7 two more times (total of three cell washes).
9. Suspend the cellular slime mold cells in appropriate buffer and use for experiments (keep on ice).

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(3) Cell conditioning by two-member culture method with bacteria (liquid shaking culture method)

(Preparation) Standard strain of *Dictyostelium discoideum* (AX2 strain)

Bacteria (food): *Escherichia coli B/r* or *Klebsiella aerogenes*

5LP liquid medium (lactose 5.0 g, Bacto Peptone (BD REF No.; 211677) 5.0 g / 1L Distilled H_20; autoclaved)

Sterile phosphate buffer (Na_2HPO_4-$12H_20$ 1.07 g, KH_2PO_4 0.96 g / 1 L; autoclaved)

Sterile distilled water, sterile triangular flasks, centrifuge tubes

Incubator (21°C), shaker

1. Scrape an appropriate amount of bacteria (food) (*Escherichia coli* B/r or Klebsiella aerogenes) cultured on N agar medium and suspend in 5LP liquid medium transferred into a triangular flask (the volume of 5LP liquid medium is (The volume of 5LP liquid medium is about 20-30% of the volume of the flask).
2. 2.(The volume of 5LP liquid medium is about 20~30% of the volume of the flask. 2) Scrape an appropriate amount of *Dictyostelium discoideum* standard strain (strain AX2) amoeba or spores and suspend them in 5LP medium.
3. 3. Shake the medium (125 rpm) in an incubator maintained at 21 °C for 4 to 5 days.
4. 4. Collect cells in the early logarithmic growth phase (cell density 1.0-3.0 × 10^6 cells/mL) in a centrifuge tube.
5. Centrifuge at 300-350 g (1,500-2,000 rpm) for 2 min.
6. Discard the supernatant and resuspend the cells in cold sterile phosphate buffer.
7. Repeat steps 5 and 6 two more times (total of three cell washes).
8. Suspend the cellular slime mold cells in appropriate buffer and use for experiments (keep on ice).

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(4) Cell preparation by aseptic culture (bacteria-free method)

(Preparation) Standard strain of Dictyostelium discoideum (AX2 strain)

*Since wild strains of cellular slime mold are not necessarily aseptically culturable, confirm in advance whether the strain to be used can be cultured aseptically.

(Strains derived from strains marked as "AX" can basically be cultured aseptically.)

HL5 medium (Glucose 14.3 g, Bacto Proteose Peptone (BD REF No.; 211684) 14.3 g, Bacto Yeast Extract (BD REF No.; 212750) 7.15 g, Na_2HPO_4 -12$H_2$0 1.28 g, KH2PO4 0.485 g / 1 L; autoclaved)

Antibiotic solution (1000x; streptomycin sulfate 100 mg, Benzylpenicillin potassium 70 mg / mL) filter sterilized and added to autoclaved HL5 medium. Commercially available solutions are also acceptable.

Folic acid (folic acid) and vitamin B12 (cyanocobalamin) solution (10,000 x; 2.0 mg folic acid, 6 mg vitamin B12 / mL) Neutralize with NaOH solution, check that the solution is dissolved, scalp, filter sterilize, and add to autoclaved HL5 medium. Then, filter sterilize and add to autoclaved HL5 medium.

Sterile phosphate buffer (Na2HPO4-12H20 1.07 g, KH2PO4 0.96 g / 1 L; autoclaved)

Sterile petri dish (e.g., 9 cm diameter) or sterile triangular flask, centrifuge tube

Incubator (21°C), shaker

1. Fill a sterile petri dish with 20 mL of HL5 medium (+ antibiotics, + folic acid, + vitamin solution; if HL5 is indicated below, it has already been added).
2. Scrape and suspend cellular slime mold spores (or amoeba cells) on agar (or in other liquid medium) with a platinum ear.
3. Incubate in an incubator at 21 °C for 4 to 5 days.
4. For liquid shaking culture, scrape off the cells with a Pasteur pipette, transfer into a sterilized triangular flask, and incubate at 21 ºC with shaking at 125 rpm. In this case, the volume of liquid medium should be 20% of the volume of the flask. 5.
5. Collect cells in the early growth phase (cell density of 1.0 - 3.0 × 10^6 cells / mL) in a centrifuge tube.
6. Centrifuge at 300-350 g (1,500-2,000 rpm) for 2 min.
7. Discard the supernatant and resuspend the cells in cold sterile phosphate buffer.
8. Repeat steps 6 and 7 two more times (total of three cell washes).

9. Suspend the cellular slime mold cells in appropriate buffer and use for experiments (keep on ice).

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Methods of preserving cellular slime molds

(1) Spore preservation method using silica gel

(Preparation) Cellular slime mold that has formed fruiting bodies

Silica gel (white small particles; sterilized by dry heat at 180°C for about 2 hours)

Heat-sensitive sterilized glass vials (lids dried after autoclaving)

Alternatively, sterile microtubes are acceptable (must be sterile and sealed)

0.5% dry milk solution (autoclaved and refrigerated)

1. Refrigerate the newly prepared cellular slime mold stock plates (plates on which the fruiting bodies have formed).
2. Collect the spore masses of the fruiting bodies with a sterilized platinum ear and take a small amount (about 0.5 mL) of the dry milk solution. Repeat this procedure to collect as many spores as possible. (Spores can also be directly suspended in silica gel without dry milk solution for storage.)
3. Chill the dry milk solution containing spores with ice and drop the solution into a pre-chilled silica gel container (approximately 0.2 mL / 1 g silica gel).
4. Seal the container (with lid) and shake well to make the contents uniform, then place this container in another container with desiccant and keep refrigerated.

(Method of returning the product to its original state)

To restore the spore to amoeboid cells

(Preparation) Spores stored in silica gel

Bacteria (food): *Escherichia coli B/r* or *Klebsiella aerogenes*

5LP agar medium (lactose 5.0 g, Bacto Peptone (BD REF No.; 211677) 5.0 g, Bacto Agar 15.0 g / 1L Distilled H20) *20 mL per 9 cm diameter petri dish

Incubator (21°C)

1. 0.5 mL 5LP agar medium (*Escherichia coli* B/r or *Klebsiella aerogenes*) incubated overnight at 37°C in 5LP liquid medium (5LP agar medium without Agar) Spread evenly on the agar and air-dry on a clean bench until the moisture is removed.
2. Add silica gel stock containing the spores (or add silica gel stock to the bacterial solution) and spread the spores evenly on the agar. (Or, add a few grains of silica gel to the bacterial solution, stir well, and spread on 5LP agar medium.
3. Keep at 21°C for 3 to 7 days.
4. After observing the fruiting bodies, transfer to a new 5LP bacterial medium or sterile medium (e.g., HL5).

(e.g., HL5).

(2) Preservation of amoeba cells using DMSO

Some mutant strains that do not produce spores cannot be preserved using the silica gel method. Such strains should be cryopreserved as amoeboid cells in an ultra-low temperature freezer (-80 °C or lower) or in liquid nitrogen.

(Preparation) Cellular slime mold amoebae (suspended in HL5 medium or phosphate)

Culture grade DMSO (e.g., Hybrimax; sigma, HPLC grade can also be used), Sterile distilled water, Sterile cryovials (sterile phosphate buffer Na_2HPO_4-$12H_20$ 1.07 g, KH_2PO_4 0.96 g / 1 L; autoclaved), -80 °C freezer, (liquid nitrogen)

1. make a 20 % DMSO solution with sterile water and store refrigerated (or frozen) (no filter sterilization). Cool on ice until immediately before use.
2. Prepare cell suspension. Cell suspensions can be either sterile cultures of cells in HL5 or bicellular cultures of cells suspended in sterile phosphate buffer after centrifugation to remove bacteria. The cell density should be 1.0 x 10^8 cells/mL.
3. Transfer 0.5 mL of the 20 % DMSO solution cooled on ice to a cryovials.
4. Add 0.5 mL of the suspended cells and pipet gently on ice.
5. Place in a cell-freezing container (e.g., BICELL) and place in a -80 C freezer for at least several hours. (*The goal is to slowly cool the cells to a supercooled state.) If no container is available, insert the tubes into a box with cotton folded in between, and place the box in a -80 C freezer for several hours.
6. Transfer the tubes to a cryobox or similar container and store at -80 °C or in liquid nitrogen.

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（Defrozen of cells）

(Preparation) Frozen cell stock stored in cryo tubes

HL5 medium or 5LP liquid medium

Bacteria (food) (*Escherichia coli* B/r or *Klebsiella aerogenes*)

5LP agar (5.0 g lactose, 5.0 g Bacto Peptone (BD REF No.; 211677), 15.0 g Bacto Agar / 1L Distilled H_20; autoclaved) *20 mL per 9 cm diameter petri dish

Incubator (21°C)

1. Remove the tube from the freezer and allow it to stand at room temperature.
2. When the entire tube is dissolved, slowly add an appropriate amount of HL5 medium or 5LP medium and transfer to a 15 mL centrifuge tube. Transfer to a 15 mL centrifuge tube.
3. Add HL5 medium or 5LP medium until the volume reaches 15 mL. Centrifuge at 300-350 g (1,500-2,00 rpm) for 2 min.
4. Discard the supernatant, add an appropriate volume of HL5, transfer to a sterile petri dish, and add an appropriate volume of HL5 medium. 5LP When adding 5LP medium for two-member cultures, add 1 to 2 mL of the 5LP-enriched bacterial suspension to the precipitated cells and transfer 0.5 mL of the suspension to 5LP agar medium. Seed the cells onto 5LP agar medium.
5. Incubate at 21°C for 3 to 7 days.

When returning to HL5 liquid medium, transfer the lysed cell solution directly to 15 mL of liquid medium and allow DMSO to dilute to the point that it does not inhibit cell growth. DMSO will be diluted to a level that does not inhibit cell proliferation, so it is safe to do so.

In addition, it is possible to return cells from one tube to the freezer many times by placing the tube in a chilled container that will never melt frozen stock, scraping off a little of the surface of the frozen stock with a sterilized thin spatula on a clean bench, placing the tube in fresh nutrient medium in a petri dish, and returning it to the freezer before the tube melts. Cells can be returned from the tube as many times as desired.

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Ⅲ. Observation of cellular slime molds

Cellular slime molds grow as amoeboid cells, remove nutrients, and under appropriate moisture conditions, such as on agar plates, multicellular body formation occurs, forming a fruiting body consisting of a spore and a stalk.

Observation of the developmental process of cellular slime molds

(1) Observation of development process by two-member culture with bacteria

(Preparation) Standard strain of *Dictyostelium discoideum* (strain AX2)

Bacteria (food): *Escherichia coli* B/r or *Klebsiella aerogenes*

5LP agar (lactose 5.0 g, Bacto Peptone (BD REF No.; 211677) 5.0 g, Bacto Agar 15.0 g / 1L Distilled H20) *20 mL per 9 cm diameter petri dish

Incubator (21°C)

1. 0.5 mL 5LP agar medium (Escherichia coli B/r or Klebsiella aerogenes) incubated overnight at 37°C in 5LP liquid medium (5LP agar medium without Agar) Spread evenly on the agar and air-dry on a clean bench until the moisture is absorbed.
2. Inoculate a standard strain of *Dictyostelium discoideum* (strain AX2) in the center.
3. Incubate in an incubator maintained at 21 ºC for 4 to 5 days.
4. bacteria feed and form a plaque at the center. Near the center, starvation is in progress and fruiting bodies are observed. Slug bodies, aggregates, and proliferating amoebae are observed as one approaches the periphery.

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(2) Longitudinal observation of cellular slime mold development process (on agar)

(Preparation) Standard strain of *Dictyostelium discoideum* (AX2 strain)
Bacteria (food): *Escherichia coli* B/r or *Klebsiella aerogenes*
5LP liquid medium (lactose 5.0 g, Bacto Peptone (BD REF No.; 211677) 5.0 g, Bacto / 1L Distilled H20)
Sterile phosphate buffer (Na_2HPO_4-$12H_20$ 1.07 g, KH_2PO_4 0.96 g / 1 L; autoclaved)
Sterile BSS (NaCl 0.6 g, KCl 0.75 g, $CaCl_2$-$2H_20$ 0.4 g / 1 L; autoclaved)
Incubator (21°C)
Non-nutrient agar (Bacto Agar 15.0 g / 1 L Distilled H_20)

1. pure culture in HL5 at early logarithmic growth phase (1.0 - 3.0 × 10^6 cells / mL) or 5LP 1. Collect cellular slime molds in centrifuge tubes after two-member culture in HL5 at early logarithmic growth stage (1.0 - 3.0 × 10^6 cells/mL).
2. 2.Centrifuge at 300 - 350 g (1,500 - 2,000 rpm) for 2 min.
3. Discard the supernatant and resuspend in cold sterile phosphate buffer.
4. Repeat steps 2 and 3 two more times (total of three cell washes).
5. Resuspend the cells in cold phosphate buffer or cold BSS to 1.0 x 107 cells / mL (approximate volume).
6. Pipette 5 µL of the suspension onto non-nutrient agar. At this time, be careful not to puncture the agar.
7. Air-dry the agar until it becomes dry, and incubate it in an incubator maintained at 21 °C.
8. Observe the agar over time under a stereomicroscope.

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(3) Cellular slime mold development process over time (in phosphate buffer solution)

Cellular slime molds do not form fruiting bodies in water but can aggregate to aggregation. Cellular level observations of cell aggregation can be made on a cover glass and under submerged conditions.

(Preparation) Standard strain of *Dictyostelium discoideum* (AX2 strain)

Bacteria (food): *Escherichia coli* B/r or *Klebsiella aerogenes*

5LP agar medium (lactose 5.0 g, Bacto Peptone (BD REF No.; 211677) 5.0 g, Bacto Agar 15.0 g / 1L Distilled H_20) *20 mL per 9 cm diameter petri dish

Sterile phosphate buffer

Incubator (21°C)

Non-nutrient agar (Bacto Agar 15.0 g / 1L Distilled H_20)

1. in pure culture or 5LP in HL5 in the early log phase (1.0 - 3.0 × 10^6 cells/mL) 1. Collect cellular slime molds in a centrifuge tube after two-member culture in HL5 or 5LP liquid medium (5LP agar medium without Agar) in the early logarithmic growth phase (1.0 to 3.0 × 10^6 cells/mL).
2. Centrifuge at 300 - 350 g (1,500 - 2,000 rpm) for 2 min.
3. Discard the supernatant and resuspend in cold sterile phosphate buffer.
4. Repeat steps 2 and 3 two more times (total of three cell washes). 5.
5. Resuspend the cells in cold phosphate buffer to 1.0 x 10^7 cells / mL.
6. Transfer the cells to a glass bottom dish, etc. to reach 1.0 x 10^6 cells/cm^2 (approximate volume).
7. Incubate the cells in an incubator at 21 °C.
8. Observe the cells under an inverted microscope.

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Ⅳ. Transformation methods for cellular slime molds

The research standard strain, *Dictyostelium discoideum* (AX strain), has an established transformation method. Various vectors necessary for transformation are also in place.

Transformation of cellular slime mold AX strain (electroporation method)

(Preparation) Standard strain of *Dictyostelium discoideum* (strain AX2)

HL5 medium (Glucose 14.3 g, Bacto Proteose Peptone (BD REF No.; 211684) 14.3 g, Bacto Yeast Extract (BD REF No.; 212750) 7.15 g, Na_2HPO_4 -12H20 1.28 g, KH_2PO_4 0.485 g / 1 L)

Antibiotic solution (1000x; streptomycin sulfate 100 mg, benzylpenicillin potassium 70 mg / mL) filter sterilized and added to autoclaved HL5 medium.

Folic acid (folic acid) and vitamin B12 (cyanocobalamin) solution (10,000 x; 2.0 mg folic acid, 6 mg vitamin B12 / mL) Neutralize with NaOH solution, check that the solution is dissolved, scalp, filter sterilize, and add to autoclaved HL5 medium. Then, filter sterilize and add to autoclaved HL5 medium.

Electroporation buffer (Na_2HPO_4-$12H_20$ 0.32 g, NaH_2PO_4 1.40 g, Sucrose 17.12 g / 1 L; filter sterilized)

DNA for transformation

2 mm wide electroporation cuvette

Sterile healing solution (100 mM $CaCl_2$, 100 mM $MgCl_2$; autoclaved)

Sterile petri dish (e.g., 9 cm in diameter)

Centrifuge tube

Incubator (21°C)

1. Prepare electroporation buffer. Sterilize electroporation buffer by filter sterilization. 2.
2. Collect pure cultures of cellular slime molds in HL5 at early logarithmic growth stage (1.0 - 3.0 × 10^6 cells/mL) in a centrifuge tube.
3. Centrifuge at 300 - 350 g (1,500 - 2,000 rpm) for 2 min.
4. Discard the supernatant, add cold electroporation solution to 5.0 × 10^7 cells/mL, suspend gently, and place on ice.
5. Transfer 400 µL to a cuvette, add 10 to 20 µg of DNA, and suspend gently.
6. Place the cuvette on ice for 30 minutes.

7. Apply electrical stimulation (e.g., BTX EC). (e.g., BTX ECM830, 500 V, 100 μsec, ×10: Biorad Gene Pulser, 0.85 kV, 25 μF, ×2: The lethality of electrical stimulation should not be less than 25%. (Usually about 50 %.) 8.
8. Allow to stand on ice for 5 minutes.
9. During this time, drop 4 μL of the healing solution onto a sterile petri dish.
10. Transfer the electrically stimulated cell suspension to a sterile petri dish and lightly suspend it in the healing solution.
11. Incubate at 21°C for 10 to 15 minutes.
12. Slowly add 15 mL of HL5 medium and incubate at 21 °C. If you wish to separate the cells by colonies, do so. If you wish to separate the cells by colony, dispense into a 96-well sterile plate at this point. In this case, add 40 mL of HL5 medium and dispense 100 μL / well.
13. After 12 to 24 hours, add 15 μL of 1,000-fold concentration of the selected drug (G418; 20 mg / mL, Blasticidin S 10 mg / mL: filter-sterilized); if the cells have been aliquoted into 96 wells, the Final concentration (G 418; 20 μg / mL, Blasticidin S 10 μg / mL).
14. After 4-5 days, replace the medium with the selective agent.
    If transformation is successful, colonies will be observed after 10-14 days for G418 and 7-10 days for Blasticidin S.

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Transformation of cellular slime mold AX strain (calcium phosphate method)

(Preparation) Standard strain of *Dictyostelium discoideum* (strain AX2)

HL5 medium (Glucose 14.3 g, Bacto Proteose Peptone (BD REF No.; 211684) 14.3 g, Bacto Yeast Extract (BD REF No.; 212750) 7.15 g, Na_2HPO_4 -12$H_2$0 1.28 g, KH_2PO_4 0.485 g / 1 L)

Antibiotic solution (1000x; streptomycin sulfate 100 mg, benzylpenicillin potassium 70 mg / mL) filter sterilized and added to autoclaved HL5 medium.

Folic acid (folic acid) and vitamin B12 (cyanocobalamin) solution (10,000 x; 2.0 mg folic acid, 6 mg vitamin B12 / mL) Neutralize with NaOH solution, check that the solution is dissolved, scalp, filter sterilize, and add to autoclaved HL5 medium. Then, filter sterilize the solution and add it to autoclaved HL5 medium.

Bis-Tris HL5 medium (2.1 g Bis-Tris in HL5, pH 7.1 with HCl, filter sterilized)

1.25 M $CaCl_2$ solution (filter sterilized)

18 % glycerol solution in 1 x HBS

2 x HBS solution (4.0 g NaCl, 0.18 g KCl, 0.05 g Na_2HPO_4, 2.5 g HEPES, 0.5 g glucose / 1 L distilled water, pH to 7.1 with NaOH: filter sterile.)

DNA for transformation

Sterile petri dish (e.g., 9 cm in diameter)

Centrifuge tube

Incubator (21°C)

1. Sow 10 mL of HL5 pure culture of cellular slime molds in early logarithmic growth phase (1.0 - 2.0 × 10^6 cells / mL) in a sterile petri dish.
2. Incubate at 21°C for 30 minutes.
3. Carefully remove the HL5, leaving the cells, and gently add 12.5 mL of Bis-Tris HL5 and incubate at 21°C for 30 minutes.
4. Prepare 540 µL of 1x HBS solution containing 10-20 µg DNA.
5. Add 60 µL of 1.25 M CaCl2 to make 600 µL.
6. Gently remove Bis-Tris HL5 from the petri dish and slowly add a drop of DNA solution from the center of the petri dish.
7. Cover the petri dish and incubate at 21°C for 30 minutes.
8. Gently add 12.5 mL of Bis-Tris HL5 and incubate at 21°C for 4 hours.
9. Gently remove Bis-Tris HL5 from the petri dish and slowly add 4 mL of 18% glycerol solution in 1x HBS.
10. Incubate at 21°C for exactly 5 minutes.

11. Aspirate off the glycerol solution in 1x HBS and add 15 mL of HL5 medium.
12. After 12 to 24 hours, add 15 µL of 1,000-fold concentration of the selected drug (G418; 20 mg/mL, Blasticidin S 10 mg/mL: filter sterilized). 418; 20 µg / mL, Blasticidin S 10 µg / mL).
13. After 4-5 days, replace the medium with the selective agent.

If transformation is successful, colonies will be observed after 10-14 days for G418 and 7-10 days for Blasticidin S.

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Ⅴ. Gene amplification by PCR method

It is possible to amplify cellular slime mold genes by PCR using the general genome as a template, but the cellular slime mold genome is AT-rich and requires slight modification of reaction conditions.

(Preparation) *Dictyostelium discoideum* standard strain (AX strain) genomic DNA

Two oligo DNA

Heat-resistant polymerase (e.g. TOYOBO, KOD plus)

1.5 mL microtubes, microtubes dedicated for PCR

1. prepare DNA as template for PCR (genomic DNA, plasmid DNA, etc.) (genomic DNA, plasmid DNA, etc.) 2.

2. Use a high-fidelity thermostable enzyme (e.g., TOYOBO, KOD plus). (e.g., TOYOBO KOD series, Takara Prime Star, Finnzyme Phusion, etc.)

10 × KOD plus ver2 buffer	3.0 μL
dNTP mix (2.0 mM)	3.0 μL
$MgSO_4$ (25 mM)	3.0 μL
Primers （10μM）	
5'-end	1.5 μL
3'-end	1.5 μL
Template(plasmid or genomeDNA)	0.1 ng (plasmid) 10 ng(genome)
KOD plus	0.3 μL
up to	30.0 μL with dH_20

Thermal cycler conditions; 94°C, (94°C - 2 min, 50-60°C - 20 sec, 65°C - full length kbp x 1 min) x 25, 65°C - full length kbp x 1 min, Hold

Electrophoresis of 3.5 μL of the reaction solution is performed to confirm that fragments of the desired length have been amplified. If necessary, purify and extract the genomic fragments.

If amplification is not successful, first perform a control experiment to confirm that the reagents have not deteriorated. In the case of amplification using genomic DNA as the template, adding 5% DMSO (final concentration) to the reaction solution may stabilize and amplify the reaction.

Ⅵ. Preparation of genomic DNA

(Preparation) Standard strain of Dictyostelium discoideum (AX strain)
Centrifuge tube
Centrifuge, tabletop cooling centrifuge
Incubator (60°C and 37°C)
Sterile phosphate buffer (Na_2HPO_4-$12H_20$ 1.07 g, KH_2PO_4 0.96 g / 1 L)
STE buffer (10 mM Tris-HCl, 10 mM EDTA, 400 mM NaCl, pH 8.0)
Proteinase K (10 mg / mL)
10% SDS solution
Sterile distilled water
1.5 mL micro tube
TE-saturated phenol
Chloroform
99.5 % ethanol
70.0 % ethanol
Ribonuclease A solution (10 mg / mL)

1. collect 2.0 - 5.0 × 10^7 cells of acellular slime mold cells in a centrifuge tube.
2. Centrifuge at 300 - 350 g (1,500 - 2,000 rpm) for 2 min.
3. Discard the supernatant and add 450 µL of STE buffer to suspend the cells in 1.5 mL microtubes.
4. Add 50 µL of 10 % SDS solution, cover with a lid, and invert up and down 5-6 times.
5. Add 10 µL of Proteinase K solution and invert in the same manner.
6. Incubate at 60 °C for 1-2 hours, followed by incubation at 37 °C for 2 hours to overnight.
7. Add 500 µL of TE-saturated phenol/chloroform (1:1) solution, cover with a lid, invert up and down 5-6 times, and centrifuge at 15 krpm for 10 minutes.
8. Carefully transfer 400 µL of the supernatant to a new micro tube without removing the turbid white intermediate layer.
9. Repeat steps 7 and 8 up to a total of three times (if necessary).
10. Add 1.0 mL of cold 99.5% ethanol and cool at -20 °C for 30 minutes.
11. Centrifuge at 15 krpm for 10 minutes. 12.
12. Add 1.0 mL of 70% ethanol to the precipitate and rinse the precipitate.
13. Centrifuge gently and discard the supernatant.
14. Repeat the procedure of 12.13.
15. Air dry for 10-15 minutes, add 100 µL of sterile distilled water, and suspend by tapping.

16. Add 1 µL of Ribonuclease A solution and incubate at 37°C for 1 to 2 hours.
17. Store the genomic DNA in a cool place because it will be fragmented by freezing and thawing.

<Memo>

Ⅶ. Methods for isolating cellular slime molds from soil

Isolate cellular slime molds from the soil in moist areas under shady trees or fallen leaves.

(Preparation) Agar medium for separation (Glucose 1.0 g, Bacto Peptone (BD REF No.; 211677) 1.0 g, K_2HPO_4, 1.0 g, KH_2PO_4, 1.5 g, $MgSO_4$ 1.0 g, Agar 15.0 g / 1.0 L Distilled H2O) 20 mL per petri dish (same as below)

N agar medium (10.0 g glucose, 10.0 g Bacto Peptone (BD REF No.; 211677), 0.96 g Na_2HPO_4-$12H_20$, 1.44 g KH_2PO_4, 15 g Bacto Agar / 1 L Distilled H20)

5LP agar medium (lactose 5.0 g, Bacto Peptone (BD REF No.; 211677) 5.0 g, Bacto Agar / 1 L Distilled H_20)

Bacteria (food) (Escherichia coli B/r or Klebsiella aerogenes)

Sterile distilled water

Sterile gauze

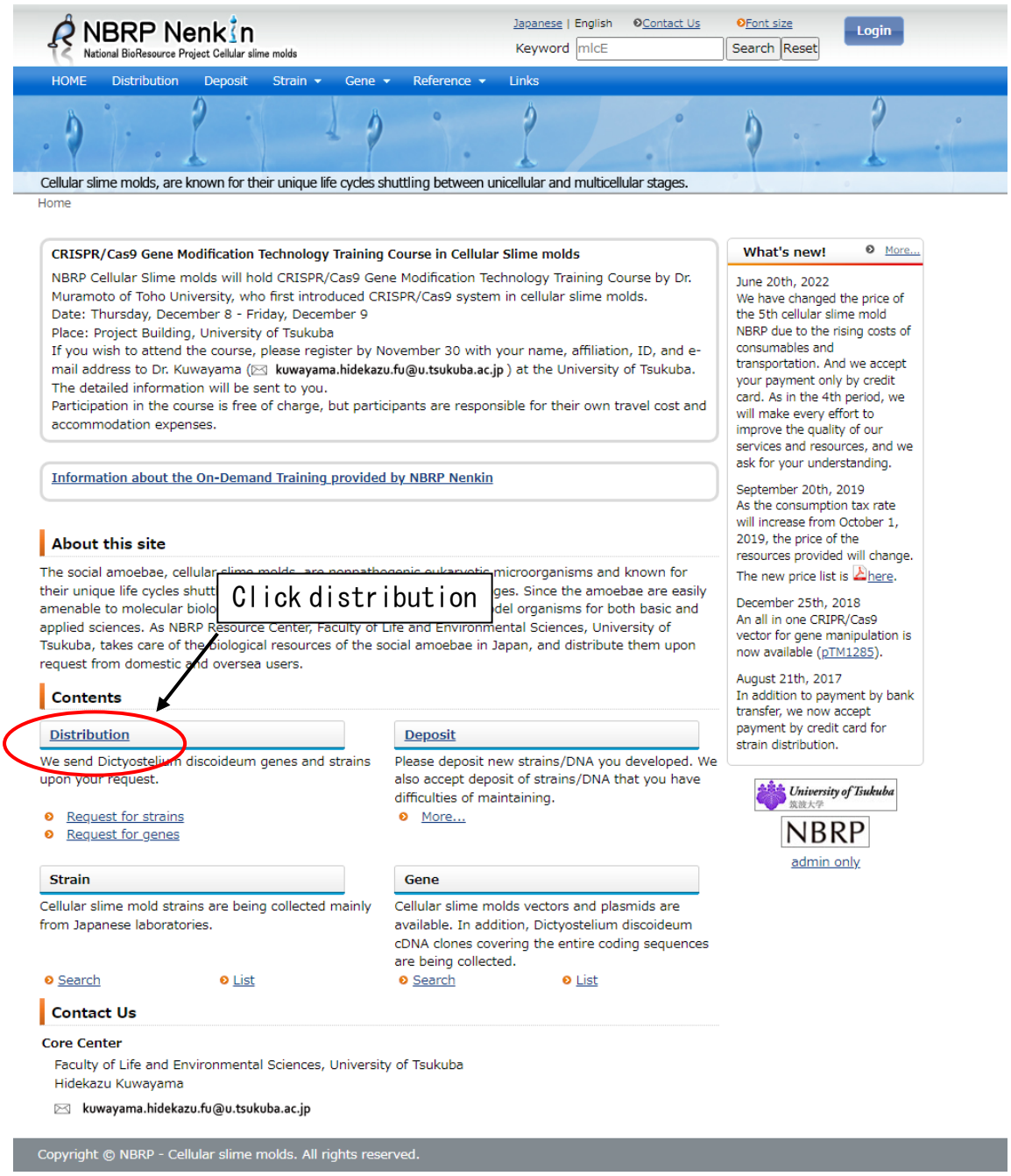
1. Collect the surface layer of soil.
2. Add 90 mL of sterile distilled water to 10 g of soil and stir well.
3. Strain through two layers of sterile gauze and add 7.5 mL of sterile distilled water to 5 mL of filtrate.
4. Add 0.4 mL of bacteria-suspended sterile distilled water (scrape bacteria off the agar plate with a platinum ear to suspend them) to 0.5 mL of the diluted filtrate from 3. Add 0.4 mL of sterile distilled water (scrape the bacteria from the agar plate using a platinum ear, etc.) and spread evenly on the isolation medium.
5. Incubate at 21 °C.
6. After 3 to 7 days, inoculate the fruiting bodies emerging on the medium onto 5LP agar coated with the bacterial suspension and incubate at 21 °C.

<Memo>

Ⅷ. How to provide cellular slime mold strains from NBRP

Cellular slime mold strains are collected and stored at the National BioResource Project (NBRP) Cellular Slime Molds and are available for a fee (although they can be provided free of charge for educational purposes at secondary schools, etc.).

How to apply for provision of cellular slime mold strains
https://nenkin.nbrp.jp/



Information required for application
E-mail address
Credit card (for payment)
Delivery method (by FEDEX only)
<Memo>

Ⅸ. Reference sites on cellular slime molds

１. http://dictybase.org/

International site for cellular slime molds. A community site focusing on Dictyostelium discoideum, a research standard strain of cellular slime molds. Genome and gene information search, cellular slime mold laboratory, and information on the International Society for Cellular Slime mold Research can also be obtained from this site.

2. http://dicty.jp/

The homepage of the Japanese Society for Cellular Mycology, where you can obtain information on the Japanese Society for Cellular Mycology, which was established in 2011, as well as information on its annual meetings. Membership is accepted at any time. There is also a link to the homepage of Japanese cellular slime mold researchers.

3. http://www.biol.tsukuba.ac.jp/hidekuwayama/index.html

This is the homepage of the Cellular Mycobacteria Laboratory at the University of Tsukuba. It introduces research contents and recruits graduate students.

Ⅹ. Reference books, bibliography

1. cellular slime molds: new developments in research

~Model Organisms, Drug Discovery Resources, Bio~.

Editors Tomoaki Abe and Yasuo Maeda, IPC (2012)

Revised edition of Model Organisms: Cellular slime molds published in 2000. Includes a chapter on experimental treatment of cellular slime molds.

2. Survival of Cellular Slime Fungi: Skilled Response to Environmental Stress (New Life Science Library: Rediscovering Organisms), H. Urushihara, Science Inc.

An introductory book on cellular slime molds that explains the ecology and research on cellular slime molds in an easy-to-understand manner for beginners.

3. Powerful Slime Fungi, by Yasuo Maeda, Tohoku University Press

An easy-to-understand explanation of the development of cellular slime molds based on his own research.

4. *Dictyostelium*.

Richard H. Kessin.

Cambridge University Press, 2001

A review of cellular slime mold research written in English.

5. Differentiation in social amoebae.

John T Bonner.

Sci. Am. 201, 152-162, 1959

A review of cellular slime molds for the general public written in old English.

6. Transformation of *Dictyostelium discoideum* with plasmid DNA.

Gaudet P, Pilcher KE, Fey P, Chisholm RL.

Nat Protocols. 2(6):1317-1324, 2007.

7. A new set of small, extrachromosomal expression vectors for *Dictyostelium discoideum*.

Veltman DM, Akar G, Bosgraaf L, Van Haastert PJ.

Plasmid. 61(2):110-118, 2009.

A paper on various gene expression vectors of cellular slime molds. Vectors are available at NBRP Cellular Slime molds.

7. A versatile set of tagged expression vectors to monitor protein localization and function in *Dictyostelium*.

   Dubin M, Nellen W.

   Gene. 465(1-2):1-8, 2010.

   Article on various gene expression vectors, especially fluorescent protein fusion expression vectors, in cellular slime molds. The vectors are available at NBRP Cellular Slime molds.

8. A user's guide to restriction enzyme-mediated integration in *Dictyostelium*.

   Guerin NA, Larochelle DA.

   J. Muscle Res. Cell Motil. 2002;23(7-8):597-604.

   Review of methods for generation, isolation and genetic analysis of mutant strains by gene insertion. The necessary vectors are available at NBRP Cellular Motil. 2002;23(7-8):597-604.

10. PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors.

    Hidekazu Kuwayama, Shinji Obara, Takahiro Morio, Mariko Katoh, Hideko Urushihara and Yoshimasa Tanaka.

    Nucleic Acids Research. 30 (2):e2, 2002

    A paper on the production of gene disruption vectors by the PCR method.

Ⅺ. Medium and others composition table

HL5 medium （sterlized by autoclave）	Proteose Peptone (BD REF No.; 211684)	14.3 g
	Yeast Extract BD REF No.; 212750	7.15 g
	Glucose	14.3 g
	KH_2PO_4	0.485g
	$Na_2HPO_4 \cdot 12H_20$	1.28g
	pure water	to 1 liter

5LP medium （sterlized by autoclave） (for 5LP agar medium. + 15.0 g Bacto agar)	Lactose	5.0 g
	Bacto Peptone (BD REF No.; 211677)	5.0 g
	pure water	to 1 liter

N medium （sterlized by autoclave） (for N agar medium. + 15.0 g Bacto agar)	Glucose	10.0 g
	Bacto Peptone (BD REFNo.; 211677)	10.0 g
	KH_2PO_4	1.44 g
	$Na_2HPO_4 \cdot 12H_20$	0.96 g
	pure water	to 1 liter

Separation medium （sterlized by autoclave）	Glucose	1.0 g
	Bacto Peptone (BD REFNo.; 211677)	1.0 g
	KH_2PO_4	1.0 g
	Na_2HPO_4	1.5 g
	MgSO4	1.0 g
	Bacto agar	15.0 g
	pure water	to 1 liter

phosphate buffer （sterlized by autoclave）	$Na_2HPO_4 \cdot 12H_20$	1.07 g
	KH_2PO_4	0.96 g
	蒸留水	to 1 liter

BSS （sterlized by autoclave） (for N agar medium.+ 15.0 g Bacto agar)	NaCl	0.60 g
	KCl	0.75 g
	$CaCl_2 \cdot 2H_2O$	0.40 g
	pure water	to 1 liter

Electroporation Buffer (sterilized through filter)	$NaH_2PO_4 \cdot 12H_20$	0.32 g
	NaH_2PO_4	1.40 g
	Sucrose	17.12 g
	pure water	to 1 liter

Healing solution （sterlized by autoclave）	$CaCl_2 \cdot 2H_2O$	1.51 g
	$MgCl_2$	0.952 g
	pure water	to100 mL

= caution =

Please refrain from eating, drinking, and smoking in the recombination laboratory.

<memo>

Name