Video Article

Mating and Tetrad Separation of *Chlamydomonas reinhardtii* for Genetic Analysis

Xingshan Jiang, David Stern
Boyce Thompson Institute for Plant Research, Cornell University

Correspondence to: David Stern at ds28@cornell.edu

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Abstract

The unicellular green alga *Chlamydomonas reinhardtii* (*Chlamydomonas*) has become a popular organism for research in diverse areas of cell biology and genetics because of its simple life cycle, ease of growth and manipulation for genetic analysis, genomic resources, and transformability of the nucleus and both organelles. Mating strains is a common practice when genetic approaches are used in *Chlamydomonass*, to create vegetative diploids for analysis of dominance, or following tetrad dissection to ascertain nuclear vs. organellar inheritance, to test allelism, to analyze epistasy, or to generate populations for the purpose of map-based cloning. Additionally, genetic crosses are routinely used to combine organellar genotypes with particular nuclear genotypes. Here we demonstrate standard methods for gametogenesis, mating, zygote germination and tetrad separation. This protocol consists of an easy-to-follow series of steps that will make genetic approaches amenable to scientists who are less familiar with *Chlamydomonas*. Key parameters and trouble spots are explained. Finally, resources for further information and alternative methods are provided.

Protocol

Part 1: Preparation of strains for gametogenesis

Note: All manipulations should be carried out at room temperature. Higher or lower temperatures will affect the speed of zygote germination and may adversely affect viability.
Important consideration: It is essential that the strains to be mated are growing robustly and are free of contaminating fungi or bacteria. If the strains have not been transferred regularly, passage on rich medium should precede generation of gametes.

1. Strains to be mated are transferred onto TAP plates (100×15 mm), 1-2 strains per plate. The cells should be grown in a concentrated strip about 1 cm in width.
2. Incubation on TAP plates should continue until a thick layer of robustly-growing cells is present, with intermediate transfers if necessary. Strains that have not been passaged recently may require one passage and a total of 1 week, whereas wild-type strains in regular use may only take a few days and no passages. Mutants that are light-sensitive may require additional time.
3. Transfer all the cells from the TAP plates to N10 plates, which are deficient for nitrogen and will begin to induce gametogenesis. Transfer the cells as a thick slab, much as they were present on the TAP plate. This will facilitate subsequent collection of the cells. After 3 days on N10, the cells are prepared to undergo gametogenesis. Cell division will be slow on N10, however cells should remain dark green. Any contaminated strains should be discarded as viable zygotes will not be obtained.

Part 2: Mating

1. Each strain is transferred into a 50 ml erlenmeyer flask and resuspended in approximately 2.5 ml sterile distilled water. This can be done either using a wire loop or a sterile stick; what is important is that the cells are well suspended and not in clumps. The exact volume of water should be adjusted so that the cell concentration, as gauged by eye, is about the same for each strain.
2. Cells are agitated under strong light (**1.260 µE/m^2/sec**) for 2 hours, during which time the cells will become motile gametes. Motility should be checked under a light microscope. If the gametes are swimming poorly, mating is likely to be inefficient. However, even poorly motile strains will mate a reduced frequency.
3. Next, equal volumes of mt+ and mt- gametes are combined into a single 50 ml flask. Gametes are left under strong light (**1.260 µE/m^2/sec**) without agitation.
4. Mating can be checked immediately under the light microscope. Pairs of gametes form rapidly, and will appear as rapidly moving cells joined at the flagella. If mating appears to be extremely rapid, samples should be taken within 30 min -1 hr for generation of zygotes. If not, mating can be checked each hour, although this is optional. Since times giving the optimal density of zygotes will vary widely between crosses, it is best to take aliquots of the mating mixture at different times after combining gametes, e.g. after 1 hr, 2 hrs, 3 hrs and 4 hrs. This is done by transferring 300 µL samples to TAP-3% Difco agar 60×15 mm plates; 3-4 spots per plate. Be careful not to agitate the mating mixture as this may disrupt mating pairs. Difco agar is used because it has fewer contaminants than lower-priced agars, and it will be easier to see zygotes under the microscope.
   The unused mating mixture can be left in the flask overnight, and the quality of the mating reaction will be obvious the next day. When mating is efficient, the zygotes will adhere to the wall or bottom of the flask and the medium will appear clear. Even agitation of the flask will fail to dislodge the zygotes. If nothing sticks to the glass when the mixture is agitated with reasonable vigor, then mating was inefficient or did not take place.
5. The TAP-Difco plates containing mating mixture are dried in a sterile hood, with the lids ajar, until any surface liquid has been absorbed. The plates are typically left in the light **(700 nE/m^2/sec)** for 18 hours, then wrapped in foil, since zygospore maturation will only take place in the dark. The strains crossed, mating time and date are written on both the plates and the foil. Plates are stored 5 to 7 days and can be kept longer, although zygote germination efficiency declines progressively.

Part 3: Tetrad Dissection

1. Vegetative cells should be scraped off dried spots of mating mixture using a dull blade, such as a scalpel. The blade can be used to expose an area of zygotes, which will stick tightly to the 3% agar. This step is usually done in the afternoon, so that the zygotes have germinated by the next morning, but have not undergone a second, vegetative division which would yield 8 progeny. *Chlamydomonas* biologists quickly learn to recognize zygotes under the microscope, since they are larger than vegetative cells, and feature a thick zygospore wall which gives a black outline. Zygospores often appear yellow but may also be somewhat green. The most important character is that they stick to agar, while vegetative cells are readily moved aside. However, if the plates are overly dry this will not be the case. Also, if the mating mixture was too dense, zygotes may form a mat which will make transfer of individual zygotes difficult or impossible. Appropriate densities are established in step 2.1, and will be quickly learned with experience.
2. Glass needles are prepared for gathering zygotes and separating tetrad progeny. These are prepared by pulling 3 mm glass rods in a small flame to generate a long, thin thread, which can be broken off. The tips of these tapered ends are smoothed or bent into hooks, by brief heating in a low flame (e.g. alcohol or a burning match). Prepare 3 or more glass needles, since they break fairly easily, especially for inexperienced users.
3. Zygotes are gathered on the 3% TAP-Difco plate using a glass needle into a small pile. A square of agar (up to 0.5 cm^2) containing the zygotes is formed and excised using a dull scalpel. Then, the agar block is placed face down on a 15 mm 1.5% TAP-Difco plate, and slid along a horizontal line about 1.5 cm from the top of the plate, to distribute the zygotes. The top horizontal line, and a grid with approximately 1.5 cm horizontal and 0.5 cm vertical spacing are etched into the back of the plates. The grid will be used the following day as a guide for separating the tetrad progeny (the grid can also be etched the day of dissection). Finally, individual zygotes are move to the intersections of the top horizontal lines with each vertical line, with about 20-30 zygotes per plate. Although only 12 or so will be dissected, not every zygote germinates, and some will divide twice before bursting, so extra zygotes are needed.
4. After distributing the zygotes, and vegetative cells that have been accidentally transferred from the 3% agar plate are killed by exposing the inverted plate for 30 seconds over a glass dish containing a thin (1-2 mm) layer of chloroform. The distance between the plate and chloroform should be about 5 cm. If too much chloroform is used, or if the distance is too small or great, one risks either killing the zygotes, or not killing the vegetative cells. The chloroform treatment must be done immediately after the zygospores have been distributed on the germination plate, to ensure selectivity.
5. Germination takes 16-20 hours for most strains, but will vary with genotype and with the age of the zygotes. For germination, the plates are placed under low light **(239 nE/m^2/sec)**, or in medium light dimmed by covering the plate with a tissue.
6. Germinated zygotes can be identified under the microscope as swollen or sometimes burst cells with daughter cells inside them. If the zygospore has not ruptured, gently touching with a glass needle will break the wall if germination has occurred. Daughter cells are gently dragged to the grid points below the zygote. It is easiest to do this by capturing the cell in a small amount of liquid medium released from the agar by the pressure of the glass needle, and dragging this “puddle” behind the needle. One form of “insurance” that one has dissected a zygote and not a vegetative cell that has twice divided, is that apart from the four daughter cells, the “skin” of the zygospore wall will remain.
7. The dissected progeny are grown under appropriate conditions until visible clonies can be picked for subsequent analysis. Plates should be monitored daily for contamination.

Note: Generation of vegetative diploids is not covered here, but essentially involves plating the mating mixture on medium which will not support the growth of either parental strain, but will support the growth of the diploid (e.g. two different auxotropic markers). Plates are kept in the light to avoid the formation of zygotes.

Discussion

Genetic analysis is simple in *Chlamydomonas*, but it requires particular techniques to generate gametes and separate tetrad progeny. With healthy strains, the techniques are readily acquired. With some mutant strains, it becomes more of an art, and readers should refer Volume 1 of the recently-published *Chlamydomonas Sourcebook* for historical notes and practical hints. Additionally, it should be noted that there are many variations on the basic theme described above, ranging from the method for inducing gametogenesis, to the pattern of zygote distribution and dissection. Ultimately, each researcher finds their comfort zone, or adapts to laboratory-specific protocols.

Certain technical details mentioned above, should be reiterated. In particular, the cells must be healthy and without any infection. It is well known that laboratory-adapted strains which have not been crossed for long periods may not mate well, and water quality may also affect gametogenesis.

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