

Deflagellation and Regeneration in *Chlamydomonas*

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[Abstract] Eukaryotic cilia/flagella are one of the only cellular structures that can be removed without injuring cells, can be highly purified for biochemical analysis, and, in many cells, can be completely reassembled within 90 min. Following amputation, the expression of many flagellar genes is up-regulated, and many are packaged and associated with intraflagellar transport (IFT) particles for transport to flagellar bases and into growing flagella. Studies of deciliation and ciliary growth provide insight to mechanisms that regulate microtubule assembly and length, mechanisms that regulate the transport of soluble cytoplasmic proteins into the ciliary compartment and their assembly into microtubules, and mechanisms that regulate trafficking of membrane proteins and lipids to the plasma membrane or to ciliary bases and their movement into and out of the cilium. These are important for motility and for signal transduction.

Deciliation methods for many cells have been developed and most require extracellular calcium ions and activation of signaling pathways that regulate microtubule severing (Quarmby, 2009). Deciliation occurs at the distal end of the basal bodies and, as soon as axonemes are severed, the membrane reseals and basal bodies begin to regenerate cilia.

Chlamydomonas is an ideal organism with which to study ciliary regeneration. Cells are easily and inexpensively cultured, flagellar amputation and regeneration is uniform in all cells in a population and growth can be assayed by observing fixed or living cells with a phase contrast microscope equipped and a 40x objective lens. Flagellar regeneration on individual living cells can be observed using paralyzed mutants immobilized in agarose. Because deflagellation leaves cells intact, the released flagella can be purified without contamination with cellular debris. The most reliable deciliation and regeneration method is the pH shock method developed by Reference 5 (also see References 4 and 11). Other methods are reviewed by Quarmby, (2009). The pH shock method is primarily used for *Chlamydomonas* but can be used for deciliation and regeneration of *Tetrahymena* cilia (Gaertig *et al.*, 2013).

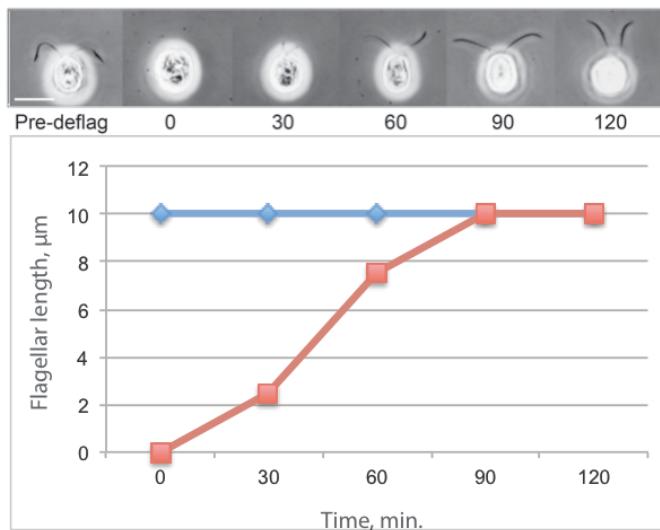


Figure 1. Typical flagellar regeneration curve showing phase contrast images of *Chlamydomonas* cells photographed before deflagellation and during regeneration. The average flagellar lengths of a population of regenerating cells is shown in red and the average flagellar lengths on a population of nondeflagellated cells is in blue.

Materials and Reagents

1. Cells

Chlamydomonas cells can be obtained from a variety of sources and pure strains of a variety of flagellar mutants can be obtained from the Chlamydomonas Resource Center (<http://chlamycollection.org/contact-us/>).

2. Media and cell culture (see Notes)

3. 0.5 N acetic acid

4. 0.5 M NaOH

5. 2% glutaraldehyde in M medium or Lugols iodine (see Recipes)

Note: For electron microscopy, one should use fresh glutaraldehyde from sealed ampoules. To measure flagellar lengths, the age of the glutaraldehyde is not critical.

Optional:

6. 2.5% low EEO agar (Thermo Fisher Scientific, FisherBiotechTM, catalog number: BP160-100)

Note: It is used (to observe flagellar growth or maintenance on individual living cells).

7. 5 mM (final concentration) colchicine (to inhibit microtubule assembly)

Note: These experiments are carried out in phosphate-buffered Minimal medium. Avoid Tris-containing buffers because they may inhibit the effects of colchicine (Margulis et al., 1969).

8. 10 µg/ml (final concentration) Cycloheximide

Note: it is used to inhibit protein synthesis.

9. VALAP (1:1:1 Vaseline : Lanolin : Paraffin)

Note: It is used to support coverslips to examine living cells without inducing deflagellation by coverslip pressure.

Equipment

1. pH meter calibrated for pH 4-7

2. *Note: Some gel-filled electrodes are not accurate across this pH range.*

3. Centrifuge and tubes

Note: For small scales, a clinical centrifuge and conical tubes.

4. Phase contrast microscope or brightfield microscope

Note: If cells are fixed with Lugol's iodine, a 40x lens is ideal for flagellar length measurements.

5. Orbital shaker

6. Magnetic stirrer

7. Stir bar

Software

1. Image J (<http://imagej.nih.gov/ij/>)

Procedure

1. Check cells with a 40x lens and a phase microscope to be certain that cells are healthy, contain two flagella, and are motile (unless paralyzed mutants are studied). Dividing cells lack flagella and are surrounded by a refractile cell wall containing 4-8 cells. Cultures with dividing cells are not synchronous and are not used for regeneration experiments.
2. Harvest cells (~5 x 10⁵ cells/ml) using a clinical centrifuge, for up to 120 ml of culture, or low speed preparative centrifuge (3 min, 1,500 x g) pour off the supernatant.
3. Suspend cells in 20-100 ml of fresh medium in a beaker by gentle swirling or gently drawing cells up and down through a large-bore pipette (I break off the tips of plastic pipettes.).

4. Check cells with a phase microscope to insure that they are uniformly flagellated and have not been deflagellated during centrifugation and resuspension.
Optional: Agarose to observe flagella on immobilized living cells.
 - a. Dissolve 2.5% low EEO agar in culture medium and maintain at 45 °C for use.
 - b. Apply cells to a clean coverslip, draw off most of the liquid, and apply ~20 microliters of agarose at 40 °C.
 - c. Rapidly invert over a clean microscope slide and seal the edges with VALAP (1:1:1 Vaseline, Lanolin, Paraffin).
 - d. Flatten the bead of VALAP around the edge of the coverslip with a warm spatula.

Note: To capture time-lapse images of regenerating flagella use a microscope equipped with a UV filter. Keep the illumination low and be certain that non-deflagellated cells maintain full length throughout the observation period (see Dentler and Adams., 1992).
5. Stir cells using a magnetic stirrer and dropwise add 0.5 N acetic acid until the pH reaches 4.5-4.0. Add acetic acid as rapidly as possible but be certain that it is well mixed as it is added to the medium. Keep at pH 4 for no longer than ~1 min and then raise the pH to 6.8-7.2 by dropwise adding 0.5 N KOH. For 100 ml of cells in M medium, use approximately 4.5 ml of 0.5 N acetic acid and 5.2 ml of 0.5 M KOH. For most purposes, it is best to monitor the pH change but, for mass-deflagellations in microtitre plates, adding defined quantities of acetic acid and KOH is useful.
6. For regeneration
 - a. Pellet cells using a clinical centrifuge, and suspend in fresh M medium to ~10⁵ cells/ml. It is not critical to suspend in fresh medium but I generally do so.
 - b. Incubate cells in light with aeration using bubblers or by gentle rotation in an Erlenmeyer flask on an orbital shaker.
 - c. Flagella generally will start to grow within 10-15 min and will be fully grown by 60-90 min.
 - d. To measure flagella on regenerating cells, fix samples at 5-10 min intervals with glutaraldehyde or Lugol's iodine.
 - i. Fix: 2% glutaraldehyde in M medium. Dilute 100 microliters of cells + 100 microliters of 2% glutaraldehyde and let settle in microfuge tubes. *Measure flagella within 1-2 days. Longer fixation will lead to clumping of cells at the bottom of the tube, which makes measurement of individual flagella very difficult.*
 - ii. Alternatively, fix by diluting 80 microliters of cells + 20 microliters Lugol's iodine. Measure flagella within 1-2 days to avoid clumping of flagellated cells.
 - iii. Regeneration can be inhibited reversibly by the addition of colchicine, an inhibitor of microtubule assembly. If cycloheximide is added at the time of deflagellation,

flagellar regeneration will be limited to half-length due to the exhaustion of a pool of proteins critical for flagellar assembly (Rosenbaum *et al.*, 1969).

7. Measuring flagella: There are a variety of ways to measure flagella and one has to balance accuracy with the ease of measuring large numbers of flagella to minimize error. Even if flagella are planar when fixed, they are curved and are viewed at various angles, depending on the orientation of the cell on the microscope slide. Very short flagella may be difficult to see if the cell is not properly oriented to reveal the flagella. It also is difficult to identify the flagellar base because it is surrounded by the cell wall, which can obscure the first micron of a flagellum above the basal body. Thus, the observer must use their judgment when measuring flagella and be consistent with all measurements. One can use complex image analysis involving the Z-axis (see Reference 9) but one also could simply deflagellate cells at various time points, photograph isolated flagella attached to a coverslip, and measure their lengths with a variety of image programs.
The easiest method to measure flagella is to use an ocular micrometer and record measurements of flagella on fixed cells by hand. I prefer to use a ccd or video camera that interacts with Image J, a free software program (<http://imagej.nih.gov/ij/>). Trace the flagella using a mouse, save the measurements, and determine the average lengths and distributions of length (using histograms) using Excel or similar spreadsheets.
8. Flagella are easily purified from pH-shocked cells following methods described elsewhere (these protocols).

Notes

1. Notes about media and cell culture
 - a. Culture media recipes are available at <http://www.chlamy.org/media.html>.
 - b. I prefer to culture cells in at room temperature in Sager and Granick (1953) minimal (M) medium (<http://www.chlamy.org/SG.html>; Harris, 1989) and synchronize cells on a 12 h dark/light cycle to insure that all cells are at the same point in the cell cycle. This is important because Chlamydomonas cells disassemble their flagella prior to division and one mutant, pf18, gradually disassembles its flagella 4-6 h after the beginning of the light cycle (Tuxhorn *et al.*, 1998). Cell density is not critical but cells grown to stationary stage ($> 10^6$ cells/ml) often are not 100% flagellated.
 - c. Autoclave media in Erlenmeyer flasks fitted with a foam plug and cotton-plugged glass Pasteur pipette for aeration with house air or an aquarium pump. For deflagellation and regeneration, cultures of 50-100 ml of cells grown to $\sim 5 \times 10^5$ cells/ml is adequate.

- d. To isolate flagella, culture 8-16 L of cells in aerated 4 L bottles and concentrate cells to 100-200 ml for pH shock.

Recipes

1. Lugol's Iodine

Mix 6% potassium iodide in distilled water and add 4% iodine crystals

Mix by stirring overnight and store indefinitely

Acknowledgments

This protocol was adapted from Lefebvre (1995), Margulis *et al.* (1969) and Witman (1986).

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