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Isolation of *Chlamydomonas* Flagella

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Abstract

A simple, scalable, and fast procedure for the isolation of *Chlamydomonas* flagella is described. *Chlamydomonas* can be synchronously deflagellated by treatment with chemicals, pH shock, or mechanical shear. The Basic Protocol describes the procedure for flagellar isolation using dibucaine to induce flagellar abscission; we also describe the pH shock method as an Alternate Protocol when flagellar regeneration is desirable. Sub-fractionation of the isolated flagella into axonemes and the membrane + matrix fraction is described in a Support Protocol.

Keywords

flagella; flagellar isolation; flagellar regeneration; cilia; axoneme

INTRODUCTION

One of the greatest attributes that the biflagellated green alga *Chlamydomonas reinhardtii* (see Fig. 3.41.1) offers for the study of cilia and flagella (the terms are used equivalently; from here on we will use “flagella”) is that the cell’s flagella can be easily and rapidly isolated from the rest of the cell, resulting in a one-step enrichment of several-hundred fold for the flagellar components. The isolated flagella can then be fractionated to yield the “9 + 2” microtubular axoneme, the flagellar membrane, components of the flagellar matrix, and individual axonemal substructures such as the inner or outer dynein arms or the radial spokes. Purified *Chlamydomonas* flagella have provided much of our knowledge about the structure and composition of flagella, including the first comprehensive proteomic analysis of a eukaryotic flagellum (Pazour et al., 2005) and of its radial spokes (Yang et al., 2006). Furthermore, isolated *Chlamydomonas* flagella or axonemes have been ideal for cryo-electron tomographic studies that have revealed the detailed structure of the flagellum (Nicastro et al., 2006), including the radial spokes (Pigino et al., 2011; Barber et al., 2012) and dyneins and associated structures (Heuser et al., 2009; Bui et al., 2012) and intraflagellar transport particles (Pigino et al., 2009). In addition, biochemical and ultrastructural comparison of isolated wild-type and mutant flagella can reveal specific defects that result from the mutation (Lechtreck and Witman, 2007; Lechtreck et al., 2009; Craige et al., 2010; Brown et al., 2012).

Chlamydomonas can be experimentally induced to shed its flagella using a variety of methods, including chemical means, pH shock, and mechanical shear. We describe here the most commonly used method in our laboratory, chemical-induced flagellar shedding by treatment of live cells with dibucaine. The dibucaine method is recommended when maximally intact flagella are required for biochemical or structural studies, or for ATP-induced reactivation of the isolated axonemes (Witman et al., 1978). As an alternate protocol, we describe the pH shock method for flagellar detachment. The pH shock method is recommended when regeneration of flagella after flagellar excision is desired. Mechanical

detachment of flagella is used less commonly but provides another option when chemical or acid treatment is undesirable (Rosenbaum et al., 1969).

For information about culturing *Chlamydomonas*, a good resource is *The Chlamydomonas Sourcebook, Volume 1* (Harris, 2009). We use an environmental room maintained at 23°C with a 14-hr light/10-hr dark cycle. For flagellar isolation, we routinely culture ~4 liters (the protocol is easily adapted to smaller or larger culture volumes) of cells that are aerated with 5% CO₂ and 95% air. Cells are typically harvested in mid-log phase ($1-2 \times 10^6$ cells/ml). Up to 8 liters of cells can be conveniently harvested by centrifugation as described below; larger cultures are more conveniently harvested by tangential flow filtration. More detailed information on growing and harvesting large volumes of cells and isolating axonemes suitable for ATP-induced reactivation is provided elsewhere (Witman, 1986). Before beginning the procedure, check the culture by phase-contrast or differential interference contrast (DIC) microscopy to ensure that the cells are healthy, well-flagellated, not contaminated, etc. (see Fig. 3.41.1A). If the culture contains a large amount of cell debris due to cell lysis, as may happen in a culture that is too dense, the resulting flagella will not be pure.

BASIC PROTOCOL

In this protocol, we describe the use of dibucaine to induce flagellar abscission. Dibucaine-induced abscission results in highly pure flagella, the flagella are relatively intact compared to flagella isolated by other methods, and the flagella can be demembrated and reactivated (Witman et al., 1978). However, dibucaine treatment results in cell death; for studies that require cell viability following flagellar abscission, see the Alternate Protocol.

Materials

Cells

10 mM HEPES, pH 7.4 (at room temperature)

HMDS-EGTA (see recipe)

HMDS (see recipe)

25 mM dibucaine (see recipe)

HMDS-25% sucrose (see recipe)

Sorvall RC-3B centrifuge with an H-6000A rotor

250-ml screw-cap bottles

50-ml disposable conical tubes, sterile

10-ml plastic serological pipets

Light microscope

25-ml serological pipets

50-ml round-bottom polycarbonate tubes (Nalgene, cat. no. 3117-9550)

1. Harvest the cells by centrifuging for 5 min at 2000 rpm ($1100 \times g$), room temperature. Avoid braking the rotor as braking tends to cause a vortex that disrupts the cell pellet. Carefully decant or aspirate off the supernatant.

We use a Sorvall RC-3B centrifuge with an H-6000A rotor and 250-ml screw-cap bottles.

2. Resuspend the cells in 10 ml of 10 mM HEPES, pH 7.4, at room temperature, and transfer into a clean 50-ml conical tube (resuspended cells from three to four 250-ml bottles can be combined at this step). Bring to 45 ml with 10 mM HEPES.
3. Centrifuge for 5 min at 2000 rpm ($1100 \times g$), room temperature. Discard the supernatant, resuspend the cells in 10 mM HEPES at room temperature to wash them. Cells can be further concentrated to a maximum of about 4 ml packed cells per tube. Bring the volume up to 45 ml with 10 mM HEPES.
4. For each tube of cells, prepare ahead of time and have on ice 20 ml HMDS-EGTA. This will be used in step 7.
5. Centrifuge as in step 3 and gently resuspend the cell pellet in ice-cold HMDS to a total volume of 10 ml. If the cell pellet after centrifugation is greater than 4 ml, the suspension should be divided into additional tubes and the total volume per tube brought to 10 ml with ice-cold HMDS; the aim is to not exceed the equivalent of 4 ml packed cells per tube. Following removal of the tubes from the centrifuge, set the centrifuge temperature to 4°C.
6. Deflagellate cells by adding 2 ml of 25 mM dibucaine to each tube of cells and pipet up and down ~10 times using a 10-ml plastic serological pipet. Within 2 min the flagella should be detached from the cells (check with a light microscope).

Pipetting should be done with enough force to ensure that the flagella are excised, but note that excessive pipetting force can cause cell lysis (see Troubleshooting). It may take some practice to achieve the optimal pipetting force.
7. Working quickly, pour 20 ml premeasured ice-cold HMDS-EGTA into each tube. Centrifuge for 5 min at $1800 \times g$, 4°C, to remove cell bodies.
8. Collect the supernatant using a 25-ml serological pipet. Sweep the sides of the tubes to collect any flagella that are stuck to the sides. Transfer the supernatant into clean 50-ml conical tubes (the same tubes used to premeasure the HMDS-EGTA in step 7 can be used).
9. Using a 10-ml serological pipet, underlay the flagellar suspension with 9 ml ice-cold HMDS-25% sucrose.
10. Centrifuge for 10 min at 2800 rpm ($2400 \times g$), 4°C. Collect the supernatant down to the sucrose interface using a 25-ml serological pipet; sweep the sides of the tube to collect adherent flagella. Transfer the supernatant into 50-ml round-bottom polycarbonate tubes. Check the purity by phase contrast or DIC microscopy (see Fig. 3.41.2).

Most of the flagella will accumulate at or near the 4%/25% sucrose interface; a small amount (up to a few milliliters) of the heavier sucrose layer may be collected to ensure that all of the flagella at the interface are recovered.
11. Concentrate the flagella by centrifuging for 20 min at $30,000 \times g$ (16,000 rpm in a Sorvall RC-5B centrifuge using an SS-34 rotor), 4°C. Carefully pour off the supernatant. The flagellar pellet should be white with little to no green color in it (see Fig. 3.41.3).

Chloroplast contamination due to cell lysis results in a flagellar pellet that is uniformly green, whereas incomplete removal of cells during step 10

results in a flagellar pellet with a dark green center surrounded by a white halo.

SUPPORT PROTOCOL FRACTIONATION OF ISOLATED FLAGELLA INTO AXONEMES AND MEMBRANE + MATRIX FRACTIONS

Isolated flagella can be further fractionated to yield membrane-free axonemes and the detergent-soluble membrane + matrix (the flagellar “cytoplasm”). Axonemal dyneins remain attached to the detergent-extracted axonemes; intraflagellar transport particles and motors are present in the membrane + matrix fraction.

Additional Materials (also see Basic Protocol)

Isolated flagella (see Basic Protocol)

HMDEKP buffer (see recipe)

10% (v/v) NP-40 alternative (Calbiochem, cat. no. 492018)

1. Resuspend isolated flagella (from step 11 of Basic Protocol) in approximately 1/8000th the initial culture volume using ice-cold HMDEKP buffer. For example, for an initial volume of 8 liters, resuspend the isolated flagella in 0.9 ml of HMDEKP. Avoid bubbles.
2. Add 10% (v/v) NP-40 alternative to a final concentration of 1% and incubate on ice for 10 min.
3. Centrifuge for 20 min at 30,000 × g, 4°C. Recover the supernatant (the detergent-soluble membrane + matrix fraction) and pellet (the detergent-insoluble axoneme fraction).

ALTERNATE PROTOCOL FLAGELLAR ISOLATION BY THE pH SHOCK METHOD

Chlamydomonas are capable of quickly and synchronously regenerating their flagella after acid-induced flagellar abscission, which provides an excellent model system for the analysis of flagellar assembly. The dibucaine method described in the Basic Protocol results in cell death; as a result, the deflagellated cells do not regenerate their flagella. In this Alternate Protocol we describe the most commonly used method—pH shock—for flagellar abscission when flagellar regeneration is desired. Upon a rapid drop from neutral pH to pH 4.5, *Chlamydomonas* cells will synchronously and rapidly (within seconds) shed their flagella. After ~60 sec, the pH is returned to neutral and the cells begin regenerating their flagella, which re-grow to nearly full-length in about an hour. Flagellar isolation steps are identical to those described in the Basic Protocol.

Additional Materials (also see Basic Protocol)

0.5 N acetic acid

0.5 M KOH

Small beaker

Stir bar and magnetic stirrer

pH meter

1. Concentrate and wash cells in 10 mM HEPES, pH 7.4, as described in Basic Protocol (see steps 1 to 3).

The cells should be concentrated down to 1/40th–1/50th of original culture volume (e.g., for 8 liters of culture, concentrate down to a volume of 200 ml).

2. Place the concentrated cells in a small beaker and stir rapidly using a stir bar and magnetic stirrer. While monitoring the pH of the cell suspension with a pH meter, rapidly reduce the pH to 4.5 by dropwise addition of 0.5 N acetic acid.

Addition of the acid should be carried out as quickly as possible without overshooting the target pH. When the pH reaches 4.5, start a timer. Have 0.5 M KOH on hand for the next step.

3. After 45 to 60 sec have passed, quickly neutralize the pH to 7.0 by dropwise addition of 0.5 M KOH.

These deflagellated cells may be used directly to monitor flagellar regeneration if the presence of detached flagella will not interfere with subsequent steps, e.g., the measurement of flagella length. However, if the goal is to isolate flagella by this method, then proceed with step 4.

4. Transfer the cells into 50-ml conical tubes (30 to 35 ml of cell suspension/tube).
5. Centrifuge for 10 min at $1800 \times g$ (2500 rpm; Sorvall H-6000A rotor), 4°C, to remove cell bodies.
6. Proceed with steps 8 to 11 of the Basic Protocol.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.

Dibucaine-HCl, 25 mM

Dissolve dibucaine (Sigma, cat. no. D0638) in ddH₂O

Prepare fresh immediately before use (room temperature)

HMDEKP

30 mM HEPES, pH 7.4

5 mM MgSO₄

1 mM DTT (see note)

0.5 mM EGTA

25 mM KCl

Protease inhibitors (Complete Protease Inhibitor tablets; Roche, cat. no. 11 836 170 001; see second annotation)

Keep on ice

Prepare fresh

For compatibility in certain downstream applications (e.g., immunoprecipitations), it may be necessary to omit the DTT.

In the original protocol (Witman et al., 1978), the “P” in HMDEKP referred to 0.5% polyethylene glycol, mol. wt. 20,000, which was necessary to achieve good reactivation;

for large-scale preparations where reactivation is not a goal, we routinely omit the polyethylene glycol and instead include the protease inhibitor.

HMDS

10 mM HEPES, pH 7.4
5 mM MgSO₄
1 mM DTT
4% (w/v) sucrose
Keep on ice
Prepare fresh

HMDS-EGTA

HMDS (see recipe) + 0.5 mM EGTA
Keep on ice
Prepare fresh

HMDS-25% sucrose

10 mM HEPES, pH 7.4
5 mM MgSO₄
1 mM DTT
25% (w/v) sucrose
Keep on ice
Prepare fresh

COMMENTARY

Background Information

Early methods for isolating *Chlamydomonas* flagella induced flagellar abscission by treating cells with cold (−12°C, 40 min) (Jones and Lewin, 1960), plus a solution containing 70% glycerol (−20°C, 1 hr) (Brokaw, 1960) or cold plus solutions containing ethanol and Ca²⁺ (Jacobs and McVittie, 1970; Witman et al., 1972). The pH-shock method described here as the Alternate Protocol was developed by Witman et al. (1972) to address a need for a procedure that was quicker, milder, and easier, especially when working with large volumes of cells. An added advantage of the pH-shock method was that cells survive the deflagellation and can regenerate new flagella. Disadvantages of the pH-shock procedure are that the membranes and internal structure of some of the flagella are disrupted, and that following demembration, the axonemes are not readily reactivated by addition of ATP. Allen and Borisy (1974) simultaneously detached and demembrated flagella by treating the cells with the non-ionic detergent nonidet P40; the resulting axonemes could be reactivated, but membrane and matrix components were lost and the isolated axonemes tended to be contaminated with cell debris, especially in the large-scale preparations needed for biochemical studies. The dibucaine procedure described here as the Basic Protocol was adapted by Witman et al. (1978) from the method of Thompson et al. (1974) for deciliation of *Tetrahymena*. The dibucaine procedure yields highly pure flagella, even from large volumes of cells; the flagella are relatively intact compared to flagella isolated by most other methods; and the method permits isolation of both membrane + matrix and axoneme

fractions. Moreover, the axonemes are excellent starting material for the purification of axonemal components such as dyneins (King et al., 1986; King, 2009) and radial spokes (Yang et al., 2001; Kelekar et al., 2009). Finally, flagella isolated by the dibucaine method can be demembrated by non-ionic detergents to yield axonemes that can be reactivated (Witman et al., 1978), at least when isolated from small (100 to 1000 ml) cultures of cells. Both pH-shock and dibucaine induce flagellar detachment by increasing the intraflagellar Ca^{2+} concentration; the mechanism of flagellar abscission and the signaling pathways involved are discussed in detail by Quarmby (2009). Slight variations of the pH-shock or dibucaine methods are now used routinely in many laboratories for detachment and isolation of *Chlamydomonas* flagella.

Critical Parameters

Prior to beginning the procedure, ensure that the cells are healthy, free of contamination, and not so dense as to have accumulated cellular debris. Once the concentrating and washing steps are initiated, the entire procedure should be carried out without stopping or pausing for significant periods of time. Effort should be made to work quickly once the deflagellating agent is added to the cells. All tubes (except plastic disposables), reagent bottles, etc. should be cleaned and rinsed well with ddH₂O to avoid detergent contamination. Cell pellets should be resuspended gently to avoid cell lysis during the procedure.

Troubleshooting

After the deflagellating agent (e.g., dibucaine, acid) is added, an aliquot of cells should be immediately observed by light microscopy to ensure that the cells have deflagellated; this must be done quickly so the cells are not left too long under the deflagellating conditions. Incomplete deflagellation can result from exceeding the recommended packed-cell volume to dibucaine ratio (e.g., if the 4-ml cell pellet/tube limit is exceeded). In the pH shock method, the pH must be rapidly lowered (within seconds) to cause efficient and synchronized deflagellation. A commonly encountered problem is chloroplast contamination of the purified flagella (see Fig. 3.41.3). The green color imparted by chlorophyll can be used as a crude but sensitive indicator of the overall purity of the isolated flagella. Chlorophyll contamination in the isolated flagella may result from lysis of cells during growth (e.g., if the culture is too dense) or from accidental cell lysis during or before the deflagellation step. Cell wall-deficient strains are especially prone to lysis; these strains require gentle handling during cell culture (e.g., gentle aeration), concentration, and washing. Care should be taken to resuspend all cell pellets gently during the washing and concentrating steps. In the dibucaine protocol, vigorous and repeated pipetting of the cells during the de-flagellation step is recommended to ensure that all of the cells deflagellate. If chlorophyll contamination is encountered, excessively vigorous pipetting is a possible cause of unintended cell lysis.

Anticipated Results

For wild-type cells, one can expect to obtain a yield of ~1 mg of flagella for every liter of culture processed. The pelleted flagella should appear milky white; a green or yellow tint in the flagellar pellet indicates cell lysis during the procedure (see Fig. 3.41.3 and Troubleshooting).

Time Considerations

When starting with 8 liters of culture, the procedure will require about 2 to 3 hr to complete. Much of the time is spent concentrating and washing the cells, and larger culture volumes will require even more time. Once the cells are concentrated, all steps should be carried out quickly, as cells will deflagellate and/or die if left concentrated for long periods.

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Figure 3.41.1.

Chlamydomonas reinhardtii. (A) Phase-contrast image of *Chlamydomonas* cells. Bar = 10 μm . (B) Differential interference contrast image of *Chlamydomonas* cell. Bar = 5 μm . (C) Transmission electron micrograph (TEM) of longitudinal section of *Chlamydomonas* showing the two flagella and their associated transition zones and basal bodies at the apical end of the cell. Bar = 1 μm . (D) TEM of a cross-section of the *Chlamydomonas* flagellum. (E) Schematic of cross-section of the *Chlamydomonas* flagellum. Abbreviations: M, flagellar membrane; IFT, intraflagellar transport particle; O, outer dynein arm; I, inner dynein arm; R, radial spoke; C, central pair of microtubules. D and E are from Pazour et al. (2005). (F) Silver-stained SDS-polyacrylamide gel (4% to 15%) of isolated *Chlamydomonas* flagella. Bracket marks the band corresponding to tubulin.

Figure 3.41.2.
DIC image of purified *Chlamydomonas* flagella. The small particles in the background are pieces of membrane that have detached from some of the flagella. Scale bar = 10 μm .

Figure 3.41.3.

Pellets of purified *Chlamydomonas* flagella. ~1.5 liters of cell culture were processed to yield each of the pellets shown. The pellet on the left has a green tint resulting from chlorophyll contamination due to unintentional cell lysis during the procedure (see Troubleshooting section). The pellet on the right has a typical milky white appearance and is devoid of chlorophyll. Scale bar = 1 cm.