EMBO Practical Course

Molecular Genetics of Chlamydomonas

Geneva, September 18-28, 2006

Laboratory protocols

	Group A	Group B
Monday September 18 1300-1400 1400-1445 1445-1530 1530-2000	L1: Introduction to Chlamydomonas (Rochaix) L2: Chlamydomonas genetics (Girard-Bascou) L3: Transformation of Chlamydomonas (Goldschmidt-Clermont) P1: Crosses P2: Cp and nu transformation	
Tuesday September 19 900-1200 1200-1300 1400-1900 1900-2000	P3: DNA analysis L4: Biogenesis of the photosynthetic (Choquet) apparatus I P3: DNA analysis Participant presentations	
Wednesday September 20 900-1100 1100-1200 1200-1300 1400-1900 1900-2000	P4: Cell fractionation: cp, mit, thylakoids L5: Biogenesis of the photosynthetic apparatus II (Wollman) L6: Proteomics of membrane proteins (Rolland) P4: Cell fractionation: cp, mit, thylakoids Participant presentations	
Thursday September21 900-1100	Protein analysis	
1100-1200	Participant presentations	
1200-1300	Participant presentations	
1400-1800	P6: A1 Pulses A2 Membranes for green gels and TMBZ	P5: membrane protein analysis
1800-1900	L7: Fluorescence measurements in intact cells (Finazzi)	
1900-2000	L8: Spectroscopy of intact cells (Rappaport)	
Friday September 22 900-1100	L9: Use of genomic information (Grossman and Vallon)	
1100-1300	P6: A1 Autoradio and blots A2 BNG and TMB	P8: genome analysis
1400-1800	P7: A1 RT Fluo, 77K, green gels A2 Spectro 515, 77K	B1 Pulses B2 Membranes for green gels and TMBZ

1800-2000	L10: Flagellar function and assembly (Witman)	
Saturday September 23 8.30-12.30 1400-1800	P7: A1 Spectro 515, P700 A2 RT Fluo, 77K, green gels P5: membrane protein analysis	P6: B1 Autoradio and blots B2 BNG and TMBZ P7: B1 RT Fluo, 77K, green gels B2 Spectro 515, 77K
Sunday September 24 900-1300	P8: genome analysis	P7: B1 Spectro 515, 77K B2 RT Fluo, 77K, green gels
Sunday afternoon 1400-15.30 15.30-end of day	Analysis of experimental results FREE	
Monday September 25 900-1100	P9: Nutrient stress	P10: Flagellar assembly/Immunofluo
1100-1200	L11: Metal stress (Merchant)	
1200-1300	L12: Nutrient stress (Grossman)	
1400-1800	P9: Nutrient stress	P10: Flagellar assembly/Immunofluo
1800-1900	P11: Tetrad analysis I	
Tuesday September 26 900-1100	P11: Tetrad analysis II	P9: Nutrient stress
1100-1300	L13: Photomovement and electrophysiology on Chlamydomonas (Hegemann)	
1400-1800 1800-1900	P12 : Photomovement and electrophysiology	P9: Nutrient stress P11: Tetrad Analysis I
Wednesday September 27 900-1100	P10: Flagellar assembly/ Immunodetection	P11: Tetrad analysis II
1100-1200 1200-1300	L14: Reverse nuclear genetics (Cerutti) L15: prospects for reverse nuclear genetics (Hegemann)	
1400-1630 1630-1900	P10: Flagellar assembly/Immunodetection	P12: Phototaxis
Thursday September 28 900-1200	Analysis of results	

Persons in charge of practicals:

P1 Crosses: Jacqueline Girard-Bascou with Isabelle Howald, Linnka Lefèbvre-Legendre
P2 Nuclear and chloroplast transformation: Michel Goldschmidt-Clermont, Linnka Lefèbvre-Legendre and Jean-David Rochaix
P3 DNA analysis: Mounia Heddad, Adrian Willig, Christian Delessert, Michèle Rahire, Jean-David Rochaix
P4: Cell fractionation: Mauro Ceol, Stéphane Miras, Thomas Gieler
Protein analysis: Vroni Winter, Mounia Heddad, Sylvain Lemeille
P5: Envelopes: Norbert Rollland
P6: Analysis of thylakoid membranes: Francis-André Wollman, Yves Choquet and Olivier Vallon
P7:Spectroscopy of intact cells: Fabrice Rappaport and Giovanni Finazzi
P8: Genome analysis in silico: Olivier Vallon and Arthur Grossman
P9: Nutrient stress: Sabeeha Merchant and Arthur Grossman with Christian Delessert
P10: Flagellae and immunofluorescence: George Witman
P11: Tetrad analysis: Jacqueline Girard-Bascou with Isabelle Howald, Linnka Lefèbvre-Legendre
P12: Photomovement and electrophysiology: Peter Hegemann and Peter Berthold

There will be 15 lectures L1-L15

The number of students will be limited to 21.
For some practicals the students will be divided in two groups of 10 and 11 persons: A and B. In some cases A and B will be split in two smaller groups of 5/6 students (A1, A2; B1, B2)

Participants will present their current work in a short 10 min presentation on September 19, 20 and 21.

P1/P11. Genetic Analysis of *Chlamydomonas reinhardtii*

Jacqueline Girard-Bascou, IBPC, Paris, France
Isabelle Howald and Linnka Lefébvre-Legendre, Geneva

Table of Contents

1. General introduction

Here are presented protocols that I use for the genetic analysis of photosynthetic mutants of C*hlamydomonas* since several years. These protocols have been designed to be simple and efficient in most cases. However, problems arise occasionally with the classical genetic analysis. For each protocol, the most common difficulties are mentioned and advice on how to overcome the problems is presented in TROUBLESHOOTING.

Several tools are necessary. I choose a good scalpel, penholder, small surgical instruments or a small dentist spatula and needle glass prepared each time (to preserve needle glass, they are pricked in modeling clay). These tools should be kept in a safe place and reserved exclusively for that purpose.

2. General guidelines for gametogenesis

Classically gametes are obtained after nitrogen starvation, but a prolonged nitrogen starvation can also induce death and dead cells are evidently not able to mate. It is recommended *first* to starve cells in exponential growth rather than in stationary phase, *second* to use TAP medium with only 1/10 of the normal amount of nitrogen rather than medium without nitrogen (stringent starvation) to allow progressive differentiation of all the cells in gametes, *third* to prepare cells on agar medium rather than in liquid medium to avoid centrifugation for obtaining high concentrations of cells. Gametes are then transferred to tubes or Erlenmeyer flasks containing sterile water to obtain between 2×10^6 to 2×10^7 cells/ml. Erlenmeyer flasks can be stirred for 30 min to allow gametes to swim vigorously. Gamete cells can be distinguished from vegetative cells under the microscope by their smaller size and because they swim more vigorously. For arginine requiring strains, use "CA" medium which is a minimal medium without nitrogen supplemented with 30 mg/ l of arginine (same conditions of timing as with N/10 medium)

3. General guidelines for crossing

There are two possibilities : either you resuspend gametes of the two mating types, up to a concentration of 5×10^6 to 5×10^7 cells/ml, into sterile water together, directly from the plates, or you mix the solutions of gametes prepared separately (in this case you can control the gametic state under the microscope before the crossing). Remember that the transfer of cells from agar plates to liquid cultures is achieved by first streaking the cells on the wall of the flask or tube just above the liquid and by mixing them progressively with the liquid solution.

You can use tubes (10 or 12 cm long) or Erlenmeyer flasks (50 ml). The resuspended cells may be stirred some minutes to obtain a homogenous cell suspension. However, afterwards the tubes or Erlenmeyer flasks are exposed to medium intensity light (2000 lux) without stirring.

Add sterile water: 1 to 2 ml per tube and between 3 to 10 ml in Erlenmeyer flasks depending on the amount of cells. A large air/solution area is preferred. This may be achieved by tilting the tubes.

4. Mating type test

General guidelines

The idea is to determine the mating types of new strains with the standard WT strains (the WT strains that you use commonly for your experiments in your laboratory) of the two mating types (+ and -) and to observe the next day the clumping reaction of zygotes in one of the two test tubes. The mating type of the new strains will be the opposite of that of the WT strain which induces clumping of the cells. This reaction is very easy to detect when it proceeds well. The zygotes stick together and adhere to the wall or the bottom of the tube and the medium appears clear. In the other tube, the cells usually remain in suspension, and the medium remains green as at the beginning of the experiment. However, sometimes the cells settle to the bottom of the tube. But this deposit is homogeneous and the cells can be resuspended by a light agitation.

It is recommended to always use the same tester strains to determine sexual compatibility between all your strains. I sometimes observe that it is difficult to cross strains from different laboratories. This may be due to different genetic backgrounds (due to the accumulation of non-selected spontaneous mutations). I have also observed that the sterility (or fertility) can be either a characteristic of a specific parental strain or of a specific cross.

Standard protocol

1) *Preparation of gametes* : transfer a ~ 1 cm x 3 cm patch of fresh cells to be tested to a TAP or TARG plate (TARG is used for arginine requiring strains) three to four days before transferring cells to gametogenesis plates . Transfer in the same way each WT tester on TAP plates. The amount of WT cells will be about half of the total amount of all cells to be tested for the mating type. Put all the plates including the WT plates under low light (200 to 300 lux).

Three to four days before the day of the test , transfer cells from the TAP/TARG plates to gametogenesis plates N/10 or CA plates (CA is used for arginine requiring strains). Concentrate the cells in approximately half the area used before.

2) *Crossing* :

a) Set up 10 or 12 cm-long sterile glass test tubes for mating-type tests, two tubes for each strain to be tested, and one additional tube for the control of the two tester strains. Add 1 ml sterile water to each tube for the strains to be tested. The aim is to have a reasonably dense solution (green culture, approximately 5 x 10^6 cells/ml). For the tester strains, resuspend cells in a volume which is equal to the total volume of all strains to be tested, with a final aliquot left for the control. Try to obtain equal concentrations of cells for all strains by varying the amount of cells or the amount of water used.

b) Resuspend about one loopful of cells to be tested from the N/10 plate to each 1 ml H_2O in the test tube (note on each tube the name of the strain and the tester added). Vortex to resuspend well.

c) Resuspend tester cells from the N/10 plate in test tubes to reach the same cell density (estimated by eye). Vortex to resuspend well.

d) Add 1 ml of tester cells to each tube containing the cells to be tested. Mix well by vortexing. Prepare a tube with the two testers as a control.

e) Put the tubes on a rack and tilt the rack as for making slants to have a larger liquid/air interface. Put the cells under high light (2000 to 3000 lux).

3) *Analysis of the test the following day* :
a) First, check the mating efficiency by looking at cells in the tubes without shaking in an upright position. Settled cells are homogeneous and have not mated. Mated cells stick to the glass and show spots (like tiger's skin) on the surface contacting the glass.
b) Confirm the mating by moving the tubes and finally by vortexing. Cells that have not mated resuspend well after vortexing. Mated cells clump in the test tube even after vortexing (some zygotes can remain fixed on the glass). When the cross is very efficient the medium will be clear and contains a zygote pellicle (a "zygote skin" or a "green fish"). This should occur after mating of the two tester strains.

TROUBLESHOOTING
Problems and possible causes and solutions

1. Infected cells or unhealthy cells : There is no clear clumping reaction in either of the two tubes. First check the cross between the two testers. If it is not efficient, the reason is clear, either of the strains has been infected or the strains are not healthy, i.e. there is no vigorous growth. You have to repeat all the tests with healthy cells. Second, if the control cross proceeded well, this can be due either to partial or total sterility of the tested strain. If you have several strains of the same genotype, you can eliminate the strains that mated poorly. In this way you also select for fertile strains.

2. Partial Sterility of a strain : If one important strain appears to be sterile in this test, it is necessary to identify the cause of sterility. There may be a deficency in swimming in the vegetative and/or gamete state, a defect in agglutination, a defect in fusion or a defect in the maturation of zygotes.
First, test the swimming of the gametes by transferring them (in 2 or 3 ml water) in an Erlenmeyer flask of 50 ml. Agitate during 30 min to 1 h. Then look under a microscope. Good gametes are swimming more vigorously and are smaller than vegetative cells.
Second, take two hematimeters and introduce on one side the strain to be tested. Introduce on the other side of the hematimeters either WT+ or WT- gametes. Watch under the microscope at the interface of the two strains the reaction of agglutination. Practice by observing this reaction with the two WT testers before. During agglutination, the gametes of opposite mating types interact with there flagella. In this way you can also identify the mating type of a strain (observation of the agglutinating process with one tester).
Third, it is possible to activate gametes of a strain by a treatment with dibutyryl-cAMP (10 mM) and iso-butyl-methyl-xanthine (1mM) during 30 minutes before crossing (Pasquale and Goodenough 1987).

5) Haploid progeny in tetrads
Step 1. Transfer a patch of ~ 1cm x 3 cm fresh cells to a fresh TAP plate three to four days before transferring to a TAP(1/10 N) plate.
Step 2. Transfer all cells from the TAP plate to TAP(1/10 N) plate three to four days before the day of mating. Concentrate the cells in a small area (~ 1cm x 2cm).
Step 3. Day of the mating:
a) Optional : Check the fluorescence of the gametes (cells on the TAP 1/10 N plate). Compare with the fluorescence of vegetative cells (cells on the TAP plate). For wild type

cells, the fluorescence pattern of the gametes looks like a leaky mutant of the cytb6/f complex due to the degradation of the complex during gametogenesis.

b) Use a 50 ml sterile Erlenmeyer flask to set up the mating. The flask will provide a large contact area between the cell solution and air during the mating. Resuspend each strain in 2~5 ml sterile H_2O to achieve a cell density between $5x10^6$ ~ $2x10^7$ cells/ml. Mating will be impeded at a higher density (probably due to reduced motility or respiration), and at lower cell density (probably due to insufficient autolysin secreted by gametes which is necessary to remove the gamete walls). Put the flasks on a shaker for at least 30 min.

c) Check the mobility of cells under the microscope. Active gametes should be jiggling and swimming. Put the flask on the shaker for longer time if cells are not active. Or, check the mating ability by putting aliquots of the cells to be mated on each side of a hematimeter and look for active aggregation at the interface of two strains.

d) Set up the mating by mixing the two parental cells in a single flask. Mix by shaking gently. Put the flask under light (2000 to 3000 lux) without shaking.

e) Check the mating after one, two or three hours. Mated cells are aggregated, initially giving rise to a granular appearance, and subsequently they begin to stick to the glass on the bottom and at the top of the medium in a ring. Plate 4 x 1~2 drops of cells (with a Pasteur pipette) onto a 3% agar TAP plate (55 mm x 13 mm) after shaking the flask gently. Wait and check every 1~2 hr if cells do not mate. Or, plate aliquots of cells every 1~2 hr if they do not appear to mate well.

f) Put the plates under bright light overnight (2000 to 3000 lux).

Step 4. Day following the mating.

Wrap the plates individually with foil. Write the name of the cross and the date. Store the plates in the dark (in a box).

Step 5. After at least six to seven days (up to one month, but sometimes the best is the second week) in the dark:

Scrape regularly vegetative cells from the plate with a dull scalpel (put the plate vertically to scrape not too strongly). The characteristics of zygotes are: round large cells with a black cell wall, yellow and never green, homogeneous without appearance of cell division, and firmly bound to the agar (the degree to which they stick may vary, but it is the most important feature).

Step 6. Under a dissecting microscope (magnifying 20 x) Collect zygotes with a scraper (a small surgical instrument or a small dentist spatula can be used), and transfer on a block of agar to a regular (1.5% agar) TAP plate with a penholder. Invert the block to transfer zygotes, and distribute zygotes along a line (one-third of the plate, etched into the bottom of the plate), using a glass needle** (magnifying 40 x). Treat the plate during 25 to 30 sec. with vapors of chloroform if there are vegetative cells around the zygotes. Put the plates under medium light (or obscurity in an aluminum paper) overnight (16 h to 20 h). The germination of zygotes varies from strain to strain. Adjust light intensity and/or incubation time if necessary.

Comments :If the zygotes give rise to 8 products instead of 4, repeat the experiment and check the plates immediately after 16 h light or use older zygotes (one or two days more). In some rare cases the cell wall of the zygote is only released after a post meiotic division. In this case either dissect the eight cells (on two lines) or change one parental clone by another.

Step 7. Dissect tetrads the next day with a glass needle. The germination is completed by the rupture of the zygote wall and the release of the four products of meiosis. If the rupture is not achieved, you can touch the zygote with a glass needle to release the four products. Often one product remains in the zygote wall. Sometimes you see five objects. In this case the four cells are bright but not the zygote wall. Etch a grid of four horizontal lines parallel to the first line,

and a perpendicular line for each tetrad, about 10 to 15 per plate. Transfer each of the four cells of a tetrad at each of the four intersections.

* *For the 50 ml flask, the minimal amount of H_2O is 1 ml, the maximal amount is 10 ml. The best amount is 5~6 ml. But 1 to 3 ml of cells give rise to a good yield of zygotes.*

*** The glass needle are prepared by pulling hollow glass tubes (3 mm in diameter) in the flame of a Bunsen burner. A deep hook is made on the stretched part with the small flame.*

6) Bulk haploid progeny

Protocol 1 : proceed until step 6 until you obtain many zygotes. Transfer about 50 zygotes in the middle of a standard TAP plate. Put under high light during a night. The next day add 100 to 200 µl of sterile water on the germinated zygotes and spread all around the plate.

Protocol 2 : proceed until step 5. Under the dissecting microscope (20 x magnifying) choose a surface with many zygotes (about 500). Scrape off vegetative cells gently from this surface with a glass loop. Do not collect zygotes. Treat all the plate with 25 to 30 sec. vapors of chloroform. With a sterilized penholder, transfer the block of agar with bound zygotes in a tube with 2 ml TAP liquid medium. Put the tube in high light without stirring. After 24 to 48h, vortex the tube during 1 to 2 minutes and plate 100 to 200 µl of the suspension on standard TAP plates (5 plates) avoiding the piece of agar containing the non germinated zygotes.

7. Selection of vegetative diploid cells

During a cross, 0,5 to 5% of the mated gamete pairs give rise to vegetative diploid cells. Selection of these vegetative diploid cells should be done by using complementing auxotrophic recessive mutations. We use commonly *arg2* and *arg7* mutations. Although these mutations are in the same gene, they complement each other well and all diploid cells are [arg+]. As *arg2* and *arg7* mutations are tightly linked, if some zygotes germinate precociously, only very few [arg+] recombinant progeny will appear.

Parental gametes are prepared in CA plates. Three hours after the mixing of the gametes, 100 µl of the mixture undiluted or diluted 10 fold are plated on TAP plate (5 plates of each). Do the same one hour after. You can plate earlier or later depending on the rapidity of the mating. The plates are then piled in very low light (but not obscurity). Large diploid colonies appear 12 to 14 days after. They should have all the same color and diameter (as most spontaneous mutations affecting these characters and often present as a genetic background in our strains are recessive mutations).

The diploid state can be controlled either by a mating test as diploid cells are predicted to be all mating type minus (at least 7 to 12 colonies have to be tested) or by a PCR test for the presence of genes specific of the mt- and mt+ loci (Werner R. and Mergenhagen D., Plant Molecular Biology Reporter 16 : 295-299, 1998).

P2. Transformation of Chlamydomonas

Michel Goldschmidt-Clermont and Linnka Lefèbvre-Legendre (Geneva)

A. Glass bead method for nuclear transformation of *Chlamydomonas reinhardtii*

Materials.

- Cell-wall deficient (e.g. cw15) host cell strain. (*If you need to use a strain with a wild-type cell-wall, the cells must be treated with autolysin prior to vortexing with glass beads (step 7).*)
- Sterile liquid growth medium (permissive for the host cell line). (*Approximately 35mL of culture / transformation plate.*)
- Sterile liquid growth medium (corresponding to selective conditions). (*This will be used to wash the cells by centrifugation before transformation. Use appropriate medium(minimal, arginine free, etc) depending on the selection for transformants that will be applied.*)
- Prepare glass tubes (3 mL) with 0.3g glass beads (Thomas Scientific), sterilize by baking in oven. *(A convenient scoop can be made from the bottom of an Eppendorf tube and a blue pipetman tip, glued by gently melting the tip).*
- Sterile centrifugation bottles and tubes.
- Sterile, cotton-plugged 5 mL pipets.
- Plates with appropriate solid medium for selection of the transformants.
- DNA with selection marker. (*Circular, supercoiled DNA can be used, but in cases where single insertions are desirable (e.g. insertional mutagenesis), a linear DNA fragment is preferable. The amount of DNA used will also influence the number of insertions (approx. range: 0.2 – 1.0 ug / transformation*).

Protocol.

1. Grow cells in appropriate medium (permissive) to a density of ~2 x 10^6/ mL.

2. Collect cells by centrifugation in sterile centrifugation bottles at room temperature (3500 g x 10 min). Discard supernatant.

3. Resuspend cells in 1/25 – 1/50 initial volume in selective medium with a cotton-plugged pipet. Transfer to a sterile centrifugation tube.

4. Collect cells by centrifugation at room temperature (3500 g x 10 min). Discard supernatant

5. Resuspend cells at approximately in 1/70 initial volume in selective medium (approximately 3.0 x 10^8 cells / mL.
Count a 1/100 dilution with the hemacytometer under the microscope. Adjust the volume to obtain a concentration of 2. x 10^8 cells / mL.

6. To a tube containing 0.3g glass beads (sterilized by baking) add:
   - 0.3 mL cell suspension.
   - ~ 0.5 – 1.0 ug DNA.

7. Vortex at full speed for 15 seconds.

8. Pour the contents of the tube on a selective plate, gently tilt and rotate the plate to spread the medium evenly.

9. Allow the liquid to dry (protect from light to avoid phototactic movements of the cells). Seal the plates with Parafilm (Micropore tape for minimal medium), and incubate under appropriate conditions for selection. (*If cells were grown under auxotrophic conditions (acetate, dark) and will be selected for photoautotrophic growth (minimal, light*), *put the plates in dim light for 16 – 24 hours before transferring to light. Colonies will appear within 1-3 weeks depending on the selection applied.)*

References:

Kindle, K. (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. USA **87**: 1228-1232.

B. Electroporation method for nuclear transformation of *Chlamydomonas reinhardtii*

Materials

- Cell-wall deficient host cell strain
- Sterile centrifugation bottles and tubes
- Electroporation cuvettes
- Plates with appropriate solid medium for selection of the transformants
- DNA with selection marker
- TAP, 40mM sucrose
- TAP, 40mM sucrose, 0.4% PEG 8 000 - Starch 20%

Starch 20% preparation

20 g starch in a centrifuge tube
Wash with ethanol 100%
Wash with water
Repeat 2 times
Resuspend in 100 ml Ethanol 70%
Aliquots of 20 ml and keep at room temperature
The day of transformation, centrifuge an aliquot 1 minute at 1 000 rpm
Wash 4 times with TAP + sucrose 40 mM
Resuspend in 20 ml of TAP + sucrose 40 mM + PEG 8 000 0.4%

Protocol

1. Grow 250 ml of cells to a density of 2 x 10^6 cells/ml

2. Collect cells by centrifugation at room temperature at 3 500 rpm for 5 minutes in sterile centrifugation bottles. Discard supernatant

3. Resuspend in 1.25 ml of TAP, 40mM sucrose

4. Incubate on ice 10 minutes

5. Transfer 250 µl of cells in a cuvette containing 1 µg of DNA

6. Incubate at room temperature 5 minutes

7. Electroporate 0.75 kV, 25 µF, no R, 6 msec

8. Incubate at room temperature 10 minutes

9. Add 1 ml of starch 20% and pour the contents of the cuvette on a selective plate, gently tilt and rotate the plate to spread the medium

10. Allow the liquid to dry (protect from light), seal the plates with parafilm and incubate under appropriate conditions for selection of transformants.

C. Chloroplast transformation of *Chlamydomonas reinhardtii*

Materials.

- Host cell strain.
- Sterile liquid growth medium (permissive for the host cell line). (*Approximately 10 mL of culture / transformation plate.*)
- Sterile liquid growth medium (corresponding to selective conditions). (*This will be used to wash the cells by centrifugation before transformation. Use appropriate medium(eg minimal) depending on the selection for transformants that will be applied.*)
- Sterile centrifugation bottles and tubes.
- Sterile, cotton-plugged 5 mL pipets.
- Plates with appropriate solid medium for selection of the transformants.
- DNA with selection marker. (*1ug / uL, 1.0 ug per sample, sufficient for up to 7 plates*).
- 100 mg/mL tungsten powder in sterile 50 % glycerol. (*25 uL per sample*)
- 2 M $CaCl_2$, sterile (*25 uL per sample*)
- 100mM spermidine (base), filter sterilized. (*10 uL per sample*)
- Filter holders for Helium gun.(*Sterilize by washing with Ethanol, air dry in sterile hood*)
- Sterile microfuge tubes and tips.

Protocol.

1. Grow cells in appropriate medium (permissive) to a density of ~2 x 10^6/ mL.

2. Collect cells by centrifugation in sterile centrifugation bottles at room temperature (3500 g x 10 min). Discard supernatant.

3. Resuspend cells in 1/30 initial volume in selective medium with a cotton-plugged pipet. Transfer to a sterile centrifugation tube. (*Steps 3 and 4 can be omitted if the media for the culture and for selection on the plates are compatible*).

4. Collect cells by centrifugation at room temperature (3500 g x 10 min). Discard supernatant

5. Resuspend cells in 1/30 initial volume in selective medium (approximately 6 x 10^7 cells / mL).

6. Plate 0.3 mL of cell suspension evenly on selective plate.

7. Allow the liquid to dry (protect from light to avoid phototactic movements of the cells).

8. Sonicate the tungsten suspension briefly (the tube is attached with a stand and clamp so as to touch the tip of the sonication probe immersed in a beaker of water).

9) In a sterile microfuge tube placed on ice, add in order:
- 25 uL 100 mg/mL tungsten (in 50 % glycerol)
- 2 uL DNA (0.5 mg / mL)
- 25 uL $CaCl_2$, 2 M.
- 10 uL Spermidine base, 0.1 M.

10. Incubate on ice for 10 min.

11. Spin 1-2 min in microfuge.

12. Remove 25 uL of the supernatant. Resuspend the rest by vortexing and a brief sonication (2-3 sec) as above.

13. Apply 8 uL to a filter holder, attach to Helium outlet. Place a plate in the apparatus and proceed with bombardment. (*Parameters that can be optimized include: Helium pressure, opening time of the valve, pressure in the chamber, distance from the sample holder to the plate*).

14. Seal the plates with Parafilm (Micropore tape for minimal medium), and incubate under appropriate conditions for selection. (*If cells were grown under heterotrophic conditions (acetate, dark) and will be selected for photoautotrophic growth (minimal, light*), *put the plates in dim light for 16 – 24 hours before transferring to light. A ring of colonies will appear within 1-3 weeks depending on the selection applied.)*

References.

Boynton et al (1988) Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. Science 240: 1534-1538.

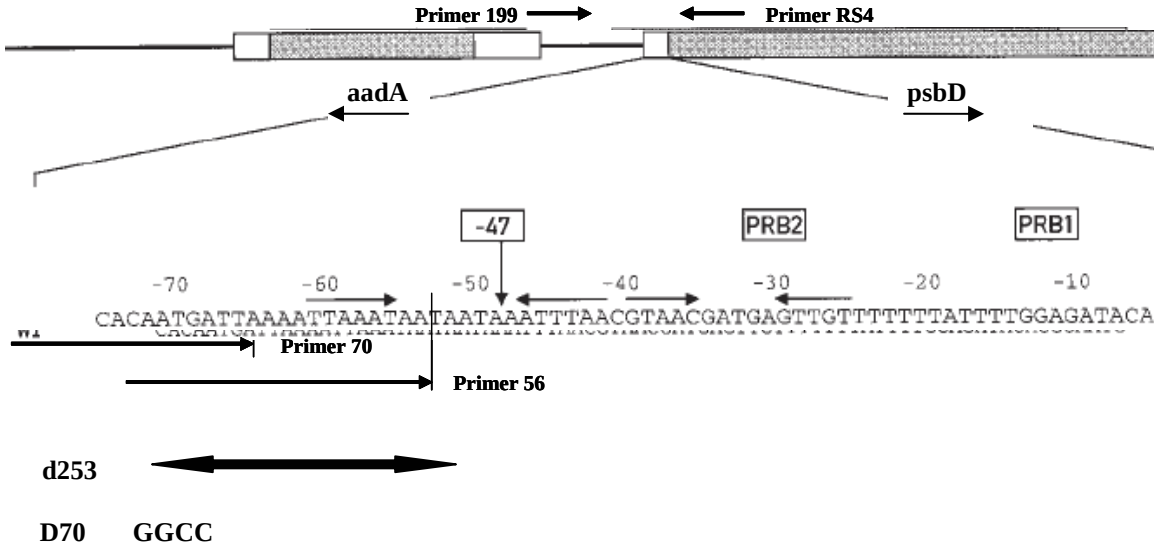
Finer et al. (1992) Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Reports 11: 323-328.

P3. DNA Analysis

Mounia Heddad, Adrian Willig, Christian Delessert, Michèle Rahire and Jean-David Rochaix (Geneva)

DNA-Extraction from Chlamydomonas cells

In this practical you will isolate DNA by three different methods. The first allows you to prepare DNA that can easily be digested with restriction enzymes and that is suitable for DNA blotting experiments. The second method allows one to obtain DNA that is sometimes refractory to restriction enzyme digestion but that is well suited for PCR analysis. The third method is a rapid PCR method that is useful for map-based cloning. You will receive the following strains for DNA extraction:
WT (wild-type)
cw15 (cell wall deficient)
S1D2 (polymorphic strain)
p10814 (chloroplast transformant with aadA cassette upstream of psbD)
p253 (same as p10814 but with small deletion -68-47 in psbD 5'UTR)



1. DNA Extraction with CsCl-EthB gradient

- 50-100 ml Chlamydomonas culture in TAP (~ 10^7 c/ml), harvest by centrifugation (3500 rpm for 10 min)
- Wash pellet with 1.5 ml H_2O and transfer to 2 ml Eppendorf tube

- Centrifuge 1 min max speed and remove supernatant (at this stage cell pellets can be frozen at -70°C and stored at -20°C)
- Resuspend pellet with 0.45 ml resuspension buffer
- Transfer to 15 ml tube (for HB 4 rotor) and add 1 ml of SDS-extraction buffer (SDS-EB)
- Mix gently and incubate at 55 ^{o}C for 1hr.
- Add 1.55 g CsCl, close tubes well and mix gently by inverting the tubes
- Add 100 µl of EtBr (10 mg/ml) and mix as before
- Centrifuge for 10 min in HB 4 at 20°C to pellet cell debris
- Transfer supernatant to small ultracentrifuge tubes for TLV 100 rotor. If necessary, fill the tubes with the "fill-up" solution and balance tubes well
- Seal tubes, check them for closeness and centrifuge in TLV 100 rotor for 5 h at 90 000 rpm at 20°C
- The DNA-band appears horizontally and is stained with EtBr
- First, fix the tube so that you have both hands to work. Puncture the tube at the top so that air can get out
- Remove the DNA-band by puncturing the tube on the side with a needle connected to a 1 ml syringe. Pull a little bit of air into the syringe before puncturing the tube. The needle should be inserted just above the band. Move the needle so that its opening is just below the band and pull it slowly into the syringe. The removed volume should be as small as possible (100-250 µl)
- Transfer the CsCl solution contaning the DNA in a 2 ml Eppendorf tube
- Add TE buffer to 0.5 ml
- Extract DNA 4x with 0.5 ml butanol saturated with H_2O and CsCl. After every extraction step, remove the butanol phase from the top (takes red color from the EtBr) and add new saturated butanol
- Precipitate DNA with 3 Vol of 70% EtOH
- Centrifuge, resuspend pellet in 250 µl TE, 10 µl NaCl 5M, 3 Vol EtOH 100%
- Centrifuge, resuspend pellet in 50 µl TE, quantify.

Resuspension buffer: 100 mM Tris pH 8
40 mM EDTA

SDS-extraction buffer (SDS-EB): 100 mM Tris pH 8
40 mM EDTA
400 mM NaCl
2% SDS

Butanol saturated with H_2O and CsCl

TE: 10 mM Tris-HCl pH 7.5, 1mM EDTA

Ref : D Weeks *et al*, Analytical Biochemistry 152, 376-385 (1986)

2. Rapid mini preparation of Chlamydomonas DNA

- Collect 10 ml of cells at 5 x 10^6 cells/ ml by centrifugation in a 15 ml Corex tube at 3000 g for 5 min.
- Resuspend pellet in 0.35 ml of 50 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl
- Transfer the cells to an Eppendorf tube (1.5 ml)
- Add 50 µl proteinase K at 2mg/ml
- Add 25 ml of 20% SDS, and incubate for 2 h at 55 ^{0}C.
- Add 2 µl of diethylpyrocarbonate, incubate for 15 min at 70 ^{0}C.
- Cool the tube in ice briefly, the add 50 µl of 5 M potassium acetate
- Mix by shaking the tube thoroughly, leave on ice for 30 min or more
- Centrifuge for 15 min in a microcentrifuge tube
- Transfer the supernatant into another Eppendorf tube
- Extract the supernatant with an equal volume of phenol
- Fill the tube to the top with ethanol at room temperature and centrifuge 2 min
- Rinse with 70% ethanol and centrifuge for 1 min
- Pipette off supernatant and discard
- Dry the pellet and resuspend in 50 µl of TE pH 7.5, 1 µg/ml pancreatic RNase. Use 10-15 µl for one restriction enzyme digestion.

- Buffers and solutions: 50 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl

3. Fast method for PCR: CHELEX DNA extraction

- Scrap Chlamydomonas cells from a plate with a yellow tip and resuspend in 20 µl H_2O
- Add 20 µl 100% ethanol
- Mix well by vortexing
- Add 200 µl 5% Chelex
- Incubate 10 min at 98° C
- Centrifuge at room temperature for 10 mins
- Use the supernatant for PCR (use 1µl per PCR reaction)

Chelex preparation: 5% (w/v) in H_2O

Analysis of DNA

Restriction enzyme analysis

Nuclear DNA is poorly cut by EcoRI whereas chloroplast DNA contains many EcoRI sites. It is thus possible to detect the chloroplast restriction fragments from a total DNA EcoRI digest.

PCR

Because the GC content of nuclear and chloroplast DNA of Chlamydomonas differ considerably, the PCR conditions for amplifying nuclear and chloroplast DNA are considerably different.

Nuclear DNA

Chloroplast DNA
10 ng DNA in 36 µl H_2O
5 µl 10 x PCR buffer
2.5 µl 2.5 mM dNTPs
1 µl 5 mg/ml BSA
3 µl oligo I (100µg/ml)
3 µl oligo II (100µg/ml)
1 U Taq polymerase
30 cycles 2min @ 94°C, 2min @ 40°C, 2min @ 72°C

P5. Fractionation of membranes for proteomic analyses

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Content

5. References

Abstract
Proteomics is a very powerful approach to link the information contained in sequenced genomes, like *Chlamydomonas*, to the functional knowledge provided by studies of cell compartments. However, membrane proteomics remains a challenge. One way to bring into view the complex mixture of proteins present in a membrane is to develop proteomic analyses based (a) the use of highly purified membrane fractions and (b) on fractionation of membrane proteins to retrieve as many proteins as possible (from the most to the less hydrophobic ones). To illustrate such strategies, we choose two types of membranes, the thylakoid membrane and the chloroplast envelope membranes. Both types of membranes can be prepared in a reasonable stage of purity from *Chlamydomonas*. This practical course will be restricted to the description of methods for the fractionation of these membrane proteins according to simple physico-chemical criteria (i.e. chloroform/methanol extraction, alkaline or saline treatments) for further analyses using modern proteomic methodologies.

1. Introduction

Membrane proteins play a crucial role in many cellular and physiological processes. They are essential mediators of material and information transfer between cells and their environment, between compartments within cells, and between compartments comprising the different tissues. The functional diversity of proteins in a cell actually is strongly related to the diversity of their physicochemical properties. This is even more obvious in membranes because of their hydrophobic nature. Ion channels or receptors, for instance, are "integral" or "intrinsic" membrane proteins, often containing several transmembrane □-helices linked together by loops located outside the membrane in an aqueous environment. Such proteins are amphipathic, in that they contain both hydrophobic and hydrophilic regions, their overall hydrophobicity relying on the proportion between loops and □-helices. In some cases, aminoacids in the loops are modified by oligosaccharides thus increasing their hydrophilicity. The secondary structure of few membrane proteins consist of □-sheets, thus forming □-barrels through which hydrophilic molecules can cross the membrane. Porins are the most conspicuous example of this type of membrane proteins, which are much less hydrophobic than proteins containing □-helices. Not all membrane proteins have transmembrane domains. Some proteins are embedded within only one bilayer of the membrane (monotopic proteins). Other types of proteins are anchored to the membrane owing to a hydrophobic moiety (fatty acid or isoprenoid chain, for instance) that is embedded in the lipid phase of the membrane. These non-transmembrane proteins as well as integral proteins may be more or less tightly bound through ionic or hydrophobic interactions to other membrane proteins, the so-called class of "peripheral membrane proteins".

Once isolated from its cellular context, a membrane therefore remains an extremely complex mixture of some very hydrophobic or hydrophilic proteins, of basic or acid proteins, of low or high molecular mass proteins, of major or low abundance proteins. Membrane proteins are extremely difficult to separate from each other and to analyze for further functional studies, essentially because of the presence of lipids. Therefore, innovative tools and methods were developed for the study of membrane proteins. One way to bring such proteins into view is to develop proteomic analyses based on subcellular compartmentation and/or physico-chemical criteria.

The purpose of this practical course is to describe rather simple procedures that have been developed to set up membrane proteomic studies in plants and especially in *Arabidopsis* (1-5) and that are now used for *Chlamydomonas*. To illustrate such strategies, we choose two types of membranes, the thylakoid membrane from *Chlamydomonas* and the chloroplast envelope

membranes from spinach leaves, each one providing a very unique lipid environment to membrane proteins. Furthermore, both types of membranes can be prepared in a reasonable stage of purity from plants and *Chlamydomonas*. This practical course is restricted to the description of methods for the fractionation of these membrane proteins according to simple physico-chemical criteria for further analyses using modern proteomic methodologies (for review, see ref 6).

2. Materials

2.1. Biological Materials

2.1.1. Thylakoid membranes from Chlamydomonas

Chlamydomonas thylakoid membranes will be prepared in P6. Measurementsf§ of protein and pigment contents will be performed (*see* **Note 1**).

2.1.2. Spinach chloroplast envelope

Chloroplast envelope membranes will be prepared from spinach leaves in Grenoble. Measurement of protein and pigment contents will be performed during the practical course.

2.2. Material

2.2.1. Material for membrane treatment

1. Centrifuge (Eppendorf centrifuge 5415D or equivalent) placed in a cold room with 1.5 ml plastic tubes
2. Branson sonifier model 250 (or equivalent), with 3 mm microtip and ice bucket
3. Nitrogen (or Argon) gas supply (cylinder) with gas pressure regulator connected to a Pasteur pipette via a plastic tube.

2.2.2. Other materials

1. UV-visible spectrophotometer (Kontron, Uvikon 810 or equivalent), with 1-cm (disposable, glass or UV silica) cuvettes, for pigment analyses
2. Nitrocellulose membranes (BA85, Schleicher & Schuell or equivalent), for western blots
3. Gel electrophoresis apparatus (BioRad Protean 3 or equivalent), with the different sets of accessories (a) for protein separation by electrophoresis (combs, plates, and casting accessories) and (b) for protein transfer on nitrocellulose membranes (central core assembly, holder cassette, nitrocellulose filter paper, fiber pads, cooling unit),

2.3. Media for membrane treatments

2.3.1. Media for detergent extraction

- Solubilization solution: 50 mM MOPS/NaOH pH 7.8, 1 mM DTT) containing either 1% (v/v) Triton X-100 or 0.1 M CHAPS (*see* **Note 2**).

2.3.2. Media for chloroform/methanol extraction

1. Chloroform/methanol mixtures in the following proportions: 0:9, 1:8, 2:7, 3:6, 4:5, 5:4, 6:3, 7:2, 8:1, 9:0 (v/v)
2. Cold (-20°C) acetone for a 80% final concentration, in water

2.3.3. Media for alkaline or salt washing of membranes

1. Na_2CO_3: 0.1 M final concentration (1M stock solution)
2. NaOH: 0.1 M or 0.5 M final concentration (2 M stock solution)
3. NaCl: 1 M final concentration (2 M stock solution)

2.4. Solutions for SDS-PAGE and protein transfer on nitrocellulose

1. Acrylamide stocks: 30% (w/v) acrylamide – 0.8 % bisacrylamide: 300 g acrylamide, 8 g bisacrylamide, H_2O to 1 liter. 60% (w/v) acrylamide – 0.8 % bisacrylamide: 600 g acrylamide, 8 g bisacrylamide, H_2O to 1 liter and store in amber bottles at 4°C
2. SDS stock solution: 10% (w/v) SDS: 10g SDS, H_2O to 1 liter and store at room temperature
3. Gel buffers: 4 x Laemmli stacking gel buffer (0.5 M Tris-HCl, pH 6.8): 363 g Tris, H_2O to 900 ml, adjust to pH 8.8 at 25°C with concentrated HCl, make up volume to 1 liter and store at room temperature. 8 x Laemmli resolving gel buffer (3 M Tris-HCl, pH 8.8): 60.6 g Tris, H_2O to 900 ml, adjust to pH 6.8 at 25°C with concentrated HCl, make up volume to 1 liter and store at room temperature.
4. Stacking gel (5% acrylamide): 5 ml 30% acrylamide – 0.8 % bisacrylamide stock solution, 7.5 ml 4 x Laemmli stacking gel buffer, 17.1 ml H_2O, 40 □l TEMED, 4 ml 10% ammonium persulfate (10 g ammonium persulfate, H_2O to 100 ml, stored at 4°C, prepare fresh every month), total volume: 30 ml.
5. Single acrylamide concentration gels (10, 12 or 15% acrylamide):
   - for 10% acrylamide gel: 33.3 ml 30% acrylamide – 0.8 % bisacrylamide stock solution, 12.5 ml 8 x Laemmli resolving gel buffer, 54 ml H_2O, 20 □l TEMED, 0.2 ml 10% ammonium persulfate, total volume: 100 ml
   - for 12% acrylamide gel: 40 ml 30% acrylamide – 0.8 % bisacrylamide stock solution, 12.5 ml 8 x Laemmli resolving gel buffer, 47.3 ml H_2O, 20 □l TEMED, 0.2 ml 10% ammonium persulfate, total volume: 100 ml
   - for 15% acrylamide gel: 50 ml 30% acrylamide – 0.8 % bisacrylamide stock solution, 12.5 ml 8 x Laemmli resolving gel buffer, 37.3 ml H_2O, 20 □l TEMED, 0.2 ml 10% ammonium persulfate, total volume: 100 ml
6. Protein solubilization: 4X stock solution: 200 mM Tris / HCl, pH 6.8, 40% (v/v) glycerol, 4% SDS (v/v), 0.4% (v/v) bromophenol blue, 100 mM dithiothreitol
7. Gel reservoir buffer: 38 mM glycine, 50 mM Tris, 0.1% SDS (about 400 ml in each reservoir)
8. Gel staining medium: 10% (v/v) acetic acid, 25% isopropanol, 2.5 g / l Coomassie brilliant blue R250, in water
9. Gel destaining medium: 7% (v/v) acetic acid, 40% ethanol, in water
10. Protein transfer medium (for western blots): Gel reservoir buffer (see above) diluted with ethanol to obtain 20% (v/v) final ethanol concentration. Final concentration: 30.4 mM glycine, 40 mM Tris, 0.08% SDS (about 800 ml)

3. Methods

3.3. Assessment of organelle or membrane purity (see Notes 3 and 4)

On a routine basis, three types of markers are used to characterize the different fractions (organelles, membranes…) prepared: enzymatic markers, immunological markers and lipid/pigments markers. Pigments (chlorophyll and carotenoids) are the most conspicuous markers from chloroplast membranes.

3.3.1. Immunological markers

3.3.1.1. Antibodies used

1. anti-ceQORH antibody (7) raised against a protein from the inner envelope membrane of *Arabidopsis* chloroplast (used at 1/10000)
2. anti-LHCP antibody (8) raised against a thylakoid membrane protein from *Chlamydomonas reinhardtii* chloroplast (used at 1/5000)

3.3.1.2. Western blot analyses

Western blots are performed after separation of membrane proteins by SDS-PAGE (see below for a description of the method). After gel migration, the proteins are transferred to a nitrocellulose membrane using the Gel transfer apparatus (BioRad Protean 3 Mini Trans-Blot module or equivalent).

1. Prepare the cassette as follows: add successively 1 fibber pad, 3 nitrocellulose filter papers, the gel, a nitrocellulose membrane (BA85, Schleicher & Schuell or equivalent), 3 nitrocellulose filter papers, 1 fibber pad, and then insert the sandwich in the holder cassette (the membrane should be placed beside the + electrode).
2. Insert the cassette in the central core assembly unit (together with the cooling unit).
3. Perform the transfer for 2 hours at 80 V in protein transfer medium.
4. Recover the nitrocellulose membrane.
5. Follow the instructions for saturation and incubation of the membrane with primary and secondary antibodies (*see* **Note 5**) provided by the manufacturers.

3.3.2. Lipids and pigments

*3.3.2.1. Determination of the chlorophyll content (see **Note 6**) of a fraction*

Media: 80% (v/v) acetone, in water.

Procedure (adapted from Arnon, 9): Add 10 µl of the extract to be analyzed to 1 ml 80% (v/v) acetone in a 1-ml Eppendorf tube. Vortex and incubate for 15 min on ice and in the dark. Centrifuge for 15 min at 16,000 g. Pour in a 1-ml spectrophotometer glass cuvette. Measure the absorbance at 652 nm against a tube containing 80% (v/v) acetone for the zero. A ratio of $OD_{652}/36 = 1$ corresponds to 1 mg chlorophyll ml^{-1}.

3.3.2.2. Pigment extraction and analyses

Lipid and pigment extraction (adapted from Bligh and Dyer, 10)

1. In order to form one liquid phase and subsequently extract the lipid, mix 200 µl of membrane suspension with 750 µl of a methanol/chloroform (2:1, v/v) mixture. Homogenize with a vortex, then add 250 µl water and 250 µl chloroform. Homogenize with a vortex.
2. Centrifuge the mixture for 10 min at 14000 g in order to get a two-phase system. Discard the upper phase with a pipette.
3. Remove the lower phase (*see* **Note 7**) by aspiration with a Pasteur pipette. Dry it under a stream of argon (or nitrogen). The residue is dissolved in a minimal volume of chloroform or 80% acetone.

Pigments analyses

1. Dissolve the lipid extract (prepared as in 3.3.3.1) in 80% acetone (1ml, final volume). Pour the solution in a 1-ml spectrophotometer cuvette.
2. Record the absorption spectrum between 350 and 750 nm. Carotenoids are responsible for a series of peaks in the 400-500 nm region of the spectrum whereas chlorophylls show in addition a sharp peak with a maximum in the 650-700 nm region (*see* **Note 8**).

3.4. Differential extraction of membrane proteins (see Note 9).

3.4.1. Protein solubilization with detergents

1. Dilute the membrane proteins (0.2 mg) in 0.2 ml of solubilization solution (50 mM MOPS/NaOH pH 7.8, 1 mM DTT) containing either 1% (v/v) Triton X-100 or 0.1 M CHAPS (*see* **Note 10**).
2. After 30 min incubation on ice, centrifuge the mixture for 15 min (4°C) at 15000 *g* (Eppendorf centrifuge 5415D or equivalent with 1.5 ml Eppendorf tubes) to separate two

fractions: the supernatant containing proteins solubilized by the treatment and the pellet containing the insoluble proteins.

3. Solubilize the insoluble protein pellets in 50 µl of the following solution: 50 mM MOPS/NaOH pH 7.8, 1 mM DTT.

4. Analyze the proteins by SDS-PAGE (see below).

*3.4.2. Membrane protein solubilization with chloroform/methanol mixtures (see **Note 11**)*

1. Dilute slowly one volume of the membrane preparation (0.5 to 1 mg protein in 0.1 ml of original buffer) (*see* **Note 12**) in 9 volumes of cold chloroform/methanol (5/4, v/v) mixtures in Eppendorf tubes (1.5 ml) (*see* **Note 13**).

2. Store the resulting mixtures for 15 min on ice before centrifugation (4°C) for 15 min at 15,000 g (Eppendorf).

3. Recover the organic phase (the white pellet containing less hydrophobic proteins is discarded). The pellet contains the chloroform/methanol-insoluble proteins (or organic solvent insoluble fraction). The supernatant contains the chloroform/methanol-soluble proteins (or organic solvent soluble fraction).

4. Then evaporate (*see* **Note 14**) the organic phase under nitrogen (to 200 µl for large amounts of proteins or 100 µl when original protein concentration is limited). Directly precipitate the proteins by adding 4 volumes (800 µl or 400 µl) of cold (-20°C) acetone (80% final acetone concentration) directly to the remaining volume of chloroform/methanol.

5. Store the resulting mixtures for 15 min on ice before centrifugation (4°C) for 15 min at 15,000 g (Eppendorf).

6. Eliminate the organic supernatant, dry the protein pellet (*see* **Note 15**) on the bench and not under nitrogen. Be sure that there is no more acetone (*see* **Note 16**). Resuspend (*see* **Note 17**) the protein pellets in 20 µl of concentrated SDS/PAGE buffer (4X) and store the protein mixtures in liquid nitrogen.

7. Analyze the proteins by SDS-PAGE (various volumes on separates lanes).

3.4.3. Alkaline or salt washing of the membrane fractions

1. Dilute slowly one volume of the membrane preparation (0.5 to 1 mg protein in 0.1 ml) to 0.5 ml with Na_2CO_3, NaOH or NaCl stock solutions to obtain 0.1 M, 0.5 M or 1 M final concentrations, respectively (*see* **Note 18**).

2. Sonicate the resulting mixtures 2 to 5 times 10 sec., the power set at 40% duty cycle, output control 5, in ice.

2. Store the mixtures for 15 min on ice before centrifugation (4°C) for 20 min at 15000 *g* (Eppendorf centrifuge 5415D, or equivalent, with 1.5 ml Eppendorf tubes).

3. Recover insoluble proteins as pellets (*see* **Note 19**), resuspend them in 20 µl of SDS/PAGE buffer (4X). Store the protein extracts in liquid nitrogen.

4. Analyze the proteins by SDS-PAGE (see below).

*3.5. Separation of membrane proteins by 1D SDS-PAGE (see **Note 20**)*

1. Prior to the experiment, prepare slab gels for protein electrophoresis (*see* **Note 21**):

- Prepare the gel apparatus according to the manufacturer specifications (*see* **Note 22**)

- Prepare the different gel solutions (stacking gel, 10%, 12% or 15% separation gel). The volumes to be used are determined by gel dimensions, and therefore by the specifications of the apparatus.

2. Heat the protein samples at 95°C for 5 min to solubilize the proteins. Add bromophenol blue dye in the samples. Place protein samples (20 µl) into gels slots by means of a pipette.

Mr markers (prestained SDS-PAGE markers low range from Bio-Rad or equivalent) are placed in another slot.

3. Set the conditions for the electrophoresis at 150 volts. Run gels for 1 hour at room temperature (until the bromophenol blue dye reaches the lower part of the gel) (*see* **Note 23**).

4. After electrophoresis, remove the gels; place them in plastic boxes in presence of staining solutions. Shake the box gently for 30 min. Pour off the staining solution and replace it by destaining solution. Shake the box gently for 15 min. Repeat the washing step once or twice.

5. *In gel* protein digestion for proteomic analyses (*see* **Note 24**).

4. Notes

1. Protein contents of membrane fractions are estimated using the Bio-Rad protein assay reagent (11).
2. A wide variety of detergents can be used: Triton X-100, CHAPS, Triton X-114, etc... (see ref. 12).
3. The use of Percoll-purified chloroplasts is very efficient to limit contamination of envelope membranes by extraplastidial membranes, as demonstrated by the absence of phosphatidylethanolamine and of different marker enzymes or proteins (13). Therefore, at this stage, the major possible contaminants of envelope preparations are soluble stroma proteins and small pieces of thylakoid membranes. Such cross contamination have been extensively analyzed by Ferro et al (2). Being the most likely source of membrane contamination of the purified envelope fraction, thylakoid cross-contamination needs to be precisely assessed. The yellow colour of purified envelope vesicles first indicates that this membrane system contain almost no chlorophyll and therefore very few contaminating thylakoids. Indeed, by western blot analyses using antibodies raised against LHCP, Ferro et al. (2) demonstrated that several independent *Arabidopsis* envelope preparations appeared to contain between 1 and 3% thylakoid proteins.
4. A thorough study of membrane purity is essential for a precise determination of the subcellular localization of the proteins of interest. An example of a protein previously expected to be located in the plasma membrane, but actually residing to the inner envelope membrane is given by Ferro et al. (1).
5. Several dilutions of the primary antibodies should be tested to identify the best signal/noise ratio.
6. The chlorophyll content was 170 mg per mg protein in chloroplasts purified from *Arabidopsis* leaves and 84 mg per mg protein in crude leaf extract (enrichment of 2). By comparison, chlorophyll concentration in crude protoplast extract is about 4.5 mg chlorophyll mg^{-1} protein (4).
7. The chloroformic (lower) phase contains lipids and pigments.
8. When correctly prepared, chloroplast envelope membranes do not contain chlorophylls, but only carotenoids. Plasma membranes, when highly purified, are expected to contain no trace of chlorophyll or carotenoids.
9. Because of the high functional value of a precise subcellular localization, we therefore focus in this article on the proteins that are the most tightly associated with the membranes. Therefore, in all cases, we analyze fractions containing the most hydrophobic proteins: i.e. the chloroform/methanol soluble proteins or the proteins remaining in the membrane after its treatment by NaOH. The discarded fractions contain a large variety of rather hydrophilic proteins, some of high interest. However, since many of them are also present in the cytosol, or in the chloroplast stroma, or any soluble extract from plant tissues, their subcellular localization cannot be precisely determined. They are of strong interest in

several cases: for instance for analyses of the protein content of the thylakoid lumen (14-16).

10. A wide variety of detergents can be used: triton X-100, CHAPS, triton X-114, sulfobetains, etc... The reader is referred to articles by Santoni et al. (12,17) for detailed analyses of membrane treatment by detergents.
11. First, be sure that the membrane preparation does not contain too many hydrophilic proteins deriving from contamination of the membrane fraction with soluble compartments (this protocol is not to be used on a crude cell extract for example, but can be used on a crude membrane extract). Hydrophilic proteins will precipitate during the process. A too large amount of these hydrophilic proteins would co-precipitate your hydrophobic proteins.
12. Most of the time, we use MOPS 10 mM pH 7.0 as a buffer.
13. The volume ratio between chloroform and methanol for an optimal extraction can be determined by comparing the polypeptide profile of the organic phase soluble proteins prepared as follows: membranes (5 mg proteins in 1 ml storage buffer) are divided in 10 fractions of 0.1 ml (in 1.5 ml Eppendorf tubes). The membrane fraction is then slowly diluted by addition of 0.9 ml of cold chloroform/methanol solutions (0:9, 1:8, 2:7, 3:6, 4:5, 5:4, 6:3, 7:2, 8:1 and 9:0, v/v). In general, the total volume of the mixture is 1 ml. If necessary, this can be increased to a much higher value when more membrane material is available.
14. Do not completely dry the sample!
15. Due to acetone precipitation (and removal of pigments), the pellet turns white, be careful in order not to loose it.
16. Trace amounts of solvent strongly limits protein resuspension.
17. Be patient, wash tube walls and avoid bubbles!
18. Treatment of membranes with these various compounds do not results in the extraction of the same proteins (2,4,5). Na_2CO_3 or NaCl extract proteins that are rather weakly associated with the membrane whereas NaOH removes proteins that are more tightly associated. It is therefore recommended to try several of these compounds to achieve more comprehensive analyses.
19. The supernatant contains the proteins removed from the membrane by alkaline or salt treatment, i.e. the less hydrophobic membrane proteins.
20. Classical proteomic methodologies based on the use of 2-D gel electrophoresis proved to be rather inefficient on membrane proteins. In general, almost no highly hydrophobic proteins, as defined by average hydrophobicity values, are found on 2-D gel separations of membrane proteins. Adessi et al. (18) observed that loading 2-D gels with high amounts of membrane proteins, resulted in the severe loss of hydrophobic proteins and therefore in the artifactual enrichment of the less hydrophobic components (and hydrophilic contaminants of the purified membrane fraction). In this case, hydrophobic proteins probably precipitated at their isoelectric point in the first dimension, thus preventing any further migration and separation in the second dimension (18). In contrast, 2D-gel electrophoresis is very efficient to analyze peripheral membrane proteomes (see 14-16,19). Strategies for membrane proteomics based on 2D-electrophoresis combined with a wide diversity of detergents have been extensively analyzed by Santoni et al. (12,17).
21. We use routinely the procedure described by Chua (20) to separate membranes proteins by SDS-PAGE. This article describes in detail all stock solutions, medium for stacking and separation gels.
22. We used a Bio-Rad apparatus, with 7-cm long gels.

23. For some analyses, protein migration can be stopped just between the stacking and the separating gels so that proteins are concentrated in a very thin band for further nanoLC-MS/MS analyses.
24. Gel pieces can be stored in the cold room until proteomic analyses. Because the description of different methods for mass spectrometry (Maldi-Tof, nanoLC-MS/MS, …) are out of the scope of this article, the readers are referred to the description by Ferro et al. (3) for detailed conditions for *in gel* digestion and further MS analyses.

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P6/P7. Assembly of photosynthetic complexes and spectroscopic analysis

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The goal of practical P6 and P7 is to analyse by a combination of various approaches, the phenotype of five *C. reinhardtii* mutant strains, A-E, each altered in a different photosynthetic function, in comparison to the reference wild-type strain (F). Analysis will include i) pulse-labelling experiments to identify alterations in the translation pattern of chloroplast genes, ii) immunoblot analyses, using specific antibodies, of the accumulation of subunits from photosynthetic protein complexes, iii) Coomassy and TMBZ staining of thylakoid membrane polypeptides to identify deficiency in polypeptides and cytochrome-binding proteins accumulation, respectively iv) non-denaturing gel electrophoresis, "green gels", to study the assembly of photosynthetic protein complexes in the mutant strains (practical P6), coupled to spectroscopic functional analyses performed on whole cells and isolated photosynthetic complexes (practical P7). This will ultimately lead to the identification of the photosynthetic function affected in each mutant strain and to a critical discussion/comparison of their phenotypes.

Short-time ^{14}C acetate pulse labelling experiments.

The aim of this experiment is to visualise specifically the newly synthesised products of chloroplast genes, as cycloheximide, preventing cytosolic translation, is added at the beginning of the experiment.

Start from cells in "good physiological state", freshly replated on TAP plates and inoculate a 200ml culture. Let it grow to ~ 2-3 x 10^6 cells.mL^{-1} (above 5 x 10^6 cell.ml^{-1} cells enter in stationary phase and will not incorporate acetate very well)

The day before the experiment, dilute the cells on fresh liquid TAP medium to 0.5-1 x 10^6 C.mL^{-1} and let them grow for the night (they should reach 2 x 10^6 the next morning).

then:

1. Centrifuge the culture 5500 rpm, 5' at RT
2. Resuspend the cell pellet in 50-100ml of MM-Tris to wash away remaining acetate
3. Centrifuge again 5500 rpm, 5', RT
4. Resuspend the cells in MM-Tris (to ~2-4 10^7 Cells.ml^{-1}) and transfer 5 mL into a small erlen (50ml)
5. Let the cells recover and intracellular pool of organic carbon deplete for 1-2 hours under dim light (~1000 lux) under strong agitation (200-300 rpm) on a rotatory shaker (good aeration $\Rightarrow$ active respiration $\Rightarrow$ active metabolism and good uptake of acetate)
6. Add 5 µL of inhibitor of cytosolic translation (cycloheximide –stock solution 10 mg.mL^{-1} in H_2O, kept at room temperature-), then 125 µL of ^{14}C Na Acetate (Amersham ref CFA13, 51-62 mCi/mM; 200 mCi/mL; 5 µCi.mL^{-1} final). Mix briefly by gentle swirring.
7. Allow pulse-labelling to proceed for 5'
8. Transfer the cell suspension into an 50 mL Corning tube containing 35 mL of ice-chilled TAP medium with 50 mM unlabelled Acetate
9. Centrifuge 5', 7500 rpm at 4°C.
10. Discard the radioactive medium and resuspend the cells in 2 mL Hepes ice-chilled washing buffer.
11. Transfer the cell suspension in two ependorfs (on ice) and centrifuge in a microfuge at maximal speed for 1'.
12. Remove the supernatant and resuspend cells (using vortex) in ~50 µL of 0.1 M DTT/0.1 M Na_2CO_3 + 1 mM PMSF
13. Freeze the tubes in liquid nitrogen.

For electrophoretic analysis of the samples:

1. Thaw on ice one tube (out of two) for each strain
2. Puncture the cap of the ependorf with a syringe needle. Add 30µL of (SDS10%, 30% sucrose) to each tube, vortex briefly and immediately denature the proteins for 90' in boiling water.
3. Cool down on ice
4. Centrifuge 15' at maximal speed in a microfuge at 4°C to pellet insoluble material.
5. For chlorophyll concentration determination, dilute 4 µL of the supernatant with 800 µL H_2O and measure the optical density at 680 nm (optical path: 1cm). An OD_{680} of 0.11 corresponds to 1 µg.μL^{-1} of chlorophyll in the sample. 15 µg of chlorophyll are typically loaded on a lane. Lower amount (10 or even 7.5 µg of chlorophyll per lane) will increase the

resolution of the gel but also the duration of exposure on a PhosphorImager screen (up to a month).
The volume of sample to load on a lane is, thus, v (µL) = 15 x 0.11/OD_{680} = 1.65/OD_{680}

HEPES washing buffer

Stock Solution	volume	[final]
1M HEPES pH 7.5	1 mL	20 mM
0.5 M EDTA pH 7.5	1 mL	10 mM
100 mM PMSF	100 µL	0.2 mM
500 mM Benzamidine	100 µL	1 mM
500 mM e amino caproic acid	500 µL	5 mM
ddH_2O	q.s.p 50 mL	

0.1M DTT/0.1M Na_2CO_3

Stock solution	volume	[final]
1M DTT	100 µL	0.1M
1M Na_2CO_3	100 µL	0.1M
H2O milliQ	800 µL	

Stock solutions: 1.0 M DTT (dithiothreitol) in sterile ddH_2O. 100 µL aliquots stored at –20°C. DTT is a reducing agent required to reduce disulfide bonds in proteins. It degrades rather easily and therefore is added to samples from a freshly thawed stock tube.

1.0 M Na_2CO_3 in ddH_2O. (stored at RT).

Preparation of stock solutions for Acrylamide gels

Acrylamide/bis-acrylamide monomer stock solution (60% acrylamide/0.8% bisacrylamide).
(*WARNING: non-polymerized acrylamides are neurotoxins! Wear gloves, Avoid skin contact and inhalation.) 30% acrylamide/0.8% bis-acrylamide stocks are commercially available, but not the 60-0.8% stock solution that has to be prepared in the lab. Wear a dust mask when weighing acrylamide powder.
For one litter
Weight 600g of acrylamide powder and 8 g of Bis-acrylmaide in a graduated cylinder. Add water up to 850 mL (be careful, acrylamide will expand upon dissolution). Mildly heat the solution (30-40°C) by placing the cylinder in a water bath to help dissolution (may require a couple hours). Add a magnetic stir bar and stir gently until the crystals dissolve. Adjust volume to 1L. Add 2-4 g of activated charcoal powder and keep stirring for another hour. Vacuum-filtrate the solution, once on a filter paper, then on a 1.2 µm nitro cellulose filter, last of 0.22 µm nitrocellulose filter.
Store stock solution in dark at 4°C. Acrylamide will crystallise but turns back in solution upon heating. *(Such solutions are usually good for up to a year.)*

If needed, prepare the 30-0.8% solution similarly, starting from 300 g of acrylamide powder.

3M Tris-Cl pH 8.8 stock solution.
Dissolve 363 g of Tris-base in ddH_2O Keep the volume to 800-900 mL. Adjust the pH to 8.8 with concentrated HCl, (requires about 50mL). Warning: ***the pH should be measured at 25°C***. As dissolution of Tris is endothermic, you will have to heat the solution in a water bath. Adjust volume to 1L. Vacuum filter on .22 µm nitrocellulose filter.
Store at 4°C.

Tris-Cl 0.5M, pH 6.8

Dissolve 60.5 g of Tris-base in ddH_2O. Keep the volume to 800-900 mL. Adjust the pH to 6.8 with concentrated HCl, (requires a few mL). Adjust volume to 1L. Vacuum filter on .22 µm nitrocellulose filter.
Store at 4°C.

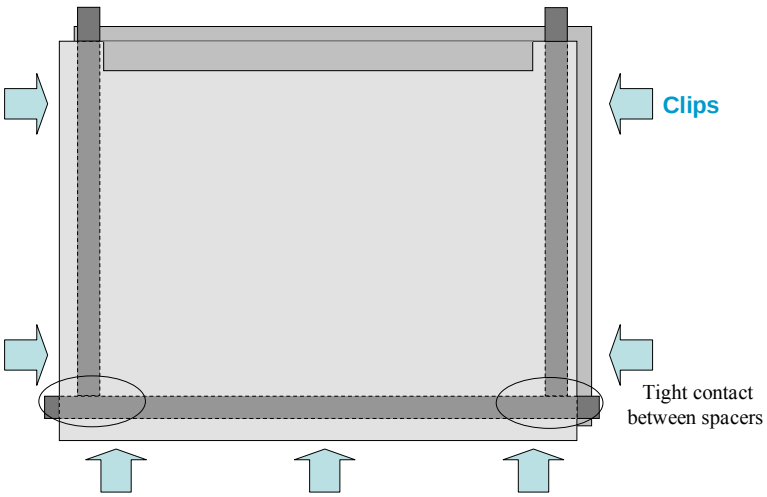
Gradient 12-18% polyacrylamide, 8M Urea gels allow a high resolution of polypeptides.

35 x 27 cm, 1mm thick gels.

1. Clean two glass plates (the rectangular and the notched one) with ddH_2O, then with Glassex and rinse with EtOH

2. Wash one long spacer, two smaller ones and one comb with ddH_2O then Glassex.

3. Assemble the gel sandwich. Place spacers at about 1 cm from the edge of the rectangular glass plate (the three spacer should be in tight contact), overlay with the notched glass plate and clamp with 7 clips (3 at the bottom and two on each side).



4. Add about 250 µL of 10% APS solution to 20ml of PLUG solution. Pour the PLUG solution with a Pasteur pipette along the external side of the three spacers.

5. Once PLUG is polymerised, install the assembled glass gel sandwich in a vertical position and hold it with a forceps.

6. Prepare the 12% and 18% acrylamide, 8M Urea solutions into 2 separate 50 mL flasks. Add reagents in order. Wear gloves. Mix reagents by gentle swirling and heat mildly on heating magnet stirring unit until urea is completely dissolved. Avoid aeration or introduction of air bubbles.

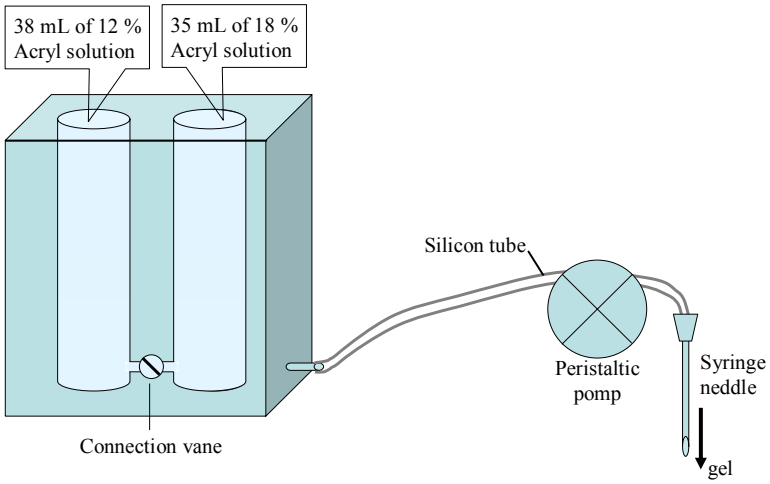
Stock Solution	**18%**	**12%**
Acryl. 60%, 0.8% bis acryl. **(ml)**	**12**	**8**
Tris-Cl pH 8.8 3M (ml)	**5**	**5**
Urea (g)	**19.4**	**19.4**
Sucrose (g)	**5.3**	**-**
H_2O (ml)	**7**	**12.8**

7. Rinse abundantly the gradient maker apparatus with ddH_2O. Close the communication vane. Set up the speed of the peristaltic pomp to 70 and press Stop.

8. Cool down 12 and 18% acrylamide solutions to RT.

9. Add the polymerisation catalysts to each gel solution.
   TEMED (N,N,N',N'-tetramethyl-ethylene-diamine): 6.4 and 9.6 µL in 18% and 12% solution, respectively
   24µL of APS 10% in each solution.

10. Pour 38 mL of 12% solution in the left chamber of the gradient maker and 35ml of 18% solution in the right chamber.



11. Start pouring the resolving gel by pressing the start button of the peristaltic pomp. Open the communication vane when the 18% solution reaches the spacer on the right.

12. Stop pouring when the resolving gel is at about 3-4 cm from the top of the gel.

13. Add ~1ml of iso-Butanol on top of the gel. Place the comb and clamp it with two clips.

14. Let the polymerisation proceed for at least two hours. A line between the polymerised gel and the polymerisation water should appear.

15. Prepare the stacking gel solution

Stacking gel

Stock solutions	for 1 gel	for 2 gels
Acryl 30%, 0.8% bis acryl.	5	10
Tris-Cl pH 6.8 0.5M	7.5	15
H_2O	17.3	34.6

16. Remove the comb, pour off iso-Butanol and polymerisation water, rinse the surface of the gel with ddH_2O. Wipe water off with a 3MM Whatman paper, being careful not to damage the top of the gel.

17. Add the catalysts (TEMED 20 µL; APS 200µL) to the stacking gels and pour it.

18. Introduce the comb. Avoid air bubbles, the top of the teeth should be at the level of the edge of the notched glass plate. Fix with two clips.

19. Let the stacking gel polymerise for at least half an hour.

20. Remove clips from the bottom of the gel, remove the bottom spacer and rinse with ddH_2O. Wipe off water with a 3MM Whatman paper.

21. Fill up the space between the two glass plates with plug (same as in 4).

22. Remove the clips and clean the notched glass plates with ddH_2O.

23. Wash two silicon tubes with ddH_2O and Glassex.

24. Wash the electrophoresis unit with Glassex and install the polymerised gel sandwich. Intercalate the two silicon tubes between the notched plate and the electrophoresis apparatus. Clamp the sandwich with two clips on each side.

25. Prepare and pour the two migration buffers (Upper and lower) in the respective buffer tanks

Migration buffers

Stock solution	Lower	Upper
Tris-Glycine x5	100 mL	360 mL
EDTA pH 7.5 0.2M		4.5 mL
SDS 20% Pierce		4.5 mL
H_2O	q. s. p 500mL ;	q. s. p 900mL

26. Remove the comb.

27. Load samples carefully into the wells with a Hamilton syringe. Avoid bubbles. The samples should sink to the bottoms of the wells. Up to 80 µL can be loaded in a well, but smaller volumes (of more concentrated samples, typically 10-20 µL) allow a better resolution.

28. Connect the gel apparatus to the power supply. SDS is negatively charged, therefore proteins migrate toward the positive (+) pole. Attach the positive lead to the bottom of the gel. For one gel use 18 mA (16 mA for green gels) constant current. Allow the chlorophyll front to migrate to the bottom (about 18 hrs).

Cold 12% acrylamide PAGE to separate chlorophyll-protein complexes.

Run gel in the cold-room (4°C). Same as above but omit EDTA in the Upper migration buffer and replace steps 6-11 by:

6. Prepare the 12% acrylamide solution into a 100 flask. Add reagents in order. Wear gloves. Mix reagents by gentle swirling. Avoid aeration or introduction of air bubbles.

12 % Acrylamide gels.

Stock solution	**V (mL)**
30% acryl., 0.8% bis acryl.	**32**
Tris-Cl 3M pH 8.8	**10**
ddH_2O	**38**
V_{total}	**80**

7. Add the polymerisation catalysts.
   TEMED: 20 µL
   10% APS: 200 µL.

8. Pour gently resolving gel from the becher into the glass sandwich.

PLUG

Acryl 30% 200 mL

H2O	300 mL
TEMED	0.5 mL

Notes:
non-polymerized acrylamides are neurotoxins! Wear gloves, avoid skin contact and inhalation.
TEMED is toxic. Avoid breathing vapours.
10% APS (ammonium persulfate): made fresh, stored up to a month at 4°C

5X Tris- Glycine

Tris base	60.5 g
Glycine	288 g
H2O q.s.p.	2 L

pH should be between 8.6 and 8.8

protease inhibitors

	Stock solution	Working concentration	dilution
EDTA	0.2 M pH 7.5	1mM	x 200
PMSF	0.1M ds EtOH	100 µM	x 500
Benzamidine-Cl	500 mM dans H_2O	5 mM	x 100
e-amine caproïque	500 mM dans H_2O	5 mM	x 100

Note: **a)** PMSF is toxic (wear gloves)**,** b) PMSF should be added immediately before use as it degrades in aqueous solution.

Membrane purification

(*Adapted from : Chua and Bennoun (1975) Proc Natl Acad Sci U S A. 72:2175-9*)

All steps are carried out at 4°C

- Centrifuge 200 ml culture, 5 000 rpm 5 mn, discard supernatant, wipe off remaining liquid
- Resuspend in 5 ml HEPES 2
- Place in cold French press cell, remove all air
- Break at 6 000 psi (400 psig on Aminco, with 1'' diameter cell on "high" ratio)
- Centrifuge in SS34 or equivalent, 10 000 rpm10 mn
- Remove supernatant (pipet), wipe sides of tube
- Resuspend pellet in 3 ml HEPES 3 with Potter
- Place at the bottom of a SW41 Ultra-clear tube
- Overlay with 4 ml HEPES 4, then 4 ml HEPES 5
- Centrifuge 1h at 40 000 rpm
- Using a needle and syringe, collect membranes at the interface between HEPES 4 and 5, and in the HEPES 4

- Dilute into HEPES 6 (at least 5 volumes), centrigue in SS34 at 20 000 rpm 20 min
- Resuspend in HEPES 6 or DTT/carbonate

BUFFERS:

Stocks : 1M HEPES-KOH pH 7.5
2 M Sucrose
0.2M EDTA pH7.5
1 M $MgCl_2$

			Add:
HEPES 2 :	5 mM HEPES	:	5 ml HEPES
	10 mM EDTA	:	50 ml EDTA
	0.3 M sucrose	:	150 ml sucrose
	H_2O	:	complete to: 1 l
HEPES 3 :	5 mM HEPES	:	1 ml HEPES
	10 mM EDTA	:	10 ml EDTA
	1.8 M sucrose	:	180 ml sucrose
	H_2O	:	complete to: 200 ml
HEPES 4 :	5 mM HEPES	:	1 ml HEPES
	10 mM EDTA	:	10 ml EDTA
	1.3 M sucrose	:	130 ml sucrose
	H_2O	:	complete to: 200 ml
HEPES 5 :	5 mM HEPES	:	1 ml HEPES
	0.5 M sucrose	:	50 ml sucrose
	H_2O	:	complete to: 200 ml
HEPES 6 :	5 mM HEPES	:	5 ml HEPES
	10 mM EDTA	:	50 ml EDTA
	H_2O	:	complete to: 1 l

To all solutions, protease inhibitors are added just before the experiment :
Phenylmethyl sulfone fluoride (PMSF): 200µM (from 100 mM stock in ethanol)
Benzamidine : 1 mM (from 100 mM stock)
ε-aminocaproic acid: 5 mM (from 500 mM stock)

solubilisation of membrane samples for loading on "green gel", run at 4°C.

1. to 80 µL of membrane samples add 20 µL of 5% SDS/30% sucrose (colourless) (final conditions for solubilisation: 1 mg chloro.ml^{-1}, 1 % SDS)
2. Vortex and transfer immediately to cold room.
3. Load 20 µL/lane (20 µg chloro.).

Coomassie staining

Carefully place the gel into a tray that contains 0.1 % Coomassie Blue R-250, 40% methanol, and 7% acetic acid. Stain with gentle agitation for at least one hour. Replace staining by distaining solution (40% methanol, 7% acetic acid). Gentle agitation from one hour to overnight. Change distaining solution if necessary, until bands become visible.

TMBZ staining

Incubate gel in solution A, freshly made up from :

- 250 mg tetramethyl-benzidine (6.3 mM final) in 120 ml methanol; dissolve in darkness at room temperature, then add
- 70 ml of Na acetate, 1M, pH5.0 with acetic acid (175 mM final)
- 210 ml H2O

Gently shake gel at room temperature for 45-60 min
Add 1.5 ml H_2O_2 (30 mM final)

Staining will develop over 10-15 mn, and can be enhanced by placing gel at 4°C
Further staining with Coomassie Blue or silver is possible after rinsing out precipitated solution

Semi-dry electrotransfer of proteins for immunoblot analysis

1. Before the experiment cut 10 pieces of 3MM Whatman paper, 1 piece of Ceralane and 1 nitrocellulose membrane (Hybond-C extra Amersham) to the size of the gel. Add 1.05g of ε-amino caproic acid to 200mL of **transfer buffer 1** and prepare 3 trays with 200, 400 and 200 mL of **transfer buffer 1, 2 and 3**, respectively.

2. Rinse anode (+) with ddH_2O. Do not let it dry.

3. Soak three sheets of Whatman paper (of the same size than the gel) into **transfer buffer 3**, place them on anode.

4. Prepare the Transfer pack on a clean rectangular glass plate.

5. Soak 2 sheets of Whatman paper (of the same size than the gel) into **transfer buffer 1** and place them on the glass plate. Soak the piece of Ceralane into **transfer buffer 1.**

6. Place the gel, cut to the desired size with a pizza knife and briefly immerged into **transfer buffer 2**, on these sheets. Long incubations of the gel in **transfer buffer 2** (more than one minute or two) will result in gel expansion.

7. Soak the nitrocellulose membrane in **transfer buffer 2**. Overlay the surface of the gel with a few mL of **transfer buffer 2** and place the membrane on the gel, carefully avoiding trapping air bubbles.

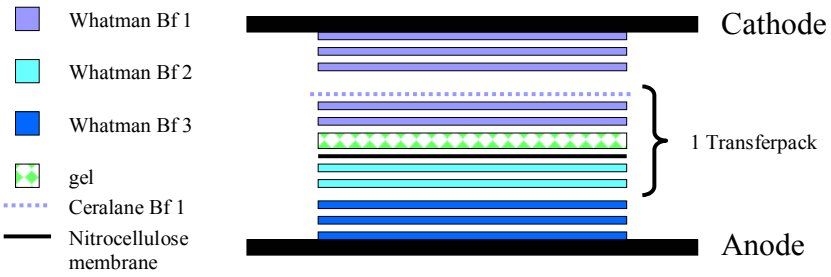
8. Cut overhanging gel with the pizza knife.

9. Overlay with 2 pieces of Whatman paper, first soaked into **transfer buffer 2**. Roll a pipette through the transfer pack to catch away any remaining bubbles.

10. Take the transfer pack from the glass plate, turn it upside down, and lay it down of the buffer 3-soaked papers on the anode. Add Ceralane, soaked in **transfer buffer 1** in step 5, on top of the transfer pack. Up to three transfer packs can be superposed that way.

11. Soak 3 pieces of 3MM Whatman paper (of the same size than the gel) into **transfer buffer 1** and place them above the transfer pack(s).

12. Rinse cathode (-) with ddH_2O and place it on top of the whole



13. Transfer for one hour at 0.8 mA/cm^2 of membrane.

14. After transfer, disassemble the transfer pack. Fix and stain the membrane into a 0.2% Ponceau red, 3% TCA solution (can be recycled for further use). Rinse anode and cathode with ddH_2O

15. Distain the membrane into ddH_2O.

16. Mark the position of the major bands with a pencil.

17. If not processed in the next few days, the membrane should be dried, sealed in a plastic bag, and kept at -20°C until utilisation.

Transfer buffers:

composition:

Tampon 1: 40 mM 6 amino-N-caproic acid/25 mM Tris-Cl pH 9.4 / 20 % isopropanol

Tampon 2: 25 mM Tris-Cl pH 10.4 / 20 % isopropanol

Tampon 3: 0.3 M Tris-Cl pH 10.4 / 20 % isopropanol

préparation :

	Tampon 1		Tampon 2		Tampon 3
volume	**200 mL**	**300 mL**	**400ml**	**500ml**	**200ml**
1 M Tris-Cl pH 9.4 (ml)	**5**	**7.5**	**-**	**-**	**-**
1 M Tris-Cl pH 10.4 (ml)	**-**	**-**	**10**	**12.5**	**60**
6 amino-N-caproïque (g)	**1.05**	**1.575**			
isopropanol (ml)	**40**	**60**	**80**	**100**	**40**

ECL revelation of blots

All steps are performed at room temperature.

1) Saturate the membrane 1 hr in PBS-TM.

2) Place the filter in a plastic bag. Add the antibody diluted in PBS-TM (about 150 µL per cm^2 of membrane). Seal the bag, avoiding trapping bubbles. Incubate with vigorous agitation for one hour.

3) Wash once in PBS-T for 15', then twice for 5'.

4) Place the filter in a plastic bag. Add a 1/10 000 dilution of the secondary antibody in PBS-TM (same volume than in 2). Seal the bag, avoiding trapping bubbles. Incubate with vigorous agitation for one hour.

5) Wash once in PBS-T for 15', then twice for 5'.

6) Wipe the membrane but do not let it dry. Transfer it into a new plastic bag. Add the 1:1 mix of detection solutions A and B (Amarsham kit) -62.5 µL of mix/cm^2 of membrane are enough- and let incubate for 1-2'.

7) From that point, work as quickly as possible to minimise the delay before exposition. Wiep the membrane without drying it, then wrap it into Saran. Expose first for 30'' on ECL optimised radiography film. Depending on the result of this first exposure, adjust the length of the second, longer, exposition. Remember that light is mostly emitted within the first two hours.

solutions

10 x PBS (for 1l)
80 g NaCl
2g KCl
14.6 g Na_2HPO_4
2g KH_2PO_4

PBS-T

1 x PBS + 0.1% Tween 20 (5 ml of 20% stock solution for 1 L)

PBS-TM

1 x PBS-T + 3% low-fat powder milk

Spectroscopic measurements

The function of the photosynthetic chain may be assessed using time-resolved absorption spectroscopy. This technique provides a mean to follow the various electron transfer steps within the different complexes and thereby to characterize their function. More importantly, it may be applied *in vivo*, provided the sensitivity is such that it allows the measure of absorption changes which usually do not exceed one thousandth of the sample absorbance. Such a requirement may be met when using a pump and probe approach: an exciting flash is used to trigger and thus synchronize the photochemical reactions and a detecting flash probes the resulting absorption changes at a discrete wavelength and a discrete time after the exciting flash. The use of weak probing flashes permits to shine enough photon

on the sample to allow the accurate measure of the light intensity which is transmitted throughout the sample while keeping the incident intensity low enough to avoid a significant exciting effect of the detecting flash that would, otherwise, desynchronize the photochemical reactions and thus decrease the time resolution of the experiment.

According to the Beer-Lambert's law:

$I/I_0=10^{-\varepsilon l.c}$ (1)

, where I_0 and I are the light intensity before and after the sample, respectively, ε the extinction coefficient, l the optical path length and c the concentration.

An absorption changes is equivalent to a change in ε. Provided the resulting variation in light intensity is small, one may differentiate eq.1:

$dI= -\ln (10).I_0.l.c.d\varepsilon.10^{-\varepsilon.l.c}$

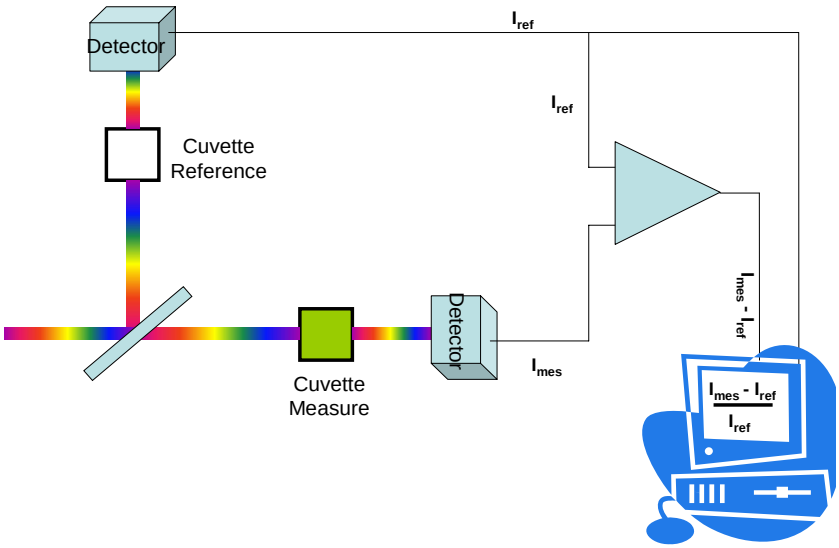
(2)

Combining eq.1 and 2 yields:

$dI/I=- \ln(10).l.c.\ d\varepsilon \cong -2.3\ dO.D.$

The ratio between the variation in light intensity and the intensity transmitted before the exciting flash is thus proportional to the absorption change.

It follows that, to asses the light induced absorption changes, both the intensity before and after the exciting flash must be measured. To this aim the detecting light is split into two beams, one being used as a reference measure and the second one being shone on the sample (see scheme and Joliot, Béal and Frilley 1980).



The light intensity is measured by photodiodes and a differential amplifier is used to determine (I_{mes}- I_{ref}) which is then divided by I_{ref}.

The main factor that governs the optimization of the experiment is the signal to noise ratio. The signal being proportional to the concentration of the sample, the former can be easily increased by increasing the latter. Yet, increasing the concentration obviously results in an increased overall absorption of the sample and a consecutive drop in the light intensity at the level of the photodiode detector (note that $I/I_0=10^{-\varepsilon l.c}$ and $dI/I=- \ln(10).l.c.d\varepsilon$ so that the light intensity after the sample decreases exponentially with the concentration, whereas the signal increases linearly). The noise of the measure is proportional to $1/\sqrt{I}$, where I is the light intensity at the level of the detector or, in other words, transmitted by the sample. As a first approximation, increasing the concentration thus results in two opposite effect: a larger signal

and a larger noise. Yet, the signal and the noise depend, respectively, linearly and exponentially on the concentration (see Fig.1).

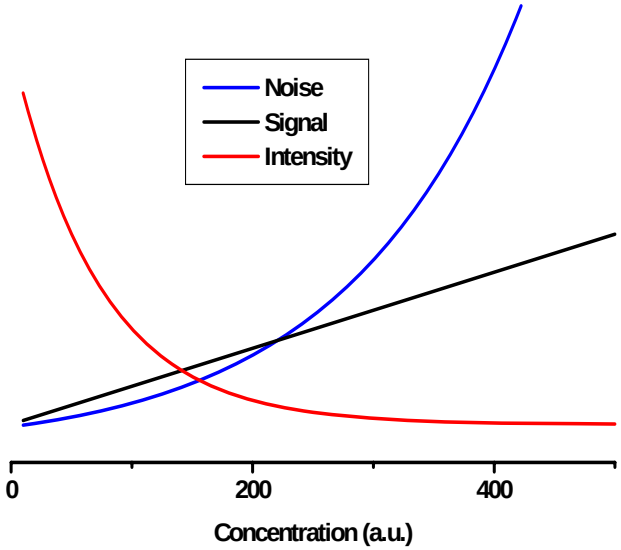


Figure 1. relationship between the concentration of the sample concentration and the extent of the signal, of the experimental noise.

This allows one to define the optimal experimental conditions: the largest possible concentration while keeping under the limit where the variation of the noise upon a concentration change is steeper than that of the signal.

Meeting these conditions determines the light intensity on the reference detector. Since the measured signal is $(I_{mes}- I_{ref})/I_{ref}$, one has to make sure that the differential amplifier is not saturated before the experiment, in other terms the light intensity on both the reference and measure detectors should be tuned so that $(I_{mes}- I_{ref})\approx 0$.

Various signals may be studied by time-resolved absorption spectroscopy. The absorption properties of most of the cofactors participating to the electron transfer depend on their redox state. As a consequence, their oxidation/reduction may be followed at specific wavelength. Besides, these specific spectroscopic signatures the transfer of charges (electron or protons) across the photosynthetic may be commonly followed by exploiting the so-called electrochromic band-shift. The transfer of a charge across the membrane results in a variation of the surface charge density on both sides of the lipid bilayer and, as a consequence, in a change in the amplitude of the transmembrane electric field. Depending on the orientation of their transition dipole, some of the pigments embedded in the light harvesting complexes, undergo a shift in their absorption spectrum in response to this electric field variation (reviewed in Witt, 1979). This shift itself results in an absorption change which is linearly related to the amplitude of the change in the amplitude of the electric field. As a consequence the various electrogenic electron transfer steps may be kinetically studied as well as the proton transfer resulting either from electron transfer at the level of the Photosystem 2 acceptor side or at the level of the cytochrome b_6f, or from the CF_0-F_1 ATP synthase turnover. Typically, the time course of this electrochromic signal after an exciting flash is the following (e.g. Joliot and Delosme 1974): i) a rise (here after named phase a), usually not resolved kinetically, reflects the charge separation at level of both photosystems, it is thus proportional to the intrinsic photochemical efficiency. The amplitude of the phase a is thus a reliable and convenient indicator of the amount of active PS1 and PS2. Combined with the use of specific

inhibitors of the PS2 activity (DCMU and hydroxylamine), it thus allows one to quantify the PS1/PS2 ratio. ii) This fast rising phase is followed by a second slower phase that reflects the electron and proton transfer steps catalyzed by the cytochrome b_6f, it typically develops in the 5 ms time range; iii) the building up of a transmembrane electrochemical difference potential, which is witnessed by these various phases, allows the synthesis of ATP at the level of the CF_0-F_1 ATP synthase. Since this synthesis consumes the proton motive force by allowing the transfer of proton across the membrane, the turnover of the ATP synthase results in a decrease of the transmembrane electric field and thus in a decrease of the electrochromic signal. As a consequence, the decay of the photo-induced electrochromic signal may be used to assess the ATP synthase activity and its physiological regulation.

Protocol

Chlamydomonas reindhardtii cells are grown at 24 °C in acetate supplemented medium under 60 $\mu Em^{-2}s^{-1}$ of continuous white light. They are harvested, during exponential growth, by centrifugation (3500rpm for 5 minutes). The pellet is resuspended at the required concentration in HS minimal medium containing 20% (w/w) of Ficoll to avoid sedimentation. The cells are vigorously agitated in the dark, to allow reoxygenation of the sample prior to the experiment. The overall photochemical activity may be measured at 518 nm, a wavelength corresponding to the peak of the elctrochromic bandshift. A saturating exciting laser flash is fired, the duration (5 ns, FWHM) of which is short enough to avoid the double turnovers that could occur if P_{680}^+ or P_{700}^+ was re-reduced during the flash. The absorbance changes reflecting the charge separation at the level of the two phtosystems are measured 100 µs after the exciting flash. This time delay is long enough to allow the occurrence of the charge separation process in its full extent and short enough to probe the amplitude of the photo-induced transmembrane electric field before cytochrome b_6f activity comes into play. In order to assess the photochemical activity of the sole PS1, PS2 inhibitors (DCMU, 20 µM and Hydroxylamine, 1 mM) may be added. As alluded to above the cytochrome b_6f activity may be assessed by measuring the transient absorption changes in the 5 ms time range.

Fluorescence measurements

Principles. **–** Measuring fluorescence emission is a widely employed technique to assess the functional status of the photosynthetic apparatus. Fluorescence corresponds to the fraction of the excited stated (which are generated in the photosynthetic antenna complexes upon light absorption) that is re-emitted as light (eq 1, Butler, 1978)

$$\Phi F \sim I\,(1\text{-}T)\,\phi F \qquad (2)$$

where I is the light intensity provided, 1-T represents the fraction of absorbed light and ϕF represents the quantum yield of fluorescence (i. e. the ratio between the rate deactivation the excited stated via fluorescence emission vs the sum of the rates of excited stated decay through all the possible deactivation processes).
Owing to thermal equilibration, fluorescence emission takes place at longer wavelengths than absorption. In chlorophyll a based system, like *Chlamydomonas reinhardtii*, fluorescence is detected in the far region of the spectrum (Fig .2).

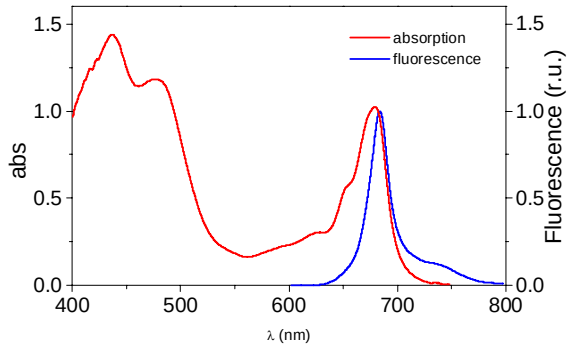


Figure 2 absorption (red) and fluorescence emission (blue) spectra of a Chlamydomonas reinardtii cell suspension.

At room temperature, fluorescence is mainly emitted by PS2. However, substantial PS1 emission can be observed at low temperature (Fig.3). Nonetheless, emission by the two photosystem can be distinguished on a spectral basis, PS1 emission being enriched at longer wavelengths.

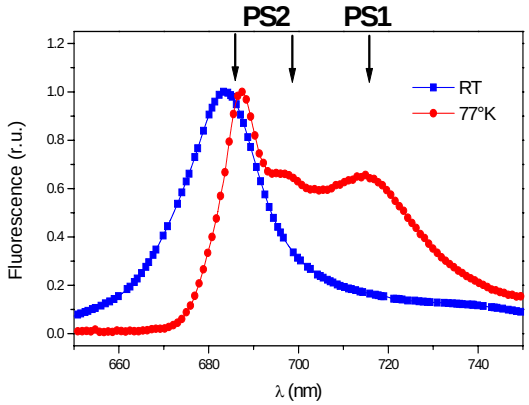


Figure 3. Fluorescence emission of Chlamydomonas cell suspension measured at room temperature (RT) and 77°K. Arrows indicate the main PSI and PSII emission peaks.

The photons re-emitted as fluorescence cannot be used for photosynthesis. Therefore, an inverse proportionality exists between the amount of light employed for photochemistry (the fist step in photosysthesis, which is taking place in the photosystems), and the one re-emitted as fluorescence. This is illustrated in Fig.

4, where a typical time course of fluorescence emission at room temperature is shown. In dark-adapted Chlamydomonas cells, illumination leads to a sudden rise to a given value (Fo), attained in the submicrosecod domain. This value represents emission by PS2 complexes in a photochemical active state, as well as the small contribution by PS1 fluorescence, which exists even at room temperature. This is followed by a second increasing phase, and by a slower decreasing one, of similar amplitude (closed squares). As a result, the steady-state level approaches the Fo one. As fluorescence is essentially emitted by PS2, the two phases can be taken as a signature of the adjustment of PS2 activity with respect to the overall electron flow process, during attainment of steady state conditions. Indeed, no such phases are seen if preilluminated cells are submitted to the same measurement (open squares).

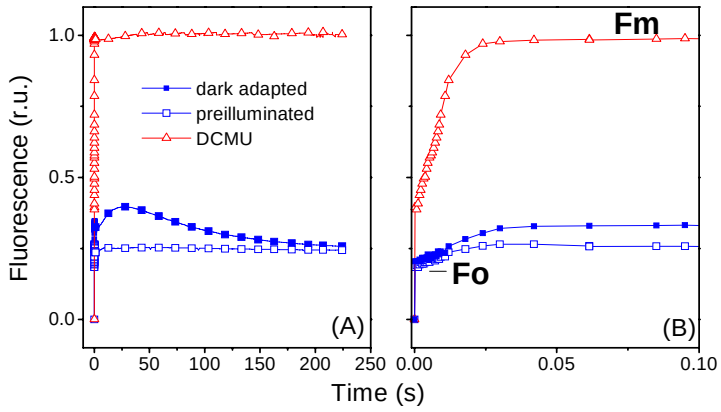


Figure 4. Time course of fluorescence emission by a cell suspension of Chlamydomonas reinhardtii. Panel B presents the same data as panel A on a expanded scale.

The inverse relationship existing between fluorescence emission and photosynthetic activity is clearly illustrated by the changes of fluorescence induced by addition of a specific inhibitor of Photosystem 2 activity, DCMU (triangles). By blocking photochemistry, this compound leads to a 3-4 fold increase in fluorescence emission (Fm level). This level increase is proportional to the fraction of photons that are normally employed for photosynthesis, and therefore emitted as fluorescence only in inhibited samples It can be demonstrated that the ratio $\frac{Fm - Fo}{Fo}$ provides a quantitative estimate of the photosynthetic efficiency of PS2 (Butler, 1978).

Analysis of electron transport efficiency by fluorescence measurements.- As stated above fluorescence increase from Fo to Fm reflects the progressive inactivation of PS2. In WT cells of Chlamydomonas, no such inactivation is seen during a dark to light transition, owing to efficient electron flow from PS2 to PS1. However, modifications of the photosynthetic apparatus that reduce the electron transfer rate may lead to a (partial) blockage of PS2, due to over-accumulation of reduced PS2 electron acceptors, and therefore to (at least partial) increase in the steady state fluorescence level. In particular, mutants with full inactivation of electron flow downstream of PS2 show a continuous fluorescence rise up to Fm during illumination (Fig. 5).

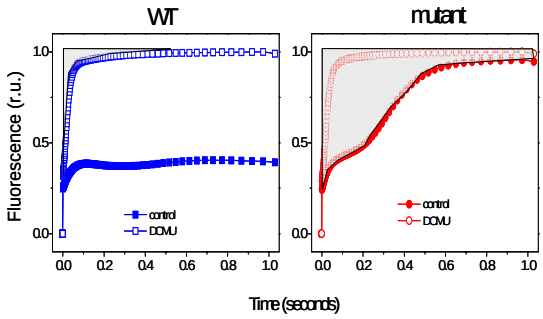


Figure 5. Time course of fluorescence emission by WT (blue) and mutant(red) cells of Chlamydomonas reinhardtii.

In the mutant cells shown in Figure 4, fluorescence rise to Fm is slower in the absence than in the presence of DCMU. This reflects the presence of some PS2 electron acceptors located between this complex and the site where the mutation has taken place. These acceptors may still allow some PS2 electron flow during illumination, thus preventing the fluorescence rise. before becoming reduced in an irreversible way (its oxidation being prevented by the mutation). The number of these acceptors can be estimated by measuring the integral of the fluorescence signal, i.e. the shadowed area above fluorescence in Fig. 5. Indeed, it is demonstrated that the fluorescence rise observed in the presence of DCMU is proportional to the transfer of one electron downstream of PS2 (Witt, 1979). Thus, the ratio of the areas ± DCMU reflects the number of electron transferred between PS2 and the site of mutation, as described below:

If the area without DCMU is $A_c = \varepsilon * N$

and the area with DCMU is $A_D = \varepsilon * 1$ electron acceptor,

where ε is the proportionality coefficient and N is the number of electron acceptors located between PS2 and the site affected by the mutation,

It follows that

$$\frac{Ac}{Ad} = N$$

In particular, N represents the size of the plastoquinone pool in mutants lacking the cytochrome b_6f complex.

Protocol

Chlamydomonas reinhardtii cells are grown at 24 °C in acetate supplemented medium under ~ 60 µE m^{-2} s^{-1} of continuous white light. They are harvested during exponential growth (3500 rpm for 5 minutes) and resuspended at the required concentration in HS minimal medium. Cell concentration can be measured as the absorbance at 680 nm of the cultures, based on a previous calibration curve between OD and the cells number.

Cells are employed at the concentration of ~ 10^6 cells /mL. Fluorescence is excited in the visible region of the spectrum ($\lambda < 600$ nm, to avoid spectral superposition between the excitation light and the fluorescence emitted). The light intensity employed for measurements provides on the average 1 photon per Photosystem 2 every 20 milliseconds. Fluorescence is recovered at 90° degrees with respect to excitation to diminish artefacts related to light diffusion. Fluorescence emission is normally detected in the near far red region of the spectrum (normally $\lambda > 690$ nm). To this end, the detector (a photomultiplier or a photodiode) is protected by a long pass filter, which cuts off the excitation light.

When required DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea is added to the algal suspension in the dark, at the concentration of 20 μM.

Quantification of changes in the Photosystem 2 antenna size.

Maximum fluorescence emission (Fm) is only attained upon full inhibition of the photosynthetic activity. As a consequence, ΦF in eq. 1 becomes constant and the intensity of the fluorescence signal measured at Fm can only be modified by changes in the amount of the absorbed light (1-T) and in the light intensity provided (I). In this case, if I is kept constant during an experiment, changes in the amplitude of the Fm (at constant intensity of the excitation) can be used to study the modulation of the PSII light harvesting capacity.
This is a useful tool to probe physiological adaptation processes in the photosynthetic apparatus, as state transitions The phenomenon of state transitions describes the reversible transfer of a fraction of the (photosystem) PS II outer antenna to PS I. It is understood as a mechanism for balancing the absorption capacity of the two photosystems under natural illumination conditions (refs). It likely occurs via the phosphorylation of PSII antenna proteins by a redox-activated kinase. Trnsition from non-phosphorylated conditions (state 1), to phosphorylated conditions (state 2) corresponds to a decrease of the PS2 absorption capacity, which is reflected by a decrease of the Fm value (Fig. 6)

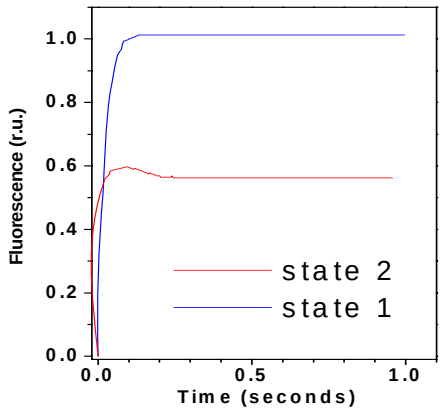


Figure 6. Fluorescence changes in Chlamydomonas cells adapted to state 1 (blue) and state 2 (red) conditions

As state transitions results in the concomitant decrease of the PS2 antenna and the increase of the PS1 antenna sizes, this phenomenon can also be studied by measuring the relative changes in the fluorescence bands associated to PS1 and PS2 at low temperature (Fig. 7)

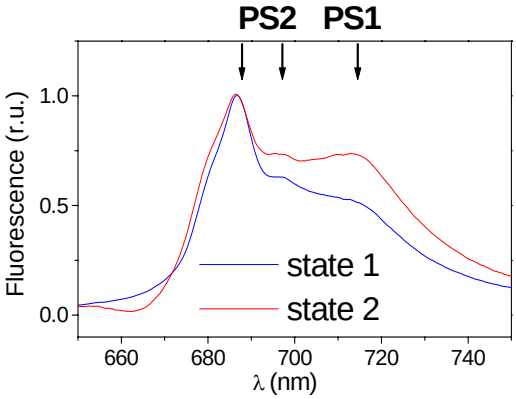


Figure 7. Fluorescence emission spectra of Chlamydomonas cells in state 2 (red) and state 1 (blue) conditions. Traces are normalised on emission at 685 nm.

Protocol

Chlamydomonas reinhardtii cells are grown at 24 °C in acetate supplemented medium under ~ 60 μE m^{-2} s^{-1} of continuous white light. They are harvested during exponential growth (3500 rpm for 5 minutes) and resuspended at the required concentration in HS minimal medium. State 2 conditions are attained in anaerobiosis in the dark. Anaerobiosis is required to promote reduction of the plastoquinone pool, which in turn is required for redox activation of the kinase. Anaerobic conditions can be induced by incubation of the algae with glucose 10mM and glucose oxidase (x units), to remove oxygen form the reaction medium. Alternatively, anaerobiosis can be attained by uncoupling mitochondrial respiration by addition of the protonophore FCCP (5μM). 10-20 minutes of dark incubation are required for full establishment of state 2 conditions. This can be probed by measuring fluorescence changes during incubation. State 1 is achieved by strong agitation in the dark, without addition of chemicals. This promotes a maximum oxidation of the plastoquinone pool (~ 50%) by enhancing respiration (Wollman and Delepelaire, 1984)

Fluorescence kinetics are measured as described above.

To measure low temperature fluorescence spectra, cells are preincubated under the same conditions described above, and then placed in a metal cuvette, which is directly bathed into a liquid nitrogen solution. Fluorescence is then excited at l < 600 nm, and detected with a CDD based apparatus.

Alternatively, transition from state 1 to State 2 can be achieved by illumination of dark adapted state 1 cells with PS2 absorbed light (e.g; 475 nm ligh ref) for several minutes; Conversely, state 1 is induced by illumination of state 2 adapted cells with PS1absorbed light (720 nm). Again, several minutes are required for full adaptation and smaller variations are measured (Zer et al. 2003.)

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P.9 Experiments for Sulfur Acclimation Processes (6 h over 1 day)

Arthur Grossman (Carnegie Institution, Stanford, USA) and Sabeeha Merchant (UCLA, USA)

Put up wild-type and mutant cells 6 or 7 days prior to use (for Sabeeha and Arthur)
There will be a wild-type and 3 mutants (*sac1*, *ars11* and maybe *sac3*); the two parental strains and three mutants, labeled A-E

After 3-4 d of growth on S+ medium, wash 25 ml of each of the A-E cultures and spot onto both S+ and S- medium (it will probably require 2 S+ and 2 S- plates per group or a total of 8 S+ and 8 S- plates – duplicates of each). We will probably want to put low levels of thiocyanate in the plates.

Allow the cells to grow for 2-3 d before spraying with X-sulfate at 9:00-11:00 on Sept 25; the color will be evaluated at 2:00-6:00 PM of the same day; the students will make up the X-sulfate solution and spray the plates.

For liquid cultures, the 5 different cell types will be transferred the night before the night before (Sept 24) to –S and +S liquid medium (cultures A-E, -S and +S). The cells should be on -S medium for at least 12 h. The students will perform an assay (in triplicate) on the cultures using ρ-nitrophenol sulfate and match it to a standard curve previously generated (by Sabeeha and Arthur).

Assay for ARS activity: *ARS activity can be assayed directly on colonies growing on solid medium by spraying plates with ~500 μl of 10 mM-5-bromo-4-chloro-3-indolyl sulfate (X-Sulfate) in 100 mM Tris-HCl, pH 7.5. Usually a blue halo forms around the colony within 1-2 h. ARS activity in liquid culture is assayed using ρ-nitrophenyl sulfate as the substrate. 50 μl of the sample is added to 500 μl of 0.1 M glycine-NaOH, pH 9.0, 10 mM imidazole, 5.0 mM ρ-nitrophenyl sulfate (Sigma) and incubated for 30-60 min at 27°C. The reaction is stopped by 2 ml of 0.25 M NaOH and the absorbance measured at 410 nm. Enzymatic activity is derived from a standard absorbance curve of ρ-nitrophenyl sulfate in 0.20 M NaOH. If whole cultures are used the cells are pelleted before measuring the absorbance.*

Reagents:
1M mM Tris-HCl, pH 7.5
1 M glycine-NaOH, pH 9.0
10 mM-5-bromo-4-chloro-3-indolyl sulfate (X-Sulfate) in 100 mM Tris-HCl, pH 7.5
100 mM imidazole (in distilled sterile water)
50 mM ρ-nitrophenyl sulfate (Sigma) (in distilled sterile water)
2.5 M NaOH
liquid TAP medium plus and minus sulfur (8 x 250 ml of each, sterile)
~8 plates TAP-S and 8 plates TAP+S (plus thiocyanate) - put in the recipe for the TAP medium
The parental (DS66) and the mutants
perfume spray vessel

Preparation of RNA and qPCR (afternoon Sept 25)

The RNA will be prepared by Sabeeha and Arthur for the wild-type and mutant cells before the class meeting. We will use one of two methods for RNA preparation.

RNA Preparation (from Moseley, 2006)

1. Cell cultures are poured into a flask immersed in liquid nitrogen and swirled to cool.
2. Cells are pelleted by centrifugation at about 8,000 rpm in SA600 rotor or equivalent.
3. Pellets are either frozen in liquid N_2 until use, or homogenized directly in homemade Trizol reagent (Invitrogen Co., Carlsbad, CA) by continuously vortexing the suspension.
4. The homogeneous suspension is then incubated at room temperature for at least 10 min. 5. Chloroform (1.6 ml) is added to the suspension, and the tubes are shaken vigorously for 1 min and incubated at RT for an additional 2-3 min.
6. Phase separation is achieved by centrifugation at 12,000 x *g* for 15 min.
7. 0.5 vol (3 to 4 ml) of 0.8 M sodium citrate - 1.2 M NaCl is added to the aqueous phase, followed by the addition of 0.5 vol (relative to the initial volume) isopropanol to precipitate nucleic acids.
8. Precipitations were performed at RT for 10 min, and the precipitated nucleic acid collected by centrifugation at 12,000 x *g* for 10 min.
9. The nucleic acid is washed with 8 ml of 75% ethanol, dried, and dissolved in diethyl pyrocarbonate-treated, double-distilled water.
10. If necessary, poly(A) RNA can be prepared using a MicroPoly(A) Pure small-scale mRNA purification kit (Ambion, Austin, TX) and quantified fluorimetrically using a RiboGreen RNA quantification kit (Molecular Probes, Eugene OR) and a Tecan SPECTRAFluor fluorimeter (Zurich, Switzerland). We do not need the polyA purification for performing the qPCR.

Nucleic Acid Preparation Using Acid Phenol (from AG)

1. Grow cells to 500 ml, 5-10 x 10^6 cells/ml
2. Pellet the cells for 4 min at 5000 rpm in a GSA rotor
3. Add 1 ml of sterile double distilled water (autoclaved) to resuspend cells and transfer 2 ml of the suspension into a 15 ml falcon tube - keep the suspension cold.
4. Add 2 ml of lysis buffer (autoclaved) and warm the suspension, shaking it well.
5. Extract the suspension twice an equal volume of Tris buffered phenol (equilibrated with the lysis buffer) - save the aqueous phase (top phase) after each extraction.
6. Extract the aqueous phase once with phenol:chloroform, 1:1 (can add 1/25th isoamyl alcohol to improve the separation of the phases).
7. Extract the aqueous phase with 100% chloroform (this eliminates the phenol).
8. Make the aqueous phase 0.3 M NH_4OAc or NaOAc
9. Add 2.5 volumes of 100% ethanol and mix - can allow the nucleic acid to precipitate in the freezer for 1 h, although it should precipitate almost immediately.
10. Pellet the RNA at ~5000 rpm in an SS34 rotor - wash with 70% ethanol - resuspend in ~500 μl of double distilled, sterile water and check OD260:280

The procedure usually yields nucleic acid at 0.5-2 mg/ml

From step 10, one can enrich for RNA -

11. Resuspend pellet of step 9 in 5 ml of sterile, double distilled water (instead of ~500 μl) and add an equal volume of 4 M lithium chloride
12. Mix and put in the refrigerator for at least 4 h.
13. Pellet RNA by centrifugation at ~5000 rpm in SS34 rotor.

14. Resuspend RNA in 2 ml of sterile, double distilled water, add NaOAc to about 0.15 M and precipitate the RNA with 2 vol of ethanol. The ethanol precipitation can be repeated.
15. Resuspend RNA in 0.5 ml of sterile, double distilled water and measure OD260:280

Acid Phenol
Heat new bottle to 65 deg (100g)
Add 20 ml H_2O + 0.2 g hydroxyquinoline
Let cool and then add ~10 ml more H_2O till a little water remains on top of phenol so that it is completely water saturated.

Lysis buffer

Stock	vol added	final concentration
10% SDS	10 ml	2%
5 M NaCl	4 ml	400 mM
0.5 M EDTA, pH 8.0	4 ml	40 mM
1 M Tris-HCl, pH 8.0	4 ml	80 mM
H_2O	28 ml	
Total volume 50 ml		

Eliminating DNA from the RNA Preparation
DNase digestion
- Start with less than 87.5 µl of crude RNA prep (5 µg)
- Add 10 µl of DNase buffer, 2.5 µl DNase I and RNase-free water to a vol of 100 µl (do not vortex)
- Incubate at RT for 10-30 min.

RNA cleanup using the Qiagen RNeasy MinElute Kit
- Add 350 µl of Buffer RLT to the 100 µl of RNA and mix thoroughly.
- add 250 µl of 100% EtOH and mix thoroughly by pipetting
- Immediately load the sample (700 µl) to an RNeasy MinElute column with a 2 ml collection tube
- Centrifuge at max speed for 15 sec
- Discard the flow-through and transfer spin column to a new 2 ml collection tube
- Add 500 µl of Buffer RPE and centrifuge at max speed for 15 sec
- Discard flow-through and reuse the same 2 ml collection tube
- Add 500 µl of 80% EtOH to the column and centrifuge at max speed for 3 min
- Discard flow-through and collection tube
- Transfer column to a new 2 ml collection tube and centrifuge at full speed for 5 min to get rid of remaining EtOH
- Discard flow-through.
- To elute, transfer spin column to a new 1.5 ml eppendorf tube. Pipet 20 µl of milliQ water (RNase free) (preheated to 40ºC) to the center of the column
- Spin at max speed for 4 min
- OD_{260} the eluted RNA

Making cDNA from the RNA (first strand synthesis)
- Add 2-5 µg of RNA to a PCR tube
- Add the following components: 1 µl of Oligo(dT); 1 µl of dNTPs 10 mM; RNase free water until 13 µl
- Heat mixture to 65ºC for 5 min and incubate on ice for at least 1 min

- Collect the contents of the tube by brief centrifugation and add:
    4 µl of 5X buffer
    1 µl DTT 0.1 M
    1 µl RNase OUT (Recombinase RNase inhibitor)
    1 µl of SuperScript III RT
- Mix by pipetting
- Incubate at 50ºC for 50 min
- Inactive the reaction by heating at 70ºC for 15 min

Quantitative Real-Time PCR (qPCR from DyNAmo HS SYBR Green pPCR Kit, Finnzymes) (David Gonzalez-Ballester)

- Dilute to the RT reaction to 1/5 the original volume (20 µl) of RT reaction.
- Master mix reaction
    - 10 µl DyNAmo SYBR Green mix
    - 0.75 µl each primer (from a Stock of 10 pmol/µl; approximate final concentration of 0.4 pmoles/µl) (generating a mixed stock of both primer at 10 pmol/µl each, is useful to reduce pipeting)
    - 1-3 µl cDNA (previously dilute) (some low expression genes require more cDNA)
    - distilled water to 20 µl

- qPCR protocol

Amplification

- 15 min at 94ºC (recommended for the manufactured of DyNAmo)
- 10 s at 94ºC
- 30 s at desired annealing temperature
- 15 s at 72ºC
- 10 s at 80ºC,
- Plate read (increasing the measurement temperature above 72º and below the product with the lower melting point allow reducing the signal background and could avoid the signal of primer dimers)
- Go to step 2, 35-40 times
- 72ºC for 5 min

Melting curve

- 94ºC 5 min
- Melting curve from 72º to 100ºC, read every 0.5ºC, hold 5 s

Analysis of data (with Opticom Monitor 3)

- Set the same parameters for all the samples. Example:
    - Subtract baseline: Average over cycle ranger
    - Threshold: manual 0.04
    - smooth: 0
    - cycle ranger: could be adjusted to every set of replicates

One step protocol (Jeff Moseley)

1. Isolated, total RNA is treated with RNase-free DNase I (Ambion Inc., Austin, TX) and then extracted with phenol-chloroform to isolate RNA for the RT reaction.
2. Real-time quantitative PCRs (qPCR) is performed using 0.1 µg of DNase-treated total RNA and an iScript One-Step RT-PCR kit with SYBR green (Bio-Rad Laboratories, Hercules, CA).
3. The amplifications is performed using the following cycling conditions: (i) 50°C for 30 min for cDNA synthesis, (ii) 95°C for 5 min to denature reverse transcriptase, and (iii) 40 to 42

cycles of 95°C for 15 or 30 s and 60°C for 30 s, with fluorescence detection after the 60°C annealing/extension step.
4. Melting curve analysis is performed on all PCR products to ensure that single DNA species is amplified; products could be sized by agarose gel electrophoresis to verify that it is the right product.
5. Some transcripts may require a two-step qPCR analysis. For cDNA synthesis, 1 μg of DNase I-treated total RNA is reverse transcribed using a Superscript II kit (Invitrogen, La Jolla, CA) as described by the manufacturer. qPCR is performed using either a DyNAmo Hot Start SYBR green qPCR kit (MJ Research Inc., Waltham, MA) or IQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA). Cycling conditions include an initial incubation at 95°C for 10 min followed by 40 to 45 cycles of 94°C for 10 s, 55°C to 60°C for 15 s, and 72°C for 10 to 15 s. The relative expression ratio of a target gene is calculated based on the $2^{-\Delta\Delta C_T}$ method (**Livak, K. J., and T. D. Schmittgen**, 2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods **25:**402-408), uses the average cycle threshold (C_T) calculated from duplicate measurements. Relative expression ratios from at least two independent experiments should be performed. The *CBLP* gene is used as a control gene, and each primer has been designed by Primer3 software.

1-step PCR protocol, with Bio-Rad iScript One-Step RT-PCR Kit With SYBR Green
(Protocol from Jeffrey Moseley)
Per reaction: 20 λ SYBR mix
0.8 λ reverse transcriptase
1-2 μg of total RNA (DNase-treated)
0.6 λ 10 μM primer 1
0.6 λ 10 μM primer 2
Water to 40 λ
Mixes for multiple reactions can be made that includes the RNA (one RNA, many different primers), or the primers (one primer pair, many different RNA samples). The SYBR mix is quite viscous, so always make enough for at least 0.5 extra reactions.
One 40 λ reaction is split into two wells (20 λ each) to make technical replicates.
Amplification protocol:

1. Incubate at 50˚C for 30 min (reverse transcription).
2. Incubate at 95˚C for 5 min (denature reverse transcriptase)
3. Incubate at 95˚C for 15 sec (melting)
4. Incubate at 60˚C for 30 sec (annealing and extension)
5. Plate read
6. Go to line 3 for 41 more times
7. Incubate at 60˚C for 7 min (final extension)
8. Melting curve from 65˚C to 95˚C, read every 0.2˚C, hold for 1 sec
9. Incubate at 60˚C for 7 min (re-annealing, important if you want to run the PCR products on a gel).

Protocols of Anne Soisig Steunou

I-Reverse transcription with specific primer:

1-Specific primers designed by Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify a DNA fragment of ~200 nucleotides. The primer are synthesized by IDT (http://www.idtdna.com/SciTools/SciTools.aspx)
Protocol:
1-Prepare the following mix in PCR tube:
1□l RNA (100 ng)

1□l reverse primer (10 □M)
1□l dNTP (10 mM)
10□l sterile, distilled water

In the PCR machine:

-Heat mixture to 65°C for 5 min (step 1)

-Incubate at 4°C for 4 min (step 2); at this step I press the bottom pause and add the following reagents to the PCR tube:

4□l 5X first strand buffer
1□l 0.1M DTT
1□l RNase out (Invitrogen, Cat. No.10777-019)
1□l Superscript III (Invitrogen Cat. No. 18080-085)

-Press pause again

-Incubate at 55°C for 44 min (step 3)

-Inactivate the reaction by heating at 70°C for 15 min (step 4)

-Incubate at 4°C (step 5)

II- RT-PCR

-2µl of the RT reaction;
-5□l of the 10X *Taq* DNA polymerase buffer (Qiagen)
-5□l reverse primer (10 □M)
-5□l forward primer (10 □M)
-2□l dNTP (2.5 mM)
-2.5□l DMSO
-28.3□l Water
-0.2 □l *Taq* DNA polymerase (1U) (Qiagen Inc., Valencia, CA)

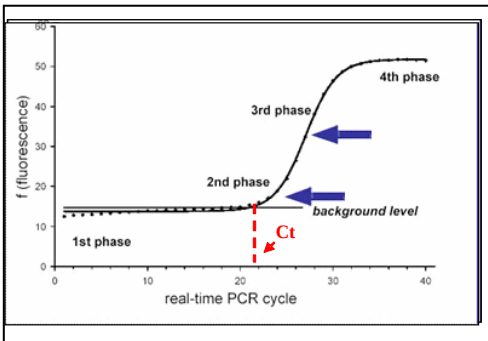
The PCR program:

1- 95°C for 1 min,
2- 94°C for 10 s,
3- 55°C for 30 s
4-72°C for 30 s
5-repeat 30 cycles from step2
6-72°C for 10 min

Amplified products are analyzed by electrophoresis in a 1.2% agarose gel.

III qPCR:

We use the Engine Opticon™ System (BioRad, South San Francisco, CA) and the DyNAmo HS SYBRGreen qPCR Kit (FINNZYMES, Espoo, Finland). If you don't have a housekeeping gene you can use absolute quantification of the cDNA.



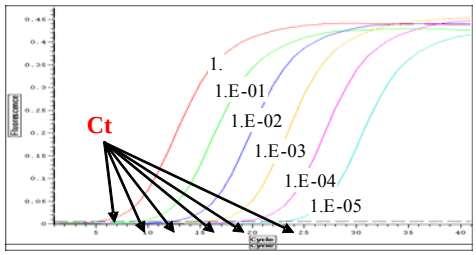
-Amplification of the cDNA by PCR follows a simoidal curve
SYBR Green I is a fluorescent reporter
-molecules that binds double-stranded DNA. We can follow the amplification by monitoring the change in in fluorescence yield

-Measurement of the Ct value for each cDNA .

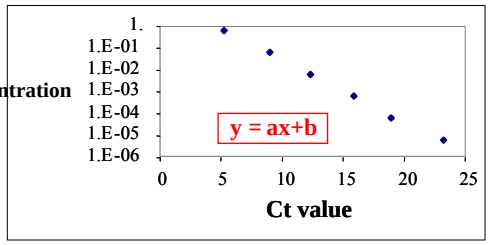
-The cycle threshold (Ct) corresponds to the PCR cycle at which the reaction generates enough fluorescence to cross the threshold.
-The Ct is inversely proportional to the copy number of the target template cDNA

Absolute quantification: Example with *nifK* gene

1-Standard Curve : a) Serial dilution of the known concentration of PCR product obtained from amplification of genomic DNA using *nifK* primers (forward and reverse).
b) PCR on each dilution of known concentration of the PCR product and measurement of Ct value.



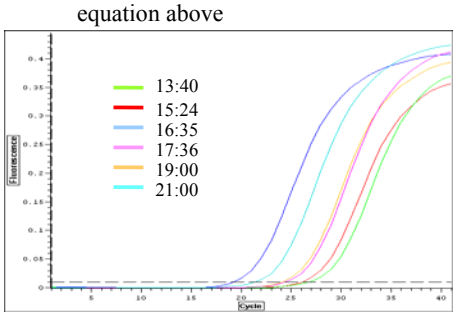
Concentration ng/ml



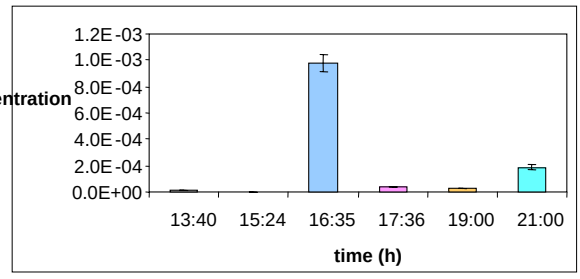
c) After determination the slope, obtain the constant and use for determining RNA concentrations.

y = log of the concentration
a = slope of the curve
x = ct value
b = constant

2- PCR of the cDNA obtained when the *nifK* reverse primer is used for cDNA synthesis. The Ct value is then measured from the PCR of the samples and used to determine the RNA concentration from the equation above



Concentration ng/ml



For absolute quantification:
Standard curve:
-PCR with the forward and reverse primer on genomic DNA. Follow the protocol described for the RT-PCR amplification except the number of cycles is reduced to 25.
-Check for the purity of the PCR product on a gel. If the product looks clean (no other products), purify using the Qiagen fragment purification kit, or purify from gel using the gel extraction purification kit from Quiagen
-Quantified the purified PCR product and do a serial dilution from 10^{-2} to 10^{-7}.

Protocol
Preparation of the primer mix (1.5 M)
-1.8□l Forward primer (10mM)
-1.8□l Reverse primer (10mM)
-8.4□l water

Preparation of the PCR mix:
-10□l master mix (from the FINNZYMES kit)
-4□l primer mix (1.5□M)
-2□l water
-2□l (either cDNA or DNA (for the standard curve))
The PCR program:
1- 95°C for 10 min,
2- 94°C for 10 s,
3- 55°C for 15 s
4-72°C for 15 s
5-*Read the plate*
5-40 cycles from step2
6-72°C for 10 min
7-Melting curve from 65°C to 95°C, read every 0.2°C, hold 1sec
8-72°C for 10 min

P10. Flagellar assembly/ Immunofluorescence Microscopy

George Witman (Worcester, USA)

Procedures for Amputation and Regeneration of *Chlamydomonas* Flagella[1]

Chlamydomonas cells readily detach their flagella at a specific site between the flagellar transition region and the flagellar shaft when exposed to certain stressful conditions. The cells then regenerate new flagella when conditions return to normal. During regeneration, the levels of mRNAs encoding proteins specific to the flagellum greatly increase, while the levels of most other mRNAs decrease. Because of this, the strong induction of a gene by deflagellation is usually taken as an indication that the gene encodes a flagellar protein. The new flagella are formed in part from newly synthesized flagellar proteins and in part by

[1] Adapted from Lefebvre, P.A. 1995. Flagellar amputation and regeneration in *Chlamydomonas*. Meth. Cell Biol. 47: 3-7.

recruitment of flagellar precursors from a pre-existing pool in the cell body. By determining the kinetics of flagellar regeneration, one can easily measure the effect of translational and transcriptional inhibitors, flagellar precursor pool sizes, various mutations, etc., on the growth of this cell organelle.

The following procedure is designed to detach flagella from cell bodies while maintaining cell viability so that the cells will regenerate their flagella. Procedures for growing *Chlamydomonas* and isolating flagella by methods that ensure maximum integrity of the flagella for reactivation and biochemical analysis are described elsewhere.[2]

1. Grow cells in minimal medium (e.g., Medium I of Sager and Granick, 1953). Cells may be deflagellated at any stage of growth, but typically are used in mid- to late-log phase. For best results, cell division should be synchronized by use of a 14 hr light/10 hr dark cycle, and the cultures used a few hours after the beginning of the light cycle.

2. Examine the cells by phase microscopy (16X objective) to be sure they are flagellated and appear healthy. Although the optics with plastic slides are not as good as with glass slides, for convenience we use plastic slides that have 10 small plastic coverslips integral with the slide (Fisher HealthCare UriSystem DeciSlide 14-375-209). Flagella are readily detached by pressure, and by detergent, which is often present on the surfaces of pre-cleaned glass slides. Therefore, if glass slides and coverslips are used, they should be carefully rinsed and dried, and a small amount of Vaseline petroleum jelly put under two edges or 4 corners of the coverslip to form a chamber for the cells and prevent deflagellation from the pressure of the coverslip. Fix an aliquot of cells at this time to establish the flagellar length at time 0 (see step 5).

3. Place the cells in a small beaker and add a magnetic stir bar. While stirring the suspension vigorously with a magnetic stirrer, immerse a pH electrode in the suspension (be careful the electrode does not come in contact with the stir bar, which will break it). The pH of the culture probably will be between 6.5 and 7.0. Quickly add drops of 0.5 N acetic acid to lower the pH to 4.5. Continue stirring for 30 seconds, then return the pH to 7.0 by dropwise addition of 0.5 N KOH. *Be careful not to undershoot pH 4.5 or overshoot pH 7.0 to avoid killing cells*. Stop stirring and examine the cells by phase microscopy; all cells should be deflagellated. Flagella detachment is independent of the volume of the suspension and of the cell concentration.

4. Collect the cells by gentle centrifugation (e.g., 5 min at 1100 x g [2000 RPM for in an IEC #253 rotor]) and resuspend in fresh medium. This removes the detached flagella, which may get in the way when measuring the regenerated flagella. If possible, place the cells on a rotary shaker with gentle agitation to keep them suspended and aerated, and illuminate them with a fluorescent light, while they are regenerating their flagella.

5. Flagella regeneration is usually completed in about 60 minutes. To prepare samples for determining a rate curve for regeneration, fix aliquots of cells before deflagellation and at 5 min intervals for at least 60 min after deflagellation. Fixation is accomplished by adding 4 drops of cells to capped microfuge tubes containing 2 drops of 10% glutaraldehyde. Transfer cells to the fixative using a plastic transfer pipette, e.g., Samco # 202-15; if glass Pasteur

[2] Witman, G. B. 1986. Isolation of *Chlamydomonas* flagella and flagellar axonemes. Methods Enzymol. 134: 280-290.

pipettes are used, be sure to rinse them first to remove detergent. Use a fresh pipette each time and be careful not to expose the regenerating cells to glutaraldehyde or its vapors. Fixed cells should be stored at 4°C.

6. For flagellar length measurement, the cells are best imaged using a 40X objective, glass slides and coverslips, and DIC optics, as the phase halo around the cell body makes it difficult to tell where the flagellum begins when using phase optics. Measure flagellar lengths directly in 20-30 fixed cells from each time point using an eyepiece reticle (e.g., Edmund Optics scaled reticle, 0-10 mm scale, or Pyser-SGI eyepiece graticule NE1, 10 mm in 0.1 mm). The eyepiece reticle can be calibrated using a stage micrometer. Alternatively, capture digital images of the cells and measure flagellar lengths using ImageJ software; as with direct measurement, an image of a stage micrometer should be used for calibration.

Helpful tips: a) Allow the cells to settle in the tube for 2-3 hours before measuring flagella, and then take cells from the bottom of the tube to increase the number of cells in the field of view. b) Let cells settle on the slide for a few minutes so all movement ceases before measurements are made.

Immunofluorescence microscopy of *Chlamydomonas*

The goal of immunofluorescence microscopy is to label and observe a specific protein in its native location in the cell. There are many protocols that attempt to accomplish this. The one described below uses a methanol fixation that works well with many proteins, but the best protocol for a given antigen and antibody needs to be determined empirically. An excellent source for other methods, as well as a variation on this one, is an article by Sanders and Salisbury[3] that focuses on immunolabeling of cilia and flagella. Double immunofluorescence – labeling with two antibodies from two different species (e.g., mouse, rabbit) -- may be used to localize two different proteins simultaneously, or, when applied to a strain expressing a GFP-tagged protein, to localize three proteins simultaneously. Single or double immunofluorescence also may be combined with DAPI labeling to reveal the nucleus.

1. Use coverslips appropriate for the microscope that will be used to observe the labeled cells. We use 18 X 18 mm No. 1½ coverslips, but some microscopes may give better optics with No. 1 coverslips. Wash the coverslips in a detergent suitable for cleaning glassware, and rinse thoroughly with deionized H_20. Air dry the coverslips overnight or dry in an oven. The detergent can be re-used.

For washing and rinsing coverslips, and for immersing them in methanol, acetone, and ethanol, we place the coverslips in coverslip racks (e.g., ceramic racks by Coors U.S.A. or Invitrogen C-14784 coverslip mini-racks) and place the racks in a glass rectangular staining dish with cover (e.g., Wheaton 900203) containing the liquid in which the coverslips will be immersed. Use EM forceps to hold and manipulate the coverslips.

2. Just before use, wet one side of each coverslip with a drop of 1% polyethylenimine (30 sec). This can be done while holding the coverslip in forceps or by floating the coverslip on an 80-µl drop of polyethylenimine on a piece of Parafilm. From here on, you must keep track

[3] Sanders, M. A. and J. L. Salisbury. 1995. Immunofluorescence microscopy of cilia and flagella. Meth. Cell Biol. 47: 163-169.

of which side of the coverslip was treated. Rinse each coverslip with a gentle stream of deionized H_2O and wick off excess water with a Kimwipe or piece of filter paper.

3. If the cell concentration is low, collect the cells by centrifugation and resuspend them in a smaller volume of medium or 10 mM Hepes, pH 6.8. Place a drop of cells in growth medium or Hepes on the treated side of each coverslip. Allow cells to adhere for 30 sec to 5 min. Motile cells require less time than non-motile cells. Adherence can be monitored under a dissecting microscope. The goal is to get an even coating of cells. However, if the cells are left too long, the flagella will begin to swell at their tips and an artificial distribution of some proteins will result. If the cells shed their flagella, try resuspending a fresh batch of cells in microtubule-stabilizing buffer, which contains EGTA and prevents deflagellation.

4. Wick off excess medium (do not allow to dry completely) and immerse the coverslips in methanol prechilled to –20°C. Leave the coverslips in the methanol in the freezer for 10 minutes. The methanol both fixes and permeabilizes the cells.

5. Quickly transfer the coverslips to –20°C acetone. This reduces the cell body autofluorescence that is a problem with *Chlamydomonas*. After 6 minutes, remove the coverslips from the acetone and allow to air dry. From here on, all operations are carried out at room temperature unless stated otherwise.

6. Place the coverslips in blocking buffer [5% (w/v) BSA, 1% (v/v) Fish Skin Gelatin (Sigma), 10% (v/v) goat serum (Sigma), in PBST (PBS + 0.05% Tween)] for at least 30 min.

7. Dilute the primary antibody (for example, Sigma's mouse monoclonal anti-α-tubulin antibody clone B-5-1-1) in blocking buffer in a small plastic tube. If a second primary antibody will be used, it should be mixed with the first antibody at this time. To prevent evaporation, incubation of the coverslips with the antibody is carried out in a plastic Petri dish (15 X 1.5 cm), the bottom of which is covered with a moist piece of 15-cm diameter filter paper. Small pieces of Parafilm are placed on top of small pieces of sponge or cardboard on top of the filter paper, an 80-µl drop of antibody solution is placed on each piece of Parafilm, and a coverslip is placed, cells down, on top of each drop. Seal the Petri dish with Parafilm and incubate 2-4 hours at room temperature or overnight at 4°C. The incubation time depends on the antibody concentration and the affinity of the antibody for its antigen. For the anti-α-tubulin antibody, 90 min at room temperature is probably adequate.

8. Place the coverslips in a rack and wash four times, 5 min per wash, with PBST.

Optional: reblock coverslips for 5 min.

9. Incubate the coverslips as above with secondary antibody [for example, Alexa Fluor 488 $F(ab')_2$, fragment of goat anti-mouse IgG(H+L), A11017, Molecular Probes diluted 1:2000] in blocking buffer for 1.5 - 2 hour at room temperature in the dark. If two primary antibodies were used in step 7, two secondary antibodies (e.g., anti-mouse, anti-rabbit, each conjugated to a different fluor) are mixed together in a small plastic tube before aliquoting onto the parafilm. If DAPI staining of nuclei is desired, add 5 µg/ml DAPI to the antibody mix in the tube. Helpful hint: since *Chlamydomonas* tends to have red autofluorescence, the most important primary antibody should be detected using a secondary antibody labeled with a green fluor (e.g., Alexa Fluor 488).

10. Place the coverslips in a rack and wash 3 times, 5 min per wash, with PBST.

11. Immerse the coverslips in 100% ethanol for 1 min to wash away salts and dehydrate the cells, then air dry the coverslips.

12. In advance of this step, remove the mounting medium (Invitrogen ProLong Gold Antifade reagent) from the refrigerator so it can warm up to room temperature. Place a 20-µl drop of the mounting medium on a microscope slide and gently lower a coverslip, cell side down, onto the drop; avoid trapping air bubbles in the medium. Gently tap the coverslip down and remove any excess mounting medium with a Kimwipe. For immediate viewing, secure the coverslip in a few places with nail polish and allow the nail polish to dry thoroughly. Alternatively, the mounting medium may be dried in a desiccator at 4^{o}C overnight. For long-term storage, keep the slides in the dark at –80^{o}C.