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Phototaxis Assays of *Synechocystis* sp. PCC 6803 at Macroscopic and Microscopic Scales

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[Abstract] Phototaxis is a mechanism that allows cyanobacteria to respond to fluctuations in the quality and quantity of illumination by moving either towards or away from a light source. Phototactic movement on low concentration agar or agarose plates can be analyzed at macroscopic and microscopic scales representing group behavior and single cell motility, respectively. Here, we describe a detailed procedure for phototaxis assays on both scales using the unicellular cyanobacterium *Synechocystis* sp. PCC 6803.
Keywords: Cyanobacteria, *Synechocystis*, Motility, Phototaxis, Photoreceptors

[Background] The model organism *Synechocystis* sp. PCC 6803 uses retractile type IV pili (T4P) to move across moist surfaces in a jerky motion referred to as twitching motility. Two secretion ATPases (PilB and PilT) are responsible for the extension and retraction of the pilus apparatus, thus pulling the cells forward. *Synechocystis* sp. PCC 6803 harbors a variety of photoreceptors covering the entire visible spectrum. Absorption of light can stimulate either positive or negative phototaxis depending on wavelength and intensity. Recently, it was demonstrated that single cells of *Synechocystis* sp. PCC 6803 are able to directly detect unidirectional illumination by focusing the light in a sharp focal point on the distal side (Schuergers *et al.*, 2016). Moreover, it was shown that the direction of twitching motility correlates with a specific proximal localization of the motor ATPase PilB (Schuergers *et al.*, 2015). A model was proposed that the focusing leads to a local inhibition of the motility apparatus, thus determining the direction of movement of single cells as a photophobic response away from the focal light spot (Schuergers *et al.*, 2016).

Materials and Reagents

1. Sterile square (120 x 120 x 17 mm) Petri dishes (Greiner Bio One International, catalog number: 688161; supplied by VWR)
2. Sterile 1 µl inoculation loops (SARSTEDT, catalog number: 86.1567.010)
3. Sterile 1.5 ml Eppendorf tubes 3810X (Eppendorf, catalog number: 0030125150)
4. Sterile µ-dishes 35 mm high glass bottom (ibidi, catalog number: 81158)
5. Microscope coverslips 20 x 20 mm (Carl Roth, catalog number: H873)
6. Sterile syringes 50 ml (SARSTEDT, catalog number: 94.6077.137)

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7. Sterile syringe filters Filtropur S 0.2 (SARSTEDT, catalog number: 83.1826.001)
8. Sterile 50 ml Greiner centrifuge tubes (Greiner Bio One International, catalog number: 227261)
9. Serological pipettes 25 ml (SARSTEDT, catalog number: 86.1685.020)
10. Serological pipettes 5 ml (SARSTEDT, catalog number: 86.1253.025)
11. *Synechocystis* sp. PCC 6803 strain (motile wild type obtained from S. Shestakov, Moscow State University, Russia), resequenced by Trautmann *et al*. (2012)
12. Liquid paraffin, viscous (Carl Roth, catalog number: 8904)
13. Ultrapure water
14. Ethylenediamine tetraacetic acid disodium salt dehydrate ($Na_2EDTA{\cdot}2H_2O$) (Carl Roth, catalog number: 8043)
15. Sodium hydroxide (NaOH) (Carl Roth, catalog number: 6771)
16. di-Potassium hydrogen phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$) (EMD Millipore, catalog number: 105099)
17. Sodium carbonate (Na_2CO_3) (Carl Roth, catalog number: P028)
18. Boric acid (H_3BO_3) (Carl Roth, catalog number: 6943)
19. Manganese(II) chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$) (Carl Roth, catalog number: T881)
20. Zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$) (Carl Roth, catalog number: T884)
21. Sodium molybdate dehydrate ($Na_2MoO_4{\cdot}2H_2O$) (Carl Roth, catalog number: 0274)
22. Copper(II) sulphate pentahydrate ($CuSO_4{\cdot}5H_2O$) (Carl Roth, catalog number: P024)
23. Cobalt(II) nitrate hexahydrate, $Co(NO_3)_2{\cdot}6H_2O$ (Carl Roth, catalog number: HN16)
24. Ammonium ferric citrate (Carl Roth, catalog number: CN77)
25. 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid (TES) (Carl Roth, catalog number: 9137)
26. Sodium thiosulphate ($Na_2S_2O_3$) (Carl Roth, catalog number: HN25)
27. D(+)-glucose (Carl Roth, catalog number: X997)
28. Sodium nitrate ($NaNO_3$) (Carl Roth, catalog number: A136)
29. Magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$) (Carl Roth, catalog number: P027)
30. Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) (Carl Roth, catalog number: 5239)
31. Citric acid (Carl Roth, catalog number: X863)
32. Agar-agar, Kobe I (Carl Roth, catalog number: 5210)
33. UltraPure™ agarose (Thermo Fisher Scientific, Invitrogen™, catalog number: 16500500)
34. 0.5 M Na_2EDTA pH 8.0 (see Recipes)
35. 3% w/v $K_2HPO_4{\cdot}3H_2O$ solution (see Recipes)
36. 2% w/v Na_2CO_3 solution (see Recipes)
37. Trace metal mix solution (see Recipes)
38. 0.6% w/v ammonium ferric citrate solution (see Recipes)
39. 1 M TES buffer pH 8.0 (see Recipes)
40. 30% w/v $Na_2S_2O_3$ solution (see Recipes)
41. 40% w/v D(+)-glucose solution (see Recipes)

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42. 100x BG11 medium (see Recipes)
43. 2x BG11 medium (see Recipes)
44. 1x BG11 medium (see Recipes)
45. 1% w/v agar solution (see Recipes)
46. 0.6% w/v agarose solution (see Recipes)
47. Macroscopic phototaxis plate medium (see Recipes)
48. Microscopic phototaxis plate medium (see Recipes)

Equipment

1. Laboratory bottles 1,000 ml (Duran Group, catalog number: 21 801 54 5)
2. Laboratory bottles 500 ml (Duran Group, catalog number: 21 801 44 5)
3. Laboratory bottles 250 ml (Duran Group, catalog number: 21 801 36 5)
4. Laboratory bottles 100 ml (Duran Group, catalog number: 21 801 24 5)
5. Non-transparent square box (127 x 127 x 19 mm) with a one-sided opening (custom-made from polyvinyl chloride)
6. Silicon ring ($ø_o$: 30 mm, $ø_i$: 16 mm) custom-made
7. Non-transparent hollow cylinder (ø: 40 mm, h: 30 mm) with 4 holes (ø: 5 mm, h: 7 mm) positioned in increments of 90° for the insertion of LEDs (custom-made from polyvinyl chloride)
8. Balance (Denver Instrument)
9. pH meter (Mettler-Toledo)
10. UV/Vis spectrophotometer (Shimadzu, model: UV-2401PC)
11. Light source (Philips Lighting Holding, model: MASTER TL-D Super 80 18W/840 1SL/25)
12. Pipetting aid PIPETBOY acu 2 (INTEGRA Biosciences, model: PIPETBOY acu 2, catalog number: 155 017)
13. Quantum Sensor LI-190R (LI-COR, model: LI-190R)
14. Transmitted light scanner (Epson, model: Epson Perfection V700 Photo, with adjustable cover)
15. Fluorescence microscope (Nikon Instruments, model: Eclipse Ni-U, with CFI Plan Fluor 40X/0.75)
16. Digital CCD camera (Hamamatsu Photonics, model: ORCA®-05G)
17. Microcontroller board (Arduino, model: Arduino UNO R3)
18. RGB-LEDs (625 nm, 525 nm, 470 nm) 5 mm (World Trading Net)
19. Biological safety cabinet (NuAire, model: Class II Type A2)

Software

1. NIS-Elements Basic Research 4.20.01
2. Arduino 1.0.6
3. MATLAB Runtime 8.3

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4. BacteriaMobilityQuant (https://web.fe.up.pt/~dee11017/software/BacterialMobilityQuant.zip)
5. R

Procedure

A. Macroscopic phototaxis assay (Figure 1)

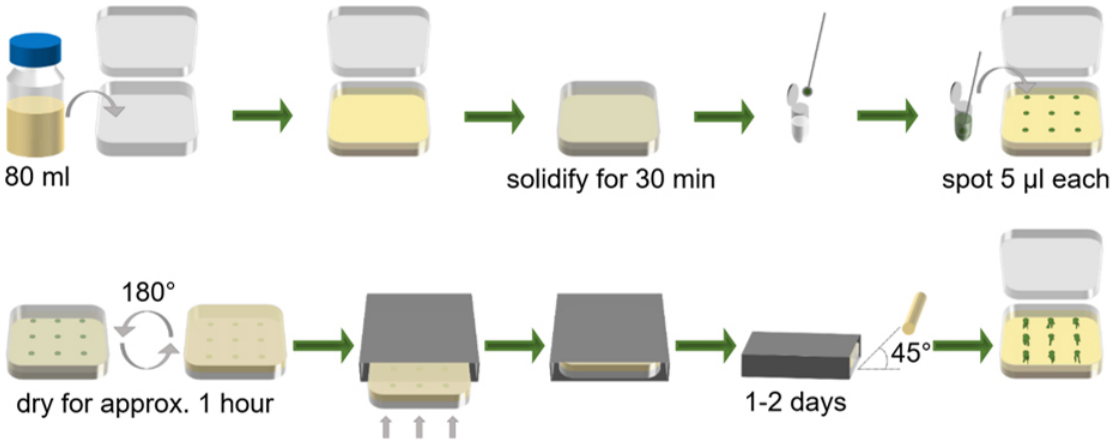

Figure 1. Macroscopic phototaxis assay. Consecutive steps as described in steps A1-A8.

1. Prepare 80 ml of the macroscopic phototaxis plate medium (see Recipes).
2. Mix very gently to avoid any air bubbles.
3. Pour the medium carefully into a square Petri dish and close the lid to avoid over-drying of the phototaxis plate. Let it solidify on an even surface. Plates should always be freshly prepared.
4. Scrape cyanobacterial cells with a sterile 1 µl inoculation loop from freshly grown agar plates or from a macroscopic phototaxis plate (see Note 5) and resuspend in 50 µl 1x BG11 medium (see Recipes) in a sterile 1.5 ml Eppendorf tube by vigorously twisting the loop (approximately OD_{750} 40 or very dark green color).
5. Place the phototaxis plate on a template with three equally spaced parallel rows drawn over the surface area of the plate. Spot 5 µl of the cell suspension in triplicates in each row. Close the lid and allow the droplets to soak into the plate. This step may take up to an hour depending on the surface of the plate and the density of the cells.
6. Place the phototaxis plate upside down in a non-transparent square box with a one-sided opening. Align the rows of the phototaxis plate parallel to the opening.
7. Incubate the phototaxis plate at 30 °C under a unidirectional white light source with an angle of about 45° (*e.g.*, Philips MASTER TL-D Super 80 18W/840 1SL/25) at approximately 35 µmol photons m^{-2} sec^{-1} measured with a quantum sensor at the front side of the phototaxis box.
8. After 1-2 days the finger-like projections of the motile cells can be recorded by scanning the plate or taking a picture (Figure 2). For reproducibility, all results should be recorded after the same time.

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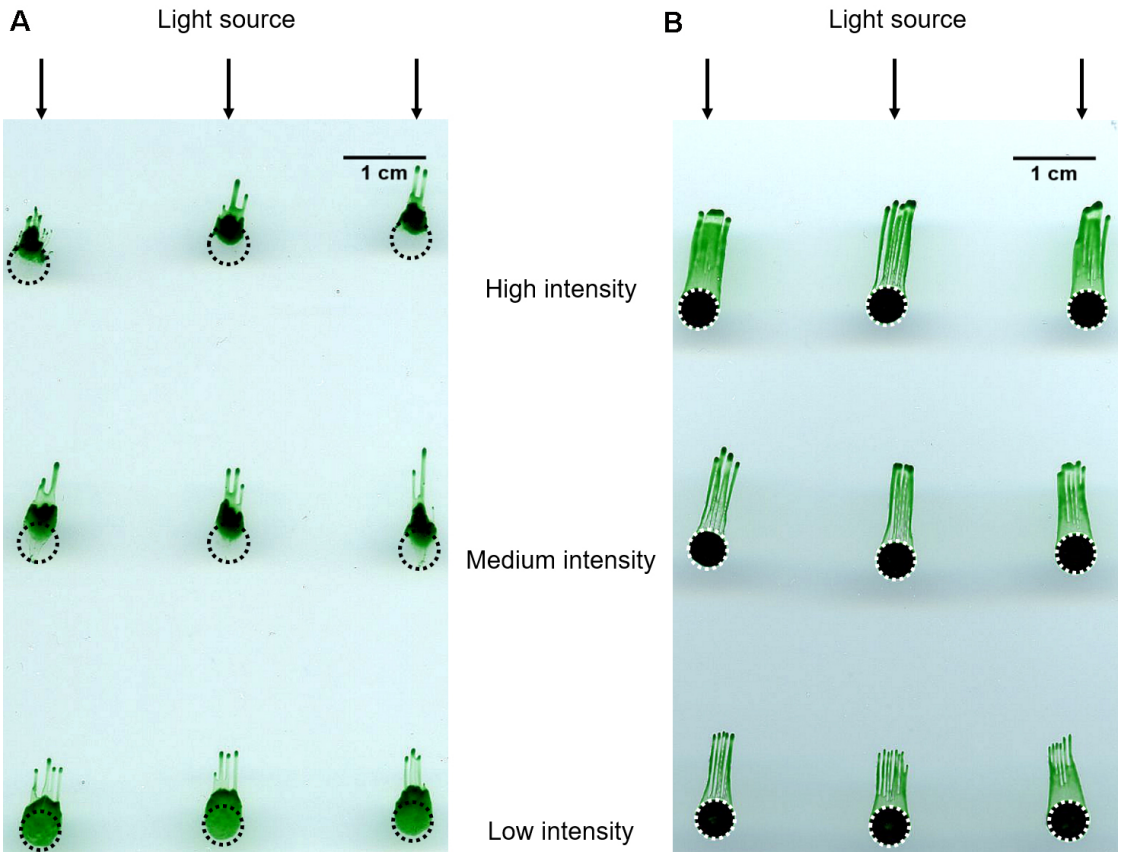

Figure 2. Macroscopic phototaxis plates. Finger-like projections of motile wild-type cells after A) one day of incubation on agar-agar, Kobe I and B) one week of incubation on standard Bacto™ agar under unidirectional white light (35 µmol photons m^{-2} sec^{-1}).

B. Microscopic phototaxis assay (Figure 3)

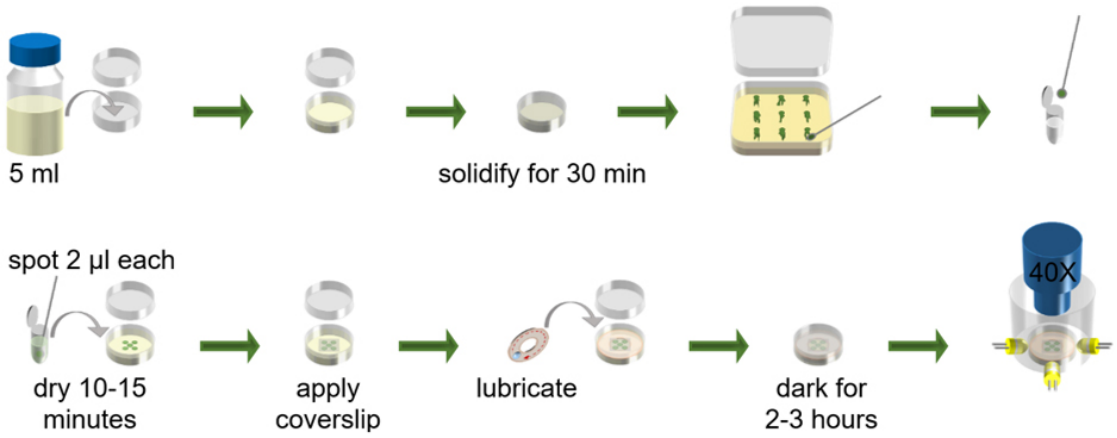

Figure 3. Microscopic phototaxis assay. Consecutive steps as described in steps B1-B12.

1. Prepare 50 ml of the microscopic phototaxis plate medium (see Recipes).
2. Mix very gently using a pipetting aid to avoid any air bubbles.

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3. Pour 5 ml of the medium carefully into each sterile glass bottom µ-dish. Use a pipetting aid and close the lids to avoid over-drying of the phototaxis plates. Let the medium solidify on a level surface to prevent unevenness. As above plates should always be freshly prepared.
4. Scrape motile cyanobacterial cells with a sterile 1 µl inoculation loop from a fresh macroscopic phototaxis plate and resuspend in 110 µl 1x BG11 medium (see Recipes) in a sterile 1.5 ml Eppendorf tube by vigorously twisting the loop (approximately OD_{750} 1.0 or very light green color). Be careful not to scrape any agar from the surface. The density of the cells is crucial for the outcome of the experiment. If the density is too low, the cells will not move properly. If the density is too high, the cells will obstruct each other and tracking will be negatively affected.
5. Spot 5 x 2 µl droplets of the cell suspension in the center of each plate and let dry for 10-15 min. Avoid over-drying and exposure to intense light (*e.g.*, sunlight on the bench).
6. Carefully place a coverslip on top of the cells immediately after all the liquid has been soaked in. Avoid air bubbles but do not press down with force. If the coverslip is applied too early or pressed down with force, the cells will float and Brownian movement will be observed, predominantly. If the coverslip is applied too late, over-drying of the surface will hinder cell movement.
7. Lubricate one side of a silicon ring with liquid paraffin and place the ring on the plate. The ring should cover all of the exposed agarose surface to minimize evaporation and prevent jittering of the sample while imaging. Be careful not to contaminate the surface of the coverslip with the liquid paraffin.
8. Incubate the samples in a dark environment for 2-3 h.
9. Place the phototaxis plate on the stage of an upright microscope equipped with a 40x objective and cover it with a non-transparent hollow cylinder with 4 holes positioned in increments of 90°. Insert the LEDs into the holes and align the pins horizontally to the stage. Make sure that the LEDs touch the center of the plate and focus on the cells. The LEDs are positioned at the same height as the agarose surface.
10. Start the microscope software NIS-Elements Basic Research 4.20.01. Dim the condenser light of the microscope so that it is barely visible. Typically, the histogram displayed in the microscope software should be in the lower range for acquisition times > 100 msec. If the intensity of the condenser light is too high, the cells will show impaired directional motility. If the intensity of the condenser light is too low, tracking will be negatively affected.
11. Switch on the RGB-LEDs as desired using a microcontroller board (*e.g.*, Arduino UNO R3 with the software Arduino 1.0.6). For a standard phototaxis assay directional white light illumination from RGB-LEDs (470/525/625 nm at equal intensities) with a total intensity of 10 µmol photons m^{-2} sec^{-1}, measured with a quantum sensor, is used.
12. Acquire images as necessary. For a standard 5 min time lapse video a frame rate of 1 frame every 3 sec at an acquisition time of 200 msec is used (Video 1).

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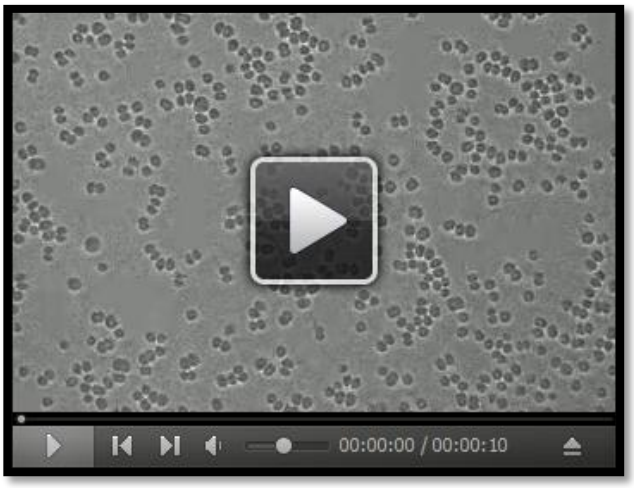
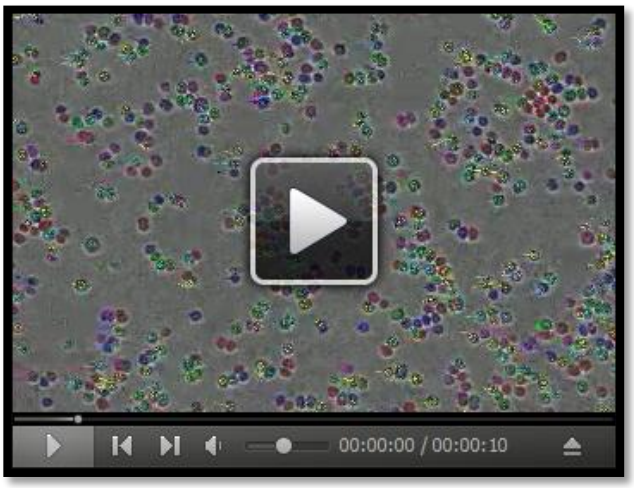
Video 1. Time lapse video of wild-type cells illuminated with 10 µmol photons m^{-2} sec^{-1} from the right side



Data analysis

1. Import your data into the BacteriaMobilityQuant software. Adjust settings such as acquisition time and scale in the parameters → update tab. Run the program. The outlines of the cells are detected separately for each frame (Figure 4A) before the actual tracking of the cells (Figure 4B).
   Note: BacteriaMobilityQuant requires MATLAB Runtime 8.3 to be installed on your PC.
2. The program returns two .txt files containing the tracking information of the cells. Trajectories are displayed in a .png file (Figure 4C) and a movie of the tracked cells is saved in .avi format (Video 2).

Video 2. BacteriaMobilityQuant cell tracking of wild-type cells illuminated with 10 µmol photons m^{-2} sec^{-1} from the right side



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3. Data analysis of the raw tracks can be performed using the software R. Some form of data cleansing is recommended to discard irrelevant tracks from the dataset (Figure 4D). To avoid artifacts due to possible mismatching of cells between frames by the tracking algorithm we only consider cells that can be tracked for at least 25 consecutive frames with an average velocity below 0.4 µm sec^{-1} and a maximum displacement of less than 8 µm between two frames (3 sec). Moreover, for tracking we discard the subgroup of immotile cells with an average velocity below 0.05 µm sec^{-1}. The best values depend on overall cell speed and frame rate and should be evaluated carefully. If desired, directional statistics of the data like a Rayleigh test of uniformity can be performed using the CIRCULAR package (Agostinelli and Lund, 2013) implemented in R.

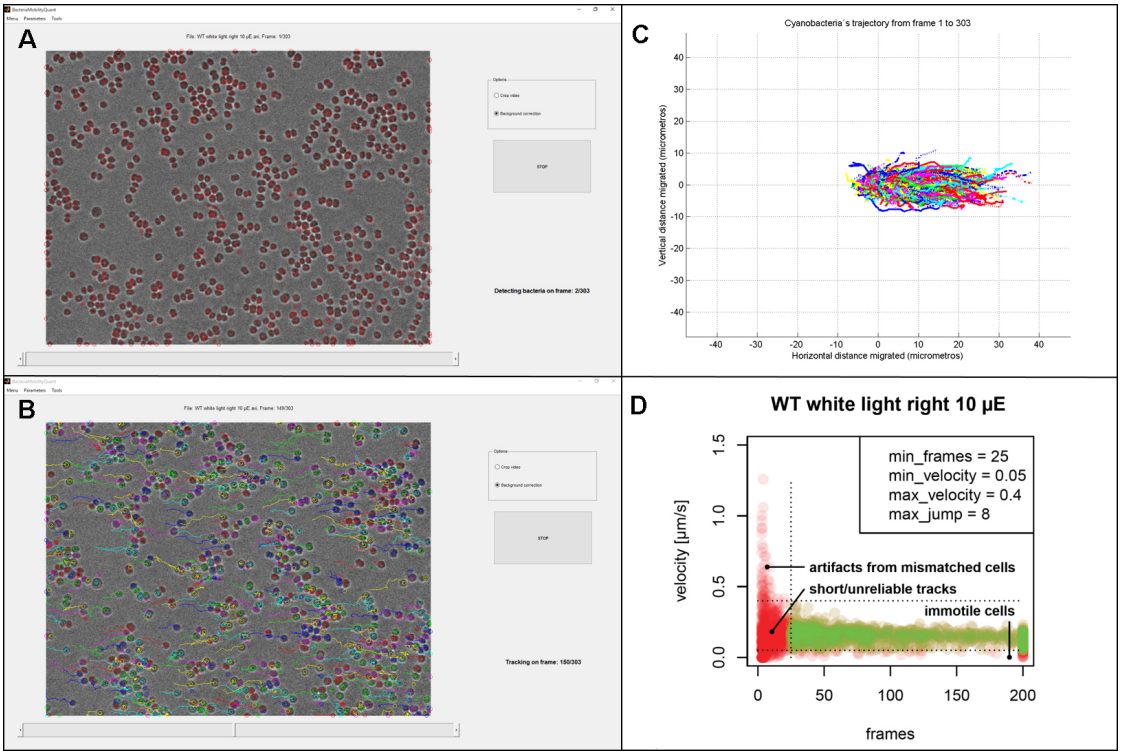

Figure 4. BacteriaMobilityQuant analysis of wild-type cells illuminated with 10 µmol photons m^{-2} sec^{-1} from the right side. A. BacteriaMobilityQuant cell detection; B. BacteriaMobilityQuant cell tracking; C. BacteriaMobilityQuant cell trajectories; D. Data cleansing with R to remove unreliable tracks and immotile cells.

Notes

1. All solutions are prepared using ultrapure water (resistivity > 18.18 MΩ cm at 25 °C) and analytical grade reagents.
2. 1% w/v agar solution and 0.6% w/v agarose solution should be prepared freshly prior to each phototaxis assay.

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3. For reproducibility, it is highly recommended to use agar-agar, Kobe I for the macroscopic phototaxis plates and UltraPure™ agarose for the microscopic phototaxis plates. In our group, a significant increase in motility was observed when changing from standard Bacto™ agar to agar-agar, Kobe I (Figure 2).
4. For reproducibility, $Na_2S_2O_3$ should be used in the plates (Thiel *et al.*, 1989) (see Recipes) and all results should be recorded after the same time.
5. For the macroscopic phototaxis assays, we achieve the best results when we take cells from a freshly grown agar plate and incubate them on a phototaxis plate under unidirectional white light for three days. The front line of the cells can now be used for the actual phototaxis assay.
6. Microscopic phototaxis assays are conducted approximately 2-3 h after spotting the cells on the agarose plates and letting them adjust to the surface in a dark environment. In this phase of motility cells show a strongly biased movement towards a directional light source.
7. For the microscopic phototaxis assays, it is also possible to use an inverted microscope if the protocol is adjusted accordingly.
8. During the microscopic phototaxis assay pay attention to the orientation of possible air bubbles between plate and coverslip. Never place air bubbles between light source and bacteria to prevent scattering.
9. Microscopic phototaxis assays are carried out at room temperature (approximately 22 °C).
10. Every experimental step involving the handling of cyanobacterial cell cultures must be performed in a biological safety cabinet to prevent contamination.

Recipes

1. 0.5 M Na_2EDTA, pH 8.0 (1,000 ml)
   186.12 g $Na_2EDTA{\cdot}2H_2O$
   Add 800 ml ddH_2O
   Adjust to pH 8.0 with NaOH
   Note: Use highly concentrated NaOH, Na_2EDTA dissolves only when the pH of the solution is adjusted to 8.0.
   Add ddH_2O to 1,000 ml
   Autoclave and store at room temperature
2. 3% w/v $K_2HPO_4{\cdot}3H_2O$ solution (50 ml)
   1.5 g $K_2HPO_4{\cdot}3H_2O$
   Add ddH_2O to 50 ml
   Filter sterilize using a syringe and a filter
   Store at 4 °C
3. 2% w/v Na_2CO_3 solution (50 ml)
   1.0 g Na_2CO_3
   Add ddH_2O to 50 ml

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Filter sterilize using a syringe and a filter

Store at 4 °C

4. Trace metal mix solution (500 ml)

   400 ml ddH_2O

   1.43 g H_3BO_3

   900 mg $MnCl_2{\cdot}4H_2O$

   110 mg $ZnSO_4{\cdot}7H_2O$

   195 mg $Na_2MoO_4{\cdot}2H_2O$

   39.5 mg $CuSO_4{\cdot}5H_2O$

   24.7 mg $Co(NO_3)_2{\cdot}6H_2O$

   Add ddH_2O to 500 ml

   Filter sterilize using a syringe and a filter

   Store at 4 °C

5. 0.6% w/v ammonium ferric citrate solution (50 ml)

   300 mg ammonium ferric citrate

   Add ddH_2O to 50 ml

   Filter sterilize using a syringe and a filter

   Store at 4 °C

6. 1 M TES buffer pH 8.0 (50 ml)

   30 ml ddH_2O

   11.45 g 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid (TES)

   Adjust to pH 8.0 with NaOH

   Add ddH_2O to 50 ml

   Filter sterilize using a syringe and a filter

   Store at 4 °C

7. 30% w/v $Na_2S_2O_3$ solution (50 ml)

   15 g $Na_2S_2O_3$

   Add ddH_2O to 50 ml

   Filter sterilize using a syringe and a filter

   Store at 4 °C

8. 40% w/v D(+)-glucose solution (50 ml)

   20 g D(+)-glucose

   Add ddH_2O to 50 ml

   Filter sterilize using a syringe and a filter

   Aliquot in 1.5 ml Eppendorf tubes and store at 4 °C

9. 100x BG11 medium (500 ml)

   74.79 g $NaNO_3$

   3.75 g $MgSO_4{\cdot}7H_2O$

   1.80 g $CaCl_2{\cdot}2H_2O$

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0.30 g citric acid
0.28 ml 0.5 M Na_2EDTA pH 8.0
Add ddH_2O to 500 ml
Autoclave and store at 4 °C

10. 2x BG11 medium (1,000 ml)
    900 ml ddH_2O
    20 ml 100x BG11 medium
    2 ml 3% w/v $K_2HPO_4{\cdot}3H_2O$ solution
    2 ml 2% w/v Na_2CO_3 solution
    2 ml trace metal mix solution
    Add ddH_2O to 998 ml
    Autoclave
    2 ml 0.6% w/v ammonium ferric citrate solution
    Store at room temperature
11. 1x BG11 medium (1,000 ml)
    900 ml ddH_2O
    10 ml 100x BG11 medium
    10 ml 1 M TES buffer pH 8.0
    1 ml 3% w/v $K_2HPO_4{\cdot}3H_2O$ solution
    1 ml 2% w/v Na_2CO_3 solution
    1 ml trace metal mix solution
    Add ddH_2O to 999 ml
    Autoclave
    1 ml 0.6% w/v ammonium ferric citrate solution
    Store at room temperature
12. 1% w/v agar solution (200 ml)
    2 g agar-agar, Kobe I
    Add ddH_2O to 200 ml
    Autoclave and use freshly
13. 0.6% w/v agarose solution (100 ml)
    0.6 g UltraPure™ agarose
    Add ddH_2O to 100 ml
    Autoclave and use freshly
14. Macroscopic phototaxis plate medium (80 ml)
    0.8 ml 1 M TES buffer pH 8.0
    0.8 ml 30% w/v $Na_2S_2O_3$ solution
    0.4 ml 40% w/v D(+)-glucose solution
    39 ml 2x BG11 medium
    39 ml molten 1% w/v agar solution

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Mix and pour very gently to avoid any air bubbles and unevenness of the surface
Avoid drying of the plate

15. Microscopic phototaxis plate medium (50 ml)
    0.5 ml 1 M TES buffer pH 8.0
    0.5 ml 30% w/v $Na_2S_2O_3$ solution
    0.25 ml 40% w/v D(+)-glucose solution
    24.375 ml 2x BG11 medium
    24.375 ml molten 0.6% w/v agarose solution
    Mix and pour very gently using a pipetting aid to avoid any air bubbles and unevenness of the surface
    Avoid drying of the plates

Acknowledgments

This protocol is based on the methods described in Schuergers *et al*. (2016) and Schuergers *et al*. (2015). BacteriaMobilityQuant software for cell tracking was developed by Tiago Esteves and Maja Temerinac-Ott (Schuergers *et al.*, 2016). BG11 medium is prepared according to Rippka *et al*. (1979). This work was funded by the Excellence Initiative of the German Research Foundation (GSC-4, Spemann Graduate School).

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