AUG 31, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.5qpvobq59l4o/v1

Protocol Citation: Carly M Moreno, Shady Amin 2023. Phycopick protocol for isolating single phytoplankton cells. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvobq59l4o/v1

Protocol status: Working
We use this protocol and it's working

Created: Apr 29, 2022

Phycopick protocol for isolating single phytoplankton cells

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ABSTRACT

Interactions between bacteria and eukaryotic phytoplankton have major influences on marine ecosystems and the global carbon cycle. Metabolite exchanges between specific bacteria and phytoplankton species result in beneficial or antagonistic associations that influence the physiology and productivity of both groups and occur within the microenvironment of the phytoplankton host, termed the phycosphere. Current sampling techniques to study these interactions are limited to sampling batch cultures or large-volume seawater, which obscures distinction between phycosphere associated bacteria and free-living bacteria. Single-cell isolation methods, such as fluorescence-activated cell sorting (FACS), have been used to isolate phytoplankton and bacteria; however, FACS only enables isolation of bacteria tightly associated with phytoplankton cells but not those loosely associated or ones that track the phycosphere using chemotaxis. Such techniques also are not amenable to large phytoplankton cell sizes or chains. Here, we present a step-by-step protocol for the isolation of phytoplankton cells and the bacteria within their phycosphere using the PhycoPick, a microosmotic pump manufactured to enable robust micro-isolation and transfer of small particles without pulsation. The PhycoPick is able to isolate volumes in the pico-to-nanoliter range, which is ideal for capturing bacteria in the phycosphere while avoiding capturing free-living bacteria. The phycospheres are carefully isolated through intuitive manipulation of the micropump inflow and outflow controls to enable the isolation of a single phytoplankton cell or chain along with the small volume of water surrounding it. These phycospheres can then be used for downstream analyses, including phytoplankton cultivation, bacterial enumeration and cultivation, amplicon and shotgun metagenomics sequencing of these communities, and targeted metabolomics of phycosphere metabolites using mass spectrometry. The PhycoPick is a powerful system to query the interactions between these microeukaryotic hosts and their bacteria in culture or microbial communities in aquatic environments.

Last Modified: Aug 31, 2023

PROTOCOL integer ID: 61688

GUIDELINES

This protocol describes the steps required to isolate single phytoplankton cells and their associated phycospheres, from culture medium or seawater, using a tandem NepaGene microscope and pipette system, herein referred to further as a Phycopick. Once the cell is picked, it can be manually sorted onto microscope slides or PCR strip tubes for different downstream applications, e.g. bacterial enumeration or genomic analyses.

BEFORE START INSTRUCTIONS

Required materials

- 12 or 24-well plates
- Microscope slides, small petri dishes, or PCR strip tubes
- 5 mL syringe
- 70% reagent grade ethanol
- MilliQ water
- Lysis buffer or cell storage solution

Specific Nepa Gene products and manuals required for the Phycopick protocol

- CompassZ Zrobo-mounted Video Microscope (3-OMN-Z1)
- Zrobo Controller (1-TKRK-C1)
- PicoPipet Controller (1-CONTHR-3)
- Silicone tubing, 1.5mm (1-ST-D)
- S-shaped glass micropipettes tips (30 or 50 µm) (1-GT30-10 or 1-GT50-10)

Instrument set up

1 1. Flush the glass micropipette tip by removing the PicoPipet reservoir cap, attaching a small syringe filled with ultrapure MilliQ water, and pushing enough water through the PicoPipet head and glass micropipette tip to ensure there are no air bubbles in the system. Remove the syringe and reattach the reservoir cap.

1.1 Note: Any air bubbles in the head of the PicoPipet or the glass micropipette tip will result in uneven, pulsating flow.

2 Attach the PicoPipet Controller cables to the PicoPipet head and mount on the PicoPipet holder of the CompassZ Zrobo-mounted video microscope.

3 Prepare samples by transferring 1 mL of culture or seawater in a sterile 12 or 24-well plate.

3.1 Note: To pick individual cells, or small chains of cells, the cell density of the sample should not be too high nor too low as both could make the experiment unfeasible. High-density cultures will inadvertently lead to capturing more than one cell or chain; conversely, low-density cultures, or oligotrophic seawater samples, will prevent the user from isolating cells in a reasonable amount of time. For phytoplankton cultures, cell concentration should be between 5000-10,000 cell/mL.

3.2 Note: For cultures or seawater, phytoplankton cells less than 30 or 50 µm can be captures depending on the size of the glass micropipette used.

4 Fill a well with 70% ethanol for sterilization of the pipette tip between samples.

5 Power on the PicoPipet controller, the Zrobo Controller, and CompassZ Zrobo-mounted video microscope.

6 Turn the small knob (coarse scale flow) on the controller clockwise (+) to increase outflow to 30 to visually inspect water coming out of the glass micropipette tip forming a small bubble. Turn the small knob counterclockwise back to 0 or press the small knob down to return outflow back to 0. If water is not coming from the pipette tip or if any air bubble formed within the pipette system, repeat flushing of the pipette system from Step

6.1 Note: the value for outflow and inflow are unitless and are meant to help the user intuitively adjust the current for smooth flow.

7 On the CompassZ Zrobo-mounted video microscope, move the X, Y and Z knobs to the middle of their respective tracks.

8 Focus the CompassZ Zrobo-mounted video microscope on the glass pipette tip by first

decreasing magnification to its lowest objective and gently adjusting the tip into view in the center of the screen. To increase magnification, the Zoom Ring on the microscope can be adjusted. Any change in magnification will require adjustments to the small Z knob to refocus.

9 For larger z-direction movement, for example to automatically move the pipette tip completely out of the sample and then into a microcentrifuge tube, the user can program different heights using the Zrobo controller. We recommend 4 different heights, 4, 15, 25, and 65 mm. These heights will be used in the rest of the protocol but are a suggestion and can be programmed to other values as needed.

Glass micropipette tip sterilization

10 Lower the PicoPipet system using the Zrobo controller down to 4 mm and submerge the glass micropipette tip in 70% ethanol to sterilize. Note: lowering the glass tip into any liquid will require slight refocusing with the Zoom Ring and Z knob.

11 Turn the small knob clockwise so that outflow of MQ-water is visible on the screen as a plume. Due to the large difference in solvent density between 70% ethanol and MQ-water inside the pipette, a liquid plume should be easily visible on the microscope screen. It is helpful to move the glass micropipette tip back and forth with the metal joystick to see the plume trail. To sterilize the inside of the pipette tip, wash the pipette tip by increasing the inflow counterclockwise so that ethanol is flowing into the tip. Wait ~3 seconds and reverse the flow to outflow so that ethanol is pushed out. Repeat process 3 times.

12 Decrease outflow with large knob (fine scale flow) so that only a very small outflow is visible as a faint plume. Use the Step Select button to make smaller voltage increments (0.5, 0.01, 0.001) to make very small adjustments in outflow. Decrease outflow as much as possible but ensure that there is no inflow to avoid inadvertently capturing multiple cells

12.1 Note: This step will have to be repeated occasionally as the PicoPipet controller drifts.

13 Raise the Picopipet system out of the ethanol to 65 mm and then lower to 4 mm into a well with a sample.

Isolating single cells

14 Refocus the glass micropipette tip. Once a cell is near the tip increase inflow slowly (large knob)

to gently draw up a cell or small chain into the pipette tip.

14.1 Note: Ideally, the inflow/outflow is accurately adjusted so that the user can see the cell enter the tip and 'float' within the tip. The inflow should not be so high that many cells are drawn up or that they disappear very quickly from view, although this is sometimes unavoidable.

15 Once the cell is captured in the pipette tip, immediately press the small knob to return to 0 flow to prevent further inflow and lift the PicoPipet system with the Zrobo controller.

16 Lower the stage to 4 and further adjust the height with Z knob so that the tip of the pipette is just in contact with a microscope slide or PCR tube. Increase outflow to expel a 1-2 µL in lysis solution or cell storage solution of user's choice.

17 Raise the stage to 65 and then lower to 4 mm in ethanol and re-sterilize the pipette tip.