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TITLE:

Effective Lysis of Cyanobacterial and Green Algal Single Cells for Whole Genome Amplification in Microfluidics

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KEYWORDS:

Plant cell, bacterial, microbial, multiple displacement amplification, cell lysis, single cell

SUMMARY:

Here we present a protocol to lyse cyanobacteria and green algae single cells that allows for subsequent single-cell whole genome amplification in a microfluidic platform with a 100% success rate.

ABSTRACT:

Single-cell sequencing is becoming popular for analyzing the genome of a single cell within a heterogenous cell population. It often relies on microfluidic tools to perform single cell isolation and nanoscale chemical reactions to lyse the cell and amplify its genome. However, single-cell sequencing has mainly been applied to human cells and certain bacterial species that are easy to lyse. It is still rare to use single-cell sequencing in environmental studies, as many species of vital environmental significance such as cyanobacterial and green algal species have complex

and rigid cell wall structures. To extend single-cell sequencing to these hard-to-lyse species, it is essential to develop an effective lysis method compatible with microfluidic tools and amplification chemistry. Here, we present a lysis method proven effective for cyanobacterial and green algal species for subsequent microfluidic-based single-cell whole genome sequencing (SC-WGA). The protocol combines thermal and chemical lysis mechanisms and has achieved >25 ng DNA for 100% of the single cells after on-chip amplification. *Nostoc* was chosen as a cyanobacterial model for the protocol development. The optimized protocol was directly applied to *Gloeocapsa*, another cyanobacterial species, and *Sphaerocystis*, a eukaryotic green alga, without modifications and achieved a 100% success rate.

INTRODUCTION:

Single-cell whole genome sequencing (SC-WGS) has been popular for studying the genetic heterogeneity of complex cell communities on a cellular level¹⁻³. Generally, SC-WGS requires single-cell isolation, lysis, and amplifying the femtograms to picograms of genomic DNA to generate enough DNA for standard library preparation (>25 ng)⁴⁻⁵. Microfluidics is an ideal tool for SC-WGA, as it handles nanoscale of fluid precisely⁶⁻¹², permitting single cell isolation into microchambers for lysis and genome amplification¹³⁻¹⁴. Multiple displacement amplification (MDA)¹⁵ has been a common chemistry technique in microfluidic-based SC-WGA. It uses ϕ 29 DNA polymerase and random primers to produce copies of template DNA with relatively high fidelity and low error rates, and it is easy to implement in most microfluidic systems¹⁶⁻¹⁸.

So far, SC-WGS has been mostly performed on human cells, as they can be simply lysed and processed following most SC-WGA kit instructions. Bacterial SC-WGA is more challenging due to rigid and multi-layered cell walls¹⁹ and 1000x less the amount of starting DNA²⁰. Even so, efforts have been directed to bacterial SC-WGA, as these microorganisms²¹ are being increasingly recognized as critical factors in human microbiome²²⁻²³. However, SC-WGS is still rare in species significant to environmental studies including cyanobacteria²⁴⁻²⁵ and green algae²⁶⁻²⁷ due to complex and thick cell structures that are often enveloped in extracellular matrix and thus hard to lyse.

Standard methods for mechanically disrupting rigid species such as bead-beating, sonication, and lyophilization are not compatible with microfluidic tools²⁸. Chemical lysis is better suited for SC-WGA in microfluidics; however, common chemical lysis agents such as sodium dodecyl sulfate and sarkosyl have substantial deleterious effect on polymerase activities in MDA chemistry²⁹. Other effective chemicals including phenol and spermine are toxic and need to be used in a fume hood; thus, they are not generally applicable in microfluidics¹⁹. Freeze-thawing followed by alkaline treatment is efficient for cyanobacteria lysis for SC-WGA in well plates³⁰, but it requires placing the plates in a -80 °C freezer for 1 h and thus is not implementable in microfluidic systems with various control units.

A common alternative is to pre-lyse a cyanobacterial or green algal cell population in-tube using ionic surfactants followed by wash steps before injecting the cell suspension into microfluidic chips^{29, 31}. However, this approach is not applicable for cells in low abundance and will likely release DNA from the bulk cell into the extracellular milieu, leading to contamination in the SC-

WGA step. Multi-round amplification is another common option when cells cannot be sufficiently lysed, but it often leads to increased bias in the sequencing data.

To enable SC-WGA for cyanobacterial and green algal cells without the aforementioned concerns, present here is a method effective for the lysis of single cyanobacterial and green algal cells for subsequent MDA-based SC-WGA in microfluidic devices, which generates >25 ng of genomic DNA per cell. This approach is based on three major cell lysis methods including thermal^{30,32-33}, enzymatic³⁴⁻³⁵, and chemical lysis³⁶⁻³⁸. In this work, the species *Nostoc* was used as a model because of substantial lysis difficulties encountered in earlier studies as a cyanobacterial species^{19,39}. Its cell wall primarily consists of an external layer of exopolysaccharide and polymerized proteins, outer membrane, thick peptidoglycan layer, and inner cytoplasmic membrane⁴⁰. Our lysis method was designed to systematically degrade these cells from their outermost to innermost layers without suppressing MDA chemistry. This protocol was tested on another cyanobacterial species, *Gloeocapsa*, and green algal species (Chlorophyta) *Sphaerocystis*, whose cell wall is often composed of microfibrillar polysaccharides and enveloped in polysaccharides⁴¹⁻⁴². Despite the existence of various novel high-throughput microfluidic-based single cell isolation technologies such as droplet-based fluorescent-activated cell sorting¹³ and in-gel single cell trapping in virtual microfluidics⁴³, laser tweezers were chosen for single cell trapping and transport due to its higher precision and single-cell confidence, which is especially important for isolating target cells in samples with complex components. 100% success was achieved for all three species following the optimized protocol without further efforts. We believe that this effective lysis method may enable SC-WGA of cyanobacterial and green algal cells in microfluidics for a wide range of single cell genomic studies in environmental research.

PROTOCOL:

1. Preparation of desiccated cell species (*Nostoc*, *Gloeocapsa*, *Sphaerocystis*)

1.1) Add 300 µL of sample diluent [0.08% poloxamer 407 in phosphate-buffered saline (PBS)] to 2 mL tubes that contain desiccated samples of *Nostoc*, *Gloeocapsa*, and *Sphaerocystis*, respectively, to re-suspend the cells.

NOTE: The desiccated samples can be identified visually in the tube as particles before re-suspension and are aggregated to the bottom of the tube after addition of the sample diluent.

1.2) Agitate the cells clusters of *Nostoc* in the tube with a micro-pestling tool for 10 s.

1.3) Repeat step 1.2 until the *Nostoc* is fully suspended and the suspension looks cloudy without visible large particles.

1.4) Agitate the cells clusters of *Gloeocapsa* in the tube with a micro-pestling tool for 20 s.

1.5) Repeat step 1.4 until the *Gloeocapsa* is fully suspended and the suspension looks cloudy

without visible large particles.

1.6) Place the tube that contains *Sphaerocystis* in a water bath sonicator for 1 min.

1.7) Repeat step 1.6 until the *Sphaerocystis* is fully suspended and the suspension looks cloudy without large particles.

2. Lysis buffer preparation

2.1) Add 1 μL of 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 1 μL of 40 KU/ μL lysozyme, and 40 μL of 1 M dithiothreitol (DTT) to 158 μL of nuclease-free water in a 0.5 mL tube.

2.2) Mix by briefly vortexing and make 10 aliquots of 20 μL in 0.2 mL PCR tubes, then store in a $-20\text{ }^{\circ}\text{C}$ refrigerator.

3. Set-up of microfluidic device for single cell experiment

3.1) Take a microfluidic device microfabricated using polydimethylsiloxane (PDMS) according to the procedure described in our previous work²¹. Place it in a Petri dish and transfer it to a UV hood for 45 min for sterilization.

NOTE: The device consists of a flow channel layer on the top and control channel layer on the bottom separated by a thin PDMS membrane. The samples are introduced into the device and reactions are performed in the flow channels, and the control channels are used for exerting positive pressure to form microvalves. Each device has 10 parallel reaction lines for experimental reactions and two reaction lines for negative controls (**Figure 1A**).

3.2) Inject 0.4 mL of de-ionized water into the tube of the pneumatic control system using a 1 mL syringe. Set the pressure of pneumatic control system to 25 psi by turning the knob on the first regulator of the pneumatic control unit that connects to a house gas outlet.

3.3) Quickly close and open the valve that connects to the tube in the pneumatic control system via the microfluidic graphic user interface (GUI) to remove all the air at the end of the tube.

3.4) Connect the end of the tube via a bent cannula to the corresponding control channel inlet port on the microfluidic device. Close the microvalves via a microfluidic GUI for the de-ionized water to exert positive pressure on the microfluidic valve membranes (**Figure 1B**).

3.5) Repeat steps 3.2 and 3.3 with another tube in the pneumatic control system until all the relevant tubes are connected to the microfluidic device.

3.6) Wait 10-15 min until the control channels in the microfluidic device are filled with de-

ionized water. Place the microfluidic chip under the microscope and observe the control channels using a 10x objective to ensure that there are no air plugs in the control channels.

3.7) Inject 0.4 mL of chip diluent (0.04% poloxamer 407 in PBS) into a connected tube set using a 1 mL syringe. Connect the tube set to a microfluidic manifold that connects to a port on the pneumatic control unit.

3.8) Set the pressure of the pneumatic control system to 2–5 psi by turning the knob on the second regulator of the pneumatic control unit. Insert a bent cannula at the end of the tube.

3.9) Quickly close and open the valve that connects to the tube in the pneumatic control system via the microfluidic GUI to remove all the air at the end of the tube. Insert the tube into the reagent port on the microfluidic device via the bent cannula.

3.10) Insert a short tube via a bent cannula at the sample inlet and outlet ports respectively on the microfluidic device. Open the reagent gate, sample inlet gate, and sample outlet gate by clicking on corresponding buttons in the microfluidic GUI.

3.11) Introduce the chip diluent into the sample channel through the reagent inlet port by pressurizing the tubing at 2–5 psi via the second regulator in the pneumatic control system, until fluid exits the short tubes at the sample inlet and outlet ports.

3.12) Close all gates via microfluidic GUI and wait 30 min prior to experiments to prevent the cells from sticking to the PDMS channel surface during the cell sorting.

NOTE: The protocol can be paused here.

4. Cell sorting in microfluidic device using laser tweezers

4.1) Pipette 10 μ L of the cell suspension of *Nostoc*, *Gloeocapsa*, or *Sphaerocystis* made in steps 1.3, 1.5, or 1.6, respectively, using a gel-loading pipette tip. Insert the pipette tip into a new tube and inject the cell suspension followed by inserting a bent cannula.

NOTE: The cell suspension should look cloudy with no large clusters in the tube.

4.2) Disconnect the short tube from the sample inlet port. Insert the tube with cell suspension into the sample channel port via the bent cannula.

4.3) Open the gates of sample inlet and outlet via the microfluidic GUI and pressurizing the tubing at 2–5 psi via the second regulator of the pneumatic control system. Check under a 60x objective to ensure that there are visually identifiable target cells in the sample channel.

NOTE: *Nostoc*, *Gloeocapsa*, or *Sphaerocystis* can be distinguished visually from common contaminations such as *Escherichia coli* based on size and morphology.

221
222 4.4) Center the microfluidic device on the microscope stage and tighten the screws on the
223 clamps to ensure proper contact between the bottom coverslip of the microfluidic device and
224 the objective.

225
226 4.5) Place a laser warning sign on the outside of the door and close the door. Put on laser
227 protection goggles. Turn on the laser tweezers system power supply and software, and close all
228 the interlocks required to ensure safety during laser operation.

229
230 4.6) Turn on the laser beam and shutter through the software. Adjust the power bar in the
231 software to trap and move the cells around to ensure laser trapping functionality. Start with
232 low power and increase the power if necessary.

233
234 4.7) Trap a target cell by turning on the laser beam and shutter through the laser tweezer
235 software. Move it to the entrance of a closed cell isolation chamber using the motorized
236 microscopic stage controller according to the real-time microscopic view displayed via the laser
237 tweezer software (**Figure 1C**).

238
239 4.8) Open the gate of the chamber via a microfluidic GUI and move the cell inside of the
240 chamber as described in step 4.7.

241
242 4.9) Move any visually identifiable contaminating cells from the chamber. Turn off the
243 shutter of the laser beam to release the cell, then close the gate of the chamber via microfluidic
244 GUI.

245
246 4.10) Repeat steps 4.7–4.9 to isolate more single cells into separate chambers.

247
248 4.11) After the cell sorting is completed, turn off the laser beam and shutter, close the
249 software window, and shut down the laser power supply.

250
251 CAUTION: Laser protection goggles must be worn at all times during laser operating. Make sure
252 that all interlocks are closed before turning on the laser beam.

253 254 5. SC-WGA in microfluidic device

255
256 5.1) Disconnect the tubes from the sample channel inlet and outlet ports and insert a new
257 set of short tubes into these ports via bent cannula. Change the microscope objective to 10x
258 and prepare for cell lysis and genome amplification. The overview of the process is illustrated in
259 **Figure 3**.

260
261 5.2) Remove the microfluidic device from the microscope stage and place it on a cold block
262 that has been stored in -20 °C for 1 min, remove the device from the cold block, and place it
263 onto a 65 °C hot plate for 1 min. Repeat the cold-heat cycle 3x.

5.3) Open the reagent gate, then sample the inlet and outlet gates via the microfluidic GUI, pressurizing the reagent tubing at 2–5 psi via the pneumatic control system to flush out redundant cells in the channels using chip diluent for 3 min.

5.4) Close all gates via the microfluidic GUI and disconnect the tubes from the sample channel inlet and outlet ports, then insert a new set of tubes.

5.5) Take a 20 μ L aliquot of customized lysis buffer and thaw on ice. Add 3.5 μ L of 10% polysorbate 20 to the lysis buffer. Mix by briefly vortexing and spin down on a microcentrifuge.

5.6) Disconnect the tube from the reagent port. Pipette 20 μ L of customized lysis buffer using a gel-loading pipette tip.

5.7) Insert the pipette tip into a new tube and inject the buffer, then insert a bent cannula into the tube. Connect the tube to the microfluidic manifold that connects to a port on the pneumatic control unit.

5.8) Insert the tube with customized lysis buffer into the reagent port on the microfluidic device via a bent cannula. Open the reagent gate via the microfluidic GUI while pressurizing the tube at 2–5 psi via the pneumatic control unit.

5.9) Open the sample inlet and outlet gates briefly to flush the channel and close these gates. Check under the microscope to ensure all air bubbles are removed.

5.10) Open the cell isolation gate and lysis chamber 1 gate via the microfluidic GUI until the lysis buffer fills the lysis chamber 1. Close all gates via the GUI and move the microfluidic chip to a hot plate set at 37 °C and keep the chip on the hot plate for 2 h.

5.11) Use tape to stick the bottom of the device to the surface of the hot plate to maximize contact between the device and hot plate. Prepare for the introduction of reagents into the microfluidic device for reactions. The overview of the process is illustrated in **Figure 1D**.

5.12) Take a 20 μ L aliquot of D2 lysis buffer (alkaline lysis buffer) from the -20 °C block and thaw on ice. Add 3.5 μ L of 50% polysorbate 20 to the D2 lysis buffer. Mix by briefly vortexing and spin down on a microcentrifuge.

5.13) Pipette 20 μ L of D2 lysis buffer using a gel-loading pipette tip. Insert the pipette tip into a new tube and inject the buffer followed by inserting a bent cannula into the tube.

5.14) Remove the microfluidic chip from the hot plate to the microscope stage. Disconnect the tube from the reagent port.

5.15) Insert the tube with D2 lysis buffer into the reagent port on the microfluidic device via a bent cannula. Connect the tube set to a microfluidic manifold that connects to a port on the

pneumatic control unit.

5.16) Open the reagent gate via microfluidic GUI and pressurizing the tube at 2-5 psi via pneumatic control unit. Open the sample inlet and outlet gates briefly to flush the channel and close these gates. Check under the microscope to ensure all air bubbles are removed.

5.17) Open the cell isolation gate, lysis chamber 1 gate, and lysis chamber 2 gate until the D2 lysis buffer fills lysis chamber 2 as observed under the microscope. Close all gates and place the microfluidic chip to a hot plate set at 37 °C and keep the chip on the hot plate for 2 h.

5.18) Use tape to stick the bottom of the device to the surface of the hot plate to maximize contact between the device and hot plate.

5.19) Take a 20 µL aliquot of neutralization buffer from the -20 °C block and thaw at room temperature (RT). Briefly vortex and spin down on a microcentrifuge.

5.20) Pipette 20 µL of neutralization buffer using a gel-loading pipette tip. Insert the pipette tip into a new tube and inject the buffer followed by inserting a bent cannula into the tube.

5.21) Remove the microfluidic chip from the hot plate to the microscope stage. Disconnect the tube from the reagent port.

5.22) Insert the tube with neutralization buffer into the reagent port on the microfluidic device via a bent cannula. Connect the tube to the microfluidic manifold that connects to a port on the pneumatic control unit.

5.23) Open the reagent gate while pressurizing the tube at 2–5 psi. Open the sample inlet and outlet gates briefly to flush the channel, then close the gates. Check under the microscope to ensure all air bubbles are removed.

5.24) Open the cell isolation gate, lysis chamber 1 gate, and lysis chamber 2 gate, then stop the buffer gate until neutralization buffer fills the stop buffer chamber as observed under the microscope. Close all gates and incubate the chip at RT for 10 min on the microscope stage.

5.25) Take a 2 µL aliquot of DNA polymerase, 29 µL aliquot of reaction buffer, and 9 µL aliquot of nuclease-free water from the -20 °C block, then thaw on ice. Add 29 µL of reaction buffer to 9 µL of nuclease-free water, briefly vortex, and spin down on a microcentrifuge.

5.26) Add 2 µL of DNA polymerase to the mixture, briefly vortex and spin down on a microcentrifuge. Add 10 µL of 25% glycerol into the mixture, briefly vortex, and spin down on a microcentrifuge and keep on ice.

5.27) Pipette 20 µL of the prepared DNA polymerase using a gel-loading pipette tip. Insert the pipette tip into a new tube and inject the buffer followed by inserting a bent cannula into the

353 tube.

354
355 5.28) Remove the microfluidic chip from the hot plate to the microscope stage. Disconnect
356 the tube from the reagent port. Insert the tube with prepared DNA polymerase into the reagent
357 port on the microfluidic device via a bent cannula.

358
359 5.29) Connect the tube to the microfluidic manifold that connects to a port on the pneumatic
360 control unit. Open the reagent gate via the microfluidic GUI while pressurizing the tube at 2–5
361 psi via the second regulator in the pneumatic control unit.

362
363 5.30) Open the sample inlet and outlet gates briefly to flush the channel and close the gates.
364 Check under the microscope to ensure all air bubbles are removed.

365
366 5.31) Open the cell isolation gate, lysis chamber 1 gate, lysis chamber 2 gate, stopping buffer
367 gate, and reaction chamber gate until reaction buffer mixture fills the reaction chamber as
368 observed under the microscope. Close all gates and move the microfluidic chip to a hot plate
369 set at 32 °C for 16 h.

370
371 5.32) Use tape to stick the bottom of the device to the surface of the hot plate to maximize
372 contact between the device and hot plate.

373 374 **6. Collecting on-chip SC-WGA products**

375
376 6.1) Incubate the microfluidic device on a hot plate set at 65 °C for 3 min to terminate the
377 reaction. Add 10 µL of polysorbate 20 to a 1.5 mL tube of nuclease-free water.

378
379 6.2) Use a 1 mL sterile syringe to aspirate 0.5 mL of nuclease-free water from the 1.5 mL
380 tube and inject it into a new tube set. Connect the tube set to the microfluidic manifold that
381 connects to a port on the pneumatic control unit.

382
383 6.3) Remove the microfluidic chip from the hot plate to the microscope stage. Disconnect
384 the tube from the reagent port.

385
386 6.4) Insert the tube with nuclease-free water into the reagent port on the microfluidic chip
387 via the cannula. Open the reagent gate while pressurizing the tube at 2–5 psi via the second
388 regulator pneumatic control system.

389
390 6.5) Open the sample inlet and outlet gates to flush the channel for 3 min and close the
391 gates. Check under the microscope to ensure all air bubbles are removed.

392
393 6.6) Insert 20 µL gel-loading pipette tips into all product outlet ports on the microfluidic
394 device. Open all gates except the sample inlet and outlet gates so that the nuclease-free water
395 flushes the amplified product out of the chip and into the pipette tips.

6.7) Take out the pipette tips and transfer the fluid with a pipette to 0.2 mL PCR tubes. Perform a DNA quantification assay to measure the amount of DNA in each sample amplified from a single cell.

6.8) Store the samples in -20 °C for long-term storage until sequencing.

REPRESENTATIVE RESULTS:

The protocol was developed in our optofluidic platform at the Mayo Clinic²¹. This platform consists of a microscope, optical tweezers, and microfluidic chip that support the serial addition of reagents (**Figure 1A–D**). **Figure 2** illustrates the cell wall structures of the cyanobacterial and green algal species tested using the protocol. **Figure 3** shows the overall workflow of the lysis and SC-WGA in microfluidic devices.

Figure 4A shows the effects of different temperature ranges and cycles of heat-shock treatment on the lysis for subsequent SC-WGA of *Nostoc*. **Figure 4B** shows the combined effect of heat-shock treatment, lysozyme, and DTT on the lysis and WGA of *Nostoc*. Optimal results (100% lysis and amplification rates, >25 ng DNA) were achieved using 200 U/μL lysozyme and 200 mM DTT at 37 °C for 2 h, followed by D2 lysis buffer at 37 °C for 1 h.

Figure 5 shows results from validating the optimized protocol using *Gloeocapsa* and *Sphaerocystis*, which are two hard-to-lyse species. **Figure 5A** shows images of these three species under microscope. **Figure 5B** shows 100% success rates (100% lysis and amplification rates, >25 ng DNA) achieved for both species without the need for additional protocol modifications. **Figure 5C** shows that the DNA amplified from *Nostoc*, *Gloeocapsa*, and *Sphaerocystis* using the protocol was not degraded based on electrophoresis results.

FIGURE AND TABLE LEGENDS:

Figure 1: Optofluidic SC-WGA platform. (A) The platform consists of a high-resolution microscope, laser tweezing system, and PDMS-based microfluidic device with 10 parallel reaction lines and two negative control reaction lines. (B) The microfluidic device consists of flow channels in the top layer and control channels in the bottom layer, separated by a thin PDMS membrane. Pressuring control channels into flow channels at the junction forms closed microchambers. (C) Time-elapsd images of the trapping and moving of a single *Nostoc* cell into a microchamber. The inset shows the location of the cell in the channel. (D) An illustration of on-chip SC-WGA procedure, which involves the sequential opening of valves for single cell isolation and the addition of lysozyme, DTT mixture, D2 lysis buffer, neutralization buffer, and master mix. This figure is reprinted with permission²¹.

Figure 2: Illustration of cell wall structures of cyanobacterial and green algal species.

Figure 3: General workflow of single cell isolation, lysis, and on-chip genome amplification. The green box highlights the optimized lysis protocol.

Figure 4: Optimization of heat-shock, lysozyme, and DTT effects on *Nostoc* lysis for SC-WGA.

(A) The temperature and cycle effects on *Nostoc* lysis for SC-WGA. After heat-shock, 200 U/ μ L lysozyme with 0.5 mM EDTA was added and incubated at 37 °C for 2 h. The D2 alkaline lysis buffer was then added and incubated at 65 °C for 2 h. (B) The DTT effect on *Nostoc* lysis for SC-WGA. After heat-shock, a mixture of 0.5 mM EDTA, 200 U/ μ L lysozyme, and DTT of various concentrations was added and incubated at 37 °C for 2 h, followed by addition of D2 alkaline lysis buffer (n = 10 single cells per condition; error bars represent standard deviation). This figure is reprinted with permission²¹.

Figure 5: Protocol validation results. (A) Microscopic images of *Nostoc*, *Sphaerocystis*, and *Gloeocapsa*. The black boxes indicate additional cells from another capture to representatively illustrate cells in the suspension. (B) Use of the optimized lysis protocol on *Sphaerocystis* and *Gloeocapsa* for lysis and SC-WGA (n = 10 single cells per species per condition; error bars represent standard deviation). Each set of experiments was repeated 3x. The sterile PBS as a negative control did lead to measurable DNA after amplification (not shown). (C) The Tapestation results of amplified DNA from single *Nostoc*, *Gloeocapsa*, and *Sphaerocystis* cells. The DNA was not degraded. Three single cells from each species are shown. This figure is reprinted with permission²¹.

DISCUSSION:

During the process of single cell isolation in a microfluidic device using laser tweezers, it is essential to ensure that no undesired cells are in the cell isolation chambers prior to adding lysis buffers. Undesired cells should be moved out of the chamber to minimize contaminating DNA caused by these cells. Earlier studies have shown that lasers with wavelength between 1250 nm and 1550 nm with 100 mW power can increase the temperature and rupture the cell membrane⁴⁴. However, other evidence has shown that a 1064 nm laser with 50 mW power can exert marginal cell viability effects^{14, 45-46}. Thus, we do not predict that the laser tweezing in this work significantly contributed to the cell lysis efficacy.

Performing heat-shock at different temperatures resulted in different SC-WGA rates and yields for the species *Nostoc*. For typical bacterial species, less aggressive heat-shock (e.g., narrower temperature ranges and decreased cycles) can be used to disrupt cell walls. However, in species having thicker and more complex cell wall structures such as cyanobacteria and green algae (or other cells with equally complex structures), a larger temperature range is necessary to facilitate cell lysis. Temperatures of -20 °C and 65 °C achieved the optimal results without compromising DNA quality for these species.

Lysozyme and DTT played a crucial role in disrupting the cell wall of bacterial species. Lysozyme is known for attacking the peptidoglycan layers, and the DTT degrades the extracellular polysaccharide matrix^{29,35,47}. Generally, the results show that single cell lysis and amplification efficiency increase with the concentration of both agents. This protocol tested as much as 200 U/ μ L lysozyme combined with 200 mM DTT, which achieved optimal SC-WGA results when incubated at 37 °C for 2 h.

This protocol succeeded in performing lysis of the chosen cyanobacterial and green algal species including *Nostoc*, *Gloeocapsa*, and *Sphaerocystis*. All three species reached 100% SC-WGA success rates and produced an average of 66.5 ng, 73.0 ng, and 42.8 ng of DNA, respectively. Apart from the sufficient lysis, ploidy of the tested organisms may also lead to a relatively high yield of DNA amplification from a single cell. Others have found that cyanobacterial species are typically polyploid, and genome copy numbers are in the range of 40 to 200⁴⁸. Certain types of green algae are proven to be polyploid⁴⁹, but there are no signs that the species tested in this study are polyploid. Besides, unlike typical lab-cultured bacterial species previously tested²¹, an average of 7.8 ng, 18.1 ng, and 8.7 ng of DNA was measured after amplifying the extracellular milieu of these species. However, no detectable DNA was found in the sterile PBS after amplification.

In addition, the DNA amplified from single cyanobacterial and green algal cells displayed a reasonable quality based on the TapeStation results and was easily distinguished from the extracellular milieu. First of all, unlike lab-cultured pure species, environmental samples generally are more complex in nature and can contain contaminating species or undesired DNA. In addition, it is also likely that DNA amplified in the extracellular milieu was initially from the extracellular DNA in the cell suspension. The initial extracellular DNA was most likely released from the cells during mechanical dissociation of the clustered cells into single cell suspensions prior to introduction into the microfluidic device. Possible ways to distinguish between exogenous and cell DNA released into the fluid include performance of qPCR or sequencing of the sample.

Although the process of cell dissociation may lead to undesired extracellular DNA in the cell suspension, it is important to sufficiently disrupt the cell clusters prior to microfluidic experimentation. Cell clusters that remain in the fluid can often clog the microscale channels and thus lead to experimental failures. Different mechanical cell dissociation approaches were used to break these clustered species into suspensions that contained a reasonable number of single cells for microfluidic processing. For *Nostoc*, pestling in a 2 mL tube was generally sufficient to obtain enough single cells, while *Gloeocapsa* and *Sphaerocystis* required prolonged pestling followed by mild sonication. For these species, the dissociation process was visually observed through the 2 mL tube, in which the visually discernable clusters were disappearing and buffer turned cloudy, which was further verified under a microscope.

This protocol serves as a guideline for the lysis of single cyanobacterial or green algal cells and other hard-to-lyse species for subsequent SC-WGA in microfluidic devices. For species whose cell wall structures closely resemble the species tested in this work, the protocol can be adopted without further modifications. Due to the vast diversity of cells species, especially bacterial and plant cell species that are usually challenging to lyse, modification of the protocol may be required to achieve successful SC-WGA. However, as chemicals are chosen to sequentially attack the cyanobacteria, green algae, and bacterial cell wall in general, it is likely that only the parameters such as incubation time and temperature need to be adjusted, without the need to finding alternative chemicals compatible with MDA chemistry in microfluidics. Beyond SC-WGA, recent efforts have been focused on developing microfluidic

technologies for library construction for sequencing⁵⁰⁻⁵². The ability to effectively lyse single cells from rigid species and sufficiently amplify the genome for standard library construction will allow for the integration of multi-step processes such as single cell isolation, genome amplification, and library preparation into a single device.

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The authors have nothing to disclose.

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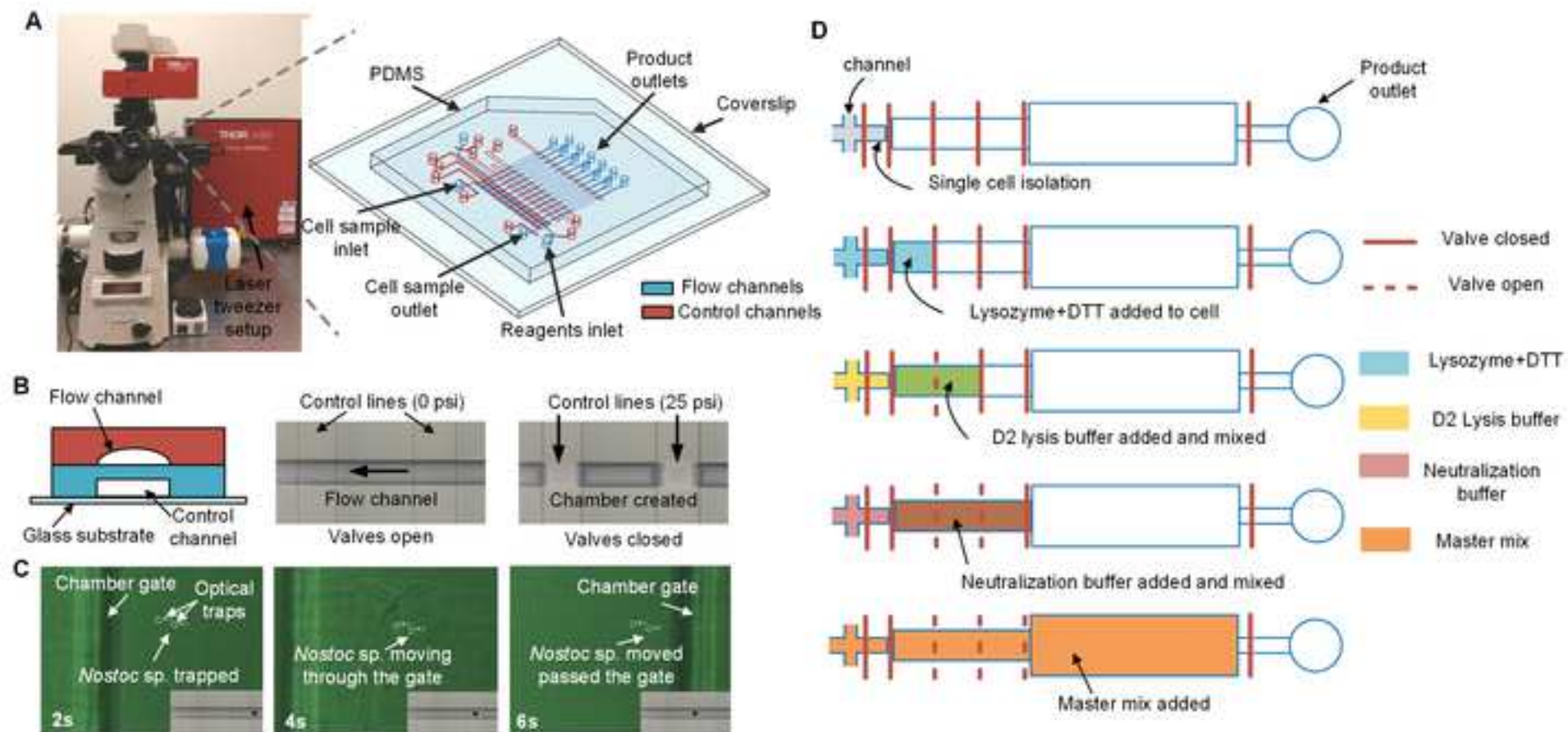


Figure 2

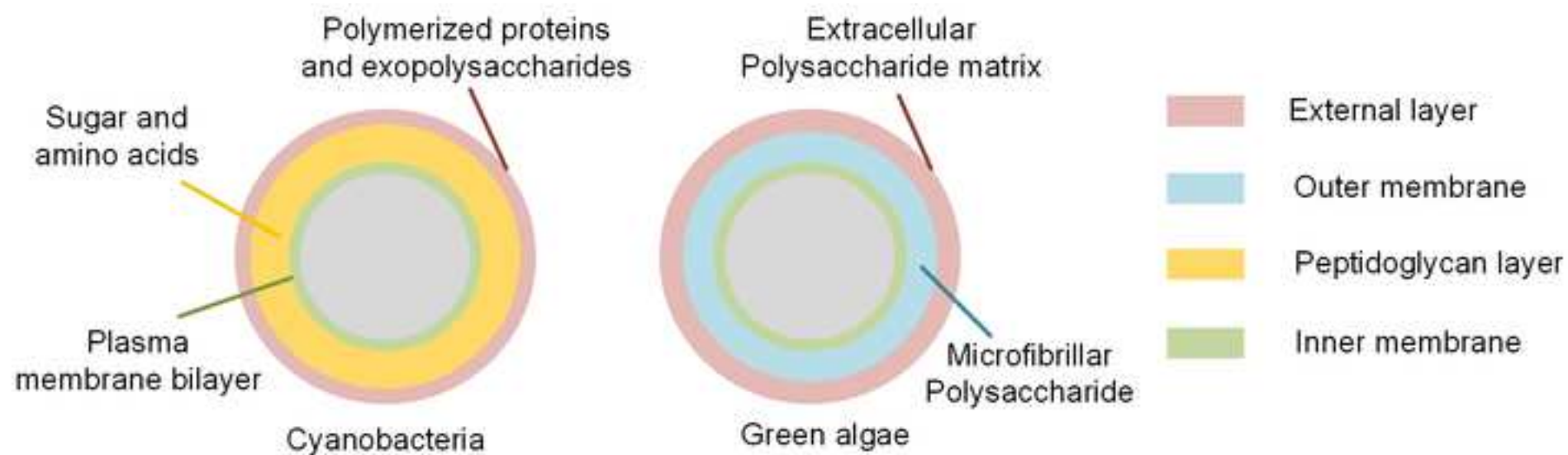
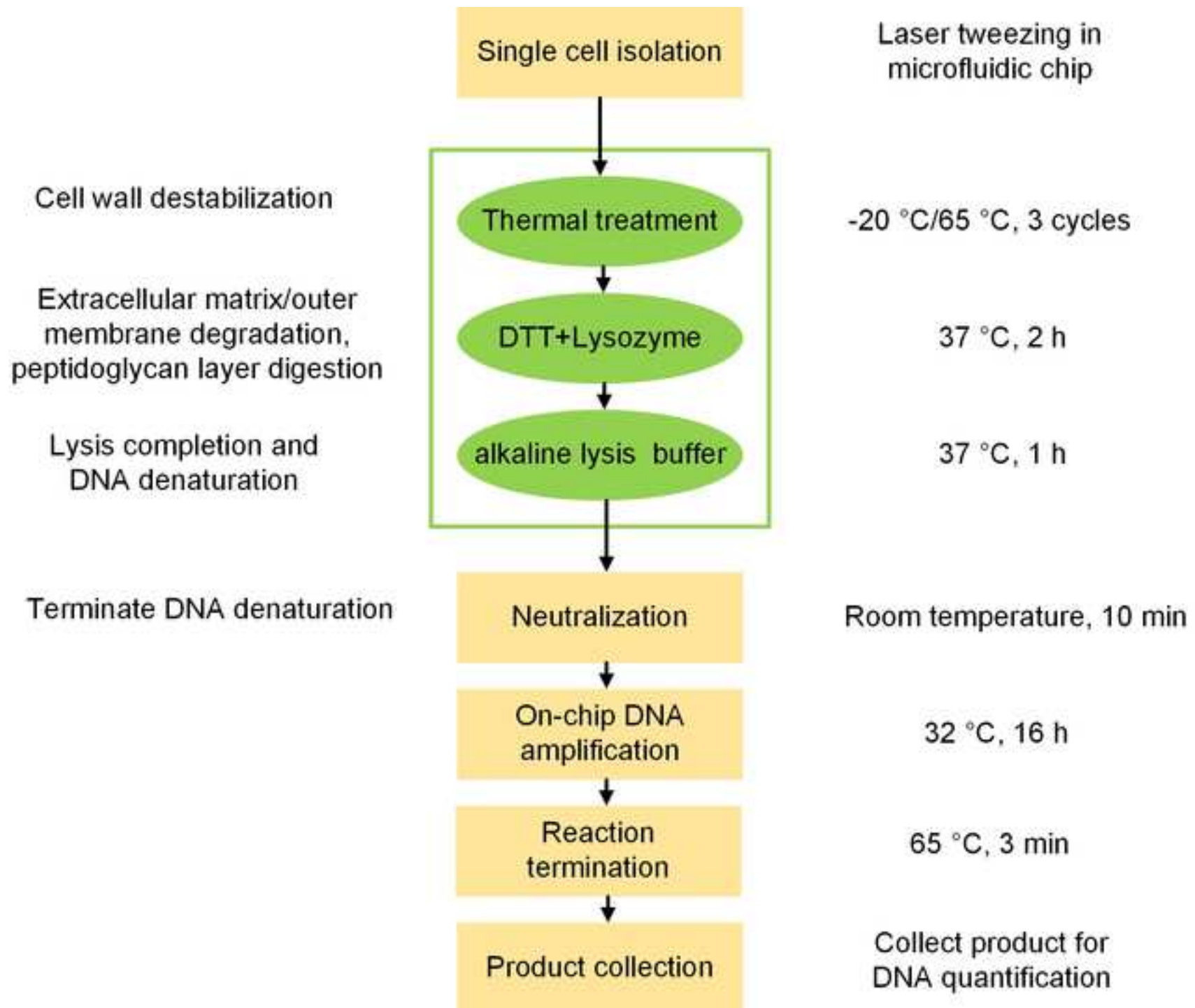


Figure 3



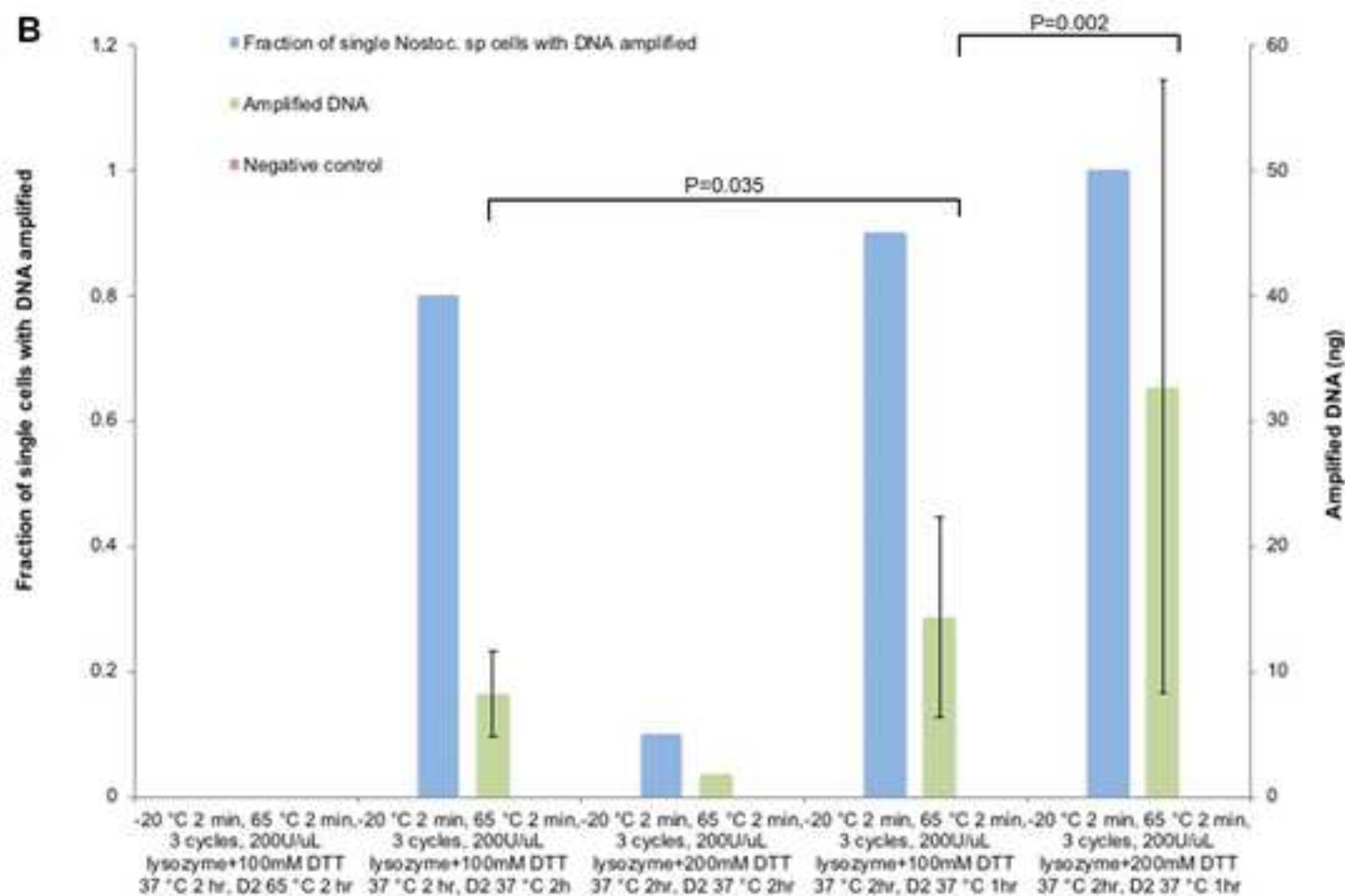
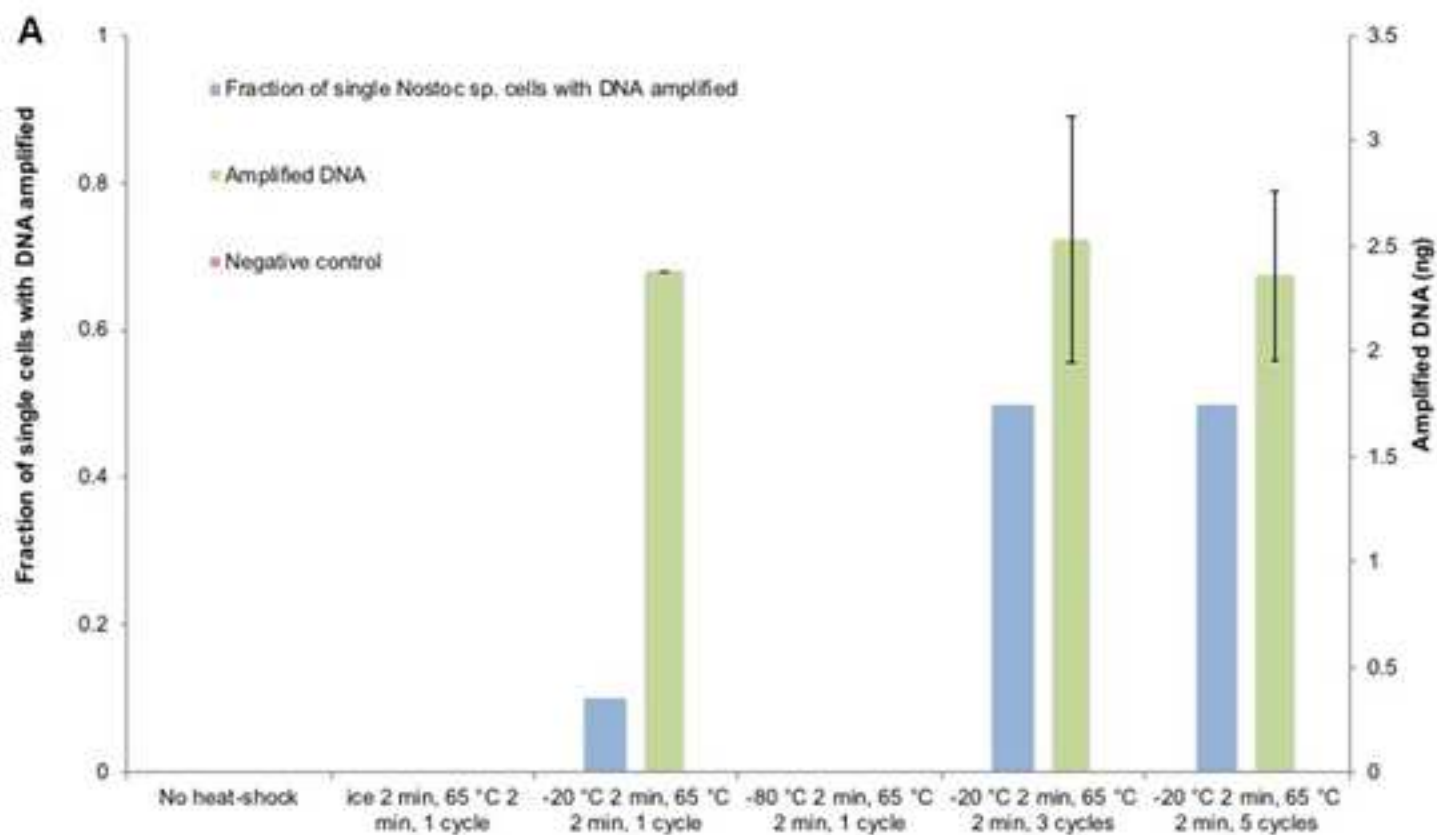
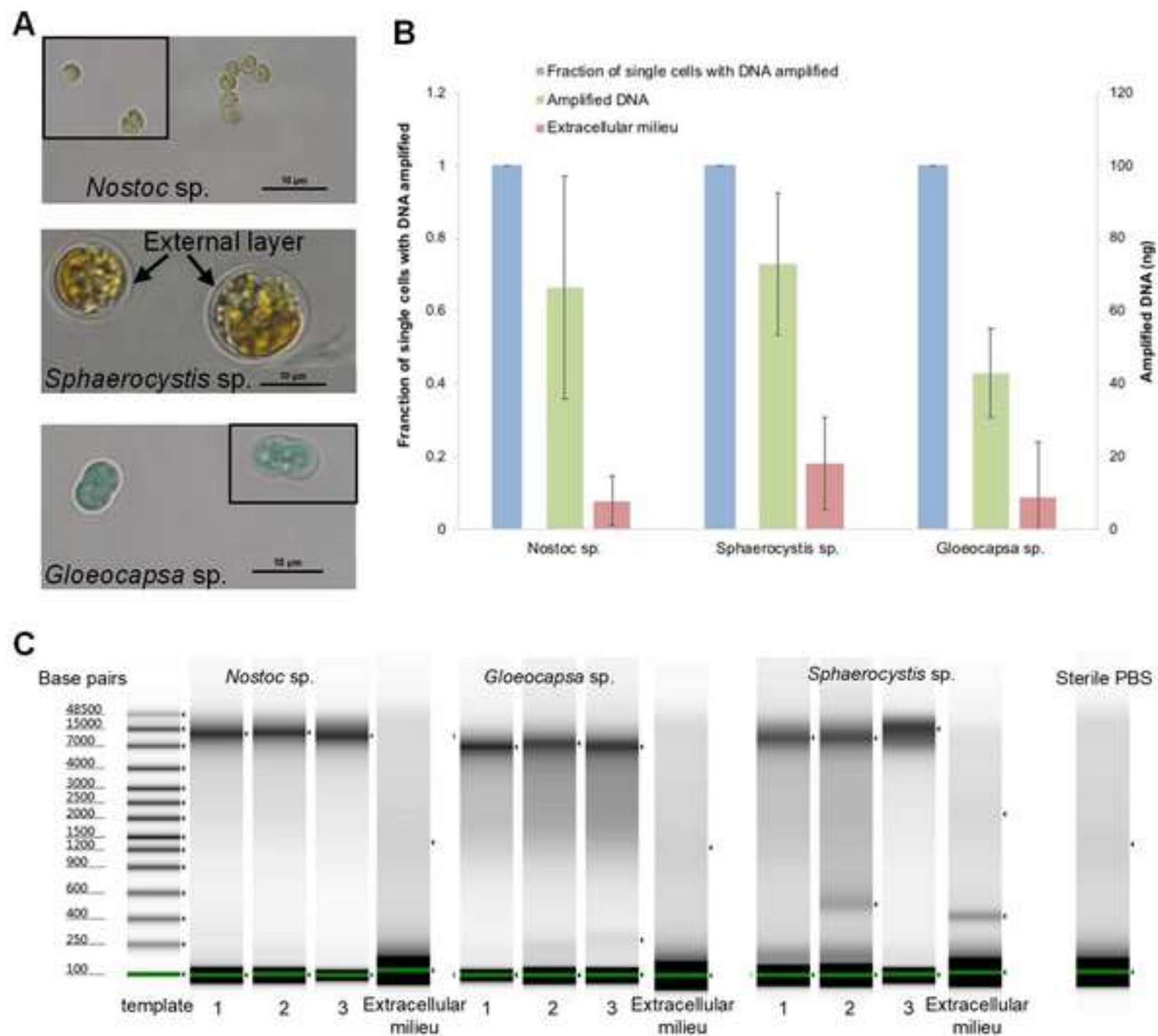


Figure 5

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
DTT	Bio-rad	1610611	
EDTA	Thermo Fisher	15575020	pH 8.0
Lysozyme	Epicentre	R1804M	
MATLAB			microfluidic user interface
Microscope	Nikon		Eclipse/Ti
Nuclease-free water	Thermo Fisher	AM9938	
Optical Tweezers	Thorlabs	OTM 211	
PBS	Thermo Fisher	10010023	pH 7.4
PDMS	Dow Corning	Sylgard 184	
Pluronic F-127	Sigma Aldrich	9003116	
Single cell WGA kit	Qiagen	150343	Include D2 buffer and neutralization buffer
Tapestation	Agilent	2200	
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Response: Edits made in the revision were tracked in the Word file without altering the format.

2. Please address all the specific comments marked in the manuscript.

Response: We have added additional information according to the editor's comments throughout the protocol section. Specific edits are marked in the revised manuscript using track-change function. Specific major edits are as following:

- 1) Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to..."

Response: The short summary was edited as following (Line 34-36): ["Here we present a protocol to lyse cyanobacteria and green algae single cells that allows for subsequent single cell whole genome amplification in microfluidic platform with 100% success rate."](#)

- 2) How much sample? Do you check the O.D.? Starting concentration?

Response: We processed the desiccated sample in a small amount, so we did not check O.D. as in cultured cells. Therefore, we modified the text as following (Line 120-124): ["Add 300 µL of sample diluent \(0.08% poloxamer 407 in Phosphate Buffer Saline \(PBS\)\) to 2 mL tubes that contain desiccated samples of *Nostoc* sp., *Gloeocapsa* sp. and *Sphaerocystis* sp respectively to re-suspend the cells. The desiccated samples can be identified visually in the tube as particles before re-suspension and are aggregated to the bottom of the tube after the addition of sample diluent."](#)

- 3) How do you do so? Any knob turns etc?

Response: The text was revised as following (Line 162-163): ["Set the pressure of pneumatic control system to 25 psi by turning the knob on the first regulator of the pneumatic control unit that connects to a house gas outlet."](#)

- 4) What kind of cell suspension? Please include a one liner note stating the same. Also is there any cell count to look for?

Response: The text was revised as following (Line 206-207): ["Pipette 10 µL of cell suspension of *Nostoc* sp., *Gloeocapsa* sp. or *Sphaerocystis* sp. made in step 1.3, 1.5 or 1.6"](#)

respectively using a gel-loading pipette tip. Insert the pipette tip into a new tube and inject the cell suspension followed by inserting a bent cannula.”

- 5) How do you visually identify target cells? Are there any other kind of cells as well? Please clarify.

Response: A note was added as following (Line 219-220): “Note: *Nostoc* sp., *Gloeocapsa* sp. or *Sphaerocystis* sp. can be easily distinguished visually from common contaminations such as *Escherichia coli* based on their size and morphology.”

4. Please proofread the manuscript carefully for any spelling or grammar issues.

Response: We fixed typos found in the manuscript.

5. Once done please ensure that the highlighted section is no more than 2.75 pages including headings and spacings. Please highlight complete step and ensure that this forms the most cohesive story of the protocol.

Response: The total highlighted part does not exceed 2.75 pages.

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