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Raman-Activated Cell Counting for Profiling Carbon Dioxide Fixing Microorganisms

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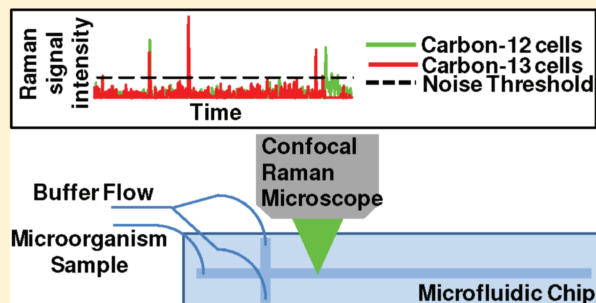
ABSTRACT: Raman microspectroscopy is a label-free and nondestructive technique to measure the intrinsic chemical profile of single cells. The naturally weak Raman signals hampered the application of Raman spectroscopy for high-throughput measurements. Nearly all photosynthetic microorganisms contain carotenoids that are active molecules for resonance Raman at a 532 nm excitation wavelength. Hence, the acquisition time for a single photosynthetic microorganism can be as short as 1 ms. The carotenoid bands in Raman spectra of photosynthetic microorganisms utilizing $^{13}CO_2$ shifted when compared to the spectra of cells utilizing $^{12}CO_2$. Here, a mixture of ^{12}C- and ^{13}C-cyanobacterial cells were counted using a microfluidic-device-based Raman-activated cell counting procedure to prove the concept that Raman spectroscopy can be used as a high-throughput method to profile a cell population.



■ INTRODUCTION

Photosynthetic microorganisms are one of the major sinks of atmospheric CO_2 and the main primary producers in marine ecosystems. Photosynthetic microorganisms may also harbor many previously unknown enzymes and molecular complexes that have extraordinary value in the biotechnology industry and renewable energy research. They are a vastly diverse group of microorganisms that consist of prokaryotic and eukaryotic species. Like other environmental microorganisms, most of them tend not to grow in laboratories, and a small amount of seawater sample may contain previously unknown species of photosynthetic microorganisms. These facts leave researchers no choice but to study individual cells in order to gain an unbiased view of the photosynthetic microbial communities.

The technological advances in Raman spectroscopy and microfluidic devices have been combined to drive the development of single-cell research.[1−3] The characterization of individual microorganisms is being used by an increasing number of researchers in order to study unculturable microorganisms at the single-cell level and individual cell phenotypes among genetically identical cells.[4−8] However, due to the small size and heterogeneity of many naturally occurring cells, developing high-throughput single-cell techniques remains a challenge. Raman spectroscopy offers a unique opportunity for single-cell analysis because it provides an intrinsic and label-free chemical profile of a single cell with 0.5−1 μm spatial resolution. When combined with a microfluidic device system, Raman spectroscopy can be used to achieve a high-throughput Raman-activated cell sorting (RACS) system to survey natural microbial communities or to study gene expression variance in cells of the same genetic identities without artificial interference such as external tagging of cells or fluorescent protein gene insertion.

Raman spectra are generated by detecting inelastically scattered light from a sample. Analogous to infrared spectra, the bands in Raman spectra match the vibrational frequencies of the chemical bonds present in the sample. The Raman spectrum of a single microorganism contains a large number of bands due to the complex chemical composition of a cell; therefore, it can serve as the chemical fingerprint of a cell, which differs depending on the species and physiological states of cells.[9,10] Visible and near-infrared lasers are usually used in Raman spectroscopy for activating biological samples. They do not cause any significant chemical or biological change in interrogated cells, which can be recovered for attempted cultivation or DNA analysis.[3,11]

However, Raman scattering is a relatively weak process that happens once for every 10^6−10^8 incident photons.[12] In order to acquire a signal that has a reasonably high signal-to-noise ratio from a single microbial cell, a spontaneous Raman spectrum usually requires an acquisition time of 4 to 5 s acquisition time with a well-optimized confocal Raman microspectroscope.[13] An acquisition time of a few seconds is difficult to achieve in a microfluidic device because it is difficult to stabilize a slow flow rate that maintains a cell within the Raman detection region for such a relatively long time. Even if it is technically possible, the

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throughput of the resultant RACS system would be very low, precluding it for useful applications.

Reducing the Raman spectra acquisition time is thus vital to achieve high-throughput RACS systems. Surface-enhanced Raman scattering (SERS) and resonance Raman (RR) spectroscopy are widely used to enhance the Raman signal and reduce the Raman acquisition time. SERS is mainly an electromagnetic enhancement that occurs in the vicinity (about <10 nm) of gold or silver nanoparticles or patterned surfaces.[14−16] SERS has been proven to enhance the Raman signal by up to a factor of 10^{14} and is able to detect single molecules.[17] SERS has been combined with microfluidic devices for several applications.[18−21] The RR effect can enhance the Raman signal from RR-active molecules by up to 10^6.[22] RR has shown great potential as a rapid label-free imaging technique for photosynthetic microorganisms.[6] The RR enhancement requires no special treatment of the sample, while SERS experiments involve treating microorganisms with gold or silver nanoparticles.

Carotenoids are highly diverse (over 600 types in carotenoids groups) and RR-active molecules. Nearly all photosynthetic microorganisms contain carotenoids that are essential for light harvesting, singlet oxygen quenching, and the structure of a photosynthetic pigment−protein complex.[6] Due to the greatly enhanced Raman signature of carotenoids in photosynthetic cells, a Raman spectrum of a single cell, recorded with a 1 ms acquisition time, can be used to quantify the CO_2 fixation rate of the cell.[6]

In this article, a combination of RR and microfluidics is reported to perform Raman-activated cell counting (RACC). We profile an artificially mixed microbial community to quantify the ratio of ^{12}C- and ^{13}C-containing cells. This study paves the way toward the development of a high-throughput RACS system that can characterize photosynthetic microbial communities and isolate photosynthetic cells of interest.[13]

■ EXPERIMENTAL METHODS

Chemicals, Microbial Strain, and Growth Conditions. All chemicals and growth media used in this study were purchased from Sigma-Aldrich, U.K., unless otherwise stated. *Synechocystis* sp. PCC 6803 was used as the model strain in this study. *Synechocystis* sp. PCC 6803 was grown in BG-11 media supplemented with 5 mM ^{12}C- or ^{13}C-sodium bicarbonate as the sole carbon source. The cell concentrations of ^{12}C- and ^{13}C-cells were counted by fluorescence microscopy before mixing them for Raman measurement. Naturally dissolved CO_2 in BG-11 media was removed by a degasification step prior to the incubation of the PCC 6803 strain, as previously described.[6] The *Synechocystis* sp. PCC 6803 strain was grown at 30 °C and 50 μmol photons m^{-2} s^{-1} on an orbital shaker (150 rpm) for 4 days (Innova 44 illuminated rotary incubator, New Brunswick Scientific, Cambridge, U.K.).

Fabrication and Operation of the Microfluidic Devices. The microfluidic chip was fabricated using a conventional soft-lithographic technique.[23] The chip was fabricated in polydimethylsiloxane (PDMS), where one-dimensional hydrodynamic focusing was implemented, whose design is shown in Figure 1. Laminar microfluidic flow was achieved by pressure-driven flow using syringe pumps (Pico Plus, Harvard Apparatus). The microfluidic channel had a height of 50 μm and a width of 100 μm (Figure 1). Cells were injected into the chip through the sample inlet, and the buffer solution (water) was injected into the chip through the buffer inlet. The ratio of the flow speed of the buffer stream and that of the sample stream was fixed at 4:1. The total flow rate at the Raman detection region was 17.7 nL/min. At the flow-focusing region, which is designed as a cross junction, the sample stream was focused down by the higher flow rate of the buffer stream.

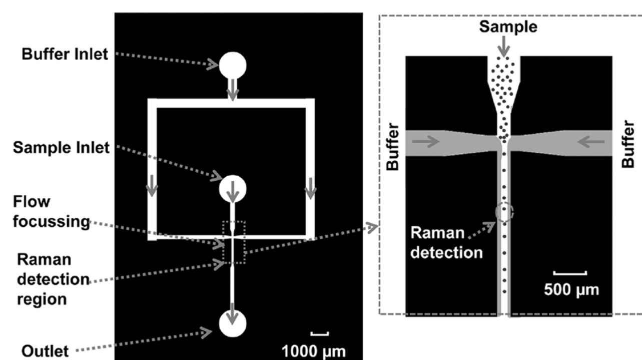

Figure 1. Design of the microfluidic chip, with one-dimensional flow focusing for high-throughput Raman-activated flow cytometry. Channels are shown in white. The buffer stream focused the sample stream into a nearly single file cell flow. (Inset) Flow focusing and subsequent Raman detection region (cell samples shown in the figure are not to scale). Cells were injected into the microfluidic chip through the sample inlet, and buffer solution (water) was injected through the buffer inlet to achieve one-dimensional flow focusing before the Raman detection region. At the Raman detection region, the channel had a width of 100 μm and height of 50 μm. The total flow speed at the Raman detection region was 17.7 nL/min, and the ratio of the flow speed between the sample stream and buffer stream was 4:1.

Total Cell Counting of the ^{12}C- and ^{13}C-Cells. In order to determine the ratio of the numbers of the ^{12}C- and ^{13}C-cells in the mixed sample, the number of cells in both samples was determined before mixing them together for the RR microfluidic device experiment. A volume of 20 μL of a mixed ^{12}C- and ^{13}C-cells suspension was dried separately onto 0.2 μm membrane filters (Millipore, U.S.A.) by a vacuum filtration device. The images of the cells on the membrane filters were recorded by a light microscope (Carl Zeiss, U.K.) with Cy5 fluorescence and a 40× dry objective (NA = 0.60, Carl Zeiss, U.K.). The number of cells in each image was manually counted in the ImageJ software package (http://rsb.info.nih.gov/ij/).

Raman-Activated Cell Counting of the ^{12}C- and ^{13}C-Cells. The mixture of the ^{12}C- and ^{13}C-cells (the mixing ratio was 1:1 by volume) was washed with phosphate buffered saline three times before loading to the microfluidic device. RR spectra were acquired continuously when the cells were flowing through the Raman detection region of the microfluidic channel. RR spectra were acquired using a confocal Raman microscope (LabRAM HR, HORIBA Scientific, U.K.) equipped with a 532 nm Nd:YAG laser (Torus Laser, Laser Quantum, U.K.) and a 50× air-dry objective (NA = 0.55, Leica Microsystems, U.K.). The laser beam was targeted to the center of the microfluidic channel. The laser power on a single cell was ∼18 mW. The detector was a −70 °C cooled CCD detector (Andor Technology, U.K.). The confocal pinhole was set to 100 μm. Each Raman spectrum was acquired between 2172 and 557 cm^{-1}, with a spectral resolution of 1.5 cm^{-1}. LabSpec software (HORIBA Scientific, U.K.) was used to control the Raman system and acquire Raman spectra. Raman spectra were acquired and recorded every 36.6 ms, including the acquisition time of 10 ms. There was around 26.6 ms of

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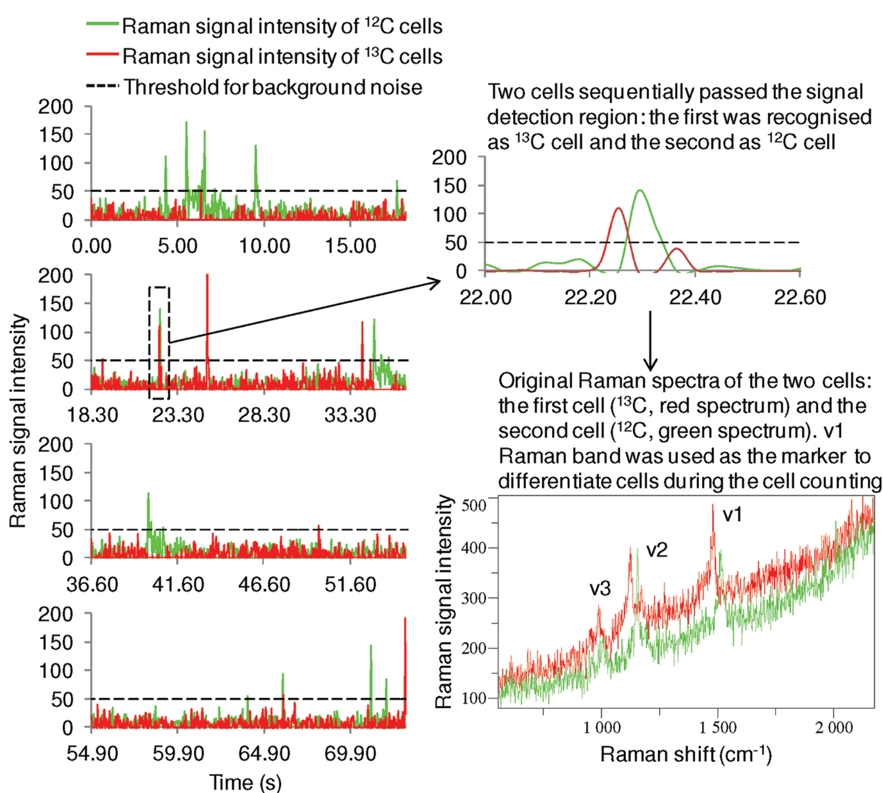

Figure 2. An example of the RR microfluidic device counting of photosynthetic microorganisms. As the cells of the model strain *Synechocystis* sp. PCC 6803 flow through the Raman detection area of the microfluidic device, RR spectra were acquired continuously about 27 times every second. The $\nu 1$ RR band was used to differentiate ^{12}C- and ^{13}C-cells. The intensity of this band was plotted against the temporal axis and displayed in green and red for ^{12}C- and ^{13}C-cells, respectively. A part of the figure near 23.3 s was enlarged to show a ^{13}C- and ^{12}C-cell passed through the Raman detection area sequentially only about 0.1 s apart from each other; the untreated RR spectra of those two cells show a distinctive red shift of all of the carotenoids RR bands.

"dead time" in every Raman signal recording cycle for the system to process and save data. Detected cells were differentiated based on the position of the $\nu 1$ RR band of carotenoids (Figure 2). Cells containing ^{13}C had distinctively red-shifted RR bands.

■ RESULTS AND DISCUSSION

The photosynthetic cells of *Synechocystis* sp. PCC 6803 were driven by the syringe pump to flow through the Raman detection area of the microfluidic channel, while the confocal Raman microscope acquired Raman spectra continuously about 27 times every second (Figure 1). *Synechocystis* sp. PCC 6803 cells were 1−2 μm spherical cells and were converged in the middle of the microfluidic channel. Discrimination of ^{12}C- or ^{13}C-cells was determined by the position of the $\nu 1$ RR band of the RR spectra (Figure 2). The $\nu 1$ RR bands of cells containing ^{13}C shifted distinctively to lower wavenumbers, which can serve as a quantitative marker of single cells' ^{13}C content.[6]

The total numbers of ^{12}C- and ^{13}C-cells counted by fluorescence microscopy were 1125 and 762. Because the fluorescence microscopic counting was performed under the same conditions for both samples and they were mixed 1:1 by volume afterward, the real ratio of the number of ^{12}C-cells to ^{13}C-cells was 1.48. This ratio was measured as 1.56 (316 ^{12}C-cells and 203 ^{13}C-cells) in the RR microfluidic device counting. The counting result of the RR microfluidic device is 95% accurate in this instance compared to fluorescence microscopic counting. The good agreement suggests that the concept of the combination of RR spectroscopy and microfluidic devices can be a novel method to rapidly profile the ^{13}C distribution in photosynthetic microbial communities in a nondestructive manner.

If a photosynthetic microbial community is given $^{13}CO_2$ as the carbon source, this method can rapidly monitor the uptake of $^{13}CO_2$ and the kinetics of CO_2 fixation. Because of the nondestructive nature of the method, the measured cells can be further used for nucleic acid amplification or attempted cultivation. The CO_2 fixation data provided by this method are easily comparable among different photosynthetic microbial communities to investigate the effect of environmental factors on the CO_2 fixation such as temperature, lighting condition, water salinity, depth of water, and so forth. RR spectroscopy is very sensitive to stable isotope labeling; therefore, a very small amount of sample (e.g., 50 μL of seawater) is needed to achieve the kinetic monitoring of photosynthetic microbial communities. In contrast to other methods that are also sensitive enough to detect ^{13}C incorporation at the single-cell level, for example, NanoSIMS, RR spectroscopy does not require any special sample preparation; it is also nondestructive, easier to operate, and inexpensive.

The method reported in this article is intended to serve as the proof of concept study and a stepping stone toward a high-throughput RACS system.[13] The flow velocity inside of the microfluidic chip in RACS should be ideally adjusted to equal the quotient of the size of the laser spot divided by the Raman acquisition time. A time of 1 ms has been proven to be enough to record a sufficient Raman signal to differentiate the ^{13}C-

single cells from the ^{12}C-single cells.[6] Considering the time needed to record data, communicate between different parts of the instrument, and so forth, measuring and sorting two cells per second is a conservative estimation of the throughput of the proposed RACS system. The resultant throughput means we may survey 14 400 single cells in 2 h, which is more than enough to characterize a photosynthetic microbial community and collect enough replicates of different species. The main technical difficulty in developing the proposed RACS system is the reliable synchronization of the Raman spectrometer, the controlling computer, and the sorting mechanism (e.g., optical force sorting, pressure-driven closure of the microfluidic channel, and so forth). There is scope for improving the efficiency of the hydrodynamic focusing in the microfluidic chip. As described above, the ratio of the flow rate of the buffer channel (water) to that of the sample channel (cell suspension) was set to 4:1. This was done to balance between the efficiency of data recovery (detecting a large number of cells) and the data quality (good signal-to-noise ratio). A higher ratio of flow rates will focus cells more tightly to the center of the channel; therefore, more cells will flow through the focal area of the laser, and more spectra of cells will be recorded. One can usually achieve a higher ratio of flow rates by increasing the flow rate of the buffer channel because the flow rate of the sample channel is usually already very low, and reducing it caused unstable flow with the experimental devices used in this study. However, increasing the buffer flow will decrease the time a cell remains within the focal area of the laser and therefore reduce the quality of the resulting spectrum. One can reduce the spectral acquisition time to match this shortened time, but it is unlikely that the signal-to-noise ratio will improve without upgrading the optical part of the system. With advancement in microfluidic technology, it will be possible to use more sophisticated microfluidic pumps to achieve a more stable flow at a slow flow rate regime.

In summary, this article reports a novel method to combine RR spectroscopy and microfluidic devices in order to rapidly profile the ^{13}C in photosynthetic microbial communities. This provides the foundation for developing a high-throughput RACS system to monitor and sort single cells of natural photosynthetic microbial communities without culture bias.

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Notes

The authors declare no competing financial interest.

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