Multiple-trap laser tweezers Raman spectroscopy for simultaneous monitoring of the biological dynamics of multiple individual cells

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We report the development of a multiple-trap laser tweezers Raman spectroscopy (LTRS) array for simultaneously acquiring Raman spectra of individual cells in physiological environments. This LTRS-array technique was also combined with phase contrast and fluorescence microscopy, allowing measurement of Raman spectra, refractility, and fluorescence images of individual cells with a temporal resolution of ~5 s. As a demonstration, we used this technique to monitor multiple *Bacillus cereus* spores germinating in a nutrient medium for up to 90 min and observed the kinetics of dipicolinic acid release and uptake of nucleic acid-binding stain molecules during spore germination.

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The ability to monitor molecular events in individual living cells in a physiological medium is essential for the analysis of biological dynamics and heterogeneity between individual cells [1,2]. Raman spectroscopy has been widely applied for the analysis of molecular composition of single cells and tissues because of its high sensitivity and rapid response [3,4]. The combination of Raman spectroscopy with optical tweezers leads to laser tweezers Raman spectroscopy (LTRS), allowing noninvasive real-time analysis of single living cells suspended in an aqueous medium [5–8]. Single-trap LTRS uses a single laser beam for both trapping and Raman spectroscopy [5–8] or uses one beam for trapping and another beam of a different wavelength for Raman excitation [9,10]. Recently, LTRS has been used to monitor the dynamic processes of nutrient germination [7,8], heat activation [11], and inactivation [12] of individual bacterial spores of *Bacillus* species. LTRS has also been applied for cancer detection [13,14] and microfluidic cytometry [15,16] and has been combined with surface-enhanced Raman scattering [17].

The major limitation of the current LTRS technique for monitoring biological dynamics of individual cells is the low experimental efficiency, because single-trap LTRS can only analyze one captured cell at a time. Consequently, this technique becomes somewhat cumbersome when hour-long observations are desired and results from large numbers of cells are required. To improve the measurement efficiency of LTRS, we developed a dual-trap LTRS system for monitoring two closely separated cells, in which a single laser beam was separated into two beams with a beam splitter to form dual traps and two trapped cells were projected at the different locations on the entrance slit of an imaging spectrograph such that the Raman spectra of these two cells can be acquired simultaneously [12,18]. Multiple-beam optical traps or optical tweezers arrays have been developed [19,20] but have not yet been combined with Raman spectroscopy.

In this Letter, we report the development of multitrap LTRS that allows simultaneous acquisition of Raman spectra of multiple individual cells in an aqueous medium. They are created by rapidly scanning a single laser beam with two galvo mirrors in the x and y directions with a programmable pattern [20]. Synchronous steering of another galvo mirror and the vertical separation of their spectra on the CCD detector can be controlled independently, and thus the cross talk between different spectral channels can be effectively avoided. In addition, our LTRS array also combines with phase contrast and fluorescence microscopy to allow simultaneous acquisition of Raman spectra and refractility and fluorescence images of multiple individual cells during prolonged observation.

The experimental setup is shown in Fig. 1(a). The single laser beam from a diode laser at 780 nm was reflected by a dichroic mirror (DM_2) and a pair of galvo mirrors (GM_1 and GM_2) and passed through a pair of lenses. The laser beam was introduced into the objective (60×, NA = 1.40) of an inverted microscope (Nikon Ti) with a hot mirror (DM_1) that reflects IR and transmits visible light. The backward Raman scattering light from the trapped particles was collected, reflected by another galvo mirror (GM_3), and focused onto the entrance slit of an imaging spectrograph (LS-785, Princeton Instruments), which was detected by a CCD detector (PIXIS 400BR, Princeton Instruments). A narrowband LED (455 nm) was used as the light source for the external phase-contrast microscopy [8] and as the excitation source for fluorescence microscopy with an imaging camera. Multiple traps were created by rapidly scanning GM_1 and GM_2, driven with programmed step-voltage waveforms V_1 and V_2. The pattern of multiple traps can be changed by the waveforms of V_1 and V_2. For example, to create a 1×4 array of traps, V_1 was set to zero and V_2 was set up as a staircase waveform with four steps. To create a 2×2 array of traps, voltage waveforms, as shown in the insert of Fig. 1(a), were applied. The small-step response of the

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galvo mirrors was <200 μs, and a scan rate of 50–100 Hz was applied so that the small trapped particles (~1 μm in diameter) would not move as the laser beam was scanned rapidly across the array pattern. Images a–d in Fig. 1(b) show the images of 2, 4, 6, and 8 polystyrene beads (2 μm in diameter), respectively, trapped in water by 1D tweezers arrays. The average laser power was ~4 mW for each trap. Images e–g show 4, 9, and 10 polystyrene beads trapped by 2×2, 3×3, and A-pattern 2D arrays of optical tweezers, respectively. By properly programming the waveforms for GM_1 and GM_2, an arbitrary pattern of optical traps could be created.

To simultaneously record the Raman spectra of multiple trapped particles, the galvo mirror GM_3 in the front of the spectrograph was synchronously scanned with a staircase waveform V_3, which demultiplexed the Raman scattering light from each trap onto different vertical positions of the entrance slit of the spectrograph and imaged on a thermoelectrically cooled 1340×400 pixel CCD camera. Figure 2(a) shows the image of the CCD chip showing Raman spectra from four trapped polystyrene beads in a 1×4 LTRS array. The spectra of these beads were separated vertically by about 15 pixels with a pixel size of 20 μm × 20 μm, much larger than the width of the individual spectra (about 5 pixels). This demonstrated that the Raman spectra of multiple particles in optical tweezers arrays can be recorded simultaneously without cross talk. By selective vertical binning on the CCD chip (~5 pixels per spectrum), Figs. 2(b) and 2(c) show Raman spectra of four trapped polystyrene beads and four trapped dormant *Bacillus cereus* spores, respectively. The number of realizable optical traps is limited by the row number of CCD pixels and the width of a single spectral image. Given 400 vertical CCD pixels, the maximum number of multiple traps of the LTRS array could be 80. The trapping stability of a particle was determined by the drift distance of the particle in the medium during

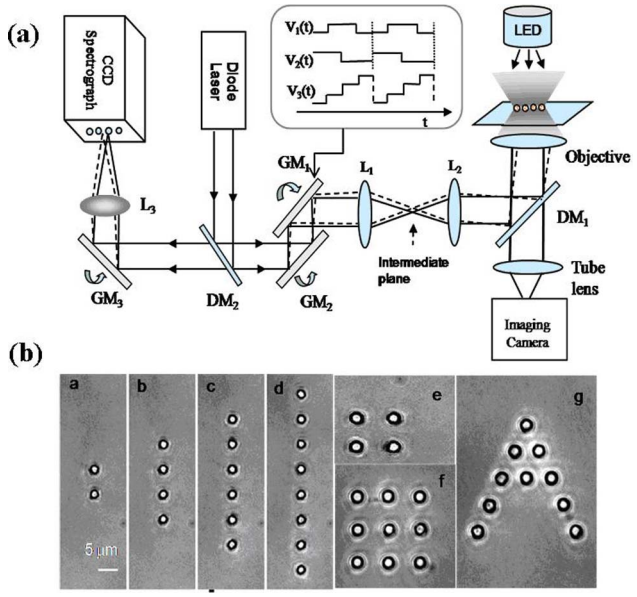

Fig. 1. (Color online) (a) Experimental setup: GM_1, GM_2, GM_3, galvo mirrors; DM_1, DM_2, dichroic mirrors; M_1, M_2, mirrors; and L_1, L_2, L_3, lenses. (b) The images of individual polystyrene beads trapped in 1D and 2D arrays with a separation of ~6 μm.

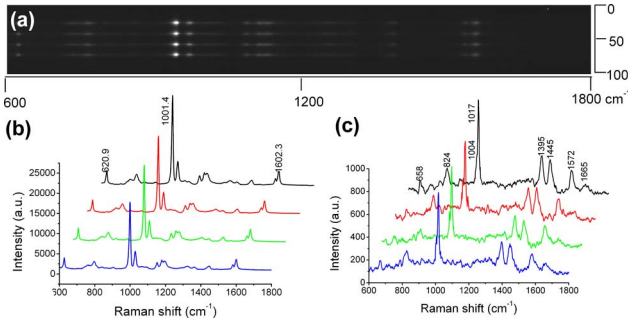

Fig. 2. (Color online) (a) Raman spectral image on the CCD chip of four trapped 2 μm polystyrene beads, (b) Raman spectra of four trapped polystyrene beads by selective binning of the CCD pixels, and (c) Raman spectra of four trapped dormant *B. cereus* spores. The average laser power for each trap was 4 mW, and the acquisition time was 5 s. The bands at 658, 824, 1017, 1395, and 1572 cm^{-1} are from CaDPA.

the period that the laser was steered to other particle positions. In our system, the spores will lose confinement from the traps when this period is longer than ~0.5 s.

Application of LTRS arrays was demonstrated for monitoring the dynamic germination of multiple bacterial spores for up to 90 min after the addition of the nutrient L-alanine. Spores of *B. cereus* T were prepared and heat-activated as described in [8]. A small aliquot of spores (~5 μl, ~10^6 spores/ml) was added to a germination medium containing 10 mM L-alanine in a 25 mM Tris buffer (pH 8.3) in a sample holder at 37 °C. The germination medium also contained 0.5 μM SYTO 16, a membrane-permeant nucleic acid dye that exhibits strong fluorescence at 530 nm upon binding to nucleic acids. Following the addition of spores, four spores were randomly captured in a linear LTRS array with an average power kept at 4 mW for each trap. Raman spectra, phase-contrast images, and LED-excited fluorescence images (at 530 nm) of these spores were continuously acquired with an acquisition time of 5 s for a total period of 90 min. Figures 3(a) and 3(b) show that before spores germinated, e.g., at 3 min after the addition of the germinant, the phase-contrast images were bright and there was no fluorescence, indicating that the SYTO 16 stain cannot enter the core of dormant spores and bind to the spore nucleic acids. After spores germinated, e.g., at 60 min, the phase-contrast images became dark and the fluorescence images were bright, indicating that SYTO 16 entered the spore core and bound to nucleic acids. Figures 3(c) and 3(d) show the intensities of the CaDPA-specific Raman band at 1017 cm^{-1} and fluorescence intensities of the four individual spores as a function of incubation time. The interesting results obtained from this dynamic measurement included (i) the germination of individual spores in response to the germinant was heterogeneous even in the nearly identical microenvironment of the optical trap array; (ii) after complete CaDPA release, SYTO 16 molecules entered the spore core and generated a rapid increase in fluorescence intensity; and (iii) after the fluorescence intensity reached the maximum value, it slowly decreased to a relatively stable value. Note that the data from four individual spores were obtained in one 1.5 h experiment by using

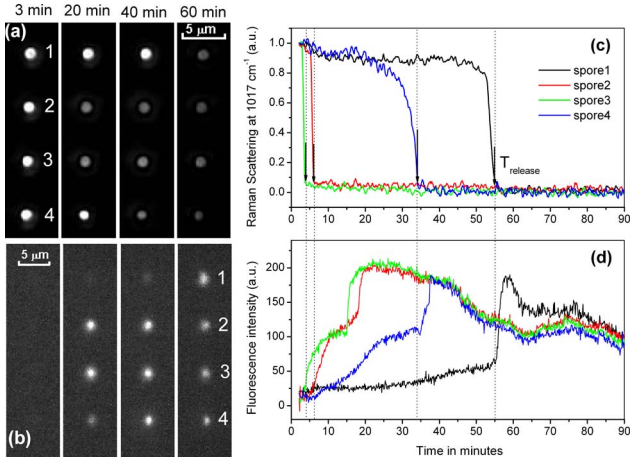

Fig. 3. (Color online) Simultaneous monitoring of four trapped *B. cereus* spores germinating in a mixture of 10 mM L-alanine, 25 mM Tris buffer (pH 8.3) and 0.5 μM SYTO 16. (a) Phase-contrast images and (b) fluorescence images of trapped spores at different incubation times. (c) Raman scattering intensities at 1017 cm^{-1} and (d) fluorescence intensities are shown as a function of incubation time. The vertical dotted lines and arrows mark the times of $T_{release}$ for each spore.

the LTRS array, and this would have taken 6 h using single-trap LTRS.

In summary, we have demonstrated that multitrap LTRS is capable of trapping multiple particles arranged in a 1D or 2D pattern and acquiring their Raman spectra simultaneously. This LTRS array was stable for long observation periods and was very efficient for cellular dynamic studies. We demonstrated the analysis of 4 to 10 individual cells and small beads in this study, and this number can be increased to 40 to 80 when using a high-power laser and fast scanning mechanism. Rich information on individual cells can be obtained with the LTRS array when combined with phase-contrast and fluorescence microscopy. It may also be very effective for flow cytometry applications.

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