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Single-Cell Analysis by a Scanning Thermal Lens Microscope with a Microchip: Direct Monitoring of Cytochrome *c* Distribution during Apoptosis Process

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We developed a microsystem for cell experiments consisting of a scanning thermal lens microscope detection system and a cell culture microchip. The microchip system was good for liquid control in microspace, and this results in secure cell stimulation and coincident in vivo observation of the cell responses. The system could detect nonfluorescent biological substances with extremely high sensitivity without any labeling materials and had a high spatial resolution of ∼1 μm. This system was applied to monitoring of cytochrome *c* distribution in a neuroblastoma−glioma hybrid cell cultured in the microflask (1 mm × 10 mm × 0.1 mm; 1 μL) fabricated in a glass microchip. Cytochrome *c* release from mitochondria to cytosol during the apoptosis process was successfully monitored with this system. The cytochrome *c* detected with this system was estimated to be ∼10 zmol. We concluded that the system was suitable for measuring the distribution of chemical substances in a single cell because the microchip is good for liquid handling in microspace and the thermal lens microscope has high sensitivity and spatial resolution.

Some of the most important studies in modern life science have focused on the reactions, signal transductions, and other chemical processes that occur in a cell in order to understand mechanisms of biological phenomena. Most conventional studies have been based on determination of analyte from cell-free extracts by chromatography or the western blotting technique. However, these studies often could provide only restricted information on a certain biological phenomenon, and additionally, they were labor-intensive, requiring troublesome pipetting procedures. Therefore, a simple and easy cell analysis system has been welcomed.

Recently, microchip techniques, which are called micro total analysis systems (μ-TAS)[1] or labs-on-a-chip,[2] have been developed, especially in chemical analysis fields and have become of major interest. We have demonstrated many applications of integrated microchemical systems, including flow injection analysis,[3,4] solvent extraction,[5−7] immunoassay,[8,9] organic synthesis,[10] laser reaction control,[11] and enzyme reaction use.[12] In these microchip-based systems, scale merits of a microspace, i.e., a rapid and efficient reaction, low reagent consumption, and short reaction time, were taken full advantage of.

Microchip techniques also appear to provide some advantages for cellular biochemical analysis systems, because the scale of the liquid microspace inside the microchip is fitted to the size of the cells. For instance, by using a microflask fabricated in a microchip, rapid and secure exchange of media or reagents will be achieved by simple operations under continuous measurements.

In the conventional experiments using a culture dish, added reagents were accessed to cells after a long diffusion time, and then rapid chemical stimulation was difficult. On the other hand,

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(1) Harrison, D. J.; van den Berg, A. *Micro Total Analysis Systems '98*; Kluwer Academic Publishers: Dordrecht, 1998.
(2) Graves, D. J. *Trends Biotechnol.* **1999**, *17*, 127−134.
(3) Sato, K.; Tokeshi; M.; Kitamori, T.; Sawada, T. *Anal. Sci.* **1999**, *15*, 641−644.
(4) Sorouraddin, H. M.; Hibara, A.; Proskrunin, M. A.; Kitamori, T. *Anal. Sci.* **2000**, *16*, 1033−1137.
(5) Tokeshi; M.; Minagawa, T.; Kitamori, T. *Anal. Chem.* **2000**, *72*, 1711−1714.
(6) Tokeshi; M.; Minagawa, T.; Kitamori, T. *J. Chromatogr., A* **2000**, *894*, 19−23.
(7) Minagawa, T.; Tokeshi, M.; Kitamori, T. *Lab on a Chip* **2001**, *1*, 72−75.
(8) Sato, K.; Tokeshi; M.; Odake, T.; Kimura, H.; Ooi, T.; Nakao, M.; Kitamori, T. *Anal. Chem.* **2000**, *72*, 1144−1147.
(9) Sato, K.; Tokeshi, M.; Kimura, H.; Kitamori, T. *Anal. Chem.* **2001**, *73*, 1213−1218
(10) Hisamoto, H.; Saito, T.; Tokeshi, M.; Hibara, A.; Kitamori, T. *Chem. Commun.* **2001**, 2662−2663.
(11) Tanaka, Y.; Slyadnev, M. N.; Hibara, A.; Tokeshi, M.; Kitamori, T. *J. Chromatogr., A* **2000**, *894*, 45−51.
(12) Tanaka, Y.; Slyadnev, M. N.; Sato, K.; Tokeshi, M.; Kim, H.-B.; T.; Kitamori, T. *Anal. Sci.* **2001**, *17*, 809−810

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the microfluidic system seems to realize rapid chemical stimulation by pressure-driven flow.

The rapid and secure exchange of media and reagents is very advantageous to time-resolved analysis. Moreover, a glass microchip is favorable for optical detection under a microscope, because the chip can be fabricated transparently with flat surfaces. Therefore, it seems useful to develop a microchip-based culture flask in which all procedures for cell analysis, i.e., cell culture, chemical stimulation, and measurement, can be performed. There are, however, very few papers about biochemical analysis of a cell cultured in a microchip.[13]

The detection scheme is also important for cell analysis. In some cases, only the distribution of analytes has a specific meaning, and it is very difficult to find a change of distribution by in vitro analysis of total cell-free extracts. For distribution analysis, single-cell imaging using a confocal fluorescent microscope was developed in combination with fluorescent probe molecules. Although this technique is very useful, it is necessary to inject fluorescent labeling molecules into the cells and some of the labeling materials themselves may possibly affect the analyte distribution and biochemical pathways in cells. In such cases, it is hard to clarify whether the probing molecule has any influence on the cell. Therefore, a novel technique for in situ analysis of intact single cells is welcomed. To overcome this drawback of the fluorescent technique, we developed a thermal lens microscope (TLM) for cell analysis, since the TLM can detect absorption substrate without the use of labeling materials.

The thermal lens effect is caused by radiationless relaxation of absorption substrates, and the spectroscopy utilizing the effect has wide applicability and high sensitivity. The effectiveness and usefulness of this spectroscopy were demonstrated by some researchers.[14−17] TLM is a novel thermal lens spectroscopy using a microscope, which offers almost the same applicability as absorptiometry with extremely high sensitivity.[18−20] Moreover, TLM is suitable for two-dimensional imaging of a molecular distribution with ~1-μm resolution, because two-point spatial resolution of TLM is restricted to the size of the focused excitation beam spot.[19] In addition, it is known that TLM is not strongly affected by scattering of the laser beam by membranes and particles in cells.[21] We have reported some applications of TLM to imaging of biological samples such as sliced tissues or the outside of a cell membrane using several probing materials.[22,23] There are, however, no reports about molecular distribution imaging inside of a cell, nor analysis of intact live cells with TLM.

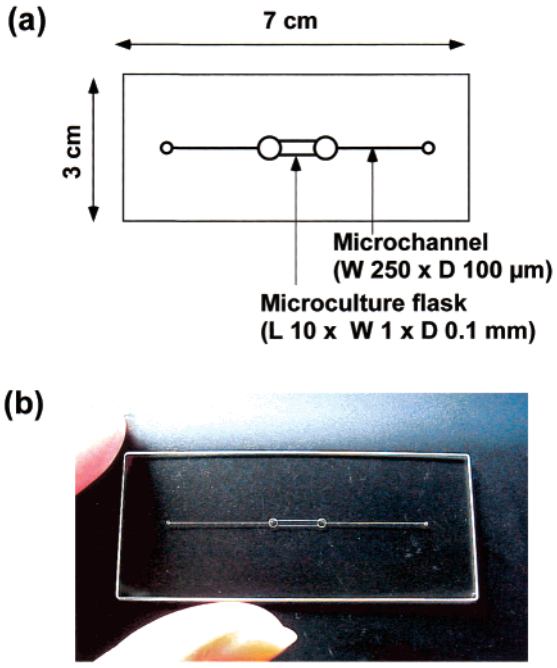

Figure 1. Microchip for a cell culture. (a) Illustration of the chip. (b) Photograph of the chip.

In this paper, we present a novel cell experimental system, which consists of TLM and a microchip, and describe the direct imaging of the cytochrome *c* (cyt) distribution in a cell cultured in a microchip. We succeeded in fabricating a cell culture microchip, and then culturing and stimulating neuroblastoma−glioma hybrid cells in the chip, and monitoring changes of the cyt distribution during the apoptosis process, which is one of the most important topics in the field of cellular science. Release of cyt from mitochondria to cytosol is thought to be a key process for signal transductions in a kind of apoptosis process.[24] However, direct measurement of cyt release in an intact live cell without the use of probe molecules has not been previously reported.

EXPERIMENTAL SECTION

Microchip Fabrication. The cell culture microchip is illustrated in Figure 1. The chip was fabricated by the same method as that of previous papers.[3,25] The chip was composed of three quartz glass plates (30 mm × 70 mm), i.e., the cover, middle, and bottom plates with thicknesses of 170, 100, and 500 μm, respectively. Two small access holes for an inlet and an outlet and two large holes for cell introduction were mechanically bored into the cover glass. Microchannels and the microculture flask were made in the middle plate using a highly focused and intensified CO_2 laser beam. Three plates were attached by using an optical contact; that is, the plates were polished to an optical smoothness and then laminated together without any adhesive in an oven at 1150 °C. The channels were 250 μm in width and 100 μm in depth, and the dimensions of the microflask were 100 μm, 1 mm, and 1 cm in depth, width and length, respectively, i.e., 1 μL in volume.

(13) Hediger S.; Fontannaz J.; Sayah A.; Hunziker W.; Gijs, M. *Sens. Actuators* **2000**, *B63*, 63−73.
(14) Krattiger, B.; Bruno, A. E.; Widmer, H. M.; Dandliker, R. *Anal. Chem.* **1995**, *67*, 124−130.
(15) Burgi, D. S.; Dovichi, N. J. *Appl. Opt.* **1987**, *26*, 4665−4669
(16) Nickolaisen, S. L.; Bialkowski, S. E. *Anal. Chem.* **1986**, *58*, 215−220.
(17) Skogerboe, K. J.; Yeung, E. S. *Anal. Chem.* **1986**, *58*, 1014−1018.
(18) Tokeshi, M.; Uchida, M.; Uchiyama, K.; Sawada, T.; Kitamori, T. *J. Lumin.* **1999**, *83−84*, 261−264.
(19) Uchiyama, K.; Hibara, A.; Kimura, H.; Sawada, T.; Kitamori, T. *Jpn. J. Appl. Phys.* **2000**, *39*, 5316−5322.
(20) Tokeshi, M.; Uchida, M.; Hibara, A.; Sawada, T.; Kitamori, T. *Anal. Chem.* **2001**, *73*, 2112−2116.
(21) Harada, M.; Shibata, M.; Kitamori, T.; Sawada, T. *Anal. Sci.* **1999**, *15*, 647−650.
(22) Kimura, H.; Nagao, F.; Kitamura, A.; Sekiguchi , K.; Kitamori, T.; Sawada, T. *Anal. Biochem.* **2000**, *283*, 27−32.
(23) Kimura, H.; Kojima, H.; Mukaida, M.; Kitamori, T.; Sawada, T. *Anal. Sci.* **1999**, *15*, 1101−1117.
(24) Liu, X.; Kim, C. N.; Yang, J.; Jemmerson, R.; Wang, X. *Cell* **1996**, *86*, 147−157.
(25) Sato, K.; Kawanishi, K.; Tokeshi, M.; Kitamori, T.; Sawada, T. *Anal. Sci.* **1999**, *15*, 525−529.

Reagents and Cell Preparations. All reagents were purchased from Sigma unless otherwise stated. Staurosporine was dissolved in DMSO (Wako Pure Chemicals, Osaka, Japan) to 1 mM and then diluted with Hank's balanced salt solution (HBSS; BioWhittaker, Walkersville, MD) to 2 μM.

NG 108-15 neuroblastoma−glioma hybrid cell was kindly supplied by Dr. Kazuki Sasaki (National Cancer Center, Tokyo, Japan). Cells were grown to 80% confluency on conventional poly(L-lysine)-coated polystyrene dishes with Dulbecco's modified Eagle medium without phenol red (ICN Biomedicals, Aurora, OH) with 10% fetal bovine serum (GibcoBRL Life Technologies, Carlsbad, CA) as a stock culture.

Instrumentations. The details of the TLM system used were described before.[5,18−20] A YAG laser (CrystaLaser, GCL-100-S, 532 nm, 100 mW) was used as an excitation laser, and the beam was introduced into the optical microscope (Nikon, custom-made). The excitation beam was modulated by a light chopper (NF Electronic Instruments, Yokohama, Japan; model 5584A) with a modulation frequency at 1.03 kHz. A He−Ne laser (Melles Griot, Carlsbad, CA; model 05LHP171, 15 mW) was used as a probe laser. Its beam was introduced from the opposite direction of the excitation laser into the microscope. Both laser beams were coaxially aligned by a dichroic mirror and a mirror in the body tube of the microscope and then introduced into an objective lens (Nikon, CF IC EPI Plan 20×, NA 0.46). The transmitted beams were collected by a condenser lens. The beams were filtered, and only the probe beam was transmitted. The beam collected by the photodiode was filtered by a dual-channel programmable filter (NF Electronic Instruments, model 3627) with high-pass filter at 750 Hz and low-pass filter at 1250 Hz. The signal was amplified with a lock-in amplifier (NF Electronic Instruments, model LI-515).

The cell culture chip was mounted on a 3D scanning stage (Sigma Koki, Saitama, Japan; model SGSP20−85), which could be controlled in 1-μm steps in the *X*, *Y*, and *Z* directions with stage controller (Sigma Koki; model Mark-102). A personal computer was used for control of the stage and data acquisition.

The liquid flow was controlled by microsyringe pumps (KD Scientific, Boston, MA; model 210) and Hamilton gastight syringes with an untreated fused-silica capillary tubing (GL Sciences, Tokyo, Japan; 320 μm i.d., 450 μm o.d.) and capillary column connectors (GL Sciences). The flow rate of the solution was adjusted to 1 μL/min for solution exchange. The syringe needle was connected to a custom-made Teflon screw with an O-ring (0.74-mm i.d., 2.78-mm o.d.) through a fused-silica capillary tube using epoxy-based glue (Ciba-Geigy, Alraldite). It screwed down a custom-built poly(vinyl chloride) holder. Details of the connection systems were described previously.[5]

Analytical Procedure. Fused-silica capillaries were connected to the inlet and outlet of the microchip. The microchip with capillaries attached was put on the stage of the TLM. The inlet capillary was then connected to the syringe, and the outlet capillary was connected to a waste reservoir. The cell introduction holes were closed with cellophane tape. The solution exchange could be achieved simply by switching the microsyringe pumps. First, the medium in the microflask was replaced with HBSS, and then staurosprine solution was introduced into the flask for the stimulation.

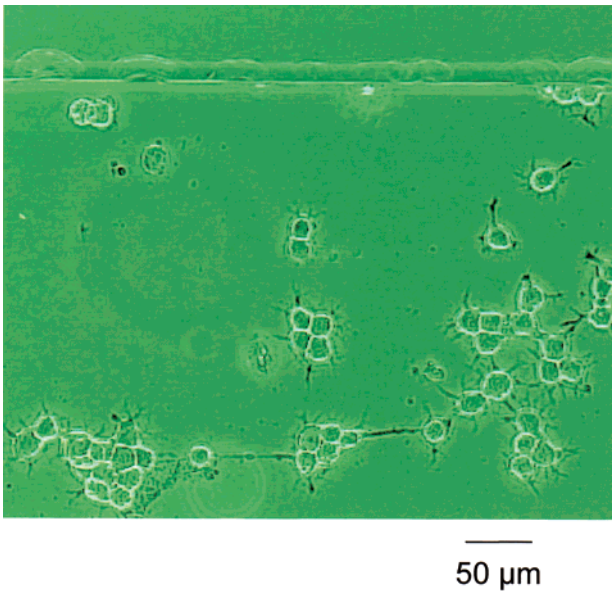

Figure 2. NG-108-15 cells cultured in microflask.

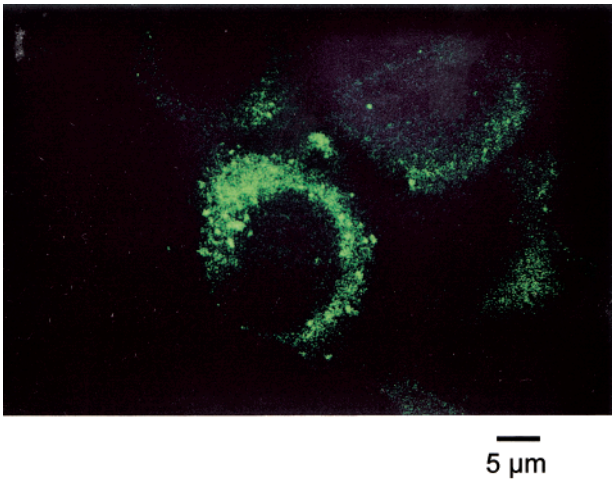

Figure 3. Fluorescent photograph of mitochondria in a cell stained by Mito Tracker Green.

For measurement of cyt in a cell, the TLM excitation beam was focused in the cell, and then signal intensities were acquired using a personal computer. Because cyt has a strong absorption around 532 nm ($\epsilon \approx 10\,000$ at 532 nm) and is a major component that has strong absorption at 532 nm, the emission line of 532 nm was selected as the excitation beam for cyt measurements; the possibility that other minor components were also detected simultaneously remained.

Active mitochondria in a cell were stained with Mito Tracker Green (Molecular Probes, Eugene, OR) solution according to the manufacturer's instruction.

RESULTS AND DISCUSSION

Cell Culture in a Microchip. The microchips for cell culture were washed with 0.1% NaOH and rinsed with pure water, followed by autoclaving at 120 °C for 15 min. The microflask of each chip was coated with 0.01% poly(L-lysine) solution for 1 h and then rinsed with sterile water. Cells grown on a polystyrene dish were suspended with a small amount of the fresh medium, and the

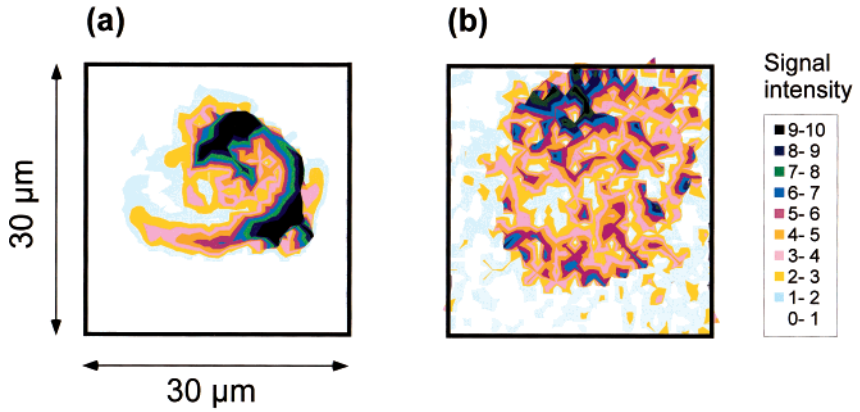

Figure 4. Scanning thermal lens microscope images of a single cell (a) before apoptosis and (b) 4 h after introduction of staurosporine. The signal intensities were taken at 30 × 30 points in 1-μm steps.

suspension was dropped onto the cell introduction hole of the chip. This chip was put into a covered 10-cm dish with a cover. which was filled with 10 mL of the medium and incubated in a CO_2 incubator at 37 °C with 5% CO_2. The medium in the microflask was replaced with the outside medium by gentle inclination of the dish twice a week. Under these conditions, cells adhered on the microflask wall and successfully cultivated in the glass microflask as shown in Figure 2. Cells were used for measurements mainly after cultivation of 5 to 10 days.

Fluorescent Photograph of Mitochondria. A fluorescent photograph of the live NG 108-15 with Mito Tracker Green is shown in Figure 3. This is a distribution of mitochondria in a live cell. Mitochondria were not located at the center part of the cell, where the nucleus was located. Because cyt is contained only in mitochondria in a live cell, cyt should also be located outside of the nucleus. Then we expected the cyt distribution before apoptosis to show a distribution similar to that of the mitochondria (Figure 3). The cyt does not have fluorescence and it cannot be observed in situ by fluorescent photograph.

Two-Dimensional Imaging of cyt. The two-dimensional distribution of cyt in a single cell cultured in the microchip was acquired by the TLM with 3D scanning stage. The microchip was held on the *XYZ* stage, and the microculture flask was filled with HBSS, which is an isotonic solution and contains enough nutrition to allow the cells to live for several hours. Using a CCD camera, we fixed the *Z*-axial position of the stage as the focal position of the excitation laser and cells in the chip were at the same level. In this experiment, the excitation laser power was set at 3 mW and the probe laser power was 5 mW. Under these conditions, cells were not affected by the laser radiation. The TLM stage was moved in 1-μm steps and signals were taken at 30 × 30 points. Each signal intensity was acquired after 5 s to ensure a stabilized signal. Figure 4a shows the two-dimensional signal intensity image of a live single cell obtained with TLM; this was possibly the distribution image of cyt in a cell as it was similar to the mitochondrial distribution (Figure 3). We thought it reasonable to consider the signal was mainly derived from cyt localized in mitochondria.

Four hours after introduction of staurosporine, which was prepared to 2 μM in HBSS, into the microflask to induce apoptosis, we measured the cell under the same conditions as the previous experiment. These results are shown in Figure 4b. In comparison

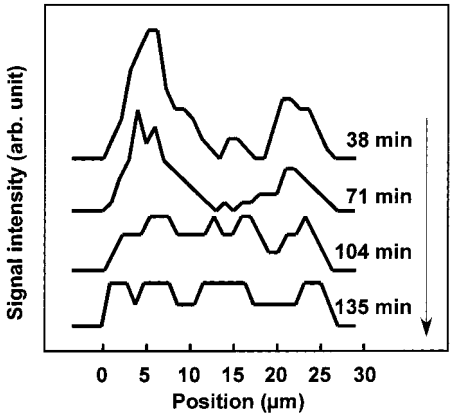

Figure 5. One-dimensional scanning images of a cell during apoptosis. The signals were taken at the times after staurosporine introduction indicated in figure.

with Figure 4a, the distribution of signal intensities was rather delocalized. This distribution change of the signal intensities indicated the diffusion of cyt from mitochondria to cytosol during apoptosis signal transduction. The probing volume can be estimated by the theory of thermal lens measurement.[14] The probing volume is the same as the confocal volume, and the value for our experimental setup was calculated to be 13.5 fL. Considering the probe volume and the calibration curve of thermal lens signal to the concentration of cyt (data not shown), the absolute amount of cyt detected with this system was estimated to be at the ~10 zmol level for each spot. We concluded that the distribution change of cyt in a live intact cell was successfully monitored using TLM without any labeling materials.

Rapid Monitoring of cyt Distribution Change. A two-dimensional scanning imaging required more than 1 h for 900 measurement points. It is difficult to achieve real-time monitoring of rapidly changing phenomena with the two-dimensional scanning method described above. To realize rapid analysis, we measured the cell during apoptosis by one-dimensional scanning, which needs a shorter time.

First, under the conditions that the excitation laser power was 3 mW, the probe laser power was 5 mW, and the scanning speed of the microscope stage was 15 s/μm, we measured a certain cell repeatedly by one-dimensional scanning without any stimulation. From these measurements, similar data were acquired repeatedly. The results clearly showed that the method had a high repeat-

ability and laser radiation itself hardly affected the cell. Considering the equation of thermal lens theory,[26] the temperature rise by laser radiation is estimated to 4×10^{-3} K.

Under the same conditions as the previous experiments, we measured a cell repeatedly after staurosporine introduction. The introduction time is reliably known because the culturing microflask is very small and the reagent can be rapidly exchanged by pressure-driven flow. The diffusion time of the stimulating chemical species in the microflask is negligible for the present analysis. The results of this experiment are shown in Figure 5. The signal patterns were obtained 38, 71, 104, and 135 min after staurosporine introduction. The signal profiles changed after 104 min. This change seemed to be derived from staurosporine introduction because no change was monitored in this time frame without its introduction. The results indicated that the cyt was secreted from mitochondria to cytosol in 30 min after a rather long incubation time (71 min). We were able to monitor the distribution change of cyt in a single cell during apoptosis signal transduction without using any labeling substrate.

We developed a novel cell analysis system by combining TLM and a microchip. The system incorporated all processes of a cellular biochemical analysis, i.e., microscale cell culture, chemical stimulation, and sensitive optical detection under a microscope. As described above, this system can image the distribution of absorption substrates in a single cell without any labeling substances and should have wide applicability. This system can also realize quick and secure chemical stimulation under continuous monitoring, and it seems suitable for time-resolved analysis. Since the system does not require labeling substances, which may possibly to affect cell metabolisms, real phenomena of an intact cell can be obtained.

(26) Bialkowski, S. E. *Photothermal Spectroscopy Methods for Chemical Analysis*; John Wiley and Sons: New York, 1996.

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