Time-resolved observation of surface-enhanced Raman scattering from gold nanoparticles during transport through a living cell

Katsumasa Fujita
Osaka University
Department of Applied Physics
2-1 Yamadaoka
Suita, Osaka 565-0871 Japan

Sawako Ishitobi
Keisaku Hamada
Osaka University
Frontier Biosciences
1-3 Yamadaoka
Suita, Osaka 565-0871
Japan

Nicholas I. Smith
Osaka University
Department of Applied Physics
2-1 Yamadaoka
Suita, Osaka 565-0871 Japan

Atsushi Taguchi
RIKEN
2-1, Hirosawa
Wako, Saitama 351-0198
Japan

Yasushi Inouye
Osaka University
Frontier Biosciences
1-3 Yamadaoka
Suita, Osaka 565-0871
Japan

Satoshi Kawata
Osaka University
Department of Applied Physics
2-1 Yamadaoka
Suita, Osaka 565-0871 Japan
and
RIKEN
2-1, Hirosawa
Wako, Saitama 351-0198
Japan

Abstract. We perform time-resolved observation of living cells with gold nanoparticles using surface-enhanced Raman scattering (SERS). The position and SERS spectra of 50-nm gold nanoparticles are simultaneously observed by slit-scanning Raman microscopy with high spatial and temporal resolution. From the SERS observation, we confirm the attachment of the particles on the cell surface and the entry into the cell with the subsequent generation of SERS signals from nearby molecules. We also confirm that the strong dependence of SERS spectra on the position of the particle during the transportation of the particle through the cell. The obtained SERS spectra and its temporal fluctuation indicate that the molecular signals observable by this technique are given only from within a limited volume in close proximity to the nanoparticles. This confirms the high spatial selectivity and resolution of SERS imaging for observation of biomolecules involved in cellular events *in situ*.
[DOI: 10.1117/1.3119242]

Keywords: Raman spectroscopy; microscopy; surface plasmons; particles; cells.

Paper 08299R received Aug. 26, 2008; revised manuscript received Jan. 24, 2009; accepted for publication Feb. 15, 2009; published online Apr. 23, 2009.

1 Introduction

Raman spectroscopy has been employed in sophisticated experiments to investigate molecular structures, and the conditions of molecules in their environments. Since Raman scattering spectra can give molecular vibrational information of scattering objects, it enables us to observe and investigate molecules in biological samples without any labeling, offering chemical information of biomolecular microenvironments in cells.[1] With the recent developments in Raman microscopy, the imaging of the distributions of biomolecules such as DNA and proteins has been demonstrated without labeling.[2,3] The dynamic changes in molecular distributions of biomolecules

Address all correspondence to: Katsumasa Fujita, Osaka University, Department of Applied Physics, 2-1 Yamadaoka, Suita, Osaka 565-0871 Japan. Tel: +81-6-6879-7847; Fax: +81-6-6879-7330; E-mail: fujita@ap.eng.osaka-u.ac.jp

1083-3668/2009/14(2)/024038/7/$25.00 © 2009 SPIE

during immune processes, cell death, and cell division have also been measured.[4–6]

The benefits of Raman microscopy in biomolecular imaging can be further magnified by applying surface-enhanced Raman scattering[7] (SERS). Since SERS dramatically enhances Raman scattering, it can be used to increase the temporal resolution in Raman imaging modes, which is especially significant for observation of living samples where the distributions and conditions of the molecules change during various biological processes. In SERS imaging of living cells, metal nanostructures such as gold nanoparticles have been used to generate the enhancement of Raman signals.[8–11] These results show that SERS with metal nanoparticles has the potential to be applied in molecular imaging of living samples with nanometer-scale resolution to provide structural and environmental information of the molecules. The strong enhancement of Raman signals by metal nanoparticles has also been applied for measuring intracellular pH (Ref. 12), monitoring neurotransmitter release,[13] cancer diagnostics,[14] and drug detection in cells.[15] The effectiveness of using metal particles for high-sensitivity detection of biomolecules has also been proved with other optical phenomena, such as harmonic generation and multiphoton excitation.[16,17]

In this paper, we report SERS observation of living cells with gold nanoparticles at high temporal and spatial resolution. To explore the feasibility of SERS imaging in live cell observation, it is important to investigate the behavior of SERS resolved both temporally and spatially since SERS agents, such as metal nanoparticles, change their positions in living cells, which strongly influences the SERS spectra to be measured. Although the effectiveness of metal nanoparticles as SERS agents is widely recognized, the temporal and spatial behavior of the resulting SERS spectra has not been investigated with metal nanoparticles in living cells. This is because conventional Raman microscopy cannot provide spatial and temporal resolution that is high enough to monitor the dynamic spectral behavior. Recently, we developed a slit scanning Raman microscope that offers diffraction-limited resolution and high temporal resolution with parallel detection of Raman scattering, which can trace the dynamics of biomolecules in living cells.[6,18] In this research, we applied this technique to monitor the behaviors of SERS spectra in cells, which is, to our knowledge, the first demonstration of temporally resolved imaging of dynamic changes in SERS signals from metal nanoparticles in living cells with simultaneous spatial resolution sufficient to observe the movement of nanoparticles in the cell.

2 SERS Imaging of Living Macrophage Cells

For the SERS observation, we used macrophage cells that exhibit endocytosis, by which we can introduce the nanoparticles into living cells. We used 50-nm-diameter gold nanoparticles as SERS agents.[8–10] The macrophage cells were cultured in a glass bottom dish in a Dulbecco modified Eagle medium (DMEM) solution, and the culturing medium was replaced by a HEPES-buffered Tyrode's solution composed of (in millimolars) NaCl, 150; glucose, 10; HEPES, 10; KCl, 4.0; $MgCl_2$ 1.0; $CaCl_2$, 1.0; and NaOH, 4.0. The replacement of the culturing media was done for SERS observations to avoid Raman scattering from molecules included in the culturing medium.

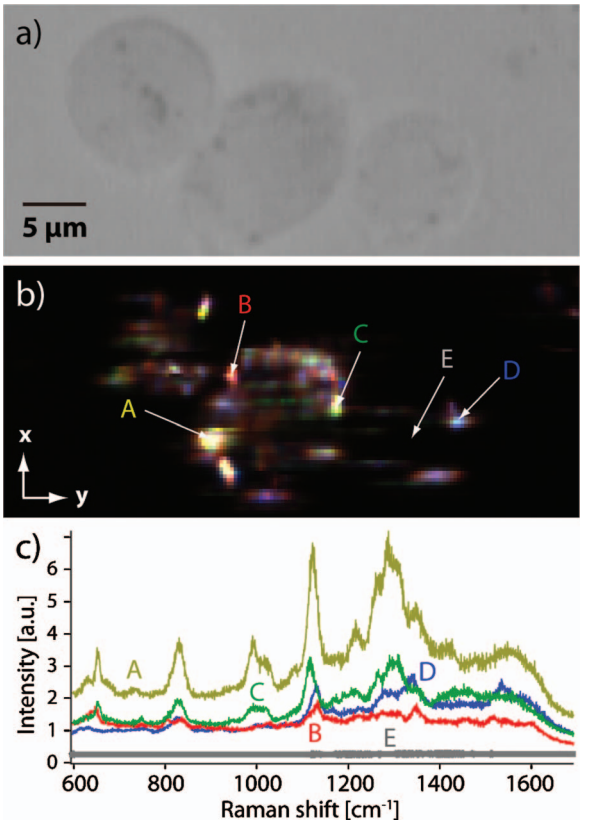

Fig. 1 (a) Bright-field and (b) a SERS image of macrophage cells with gold nanoparticles. Raman spectra in the spots indicated as A to E are shown in (c). For taking the SERS image, the samples were irradiated with 785-nm continuous-wave laser light of intensity of 7 mW/μm^2, and the total exposure time was 1 min for the image with 70(x) $\times$164(y)pixels.

Figure 1(a) shows bright-field and Fig. 1(b) shows Raman images of macrophage cells with the gold nanoparticles. Figure 1(c) shows the Raman spectra obtained from different positions, as labeled in Fig. 1(b). The cells were cultured for 24 h in DMEM solution containing gold particles at a concentration of 1.3×10^{10} particles/ml. During this time, particles enter the cells through endocytosis. Both images were obtained by a slit-scanning Raman microscope (Nanophoton, RAMAN-11) equipped with a 785-nm laser as a light source, a water-immersion objective lens of 1.0 numerical aperture (NA) for sample observation, and a cooled CCD camera (Princeton Instruments, Pixis 400BR) for detection of Raman scattering signals. Figure 1(b) was constructed using the distribution of relative intensities of Raman peaks at 640 cm^{-1} (C-C twisting in tyrosine), 1100 cm^{-1} (PO_2^- stretching in DNA or C-C stretching in lipid), and 1530 cm^{-1} (guanine or adenine) and setting them to respective red, green, and blue color channels in the image.

In Fig. 1(b), strong Raman scattering is confirmed at several spots in the cells. The intensities of the strong Raman spectra [A, B, C, and D in Fig. 1(c)] are orders of magnitude higher than the Raman signals emitted from the cell body [spectrum E in Fig. 1(c)]. Unlike typical Raman images of

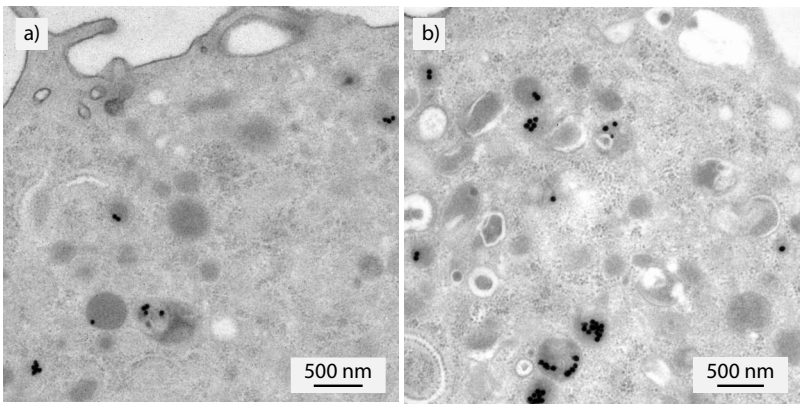

Fig. 2 TEM images of macrophage cells incubated with gold nanoparticles for (a) 2 and (b) 24 h, respectively.

biological cells,[3,6] Fig. 1(b) shows that strong Raman scattering is localized at several positions due to the SERS effect from the gold nanoparticles in the cells. We also confirmed uptake of gold particles by transmission electron microscopy (TEM) observation of macrophage cells. Figures 2(a) and 2(b) show the TEM images of the macrophage cells that were processed for TEM observation after being incubated with gold particles for 2 and 24 h, respectively. By TEM observation, we confirmed that the gold particles were taken into the cells during the incubation time. These results also confirm the aggregation of gold nanoparticles in the cells, which has also been reported by Kneipp et al.[9] These aggregated gold nanoparticles result in a red shift of the plasmon resonance frequency, which may be responsible for the bright SERS spots shown in Fig. 1, which are clearly visible even though the light source for SERS excitation was near-IR.

We also compared Raman spectra obtained with and without gold nanoparticles. We prepared two types of samples: macrophage cells incubated in the DMEM solution for 3 h with gold nanoparticles as well as cells incubated in the solution without gold particles. Figure 3 shows the Raman spectra obtained (curve a) with and (curve b) without gold nanoparticles. The light exposure for Fig. 3 [curves (a) and (b)] are 10 mW/μm^2 for 1 s and 680 mW/μm^2 for 60 s, respectively. A 1.0 NA water-immersion objective lens was used for both measurements. In Fig. 3, the number of Raman peaks observed with gold nanoparticles was notably smaller than that obtained without gold particles. This shows that the number of molecular species observed simultaneously is smaller in SERS with gold nanoparticles than that obtained simply with a laser spot. This indicates that the sample volume subject to SERS is significantly reduced and, as the result, higher spatial selectivity and resolution may be provided in SERS imaging compared to those in typical Raman imaging.

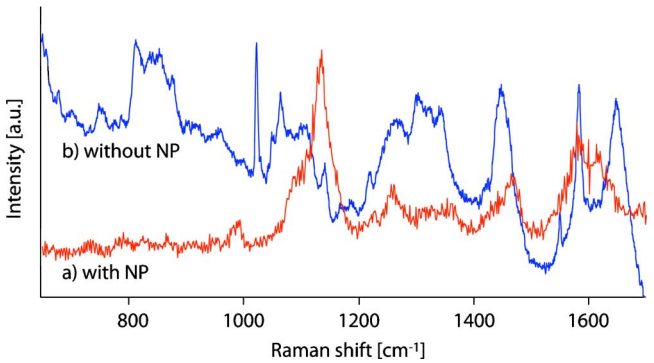

Fig. 3 Raman spectra obtained from a living macrophage cell (curve a) with and (curve b) without gold nanoparticles (NP). The light exposure for curves (a) and (b) are 10 mW/μm^2 for 1 s and 680 mW/μm^2 for 60 s with 785-nm continuous-wave laser light, respectively.

3 Time-Resolved SERS Observation

To confirm the entry of nanoparticles into cells, we used time-lapse observation of the spatial and temporal behavior of the SERS signals from the nanoparticles during the endocytosis process of a macrophage cell. After obtaining the dark-field and Raman images as in Figs. 4(a) and 4(b), gold 50-nm-diameter nanoparticles were added to the cells with the resultant concentration of 1.3×10^{10} particles/ml. The particles were added to the culture dishes and SERS observations were recorded every 2.5 min. The total exposure time for each image was 2 min with 60 scanning lines. For this time-lapse observation, we used slit-scanning Raman microscopy, which enables a higher image acquisition rate than a single-focus scanning microscope to observe behavior of SERS spots with a temporal resolution of a few minutes.[6] A titanium sapphire laser operated in cw mode at a 785-nm wavelength was used as the light source. The laser light was introduced to a slit-scanning Raman microscope equipped with a water-immersion objective lens with NA of 1.0, a spectrometer (Bunkoh Keiki, MK-300), and a cooled CCD camera (Princeton Instruments, Pixis 400BR). For Figs. 4(b)–4(e), the images were reconstructed using distributions of total Raman intensities between Raman shifts of 600 and 1750 cm^{-1} and normalized to the same intensity. Before constructing images, we subtracted the background signals (the combined non-Raman contributions such as fluorescence) from the Raman spectra at each pixel by a modified polyfit fluorescence removal technique.[19]

We confirmed that a SERS signal was produced within the first 2.5 min following the introduction of the gold nanopar-

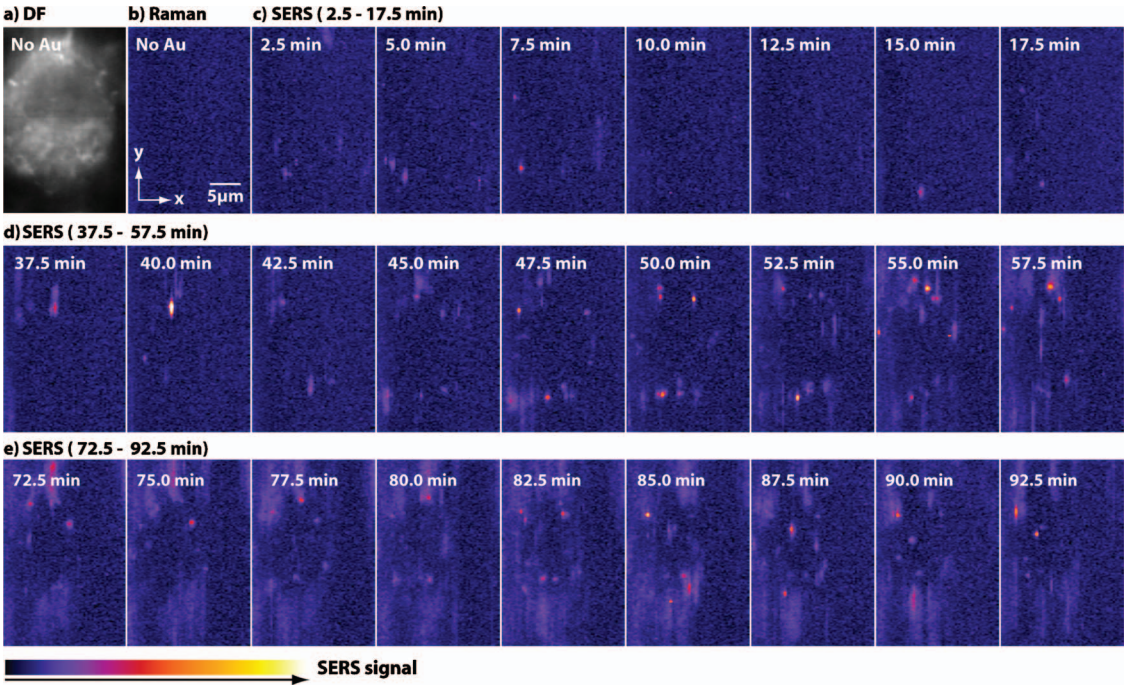

Fig. 4 Time-lapse SERS image sequences of a macrophage cell with gold nanoparticles. The cell was immersed in Tyrode's solution and gold nanoparticles were added to the solution after taking the images using (a) dark-field and (b) Raman imaging modes. Each image contains 60(x) $\times$ 200(y)pixels. The sample was irradiated with a light intensity of 15 mW/μm^2.

ticles into the solution, as evident from the strong Raman scattering spots in Fig. 4(c), which appeared only after the addition of nanoparticles. In Fig. 4(c), we can see that several gold nanoparticles attached to the cellular surface and exhibited strong SERS. The number and positions of SERS spots changes with time, which indicates that the positions of gold nanoparticles move, associated either with certain cellular activities and/or Brownian motion of the particles. About 30 min after the introduction of the particles, more particles attached on the cell surface and started to enter the inside of the cells as seen in Fig. 4(d). In Fig. 4(e), we can observe the SERS spots internalized after sufficient incubation time, and which then exhibit movement inside the cell. The number of SERS spots increased during the observation, showing that the cell takes in a larger number of particles with an increase in incubation time. In these SERS images, several spots can be seen to extend in the y direction. These elongated spots are SERS signals from out-of-focus planes. This is due to the use of slit-scanning detection system, and is not seen in typical single-point confocal optical systems. In Fig. 4(d), elongated SERS spots occur due to gold nanoparticles on or near the cellular membrane at out-of-focus positions, making the membrane areas much brighter than regions inside the cell. In the intracellular regions, SERS signals are also observable from the gold nanoparticles that have entered the cell by endocytosis.

We also confirmed SERS spectra in each frame, and selected frames are shown in Fig. 5. Different SERS spectra were obtained at different positions and times in the cells to give local molecular information in the cells. Table 1 shows the molecular vibration and species that can be assigned from the Raman peaks in Fig. 5. In these results, we can observe that the nanoparticles provide information of nearby molecules as SERS spectra, and we can see that the observed molecules change over time, since the molecular distributions undergo changes associated with cellular motion or endocytosis. During endocytosis, the nanoparticles can be expected to be surrounded by lipid molecules.[22] In our results, even though it is clear that the particles are internalized in the cell, we did not find a strong contribution from molecular vibrations that result from lipid molecules surrounding the nanoparticle. Similar results have also been seen in other reports of SERS measurement with gold nanoparticles introduced by endocytosis.[9,10] The reason why the lipid vibrational modes were not observed is not yet clear. However, this result indicates that we can detect molecules in cells with SERS agents introduced by endocytosis without the influence of strong background Raman signals from the surrounding lipid molecules. In our experiments, we can not currently confirm that these spectra are generated from a single gold particle. Note, however, that the SERS signals typically appeared as peaks with a height of around 10 to 40 photons/s above the background in most spectra, indicating that the enhancement factor for SERS did not change drastically in this observation for each particle and condition. In some spectra, we also observed much stronger SERS peaks as seen in Fig. 5(d), which may be due to aggregation effects of gold particles, as reported previously.[9]

4 Temporal Fluctuation of SERS Spectra in Living Cells

To determine the minimum required temporal resolution for a SERS microscope, we measured the time-variation in gold

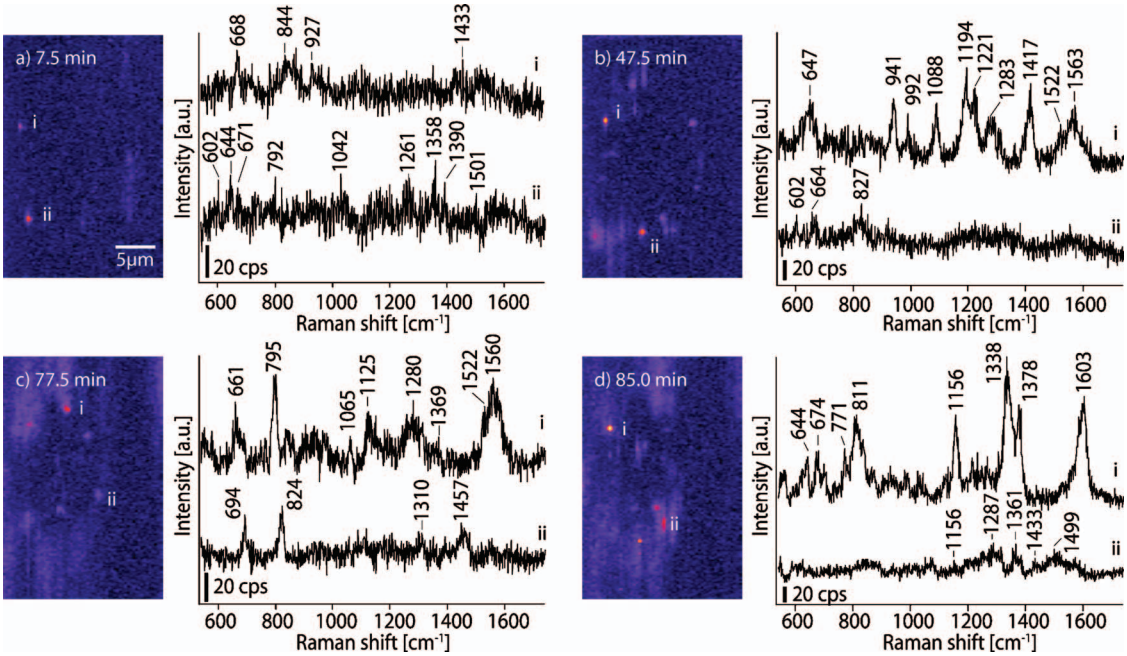

Fig. 5 SERS spectra obtained in selected frames of the time-lapse SERS observation shown in Fig. 4. The bar in the spectra indicates 20 photon counts/s.

nanoparticle SERS spectra from a single spot in a living macrophage cell, as shown in Fig. 6. We measured SERS spectra every 0.63 s with an exposure time of 0.5 s. The sample was observed after incubation with gold nanoparticles for 1 day. In the resulting spectra, Raman peaks assigned to tyrosine (645 cm^{-1}) and RNA (phosphate, 811 cm^{-1}) were confirmed in all spectra, however, the peaks were slightly shifted in each spectra. This phenomenon has also been reported with a human osteosarcoma cell,[10] and has been observed[23,24] in single-molecule detection by SERS, where it was thought to result from the fluctuation of individual molecules on the surface of the particle. While the temporal resolution in those experiments was lower than in the measurements presented in this paper, the overall agreement between the two measurements indicates that the fluctuation of the SERS signal is a physical effect at the nanoparticle level, and may be a fundamental limitation of all nanoparticle-based SERS imaging systems. It can also be assumed that in addition to the molecular level fluctuations, the motion of the particle will influence the temporal behavior of the SERS spectra. The heating effect by the laser irradiation may also cause an environmental or chemical condition change of the molecules, which also influence the SERS spectra.[25] In Fig. 6, Raman peaks assigned to proline (1134 cm^{-1}) and guanine (1325 cm^{-1}), which were observed at the beginning of the measurement, disappeared after around 1 and 5 s during the measurement, respectively. Some other peaks, such as the peak at 520 cm^{-1}, appeared in only one spectrum. This is presumably caused by detachment of the molecules from SERS active sites on the particles due to diffusion of the molecules or the motion of the gold nanoparticles.

5 Discussion and Conclusions

In this paper, we have shown the observation of the temporal and spatial behavior of SERS signals from a living macrophage cell with gold nanoparticles. The SERS signal was obtained immediately after introducing gold nanoparticles into the cell dish. We confirmed that SERS spectra change drastically depending on the position and the measurement time. These results show the feasibility of a gold nanoparticle as a SERS probe for imaging cellular components in live cell conditions.

Simultaneous measurement of positions and spectra of SERS spots, as shown in this paper, can be used to realize SERS imaging of the inside of living cells. For this purpose, the temporal resolution of the observations presented here is not yet high enough to unambiguously track the motion of all the particles in our living cell samples. In addition, the SERS spectrum obtained from a single spot in a living cell with gold nanoparticles changes shape in less than 1 s, as shown in Fig. 6. Depending on the rate of temporal change of the spectra, we must employ a spectrum detection scheme that provides a temporal resolution high enough to detect the complete behavior of SERS spectra. The limitations of the signal strength and the ambiguities in deciphering the relative contributions of noise, particle movement, binding statistics, and chemical changes to the detected signal are significant. However, the results presented here indicate that time-resolved imaging of dynamic biological processes with high spatial, temporal, and spectral resolution will be possible in the near future. Although the single point fluctuation measurements show that a further increase in temporal resolution would be desirable, some of the molecular peaks were seen to fluctuate more slowly than others. For the peaks that fluctuated more slowly

Table 1 Temporal assignment of Raman peaks shown in Fig. 5.

Raman Shift (cm^{-1})	Assignment
602, 644	C: ribose-phosphate
647	Tyr: γ_T(CC)
661-694	ν(CS)
674	G: base
762	C: ribose-phosphate
771	alanine, lipids
792, 795	C: ring breathing
811	phosphate: ν(OPO)
824	valine, histidine
827	proteins, Try: δ(CCH) aliphtic, Tyr: ring
844	C: ribose-phosphate
927	proline: ring ν(CC)
941	glutamate, A
992	C: base, proline
1042	A, C: ν(CO)
1065	lipids: ν(CC), arginine
1088	histidine, phosphate
1125	A, valine
1156	nucleotides: ribose-phosphate, protein: ν(CN)
1194	C: base
1221	G: ν(CC)/ ν(CN)
1261	protein: amide III
1280	C: base
1283	glutamate
1287	proline
1310	A: ring, proteins: $\gamma_T(CH_2, CH_3)$
1338, 1358	proteins: $\gamma_T(CH_2, CH_3)$, tryptophan
1361	alanine, try, G: base
1369	T, lipid: $\gamma_w(CH_2)$
1378	proline, alanine, arginine
1390	$\nu(COO^-)$ sym, G: base
1417	Try, glutamate, G: base, lipid: $\delta(CH_3)$
1433, 1457	lipids, protein: $\delta(CH_2, CH_3)$
1499, 1501	histidine, alanine
1522	C: base
1563	Try
1603	proteins, phenylalanine, Try

The assignment is based on Refs. 9, 10, 20, and 21. Abbreviations indicate: ν, stretching; δ, deformation; γ_T, twisting; γ_W, wagging; sym, symmetrical; asym, asymmetrical; Try, tryptophan; Tyr, tyrosine; A, adenine; T, thymine; C, cytosine; G, guanine.

in Fig. 6 (e.g., RNA, guanine, etc.), the current data acquisition rate was fast enough to resolve the time varying changes.

The simultaneous measurement of the nanoparticle position and SERS spectra also provides probe microscopy capability without requiring scanning apparatus. Cellular activities such as protoplasmic movement, protein transport, and endo- or exocytosis may be used to move the particles in living cells. The molecular signatures of these biological activities at distinct locations in position and time can be reported by SERS, enabling minimally invasive nanoendoscopy for living cells.

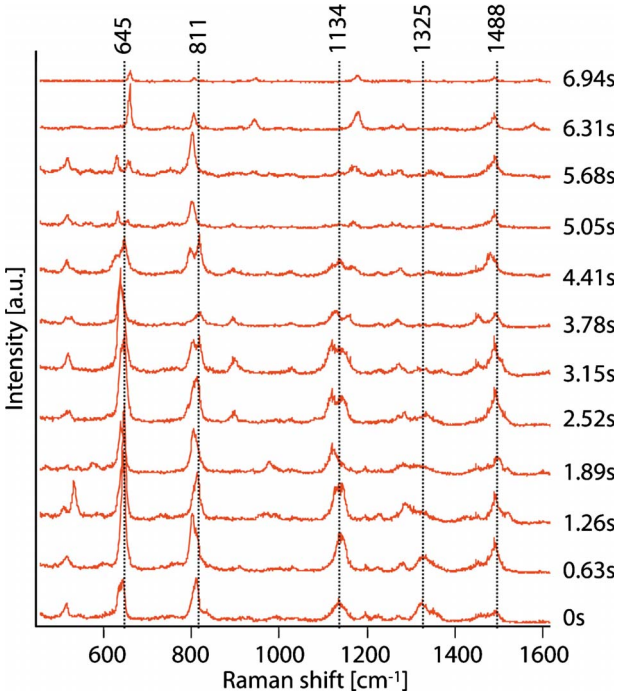

Fig. 6 Temporal behavior of a SERS spectrum obtained at a single point in a living macrophage cell. The exposure time was 0.5 s, and SERS measurements were taken every 0.63 s.

Acknowledgments

The authors thank Dr. Taro Ichimura for useful discussion about SERS effects with gold nanoparticles. The authors also thank Hirohiko Niioka for his assistance in performing the TEM observation. This research was supported by a CREST (Core Research for Educational Science and Technology) project of JST (Japan Science and Technology Corporation).

References

1. G. J. Puppels, F. F. M. de Mul, C. Otto, J. Greve, M. Robert-Nicoud, D. J. Arndt-Jovin, and T. M. Jovin, "Studying single living cells and chromosomes by confocal Raman microspectroscopy," *Nature (London)* **347**, 301–303 (1990).
2. N. Uzunbajakava and C. Otto, "Combined Raman and continuous-wave-excited two-photon fluorescence cell imaging," *Opt. Lett.* **28**, 2073–2075 (2003).
3. N. Uzunbajakava, A. Lenferink, Y. Kraan, E. Volokhina, G. Vrensen, J. Greve, and C. Otto, "Nonresonant confocal Raman imaging of DNA and protein distribution in apoptotic cells," *Biophys. J.* **84**, 3958–3981 (2003).
4. H.-J. Manen, Y. M. Kraan, D. Roos, and C. Otto, "Intracellular chemical imaging of heme-containing enzymes involved in innate immunity using resonance Raman microscopy," *J. Phys. Chem. B* **108**, 18762–18771 (2004).
5. Y.-S. Huang, T. Karashima, M. Yamamoto, and H. Hamaguchi, "Molecular-level investigation of the structure, transformation, and bioactivity of single living fission yeast cells by time- and space-resolved Raman spectroscopy," *Biochemistry* **44**, 10009–10019 (2005).
6. K. Hamada, K. Fujita, N. I. Smith, M. Kobayashi, Y. Inouye, and S. Kawata, "Raman microscopy for dynamic molecular imaging of living cells," *J. Biomed. Opt.* **13**, 044027 (2008).
7. K. Kneipp, H. Kneipp, I. Itzkan, R. R. Dasari, and M. S. Feld, "Surface-enhanced Raman scattering and biophysics," *J. Phys. Condens. Matter* **14**, R597–R624 (2002).
8. K. Kneipp, A. S. Haka, H. Kneipp, K. Badizadegan, N. Yoshizawa, C. Boone, K. E. Shafer-Peltier, J. T. Motz, R. R. Dasari, and M. S. Feld, "Surface-enhanced Raman spectroscopy in single living cells using gold nanoparticles," *Appl. Spectrosc.* **56**, 150–154 (2002).
9. J. Kneipp, H. Kneipp, M. McLaughlin, D. Brown, and K. Kneipp, "In vivo molecular probing of cellular compartments with gold nanoparticles and nanoaggregates," *Nano Lett.* **6**, 2225–2231 (2006).
10. H. W. Tang, X. B. Yang, J. Kirkham, and D. A. Smith, "Probing intrinsic and extrinsic components in single osteosarcoma cells by near-infrared surface-enhanced Raman scattering," *Anal. Chem.* **79**, 3646–3653 (2007).
11. A. Sujith, T. Itoh, H. Abe, A. A. Anas, K. Yoshida, V. Biju, and M. Ishikawa, "Surface enhanced Raman scattering analyses of individual silver nanoaggregates on living single yeast cell wall," *Appl. Phys. Lett.* **92**, 103901 (2008).
12. J. Kneipp, H. Kneipp, B. Wittig, and K. Kneipp, "One- and two-photon excited optical pH probing for cells using surface-enhanced Raman and hyper-Raman nanosensors," *Nano Lett.* **7**, 2819–2823 (2007).
13. R. J. Dijkstra, W. J. M. Scheenen, N. Dam, E. W. Roubos, and J. J. ter Meulen, "Monitoring neurotransmitter release using surface-enhanced Raman spectroscopy," *J. Neurosci. Methods* **159**, 43–50 (2007).
14. X. Huang, I. H. El-Sayed, W. Qian, and M. A. El-Sayed, "Cancer cells assemble and align gold nanorods conjugated to antibodies to produce highly enhanced, sharp, and polarized surface Raman spectra: a potential cancer diagnostic marker," *Nano Lett.* **7**, 1591–1597 (2007).
15. I. R. Nabiev, H. Morjani, and M. Manfait, "Selective analysis of antitumor drug interaction with living cancer cells as probed by surface-enhanced Raman spectroscopy," *Eur. Biophys. J.* **19**, 311–316 (1991).
16. G. Peleg, A. Lewis, M. Linial, and L. M. Loew, "Non-linear optical measurement of membrane potential around single molecules at selected cellular sites," *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6700–6704 (1999).
17. D. Yelin, D. Oron, S. Thiberge, E. Moses, and Y. Silberberg, "Multiphoton plasmon-resonance microscopy," *Opt. Express* **11**, 1385–1391 (2003).
18. D. K. Veirs, J. W. Ager III, E. T. Loucks, and G. M. Rosenblatt, "Mapping materials properties with Raman spectroscopy utilizing a 2D detector," *Appl. Opt.* **29**, 4969–4980 (1990).
19. C. A. Lieber and A. Mahadevan-Jansen, "Automated method for subtraction of fluorescence from biological Raman spectra," *Appl. Spectrosc.* **57**, 1363–1367 (2003).
20. A. T. Tu, *Raman Spectroscopy in Biology: Principles and Applications*, Wiley, New York (1982).
21. J. D. Gelder, K. D. Gussem, P. Vandenabeele, and L. Moens, "Reference database of Raman spectra of biological molecules," *J. Raman Spectrosc.* **38**, 1133–1147 (2007).
22. B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and R. Walter, *Molecular Biology of the Cell*, 5th ed., pp. 787–799, Garland Science, New York (2008).
23. S. Nie and S. R. Emory, "Probing single molecules and single nanoparticles by surface-enhanced Raman scattering," *Science* **275**, 1102–1106 (1997).
24. K. Kneipp, Y. Wang, H. Kneipp, L. T. Perelman, I. Itzkan, R. R. Dasari, and M. S. Feld, "Single molecule detection using surface-enhanced Raman scattering," *Phys. Rev. Lett.* **78**, 1667–1670 (1997).
25. H. H. Richardson, Z. N. Hickman, A. O. Govorov, A. C. Thomas, W. Zhang, and M. E. Kordesch, "Thermooptical properties of gold nanoparticles embedded in ice: characterization of heat generation and melting," *Nano Lett.* **6**, 783–788 (2006).