Optical signals based on Raman scattering, coherent anti-Stokes Raman scattering (CARS), and harmonic generation can be used to image biological molecules in living cells without labeling. Both Raman scattering and CARS signals can be used to detect frequencies of molecular vibrations and to obtain the molecular distributions in samples. Second-harmonic optical signals can also be generated in structured arrays of noncentrosymmetric molecules and can be used to detect structured aggregates of proteins, such as collagen, myosin and tubulin. Since labeling techniques using chemical and biological reactions may cause undesirable changes in the sample, label-free molecular imaging techniques are essential for observation of living samples.

INTRODUCTION

One of the main advantages in using optical microscopy for biological imaging is that optical microscopes allow us to observe a sample in living conditions. Light of low-energy photons interacts weakly with biological molecules, so that activities of biological specimens can be observed without serious damage to the specimen. Although a typical spatial resolution of an optical microscope is incomparably lower than that of some other microscopes, such as scanning probe microscopes and scanning electron microscopes, optical microscopy has taken on a critical role in research on cellular and molecular biology and medicine.

Another advantage of optical microscopy is that the spectrum of the signal light provides a multitude of information regarding the material in the sample. This allows us to investigate biological molecules in cells and tissues without chemical labeling. For example, with Raman scattering, the wavelength of light scattered by molecules depends on the vibrational frequencies of the molecules, and the spectrum of the scattered light shows information of molecular species, structures and environments. Nonlinear light scattering, such as second harmonic generation, can visualize the orientation and alignment of proteins in cells with high-contrast. These label-free molecular imaging techniques are particularly useful for observation of living specimens. Typical labeling techniques require chemical or biological modification of a sample, which can significantly interfere with the biological activities of the molecules in the sample.

In this review, the label-free molecular imaging techniques that have recently been developed in optical microscopy are introduced. Several of these techniques utilize spectroscopic methods that have been used for analysis of molecules in the fields of chemistry and material science, but not for living biological specimens due to the inefficient emission of the signal light. The recent development of high-quality lasers and high-sensitive light detectors has made it possible to detect weak signals with a signal-to-noise ratio high enough to construct images of molecular distributions.

Raman scattering microscopy

Raman scattering is light scattering where the wavelength of scattered light shifts slightly from the original due to excitation of the light-scattering molecules to a vibrationally excited state (Fig. 1). The shift of the wavelength depends on the frequency of the molecular vibration excited by the light. Since the frequency of molecular vibration strongly depends on molecular structures, conditions and environments, the resulting Raman spectra can be used to identify and investigate molecules in the sample.

Figure 2 shows a typical Raman spectrum obtained from cytosol of a living HeLa cell. The lateral axis shows the Raman shifts which correspond to the energy that has been used to excite molecules into vibrational excitation states. For each peak in the spectrum, the peak position represents the vibration mode of molecules that has been excited, and the peak intensity is proportional to the amount of excited molecules. Note that a simple ratio of intensities between different Raman peaks does not actually give the ratio of the number of molecules indicated by the Raman shifts because the Raman scattering efficiency can be varied for different vibrational modes.

As shown in Fig. 2, each Raman peak in the spectrum can be assigned to molecular vibrations in the sample. For example, the Raman peak at around 1,450 cm$^{-1}$ can be assigned to the bending vibration mode of CH$_2$ which is contained in large amounts in the hydrocarbon chains of lipid molecules. The Raman peak at 1,680 cm$^{-1}$ can be assigned to Amide-I vibration mode of peptide bonds that can be commonly seen in proteins. Raman spectroscopic approaches have been combined with microscopic imaging techniques for label-free mapping of cellular components (Matthäus et al., 2007; Puppels et al., 1990, 1993; Rück et al., 2007; Uzunbajakava et al., 2003), immune components...
response of granulocyte cells (Manen et al., 2004; 2005), and investigation of molecular dynamics in cells under cell division (Huang et al., 2004; Matthäus et al., 2006).

In Raman microscopy, Raman spectra are obtained from each position in a sample, and the distribution of intensity of Raman scattering is used to construct an image of a molecular distribution. In a typical Raman microscope, laser light is focused into a sample to obtain Raman scattering, and the focal spot scans the sample while measuring Raman spectra to construct Raman images. However, with typical Raman microscopy, it takes several hours to obtain Raman spectra from each point in the observation area because the efficiency of Raman scattering is extremely low (a typical Raman scattering cross-section is about $10^{-30}$ cm$^2$, while a typical single-photon absorption cross-section is about $10^{-16}$ cm$^2$), which makes the observation of living specimens difficult. By optimization of the laser parameters and signal acquisition, Raman microscopes with frame rates in the range of tens of minutes have now begun to appear (Rück et al., 2007).

Recently, a Raman microscope that utilizes parallel detection of Raman spectra from different points in the sample has been developed. The parallel detection system significantly improves the image acquisition time of Raman microscopes, and makes it possible to observe living samples with an imaging time of several minutes (Hamada et al., 2008).

Figure 3 shows Raman images of living HeLa cells (Hamada et al., 2008). The images in Fig. 3 were constructed by using the distributions of light intensities of Raman peaks at the Raman shifts shown in the figure. In the observation, light of 532 nm wavelength was used for illumination. The images contain 156 × 209 pixels and the image acquisition time was about 14 min. The Raman peak at 753 cm$^{-1}$ can be assigned to a vibration mode of the heme protein in cytochrome. In particular, cytochrome c has light absorption at 532 nm and exhibits resonant Raman scattering (Spiro et al., 1972). Because resonant Raman scattering gives signal intensity 2 to 3 orders of magnitude higher than that of typical Raman scattering, cytochrome c in mitochondria mainly contributes to the image contrast in Fig. 3A. The Raman peak at 1,689 cm$^{-1}$ can be assigned to a vibration mode of peptide bonds seen in the beta sheet of proteins. Beta sheets are contained in various kinds of molecules that are distributed in the entire cell body as seen in Fig. 3B. Figure 3C was constructed by using the intensity of Raman peak at 2,852 cm$^{-1}$ that can be assigned to the stretching vibration mode of CH$_2$. Since CH$_2$ is highly concentrated in hydrocarbon chains of lipid molecules, Fig. 3C shows the distribution of lipid vesicles and lipid membrane of organelles in the cell. Fig. 3D shows the color image produced by introducing Figs. 3A, 3B, and 3C into color channels of G, B, and R, respectively.

Figure 4 shows Raman images of a HeLa cell during mitosis. Each image contains 48 × 161 pixels, and the image acquisition time was about 3 min. The cell was observed every 5 min. In Fig. 4, movement of cellular molecules during the mitosis can be confirmed. The movement of the chromosomes can be clearly confirmed by the protein distribution. In these examples, biological molecules in the cell were imaged. In addition to the molecules that exist inherently in cells, exogenous chemicals, such as drugs, can also be imaged without labeling by using Raman scattering (Harada et al., 2008; Ling et al., 2002).

At the current stage of development, Raman microscopy still requires a few minutes of imaging time for observation of living specimen. Even with this image acquisition time, which is slow compared to conventional light microscopy, we can observe biological samples where the temporal changes are relatively
**Fig. 4.** Raman images of a HeLa cell under mitosis. Laser light with the wavelength of 532 nm was used to illuminate the sample by an objective lens with an NA of 1.2. Each image consists of 48 × 161 pixels and was recorded every 5 min with an image acquisition time of around 3 min.

**Fig. 5.** Jablonksi energy diagram of optical four-wave mixing (FWM) with (A) and without (B) and (C) resonance to molecular vibrations. Slow. Another drawback of using Raman scattering is that it is difficult to observe fluorescent samples. The emission wavelength of strong fluorescence overlaps that of Raman scattering, and makes detection of the Raman signals impossible. To overcome these problems, coherent anti-Stokes Raman scattering can be used as described below.

**Coherent anti-Stokes Raman scattering microscopy**

Coherent anti-Stokes Raman scattering (CARS) is a nonlinear optics phenomenon where the scattering efficiency is determined by the vibration frequency of scattering molecules, but with different selectivity than the Raman scattering described above. In CARS, light beams with two different optical frequencies (pump beam: ω_p, stokes beam: ω_s) are incident on a sample, and a strong CARS signal is produced when the difference of the frequencies of the beams matches the vibration frequency of the molecules. Figure 5A shows the energy diagram of molecules with CARS. When the difference in optical frequencies of the two incident light beams (ω_p - ω_s) matches the frequency of molecular vibration (Ω), the CARS signal with a frequency of 2ω_p - 2ω_s is produced by a four-wave mixing process resonant to the molecular vibration.

In CARS Microscopy (Cheng et al., 2004; Zumbusch et al., 1999), CARS signal is generated from the focal spot where the pump and the Stokes beams are focused by an objective lens and are temporally and spatially overlapped. Since CARS signal is generated strongly with the process resonant with molecular vibration, distribution of the molecules vibrating at a particular frequency can be mapped by measuring the CARS signal with scanning the focal spot in the sample. CARS microscopes possesses the spatial resolution in the three dimensions because CARS is a nonlinear optical phenomena and the signal is generated within the focal volume (Hashimoto et al., 2001) similarly to two photon excited fluorescence.

Duncan et al. reported the first demonstration of CARS microscopy in 1982 (Duncan et al., 1982). They observed onion skin cells using two dye lasers as the light source. Because an ultra-short pulsed laser, which is required for obtaining CARS signal efficiently, was not commercially available until late 1990s, CARS microscopes had not been applied for observation of living biological cells or tissues. In 1999, Zumbusch et al. utilized two pico-second pulsed lasers as light sources and demonstrated high-resolution CARS imaging of unstained bacteria of the type Shewanella putrefaciens and HeLa cells in living conditions (Zumbusch et al., 1999). After this report, several types of CARS microscopes have been developed and applied to biological observations, such as imaging of brain structures (Evans et al., 2007), lipid droplets (Nan et al., 2003) and organelle transport in living cells (Nan et al., 2006).

One of the advantages of using CARS for molecular imaging is that the CARS signal does not spectrally overlapped with the fluorescence background that usually appears at longer wavelength regions, and complicates the collection of standard Raman signals. As shown in Fig. 5A, the CARS signal possesses higher frequency than the incident beams. Consequently, the wavelength of the signal is shorter than those of the incident beams. The shorter signal wavelength allows the collection of the anti-Stokes Raman signal without the fluorescence background. Another advantage of using CARS is that the CARS signal detected from cells is typically larger than that by Raman scattering. With this advantage, CARS microscopy has been
applied to observe biological samples, such as the skin of a live mouse (Evans et al., 2005) at video rate. However even CARS does not provide background-free detection of signals. Light with the same wavelength as the CARS signal may also be produced without resonant effect to molecular vibrations as shown in Figs. 5B and 5C. To these non-resonant background signals, several techniques using backward scattering (Volkmer, et al., 2001), polarization (Cheng et al., 2001) and phase dependence of CARS signal (Evans et al., 2004), time-resolved CARS (Volkmer et al., 2002) have been proposed.

The images in Fig. 6 show CARS images of label-free living HeLa cells. The wavelengths of the two incident beams were chosen so that the difference in optical frequencies corresponds to 1,446 cm$^{-1}$ and 1,240 cm$^{-1}$ for Figs. 6A and 6B, respectively. The Raman shift of 1,446 cm$^{-1}$ and 1,240 cm$^{-1}$ can be assigned to the bending vibration of CH$_2$ and Amide-III vibrational mode of peptide bonds, indicating a main contribution from lipid molecules and proteins to the contrast of the images in Figs. 6A and 6B, respectively.

Since the intensity of signal light in CARS microscopy is much higher than that in ordinary Raman microscopy during observation of cells, CARS microscopy allows an image acquisition rate higher than that of Raman microscopes. Video-rate imaging by CARS has been demonstrated in observation of lipid molecules in mouse skin tissue (Evans et al., 2005). In addition, CARS microscopy offers high spatial resolution arising from the nonlinear property of CARS in a similar manner as in two-photon excited fluorescence microscopy (Denk et al., 1990). To obtain CARS spectra, the frequency difference of two beams has to be scanned. However, in the recent development of multiplex CARS microscopy, which utilizes a broadband laser for Stokes beam ($\omega_0$), CARS spectra can be obtained by a spectrophotometer (Wurpel et al., 2002).

Second-harmonic generation microscopy

Second-harmonic generation (SHG) is a wavelength-conversion effect that can be seen when light with a high intensity is incident onto a sample (Boyd, 2003). The wavelength of light generated by SHG has a wavelength exactly equal to half of the wavelength of the incoming light (Fig. 7). For SHG, the material needs to have a noncentrosymmetric structure. For biological samples, it has been reported that collagen, myosin, and tubulin molecules have noncentrosymmetric structure and can exhibit SHG with irradiation of intense near-infrared light. (Boulesteix et al., 2004; Campagnola et al., 2003)

In SHG microscopy, an image is constructed by detecting SHG from a sample. Near-infrared pulsed-laser light is focused into the sample, and second-harmonic (SH) light is generated at the focal point. The focal spot scans the sample while measuring the intensity of SH light at each position in the sample. Since SHG requires noncentrosymmetric structure, the parts that satisfy this condition in the sample can be imaged with the highest contrast. The intensity of SH light is proportional to the square of the incident light and, as a result, the spatial resolution in three dimensions can be obtained, in the same manner as seen in two-photon excited fluorescence microscopy. Scanning the focal spot in three dimensions can then be used to observe the 3D structure of the sample, highlighting the distribution of proteins that satisfy the conditions for SHG.

Figure 8 is an SH image of unstained collagen in medaka fin scale. The fiber-like structure of collagen is seen in the image. Because collagen proteins have a significant role in determining the shape of biological tissues and are found in many parts of the body such as bone, skin and tendon, imaging of unstained collagen can be applied to diagnosis of biological tissues. Figure 9 shows a SH image of unstained human heart tissue. The bright parts in the images show myosin, seen as small stripes and collagen at the top left corner of the image with a different shape from the myosin structures. Myosin has a noncentrosymmetric structure and forms bundles in tissue, which exhibit high contrast in the image (Boulesteix et al., 2004; Campagnola et al., 2003; Dombeck et al., 2004).

Figure 10 is an SH image of living HeLa cells. Green fluorescent protein (GFP) fused with Connexin 43 is expressed by the cell to make the cell membrane visible (Oyamada et al., 2002).
SHG (in purple), and GFP fused with Connexin 43 in the cell membrane by photon fluorescence. Highly concentrated tubulins were imaged by SHG (in purple), and GFP fused with Connexin 43 in the cell membrane by photon fluorescence. Highly concentrated tubulins were imaged by

The purple color indicates the SH signal from the sample and the green indicates fluorescence from GFP. In this observation, the HeLa cell is in the metaphase of cell division, and SHG emission from microtubules is obtained (Campagnola et al., 2002). On the other hand, the cells that are not in mitosis do not show SHG emission even though microtubules exist in the cytosol. The strong image contrast is due to the number of microtubules and their strong orientation during mitosis.

CONCLUSIONS

In this article, label-free molecular imaging techniques realized by laser microscopy have been introduced. The recent development of fluorescence probes has visualized various biological phenomena in living samples and contributed to the advancement of optical microscopy technique. The label-free imaging techniques provide a possibility of biological imaging that can show the activities of biological cells and tissues without disturbance by the exogenous molecules. In conventional microscopy, the contrast of images was produced by using optical properties of a sample, such as refractive index, light absorption and scattering, and fluorescence. The recent development in laser technologies have made it possible to use other optical phenomena, such as multiphoton excitation and harmonic generation as mentioned above, for biological imaging, providing a new modality in optical microscopy.

The role of optical microscopy will become more important in the future. The recent developments in high-resolution microscopy have realized an imaging spatial resolution beyond the diffraction limit of the light by careful handling of the light emission from molecules by using stimulated-emission-depletion (Hell, 2007; Hell et al., 1994), photostable fluorescence molecules (Betzig et al., 2006; Rust et al., 2006), saturated excitation (Fujita et al., 2007; Gustafsson, 2005; Yamanaka et al., 2008). With these optical techniques, optical microscopy will reveal the biological functions of molecules more clearly in the near future.

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REFERENCES

Matthäus, C., Boydston-white, S., Miljkovic, M., Romeo, M., and