High-sensitivity hyperspectral vibrational imaging of heart tissues by mid-infrared photothermal microscopy

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Abstract
Visualizing the spatial distribution of chemical compositions in biological tissues is of great importance to study fundamental biological processes and origin of diseases. Raman microscopy, one of the label-free vibrational imaging techniques, has been employed for chemical characterization of tissues. However, the low sensitivity of Raman spectroscopy often requires a long acquisition time of Raman measurement or a high laser power, or both, which prevents one from investigating large-area tissues in a nondestructive manner. In this work, we demonstrated chemical imaging of heart tissues using mid-infrared photothermal (MIP) microscopy that simultaneously achieves the high sensitivity benefited from IR absorption of molecules and the high spatial resolution down to a few micrometers. We successfully visualized the distributions of different biomolecules, including proteins, phosphate-including proteins, and lipids/carbohydrates/amino acids. Further, we experimentally compared MIP microscopy with Raman microscopy to evaluate the sensitivity and photodamage to tissues. We proved that MIP microscopy is a highly sensitive technique for obtaining vibrational information of molecules in a broad fingerprint region, thereby it could be employed for biological and diagnostic applications, such as live-tissue imaging.

Keywords Mid-infrared photothermal microscopy · Tissue imaging · Infrared spectroscopy · Raman spectroscopy · Photodamage

Introduction
Biological cells and tissues consist of a wide variety of chemical constitutes, such as proteins, lipids, carbohydrates, and nucleic acids. Since the biological processes and diseases are generally associated with specific chemical changes or alterations of their compositions, visualizing the spatial distribution of biochemical contents in cells and tissues is of importance in fundamental biology and pathology.

Label-free vibrational imaging using Raman spectroscopy or infrared (IR) spectroscopy, is a promising approach to investigate the chemical contrast inside cells and tissues [1–4]. In particular, Raman microscopy is widely used for inspecting the origin of diseases owing to the sub-micrometer scale spatial resolution, which is high enough to resolve and visualize the distribution of primary biomarkers inside cells and biological organelles in tissues [5, 6]. However, Raman microscopy long suffers from intrinsically low cross sections of Raman scattering signal, thereby, it requires long acquisition time for Raman measurement, which makes it difficult to investigate multiple cells in a frame or large areas of tissues. The low sensitivity of Raman spectroscopy also forces to employ the high laser power to acquire enough Raman signal for entire fingerprint regions, which leads to photodamage of biological samples [7]. Although coherent Raman microscopy utilizing non-linear optical phenomenon achieves the high sensitivity to acquire Raman vibrational information, which enables fast Raman imaging of biological samples, it adopts ultra-short pulse lasers with high energy concerning photodamage of samples [8–10]. Another drawback in Raman spectroscopy is interferences

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of autofluorescence, where large fluorescence signal originating from samples overwhelms weak Raman scattering from the sample. Although the use of a near-infrared laser as an excitation source can solve this issue, the scattering efficiency in the longer wavelength region is extremely low, thereby high-resolution Raman imaging consumes time. It also requires sophisticated detectors in the near-infrared region. In contrast, Fourier transform infrared (FTIR) spectroscopy is highly sensitive vibrational spectroscopy based on infrared absorption of molecules possessing much higher cross sections than that of Raman scattering [11]. FTIR spectroscopy has been applied to chemical analysis of many kinds of biological tissues in a nondestructive manner [12–14], and has revealed biological functions and processes, such as dynamic change of secondary structures of proteins [15] and uptake and metabolism of fatty acids [16] owing to rich vibrational information in the entire fingerprint regions. However, the spatial resolution of conventional IR microscopy is limited to several micrometer owing to the diffraction-limited focus spot of IR light, which is not high enough to resolve chemical heterogeneities of biochemical components in tissues.

Mid-infrared photothermal (MIP) microscopy is a cutting-edge IR spectroscopic technique that is recently demonstrated by a few groups [17–20]. MIP microscopy adopts both, IR and visible laser beams, to detect the change in the refractive index of a sample on account of a local temperature change upon IR absorption of molecules. The photothermal effect on irradiation of pulsed-IR light can be probed by the phase-sensitive detection of the visible probe light. Hence, the spatial resolution of MIP microscopy is determined by the wavelength of the probe visible beam and the objective lens to focus the probe beam. Owing to the high spatial resolution together with high molecular sensitivity based on IR absorption, chemical analysis of biological samples, such as single-cells [21, 22], and bacteria [23, 24] has been achieved without the interferences of autofluorescence. Although a few examples of tissue imaging by means of MIP microscopy have been demonstrated so far [25], they only focus on mapping the distribution of proteins or discussion on orientation of collagen fibers by monitoring amide I and amide II bands originating from proteins. Imaging at other primary IR bands originating from different molecules, such as phosphate-containing molecules and lipids would provide more insightful information on fundamental biology and diseases associated with those molecules.

In this work, we visualized the chemical contrast of heart tissues with IR vibrational information in the broad fingerprint region by means of MIP microscopy. We obtained hyperspectral IR images at multiple IR bands derived from proteins, lipids/carbohydrates/amino acids, and phosphate-containing molecules to investigate variations of chemical compositions in heart tissues. In addition, we also evaluated the detection sensitivity and the non-destructivity of the MIP technique by direct comparison of Raman spectroscopy. We experimentally demonstrated that MIP microscopy enabled one to perform vibrational analysis of tissues with the higher sensitivity and lower photodamage than Raman spectroscopy.

**Experimental**

**Materials**

Alanine crystal was purchased from Tokyo Chemical Industry. The heart of a C57BL/6 mouse was excised after euthanasia, stored at −80 °C, and sliced into 5-μm-thick sections with a cryostat microtome (Tissue Tek; Sakura Finetek Japan). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted, and ethical approval was obtained from the Institutional Animal Care and Use Committee of Tokushima University, Japan (Approval number: T2020-39).

**MIP microscopy**

Figure 1 (a) shows a schematic illustration of an optical setup for MIP imaging. For the MIP measurements, a tunable quantum cascade laser (QCL, Daylight Solutions, MIRcat-QT-2100) with a broad wavenumber range of 1000–1690 cm⁻¹ was used as a pump light source. The repetition rate of the QCL was set at 80 kHz and a pulse width was set to 500 ns. The power of the pulsed IR beam was set to less than 1 mW for all measurements. A CaF₂ window (5% reflection) was used to guide the residual IR beam to a mercury cadmium telluride (MCT) detector (Thorlabs, DET10A2) and guided to a lock-in amplifier (NF Electronic Instruments, LI5660) for the phase-sensitive detection of the MIP signal.

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Results and discussion

Figure 2a shows an optical micrograph of a heart tissue sample. The blue spectrum in Fig. 2b shows a typical MIP spectrum of the heart tissue in the broad fingerprint region. The primary IR bands of biochemical components in tissues were appeared, for example, amide I and II bands from 1550 to 1660 cm\(^{-1}\) [26], the symmetric stretching mode of COO\(^-\) around 1380 cm\(^{-1}\) [27, 28], and the symmetric stretching mode of PO\(^4-\) at 1080 cm\(^{-1}\) [29] were observed. Figure 2c-f displays the hyperspectral MIP images of the heart tissues constructed by the MIP intensity at 1380 cm\(^{-1}\), 1552 cm\(^{-1}\), 1660 cm\(^{-1}\), and 1080 cm\(^{-1}\), respectively. The image area was indicated as the yellow square in Fig. 2a. Figure 2c shows the distribution of mainly lipids, carbohydrates, and amino acids [30]. One can see that they were distributed in the entire tissue sample. We would like to mention here that the distribution of lipids can be identified by observing the IR band at 1750 cm\(^{-1}\) assigned to the vibrational mode of lipids [22]. However, unfortunately it is out of the spectral range of our QCL. Figure 2d, e shows the distribution of proteins in the tissue. Although both amide I and amide II bands originate from proteins, there is the slight difference of the contrast between these two images. This is possibly due to the difference of the chemical compositions of proteins in the tissue [31]. The amide I band appeared at 1660 cm\(^{-1}\) dominantly originates from proteins that have α helices structures while the amide I band appeared at 1640 cm\(^{-1}\) shows the β sheets-rich proteins. The amide II band was less sensitive to the protein secondary structures than amide I band, thereby Fig. 2d, e visualizes the distribution of proteins with α helices structures and proteins with various secondary structures, respectively. It should be noted that advanced correlative analysis of amide I and amide II bands would make it possible to determine the secondary protein structures [32]. The direct comparison of Figs. 2c, d or (e) revealed the regions where proteins and other biomolecules, such as lipids and nucleic acids co-exist. The red and yellow spectra in Fig. 2b display the MIP spectra recorded at different locations marked in Fig. 2c. The MIP spectra clearly showed the varied spectral features, for example, the red spectrum shows the strong peak at 1380 cm\(^{-1}\), suggesting the existence of the lipid/carbohydrates/amino acid-rich region while the yellow spectrum shows the higher intensity of the amide I band compared to the typical MIP spectrum of the tissue that is the blue spectrum in Fig. 2b. Figure 2f shows the distribution of the phosphate-containing molecules, in which the localization of the strong MIP intensity was observed at some areas, indicating the accumulation of molecules that contain phosphate groups. In heart tissues, there are a variety of

The spectral fidelity of our MIP microscopy was examined by obtaining the MIP and FTIR spectra of biomolecules. The alanine was chosen as a biomolecule sample and its MIP and FTIR spectra are shown in Fig. 1b. Great consistency between the MIP spectrum and FTIR spectrum in the broad MIR fingerprint region was obtained. The slight variation of spectral properties between them was ascribed to the polarization dependence of IR absorption.

For Raman spectroscopic analysis, we used a commercially available Raman microscopy equipped with a CW laser with a wavelength of 532 nm (Nanophoton, Raman 11).
molecules that possess phosphate groups, for example phospholipid molecules, nucleic acids, and proteins. By comparing the other hyperspectral images, the localization of MIP signal of phosphate groups overlaps with the area, where the amide I and II bands appeared. Therefore, these areas can be characterized as phosphate-containing protein-rich regions. In biological systems, phosphate-containing proteins play key roles in enzymatic activity and formation of protein complexes, resulting in various diseases \[33, 34\]. The present results would be appreciated for diagnostic applications. The spatial resolution of MIP imaging in the present work was estimated to be as high as 1.1 \( \mu \)m, by measuring the line-profile of the MIP image as shown in Figure S1 in Supporting Information. Although this is far beyond the diffraction limit of IR light, we would like to mention here that the spatial resolution of our MIP imaging will be much improved down to the sub-micrometer scale by either using a high-NA (< 0.9) reflective objective or adopting counter-propagation configuration for pump and probe beams \[20\]. It should be noted that the depth resolution in our MIP system was estimated to be about 2.2 \( \mu \)m determined by the size of the pinhole.

Apart from visualization of the chemical contrast based on IR absorption of biomolecules, we also experimentally examined the sensitivity of MIP measurement and photodamage of the tissue sample with comparison of Raman spectroscopic measurement of the same tissue sample. The sensitivity of the two techniques was compared by calculating the signal-to-noise ratio (SNR) of MIP and Raman spectra. It should be noted that the NA of objective lens used for both MIP and Raman measurement is same (NA: 0.5). Figure 3a shows a MIP spectrum of the heart tissue with the acquisition time of 25 s and 2 mW of the visible probe laser power. Figure 3b shows Raman spectra of the same tissue sample with the acquisition time of 25 s and 2, 30, and 50 mW of the visible probe laser power. The SNR of MIP and Raman spectra was obtained by the ratio of the intensity of MIP and Raman peaks to the standard deviation of the noise signal that is extracted from the areas where no peaks appear. We chose the amide I band for the calculation of SNR of MIP spectra and the \( \text{CH}_2 \) peak around 1450 cm\(^{-1}\) for Raman spectra. The SNR of the MIP spectrum of the tissue was 152 and the SNR of Raman spectra (2, 30, and 50 mW) were 8, 76, and 181, respectively. There was no notable difference of the experimental conditions that could

![Image of a heart tissue sample with MIP spectra](image_url)

**Fig. 2** a Optical micrograph of a heart tissue sample. The yellow square in the image indicates the area of MIP imaging. b Comparison of MIP spectra recorded from different positions marked in c. c–f Hyperspectral MIP images of the tissue constructed by the MIP intensity of IR bands at 1380 cm\(^{-1}\), 1552 cm\(^{-1}\), 1660 cm\(^{-1}\), and 1080 cm\(^{-1}\), respectively.
affect the SNR of the two measurements since the numerical aperture of the objective lens used for the two measurement is same. The comparison of SNR of MIP and Raman spectra with the same experimental conditions concludes that the SNR of MIP spectrum is at least 19 times higher than SNR of Raman spectrum. It should be noted that the difference of SNR between MIP and Raman measurement discussed above does not reflect the difference of the detection sensitivity between the two techniques. In MIP measurement, since the wavelength of QCL was swept with the dwell time at each wavenumber, which was 200 ms, it took 25 s to acquire the whole MIP spectra. This means that IR light interacts with molecules at each wavenumber for only 200 ms. If one discusses about the SNR by taking the effective time of optical interaction between light and molecules into account, the effective SNR of MIP is calculated to be 19,000, which is 2300 times higher than SNR of spontaneous Raman scattering measurement.

We also evaluated photodamage in MIP and Raman measurement of the heart tissue. For quantitative evaluation of photodamage in two different vibrational spectroscopies, the power of the visible probe beam and the acquisition time was set to 2 mW and 25 s for MIP measurement and 40 mW and 25 s for Raman measurement to realize the similar SNR levels (~150). Figure 3c, d exhibits MIP and Raman spectra continuously recorded 4 times. In Fig. 3c, no significant degradation of MIP spectra was seen owing to the use of the low power of the probe beam. On the other hand, Raman spectra of the heart tissue were obviously degraded in time in Fig. 3(d) and the SNR was rapidly decreased. The Raman bands of the heart tissue eventually disappeared and only broad peaks around 1300 cm$^{-1}$ and 1600 cm$^{-1}$ were observed at the end of measurement. These broad bands are attributed to amorphous carbons that were typical products of photodamaged organic molecules [35]. The present result indicates that the MIP microscopy allows vibrational analysis of the heart tissue sample with high SNR, but low photodamage to the sample. Quick acquisition of MIP signal with high SNR and low photodamage is particularly important for practical biological applications that requires fast and continuous imaging, such as chemical imaging of live-cells and live-tissues. At last, we would like to emphasize that Raman spectroscopy is sometimes much useful for chemical characterization of samples, such as nano-carbon and semiconductor materials [36–38]. It is also possible to avoid severe photodamage of biomolecules in Raman spectroscopy when measured in solution since photo-induced heat can be easily dissipated in water. In fact, many biological samples, such as cultured cells, are required to be measured in solution to investigate their original functions and behaviors. Although the IR absorption of water and the dissipation of photothermally induced heat, resulting in the small change of the refractive index of the sample would reduce the SNR of MIP signal, MIP microscopy enables IR spectroscopic measurement of cells in solution, unlike conventional IR spectroscopic techniques [22] owing to its detection scheme.
Conclusions

We presented infrared vibrational imaging of heart tissues with the high spatial resolution far beyond the diffraction limit of IR light by means of MIP microscopy. We demonstrated that the chemical contrast of heart tissues based on IR absorption of proteins, phosphate-containing molecules, and lipids and other biomolecules was visualized by hyperspectral MIP images. By taking advantages of MIP microscopy, the large area of the heart tissue was chemically mapped with the high spatial resolution far beyond the diffraction limit of IR light. Since IR vibrational spectroscopy is capable of revealing not only the distribution of biochemical constituents in tissues, but also the variation of chemical contents of proteins and lipids, both of which are involved in many biological processes and diseases, our present MIP imaging would offer a wealth of information in fundamental biology and pathological insight. Furthermore, experimental comparison of MIP and Raman measurement of the same tissue was also presented. The effective SNR of MIP was at least 2000 times higher than Raman measurement. We also verified that MIP spectra of the tissue with high SNR can be obtained without significant photodamage owing to the high sensitivity whereas Raman measurement of the tissue with the same SNR yield serious photodamage to tissues. MIP microscopy would be appreciated for high-sensitivity and high-resolution chemical imaging of biological samples because it overcomes not only the issue of photodamage of samples but also the issue of autofluorescence, both of which Raman spectroscopy suffers from. Our present work paves the way for biological and diagnostic applications that should be quickly and repeatedly performed in a nondestructive way, such as bio-imaging of live-cells and live-tissues.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s44211-022-00182-8.

Acknowledgements This research was supported in part by JSPS KAKENHI grant no. JP21K20503 and JP22K14650 (R.K.), and JST ACT-X grant no. JPMAX21B4 (R.K.), JST FOREST grant no. JPMJFR2021 (T.Y.), JST CREST grant no. JPMJCR1904 (T.T.), and JST COI-NEXT grant no. JPMJP2011 (R.K. and T.Y.). We also acknowledge the financial support from the project on the Promotion of Regional Industries and Universities by the Cabinet Office, and the Plan for Industry Promotion and Young People's Job Creation by the Creation and Application of Next-Generation Photonics by Tokushima Prefecture.

Declarations

Conflict of interest There are no conflicts to declare.

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