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Whole-Organism Analysis by Vibrational Spectroscopy

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Abstract

Vibrational spectroscopy has contributed to the understanding of biological materials for many years. As the technology has advanced, the technique has been brought to bear on the analysis of whole organisms. Here, we discuss advanced and recently developed infrared and Raman spectroscopic instrumentation to whole-organism analysis. We highlight many of the recent contributions made in this relatively new area of spectroscopy, particularly addressing organisms associated with disease with emphasis on diagnosis and treatment. The application of vibrational spectroscopic techniques to entire organisms is still in its infancy, but new developments in imaging and chemometric processing will likely expand in the field in the near future.

Keywords

infrared spectroscopy, Raman spectroscopy, parasites, yeasts, insects
INTRODUCTION

Vibrational spectroscopy has long held out the promise of deepening our understanding of biological compounds and processes (1). The distinct spectral signatures of key biological molecules have assisted the interrogation of cells, tissues, microorganisms, and pathology (2).

Applying chemometricalgorithms to vibrational spectra dramatically improves the discriminative power when analyzing complex chemical mixtures, especially in the case of biological systems. Progress along this trajectory has allowed spectroscopic interrogation of whole organisms: single- or multicellular, prokaryotic or eukaryotic, independent or endosymbiotic. In this review, we highlight the application of vibrational spectroscopy to whole-organism analysis using state-of-the-art vibrational spectroscopic techniques and instruments. Given the importance of quality data collection and analysis to this field, we have also chosen to include discussions of advanced Raman and infrared techniques underpinning the applications to whole-organism analysis.

In any such review of a field as wide and varied as the vibrational spectroscopy of whole organisms, there is need to constrain the scope of study. It is therefore inevitable that some crucial topics will end up casualties of word count limits, for example, the analysis of prokaryotes, which would warrant several reviews on its own. Figure 1 summarizes the scope of this review hereafter.

PARASITES

Malaria is by far the most comprehensively studied parasitic disease using vibrational spectroscopic techniques. The tropical disease is caused by parasites of the genus *Plasmodium*. Forty percent of the world’s population is at risk of malaria, most of whom live in the world’s poorest countries. With approximately a half a million annual deaths, most of them children under 5 years old, malaria is one of the world’s most devastating diseases. Malaria can lead to serious complications,
including cerebral symptoms. *Plasmodium* undergoes a complicated life cycle, including stages in the *Anopheles* mosquito and in humans. After transmission from the mosquito to the human host during the mosquito’s blood meal, the parasites enter the blood stream via the liver prior to entering the erythrocytes. This stage is the most important for malaria diagnosis because of the ease of collecting a peripheral blood sample through venepuncture or by a finger prick.

Malaria diagnosis is often performed in field settings, which needs to be considered when new diagnostic tools are being developed. Currently, parasite-based diagnoses are performed in the field and involve microscopy and rapid diagnosis tests. Microscopy requires frequently trained personnel and can be prone to human error bias, whereas rapid diagnosis tests have a limited sensitivity. Molecular detection methods such as polymerase chain reaction (PCR) are routinely used only for research purposes and epidemiological studies, as the need for expensive and perishable consumables makes them infeasible for point-of-care field deployment.

Vibrational spectroscopy offers nondestructive, label-free options for rapid, easy, and objective diagnosis of infectious diseases. Having proven its high sensitivity, vibrational spectroscopy has the potential to become a front-line tool in malaria diagnosis.

**Application of Infrared Spectroscopy in Malaria Research**

**Infrared instrumentation.** In recent years, advances in laser technology have introduced quantum cascade lasers (QCLs) as alternative infrared light sources for spectroscopic instruments. More traditional Globar (electrically heated silicon carbide rods) sources emit a broad spectrum of infrared frequencies, with a Fourier transform used to convert the resulting interferogram into an absorption spectrum. Such thermal sources lack spatial coherence, which poses difficulties for high spatial resolution hyperspectral imaging (3). QCLs are tunable semiconductor lasers with high intensity and spatial and temporal coherence. These characteristics respectively provide a high acquisition speed (for a single-frequency image) and high spatial and spectral resolution. The actual advantage in acquisition time depends on how many discrete frequency images are required to address the user’s specific question. As Childs et al. (3, p. 824) note: “If the information content is highly localized in the spectral domain, large increases in throughput can be achieved.”

The introduction of QCL-based instruments and their improved acquisition times to organismal analysis opens the door to spectroscopic observation of biological processes in real time. However, the spatial coherence of the laser source brings its own problems, in particular, the exaggeration of fringing effects. In 2012, Kole et al. (4) countered these effects by placing a diffuser plate in the beam path between the laser and the sample. The diffuser in question was a calcium fluoride plate, heavily scratched to induce random phase shifts in the beam. This improved the image and spectral quality of their data, though it also produced additional scattering effects. Improved design in more recent instruments has lessened the fringing effects, though it still poses a clear challenge to the use of QCL sources in infrared microscopy (5, 6).

Another approach for overcoming the spatial coherence limitations of traditional Fourier transform infrared spectroscopy (FTIR) instruments is the use of synchrotron radiation as an infrared source. In a synchrotron, a beam of electrons accelerated to relativistic speeds emits bright, highly collimated light across a large swath of the electromagnetic spectrum, from which mid-infrared frequencies can be selected. Synchrotron FTIR has typically been performed with single-point raster-scan data collection, which illuminates the target with a high-flux, spatially coherent photon beam (7). This is particularly useful when applied to live, single-cell analysis where the high throughput of the synchrotron source enables enough photons to pass through the aqueous medium, the cell under investigation, and the calcium fluoride windows sealing the cell suspension in the case of transmission measurements. However, this approach greatly increases the time
needed for collection for two reasons: First, collecting spectra one point at a time is significantly slower than a benchtop focal plane array (FPA) (a 128 × 128 array collects 16,384 spectra simultaneously); second, the spatial resolution increases as the aperture decreases. This means that for high-resolution imaging some of the high photon flux is discarded, requiring a greater number of scans to produce the same signal-to-noise ratio.

To overcome these difficulties, several attempts have been made to incorporate FPA detectors with synchrotron light sources. For example, the Infrared Environmental Imaging (IRENI) beamline at the Synchrotron Radiation Center in Stoughton, Wisconsin have extracted a 3 × 4 bundle of collimated beams, which they defocus across a 128 × 128 FPA detector in an effort to achieve an even illumination (7). Similarly, the infrared microspectroscopy and THz/far-infrared beamlines at the Australian Synchrotron combined their light extraction to produce a 2 × 2 bundle of collimated beams (8). With this they were able to illuminate a 64 × 64 region of the detector, though illumination was only achieved when confined to the central 32 × 32-pixel region of the FPA (8). While this approach has had some success, the inaccessibility of synchrotron radiation for everyday research projects means it has a limited scope for application in the field.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy has a number of advantages over the more high-end imaging and microscopic systems in that it is relatively inexpensive, portable, and very easy to use. The depth of penetration of the evanescent wave is dependent on the refractive index of the window and the sample. Providing the refractive index of the sample is consistent, the spectra do not require normalization, and it is not possible to saturate the detector. Indeed, the application of this technology to whole-cell and biofluid analysis is currently experiencing a renaissance and is no longer the technique being only applied to identify functional groups in liquids and solids (9–12).

Atomic force microscopy-infrared spectroscopy (AFM-IR) is an emerging technique for acquiring spectroscopic and topological information from a sample. The infrared absorption is typically measured not through FTIR, but by using a photothermal technique: A pulsed infrared laser is used to excite the sample directly beneath an AFM tip, and the thermal expansion and relaxation thus caused are detected by a second laser measuring the ringdown in the cantilever (Figure 2). The spatial resolution of this technique is much greater than traditional microspectroscopy—less than 100 nm (13)—as it is not constrained by diffraction limits.

**Applications of infrared spectroscopy to malaria diagnosis.** Slater et al. (15) studied the *Plasmodium* sp. metabolite hemozoin and its synthetic analogue β-hematin and assigned bands at 1,663 cm⁻¹ and 1,209 cm⁻¹ to the important carboxylate and C–O stretching vibrations from the propionate group, respectively. Based on these findings, Webster et al. (16) applied FTIR microscopy with the intense light afforded by a synchrotron to investigate *Plasmodium*-infected human red blood cells. The authors could discriminate between infected and uninfected cells, as well as between different parasitic life stages. Whereas parasites in the trophozoite stage stood out because of the hemozoin bands at 1,712, 1,664, and 1,209 cm⁻¹, other life stages differed mainly in the CH stretching region (3,100–2,800 cm⁻¹), indicating that they have a distinct lipid composition for each of the life cycle stages. Khoshmanesh et al. (12) further developed this work by using a commercial bench top ATR-FTIR instrument for the detection of *Plasmodium falciparum* in red blood cells down to 0.0001% parasitemia and for discrimination of the parasitic life stages. Because the lipid composition has proven to be significant in the detection of *Plasmodium*, Perez-Guaita et al. (17) used FPA-FTIR microscopy to image infected erythrocytes in transmission on conventional glass microscope slides. The authors were able to obtain high-quality hyperspectral images and resolve the digestive vacuole inside the parasite despite the infrared opacity of glass obscuring all spectral information below 2,000 cm⁻¹.
Figure 2
Schematic diagram of atomic force microscopy-infrared spectroscopy (AFM-IR). An IR-pulsed tunable laser source is focused on a sample near the AFM tip. The laser is tuned on a wavenumber corresponding to an absorbing band of the sample, thus resulting in a photothermal expansion of the absorbing regions of the sample. This expansion induces a transient cantilever oscillation proportional to the IR absorption. By measuring the AFM cantilever oscillation amplitude as a function of wavenumber, local absorption spectra can be obtained with sub-100-nm spatial resolution. Figure and caption adapted with permission from Reference 14. Copyright 2018, John Wiley & Sons.

These findings have provided a foundation for the development of an infrared-based point-of-care diagnostic tool for malaria. To this end, Martin et al. (18) investigated the confounding effects of various anticoagulants commonly found in blood collection tubes on the detection of Plasmodium infection and determined that lithium heparin–treated tubes were the optimal choice for future clinical trials. Roy et al. (10) developed an ATR-FTIR point-of-care test for simultaneous identification of Plasmodium, blood glucose, and urea in whole blood samples. Martin et al. (19) also demonstrated that FTIR spectra could be used to detect malaria in aqueous samples of red blood cells spiked with malaria infected. The method of sample preparation, spectral acquisition, and modeling was presented in a video format for the Journal of Visualization Experiments (19).

Resonance Raman Spectroscopy in Malaria Research
Raman microspectroscopy has several advantages over infrared techniques when analyzing organisms. Because the excitation wavelength for Raman is much shorter, diffraction limits are reduced and submicron spatial resolution can be achieved. The vibrational modes of water that can overwhelm biological infrared spectra are also Raman inactive, meaning that live cells can be analyzed nondestructively in aqueous solution with minimal sample preparation required.

However, inelastic scattering is a much less efficient process than absorption, so Raman spectra are generally noisier than infrared spectra. A number of techniques have been developed to improve the signal-to-noise ratio of Raman microspectroscopy.

Surface-enhanced Raman spectroscopy (SERS) is a method to enhance the Raman signal of an analyte by applying the sample onto a specific (often metallic) surface. The chemical and electromagnetic interactions between analyte and surface can provide a signal enhancement of greater than $10^{10}$ (20). Coherent anti-Stokes Raman spectroscopy (CARS), stimulated Raman spectroscopy (SRS), and second-harmonic generation (SHG)/third-harmonic generation (THG)
Spectroscopy are other Raman-based techniques utilizing nonlinear optical effects to enhance the Raman signal (21–23).

Tip-enhanced Raman spectroscopy (TERS) is the combination of AFM with Raman spectroscopy (24). The electromagnetic field at the end of the AFM tip is enhanced, increasing Raman scattering within a highly localized region. If this effect is large enough, the spatial resolution is also effectively increased, as the enhanced signal from near the tip swamps the signal from sample molecules further from the enhanced field.

It is important to remember when using SERS or TERS that the activation of a vibrational mode for Raman scattering is derived from symmetry-based selection rules. The interaction between analyte and surface or tip that produces the SERS/TERS signal enhancement may affect the conformational state of the analyte and thus activate or deactivate certain modes (25). Thus, SERS/TERS spectra should not be simplistically regarded as amplified versions of spontaneous Raman spectra, although they will usually be very similar.

At the 26th International Conference on Raman Spectroscopy (ICORS) in August 2018, Curtis Marcott on behalf of Photothermal Spectroscopy Corp. (Santa Barbara, California) presented preliminary data from a new instrument able to simultaneously record Raman and photothermal infrared spectra. This will no doubt make an impact on the field, as efforts to acquire the complementary information from infrared/Raman of the same sample region currently require complex hyperspectral image registration processes (26, 27).

**Raman Spectroscopy as a Diagnostic for Malaria**

Raman spectroscopy is frequently used in malaria research, as the resonance effect of the parasite’s metabolite hemozoin leads to strong, characteristic Raman spectra. To the best of our knowledge, the first resonance Raman-based investigation of isolated hemozoin was performed by Brémard et al. (28) in 1992. The authors found high-spin monomeric iron (III) protoporphyrin hydroxide in hemozoin.

Based on these findings, Ong et al. (29) studied *Plasmodium berghei*-infected mouse erythrocytes with resonance Raman microspectroscopy (excitation wavelength: 488 nm). The infected red blood cells showed a C-N-C deformation vibration at 754 cm\(^{-1}\) from hemozoin as opposed to the C-N-C deformation mode at 747 cm\(^{-1}\) from hemoglobin in healthy red blood cells. Using an excitation wavelength of 632.8 nm into the charge transfer transition from high-spin ferric hemes, Raman bands at 724, 795, and 1,090 cm\(^{-1}\) were resolved in infected erythrocytes (30). These studies show how different Raman spectra of *Plasmodium*-infected erythrocytes are obtained using lasers with different excitation wavelengths. Wood et al. (31) recorded micro-Raman spectra of hemozoin in its natural environment, i.e., the food vacuole of *P. falciparum*, with different excitation wavelengths (488, 514, 564, 633, and 780 nm). Figure 3 shows Raman spectra of hemozoin, hematin, and β-hematin as well as a Raman image of the 1,376-cm\(^{-1}\) band.

Strong signal enhancement was observed in hemozoin when using the 780-nm excitation laser due to exciton coupling between linked porphyrin subunits. This allowed for imaging of the pigment within the background intensities of the food vacuole paving the way for Raman-based drug interaction studies in cells.

Using a point-scanning technique, Frosch et al. (32) localized hemozoin in *P. falciparum*-infected red blood cells and compared the spectra to Raman spectra of isolated hemozoin, β-hematin, hematin, and hemin. Whereas hemozoin and β-hematin show similar physical and optical properties, spectra differ significantly from hematin and hemin, mainly due to an additional band at 1,655 cm\(^{-1}\) in hemozoin and β-hematin. This study contributes to the understanding of hemozoin formation, as well as a study performed by Bonifacio et al. (33), where the
distribution of hemoglobin and hemozoin is examined by resonance Raman imaging with an excitation wavelength of 514.5 nm analyzed with cluster analysis. Combining resonance Raman microscopy with partial dark-field microscopy allowed Wood et al. (34) to detect smaller hemozoin inclusions in earlier, less hemozoin-rich parasitic life stages using thin and thick films. The simplicity and speed of this approach compared to the standard diagnostic tools discussed above paved the way for a Raman-based malaria diagnosis, although the time taken to image a large field of cells is prohibitively long with current technology.

**Nonresonance Raman Spectroscopy in Malaria Research**

Most Raman-based approaches for malaria diagnosis are based on the resonance effect of hemozoin. Hemozoin, however, is more abundant in later stages of the parasite’s life cycle. Earlier stages such as the ring stage only have traces of hemozoin. Yet, the ring stage is the most common stage in the peripheral blood, which is usually used for malaria diagnosis because it can be obtained from a pinprick. Therefore, Hobro et al. (35) additionally examined malaria in plasma samples, as Raman studies in red blood cells are usually dominated by the resonance effect of hemoglobin and hemozoin. Plasma samples have a lower heme background and, combined with PCA, the authors were able to identify infections as early as one day after *Plasmodium* infection in mice. This is particularly useful for Raman-based point-of-care diagnosis where malaria infection needs
to be detected at an early stage with low parasitemia levels. Kozicki et al. (36) analyzed ring stage 
P. falciparum infections in hospitalized patients based on changes in the biochemical composition of 
whole blood samples using a 785-nm incident laser beam. For example, due to modifications of the 
erythrocyte membrane, the 1,130:1,075 cm$^{-1}$ ratio is reduced, whereas the 2,930:2,850 cm$^{-1}$ ratio 
is increased due to alterations in the structure of membrane proteins and lipids. The authors used 
confocal Raman microspectroscopy for the investigation of early signs of P. falciparum–induced 
hemoglobin degradation such as a deoxygenated state of the infected cell, which might serve as 
a first step in unraveling the mechanism of hemozoin formation and changes in the secondary 
structure of proteins. This publication shows how spectroscopic methods allow for generation of 
a biochemical fingerprint by simultaneously detecting several biomarkers in a sample instead of 
focusing on single molecules as in immunohistochemistry, for example.

**Raman Spectroscopy in Malaria-Infected Tissue**

The spleen cleans out the malaria parasites. Therefore, *Plasmodium* is found in spleen tissue along 
with their metabolite hemozoin. In a recent study (37), spleen tissue of *Plasmodium*-infected mice 
was examined with Raman spectroscopy. The overall biochemical composition of infected and 
uninfected tissue was found to be quite similar. By applying PCA, infected tissue could be dis- 
criminated from healthy tissue based on Raman signals from hemozoin. Although this study was 
performed in isolated tissue sections, it may serve as a step toward noninvasive examination of the 
tissue burden of a malaria infection, which is an indicator of the severity of the disease. However, 
this methodology is not transferrable into the real world because the penetration depth of the 
laser (532 nm) is not deep enough to penetrate skin and analyze blood capillaries. Near-infrared 
excitation wavelengths could provide the depth to scan capillaries but no studies have applied this 
approach to diagnose malaria to date.

**Raman Spectroscopy to Study Parasite–Drug Interactions**

The aforementioned publications deal with the detection of the malaria parasite or its products 
in different body sections such as red blood cells, plasma, and spleen tissue. On the one hand, 
this serves to establish Raman spectroscopy as a diagnostic tool and, on the other hand, to gain 
insight into the parasite’s biology and the host’s response, which is particularly advantageous if the 
interaction of drugs with parasite and/or host cell should be studied.

Webster et al. (38) and Kozicki et al. (9) studied the effect of chloroquine in cultured 
P. falciparum–infected red blood cells with Raman spectroscopy using 782-nm and 532-nm exi- 
tation wavelengths, respectively. The studies showed the effect of chloroquine on the molecular 
environment of hemozoin and on the oxygenation status of hemoglobin in infected and uninfected 
red blood cells. Although the chloroquine was not directly detected in these studies, there was evi- 
dence of a reduction of hemozoin bands, indicating some interaction occurring with the hemozoin 
molecules. Analyzing the effect of chloroquine on the biochemical composition of isolated live or 
dry P. falciparum–infected red blood cells sets the stage for in situ investigation of drug–pathogen 
interactions.

**Special Instrumentation**

Raman spectroscopy was combined with acoustic levitation, which allows for investigation of a 
population of cells under changing environmental conditions (39). Wide-field Raman imaging 
facilitated hemozoin detection with a high spatial resolution in early developmental stages (40).
In a multimodal approach, Perez-Guaita et al. (41) combined Raman and infrared spectroscopy for complementary analysis, and Kang et al. (42) combined confocal Raman and quantitative phase microscopy, which allowed for collection of chemical and morphological information without staining.

For studies on malaria parasites, both hemozoin (43, 44) and changes in the red blood cell membranes (43) have been targeted with SERS. The signal enhancement allows for hemozoin detection even in the ring stage, a life stage that has a very low hemozoin content (44).

TERS was applied by Wood et al. (45) to investigate hemozoin crystals in the digestive vacuole of a sectioned malaria parasite-infected cell. AFM-IR was applied by Perez-Guaita et al. (46) to simultaneously obtain morphological information and chemical information with a nanoscale spatial resolution, as shown in Figure 4. The authors show how this extraordinary setup allows for collection of infrared spectra of biological samples over the usual spatial resolution, which is limited by the wavelength diffraction limit. This has potential applications in the study of drug-pathogen interactions and the biochemical and morphological characteristics of drug-resistant *P. falciparum* strains.

![Figure 4](https://www.annualreviews.org/content/12/05/89.f04)

**Figure 4**
Characterization of a red blood cell infected with several rings using different atomic force microscopy-infrared spectroscopy (AFM-IR) discrete wavenumber value maps. (a) Visible image stained with Giemsa. (b) AFM topography image. (c) Cluster image, where each pixel is colored according to the class assigned in the k-means cluster analysis. (d) Composite image of the AFM deflection and cluster analysis. (e) False color image representing the principal component (PC) 1 score value for each pixel. (f) Normalized average intensity values for each class at selected wavenumbers (Nblue = 4,967, Norange = 3,507, NYellow = 3,208, NViolet = 1,976, Ngreen = 3,245, Nlight blue = 5,131). Average variables of each class were computed after normalizing the signal of each pixel (sum = 1). For each variable, the average value in each class was divided by the average value of all the intensity values. (g) Loading values of the first PC at selected wavenumber values. Adapted with permission from Reference 46. Copyright 2018, American Chemical Society.
Other Parasites

Although Plasmodium is the by far most frequently studied parasite using Raman spectroscopy, several other parasites have been studied with this technique, including Schistosoma mansoni (47), Trypanosoma cruzi (48), and Toxoplasma gondii (49) based on parasite-induced changes in the lipid composition of a sample. Naemat et al. (50) also studied the incorporation of isotopic amino acids into T. gondii.

Cryptosporidium parvum oocysts have been studied with Raman imaging, SERS, CARS, and surface-enhanced resonance Raman (SERRS) (51–54). In the same study, the authors used SERRS for Giardia lamblia analysis (54). These pathogens constitute dangerous contaminations in fresh water. Therefore, these studies pioneer the potential of Raman-based techniques for the biological monitoring of water.

Another interesting system that has been studied as a whole organism is the nematode Steinernema kraussei, which often serves as a model organism for multicellular organisms in biomedical research. Lau et al. (55) investigated nematodes with FTIR and Raman imaging, which allowed for discrimination of different regions based on the distribution of biological molecules such as lipids, proteins, and collagen. The authors showed that the tail is a collagen-rich domain, whereas the head is higher in proteins and nucleic acids. Hobro & Lendl (56) also showed in a proof-of-concept study the potential of an FPA-FTIR microscope for the analysis of thicker multicellular systems using the nematode as an example.

YEASTS

Yeasts are the only single-cell eukaryotic microorganisms found in the kingdom Fungi and are classified into two separate phyla. As single-cell organisms, yeasts are unique because they possess a cellular organization similar to those of higher organisms but unlike any other single-cell organisms (57).

Vibrational spectroscopy is widely used in yeast research, utilizing approaches based on both single-spectra and submicron hyperspectral imaging. Aside from standard techniques, such as ATR-FTIR (58–60), FTIR (61–65), including synchrotron-FTIR (66), and spontaneous Raman (59, 67–77), examples of the applications of advanced research techniques such as SERS (78–81), CARS (23, 82–87), SRS (88, 89), SHG/THG (23), and AFM-IR (13, 71) to yeast can be found in the literature.

Yeast research can be generally divided into three areas, depending on the aspect on which the studies are focused: (a) food-related research (e.g., ethanol production), (b) studies of clinical/pathogenicyeast, and (c) research focused on metabolic engineering (e.g., production of biodiesel, insulin, etc.).

Food-related research focuses mainly on the application of vibrational spectroscopy for monitoring the fermentation process. Recently, a Raman-based approach utilizing a noncontact probe was shown for analyte-specific, in-line monitoring of glucose and ethanol (71). Canal & Ozen (60) demonstrated the application of ATR-FTIR spectroscopy combined with multivariate data analysis to simultaneously monitor a broad range of compounds. Schalk et al. (58) showed a similar approach utilizing ATR-FTIR and multiple linear regression, but it was adapted to in-line fermentation monitoring based on a novel mid-infrared-ATR sensor. In addition to in-line monitoring, vibrational spectroscopy is proving useful for rapid food safety surveillance. Detection and identification of spoilage yeast in beverages was demonstrated using SERS (78). A near-infrared approach was also utilized for the example of kiwifruit juices, with particular focus on osmotolerant yeast, which are frequent contaminants of high-sugar foods (90). In fact, examples of the safety surveillance application of vibrational spectroscopy can be found not only in the food industry but
in environmental studies, e.g., to monitor water contamination (68). Furthermore, FTIR in particular is commonly used for detection and identification of yeast strains present in specific food products (e.g., grapes) (63, 64). The aforementioned work demonstrated the feasibility of vibrational spectroscopy for practical applications in chemical, pharmaceutical, and biotechnological industries owing to the fact that it is nondestructive and requires no sample preparation.

The second major area of yeast research concentrates on pathogenic strains in reference to both potential diagnosis and pharmaceutical development. Raman spectroscopy for classification and identification of various clinically relevant yeast species (e.g., Candida) has been commonly utilized for more than a decade (69). Recently, an ultraviolet resonance Raman (UV-RR) approach demonstrated the advantage of significant signal enhancement together with a lack of interferences from autofluorophores (67). A recently proposed high-throughput technique is based on a combination of next-generation sequencing of the internal transcribed spacer (ITS) and a D1/D2 LSU marker with FTIR spectroscopy and was shown to correctly identify 97.4% of Candida albicans strains used in the study (73). Vibrational spectroscopy techniques are also broadly used to characterize (74), study the effects (91), and map the distribution (88) of various potential antifungals. Furthermore, as biofilm formation is one of the most important factors to ensure pathogen survival, a novel Brillouin-Raman microscopy technique allowing simultaneous mechanical and chemical mapping was recently demonstrated for C. albicans and C. parapsilosis biofilms (75). The high-resolution spectral imaging represents particular advantages for studies focused on yeast cell walls (constituting the main target for antifungals) (71, 79) because it facilitates directly visualizing structurally similar cell wall components, such as α/β glucans (71). Recently, Lin et al. (89) also demonstrated a sparse spectroscopic stimulated Raman spectroscopy approach permitting rapid imaging and visualization of the intracellular composition in living C. albicans.

Finally, yeasts have a broad range of uses as probiotics and nutritional supplements. In addition, they are commonly used as genetically engineered biofactories for production of various pharmaceuticals (e.g., insulin, vaccines for hepatitis) and are considered to be the favorable source for production of triglycerides used in biodiesel production. Vibrational spectroscopic techniques are broadly applied to follow the lipid production in yeast (62, 71, 76, 92). Both infrared and Raman spectroscopy are particularly suited for lipid studies due to the high intensity of their signals, resulting in significant sensitivity of those techniques toward lipids. Infrared spectroscopy can be used for quick assessment of total lipid yield without the sophisticated sample preparation required for gas chromatography (62, 71). Confocal Raman imaging (CRI), as well as visualizing lipid bodies, enables the simultaneous detection of other substances such as carotenoids (76) and polyphosphate (71). An example of this is presented in Figure 5, where CRI of a single Saccharomyces cerevisiae cell combined with k-means cluster analysis and integration of areas under marker bands enabled the localization of several intracellular compounds (and structures). Recently, Kochan et al. (93) demonstrated the use of confocal Raman spectroscopy imaging to detect the presence and spatial localization of specific lipids, cyclopropane fatty acids, in genetically engineered yeast strains. The spatial localization of these lipids is impossible to obtain in a nondestructive manner and without complicated preparation using other techniques and at the same time has critical influence on the viability of engineered yeast strains. Visualization of lipids, and particularly lipid bodies, can be also achieved using CARS imaging, enabling an extensive acceleration of the imaging process (to a video rate) (82–86). In fact, Brackmann et al. (82) demonstrated the ability to use CARS for quantitative evaluation of production of lipid bodies. Furthermore, Segawa et al. (23) demonstrated the ability to combine CARS with other molecular imaging techniques such as SHG and THG spectroscopy to study living budding yeast cells (S. cerevisiae and Saccharomyces bayanus). Recently, AFM-IR was applied to study genetic engineering strategies in oleaginous yeast (13, 71), not only with respect to lipid bodies (13, 71) but also to demonstrate
Figure 5

Comprehensive analysis of individual cells via confocal Raman spectroscopy, demonstrated on the example of a Saccharomyces cerevisiae yeast cell. (a) Confocal imaging enables depth profiling that shows the distribution of selected components. The depth profiling was done with a step size of 300 nm. The distribution of organic matter is demonstrated on the basis of integration of bands in the range of 3,050–2,800 cm$^{-1}$ and visualizes the cell. The integration of the band at 1,444 cm$^{-1}$ enables us to visualize lipid bodies (LBs). As illustrated, the lipid body signals are present only within selected layers, corresponding to the inside of cell. (b–f) Distribution of selected components in a cell with a polyphosphate vacuole, on the basis of integration of bands at (b) 3,050–2,800 cm$^{-1}$ (organic matter), (c) 1,444 cm$^{-1}$ (lipids), (d) 2,925 cm$^{-1}$ (proteins), (e) 753 cm$^{-1}$ (heme), and (f) 1,160 cm$^{-1}$ (polyphosphate). (g, h) Cluster analysis results obtained for the cell presented in panels b–f: (g) distribution of classes and (h) corresponding spectra of each class with marked selected bands. Size of the imaged area: (a) 5.32 × 5.24 μm and (b) 5.22 × 5.17 μm. Adapted from Reference 94 under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).
impoverishment of the cytoplasmic carbohydrate pool (71). Vibrational spectroscopy techniques are also used to study yeast cells with respect to production of substances other than lipids (70, 65, 77). Such studies include investigation of factors influencing the production (65, 77) as well as monitoring and controlling the pharmaceutical production process (70). For example, Voss et al. (70) demonstrated the feasibility of Raman spectroscopy to monitor the pharmaceutical production process of a new malaria vaccine candidate in *Pichia pastoris*.

Yeasts are some of the most commonly investigated eukaryotic systems, serving as a model for all eukaryotes. As such, multiple other recent examples of the application of vibrational spectroscopy for yeast studies can be found in the literature. These include, for example, monitoring the apoptosis induced by overexpression of p53 via FTIR (61), following the cellular response to the dehydration process using synchrotron FTIR (66), investigating intracellular structural changes, following mechanisms of cell death in plasma-irradiated budding yeast using CARS (87), and making local measurements of pH at the desired position by surface-enhanced hyper-Raman and optical trapping (81). Such extraordinarily extensive use of vibrational spectroscopy techniques in itself proves their usefulness. They enable new insights into a broad range of biological processes enabling the investigation of living systems and providing comprehensive, spatially localized chemical information with resolution unattainable by other techniques without damaging the sample.

**INSECTS**

The study of insects by vibrational spectroscopy is interesting on two scales: Many are small enough that a tissue section of all or most of the insect can be hyperspectrally imaged but large enough that pathogens can be detected within them.

The *Aedes aegypti* mosquito is largely responsible for the spread of dengue fever, a viral disease that infects an estimated 390 million people globally per annum (95). Khoshmanesh et al. (11) used ATR-FTIR spectroscopy to investigate these vectors. Whole mosquito abdomina were fixed in ethanol, dried, and loaded directly onto the ATR crystal. Using automated feature selection and preprocessing optimization, they were able to classify the sex and age (two days or ten days old) of mosquitoes. Females were easily distinguished from males, primarily by high absorbance in the region of 1,150–1,000 cm\(^{-1}\), corresponding to C–O stretches in sugars and suggesting a higher carbohydrate concentration. Young (two-day-old) mosquitoes were spectrally similar to old (ten-day-old) mosquitoes but could be identified by a slightly higher protein (1,700–1,500 cm\(^{-1}\)) concentration. In addition, *Wolbachia* [an endosymbiont used to suppress the transmission of dengue, Zika, and chikungunya viruses (96, 97)] was detected within the mosquito abdomina using a support vector machine model. As Figure 6 shows, an independent test set of 35 female mosquitoes could be tested for *Wolbachia* with 95% accuracy. The regression vector highlights the importance of changing lipid concentration in this classification, with the C–H stretching region (2,800–3,000 cm\(^{-1}\)) and ester carbonyl band (1,743 cm\(^{-1}\)) playing a significant role in the model.

*Drosophila melanogaster* has also been spectroscopically investigated. Gautam et al. (98) were able to classify the pathophysiology of mutant flies from Raman spectra of wing muscles. The effect of diet on cuticular composition of the *Ectatomma brunneum* ant was observed by Bernadi et al. (99) using infrared photoacoustic spectroscopy, a technique that detects the pressure wave coming from the absorption-based thermal expansion of a surface (100).

**FUTURE DIRECTIONS**

There is no doubt that QCLs will continue to have a big impact on the spectroscopy of small organisms, particularly when the target molecule for analysis features a prominent or unique
Figure 6

(a) Average attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of ~200 spectra from infected (blue) and uninfected (red) methanol-dried female *Aedes aegypti* lab mosquito abdomina. (b) Partial least squares discriminant analysis (PLS-DA) calibration model built from second derivative spectra using the combined spectral ranges of 3,100–2,800 cm⁻¹ and 1,800–700 cm⁻¹ of infected (blue) and uninfected samples (red) from ~200 female mosquito abdomina. (c) PLS-DA independent test set projected onto the calibration model. (d) ROC plot showing the high performance of the prediction model. (e) Regression vector for PLS-DA model where red is *Wolbachia* infected and blue is uninfected. Figure and caption adapted with permission from Reference 11. Copyright 2017, American Chemical Society.
absorption band, such as hemozoin or collagen. The ability to make discrete frequency images with vastly improved throughput will allow ever larger organisms to be investigated spectroscopically, which may in turn open the door for holistic systems-based analyses (as opposed to localized analyses on, for example, parasites in individual cells). The vastly superior photon flux of QCL instruments will also likely promote live-cell analysis of microorganisms, as the feasible path length through aqueous media is increased. However, due to the problems discussed above—in particular, the convoluted and information-rich structure of biological absorption bands—it is far from certain that QCL instruments will replace more traditional FTIR-based systems in this field.

The ability to simultaneously acquire the complementary information of infrared and Raman spectra will also undoubtedly improve the discriminative power of chemometric modeling. As the feasible size of hyperspectral image data increases in both spatial and spectral dimensions, there will be a need to develop more computationally efficient workflows of data collection and analysis. Although calculations that were unthinkable just a few years ago are now performed routinely, researchers in this field may yet find themselves butting up against the processor limits of personal computers, necessitating a move toward more high-performance computing.

Finally, it must be noted that there exist many applications of vibrational spectroscopy to organisms that do not fall neatly into the scope of this review, yet they are worthy of mention for specialists in the field. For example, Moore et al. (101) discuss detection of viruses in their wider review of SERS applications in disease diagnosis, whereas Lasch et al. (102) used FTIR imaging and machine learning to identify different pathogenic bacteria. Finally, there are several publications that bring specific focus to the spectroscopy of carbohydrates (infrared/Raman) (103), lipids (Raman) (104), proteins (Raman) (105), and nucleic acids (SERS) (106).

DISCLOSURE STATEMENT

D.P.G. is the inventor on two patents (AU2016903287A0, WO2018033894A1) related to issues discussed in this review and is a minor shareholder of BTR Resources, a company related to the issues discussed here. B.R.W. is the lead inventor on the US patent WO201511717A1 (Method and System for Rapid Malaria Detection), which is briefly reviewed here.

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Contents

Wearable Sensors for Biochemical Sweat Analysis
Amay J. Bandodkar, William J. Jeang, Roozbeh Ghaffari, and John A. Rogers ............. 1

E-Cigarette Chemistry and Analytical Detection
Robert M. Strongin ................................................................. 23

Emerging Analytical Techniques for Rapid Pathogen Identification and Susceptibility Testing
Dong Jin Shin, Nadya Andini, Kuangwen Hsieh, Samuel Yang, and Tza-Huei Wang ...................................................... 41

Polyvalent Nanoobjects for Precision Diagnostics
David T. Omstead, Jenna Sjoerdma, and Basar Bilgicer ........................................ 69

Whole-Organism Analysis by Vibrational Spectroscopy
Dale Christensen, Anja Rüther, Kamila Kochan, David Pérez-Guaita, and Bayden Wood .......................................................... 89

Recent Developments in Nanosensors for Imaging Applications in Biological Systems
Guoxin Rong, Erin E. Tuttle, Ashlyn Neal Reilly, and Heather A. Clark ............. 109

Development and Applications of Bioluminescent and Chemiluminescent Reporters and Biosensors
Hsien-Wei Yeh and Hui-Wang Ai .................................................. 129

Advances in Surface Plasmon Resonance Imaging and Microscopy and Their Biological Applications
Markéta Bocková, Jiří Slabý, Tomáš Špringer, and Jiří Homola .......................... 151

Challenges in Identifying the Dark Molecules of Life
Maria Eugenia Monge, James N. Dodds, Erin S. Baker, Arthur S. Edison, and Facundo M. Fernández ................................................ 177

Metabolic Imaging at the Single-Cell Scale: Recent Advances in Mass Spectrometry Imaging
Ian S. Gilmore, Sven Heiles, and Cornelius L. Pieterse ........................................ 201

Laser Desorption Combined with Laser Postionization for Mass Spectrometry
Luke Hanley, Raveendra Wickramasinghe, and Yeni P. Yang .......................... 225
Molecular Characterization of Atmospheric Organic Aerosol by Mass Spectrometry
Murray V. Johnston and Devan E. Kerecman

Electrochemiluminescence Imaging for Bioanalysis
Jingjing Zhang, Stéphane Arbault, Neso Sojic, and Dechen Jiang

Electrochemistry at the Synapse
Mimi Shin, Ying Wang, Jason R. Borgus, and B. Jill Venton

Advanced Spectroelectrochemical Techniques to Study Electrode Interfaces Within Lithium-Ion and Lithium-Oxygen Batteries
Alexander J. Cowan and Laurence J. Hardwick

Single Nanoparticle Electrochemistry
Fato Tano Patrice, Kaipei Qiu, Yi-Lan Ying, and Yi-Tao Long

Single-Molecule Analysis with Solid-State Nanopores
Tim Albrecht

Flow Cytometric Analysis of Nanoscale Biological Particles and Organelles
Hong Lian, Shengbin He, Chaoxi Jiang, and Xiaomei Yan

High-Parameter Single-Cell Analysis
Pratip K. Chattopadhyay, Aidan F. Winters, Woodrow E. Lomas III, Andressa S. Laino, and David M. Woods

Single-Cell Protein Secretion Detection and Profiling
Zhuo Chen, Jonathan J. Chen, and Rong Fan

Well-Defined Materials for High-Performance Chromatographic Separation
Yu Liang, Lihua Zhang, and Yukui Zhang

Separation Phenomena in Tailored Micro- and Nanofluidic Environments
Mukul Sonker, Daihyun Kim, Ana Egatz-Gomez, and Alexandra Ros

Solving the Structure and Dynamics of Metal Nanoparticles by Combining X-Ray Absorption Fine Structure Spectroscopy and Atomistic Structure Simulations
J. Timoshenko, Z. Duan, G. Henkelman, R.M. Crooks, and A.I. Frenkel

Imaging and Analytics on the Helium Ion Microscope
Tom Wirtz, Olivier De Castro, Jean-Nicolas Audinot, and Patrick Philipp

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