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From structure to cellular mechanism with infrared microspectroscopy

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Abstract

Current efforts in structural biology aim to integrate structural information within the context of cellular organization and function. X-rays and infrared radiation stand at opposite ends of the electromagnetic spectrum and act as complementary probes for achieving this goal. Intense and bright beams are produced by synchrotron radiation, and are efficiently used in the wavelength domain extending from hard X-rays to the far-infrared (or THz) regime. While X-ray crystallography provides exquisite details on atomic structure, Fourier transform infrared microspectroscopy (FTIRM) is emerging as a spectroscopic probe and imaging tool for correlating molecular structure to biochemical dynamics and function. In this manuscript, the role of synchrotron FTIRM in bridging the gap towards 'functional biology' is discussed based upon recent achievements, with a critical assessment of the contributions to biological and biomedical research.

Introduction

Millions of genes have been identified through DNA sequencing, and the structures of thousands of proteins have been determined using techniques such as X-ray crystallography and nuclear magnetic resonance (NMR), with the goal of understanding biological function at the molecular level. Even beyond the determination of atomic resolution structures, there is the daunting task of understanding how macromolecules assemble and function inside a living cell, where thousands are simultaneously interacting in a complex biochemical environment. Many new approaches are being developed for this growing field of 'functional biology', including infrared spectroscopy, microspectroscopy and imaging.

Fourier transform infrared (FTIR) spectroscopy is a well-known technique that probes the vibrational modes of molecules, providing a spectrum that is structure-specific. In biological materials, the standard building blocks such as proteins, lipids, nucleic acids, and carbohydrates have unique chemical structures and thus distinctive infrared spectra [1]. FTIR is most commonly used to study bulk, homogeneous materials. However, developments in infrared sources, optics, and detectors over the past decade or so have led to the marriage of FTIR spectroscopy with light microscopy, enabling spectroscopic

microscopy within the complex environment of biological cells and tissues. Moreover, since infrared radiation is non-ionizing, compositional changes can be probed over the course of time, on time scales ranging from a few milliseconds to hours.

One drawback of FTIR microspectroscopy (FTIRM) is the long wavelength of infrared light, where the diffraction-limited spatial resolution is theoretically predicted to be $\sim 2\text{--}10\text{ }\mu\text{m}$ in the mid-infrared region ($4000\text{--}500\text{ cm}^{-1}$) [2^{*}]. In practice, however, the spatial resolution is often limited by the brightness of the conventional thermal infrared source found in laboratory FTIR spectrometers. This is where synchrotron radiation is playing an important role; since it is 100–1000 times brighter than a conventional thermal source, it enables diffraction-limited spatial resolution to be achieved [3]. Thus, for brightness-limited experiments, such as those requiring high spatial or spectral resolution, or time-resolved measurements, a synchrotron infrared source provides vastly improved data quality that has high signal-to-noise and faster acquisition times.

Infrared microscopes are generally equipped with single-pixel detectors of high responsivity, generally liquid nitrogen-cooled, broad-band or narrow-band mercury cadmium telluride (MCT) detectors. However, the most recent development in IR detectors involves the coupling of two-dimensional array detectors to an FTIR microscope [4,5]. Focal plane array (FPA) detectors consist of hundreds of infrared detector elements that enable spectroscopic imaging of large areas with high spatial resolution ($5\text{--}20\text{ }\mu\text{m}$), with significant time savings compared to conventional single-element IR detectors [6]. Despite these advantages, FPA detectors typically have a lower signal-to-noise than conventional MCT detectors and the spatial resolution can be compromised by pixel-to-pixel blurring. Improvements have been demonstrated recently, however, where FPA detectors have been coupled to synchrotron infrared beamlines, holding the promise of bringing this imaging approach below the diffraction limit with faster image acquisition times [7^{*},8^{*}].

Structural information contained in FTIR spectra

A protein's FTIR spectrum has two prominent features, the Amide I ($1600\text{--}1700\text{ cm}^{-1}$) and Amide II ($1500\text{--}1560\text{ cm}^{-1}$) bands, which arise primarily from the C O and C–N stretching vibrations of the peptide backbone, respectively. FTIR has been shown to be particularly sensitive to protein secondary structure based on the vibrational frequency of the Amide I (C O) band, which is affected by different hydrogen-bonding environments for α -helix, β -sheet, turn, and unordered structures. For example, α -helices and β -sheets have Amide I vibrational frequencies at approximately 1655 and 1630 cm^{-1} , respectively [9^{*},10]. For most proteins, a mixture of secondary structures exists, and in this case, the Amide I band represents a combination of these components (Figure 1, inset). As a first approximation, the Amide I band can be curve-fit to predict a protein's secondary structure, or database approaches based on crystallographic information have also been used [11].

FTIR spectroscopy is an attractive complementary technique to UV Circular Dichroism (UV-CD) for secondary structure determination [12], and to fluorescence microscopy for identifying protein distribution and protein–protein interactions within cells [13]. UV-CD is the most widely used method for determining protein secondary structure in solution, and

recently synchrotron-based CD has been demonstrated to improve the accuracy of the technique [12]. Importantly, conventional and synchrotron CD have helped tremendously to refine the curve fitting accuracy of secondary structure determination with FTIR spectroscopy [14]. However, CD spectroscopy is typically used as an *in vitro* technique on purified proteins, whereas spatially resolved FTIR microspectroscopy also permits secondary structure analysis within biological cells and tissues. For *in vivo* studies, fluorescence microscopy is undoubtedly the most common approach for studying protein distribution and interactions in live cells through the use of methods such as green-fluorescent protein (GFP) labeling and fluorescence resonance energy transfer (FRET) [13]. While FTIR microspectroscopy does not achieve nanoscale spatial resolution like fluorescence microscopy, it has the unique advantage of being a label-free method that can probe many biomolecules simultaneously [15].

In addition to protein structure, FTIR simultaneously provides information about sample biochemistry [1] (and references within) (Figure 1). For example, the dominant absorption features of the lipid spectrum are found in the region 2800–3000 cm^{-1} , and are assigned to antisymmetric and symmetric C–H stretching vibrations of CH_3 (2956 and 2874 cm^{-1}) and antisymmetric and symmetric C–H stretching vibrations of CH_2 (2922 and 2852 cm^{-1}). In addition, a strong band at 1736 cm^{-1} arises from ester C O groups in the lipid. Nucleic acid spectra also have C O stretching vibrations from the purine (1717 cm^{-1}) and pyrimidine (1666 cm^{-1}) bases. In addition, the region between 1000 and 1500 cm^{-1} contains contributions from antisymmetric (1224 cm^{-1}) and symmetric (1087 cm^{-1}) PO_2^- stretching vibrations.

The complexity of FTIR microspectroscopy data

While using FTIR spectroscopy for secondary structure determination is straightforward for isolated proteins in solution, evaluation becomes significantly more complicated when probing biological cells or tissues using FTIRM. Because FTIR microspectroscopy monitors the global biochemical composition in the probed volume (i.e. tens of cubic microns), the vibrational signatures recorded are a superposition of the spectra of thousands of constituents. Yet despite the complexity, it has been demonstrated that the technique is highly sensitive to slight changes in the composition. The interpretation of these composite spectra requires sophisticated data analysis, which is still evolving through the continued development of multivariate methods. These approaches are based on the principle that there exist small, but reproducible changes in the spectra that can be associated with the variations in sample properties that are investigated. There are several known statistical approaches for infrared data analysis [16], but perhaps the most frequently used is principal components analysis (PCA). It aims at determining if the variance in the spectral pattern of all the individual cells studied is correlated, or due to random fluctuations [16].

Importantly, systematic spectral variance can arise not only from biochemical differences, but also from physical effects — especially when the size of the cell is similar to the probed wavelength [2*,17*]. Specifically, Mie-type scattering can result in a broad sinusoidal oscillation in the baseline of the spectrum and can lead to distortions in both position and intensity of absorption band [2*,18]. Moreover, the Mie scattering efficiency is dependent

upon the refractive index of the sample and changes on passing through an absorption resonance, an effect called resonant Mie scattering [17*]. For example, an increase in Mie scattering is often observed as cells become more rounded, for example, due to the action of a cytotoxin. Under such circumstances, the frequency and intensity are affected and cannot be used with any certainty to evaluate cellular biochemistry [19]. Only recently has this spectral artifact been described in detail and a correction algorithm has been developed [20*] and is illustrated in Figure 2.

Tissue imaging

One particular use of structure-based FTIR microspectroscopy has been the application to neurological protein-folding diseases. The vibrational frequency of aggregated protein falls around 1620–1625 cm^{-1} , due to the distinct hydrophobic environment. Thus, they are easily distinguishable in cells and tissues and can be imaged by FTIR microspectroscopy. For example, in Alzheimer's disease, amyloid beta plaques have been imaged in human brain tissue [21–23] and shown to be different than aggregates formed *in vitro* [23,24]. And since FTIR microspectroscopy can simultaneously provide information on other biomolecules, the same datasets showed elevated phospholipids near the plaques [24] and evidence for lipid peroxidation in the white matter [25]. FTIR microspectroscopy has also been used to image the structure and distribution of prion protein aggregates [26–28] while simultaneously evaluating changes in lipid and nucleic acid composition as a function of disease severity [29].

Just over the past few years, protein structure determination with FTIR microspectroscopy has also been used in the classification of lymphoma [30], early detection of demyelination in an animal model of multiple sclerosis [31], understanding elastin and collagen structure in aortic dilation [32] and collagen cross-linking in bone development [32], and even in the evaluation of the nutritional value of cereal grain endosperm [33]. Importantly, in all cases, the observed alterations in protein structure were also correlated with other metabolic changes in the tissue.

Imaging cells in culture

For tissue analysis, FTIR microspectroscopy is typically performed on cryo-cut, dried, histological sections placed either on IR-transparent (e.g. CaF_2 , BaF_2 , ZnSe , ZnS) or IR-reflective (e.g. gold-coated or low-e) slides. However, numerous studies have also been performed on isolated cells from culture or from bodily fluids. In these cases, cells are either grown directly on IR-compatible surfaces or a cytospin is used to deposit cells on these substrates. In contrast to tissue analysis, spatially resolved imaging of the cells is not typically performed, but instead statistical analyses of cell populations are evaluated. Again, due to the small size of most biological cells (typically 5–50 μm), a synchrotron infrared source provides subcellular spatial resolution and high signal-to-noise.

The first subcellular interrogation of protein structure in single cells was demonstrated over a decade ago in a study of apoptosis [34]. Epifluorescence microscopy was used to determine the stage of apoptosis while protein oxidation and degradation were observed with FTIR microspectroscopy [35]. Since then, high-resolution FTIR microspectroscopy has

been widely used, for example to evaluate protein structure in fibroblasts, giant sarcoma cells [36], and mitotic cells [37], amyloid formation in neuronal infection [38], drug resistance in human melanoma cells [39], and epithelial cells for cervical cancer screening [40].

Most recently, particular interest has been paid to the use of FTIR microspectroscopy for studying the differentiation of stem cells. Due to the potential formation of teratomas from undifferentiated stem cells, there is a crucial need to monitor this process with a rapid, non-invasive technique [41,42]. While immunocytochemistry is highly specific, it can also be very time-consuming and expensive. FTIR microspectroscopy is complementary because it does not require any exogenous labels or stains, is quick and inexpensive, and sensitive to chemical changes in the cell. Recent studies have shown that stem cells and their derivatives exhibit different infrared signatures [43,44,45*,46]. For example, human mesenchymal stem cells were stimulated in osteogenic media and FTIR microspectroscopy clearly distinguished between the control and differentiated cells, primarily based on reduced β -sheet and elevated α -helical proteins during differentiation [44,45*]. Another study was recently performed on embryonic stem-derived neural cells (ESNCs) [47]. These cells are a promising source of neurons for a cell-based therapy for the treatment of brain tumors and other neurological diseases and disorders. FTIR microspectroscopy coupled with multivariate statistical analysis were able to distinguish between the developmental stages of mouse embryonic stem cells into three cell types: embryoid bodies (EBs), neural progenitor cells (NPCs), and ESNCs. Spectral differences were attributed to an overexpression of glycerophospholipids and a concomitant increase in α -helix rich proteins as mESCs differentiated towards ESNCs.

FTIR microspectroscopy has also been used recently to investigate the mechanisms of photodynamic therapy (PDT) in the treatment of cancer. Specifically, indocyanine green [48] or hypocrellin A [49] were used with laser illumination to generate toxic singlet oxygen that initiates cell death. In both studies, principal components analysis showed the time exposure and dose necessary to cause changes in protein conformation, which was attributed to the denaturation of cellular proteins. In addition, the PDT with indocyanine green showed a decrease in the nucleic acid phosphate absorption modes at 1240 and 1180 cm^{-1} , consistent with the fragmentation of DNA in cells undergoing apoptosis. Importantly, these changes pinpointed specific chemical changes and were observed earlier than conventional light microscopy stains.

Structural dynamics

More than a decade ago, FTIR spectroscopy was shown to be highly sensitive to time-resolved structural changes of isolated biomolecules in solution [15,50]. Since then, it has been used to study numerous systems, including the structural changes behind the gating mechanism in influenza [51,52*], picosecond ligand switching in heme proteins [53], and folding/unfolding kinetics in peptides [54].

Since infrared light is non-ionizing, FTIR microspectroscopy has the potential for imaging living cells in real time. However to date, most FTIR microspectroscopy studies have been performed on cells or tissues that have been fixed and dried. This is largely due to the

intense absorption by water in aqueous systems. Especially for studying protein structure, very strong water vibrational modes interfere with the protein Amide A, I, and II bands (Figure 3). The strong water absorption also necessitates the use of very short sample path-lengths, typically between 8 and 10 μm . However, the sensitivity of modern FTIR spectrometers, combined with the high brightness of the synchrotron source, is permitting the observation of living cells even in the presence of the high water background signals. Even so, the water background must still be accurately subtracted, which has proven to be difficult because the cell contains less water than the surrounding medium, leading to overcompensation of the water contribution (Figure 3).

Despite these challenges, several groups have taken different approaches to studying cells in aqueous media. Early on, Moss *et al.* were able to collect spectra from single cells in a flow system based on a commercial demountable cell that employed 3 mm thick CaF_2 windows separated by a 15 μm thick Teflon spacer [55]. More recently, Tobin *et al.* employed a similar device with a synchrotron source [56]. In addition, the development of microfluidic devices is now underway for introducing chemical agents such as drugs and nutrients into the cell culture, enabling real-time spectroscopic changes to be evaluated [57*]. Recent examples of microbe activity in biofilms [58*,59*] and the adaptive response of anaerobes to oxygen have been very promising.

The study of living cells by FTIR microspectroscopy has also been investigated through the use of attenuated total reflection (ATR) [60*]. When cells are grown on an ATR crystal, this method reduces the pathlength, and hence the water absorption, to around 1.0 μm , while allowing the exchange of nutrients without the requirement for a thin flow volume above the cells, which can otherwise induce large shear forces. It also improves the spatial resolution of the technique due to the high refractive index of ATR materials. However, one issue with ATR-FTIR is that the evanescent wave only probes the first 1–2 μm from the cell surface, so the nucleus may only give a small contribution to the overall signal. Given that nucleic acids play a large part in the reported changes in spectral signatures, and the nucleus may remain above this penetration depth, it remains to be seen whether ATR can be readily used as a non-invasive biomarker-free analytical technique for live cell studies.

Another challenge with time-resolved infrared imaging is the inherently low spatial resolution of FTIR micro-spectroscopy compared to conventional light and fluorescence microscopy. And since biological samples are heterogeneous on many size scales, improvements in spatial resolution are desirable for reducing the complexity of the volume probed. Due to the long wavelengths of infrared light, the spatial resolution of FTIR microspectroscopy is typically diffraction-limited. However, having an accurate knowledge of the diffraction pattern implies that a mathematical correction for diffraction is possible by deconvolution methods. But for acceptable results, deconvolution requires both high spatial oversampling and excellent S/N. High-resolution sampling can be accomplished using an FPA and a high magnification objective, but the flux incident on each pixel may be more than 100 $\times$ smaller such that the S/N is not sufficient for a successful deconvolution. Thus, coupling a synchrotron source to an FPA microscope has the potential to correct for diffraction effects and improve the spatial resolution of FTIR microspectroscopy over regions of modest size (Figure 4). Thus, the synchrotron and thermal sources play

complementary roles. With the thermal source, many millimeters can be surveyed quickly and offer excellent performance down to about 10 μm spatial resolution. With the synchrotron, the resolution limit may be extended down to around 1 μm , but over a much more limited area.

In the future, we anticipate that microfluidic devices will continue to evolve for FTIR microspectroscopy studies of cells *in vivo*. Especially coupled to the brightness of a synchrotron source and multi-pixel focal plane array (FPA) detectors, time-resolved imaging of cell composition is on the horizon. But in addition, coupling of these microfluidic devices to the bright synchrotron beam will also enable rapid-mixing studies with microsecond time resolution, opening the door to studies such as protein folding, ligand binding, macromolecular complex formation. As such, this may prove to be a powerful platform for proteomic-scale structural studies using FTIR spectroscopy, providing structural dynamics information on numerous biological systems.

Conclusions

FTIR microspectroscopy is becoming increasingly important in the translation of molecular structure to biological function in the context of cells and tissues. The ability to image protein structure in cells and tissues without the use of added contrast agents is unique. And since the technique probes a large number of macromolecules simultaneously, its sensitivity to simultaneous structural and compositional changes makes it complementary to other microscopic tools such as immunostaining and GFP-labeling. Since infrared light is non-ionizing, there is a promising future for time-resolved imaging of living cells. By utilizing the high brightness of synchrotron infrared light and recent developments in microfluidic devices, FTIR microspectroscopy provides an original approach to bridging the gap between atomic structure determination and functional biology.

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Figure 1.

(a) Infrared spectrum of a single A431 cell deposited on a CaF_2 slide and fixed. (b) Infrared spectrum of an A431 cell in aqueous solution. Note that the water contribution in the spectrum has been efficiently removed. However, the spectrum appears quite different, especially in the Amide II region (at 1550 cm^{-1}). This has been already reported [47]. The spectrum was acquired at the Australian Synchrotron Infrared beamline with the device described in [43]. A431 cells are a model cell line (epidermoid carcinoma) used in biomedical research. More specifically, they are used in studies of the cell cycle and cancer associated cell signalling pathways since they express abnormally high levels of the Epidermal Growth Factor Receptor (EGFR). The cell line was established from an epidermoid carcinoma in the vulva of an 85-year-old female patient. (Inset) FTIR spectrum of the Amide I band of a protein, which can be deconvolved into its secondary structure components.

Figure 2.

(a) Optical image of a PC3 cells (i) and an IR spectrum of the smaller cell (ii). (b) Corrected IR spectrum using an existing EMSC algorithm. (c) Corrected spectrum using new EMSC RMieS algorithm (from [20*] with permission).

Figure 3.
(—) FTIR absorbance spectrum of a typical biological cell. (- - -) FTIR transmission spectrum of water. Note that the O–H stretching and bending modes of water overlap with the Amide A, I, and II bands of the protein.

Figure 4.

Synchrotron FTIRM imaging of 6 μm -diameter polystyrene beads on a 1 mm-thick CaF_2 slide. The sample was illuminated with a low magnification objective (36 $\times$, 0.6 NA) for long working distance and data collected with a high magnification (74 $\times$, 0.6 NA) objective. A 64 $\times$ 64 pixel MCT FPA detector was used to image the beads, where the resulting field of view on the FPA was 39 μm $\times$ 17 μm . Data collection time was 10 seconds. **(a)** Bright field visible image of the polystyrene spheres. **(b)** Sample spectrum from the center of a polystyrene bead. **(c)** Peak integration at 1450 cm^{-1} . **(d)** Peak integration at 1450 cm^{-1} after point spread function blind deconvolution.