Applications of modern micro-Raman spectroscopy for cell analyses

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Raman spectroscopy assesses the chemical composition of a sample by exploiting the inherent and unique vibrational chacteristics of chemical bonds. Initial applications of Raman were identified in the industrial and chemical sectors, providing a rapid non-invasive method to identify sample components or perform quality control assessments. Applications have since increased and sample sizes decreased, leading to the onset of micro-Raman spectroscopy. Coupling with microscopy enabled label-free sample analysis and the unveiling of total chemical composition. Latter adaptations of Raman have advanced into biomedical diagnostics and research. Alongside technical developments in filter systems and detectors, spectral peak intensities and improved signal-to-noise ratios have facilitated target molecule measurement within a variety of samples. Quantitative sample analysis applications of Raman have contributed to its increasing popularity. Through these exceptional capabilites, potential Raman spectroscopy utility in biomedical research applications has expanded, exemplifying why there is continued interest in this highly sensitive and often under-used technique.

Introduction

Biological studies have traditionally focussed on the macro-molecule scale, including proteins, metabolites, and transcripts. However, what is often overlooked is the chemical architecture formulations of these various moieties. All matter is comprised of various elements, each possessing their individual macro-scopic conformations (sheets, helices and so forth), they will also possess a specific microscopic chemical formulation. Previously reserved for chemists to explore, the chemical formulations of various cellular targets have become of great interest for both diagnostic and pharmaceutical applications. With this growing interest, a technique enabling a 'systems' perspective of analytes, spanning from the whole cell down to the chemical composition of regions of interest. This is precisely where the realm of spectroscopy has found its niche.

Photons, the elemental units forming light, and their behaviour in an electromagnetic wave (light) are the basis of spectroscopy. There are multiple types of photon behaviour, with Raman scattering being one common example.

Raman scattering was first identified in 1928.[1] This form of photon scattering was found to differ in energy (Fig. 1) and in resulting signal intensity from the more commonly known Rayleigh scattering, with Raman scattering occurring in approximately every 1 in 10^6–10^8 photons.[2] Following the advent of the laser, target excitation became appreciably more efficient and compatible with smaller sample volumes. The resulting photon scattering following sample excitation is collected with a photomultiplier, or more commonly with multichannel detectors like a charge coupled device (CCD) that has since been joined to a computer for digital spectral analysis[3]

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Insight, Innovation, Integration

Fluorescent labeling and other staining methods are major tools in investigating biological samples. However, a prevailing concern surrounds how these procedures influence the sample and resulting data. On the other hand, Raman spectroscopy is an already well-established tool in many research fields to directly investigate the structure and composition of materials without the need of further staining or labeling techniques. This tutorial review explains the major technical developments over the last decades that improved the technique such that its application towards bio-relevant samples like cell cultures or even tissue samples becomes straight forward. In particular, the ability of Raman spectroscopy to assess components non-invasively and non-destructively makes it a valuable tool for bio-analysis. Its integration with commonly used microscopy set-ups further extends the number of applications and readily allows the comparison of results obtained with standard labeling methods. Several examples ranging from standard tissue characterization to cutting edge nanoscale investigations of nucleotides demonstrate the potential of Raman spectro-scopy. As also the potential for quantification expands, micro-Raman spectroscopy will certainly continue to deliver in bio-medical applications.

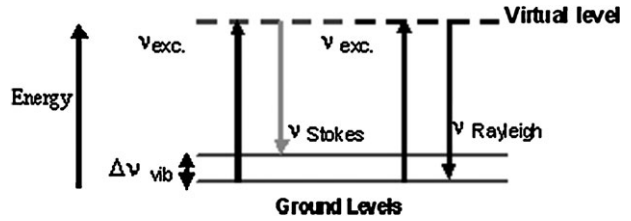

Fig. 1 Schematic of the Raman (Stokes) and Rayleigh scattering process. Stokes scattering corresponds to an inelastic scattering of photons, where the incident photon is of a higher frequency then the resulting scattered photon ($\Delta\nu_{vib} = \nu_{exc.} - \nu_{Stokes}$). Rayleigh scattering is the elastic scattering of photons, where the incident and scattered photons possess the same frequency and wavelength levels.

Raman spectroscopy originated in the chemical and materials analysis field, studying both mixtures and purified substances, identifying compositions and characterizing chemical structures.[4,5] Most appealingly, because little to no sample preparation is required, samples could be non-invasively studied. Quantification had also become possible through comparing the resulting spectral peak intensities and the development of specific algorithms for processing large spectral data sets.[6] Subsequently, from the wealth of information present in a Raman spectrum and the ability to quantitatively assess components non-invasively and non-destructively, exploratory applications involving biological matter were initiated.[7]

As biological applications grew in prevalence, their numerous analytical drawbacks became the focus of scrutiny. These samples are often present in limited quantities, possess heterogeneous composition and compartmentalized targets, require microscopy, and produce high auto-fluorescence. This results in complex spectra that often make accurate analyte quantification difficult to achieve. These areas of concern were addressed through the enhancement of associated instrumentation, better suiting biological studies and culminating with the current micro-Raman spectroscopy widely used today.[6]

Instrumentation advancements

Lasers

Modern spectroscopy could not be applied to quantitative biomedical studies, particularly for diagnostic pursuits, without the sensitivity offered by lasers due to their stability and coherence of light at a specific wavelength. Since the first solid state ruby laser,[8] technological advancements have lead to a range of lasers using a variety of gain-mediums, like the commonly used gas (helium-neon, krypton and argon ion) or diode lasers.[9,10]

Modern lasers have become more stable and provide a better control over beam power and current, enabling more reliable sample quantification. Swapping between wavelengths has also become easier, avoiding weak signals or competing processes like fluorescence, the emission of light at a longer wavelength causing signals of interested to be overshadowed.[11] The enhancement in lasers and the ability to regulate laser power, current and wavelength, combined with calibration of accompanying optics, enables robust and reproducible spectral quantification.

New filter systems

Filter systems select a wavelength from the back-scattered light (Fig. 2) before the Raman scattering is transmitted to a

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detector. Resulting spectra will possess lower background noise, only the resulting photon scattering of interest (Raman scattering in this case) and can be tailored to fit the needs of the sample (*e.g.* wavelength selection, sample translucence, intensity & duration of sample excitation). Keeping spectral noise levels low is critical for quantification, as the higher the noise level, the more data pre-treatment is needed to decrease the noise. This increase in filter specificity decreases spectral intensity variations and disruptive noise, providing more accurate and easily quantifiable spectra.

Holographic notch or dielectric edge filters are currently the most prevalent, replacing spectrographs and interferometers as filter stages. In combination with a single monochromator stage, the collection efficiency increases considerably and, when in combination with a microscope, allows efficient spectroscopic mapping. Certain applications require particular wavelengths rather than the whole spectrum, and band-pass filter systems have been developed to enable the transmission of single or even multiple pre-selected wavelengths. These optical systems are ideally suited for quantitative Raman imaging when complete wavenumber (cm^{-1}) information is not needed.

Ideally suited to bio-medical studies are acousto-optically tunable (AOT) filters.[11] These filters possess no movable parts as the band-pass selection is electrically tunable, enabling the transmitted wavelength to be modified in microseconds to accommodate sample-specific requirements. The overall diffraction efficiency reaches upward of 80%, making AOT filters compatible with fast and consistent quantitative Raman imaging.

Detectors & spectral noise

For the more common single-spectrum method of sample analysis (in contrast to the aforementioned imaging systems), filtered light is spectrally dispersed and collected on the detector (Fig. 2). Photomultiplier tubes (PMT) were the commonly used detector, but have mainly been replaced with CCD's.[11]

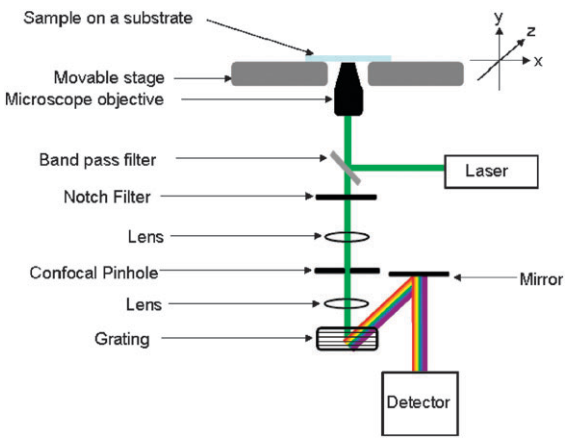

Fig. 2 Instrumentation for a Micro-Raman Spectroscopy set-up to conduct sample imaging. The collimated laser light is directed through a band pass filter with appropriate wavelength selection and is focused through a microscope objective onto the sample. The backscattered light is collected through the objective and after filtering with a Notch filter to reject elastically scattered signal, the light is transmitted through the confocal pinhole into the spectrometer. The signal is then directed onto a grating which disperses the light and eventually guided onto the detector to collect the Raman spectrum.

Autofluorescence, a significant problem for biological samples, is a type of determinate noise increasing with each spectral acquisition and obstructs the identification of spectral features and ensuing quantification.[2] Not all samples will fluoresce but in those that do, incident light is absorbed and converts nearly all of the absorbed photons into fluorescence, producing a spectral intensity up to 10^{10} higher than typical Raman scattering intensity and can saturate the CCD before Raman information can be determined. There are numerous methods to reduce fluorescence in resulting spectra and include varying sample preparation techniques,[12] alternating wavelengths,[13,14] applying time-resolved detection[15] and even sample photo-bleaching.[16] Modifications in signal magnitude, aperture, laser power and stabilization can also improve resulting spectra and the S/N ratio.

With the prevalence of fluorescence in biological samples, improving signal acquisition, minimizing noise levels and reducing the effect of cosmic rays in CCDs are areas of constant exploration. In turn, innovative methods to produce better signal acquisition are regularly being developed, thus improving spectral reproducibility, ease of use, applications and quantitation.

Micro-Raman spectroscopy

Considering that the diameter of the majority of individual cells are in the range of 6 to 40 μm, the microscope has long been an ideal platform for sample analysis, allowing the region of interest to be targeted with the use of objectives providing an appropriate numerical aperture. The incorporation of confocal microscopy with Raman spectroscopy revolutionized the scope of adaptations and in 1990 was first applied to study single cells and chromosomes.[7]

This pairing simplified biological studies and enabled a 3-dimensional image translation and magnification of the sample relative to the microscope objective. Moreover, the confocal pinhole implements a geometry that ensures only select photons are collected from the back-scatter and reach the detector. This precision enhances the ability to quantify spectra by providing spatio-temporal information of their origin, useful in identifying and quantifying specific localities.

By guiding a laser through an objective lens, a near-diffraction limited spatial resolution and increased collection efficacy can be achieved, since the laser spot size equals the wavelength divided by the numerical aperture of the objective. (Fig. 2).[11] The laser beam intensity can be increased for stronger spectral features and can provide a spatial resolution below 1μm using the right wavelength/objective combination (as previously described), enabling biologically important structures including individual nuclei, mitochondria, cilia and regions of cell-interaction to be analyzed.[31–34] The minimally invasive micro-Raman process maintains sample integrity and direct sampling in either air or aqueous environments can be carried out maintaining viability.

The innovation of micro-Raman spectroscopy has enabled the collection of detailed information pertaining to analyte

molecular structure and composition on multiple focal plains. Micro-Raman spectroscopy continues to be a growing area of research, providing a spatial resolution surpassing that of IR spectroscopy and providing quantitative chemical insight.

Benefits of modern micro-Raman spectroscopy

The versatility of micro-Raman spectroscopy can be best observed by its broad range of applications. This technique has been swiftly applied to a variety of subject matters, including the characterization of molecular structures in biological samples (*e.g.* sub-cellular and single cell components) and differentiation between cells as a result of growth cycles, physiological behavior or even physiological states (*e.g.* activation states),[35] all of which can be relatively quantified through the use of specific data processing procedures.

Given recent instrumentation advancements, high levels of technical flexibility are now available. This includes the ability to change objective lenses to suit each study and readily apply different wavelengths for analysis. Fully automated *xy* maps and line scans are now possible, maintaining the spatial and temporal resolution while enabling sample imaging. In turn, the resulting Raman spectral images can provide direct insight into the composition of the analyte with molecular resolution, providing a truly objective view of pathology or even cell functionality (Table 1).[36]

Resulting spectra from both single point and imaged samples provide a complete chemical snapshot of the sample. This includes in principle all present RNA, DNA, protein, lipid and carbohydrate content and providing a more 'holistic' approach to studying the sample. Quantitative and qualitative results can be generated by measuring emission or adsorption patterns, distinct for all functional groups and organic compounds. Micro-Raman imaging can be quantified by processing the hyper-spectral data through cluster analysis and successive color-coding. These images provide detailed information on analyte molecular structure and distribution in relation to the spatial-temporal organization.

Specific to sample preparation, numerous substrates can be used that are interchangeable with other techniques, including CaF_2 and glass. Little to no sample preparation is necessary for preservation, enabling use of the same sample for further down-stream studies. Aqueous solutions can also be used, facilitating live cell and reaction monitoring, which offers tremendous benefit for pharmaceutical studies of drug activity. Final product drug conformations can be analyzed undisturbed and their activity in its natural environment can be studied, shedding light onto compound reactions *in vivo*.[37]

Micro-Raman spectroscopy & bio-analysis

From the wealth of information in a Raman spectrum and its non-invasive and quantitative nature, biological and bio-medical applications have become vast. Studies encompass the detection of sub-cellular reactions and components (including protein and nucleic acids),[38] single cell studies,[39–41] sample imaging and tissue characterization.[42–46] As water presence is not detrimental to Raman spectra, many buffers and media used in biological sample growth and preparation possess low Raman signals, reducing convolution in the resulting data.[41] This flexibility of cell sampling can permit cells to be fixed, dried,[47] analyzed alive or even be measured *in vivo*,[48,49] circumventing the need for excisional biopsies. Minimum sample processing also permits future sample analysis, including techniques like IR spectroscopy, traditional histology and gene expression analysis for result comparison. By combining techniques, a steadfast quantitative biochemical understanding of the sample can be achieved.

With this broad range of applications, micro-Raman spectroscopy has expanded into new disciplines including biotechnology and medical diagnostics, where it has been applied as a competitive method for traditional diagnostic methods.[16,45]. Raman analysis has also become an important technique across a range of industries, encompassing chemical,[50,51] pharmaceutical[52,53] and cosmetic fields.[54,55] As the nature of both industry and medicine becomes increasingly quantitative, the pressure for novel enabling techniques to provide an appropriate level of measurement has become critical.

Raman spectroscopy is intrinsically suited to quantification, both relative and absolute, exhibiting spectral features indicative of molecular composition. Through spectral comparison and dilution studies, relative quantities can be determined. However, relative quantification in isolation is insufficient to competitively penetrate into bio-medical diagnostics. Through the use of innovative data analysis methods like hierarchical clustering analysis (HCA),[56] principle component analysis (PCA),[57] multivariate curve resolution (MCR),[58] vertex component analysis (VCA)[59] and the development of novel algorithms specific for the subject matter, the ability to extrapolate increasingly quantitative data from Raman spectra has become possible.

Quantitative bio-medical applications

Cellular components have been studied using single spectrum analysis or through region-specific imaging in a variety of samples. Using single spectrum analysis, specific proteins, organic pigments and nucleic acids have been quantified.[60] Individual cells[40,44] and nuclear components have additionally been quantitatively explored using this method.[61] Quantitative *in vivo* studies have also been carried out, applying low intensity output laser light to patients for real-time tissue characterization or cancer detection.[62,63]

Single spectrum analysis can be quantified relative to the sample, identifying differences or similarities by using already available methods like PCA or MCR. Both of these techniques implement the assumption that the spectra used from a mixture of chemicals can be displayed in a 2D space while still making use of the multivariate nature of spectra.[17] In PCA, singular-value decomposition is used to calculate basis spectra while MCR is designed to extract basis spectra similar to the original Raman spectra of the chemicals present within the sample.[64] These techniques are both useful when little is known about the sample prior to analysis as they still permit the extraction of embedded chemical information.[17] These methods allow a comparison of data relating to the composition of the sampled population and can be carried out

Table 1 Imaging techniques and their quantification capabilities. Micro-Raman spectroscopy, FISH, Immunohistochemistry, AFM, Electron Microscopy, Multi-Photon Microscopy, SIMS and MALDI-Imaging are all prevalent imaging techniques, whilst TERS is a novel spectroscopic addition to these imaging techniques

Imaging techniques	Spatial resolution	Sample preparation	Molecular probe use	Visualized target	Information obtained	Acquisition time	Cell to result time	Quantification	Ref.
Micro-Raman spectroscopy	Approx. 500 nm	Not required	N/A	Proteins, RNA/DNA, lipids, carbohydrates	Chemical composition	Milli-Seconds–Hours; dependent on sample size	Min.–Hours	Absolute & Relative	17, 18
Fluorescent *in situ* hybridization	N/A	Chemical & formalin fixation, labelling	DNA primers conjugated to a fluorophore	DNA & RNA sequences, chromosomes	Gene sequence presence	Min.–Hours	Min.–Hours	Absolute & Relative	18
Immunohistochemistry	N/A	Formalin fixation, labelling	Antibodies for cell labeling	Cell constituent localization	Proteins	Min.–Hours	Min.–Hours	Relative	19
Atomic force microscopy	Atomic resolution	Intermolecular forces & sample immobilization	N/A	Sample Mapping, including whole cell surface & individual components	Topographical & nm scale structural information	Min.	Min.	N/A	20
Electron Microscopy	Approx. 5 nm	Cryo or chemical fixation, gold sputtering, dehydration, sectioning	N/A	Proteins & Cells	Protein Structure, Cell conformations	Seconds	Min.–Hours	Relative	21
Multi-photon microscopy	15–1000 nm	Chemical fixation, sectioning	Rhodamine amide, quantum dots, fluorescent proteins, dyes	Visualization of cell structures	Structural & topographical information	Seconds	Min.–Hours	Relative	22, 23
Tip- enhanced Raman spectroscopy	5–50 nm	Tip preparation	N/A	Proteins, RNA/DNA, lipids, carbohydrates	Chemical composition	Seconds–Min.; dependent on sample and tip	Min.–Hours	Relative	24, 25
Secondary ion mass spectrometry-SIMS	50 nm	Cryopreservation	N/A	Whole cells, intracellular structures	Molecular & or elemental composition of the sample	Seconds–Min.	Min.–Hours	Absolute & Relative	26, 27
MALDI-imaging	Down to 500 nm	Sectioning & MALDI-matrix application	N/A	proteins, peptides, metabolites, biomarkers	Drug development, biomarker characterization	Min.–Hours	Min.–Hours	Relative	28–30

using commercially available software for rapid data pre-treatment and analysis.

Novel algorithms can be developed that are specific for the analysis needs of a sample, like prostatic adenocarcinoma cell lines[40] or bacterial strains.[65,66] These algorithms can then determine sample homogeneity during processing and classify all spectra into groups based on spectral similarities.

Micro-Raman imaging has proven capable of identifing specific differences in chemical composition within highly complex biological samples and has become an area of great interest. Recent examples include studies on cellular protein distribution,[49] whole-cell variations between cancerous and non-cancerous architecture[67] and the monitoring of cell activities including induced cell death.[68]

Data acquired from imaging studies can be relatively and absolutely quantified using multiple methods. In one study, the molecular architecture of oocytes was investigated and unsupervised HCA was applied to the resulting spectral images, relatively quantifying sample heterogeneity.[69] Micro-Raman imaging creates spectral images through examining the intensity of selected, individual peaks for the spectrum of each pixel. As the laser raster-scans the sample, one spectrum is acquired for each pixel. These spectra are then compared through a variety of analysis methods, including K-means[70] and Ward's algorithm[71] to achieve hierarchical clustering. This form of analysis can quantify an image through clustering image spectra based on similarities and assigning false-colors to identify the different regions in a heterogeneous sample, simplifying the analysis of otherwise complex and detailed spectra. The number of pixels forming each cluster can be compared with the total number of pixels in the image to provide a quantitative view of the analyte distribution within the spectral image (Fig. 3). Molecular composition of each cluster can also be measured by comparing peak intensities for each cluster within a sample. Using cluster analysis, spectral clustering can be carried out that is specific for the spectral region(s) of interest, like the 'fingerprint region' between 400–2000 cm^{-1} for general RNA, DNA and protein content, the CH– stretching region between 2800–3050 cm^{-1} for lipid content and the ester carbonyl band from 1720–1750 cm^{-1} specific for the type of lipid content.

Recently, a myriad of papers have been published applying micro-Raman imaging to spatially explore chemical variations in tissue samples for diagnostic purposes[36,44,45,72,73] Some examples of subject areas include prostate, lung and bladder cancers. These studies often apply HCA, as described, and can evaluate the amount of cancerous tissue present relative to non-cancerous tissue in the sample. Moreover, diseased tissue of varying degrees can be identified and quantified.

Micro-Raman spectroscopy has also seen great interest for bacterial, yeast and viral studies. Resulting from bacterial and other microorganism outbreaks in hospitals and contamination within food facilities and pharmaceutical packaging sites, the detection and identification of present bacteria and microorganisms is critical to uphold quality control standards and ensure facilities are free from potential infections. By applying micro-Raman spectroscopy and collecting a single spectrum from each analyte, individual strain variations can be detected and important phylogenetic information can be obtained and quantitatively assessed.[65,74] Using a single organism, quick, reliable and non-destructive analysis can provide spectral data pertinent to treatment or cleaning conditions.[65]

Quantitative micro-Raman spectroscopy has also been applied within the pharmaceutical industry to monitor drug uptake. Specifically, the permeation of pro-drugs into the skin can be monitored and the resulting drug concentration can be compared with that of the starting drug concentration. From the ability to monitor *in vivo* or in excised biopsies but with no sample preparation, drug conformations following permeation into the body can be accurately evaluated.[46,75]

Conclusions

Combining Raman spectroscopy with microscopy has enabled the breadth of Raman applications to expand considerably within the bio-medical sciences. This combination has lead to the quantitative, non-invasive analysis of specific localities or the imaging of whole samples, providing a resolution below the micrometre level while maintaining spatio-temporal organization. Starting from its high resolution and information-rich spectra, accurately filtering of the many

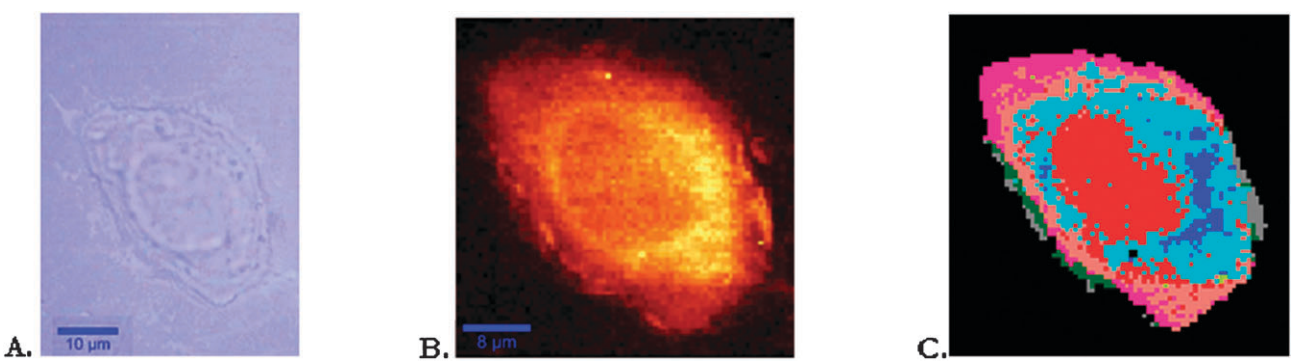

Fig. 3 Micro-Raman imaging of a single MCF-7 cell and subsequent quantitative analysis. (A & B) Visible image of an MCF-7 cell and chemical imaging specific for the intensity of CH– stretching throughout the cell (80 × 80 pixels and a 0.5 μm spot size). (C) Hierarchical clustering of the spectral image specific for the 'fingerprint region' (1800–675 cm^{-1}) displays the localities of sub-cellular components. Clustering can be used to quantify sub-cellular components, like the nucleus, which is 12% (768 pixels) of the entire cell in this example.

intrinsic spectral components of micro-Raman spectra has lead to the development of multiple algorithms to de-convolute heterogeneous samples and provide quantitative insight of even complex specimen. Although absolute quantification of raw spectral data remains challenging, relative quantification can be readily achieved.

With the path of medicine and related industries leaning increasingly towards quantification, all techniques implemented for diagnostic use must be capable of providing at least a certain degree of quantification. For single spectrum analyses, methods like band deconvolution already assist in relative sample evaluation.[76] Relative quantification with respect to sample heterogeneity *versus* molecular quantity can be achieved with imaged samples by applying methods like HCA and PCA. Through the use of hyper-spectral data clustering methods and the sensitivity of micro-Raman analysis, sample heterogeneity can be clearly visualized and detected, using specific techniques already at the single molecule limit.

Micro-Raman can reach sub-micron resolution. Techniques like tip-enhanced Raman spectroscopy (TERS) and coherent anti-Stokes Raman (CARS) are already pushing the boundaries of resolution and sensitivity limits well beyond the diffraction limit and challenges microscopic techniques that rely on fluorescent labeling. TERS in particular can provide a lateral resolution down to 10 nm, comparable to the size of the tip used[77] and has been applied to biological samples like virus[78] and cells.[79] Meanwhile, CARS can offer rapid data acquisition (pico-seconds) from the use of its two-laser confirmation. This enables a high method-intrinsic spatial resolution (the region where sample excitation is most efficient by both lasers) and 3D imaging capabilities based on varying laser penetration depth.[80] Biological applications exploiting these particular attributes have recently begun expanding from basic lipid vesicles and layers[81–83] into more complex samples, including single cells[84] and multi-cellular organisms.[85,86]

As micro-Raman spectroscopy gains acclaim in bio-medicine and significant spectral databases are developed for accurate spectral assignments, quantification capabilities are likely to develop synergistically. This continued improvement in instrumentation and spectral deconvolution will permit more rapid and reliable sample analysis, particularly for more adaptations to whole tissue samples. In turn, as the potential for quantification expands, micro-Raman spectroscopy will continue to deliver in bio-medical applications.

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