Raman spectroscopy for physiological investigations of tissues and cells

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Abstract

Raman micro-spectroscopy provides a convenient non-destructive and location-specific means of probing cellular physiology and tissue physiology at sub-micron length scales. By probing the vibrational signature of molecules and molecular groups, the distribution and metabolic products of small molecules that cannot be labeled with fluorescent dyes can be analyzed. This method works well for molecular concentrations in the micro-molar range and has been demonstrated as a valuable tool for monitoring drug-cell interactions. If the small molecule of interest does not contain groups that would allow for a discrimination against cytoplasmic background signals, „labeling“ of the molecule by isotope substitution or by incorporating other unique small groups, e.g. alkynes provides a stable signal even for time-lapse imaging such compounds in living cells. In this review we highlight recent progress in assessing the physiology of cells and tissue by Raman spectroscopy and imaging.

Keywords

Raman scattering, inelastic light scattering, single cell analysis, cell physiology, tissue physiology, coherent Raman scattering, label-free optical microscopy

1. Introduction and Background

Optical methods are commonly used to probe the physiological state of cells because of their ability to obtain detailed biochemical information from fixed and living cells without the need for direct physical contact with the cell (1). While fluorescence is perhaps the most commonly used optical technique for this purpose, it requires the application of exogenous fluorescent tags to identify and detect specific biomolecules. This procedure, however, necessitates careful control studies, because the addition of foreign organic molecules to cells has the potential to alter a cell's biochemical profile or to harm it. This is certainly also true for genetically modified organisms whose cells express fluorescent proteins as fusions with the protein of interest. The inclusion of such exogenous reporters into cells can have a direct effect on a cell's physiology (2, 3). Also, fluorescence-based approaches require decisions to be made beforehand about which molecular targets require labeling to enable their monitoring. This process can be quite tedious

and it limits the amount of biochemical information that can be obtained during a single experiment. Therefore, alternative optical techniques for probing the physiological state of cells continue to be explored with the hope that these will hold considerable promise for revealing new insights into cellular behavior while minimally affecting cells during the measurement.

Over the past 25 years, Raman spectroscopy has emerged as a highly sensitive and promising analytical technique that has been applied to a number of biomedical problems at the single cell as well as the tissue level, e.g. in cancer, infectious diseases, regenerative medicine, and cardiovascular disease. Raman spectroscopy is a laser-based spectroscopic technique for the direct detection and characterization of molecular bonds. The underlying process is the inelastic scattering of photons on molecular bond vibrations (aka „Raman scattering“), where a small fraction of photons (~ 1 in 10^8) are inelastically scattered and lose a portion of their energy to the molecular bond vibration. The difference in energy between the incident and scattered photons corresponds to the energy that was required to excite (or de-excite) the molecular vibration. Detection of these scattered photons results in a spectrum of narrow peaks, each of which can be assigned to a specific vibrational resonance of a molecular group. Each Raman peak of a particular molecular vibration occurs at a specific vibrational energy relative to the wavelength of the excitation source, which is displayed as a Raman „shift“ in units of „wavenumbers“ (in cm^{-1}). Therefore, a Raman spectrum resembles a „molecular fingerprint“ of the sample under investigation. When applied to the analysis of single cells, Raman spectroscopy provides information about the biochemical composition of cells that could otherwise only be obtained by destructive techniques, such as chromatography or mass spectrometry (4). In addition, since a molecular vibration is sensitive to its neighboring molecular bonds and molecular structure, Raman spectroscopy also provides information about the conformation of biomolecules and their interactions. When combined with confocal microscopy, single cell and subcellular chemical information can be obtained (4).

Raman spectroscopy has found extensive applications in biology and biochemistry for the characterization of the structure and interactions of biomolecules (5). Such assessments are often made based on the presence or absence of one or a few select Raman peaks. The definitive identification of specific peptides or proteins, however, typically requires that the entire spectrum of Raman-active vibrational modes is being evaluated. For example, many amino acids, such as tyrosine, tryptophan, and phenylalanine, have distinct peaks in the 600 to 1700 cm^{-1} spectral fingerprint region. Amide linkages between amino acids give rise to two Raman-active vibrations, the amide I (C=O) stretching vibration, and the amide III (C-N) stretch and (N-H) in-plane bending modes. The relative positions of these two vibrations can provide information about the conformation in which proteins are predominantly present in a sample, e.g. in alpha helix or beta sheet conformation. DNA has several distinct spectral peaks that can either be assigned to the sugar phosphate backbone or to the four DNA bases. The symmetric stretching vibration of two phosphate oxygen groups in the diphosphate ester PO_2^- group occurs between 1100 to 1150 cm^{-1} . The O-P-O stretching mode of DNA depends on DNA conformation: for DNA in B-form it is located near 835 cm^{-1} , and for A-form (DNA and RNA) it occurs at 800-815 cm^{-1} . The exact position of these peaks provides information about DNA conformation (A, B, C, or Z form) or about subtle changes to the structure of DNA. Phospholipid molecules, which make up the plasma membrane of cells, have spectral markers due to both, the head and tail

groups. For example, polar head groups have a C-N stretching vibration at 720 cm^{-1} , while hydrophobic chains have vibrational peaks in the $1000\text{--}1150\text{ cm}^{-1}$ region due to C-C skeletal modes. The intensity and location of these peaks are extremely sensitive to the structural conformation of the chains and, therefore, varies depending on whether they are *trans* or *gauche* configurations, or if the chains are saturated or unsaturated. *Cis* and *trans* structures of the C=C group can be elucidated by identification of lipid-related peaks at 1655 and 1668 cm^{-1} , respectively. These Raman signatures can be very sensitive to the biological state of the cell; and as such, spontaneous Raman spectroscopy can be a powerful approach for studying cell physiology.

Raman spectroscopy is particularly attractive for detecting and imaging the distribution of small molecules, where labeling with fluorescent dyes is not feasible because the molecules of interest are smaller or similar in size as the fluorescent molecule. Here, it is of advantage to use molecules that have one or more distinctly different molecular groups compared to typical biomolecules, so that the compound of interest can be isolated against the background of cells and tissues by the unique Raman-active vibration of this group. This condition is often met for synthetic drugs, the uptake and metabolic reaction products of which are then easy to detect by following the distribution of the Raman-active group. If small molecules do not exhibit unique Raman spectral modes, then their interaction with cells can often still be investigated by probing their effect on the cellular physiology. E.g. if the small molecule is toxic above a certain minimum concentration, then its interaction with cells will often initially lead to the release of cytochrome c from mitochondria, followed by membrane blebbing, and ultimately the disintegration of cells. Another, often utilized way to specifically detect small molecules by Raman spectroscopy is to artificially provide them with a unique Raman signature through substituting some atoms by stable isotopes, where e.g. hydrogen is replaced by deuterium (6). This leads to a dramatic change in a corresponding peak's wavenumber position because deuterium has twice the atomic mass of hydrogen. Other stable isotopes, e.g. the replacement of ^{12}C by ^{13}C , lead to subtle, but still noticeable shifts in the Raman spectra of molecules containing these elements. A particular benefit is the creation of "new" peaks in areas where there is no Raman activity for most naturally occurring compounds. For biological materials this is true for the spectral range between $1800\text{--}2800\text{ cm}^{-1}$. The deuteration of biomolecules, such as lipids, will then lead to a new peak occurring at $\sim 2200\text{ cm}^{-1}$ that can easily be identified in the otherwise flat spectral region. Such peaks are indeed so well isolated that they can also be used for highly selective imaging of the distribution of molecules carrying this signature. The rapid growth of "click chemistry", i.e. the formation of a covalent bond between alkyne and azide groups in the presence of copper through a cycloaddition reaction, has led to increasing commercial availability of compounds carrying these groups, which can now be exploited for Raman-based detection and imaging (7, 8). These "tools" were initially developed for the specific fluorescent labeling e.g. of nucleotides as part of a cell proliferation assay, or to specifically label small molecules, such as sugars and lipids in cells. The triple bonds in alkynes and azides also serve as a unique molecular group giving rise to Raman stretching vibrations around 2100 cm^{-1} , which are also ideal molecular tags for coherent Raman imaging in the form of coherent anti-Stokes Raman scattering (CARS) microscopy or stimulated Raman scattering (SRS) microscopy (9, 10).

The combination of stable isotopes together with such unique molecular groups opens up a wide range of possibilities for creating spectrally narrow optical probes.

In this review we discuss how Raman spectroscopy has evolved during the last decades to the point where it is now possible to directly probe the physiological state of isolated cells, to follow changes in their physiology, and to expand these methods to the tissue context for medical diagnostics. We will focus primarily on applications that make use of intrinsic Raman markers to assess the physiological state of cells, their interpretation, and the response of these markers to external stimuli; rather than describing the uptake of drugs by specific drug-related marker modes. Direct imaging of drug interactions and drug uptake, the characterization of microorganisms, and the use of nonlinear Raman techniques are all being discussed by other, specialized review articles in the same issue of *Advanced Drug Delivery Reviews*.

2. Probing the physiology of single cells by spontaneous Raman spectroscopy

The unique ability of Raman spectroscopy to non-destructively determine the chemical composition of organic and inorganic materials with a spatial resolution of 1 μm or less, has made it a method of choice for analyzing the biochemistry of fixed and living cells. Even more so, because chemically specific information can be obtained from minutes volumes of 1 femtoliter (10^{-15} l) or less, even individual cellular compartments and organelles can be probed and their modifications monitored with time. The nondestructive characterization of single cells by Raman spectroscopy was first demonstrated in the seminal work by Puppels et al., who managed the collection of diffraction-limited, location-specific Raman spectra from within single cells and from isolated chromosomes by micro-Raman spectroscopy (4). Since then, Raman spectroscopy has been applied to study the physiology of a wide range of cell types, such as prokaryotic and eukaryotic cells, as well as plant cells, and the interaction of drugs and other small synthetic molecules with cells (5).

The great advantage of Raman spectroscopy, namely the fact that it delivers label-free chemical signatures of samples, does, however, also create a problem of having to discriminate against undesired background contributions. Especially in the case of biological samples, such as single cells, samples typically have to be prepared on a substrate which has its own Raman signature. The addition of confocal detection optics to micro-Raman spectroscopy systems helps to suppress any such signals that emanate just outside the vertical focus of the optical system (4). Transparent substrates, such as glass, also have a much higher density than biological samples, leading to significantly higher Raman signals. Borosilicate glass in particular, also exhibits fluorescence from impurities, when excited at near-infrared wavelengths, which are often used for micro-Raman spectroscopy. In this case, the implementation of confocal detection optics often not sufficient to suppress the background signal and other solutions to this problem have to be found. Fortunately, there are a few substrates that have little to no Raman signature in the spectral fingerprint region of biological samples. Such substrates are typically made up of materials that only contain molecular bonds with vibrations in the low wavenumber range close to the laser excitation wavelength, which standard notch or edge filters will suppress.

Magnesium fluoride or calcium fluoride (MgF_2 , CaF_2) are among the most widely used materials for this purpose. They are, however, rather expensive and also quite brittle. Another possibility is the use of highly reflective substrates, such as metal-coated mirrors, which also has the benefit of increasing the signal collection for biological samples, where Raman-scattered photons are typically emitted in all directions. Due to the previously mentioned near-IR fluorescence, glass cover slips are only appropriate when shorter wavelength excitation sources are used. Here, fused silica microscope cover slips also represent a good alternative because of their relatively weak Raman signals and low autofluorescence background.

During the last decade a variation of single cell Raman spectroscopy was developed that does not require cells to be immobilized to substrates. Because biological cells are mostly transparent for near-infrared laser beams, highly focused near-IR beams can be used to immobilize single cells in suspension by optical forces. The same or additional, other laser wavelengths are then used to obtain Raman spectra from these optically trapped cells (11). The phrase "Laser Tweezers Raman Spectroscopy" (LTRS) was coined to capture all variations of such combined systems with a single name. In the case of an optical tweezers system utilizing just a single laser beam for both, Raman scattering and optical trapping, it was shown that cells are typically trapped either by their nucleus or by cytoplasmic lipid vesicles (12). This method has the added benefit, that standard glass cover slips can be utilized as substrates, because the cells can be trapped high above the substrate surface (up to several tens of microns above the substrate), where the confocal detection optics efficiently rejects any residual Raman signals from the substrate. LTRS is particularly well suited for probing single *non-adherent cells* in suspension (11, 13-15). Note that LTRS is a technique most commonly used for whole organism (cell) Raman fingerprinting to obtain biochemical information of the cell, but the tradeoff is a loss of spatial information of the distribution of chemical species in the cell. Creely et al., however, did demonstrate the ability to perform Raman imaging of a trapped cell by developing a sophisticated system involving two lasers, in which one laser source was used to trap and immobilize the cell using holographic optical tweezers and the second laser source was scanned across the specimen to image the cell (16).

To realize optical trapping at laser powers that are not substantially higher than those typically employed in micro-Raman spectroscopy, tight focusing of the laser beam by objective lenses with a high numerical aperture is key. This condition generates a high electric field gradient in the transverse direction that draws the particle to the center of the focused beam. At this equilibrium position no net lateral force is exerted on the cells due to the axial symmetry of the laser beam which in most cases has a Gaussian intensity distribution around the optical axis. The axial gradient force negates the scattering force, resulting in stable trapping in the axial direction. Even subcellular objects can be analyzed dynamically by LTRS, e.g. the swelling behavior of individual mitochondria after exposure to Ca^+ ions (17), or, at even smaller length scales the composition of individual lipoprotein particles isolated from human blood (18). By this means, Chan et al. were able to show that the distribution and composition of lipoprotein particles changes as a result of the fat content of a person's diet.

A last benefit of LTRS is that unlike fluorescence, the Raman signal strength is not diminished by photobleaching. This makes LTRS an attractive tool for the continuous realtime monitoring of dynamic events in cells that can be triggered or stimulated externally. Several examples for such dynamic experiments e.g. by triggering protein expression or by treating cells chemically or by heat are summarized below.

2.1. Characterizing bacteria, bacterial spores, and their dynamics by Raman spectroscopy

Bacterial infections are a major health issue that is caused by a wide range of different bacteria. The development of antibiotics was a significant accomplishment in modern medicine; however despite such advancements, bacterial infections are still a leading cause of death, especially in children and the elderly. In particular, hospital acquired infections (HAI, i.e. nosocomial infections) remain a major problem, leading to almost 2 million HAI in the United States and over 90,000 deaths each year (19). These numbers are exacerbated by the fact that widespread and inappropriate use of antibiotic drugs has resulted in the development of bacterial strains that are antibiotic resistant due to changes in the microbes' genome. In addition, multidrug resistant (MDR) bacteria (termed superbugs) are also developing. The World Health Organization recognizes antibiotic resistance as a “growing public health threat of broad concern”. For example, methicillin resistant *Staphylococcus aureus* (MRSA) is a bacterium that is resistant to beta-lactam antibiotics, which includes the penicillins. *Clostridium difficile* (*C. difficile*) is a pathogenic spore-forming bacterium producing multiple toxins that can lead to diarrhea and inflammation in infected patients. Both, MRSA and *C. difficile* are leading causes of nosocomial infections. New analytical techniques that can 1. detect and discriminate between different bacteria down to the species and strain level accurately and quickly, 2. monitor cell dynamics to better understand fundamental cell physiology, and 3. identify drug resistant and susceptible cells based on differences in their biochemistry and/or physiological behavior are needed to battle this growing problem in human health. The studies that are discussed in this section illustrate the application of Raman spectroscopy for bacterial cell and spore characterization in recent years, and demonstrate the potential of this technique to make an impact in the area of infectious diseases. For an even more detailed review on this topic please see the accompanying article on characterizing bacterial cells in this issue of Advanced Drug Discovery Reviews.

Individual bacterial cells are amongst the smallest biological objects of which a physiological state can be determined by Raman spectroscopy. Chan et al. first demonstrated that micro-Raman spectroscopy in combination with optical trapping could rapidly identify *individual* endospores in suspension and discriminate them from other particles of similar size which were otherwise indistinguishable from spores in high resolution transmitted light microscopy (13). Also, a distinct spectral difference can be found between dormant cells (bacterial spores) and cells in the vegetative state. In the dormant state, the Raman spectra of bacteria are dominated by signals that can be attributed mostly to calcium dipicolinic acid (CaDPA), which the cell synthesizes to preserve and protect the genetic material inside the spore. The high concentration of CaDPA inside the small volume of such cells results in a distinct vibrational

spectrum, which often overpowers Raman signals from other biomacromolecules. This is shown in Figure 1a, which recreates the process of probing bacterial spores by coherent anti-Stokes Raman scattering (CARS) microscopy (for explanations of this process please refer to the section on coherent Raman scattering further below). The spectrum shown in blue in the Figure is the spectrum of a single bacterial spore. The image in Figure 1b is the corresponding CARS image of bacterial spores immobilized on a glass cover slip surface. As a spore regenerates into the vegetative state, the spectrum initially depicting CaDPA peaks changes entirely and suddenly - a process that can easily be followed by continuously collecting Raman spectra of single spores. By combining LTRS with light scattering and imaging, the dynamics of spore germination could be followed in great detail (20, 21). Peng et al. showed in 2009 that by combining Raman scattering and elastic light scattering spectroscopy they were able to monitor the temporal relationship between the release of CaDPA, spore morphology, and the refractive index of the cells during germination of individual spores in optical traps (20). Interestingly, the rapid acquisition of successive Raman-scattered signals from the endospores enabled these researchers to show that CaDPA release occurred in two steps, a slow phase where the CaDPA concentration initially decreased by only ~15% and a second phase where the remaining CaDPA was entirely released. This also allowed them to determine the effect of different enzymes on CaDPA release rates during germination. Subsequent studies (22) found that the time required for the release of CaDPA was independent of the concentration of nutrients, the level of germinant receptors, or heat activation. Performing such experiments at the level of single cells has the advantage that single cell statistics can be determined to reveal whether there are discrete subpopulations in an ensemble, whereas typical biochemical assays only obtain ensemble averages. This led to the discovery that bacterial spore germination is a rather heterogeneous process, based on a variable time lag factor between the exposure of spores to germinants until the beginning of CaDPA release. The lag factor was found to be affected e.g. by heat activation, the number of germinant receptors per spore, and the levels of germinant (23-26).

Several studies have shown that subtle differences in the Raman spectrum of bacteria even make it possible to identify and distinguish between different types of bacteria. To name just one example, the extensive work of Gerwin Puppel's group over the past decade, the pioneering group in single cell Raman spectroscopy, has illustrated that micro-Raman spectroscopy can identify different microorganisms of clinical relevance (27-30). Many of these studies used a confocal Raman microscope to interrogate bacterial microcolonies. So, although populations of single cells were analyzed in these studies, they are not Raman studies at the single cell level in the strictest sense. More recently, Hamasha et al. showed that Raman spectra of a dried layer of bacteria can discriminate between four closely related *E. coli* strains, one of which is pathogenic (31). The sensitivity and specificity was greater than 95% for two multivariate chemometric techniques that were implemented to analyze and classify the Raman data. Lu et al. showed that Raman spectroscopy performed on a pellet of bacteria dried on a substrate could be used to rapidly detect and identify *Campylobacter* strains (a cause of bacterial foodborne disease in many developed countries) recovered from infected animals and humans from both, North America and China (32). Raman spectroscopy correlated well with multilocus sequence typing

and had a recognition rate of > 97%. Pushing the discrimination of bacterial cell types down to the single cell level, Xie et al. used LTRS to measure spectra of single bacteria cells and showed that Raman signatures of different bacterial species (*Bacillus cereus*, *Enterobacter aerogenes*, *Escheria coli*, *Streptococcus Pyrogenes*, *Enterococcus faecalis*, and *Streptococcus salivarius*) were sufficiently different for each species to be accurately identified (33).

The high spatial resolution of confocal micro-Raman spectroscopy also enables one to follow physiological dynamics in individual bacterial cells. This makes it possible, e.g. to follow the rapid onset of the overexpression of proteins in genetically modified bacteria which can be observed by inducing the expression by addition of isopropyl thiogalactoside (IPTG) to the growth solution. This was demonstrated in *E. coli* cells transfected with a vector encoding for the myelin oligodendrocyte glycoprotein. The sudden addition of IPTG to the bacterial cells leads to overexpression of the protein, which could be monitored by following the rapid increase in protein-specific Raman peaks (34).

The structural changes that occur on the molecular scale during bacterial lysis were investigated by Chen et al. by combining LTRS with light scattering. The induction of bacterial lysis was initiated by two different methods: through the addition of lysozyme or bacteriophages to bacteria (35). This experiment unveiled a direct correlation between the initial cell wall disruption and the subsequent release of intracellular material. Raman spectroscopy of individual bacterial cells also allowed the authors to follow the interaction of the lysozyme with *E. coli* cells during different stages of the process in real time, and to observe bacteriophage production. The authors found that, depending on the lysis process, the final results were different. Complete cell destruction was only observed if lysis was induced by bacteriophages.

If bacterial cells are kept in minimal medium, under essentially starving conditions, they are particularly susceptible to rapidly taking up food sources once they become available. This was recently used by Avetisyan et al. to determine how rapidly single cells of the soil-based bacteria *Sinorhizobium meliloti* take up trehalose (amongst other sugars) and how the bacteria metabolize trehalose (36). By acquiring spectra of pure trehalose and keto-trehalose, they were able to show that the bacteria take up trehalose and metabolize it to its keto-form within less than 5 minutes after addition of the sugar. Cells that were lacking the *thuEFGK* transport gene for trehalose were unable to metabolize the sugar (37). Figure 1c shows representative spectra of *Sinorhizobium meliloti* that were acquired within just 1 minute between the individual spectra and demonstrate the rapid rise of the 1734 cm⁻¹ C=O vibration that is representative of the sugar conversion to keto-trehalose.

In order to determine if LTRS could be used to detect the effects of drugs on single cells, similar work was conducted on bacterial cells after exposure to antibiotic drugs (38, 39). Here, it was found that during different phases of their growth curve, changes that occurred in *E. coli* cells could be monitored by following the temporal evolution of Raman bands associated with DNA, RNA, and proteins. Within the same stage of the growth cycle, bacterial cell spectra were found to be highly reproducible. Changes in select Raman bands were observed after addition of the antibiotic (penicillin/streptomycin, cefazolin). This enabled the discrimination of drug-exposed

cells from cells that were not exposed to the antibiotic within just hours after the initial drug application. The authors found that different drugs caused slight differences in the evolution of Raman bands for cefazolin, leading them to the conclusion that LTRS could be used to identify to which drugs the cells have been exposed. Munchberg et al. expanded on the use of single cell Raman spectroscopy (albeit not in a laser trapping configuration) to study bacterial response to antibiotics by analyzing two strains of *E. coli*. and two species of *Pseudomonas* that have different modes of action after treatment with antibiotics (40). The Raman data of treated cells allowed for species identification accuracy in the range of 85-97% after a linear discriminant analysis (LDA) model was applied. Detection of a resistant strain of *E. coli* was also demonstrated with their model. The model for predicting cell type is enhanced by incorporating spectral information associated with antibiotic induced cell stress and specific antibiotics.

2.2. Probing the physiology of individual mammalian cells by Raman spectroscopy

Single cell Raman spectroscopy of individual mammalian cells has evolved significantly since the original work by Puppels et al and the current number of such studies is far too great to fully account for all publications. Instead, we will highlight a number of select recent applications that illustrate the type of information that can be obtained by this technology. After utilizing confocal micro-Raman spectroscopy to characterize the Raman signatures of various cell types, researchers soon began studies with the aim of comparing Raman spectra of cells to determine whether they are neoplastic or normal or to discriminate stem cells from their differentiated descendants (41). Chan et al. showed that the Raman spectra of individual T cell and B cells from the Jurkat and Raji cancer cell lines carried distinct differences compared to normal T cells and B cells (42). Soon thereafter they also managed to reveal similar differences in blood samples from leukemia patients diagnosed with B cell or T cell leukemia, where over 90% of the patient cells were accurately classified by their respective cell type (43). Discrimination of a larger variety of cell types, including leukocytes, erythrocytes, leukemic cells, and cells originating from breast carcinoma tumors, using single cell Raman spectroscopy combined with support vector machines was also demonstrated (44). For urological cells, Harvey et al. showed the feasibility of Raman spectroscopy to distinguish between live prostate cancer and bladder cells, benign prostate hyperplasia cells, and primary urethral cells with >90% sensitivity and specificity (45). These studies were then followed by other work that integrated Raman spectroscopy with microfluidic systems to demonstrate the identification and differentiation of these cells in flow, which brought the concept of Raman activated cell sorting (equivalent to fluorescence activated cell sorting) to reality (46-48).

By using an elongated illumination spot, Notingher et al. used Raman microspectroscopy to detect Raman biochemical markers during the differentiation of murine embryonic stem cells that could be used to assess the differentiation status of the entire cell (49). Similarly, human embryonic stem cells could be distinguished from cardiomyocytes derived from these stem cells by a combination of Raman spectroscopy and multivariate analysis (50, 51). Schulze et al. shortly thereafter showed that the glycogen content in embryonic stem cells resulted in distinct

Raman spectral features that allowed them to distinguish human embryonic stem cells from their derivatives (52, 53).

For the analysis of gamete cells, Huser et al. recently showed that Raman spectra could be obtained from individual sperm cells, and that the vibrational signatures could be used to assess DNA packaging efficiency in the sperm heads (54). Sperm cells attached to a substrate were initially imaged by autofluorescence. Subsequently, the sperm heads were probed by confocal micro-Raman spectroscopy. A correlation was found between the packing efficiency and the shape of the sperm head. Interestingly, it was discovered that the DNA packing efficiency in sperm cells with normal head shape morphology varied greatly, suggesting that selecting viable sperm cells for *in vitro* fertilization purely based on morphology, may not be sufficient for successful outcomes. Single cell Raman spectroscopy could therefore be a powerful tool to aid in the selection of sperm cells for *in-vitro* fertilization. Mallidis et al. reported on the use of Raman spectroscopy for accurate determination of sperm DNA structure and damage sites associated with UV irradiation (55). Changes in the 1042 cm^{-1} region, attributed to the phosphate backbone, indicated DNA damage. A second region in the 1400-1600 cm^{-1} region corresponding to protein-DNA interactions also showed changes reflecting DNA damage.

To enable Raman-based imaging of living cells within just a few minutes of total image acquisition time, a line-scanning geometry and simultaneous spectral acquisition of hundreds of Raman spectra along the illuminated line, has been utilized. Hamada et al. demonstrated dynamic Raman imaging of the molecular distribution inside living HeLa cells by this approach (56). Their choice of an excitation wavelength at 532 nm which is typically detrimental to cell health (57) enabled them in this case to optimize their sensitivity for detecting cytochrome c, protein beta sheets, and lipids while still generating minimal autofluorescence background. Time-lapsed Raman images of these cells during cytokinesis were obtained by acquiring images with a 185 second total acquisition time, with an interval between images of 115 seconds.

Investigating the physiology of red blood cells (RBCs) by Raman spectroscopy has been quite popular during the last decade, mostly because these cells are relatively easy to obtain, but also because of their importance for distributing oxygen to tissues within the mammalian body. The average diameter of RBCs is approximately 8 μm . These cells also have a rather flexible membrane, which enables them to squeeze through very narrow vessels in organs such as the liver or the lungs. The high hemoglobin content enables RBCs to efficiently bind oxygen. Furthermore, the resonance Raman effect that is observed when RBCs are excited at a number of different wavelengths that match the electronic absorption bands of the high symmetry, chromophoric structure of hemes generates very strong, enhanced Raman vibrational bands, whose spectral positions and peak intensities are strongly dependent on the oxygenation state of the RBC. Wood et al. used single cell Raman spectroscopy to monitor the deoxygenation and oxygenation of hemoglobin inside human red blood cells for a duration of 30 minutes (58, 59). Photoinduced and thermal degradation of the cells was observed after prolonged exposure to the laser beam, and Raman markers indicative of heme aggregation were identified. These results suggest the potential use of single cell Raman spectroscopy for the analysis of erythrocyte disorders characterized by heme aggregation, such as sickle cell disease and

malaria. This was indeed picked up in a recent publication by Liu et al. to demonstrate their ability to assess red blood cell physiology in the case of sickle cell anemia (60).

In the past, optical tweezers were used to stretch RBCs by creating two optical traps on opposite sides of the cell and to measure their response to deformation (61-63). The spectroscopic and mechanical properties of normal and thalassemic RBCs were assessed by LTRS (64). This work showed based on differences that occurred in Raman bands associated with oxyhemoglobin and deoxyhemoglobin, that thalassemic RBCs cannot retain oxygen as well as normal cells. These RBCs also exhibit a significantly greater heterogeneity in their oxygenation behavior compared to normal RBCs. Damaging the cells by exposure to 532 nm light lead to a much stronger response in thalassemic RBCs than in normal cells as monitored by the intensity of the 1548 cm^{-1} Raman peak. In separate experiments it was found that thalassemic RBCs are less deformable than normal RBCs when stretched by two optical traps. The effect of mechanical forces on the ability of RBCs to retain oxygen was first demonstrated by Rao et al. using LTRS (65). This was achieved by combining LTRS with a dual-beam optical trap which was used to stretch RBCs while their Raman spectra were measured. Rao et al. showed that stretching an oxygenated RBC by up to 40% of its original size resulted in deoxygenation of the cell. This behavior was explained by enhanced interaction rates between neighboring hemoglobin and the cell membrane resulting as a consequence of stretching the cell.

Shortly thereafter, Liu et al. confirmed these results using single beam LTRS (66). They reported that RBCs can also fold and deform when trapped in a single optical trap. Increasing laser power appeared to induce deoxygenation of the trapped cells. Toggling the cells between oxygenated and deoxygenated states was possible by rapidly switching the optical trap between low and high laser powers. During the time scales on which these experiments were conducted, no adverse effects due to photodamage to the cells were observed. Wood et al., however, reported that trapping RBCs with as little as 5 mW laser power for more than 90 seconds caused irreversible photodamage even with near-infrared laser wavelengths (58, 59). Many similar such results obtained by utilizing optical tweezers are, however, currently being revisited due to the fact, that many of the effects that were previously believed to be induced by the mechanical forces exerted by the optical traps appear to indeed be photo-induced (67).

In an attempt to still utilize optical tweezers, while trying to avoid photo-induced damage, Raj et al. stretched RBCs by binding microparticles to opposite ends of the cells, where the particles were then manipulated by optical tweezers rather than the cell itself. This enabled Raj. et al to still interrogate RBCs by LTRS while inducing their deformation by other means (68). Interestingly, these authors observed yet another Raman peak that did not exhibit a spectral change in previous experiments to undergo a change. It was speculated that this additional change could be due to the tight connection between the cell's membrane and the cytoskeleton, which both respond in a correlated fashion during external deformation.

In addition to the effects on oxygenation, other aspects of RBC biology were also studied. Dasgupta et al., e.g. compared spectra of individual RBCs from healthy donors and patients infected with *P. vivax* malaria (69). Their results indicated a reduced oxygen-binding capacity of hemoglobin in malaria-infected cells. This result is consistent with acute respiratory syndrome

which is observed in patients infected with *P. vivax* malaria and the also often occurring anemic conditions of these patients. The oxidative stress response of individual RBCs was also probed by LTRS (70). In these experiments, oxidative stress due to exposure to hydroxyl radicals was induced in RBCs. Cells exposed to these radicals were found to show increased intensities in Raman bands at 500, 519, and 550 cm^{-1} which were interpreted to result from an increase in disulphide bridge bonds in stressed RBCs.

Very recently, LTRS was also demonstrated to enable the in vivo characterization of RBCs in microvessels (71). Single RBCs were trapped directly in arterioles and venules of a mouse ear. RBCs in arterioles were found to be more oxygenated than those in the capillaries of venules. This work opens up interesting prospects for potential in vivo studies in the future, even in humans.

Cellular dynamics in mammalian cells other than RBCs were also investigated by LTRS. Peng et al. successfully monitored the accumulation of intracellular ethanol in yeast cells. This opens up the potential to directly follow ethanol production in single yeast cells during aerobic fermentation (72). Also, the rapid assessment of oxidative stress response of single yeast cells was demonstrated by Chang et al. (73). Here, cells were exposed to hydroxyl radicals while being optically trapped and interrogated for over 40 minutes. These experiments showed a significant decrease in cellular Raman peaks at 1266 and 1651 cm^{-1} . Experiments conducted on individual liposomes produced similar results and showed that these effects can be explained by the peroxidation of C=C bonds in lipids. When ascorbic acid, an efficient antioxidant that scavenges reactive oxygen species, was added to the liposomes, the decline of intensity of these Raman peaks was significantly reduced, indicating that ascorbic acid can indeed partially prevent the oxidation of C=C bonds.

As mentioned in the introduction, the introduction of molecules carrying stable isotopes of either small molecules, or the Raman-active labelling of proteins with stable isotopes enables the detection and imaging of these molecules by spontaneous Raman spectroscopy. This was demonstrated e.g. by van Manen et al. who utilized metabolic labelling of proteins with stable isotopes in single human cells (6). Similarly, molecules carrying alkyne groups can also easily be detected and imaged inside living cells. This is particularly interesting for isolating and imaging proliferating cells in a culture, where the cells have been provided with 5-ethynyl-2'-deoxyuridine (EdU), a replacement for the popular BrdU assay for proliferating cells. During DNA synthesis this compound is integrated into the newly synthesized genomic material of daughter cells. To detect these cells by fluorescence, the "click" reaction has to be conducted, i.e. the cells typically have to be fixed and often also permeabilized in order for the complementary azide-carrying fluorophore to reach EdU in the cells' nuclei. By isolating the unique spectral signature of the alkyne group, Yamakoshi et al. were able to image these cells without these additional steps (7). Soon thereafter, they extended this approach to the visualization of a range of alkyne-carrying small molecules in living cells (8). Figures 2a and 2b show Raman spectra of fatty acids containing either 2 deuterium atoms, giving rise to a small peak at $\sim 2200 \text{ cm}^{-1}$ (Fig. 2a), and a fatty acid with a terminal alkyne group, giving rise to a strong peak at 2115 cm^{-1} (Fig. 2b).

2.3. Effects of drug-cell interactions probed by Raman spectroscopy

Label-free spectroscopy techniques have received increased attention in cell-based drug screening studies (74-76). This is, again, partly due to the added advantage of being able to non-invasively and continuously monitor cellular kinetics in real-time. This feature enables new experiments to be conducted involving e.g. the probing of the effects of combinations of drugs administered at different time points to cells to determine optimal treatment conditions and concentrations before burdening a patient with high drug doses. Current label-free cell-based technologies used to study cell-drug interactions for drug discovery monitor cellular properties based on impedance, refractive index (resonant waveguide grating, surface plasmon resonance), acoustic, or white light imaging measurements (76). These transduction signals are an *indirect* measure of various aspects of a cell's biological properties. A label-free technique that can provide biologically relevant readout signals that *more directly* reflect the biochemical properties of a cell can potentially provide more accurate, reliable cellular information resulting in a tremendous benefit to drug-cell interaction studies.

There are several unique features of Raman spectroscopy that make it an ideal technology amenable for studying drug-cell interactions. Raman spectral signatures are a new class of optical markers intrinsic to the cell that reflect the detailed biochemistry and biological state of the cell. The introduction of synthetic drugs that have their own, unique Raman spectral signature to cells in solution interrogates native effects and interactions between cells and drugs without having to contaminate the cells with other exogenous molecules.

Several pilot studies have demonstrated the potential impact of Raman spectroscopy for real-time assessment of the response of cancer cells to drugs. These publications showed that changes in the Raman profile of cancer cells exposed to drugs could be observed that reflect the biochemical activity of the cell. These changes could then be correlated to known chemical responses of the cells to drugs at the molecular level (e.g. the cleavage of certain molecular bonds) and at the cellular level (e.g. overall cell apoptosis). As an example, a recent Raman-based study showed that the spectra of single prostate cancer cells exposed to Nerium Oleander showed an increase in the protein peak at 1656 cm^{-1} , the phenylalanine peak at 1004 cm^{-1} , increases in lipid peaks at 1295 , 1340 , and 1368 cm^{-1} , and a decrease in the protein and lipid peak at 1454 cm^{-1} as a function of increasing drug concentration from 20 to $40\text{ }\mu\text{g/ml}$ (77). No noticeable changes occurred in the peaks typically attributed to nucleic acids. Yao et al. studied the treatment of gastric carcinoma cells with fluorouracil (5-FU) and characterized the spectral changes observed in the cancer cells due to the reduction in vibrational band intensities of cellular lipids, proteins, and nucleic acids signifying apoptosis (78). A 45% decrease in the 782 cm^{-1} band indicated a breakdown of the DNA bases and phosphodiester bonds. A Raman study of the cancer drug etoposide on human pneumocyte-like cells (A549 line) showed that DNA and RNA concentrations decreased by 27% after 24h and as much as 87% after 48 h of exposure to the drug, indicative of the double-strand DNA breaks that the drug is known to induce (79). An increase in lipid Raman bands with time was also observed. Another study using etoposide on human medulloblastoma (DAOY) cells also observed sharp decreases in DNA modes and protein vibrations 48 h after exposure, corresponding to cell death (80). Zoladek et al. also recently used

confocal Raman micro-spectroscopy to obtain time-dependent spectral images of living human breast cancer cells undergoing apoptosis after being exposed to the drug etoposide (81). Raman images of DNA (788 cm^{-1}) and lipids (1659 cm^{-1}) were acquired from the same cell every 2 hrs over a 6 hr period. It was observed that, relative to control cells, drug treated cells exhibited an increase in DNA band intensities due to DNA condensation and lipid band intensities reflecting the high accumulation of membrane phospholipids and unsaturated non-membrane lipids.

Raman microscopy has also been used to reveal the mechanism and efficacy of chemotherapeutic agents. Nawaz et al. characterized the spectral changes in the cell membrane and cytoplasmic regions of A549 adenocarcinoma cells exposed to cisplatin after 96 hours exposure (82). Multivariate models were established to illustrate the variation in spectral content with levels of exposure and degrees of cytotoxicological responses. Multivariate PLS regression was used to demonstrate that Raman spectroscopy can be used to predict both exposure dose and viability of cell culture.

More recently, Nawaz et al. carried out a study investigating the interaction of vincristine with a human lung adenocarcinoma cell line (A549), with the purpose of studying the interaction of the drug on the cells and characterizing the biochemical changes based on Raman spectra (83). The Raman data show that vincristine interacts with nuclear DNA, resulting in changes in Raman bands associated with DNA vibrational modes indicating intercalation and external binding. In addition, flow cytometric analysis of the expression of bcl-2 protein, an anti-apoptotic protein generated following DNA damage, shows that expression was dependent on the vincristine concentration, and that this expression drops at higher concentrations. Together, the Raman and flow cytometric data reveal that vincristine binds cellular DNA by intercalation, and bcl-2 upregulation in response to DNA damage at low doses inhibits cell death. Therefore, microtubule binding and damage is proposed as the mechanism of action at low doses, while at high doses, DNA intercalation appears to be the mode of action.

Moritz et al. have recently shown that LTRS can also be used to detect drug-induced cellular changes in human lymphocytes. They applied a chemotherapy drug system, doxorubicin, which is used in the treatment of leukemia, to individual Raji T cells and monitored the changes that occurred in the Raman spectra of these cells over a prolonged time span (between 24-72 h) for two different concentrations of the drug (84). Principal component analysis (PCA) revealed that drug-exposed cells exhibited three distinct Raman signatures correlating to different phases during drug exposure. At low drug concentrations, as well as during the initial application of higher drug concentrations, the Raman spectra exhibited increases in lipid-related Raman bands. During later time points, Raman peaks attributed to DNA vibrations became more pronounced. Even longer exposure to the drug resulted in an overall reduction of the overall cellular Raman spectra, while the phenylalanine peak at $\sim 1003\text{ cm}^{-1}$ increased considerably. These spectral changes can be interpreted by initial chromatin condensation, subsequent membrane blebbing, and, ultimately, the formation of apoptotic bodies, which occur during drug-induced cell death. The different stages which the cell undergoes and the resulting spectra associated with each step leading up to apoptosis are depicted in Figure 3. Exposure to drugs often leads to very heterogeneous cellular response which results in almost every cell showing

rather different Raman spectra. This complicates the analysis of such events and requires large data sets to be obtained and multivariate analysis methods to be applied in order to determine the effects of drug exposure on cells. Despite the variations in the individual biochemical response of cells, the collective evolution of the spectra was, however, found to remain fairly consistent for all cells.

This discussion demonstrates quite clearly, that Raman micro-spectroscopy of single cells can assess the physiological state of individual cells and it also finds clear evidence of heterogeneity, i.e. cells being in different physiological states at the same time, although having been treated in the same way. The issue of the heterogeneous response of cells has also been encountered in earlier work, e.g. in the identification of cancer cells from leukemic patients, and most other cancer-related studies that assessed several tens of cells (43). So far, however, spontaneous Raman spectroscopy is still a relatively slow technique, requiring, in the best of cases, still at least an acquisition time of at least 1s for single cells. Because of this issue, truly high throughput measurements on the order of hundreds of thousands of cells have not yet been conducted. Here, as far as spontaneous Raman spectroscopy is concerned, efforts to perform compressed sensing in the form of compressing a Raman-spectrum by phase-sensitive means or by using “adjustable gratings”, e.g. in the form of micro-mirror devices, appear quite promising because these approaches lower the data acquisition time per spectrum by 1-2 orders of magnitude (85, 86). Ultimately, even faster approaches will require the combination with multiplexed coherent Raman spectroscopy, which will be discussed in more detail below, as well as the combination with microfluidic delivery of cells to truly achieve rapid Raman-assisted cell sorting (48, 87).

In addition to being able to detect intracellular changes to the intrinsic biochemistry of a cell after being exposed to drugs, another important application of Raman spectroscopy has been its application for imaging and studying drug distribution and drug delivery systems in cancer cells. These studies are important for the increasing role of nanotechnology for the targeted delivery of drugs for a more specific and possibly personalized treatment of cancer. Chernenko et al., for example, showed that Raman microscopy could be used to image the intracellular distribution of cationic liposomes in HeLa cells (88). Three types of liposomes with three distinct cationic moieties were used in this study. Deuteration of the phospholipids was performed to yield distinct Raman peaks in the spectrally silent region of bioorganic materials ($2050\text{-}2300\text{ cm}^{-1}$), which allowed for the detection of the nanoparticles with higher sensitivity and specificity. The dynamic behavior of the uptake and internalization of these different liposomes was monitored using a combination of the deuterated Raman signals of the liposomes in combination with the intrinsic signatures of the cell itself (mitochondria, nuclei) to determine the preferential targeting of subcellular compartments such as the mitochondria due to the affinity of the cationic compounds with distinct affinities for those structures. Dorney et al. also used Raman spectroscopy to detect and image, in a label-free manner, the distribution of polystyrene nanoparticles internalized by A549 human lung adenocarcinoma cells (89). Using the Raman fingerprint of polystyrene and multivariate analysis, it was shown that Raman spectroscopy could accurately detect and locate the particles and also identify the local subcellular environment surrounding the nanoparticles. Direct detection of intracellular drug distribution

has also been demonstrated. Meister et al. showed that the uptake and cellular distribution of carbon monoxide releasing molecules (CORMs), a metal-carbonyl compound that has photoinducible cytotoxic activity towards cancer cells, could be imaged inside human colon cancer cells using the strong CO stretching vibration between 1800-2200 cm^{-1} (90). The position of the CO stretching vibration peak is ideal for imaging this compound because it falls in a spectral region where contributions from other cellular constituents are negligible. It should be noted that a fairly high concentration (2 mM) of the solution and a long incubation time (3 h) were used in this study. A major challenge for spontaneous Raman spectroscopy is the detection of molecular concentrations well below the milli-molar concentration range because of the intrinsic weakness of the Raman scattering signals. Therefore, spontaneous Raman imaging may be best suited for applications in which drug treatments at high concentrations are physiologically relevant or in situations where cells concentrate the drugs into specific cellular compartments to levels high enough to be detected. For example, El-Mashtoly et al. were able to image the spatial distribution of the drug erlotinib, an inhibitor of the epidermal growth factor receptor (EGFR), within human colorectal adenocarcinoma cells after incubation using 100 μM solution of the drug for 12 hours (91). Typically, it is challenging for spontaneous Raman spectroscopy to detect at 100 μM levels, so this presumably means that the intracellular concentration is higher due to uptake and concentration of the drug in the cell. The authors suggest that the internalization and clustering of EGFR in the cells upon addition of erlotinib may be responsible for the increase in the erlotinib concentrations, thus allowing for the drug to be detected by Raman microscopy. In this study, they used the triple-bonded alkyne vibration to image the drug distribution, which again falls in the silent region of the Raman spectrum of biological materials as mentioned earlier. A very exciting and interesting finding was that the Raman spectrum of erlotinib in the cell changes compared to a cell-free erlotinib spectrum, which indicates that the drug is metabolized to its demethylated derivative. This suggests that Raman spectroscopy could potentially be used to detect metabolic interactions of drugs with cancer cells and can provide insights into drug targeting mechanisms.

Another example of Raman spectroscopy detecting drug metabolism is a recent study by Harada et al. on the interaction of CPT-11, an anticancer reagent, with human cancer cells (92). Using a slit scan confocal Raman microscope, the authors reported that they could acquire images showing changes in the intracellular distribution of CPT-11 and also the intracellular conversion from CPT-11 to its metabolite SN-38 on the order of several minutes.

2.4. Probing the physiology of individual cells by coherent Raman microscopy

Many biological events occur on time scales that are too fast to capture with the relatively long signal interrogation times required for spontaneous Raman spectroscopy. Because of this fact a modality that could assess tissues and cells using label-free chemically sensitive imaging at video-rate would be extremely beneficial for biomedical diagnostics. In the case of Raman scattering this became possible by utilizing high-power, short-pulsed laser systems that permit coherent Raman signal generation in tissue. The first and still most popular of these nonlinear coherent Raman scattering schemes, which was used extensively for gas and flame spectroscopy, as well as biomedical applications is coherent anti-Stokes Raman scattering (CARS). In CARS a

molecular vibration of interest is driven coherently by two appropriately tuned short laser pulses that synchronize the coherent emission of Raman-scattered photons of large numbers of the same molecular bonds, resulting in a signal that is several orders of magnitude stronger than the randomly excited spontaneous Raman signal. When combined with confocal detection optics in a laser scanning microscope, CARS not only enables one to image specific molecular vibrations without the need for fluorescent dyes, it also provides 3D sectioning capability due to the nonlinear origin of its signal generation. A beneficial side-effect of the high pulse energies required to efficiently generate coherent Raman signals is that the same laser beams can also produce other nonlinear signals, such as Second Harmonic Generation (SHG), Third Harmonic Generation (THG) and Two-Photon Fluorescence (TPF), all of which can result in additional information about sample morphology and sample composition.

Although Maker and Terhune demonstrated the first successful generation of CARS signals in 1965, it took nearly 20 years until the first CARS microscope was realized by Duncan et al. Nonetheless it took yet another 17 years until coherent Raman microscopy became truly feasible and widely applicable for biomedical research. The seminal paper by Zumbusch et al. laid the foundation for a new implementation scheme for CARS microscopes using a collinear beam geometry and high numerical aperture microscope objective lenses to generate and detect CARS signals from volumes as small as 1 femtoliter (93). Cheng et al. demonstrated the label-free imaging of lipid biogenesis and visualized the chromosome distribution in NIH 3T3 cells by CARS microscopy (94). Due to their unparalleled signal strength, aliphatic CH groups in lipids, which exhibit a resonance at 2845cm^{-1} , made lipid droplets a popular target in CARS microscopy. For example, Nan et al. successfully investigated cellular lipid droplet motility by investigating the distribution of the CH_2 stretching vibration (95). Akeson et al. were the first to utilize the intrinsic Raman-scattering of glucose to follow the metabolism of glucose by yeast cells using CARS (96). Also, to enable the analysis of floating cells or non-adherent cells, Chan et al. found that the combination of CARS and optical tweezers is straight-forward (97). Also, by extending narrowband CARS microscopy to the multiplexed detection of CARS spectra, Rinia et al. were able to analyze the composition and interactions of lipids contained within cellular lipid droplets (98). It should also be noted, that similar to the detection of exogenous molecules that carry the Raman-active alkyne marker group by spontaneous Raman scattering, the similarly strong signal can also be exploited in coherent Raman scattering to detect and isolate exogenous molecules or to highlight specific molecules of interest (9, 99). Although CARS microscopy is still very popular because it is reasonably easy to implement in existing confocal microscopy setups, a particular problem intrinsic to the CARS signal generation process is the creation of a non-specific background signal. This signal is generated in any medium purely by the four-wave mixing process inherent to CARS and can only be avoided by obtaining multiple images across a Raman-active resonance where the signals are processed and subtracted to remove the non-specific background, or by implementing complicated polarization-sensitive detection schemes, time-gating experiments, or by frequency-modulation across a resonance. To avoid these complications, which still require mostly photon-counting detectors because of the weak CARS signals, stimulated Raman scattering (SRS) has become the main focus of current developments

in coherent Raman scattering microscopy (100, 101). This modality is a byproduct of the CARS signal generation process, but has to be detected as either a signal loss or signal gain of either the pump or probe wavelengths and thus requires lock-in detection schemes. It is inherently background-free, just like spontaneous Raman scattering, and can thus be used to detect and image the composition of drugs in tablets (102) or the distribution of newly synthesized proteins in cells, if strong Raman-active markers, i.e. isotope-substitution, is utilized (103). As will be discussed in more detail further below, the most significant applications of SRS microscopy are found in biomedical imaging, though.

3. Assessing the physiological state of tissues by Raman spectroscopy

3.1 Applications of spontaneous Raman spectroscopy to tissue physiology

During the last decade spontaneous Raman spectroscopy has increasingly been applied to biomedical research and medical diagnostics. Besides the sometimes difficult and cumbersome application of single cell Raman spectroscopy, it provides very useful information for the in vitro and in vivo analysis of cells in the context of tissue. This is due to the fact that probing cells by Raman spectroscopy does typically not change the cellular biochemistry and does not destroy the cells. Thus, Raman spectroscopy can be used parallel to conventional hematoxylin and eosin (H&E) staining in pathology to classify tissue. Even large tissue sections can be assessed quite rapidly by Raman spectroscopy without the need for fixation or staining, resulting in significant time savings and potentially higher specificity in identifying markers of disease.

Amongst some of the earliest applications of Raman spectroscopy to biomedical research was the characterization of atherosclerotic lesions. Of main interest, here, are finding ways to minimally invasively probe the state of atherosclerotic plaques and their progression towards disease in situ and in vivo. To achieve this, Feld and coworkers were the first to collect and analyze all major biomolecules and biominerals found in atherosclerotic tissue, such as elastin, collagen, cholesterol, cholesterol esters, lipids, carotenoids, and calcium apatite. They then applied a correlation model that successfully enabled the identification of the composition of atherosclerotic plaques based on their Raman spectra. To also enable the analysis of atherosclerotic lesions in vivo in the hospital, the same group then developed a portable Raman spectroscopy system using near-infrared wavelengths. With this system, they could demonstrate the rapid ($< 0.01\text{s}$) identification of hydroxyapatite in calcified coronary arteries. Following this success, Motz et al. reported the analysis of vulnerable plaques during surgery, where spectra were taken with 1s acquisition time and provided 79% sensitivity and 85% specificity of identifying diseased tissue (104). More recently, a number of new studies have significantly improved upon these early results. In particular, as will be discussed further below, the advent of CARS/SRS microscopy now enables the specific, label-free imaging of atherosclerotic lesions either in excised tissue, but also in vivo. Here, we will limit the discussion to spontaneous Raman spectroscopy. Notably, Ogawa et al. have implemented a high-speed version of spontaneous confocal Raman spectroscopy and used this to image rat heart tissue, which enabled them to identify cardiomyocytes, fibrotic tissue, and blood vessels by analyzing the cytochrome c and collagen content in tissue by multivariate analysis (105). Yamamoto then expanded this approach to analyze samples from human patients with cardiomyopathies that had to undergo

survival ventricular restoration (106). Similarly, by developing a Raman probe, Lattermann et al. and Matthaus et al. were able to analyze and image atherosclerotic plaques by Raman spectroscopy (107, 108). These recent, in combination with the early studies managed to determine hallmarks of atherosclerotic and infarcted tissue that could potentially be used in the near future to guide surgery.

Similarly, significant research into the possibility of using Raman spectroscopy to analyze tumors and determine tumor margins in tissue has also been conducted. A focus of early studies was cancer tissue that was fairly easily accessible, e.g. skin cancer, esophageal cancer, cancer of the cervix, and even breast cancer, where diagnostic methods still require surgical excision of tissue for assessment by pathologists. Breast cancer carcinoma tissue has been studied extensively by Raman spectroscopy to enable the minimally invasive differentiation of malignant, benign and normal breast tissue. Alfano et al. first used Raman-based methods to analyze normal breast tissue and breast cancer tissue (109). Shortly thereafter, Redd et al. showed that normal, precancerous, and cancerous breast tissue exhibited subtle, but distinguishable differences in their Raman spectra (110). Using tissue from biopsies of a significantly larger cohort of patients, Manoharan et al. then determined the spectroscopic differences of malignant and benign breast cancer tissue compared to normal breast tissue which showed that Raman spectra of normal breast tissue exhibited similar features to spectra obtained from lipids. Carcinoma tissue, on the other hand, revealed increases in protein markers, as well as modifications in the peak ratios of lipid spectra when compared to normal samples. These authors attributed these differences to changes in the chemical composition of lipids found in breast tissue (111). Shafer-Peltier et al. then followed this work up by an in-depth characterization of breast tissue samples by micro-Raman spectroscopy. This enabled them to obtain a correlation data set that can be used to analyze different tissue components in breast tissue (112). Linear combinations of the spectra of elementary biochemical compounds found in breast tissue (i.e. lipids, DNA, RNA, collagen, β -carotene, calcium hydroxyapatite, cholesterol, water, and calcium oxalate) made it possible to analyze breast cancer tissue, and to discriminate between benign, malignant, and normal tissue. More recently, Kong et al. found that selective sampling by Raman spectroscopy during breast-conserving surgery can be used during surgery to guide the surgeon to minimize the risk of having to perform multiple surgeries on the same patient (113).

This early work has since then rapidly expanded to a wide range of other cancers which are typically only accessible during the course of surgery, such as lung, liver, bone, or colon cancer (82, 114-118). Again, here, we restrict our discussion to some of the most recent work on this topic. Rashid et al. have shown that by using a combination of sophisticated multivariate analysis tools and by using samples from cervical biopsies with various degrees of cancer infiltration as control samples, they were able to assess cervical tissue that showed no morphological differences for pre-malignant changes (119). Similarly, by combining Raman spectroscopy and the analysis of tissue autofluorescence, Kong et al. were able to show that these optical analysis methods in combination with sophisticated classification models enabled them to discriminate tumor tissue and tumor cells during tissue-conserving surgery (120). Again, the hope here is that such information, when obtained by optical probes, could be used to catch tumors or

malignant infiltration of tissue at much earlier stages than what is currently possible. Based on the large number of tumors for which Raman spectroscopy has been demonstrated to provide non-invasive tumor discrimination, the prospects for such integrated optical probes that could help guide surgery, are looking very promising. One of the currently greatest weaknesses of these methodologies are that they are typically being conducted by individual research groups and cross-validation with data from other groups that might be using different laser wavelengths, different equipment, etc. has not yet been conducted in a systematic manner. The wide-spread availability of highly sensitive equipment and the accompanying expertise is no longer the limiting step, which permits the hope that multinational funding bodies, such as the European Commission or Private Foundations will soon decide to sponsor such complex studies, which could truly create the basis for a universal spectral library to assess tumors using data that have been validated across the globe.

3.2 Tissue physiology and dynamics probed and imaged by coherent Raman microscopy

We previously discussed the two main modalities of coherent Raman scattering microscopy in the context of analyzing individual cells. Here, we will briefly review some of the more recent applications of CARS and SRS microscopy as imaging tools for probing the physiology of tissues either as excised, frozen or fixed tissue, or fresh and *in vivo*, where the imaging modalities are used for diagnostic purposes or to assess dynamic changes in tissue structure and biochemistry.

Among the first tissue imaging applications of CARS microscopy were the direct visualization of glands in animal tissue *in vivo* (121) or the imaging of capillary blood flow *in vivo*, even at video rate speed (122). For several years, CARS microscopy was then primarily used to study lipid metabolism disorders at the tissue level, e.g. atherosclerosis (123), non-alcoholic fatty liver disease (124, 125), or to expand the knowledge of basic, metabolic lipid effects at the cellular and subcellular level (99, 126-128). Similarly, damage occurring to neural myelin sheets could be studied *in situ* and *in vivo* based on the same strong lipid resonance (129-131). Some of the most recent publications in this area have utilized CARS microscopy to image lipid deposits in whole *Drosophila* larvae at different developmental stages (132), and for tumor diagnostics (133).

Figure 4 shows the multitude of information that can be obtained by a single CARS imaging experiment, in this case of mouse cartilage tissue. Figure 4a shows the CARS image of the chondrocyte distribution within the tissue by imaging a general lipid and protein marker mode at 2845 cm^{-1} . Figure 4b is a byproduct of this imaging process, where the second-harmonic signal generated by collagen in the extracellular matrix between the cells was collected. Figure 4c is an overlay of the two different signals showing the cells and collagen in the tissue context.

More recently, CARS microscopy has been rivaled by Stimulated Raman Scattering (SRS), which is a byproduct of the four-wave mixing process leading to CARS and which is therefore also efficiently generated by the coherent excitation of scattering of many molecular bonds. A significant advantage of SRS microscopy is that it is not riddled by the non-resonant background signal that plagues many CARS applications (100). This method has further expanded the spread of coherent Raman imaging techniques to the fingerprint region of Raman spectra, where it

facilitates e.g. the fast and label-free imaging of protein distributions (134). SRS expands the superb chemical sensitivity of CARS microscopy, and maintains the natural confocality, meaning the signal is generated only within the focal volume. Because the SRS signal is not convoluted with potential non-resonant sample background signals the spectral interpretation is straightforward and in agreement with spontaneous Raman scattering spectroscopy. Because the signal is linearly dependent on the concentration, quantitative signal analysis can be applied much more easily than in CARS, where the signal is quadratically dependent on the concentration. Spectrally Tailored Excitation (STE)-SRS was first demonstrated by Freudiger et al. to simultaneously analyze multiple molecular vibrations. By using spectral shaping of a broadband laser excitation pulse they were able to distinguish different molecular moieties with significant overlap in their vibrational spectra (135). STE-SRS was then used to visualize the distribution of different fatty acids and proteins in living *C.elegans* worms. Similarly, Wang et al. used SRS to screen the local distribution of RNAi genes in *C.elegans* nematodes where different genes had been knocked out (136). Impressively, the SRS-based screening compared very favorably to traditional analyses using Gas Chromatography or Thin Layer Chromatography. Most recently, in an approach to demonstrate virtual histology or pathology without hematoxylin and eosin (H&E) stained tissue, Ji et al. utilized a 2-color SRS approach to image fresh brain tissue with similar contrast as that typically provided by H&E staining (137).

Just as we have already stressed for spontaneous Raman spectroscopy, modifying molecules of interest by substitution with stable isotopes or by tagging these molecules with unusual molecular groups also provides a great benefit for coherent Raman imaging. Such modifications are rather minor and will typically not inhibit the interaction of small molecules with biological molecules as the conjugation with the rather large fluorescent molecules often does. Also, molecules that are modified in such ways will not photobleach and can therefore be imaged for extended periods of time, opening up new ways for extended time live cell imaging or in vivo imaging in the near-infrared. The potential for using alkyne-tagged lipid molecules in coherent Raman microscopy was first demonstrated by Weeks et al. who at the same time showed that by using a doubly-resonant imaging methodology they could improve the sensitivity with which these Raman tags could be detected (9). Shortly thereafter they extended this approach to the identification of exogenous lipids in human monocytes that were treated with lipids and lipolysis products in order to induce the formation of early precursors to foam cells (99). This is also shown in Figure 2c, where a CARS image collected at 2845 cm^{-1} of the monocytes is depicted. Figure 2d shows the resulting doubly-resonant CARS image, where both, the 2845 cm^{-1} lipid resonance and the 2115 cm^{-1} alkyne resonance were probed simultaneously to amplify the weak signal of the alkyne group. This early work in our group was followed by studies by Wei et al. who utilized isotope substitution to image newly synthesized proteins in living cells (103), and later alkyne-tagging in combination with SRS to image the distribution of small molecules in living cells (10).

4. Conclusions and Perspective

Raman spectroscopy as a label-free tool to assess the physiology of cells and tissues has come a long way over the last 30 years, and especially the last decade has seen an almost exponential growth of applications and methodologies related to this topic. The gain in sensitivity brought about by novel technologies, the more advanced, easily accessible, and powerful multivariate analysis tools, such as k-means clustering or variations of principal component analysis have become standard in the analysis and interpretation of large multidimensional data sets obtained by spontaneous Raman imaging or multiplexed coherent Raman imaging. Similarly, the clinical or preclinical applications of Raman spectroscopy, mostly as a tool of assessing tissue status before or during surgery have also risen significantly. This rising demand has also led to a rise in the number of commercial players offering e.g. novel, compact, hand-held Raman-spectroscopy probes, which, we think, will find its way from applications related to Homeland Security or Law-Enforcement to Biomedicine. We expect this market to grow even more in the coming years, in parts owing e.g. to strong international efforts to develop novel imaging methodologies for intravital microscopy in the living brain. Also, the current efforts in developing various modes of super-resolution optical microscopy techniques that have culminated in the 2014 Nobel Prize in Chemistry, are beginning to show their effects on other, fluorescence-free imaging techniques. The limitations of fluorescent probes in optical microscopy, especially when it comes to in vivo imaging, are becoming more and more apparent and Raman scattering offers an interesting potential in this area. There is no doubt, that mechanisms that can break the optical diffraction limit, such as stimulated emission depletion or structured illumination microscopy can and will be adopted in one form or another to also extend the spatial resolution of Raman-based microscopy. Thus, the coming years might see an interesting transition in intravital imaging applications from fluorescence-based methods to hyper-spectral imaging, where Raman scattering plays an important role as a chemically sensitive imaging methodology. Currently, however, this is still somewhat limited by the rather high concentration limit required for Raman spectroscopy. While surface-enhanced Raman scattering has already demonstrated its potential for detecting even single molecules, coherent Raman microscopy and (to a lesser extent) spontaneous Raman spectroscopy, where the concentration limit scales with the acquisition time, are mostly stuck in the micro-molar concentration range. Several groups around the world are working on schemes to overcome this limit. This will also benefit the detection of novel exogenous probes, such as isotope-substituted molecules, or modifications based on alkyne or azide groups that, because of their specificity and high signal to background contrast ratio will open up a new frontier for biomedical imaging that is no longer riddled by photobleaching or the interference with physiological processes by the large molecular structures required for fluorescence. The future looks very bright, indeed, for Raman spectroscopy!

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Abbreviations

LTRS - laser tweezers Raman spectroscopy, CARS – coherent anti-Stokes Raman scattering, SRS – stimulated Raman scattering, CaDPA - Calcium dipicolinic acid, IPTG - isopropyl thiogalactoside

Figure Captions

Fig. 0: Graphical abstract

Fig. 1: Following the metabolic activity of single cells by Raman spectroscopy. a) The process of probing bacterial spores by CARS. A pump beam at 750 nm in combination with a probe beam at 812 nm probes the 1013 cm^{-1} vibration of CaDPA, resulting in a CARS signal at 697 nm. b) Corresponding CARS micrograph of a glass surface to which bacterial spores have been immobilized. c) Raman spectra of *Sinorhizobium meliloti*, a soil-based bacterium. The upper spectrum was taken 30s after the addition of trehalose to the cells. The lower spectrum was taken just another 60s later indicating the uptake and conversion of trehalose to keto-trehalose by the cell based on the sudden rise of the C=O peak at 1734 cm^{-1} . d) Transmitted light micrograph of a single bacterial cell held in an optical trap.

Fig. 2: Raman spectra of modified lipid molecules that can be utilized as optical probes for external lipids. a) A fatty acid containing 2 deuterium atoms instead of hydrogen leading to a small peak around 2200 cm^{-1} . b) A fatty acid containing a single alkyne end group, resulting in a strong signal at around 2100 cm^{-1} . c) CARS image of human monocytes that were treated with the alkyne-containing lipid in b), but imaged at the 2845 cm^{-1} general lipid mode. d) Image of the same monocytes, but imaged by simultaneously probing the 2845 cm^{-1} CH mode and the 2115 cm^{-1} alkyne vibration providing significantly higher contrast for the accumulation of the fatty acid within the cell.

Fig. 3: Raman spectra of doxorubicin-treated leukemic T cells (Jurkat T cell line), where the cells have been treated with two different concentrations of doxorubicin as indicated in the figure. a) Spectra for cells treated for 24h are indicative of chromatin condensation. b) Spectra for cells treated for 36h are indicative of membrane blebbing. c) Spectra for cells treated for 72h are indicative of apoptotic body formation.

Fig. 4: Multiplexed nonlinear optical imaging of mouse cartilage tissue. a) CARS image of mouse cartilage where the 2845 cm^{-1} CH stretching vibration has been used for image formation highlighting the chondrocytes within the tissue. b) Second harmonic generation (SHG) image of the extracellular matrix between the cells, composed mostly of collagen. c) Composite image, where both signal channels shown in a) and b) have been overlaid to create a false-color image of the cartilage tissue.

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Figure 1

Figure 2

Figure 3

Figure 4

Raman scattering

Raman spectroscopy

Coherent Raman imaging

Graphical abstract