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Chapter 3

Raman Mapping: Emerging Applications

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Additional information is available at the end of the chapter

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Abstract

Raman mapping is a noninvasive, label-free technique with high chemical specificity and high potential to become a leading method in biological and biomedical applications. As opposed to Raman spectroscopy, which provides discrete chemical information at distinct positions within the sample, Raman mapping provides chemical information coupled with spatial information. The laser spot scans the investigated sample area with a preset step size and acquires Raman spectra pixel by pixel. The Raman spectra are then discriminated from each other by chemometric analysis, and the end result is a false color map, an image of the sample that contains highly precise structural and chemical information. Raman imaging has been successfully used for label-free investigations at cellular and subcellular level. Cell compartments, cell responses to drugs and different stages of the cell cycle from the stem cell to the completely differentiated cell were successfully distinguished. This technique is also able to differentiate between healthy and cancer cells, indicating great potential for replacing conventional cancer detection tools with Raman detection in the future.

Keywords: Raman spectroscopy, Raman mapping, Raman imaging, cells, tissues, medical diagnosis, plants, algae

1. Introduction

1.1. Basic concept and working principle

Various techniques are being currently used for imaging of cells and tissues. Individually, each technique is able to address some aspects of the system under study. For example, optical microscopy is very often used for cell and tissue analysis; it is a cost-effective method which gives morphological information, but is unable to provide molecular and structural

information. Electron microscopy and atomic force microscopy are high spatial resolution techniques, able to image subcellular compartments; however, they lack chemical specificity. In most cases, fluorescence microscopy, for example, confocal scanning fluorescence microscopy, is used for cellular visualization. Fluorescence microscopy requires fluorescent labels specifically bound to the substrate under study. Subcellular structures can be visualized, but since each fluorescent label is excited by a different wavelength, the number of structures that can be visualized is limited. The need to introduce fluorophores and their limited stability and photo bleaching are drawbacks of using confocal microscopy. Nonetheless, the technique is largely used for cell imaging and imaging of cellular uptake of micro and nanoparticles. There is great need for techniques that provide chemically specific information coupled to spatial information for the visualization of, for example, cellular uptake and localization of biologically active molecules, cellular transport pathways, molecular changes in cancer vs healthy tissues, etc.

Raman mapping (Raman imaging, Raman scanning or Raman micro-spectroscopy) has recently become an emerging imaging technique in biological and biomedical research and applications. The Raman effect is based on inelastic scattering of photons when electromagnetic waves interact with atoms or molecules. The small fraction of incident photons scattered inelastically have different frequencies compared to the incident photons. The phenomenon is called Raman scattering, and the difference in frequency between the incident photons and scattered photons is the so-called Raman shift (cm^{-1}). The Raman shift is related to the vibrational levels of each specific molecule, being used as a fingerprint for molecular identification [1].

In contrast to Raman spectroscopy, which provides discrete chemical information at distinct positions within the sample, Raman mapping provides chemical information coupled with spatial information [2]. Raman mapping is a noninvasive, label-free technique, with high chemical specificity. In Raman mapping, the laser spot scans the investigated sample area with a preset step size and acquires Raman spectra at every set point. The Raman spectra are then discriminated from each other by chemometric analysis, and the end result is an image of the sample that contains highly precise structural and chemical information. Excitation wavelengths in the visible and near-infrared range give high spatial resolution (<1 μm), making Raman spectroscopy combined with microscopy an ideal tool for biological samples imaging, and especially for cell and tissue imaging. In this latter case, Raman mapping has important advantages over conventional biological assays: it is a rapid, noninvasive, label-free technique, which does not damage the cells if using suitable laser wavelengths and power.

1.2. Instrumentation and data analysis

The most important parameters to ensure the success of a Raman imaging measurement on biological samples are the wavelength and power of the laser, the resolution of the images, and the sample preparation and fixation. The intensity of the scattered radiation is proportional to the wavelength at the power of −4 ($\sim\lambda^{-4}$), meaning that shorter (blue) wavelengths are scattered more strongly than longer (red) wavelengths. Thus, shorter wavelengths generate more photons scattered inelastically, giving thus higher Raman intensities. However, shorter wavelengths typically lead to stronger auto-fluorescence from the samples, which can mask

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the Raman signal arising from the molecules of interest. Therefore, a compromise is needed. Hamada et al. studied the influence of 488, 514.5, 532 and 632.8 nm laser excitation wavelengths on the Raman signal yield and background signal for the imaging of living cells [3]. The authors found that the 532 nm excitation is a good compromise between Raman signal intensity and auto-fluorescence background because it generates strong Raman scattering signals and suppresses auto-fluorescence. Photodamage caused by light absorption of the biological samples is another important parameter to be considered for choosing the appropriate laser excitation. Puppels et al. [4] found that a 660 nm laser induces no photodamage to cells and chromosomes compared to the 514.5 nm (visible) laser. Even though Raman scattering efficiency decreases with increasing wavelength, recent advances in the design of Raman spectrometers with high optical throughput and highly sensitive CCD (charge-coupled device) detectors allow measuring spectra and obtaining reasonably high signal strength. Notingher et al. used a 785 nm laser for their measurements on live cells and tissues [5–7]. In one study, they compared the 488, 514 and 785 nm lasers with respect to photodamage of cells and found that the 488 and 514 nm lasers induce photodegradation and reduce the number of living cells; with the 785 laser, cell degradation and auto-fluorescence were low and the signal intensity was reasonably high [8]. Even though going higher than 785 nm (e.g., 1064 nm) in the laser wavelength would decrease the photodamage of the cells, it would also dramatically decrease the Raman scattering efficiency. The recent literature mentions mostly the use of 785 and 532 nm lasers for cellular mapping [6, 9]. However, when using near-infrared (NIR) lasers for Raman excitation, cooled deep depletion back-illuminated CCD detectors are preferred [6] instead of standard back-illuminated, visible-optimized CCDs, because of their higher quantum efficiencies (QE) in the near-infrared (NIR) spectral region (up to 95% with the new Low Dark Current Deep-Depletion (LDC-DD) Technology.

In some cases, for samples that cannot be detected by regular Raman scattering, signal enhancement can be induced. In some situations, it is possible to obtain resonance Raman effects. Such effects take place when the laser excitation wavelength overlaps with the absorption band of the molecules due to electronic transitions, and this can lead to increase the Raman intensity by a factor of 103–105 [1]. The phenomenon is called resonant Raman scattering. Consequently, Raman imaging of a resonant molecule can be significantly improved by choosing an excitation laser wavelength in the absorption band region of the molecule. For example, cytochrome C absorbs light at around 520 nm and shows a strong resonance Raman effect when analyzed using a 532 nm laser. This property can be used to image its intracellular distribution. Other examples of molecules that can benefit from strong Raman resonance effects are the carotenoids, chlorophylls, vitamin B12 and heme proteins [3, 10].

Another way to obtain signal enhancement in Raman spectroscopy is to use surface-enhanced Raman spectroscopy (SERS) or coherent anti-Stokes Raman scattering (CARS). In SERS, it is possible to reach high enhancements of the Raman intensity (by a factor of 10^7 or more) when the molecule of interest is adsorbed onto or in the very close vicinity of plasmonic metallic nanostructures such as silver and gold [11]. This effect significantly lowers the detection limit of molecules. CARS is a nonlinear optical effect in which two lasers, a pump laser and a Stoke laser, are overlapped and strongly focused onto the sample to generate the CARS signal. When

the difference in frequency between the pump and the Stoke lasers is tuned to the exact value of a vibrational frequency within the sample, strong enhancements of the CARS signal can occur [1].

The instrumental resolution is very important for cell and tissue mapping, in order to be able to image cellular and subcellular structures. The lateral resolution is limited by the wavelength of the laser and the numerical aperture of the objective used for the experiment, while the axial resolution is given by the instrument aperture (slit or pinhole) and the refractive index of the immersion medium. Currently available Raman spectrometers can go down to 200 nm for the lateral resolution and 500 nm for the axial resolution [2].

Cells and tissues could be fixed on specific substrates for usage over longer periods of time. The most important requirements for a substrate suitable for Raman imaging are as follows: (a) transparency in the visible and near-infrared region of the light spectrum; (b) low background signal to avoid overlapping with the Raman signals from the sample; and (c) suitability for cell culture growth or tissue fixation. Calcium and magnesium fluoride (CaF_2 and MgF_2) and quartz are the preferred substrates for Raman imaging. Glass and plastic substrates are not recommended because of high background signals [2, 12]. Zinc selenide (ZnSe) has the disadvantage of weak cell adherence [12]. A variety of cell fixation methods has been so far reported can be used: paraformaldehyde, methanol, methanol:acetone, formalin, air-drying and cytocentrifugation [2, 6, 13]. For live cell imaging, special instrument setups, in which cells are confined in a sterile chamber and kept at 37°C and a 5% CO_2 atmosphere to ensure viability [14, 15], have been reported.

After taking the pixel-by-pixel Raman spectra, the raw dataset needs to be processed in order to identify the key molecules in the sample and based on their spectral fingerprint, to generate the false color Raman images. Since no label is used, the pixel-to-pixel spectral variations are small and multivariate methods of analysis need to be employed to get the Raman images from the dataset. Several approaches are currently used: principal component analysis (PCA), self-modeling curve resolution (SMCR), K-means cluster analysis (KMCA), hierarchical cluster analysis (HCA), divisive correlation cluster analysis (DCCA), vertex component analysis (VCA), fuzzy C-means cluster analysis (FCCA) and linear discriminant analysis (LDA) [6, 16–20].

Here we aim to highlight the recent advances of Raman mapping and provide an overview on its emerging applications, which range from single cell and tissue imaging to medical diagnosis, including cancer detection. Some applications that will be discussed include:

- Stem cell research, especially stem cell differentiation [6, 21–23]
- Single cell and microorganism imaging [2, 18, 24, 25], including evidentiation of subcellular compartments [23]
- Identification of cell cycle phase [26]
- Monitoring of cell death [27, 28]
- Cellular responses to drugs [9]

- Imaging of intracellular localization of bioactive molecules and drug carriers such as colloidal nanostructures, liposomes and polymeric particles [2, 29, 30]
- Imaging of tissue physiology [31, 32]
- Medical diagnosis, including cancer detection based on the capacity of Raman mapping to detect molecular changes in cells, tissues or biofluids, that are either the cause or the effect of diseases [7, 33, 34]
- Intraoperative detection of tumor margins [7, 35]
- Cancer detection based on the ability to discriminate between normal and cancer cells [36]

2. Raman mapping for cell imaging

Several Raman peaks are used as fingerprints for the intracellular identification of nucleic acids, proteins and lipid-rich structures. In terms of chemical information, the richest part in a Raman spectrum is the region below 1800 cm^{-1}, also called the fingerprint region. Some representative Raman bands from the fingerprint region, characteristic for nucleic acids, proteins and lipids, are given in **Table 1** [6, 37, 38]. The region between 1800 and 2800 cm^{-1} is the so-called silent region, since no Raman cellular vibrations arising from functional groups appear in this region, excepting triple-bond vibrations. Finally, the high-frequency region above 2800 cm^{-1} is dominated by C–H stretching vibrations (CH, CH_2, CH_3).

Stem cells are attractive to be studied in the biomedical field because they have the ability to differentiate into any cell phenotype, and they can proliferate indefinitely [39]. Differentiation of stem cells could be used in stem cell therapy and tissue engineering. The differentiation process of stem cells needs identification of specific markers; currently, this involves the use of immunolabeling or fluorescence. Both methods have the disadvantage of being invasive. In contrast, using Raman-specific intrinsic fingerprints would be advantageous, being noninvasive, nonlabeling approach, and able to provide accurate and highly specific information. The method would be suitable also for cells that lack specific markers for separation by conventional means [21] such as cardiomyocytes.

The intracellular distribution and concentration of nucleic acids were used by Ghita et al. [6] to distinguish between undifferentiated and differentiated stem cells, more precisely from undifferentiated neural stem cells and the glial cells derived from them. They manage to differentiate with 89.4% sensitivity and 96.4% specificity. The spectral fingerprint of the nucleic acid backbone was used to detect DNA- and RNA-rich regions. The Raman spectrum of β-DNA shows a strong band at 788 cm^{-1} and a shoulder at 835 cm^{-1}, characteristic to the symmetric O-P-O stretching vibrations of the phosphate groups and to the asymmetric O-P-O vibrations, respectively. In case of RNA, the symmetric O-P-O vibrations appear shifted to 813 cm^{-1}, while the shoulder corresponding to the asymmetric O-P-O stretching disappears. Based on the spectral differences between DNA and RNA, it was found that undifferentiated neural cells have higher concentration of nucleic acids compared to glial cells. The Ram-

Raman peak position (cm^{-1})	Assignments
	Nucleic acid bands
684	Guanine ring breathing
729	Adenine ring breathing
751	Thymine ring breathing
782	Uracil ring breathing
785	Cytosine ring breathing
788	Symmetric O-P-O stretching in β-strands DNA
835	Asymmetric O-P-O stretching in β-strands DNA
813	Symmetric O-P-O stretching in RNA and α-strands DNA
1095–1098	O-P-O vibrations (DNA, RNA)
	Protein bands
938	Backbone C=C stretching
1005	Phenylalanine symmetric ring breathing
1033	Phenylalanine in plane C–H vibrations
1200–1300	Amide III band (CH, NH deformations)
1655–1662	Amide I (C=O stretching)
	Lipid bands
980	=C–H bending
1093	C–C stretching
1257	=C–H bending
1450	C–H deformation
1658	C=C stretching

Table 1. Some of the most representative Raman peaks for nucleic acids, proteins and lipids in cells.

an band at 813 cm^{-1} was the indicator of the differentiation status, and this allowed distinguishing between the two cell types. Undifferentiated neural stem cells have high concentration of RNA in the cytoplasm (as high as 4 mg/ml), while below the instrument detection limit (<1 mg/ml) in the glial cells. Based on Raman mapping, it was possible to image RNA- and DNA-rich structures in the stem cells. The RNA-rich structures, representing the stem cells cytoplasm, were imaged using the 813 cm^{-1} Raman band, and the DNA-rich part related to the stem cells nucleus was imaged using the 788 cm^{-1} Raman band. Similar results were reported by the same research group regarding the differentiation status of embryonic stem cells [22]. The differentiated cells had 75% less RNA, as monitored by the decrease in intensity of the 813 cm^{-1} peak. Basically, most prominent Raman peaks of embryonic stem cells are the ones of proteins (amide I band at 1660 cm^{-1}, amide III at 1200–1300 cm^{-1}, 1005 cm^{-1} vibration of phenylalanine and C=C stretching at 938 cm^{-1}) and nucleic acids. Dental follicle mesenchymal stem cells were also imaged using Raman mapping; several compo-

nents, and especially a high concentration of cytoplasmic RNA, were found to be a good indicator to the undifferentiated status of the cells [40].

Raman mapping was also used inside a bioreactor culture system, where human embryonic stem cells were grown and differentiated into cardiomyocytes [41]. The purpose was to monitor the cardiac differentiation of the embryonic bodies. The Raman maps were compared with immunofluorescence imaging. A positive correlation was found between Raman bands at 1340, 1083, 937, 858, 577 and 482 cm^{-1} and the expression of the α-actinin protein in the differentiated cardiomyocytes. Konorov et al. [26] obtained information on the cell cycle phase of human embryonic stem cells. The 783 cm^{-1} DNA band from a large number of cell nuclei was used as indicator of the cell cycle phase. The results were corrected for the RNA contribution at 811 cm^{-1}. As such, the authors were able to get information on the state of division of the embryonic stem cells by quantifying the DNA and RNA peaks from the Raman spectra and obtained Raman intensities similar to the fluorescence intensities of flow cytometry.

In another study, Pascut et al. [42] obtained 97% specificity and 96% sensitivity in differentiating the cardiomyocytes derived from human embryonic stem cells. The main spectral features that allowed the discrimination of cardiomyocytes were attributed to glycogen and myofibrils. The results were correlated with the immunofluorescence staining, and a good correlation was observed. The same authors investigated the potential for developing Raman-activated cell sorting of individual cells [43]. Hashimoto et al. [44] got information on osteoblast differentiation and mineralization mechanisms by monitoring fluctuations in the cytochrome C concentration. The above preliminary studies suggest that Raman spectroscopy has a great potential to become a leading method for stem cells investigation.

Raman mapping can be used as a tool to obtain molecular fingerprint information from different subcellular compartments. Based on their distinct chemical features, nucleus and cytoplasm and also other cellular organelles can be imaged. For example, in their study on follicle mesenchymal stem cells, Leopold et al. [40] were able to image the cell nucleus based on the 785 cm^{-1} band characteristic for the DNA O-P-O vibrations. Lipid characteristic peaks, such as the 1446 cm^{-1} peak characteristic to CH_2 vibrations, made it possible to highlight the smooth endoplasmic reticulum in the Raman images, which is known to be the source of intracellular lipid synthesis. Based on characteristic Raman vibrational peaks of lipids, proteins and nucleic acids, Krafft et al. [45] were able to reconstruct the main cellular components: the nucleus, the contour of the cell and the organelles. They focused mostly on the 2800–3000 cm^{-1} region, where CH_2 and CH_3 vibrations from proteins, lipids and nucleic acids are present. In both studies, the cellular organelle identification was carried out based on the score plots obtained from the principal component analysis. From the score plots, false color Raman maps were generated, highlighting the subcellular compartments. Raman images of subcellular organelles were also reported by Krauß et al. [23]. The authors have also correlated well their results with fluorescence microscopy.

Cytochrome C, protein and lipid-rich structures were evidenced in different Raman images on HeLa cells [3] by irradiating the cells with 488, 514, 532 and 633 nm lasers. The HeLa cells gave Raman spectra with peaks at 1000 cm^{-1} (breathing of phenylalanine), 1451, 2850, 2885, 2935 cm^{-1} (CH_2 deformation, CH_2 and CH_3 stretching) and 1660 cm^{-1} (amide I bands mode of

peptide bonds). When irradiated with the 532 nm laser, resonant peaks at 753, 1127, 1314 and 1583, characteristic to cytochrome C, were obtained. Raman images highlighting the Cytochrome C, protein β-sheets and lipids were created using the 753 cm^{-1} peak (pyrrole ring of cytochrome C), the 1686 cm^{-1} peak (amide I vibration of peptide bond in protein) and the 2852 cm^{-1} peak (CH_2 stretching vibration of hydrocarbon chain of lipids). Since cytochrome C is essential for the electron transfer in mitochondria, the Raman images of cytochrome C should also point out the distribution of mitochondria in the cell. In addition, Raman spectra from the nuclei showed no resonance, being similar for all excitation wavelengths. Matthäus et al. [46] reported on obtaining Raman maps pointing out the location of different cellular structures in HeLa cells, with emphasis on mitochondria. They performed hierarchical cluster analysis and found localization of mitochondria in the perinuclear region, which was supported by correlation with fluorescence maps.

In some cases, Raman imaging requires the use of tags. This happens when molecules cannot be detected by regular Raman scattering, either because they are in very low amounts, or because their Raman signal overlaps with other compounds and cannot be distinguished clearly. There are two approaches for using tags for Raman imaging: (a) using surface-enhanced Raman scattering, which implies binding of tags onto the plasmonic nanoparticles surface that can be further used for intracellular identification of analytes [47–50], and (b) taking advantage of the silent region in the Raman spectra of cells between 1800 and 2800 cm^{-1}. In this region, most of the biologically active molecules show no Raman vibrations, so functional molecules with Raman fingerprint in this region could be useful as tags for detection of molecules, which do not give clearly distinguishable Raman peaks in the intracellular medium. This also has the advantage of avoiding the overlap with any endogeneous molecules. Tags suitable for the silent region detection are alkynes, azides, deuterium and nitrile. Palonpon et al. [10] utilized alkyne-tagged EdU (5-ethynil-2-deoxyuridine), for the detection of DNA accumulation and synthesis in cells. EdU is rapidly incorporated in the DNA during the replication process, accumulates in the nucleus and is thus suitable for acquiring information about DNA synthesis in cells.

Silver nanoparticles (AgNPs) prepared by reduction with hydroxylamine according to the Leopold and Lendl method [51] were used for mapping sub-membrane hemoglobin in erythrocytes (red blood cells) [52]. Erythrocytes contain cytosolic and sub-membrane hemoglobin. Although hemoglobin exhibits strong Raman scattering, Raman spectra of erythrocytes generally have mostly contribution from cytosolic hemoglobin, since the sub-membrane hemoglobin is negligible in amount. To trace this sub-membrane hemoglobin, the authors used SERS-active AgNPs that were internalized in the cells and accumulated in the cell membrane. SERS images showed the erythrocytes that come in contact with the AgNPs. Lee et al. [53] used SERS to detect different human breast cancer cell lines phenotypes and to quantify the proteins on the cell surface. For the purpose of SERS enhancement, silica-encapsulated hollow gold nanospheres conjugated with specific antibodies were used. The expression of epidermal growth factor (EGF), ErbB2 and insulin-like growth factor-1 (IGF-1) receptors was determined in the MDA-MB-468, KPL4 and SK-BR-3 cell lines by SERS mapping. Different distributions of growth factors were clearly identified and distinguished from their corresponding SERS

mapping images. Taking advantage on the characteristic wave number of the carbonyl group vibration that lies within the silent region of the Raman spectra, Kong et al. [54] developed osmium carbonyl clusters for cancer cell imaging. The clusters were conjugated with PEG-coated AuNPs and further functionalized with antibody for epidermal growth factor receptors (anti-EGFR). AuNPs were used for carbon monoxide Raman signal enhancement, while functionalization with antibody was needed since the EGFR is highly expressed in many cancer cell lines. Both EGFR-positive and EGFR-negative cancer cells were used. The nanoparticle conjugates were imaged after cellular uptake based on the CO absorption signal at 2030 cm^{-1}, and the results showed the specificity and efficient targeting of CO-nanoparticle conjugates to EGFR-positive cells.

For toxicology studies, it is important to be able to distinguish between healthy and apoptotic cells and also to gain information on the molecular changes associated with apoptosis. Zoladek et al. [28] used Raman imaging for understanding changes associated with apoptosis in the MDA-MB-231 human breast cancer cells. Cells were exposed to the apoptotic drug etoposide, and Raman spectra were recorded 2, 4 and 6 h after exposure. An 1.5-fold increase in the DNA content was observed after 6 h, and the change was assigned to DNA condensation. The most drastic change was in the lipid profile; a high concentration in membrane phospholipids and unsaturated non-membrane lipids was observed in apoptotic cells. The Raman images of the lipidic areas of the cells were generated based on the 1005 and 1659 cm^{-1} peaks ratio. The 1005 cm^{-1} peak with contribution from phenylalanine is not affected by etoposide exposure, while the 1659 cm^{-1} C=C stretching vibrations from lipids show strong increase upon etoposide exposure, indicating an increased degree of unsaturation of lipids for the apoptotic cells. Okada et al. [55] used resonant Raman scattering for imaging the intracellular distribution of cytochrome C and observing dynamic changes of its 750 cm^{-1} band associated with cell apoptosis.

The cellular uptake of nanoparticles and drugs and the cellular responses to drugs are important aspects to be investigated for molecular biomedical applications. Cellular uptake and localization of polyethylene glycol-coated gold nanoparticles in human prostate cancer cells (LNCaP Pro 5) were visualized based on their photoluminescence peak (180–1800 cm^{-1}). In the Raman images of LNCaP Pro 5 cells with AuNPs internalized, cell nucleus and nucleoli are visible, as well as spots generated from the photoluminescence peak of AuNPs. The nanoparticles are located at different positions inside the cells, depending on the time elapsed from exposure. Two hours after exposure, the AuNPs are located in the cell membrane, 12 h after they are located in the cytoplasm, and after 24 h, AuNPs are imaged in the perinuclear region [29].

3. Raman mapping for tissue imaging and medical diagnosis

There is clear indication that Raman spectroscopy could provide insights into drug targeting mechanisms and could be used for detection of metabolic interactions of drugs with cancer cells. In their attempt to detect physiologically relevant cellular responses to drugs, El-Mashtoly et al. [9] used Raman imaging to quantify the effect of the epidermal growth factor

inhibitor panitumumab on colon cancer cells expressing Kirsten-ras mutations (oncogenic and wild-type). It is known that oncogenic K-ras mutations block the response to anti-epidermal growth factor therapy such as panitumumab, while cells expressing wild-type mutations respond to the treatment; all these facts were nicely confirmed by the Raman mapping results. The authors used hierarchical cluster analysis on the 700–1800 cm^{-1} and 2800–3050 cm^{-1} regions for the identification of subcellular components such as cellular membrane, cytoplasm, nucleus and lipid droplets. They found that the oncogenic mutated K-ras cells showed no response to the drug, while the wild-type mutated cells have strong cellular responses to panitumumab treatment, as demonstrated by Raman intensity changes and wave number shifts. The panitumumab-induced changes are strongest on the lipid droplets, suggesting that lipid droplets might play a crucial role in anticancer therapy. The results were confirmed by fluorescence spectroscopy. In another work, El-Mashtoly et al. [30] were able to image the spatial distribution of the erlotinib, another inhibitor of the epidermal growth factor receptor, in colon adenocarcinoma cells upon 12 h of incubating the cells with 100 μM erlotinib solution. Normally, erlotinib cannot be detected by Raman spectroscopy at 100 μM concentrations, which basically implies that its intracellular level was higher due to most likely concentration of the drug in the cell. The authors used the C=C alkyne vibration from the silent region of the Raman spectrum (2085–2140 cm^{-1}) to image the erlotinib distribution and found that the drug was mostly concentrated at the cell borders.

Cells, tissues and bio-fluids can be imaged by Raman micro-spectroscopy. Based on the hypothesis that molecular changes associated with different diseases can be quantified by Raman spectroscopy, the method has been used in medical research and diagnosis during the last years. On one hand, *in vitro* and *in vivo* analysis of tissue is important to be able to distinguish between healthy and tumor cells, and on the other hand, in the medical diagnostics field, there is imperative need for research directed toward identifying noninvasive methods for tumor analysis and toward determining the exact tumor margins. There are several papers reporting the use of Raman spectroscopy and imaging in these directions. Present research still requires comparison with conventionally used staining methods used in histopathology. The gold standard method for tumor pathology and classification is the hematoxylin and eosin (H&E) staining. As opposed to the H&E which involves tissue staining and fixation, Raman micro-spectroscopy is a nondestructive, nonlabeling method. Also, histopathology cannot be used intraoperatively as it requires long incubation times. NIR and the visible 532 nm laser are reported for tissue imaging related to cancer research [7, 30].

The major drawback of Raman technique that limits its application in the medical field is the low efficiency of the inelastic scattering process. Different strategies have been developed to overcome this difficulty, based on, for example: (a) using nonlinear imaging modes such as CARS; (b) acquiring selective sampling of the analyzed probe (e.g., tissue auto-fluorescence can be used to determine the characteristics of the tissue sample and to further use the information to prioritize the sampling points for Raman spectroscopy); (c) multimodal integration of Raman with other techniques such as auto-fluorescence; (d) use of fiber-optic probes for hand held instruments; and (e) use of plasmonic metallic nanoparticles suitable for surface-enhanced Raman scattering.

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In vivo and *in vitro* cancer diagnosis based on Raman imaging was so far focused on brain, breast, lung, skin, prostate, colorectal, esophagus and bone cancer [33]. The group of Notingher et al. are pioneers in using Raman imaging for tumor diagnosis, in particular for detecting tumor margins. Multimodal spectral imaging, combining auto-fluorescence imaging and Raman micro-spectroscopy, was used [34, 35, 56] to distinguish between healthy and cancer cells in different carcinoma tissues during intraoperative or postoperative evaluations, for the purpose of accurately detecting the tumor margins. Multimodal spectral imaging is required to reduce the acquisition times needed for raster scanning. Instead of raster scanning the sample, selective sampling is achieved based on integrating collagen auto-fluorescence imaging with Raman imaging. First auto-fluorescence images are used to determine the features of the tissue, and then, the information is used to prioritize and decide the sampling points for Raman spectroscopy. The tissue areas with auto-fluorescence are those containing collagen and are thus identified as healthy dermis and excluded from the Raman measurements. In this way, a dramatic decrease in the acquisition time is achieved: autofluorescence Raman typically requires ~100-fold fewer Raman spectra compared to raster scanning [35]. The high speed of fluorescence imaging relies on the capability to image large tissue area, in contrast to Raman imaging, which requires a pixel-by-pixel readout. For example, an integrated system based on Raman scattering and auto-fluorescence imaging was used by Kong et al. [35] to diagnose basal cell carcinoma tumor margins during tissue-conserving surgery. The major challenge in tissue-conserving surgery is to completely remove the tumor, with minimal loss of healthy tissue. Auto-fluorescence images were necessary in order to prioritize the sampling points for Raman. By using k-means cluster analysis and comparing the images obtained from clustering with the histopathology images, it was possible to diagnose the tumor with 100% sensitivity and 92% specificity. As such, it was possible to assign tissue areas corresponding to the tumor, epidermis, dermis, fat, inflamed dermis, sebaceous gland and muscles. The tumor areas show more intense DNA peaks at 788 and 1098 cm^{-1} compared to healthy tissue. The spectra of the dermis were characterized by collagen-specific peaks at 851 and 950 cm^{-1}. It was possible to achieve shorter diagnosis times than those required by histopathology.

Selective sampling for intraoperative diagnosis during the breast cancer conserving surgery leads to a diagnosis of mammary ductal carcinoma with 95.6% sensitivity and 96.2% specificity [56]. As in the study above, discrimination between healthy and tumor areas was based on increased concentrations of nucleic acids (bands at 788, 1098 cm^{-1}) and decreased levels of collagen and fats (851 and 950 cm^{-1} bands) in the tumor regions. Tissues from 60 patients were deposited on MgF_2 plates; 20 μm tissue sections were sampled and analyzed by Raman micro-spectroscopy, and adjacent sections of 70 μm were stained with H&E. To reduce acquisition times needed for raster scanning, selective sampling was achieved based on integrated auto-fluorescence imaging and Raman. This procedure is also known as multimodal spectral histopathology. By comparing the Raman images obtained from the k-means cluster analysis with the ones obtained from H&E staining, the tumor *vs* healthy breast tissue assignment was successfully carried out. The images from the tumor regions showed large number of cells with enlarged nuclei. Compared to regular raster scanning Raman that would require 10,000 spectra/mm^2 and 5 h analysis time, the multimodal spectral imaging drastically reduces the

analysis time by reducing the number of Raman spectra acquired to 20 spectra/mm^2, which needs 17 min for reaching diagnosis. In a recent study [7], the same group reported on face and neck basal cell carcinoma analysis by selective sampling Raman with 95.3% sensitivity and 94.6% specificity. The results are promising; the method can significantly decrease the diagnosis time. However, it requires strong computing power for the calculations needed after measurement of each Raman spectrum, and this can still be considered a drawback.

Cancer and pre-cancer cells, erythrocytes and lymphocytes were successfully assigned to colon cancer tissue sections by combining Raman imaging with histopathology (H&E staining) and with immunohistochemistry [13]. Hierarchical cluster analysis was used in the spectral region 700–1800 cm^{-1} and 2600–3100 cm^{-1}. The tumor protein p53 is normally highly expressed in cancer and pre-cancer cells because it is a tumor suppresser. The possibility of obtaining Raman imaging of tumor and pre-tumor cells by highlighting p53 active areas was confirmed. By comparing the obtained false color Raman maps with the images given by the anti-p53 immunohistochemical stained image, it was found that the sample auto-fluorescence matches the fluorescence from the anti-p53 stained tissue, proving that the Raman imaging can be used for assigning the p53 active areas of the tissues. The p53 active areas represent more specifically the cancer cell nuclei.

Using SERS-active nanoparticles for intraoperative detection of tumor margins is another promising direction of research in Raman imaging. With this purpose in mind, Wang et al. [48] developed multi-receptor-targeted SERS-active nanoparticles that are topically applied at the surface of tissues excised during breast cancer lumpectomy and that enable quantitative molecular phenotyping at the tumor surface for the purpose of diagnosis. The nanoparticles are tagged with multiple antibodies to achieve as high accuracy as possible and to be able to eliminate influence of nonspecific binding of the nanoparticles. Bovin serum albumin (BSA) was also used to limit nonspecific accumulation of nanoparticles within cells. Antibodies for the epidermal growth factor receptor (EGFR) or the human epidermal growth factor receptor 2 (HER2) and a negative control antibody were conjugated to the nanoparticle surface; a fluorophore was also used to conduct flow cytometry for result confirmation. By targeting the SERS-active nanoparticles to various tumor biomarkers simultaneously and recording the SERS spectra, followed by computational demultiplexing to determine the relative concentrations of the individual SERS nanoparticles, it is possible to detect residual tumors at the surgical margins. The results of the study demonstrated the ability to perform successful Raman imaging on the tissues and to accurately quantify relative tumor biomarker expression levels (high levels of HER2 expression were found, characteristic for breast tumors), in less than 15 min.

4. Raman mapping in plant and algae research

There is a growing interest in getting a more comprehensive understanding of the chemical composition of various plant tissues. Investigations on structural aspects of plant cell wall components, on the chemistry of plant metabolites and relevant plant molecules, are feasible

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using Raman mapping. NIR-FT Raman is suitable for imaging of large plant structures such as leaves, seeds and fruits, while the higher resolution visible lasers allow investigation of smaller plant structures.

For example, using the 633 nm laser, it was possible to image the distribution of cell wall components such as cellulose and lignin in a 55-year-old black spruce wood (*Picea mariana*) [57]. Raman images of cellulose and lignin were accurately generated. Cellulose gives three distinct peaks at 380, 1098 and 2900 cm^{-1}, whereas lignin has two overlapping bands at 1600 and 1650 cm^{-1}. The distribution of lignin was generated using both the 1600 and 1650 cm^{-1} bands, while the cellulose distribution maps were found to be most reliable when generated using the 2900 cm^{-1} band which has contribution from lignin alone, without other chemical interferences. Lignin-to-cellulose ratio was also determined, and it was found to differ in different areas of the plant cell wall. Because the 1650 cm^{-1} line had as well contribution from coniferaldehyde and coniferalcohol, it was possible to also image the coniferaldehyde and coniferalcohol distribution, which followed that of lignin. Sun et al. [58] have also used Raman mapping to get information on the lignin and cellulose polymers distribution and composition in *Eucalyptus globulus* and corn stover. They have imaged the lignin and cellulose within different areas on the plant cell walls, from the epidermis to the pith area. Based on the Raman spectral fingerprints, significant compositional differences between *Eucalyptus globulus* and corn stover were observed, but also between different types of cells within the same plant. Schmidt et al. [59] acquired sub-micrometer lateral resolution Raman images of *Arabidopsis thaliana* stem cross sections using the visible 532 nm laser and obtained information on the spatial distributions of cell wall polymers. As such, the distribution of carbohydrates (mainly cellulose) and lignin was obtained. The spatial distribution of polymers was obtained by integrating the C–H intensities between 2820–2935 cm^{-1} for cellulose and 1550–1700 cm^{-1} for lignin. Intense cellulose signals were identified within the secondary walls, whereas lignin was mainly found in the cell corners and in very little amounts in the secondary walls. However, since lignin distribution was not homogeneous, some secondary walls were strongly lignified (ensuring waterproofing). Richter et al. [60] took images from different tissues at different positions within the leaf of *Phormium tenax* and managed to visualize (using the 532 nm laser line) pectin and lignin distribution and to determine the cellulose microfibril angle on the cell walls.

Carotenoids are another promising class of compounds that can be analyzed and imaged through Raman mapping. They are organic pigments, conjugated double bond chains found in plants and other photosynthetic organisms, including bacteria and fungi. Carotenoids have important physiological roles, making them important molecules in plant biology, food science and pharmacology. In plants and algae, carotenoids protect from photodamage and absorb energy to be used in photosynthesis, whereas in human body they are potent antioxidants, and some of them are vitamin A precursors. The human body is unable to synthesize carotenoids, so they must be introduced through the diet, from carotenoid-rich foods (e.g., carrots, tomato, maize, kiwi, cucumber, spinach, broccoli, etc). Information about carotenoid distribution in different plants and plant tissues is limited. Brackmann et al. [61] used coherent anti-Stokes Raman scattering (CARS) to gain information on β-carotene distribution in sweet

potato, carrot and mango. The β-carotene distribution was probed using the C=C vibrational peaks at 1520 cm^{-1}, characteristic for β-carotene. Heterogeneous rod-shaped bodies with high carotenoid density were identified in sweet potato and carrot, while in mango carotenoid-filled lipid droplets were identified as homogeneous aggregates. Raman imaging would also be suitable for other types of carotenoids such as lutein and lycopene, since they all have similar vibrational Raman bands at 1500–1535 cm^{-1} (C=C stretching), at 1145–1165 cm^{-1} (C–C stretching) and at 1000–1010 cm^{-1} (C–CH_3 deformation) [62, 63]. The Raman bands are similar to all carotenoids, but shifted in position according to the number of conjugated bonds, the side groups and to the interaction of carotenoids to other plant constituents. Raman mapping was proved to be useful in evaluating the individual distribution of 7-, 8- and 9-double bond conjugated carotenoids in the intact tissues of *Calendula officinalis* [63]. The Raman images were generated based on the peak at round C=C stretching vibration at 1520 cm^{-1}. This band was shifted at 1536, 1530 and 1524 cm^{-1} for the 7-, 8- and 9-conjugated double bond carotenoids, respectively.

Roots of different carrot cultivars were screened for their individual carotenoid distribution. The β-carotene signal at 1520 cm^{-1} was used for integration. The level of β-carotene was heterogeneous across root sections of orange, yellow, red and purple carrots. In the secondary phloem, the level of β-carotene increased gradually from periderm toward the core, but declined fast in cells close to the vascular cambium. Lutein and α-carotene were deposited in younger cells, while lycopene in red carrots accumulated throughout the whole secondary phloem at the same level [64]. Raman mapping was also applied for studies of *Pelargonium hortorum* to illustrate the heterogeneous distribution of the individual carotenoids in the leaves [65].

Plant polyacetylenes are another class of compounds that can be identified based on their C=C stretching vibration in the 2100–2300 cm^{-1} range. Using the Raman peaks at 2258 and 2252 cm^{-1} characteristic to the most common polyacetylenes falcarinol and falcarindiol, Baranska et al. [66] showed that polyacetylenes are mainly located in the outer section of the carrot roots.

Algae species are important candidates for industrial lipid and biofuel production. Sharma et al. [67] used Raman mapping for lipid analysis of microalgae. Characterization of lipid contents in cells obtained by mutagenesis showed that they managed to obtain mutants with increased lipid content. They have generated Raman images of the lipid-rich, carotenoid-rich and protein-rich areas on the *Chlamydomonas reinhardtii* microalgae based on the characteristic peaks at 1003 cm^{-1} (proteins), 1445 cm^{-1} (lipids) and 1520 cm^{-1} (carotenoids).

Apart from mutations, the growth media can also induce generation of different metabolites. *Chlorella sorokiniana* and *Neochloris oleoabundans* represent two good candidates for biofuel production. The species were Raman mapped at 532 nm for identification of carotenoid and triglyceride production, and in consequence, the maps were generated based on the signal intensity in the 1505–1535 cm^{-1} for carotenoids and 2800–3000 cm^{-1} for triglycerides (CH_2 stretching) [68]. Both healthy algae and nitrogen-starved algae were examined. Only carotenoids could be mapped in the healthy cells. The maps showed distinct locations where the carotenoids are concentrated as they are normally located in the chloroplasts. Triglyceride production was observed under nitrogen-starvation conditions, and it was possible to image

the lipid-rich regions within the starved algae. He et al. [69] reported similar results of triglyceride accumulation upon nitrogen starvation of *Coccomyxa* sp. algae. The triglycerides were imaged through the Raman lipid characteristic peaks at 1440, 1650 and 2840–2950 cm^{-1} (alkyl C–H bending, C=C stretching and CH_2 stretching, respectively).

Some algae are able to produce large amounts of carotenoids when irradiated with light under specific conditions (e.g., *Hematoccocus pluvialis* which produces large amounts of zeaxanthin). Grudzinski et al. [70] analyzed two algal strains, *Chlorella protothecoides* and *Chlorella vulgaris*, with respect to carotenoid production upon light-induced yellowing. They found the yellow coloration to be associated with xanthophyll formation, especially zeaxanthin. Under strong light exposure conditions, newly formed carotenoids were identified as a cell nucleus. It was possible to determine that zeaxanthin is the major carotenoid by performing Raman mapping both at 488 and at 514 nm. Both wavelengths are in resonance with xanthophyll pigments, but 514 nm is in resonance with zeaxanthin only. The cell nuclei give particularly high signal assigned to carotenoids when imaged under the 514 nm laser and low signal when imaged under the 488 nm laser, proving that zeaxanthin is the major synthesized carotenoid.

5. Conclusions

Raman mapping is a powerful technique for label-free, noninvasive investigations of tissues, cells and microorganisms. The resulted Raman maps contain not only spatial information but also valuable structural and chemical information on the analyzed samples. Raman imaging has been successfully used for investigations at cellular and subcellular level, including identification of nucleus, nucleoli, mitochondria and lipid-rich structures. Cell responses to drugs and different stages of the cell cycle from the stem cell to the completely differentiated cell were as well distinguished. In addition, Raman mapping has a great potential for becoming a leading method in a wide range of biomedical applications, owing to its high chemical specificity, good resolution and to the fact that it is a noninvasive to tissues and cells. It is possible to achieve accurate detection of healthy and cancer tissues. At the moment, for the purpose of medical diagnosis, the results of Raman imaging need most often to be compared with currently used diagnosis tools (PCR, histopathology and immunohistopathology). But since it has already been proved that Raman images are sensitive indicators for cancer detection, there is strong indication of the possibility to replace the conventional tools with Raman detection in the future.

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