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Authors	de Carvalho, Luis Felipe das Chagas e Silva;Saito Nogueira, Marcelo
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## New insights of Raman spectroscopy for oral clinical applications

Luis Felipe das Chagas e Silva de Carvalho<sup>a,c,d,e</sup> and Marcelo Saito Nogueira<sup>b,d,e</sup>

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Oral injuries are currently diagnosed by histopathological analysis of biopsy, which is an invasive procedure and does not give immediate results. On the other hand, Raman spectroscopy technique is a real-time and minimally invasive analytical tool, with notable diagnostic capability. At the current stage, researchers are widely aware of the diagnostic potential of the technique and how it is considered promising for providing biochemical information in real-time and without damaging the tissue. The problem originates from the lack of relevant studies and clinical trials that could show the actual use of Raman spectroscopy to help patients. Our goal here is to narrow the relationship between physicists, chemists, engineers, computer scientists, and the medical community, and in fact discuss the potential of Raman spectroscopy as a novel clinical analysis method. In the present study, we focused in the use of Raman spectroscopy as a daily clinical practice. In this context, additional studies and *in vivo* tests should be performed with the same approach of a real application. We want to show to the scientific and industrial community what is really necessary for this, starting from a clinical point of view. Using our previous experience publishing in different oral pathologies and types of samples, we also aim to discuss about the current state, potential, and what is required to implement Raman spectroscopy for oral clinical applications.

## Introduction

Many researchers have conducted studies to introduce Raman spectroscopy as new approaches for clinicians. However, very little has strong potential to help patients. At this stage, the specific point is how many capabilities we could use in our daily clinic. First, we need to share the applications for different purposes. As an example, a cell culture study which reports the difference between nucleus and cytoplasm are very important to show the big potential of the technique, but it does not have any clinical applications in terms of *in vivo* diagnostics or surgical guidance. Even though the technique (Raman spectroscopy) is the same, we have showed that if the instrument, probe, samples, etc is different, the final spectrum is totally different and cannot be related without taking into consideration optical properties of biological tissues<sup>1-6</sup>.

In this way, we should primarily target diverse clinical applications. This includes on-site clinical applications and surgical guidance, involving the use of *in vivo* portable Raman systems or rapid assessment of *ex vivo* samples in the Hospital, very similar to evaluation of a frozen biopsy. Second, aiding the pathologists in their laboratories, it would be possible to use a Micro-Raman system for cell cultures or histological samples to obtain molecular information, which cannot be assessed by using standard histological analysis.

Those information is extremely valuable and important to be publicized to clinicians so that they could contribute by improving the ideas about what patients really need. Obviously, many initial studies were performed by physicians without the presence and opinions of clinicians because they are really preliminary. However, at the current stage, we consider many of these studies not suitable for clinical translation, as, from our clinical experience, their published results will never be applied in the clinic. This occurs due to the simple fact that these studies do not have any applicability.

Also, two important professionals should be supported in this whole process, apart from clinicians, physicians and chemists: biomedical engineers and computer scientists. We should incorporate these professionals in the team in order to give research groups a chance to interact with medical device companies that build Raman systems and to develop customized instruments for clinical applications. Computer scientists and related professionals are required in order to create and adapt scripts (in MATLAB or other software) for control, automation, generation of user-friendly interfaces, fast data acquisition and processing to help patients in terms of diagnostic and treatment monitoring.

This paper aims to discuss about the current state, potential, and what is required to implement Raman spectroscopy for oral clinical applications.

## Oral diseases and optical biopsy

Oral health has a strong impact in the systemic health and quality of life. It is defined as a "state of being free from mouth and facial pain, oral and throat cancer, oral infection and sores, periodontal (gum) disease, tooth decay, tooth loss, and other diseases and

<sup>a</sup> Departamento de Odontologia, Universidade de Taubaté - Brazil<sup>b</sup> Tyndall National Institute, University College Cork, Lee Maltings Complex

Dyke Parade, Cork, Postcode: T12R5CP

<sup>c</sup> Centro Universitário Braz Cubas - Brazil<sup>d</sup> Corresponding authors: [luisfelipecarvalho@hotmail.com](mailto:luisfelipecarvalho@hotmail.com) and [marcelosaitonogueira@gmail.com](mailto:marcelosaitonogueira@gmail.com)<sup>e</sup> Co-first authors with equal contribution

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disorders that limit an individual's capacity in biting, chewing, smiling, speaking, and psychosocial wellbeing" <sup>1</sup> by the World Health Organization (WHO). WHO has promoted health improvements and oral disease prevention by building an oral health database <sup>2</sup> through the "WHO Oral Health Country/Area Profile Programme" in the Country/Area Profile Project CAPP and by implementing the directions of the Global Oral Health Programme <sup>3,4</sup>

Global initiatives allowed the development of preventive dentistry. In preventive dentistry, important initiatives for implementation of oral health programmes and integrated interventions were discussed in the last 11th International Association for Dental Research (IADR) World Congress on Preventive Dentistry<sup>5</sup>. Among the oral health topics, the prevention of oral disorders and conditions such as dental cavities (60–90% of school children and nearly 100% of adults have dental cavities worldwide), periodontal disease (severe cases found in 15–20% of middle-aged (35–44 years) adults), tooth loss (globally, about 30% of people aged 65–74 have no natural teeth), oral cancer (incidence ranges from one to 10 cases per 100 000 people in most countries), fungal, bacterial or viral infections in HIV, oro-dental trauma, noma, and cleft lip and palate are highlighted <sup>1</sup>.

In order to prevent oral disorders, many programmes have focused on optical technologies, which can provide a fast, non-invasive, molecular-sensitive, and *in situ* analysis of biological tissues. Optical techniques have the potential to be integrated in medical/dentist tools and can be relatively low cost compared techniques such as magnetic resonance imaging. These techniques are widely exploited in pre-clinical and clinical studies to evaluate the potential for implementation of an "optical biopsy" <sup>6–23</sup> or "spectral cytopathology"<sup>24</sup>. These latter concepts refer to the use of optical technologies as tools for clinical diagnostics, having a strong association with the current gold standard for disease diagnosis: the biopsy followed by histopathology analysis. In a conventional clinical setting, patients need to wait for a clinical-laboratory diagnosis, which may be time-consuming. This includes a set of examinations including biopsies and biofluid analysis. The waiting time can be extremely dangerous for certain oral injuries, since most of them are diagnosed in a later stage. This makes the treatment of oral diseases more expensive and difficult, compromise patients' prognosis, and, consequently, increases the demand for doctors and dentists attention. With this in mind, optical biopsies and biofluid analyses can be accelerated by using optical diagnosis technologies, as Raman spectroscopy.

### Raman spectroscopy

Two of the most powerful optical techniques are Raman spectroscopy and imaging (or mapping). They have the advantage of being extremely molecular specific, since the captured signal has features of molecular bonds. Both optical techniques rely on the collection of Raman scattered photons, i.e., photons that undergo inelastic scattering through energy exchange with vibrational or rotational modes of molecular bonds<sup>25</sup>. Many studies in *ex vivo* and *in vivo* biological tissues have reported the success of the proof-of-concept for the use of Raman spectroscopy and imaging for identification of oral diseases such as cancer or inflammatory

diseases<sup>26</sup>. In addition, *in vitro* studies report changes in a subcellular and cellular level, which can be associated with what is observed in a clinical study<sup>24</sup>. However, the biological variability is a factor that must be considered and are not consistently monitored. Advancements in instrument probe designs and automated analysis are still under development <sup>25,27,28</sup>.

Within this context, Raman spectroscopy can be applied in many areas, such as the diagnosis of early and difficult diseases, but there is a need for the communication between basic science (physicists, chemists, engineers and others) and applied science (doctors, dentists, among others) so that researches have a higher usability and are not lost with time. For example, an *in vivo* Raman system will be used clinically when we need a fast real-time response, and an imaged micro-Raman system may assist the pathologist in a laboratory for more elaborate analyses that are difficult to perform. The pathologist often needs complementary information from immunohistochemical techniques and immunofluorescence, since histology alone is not enough.

Many clinicians are not aware of Raman spectroscopy, nor of its diagnostic ability. This hinders the progression of Raman spectroscopy in the clinical environment. The importance of presenting research in medical conferences, and scientific journals with medical applications is certainly a viable alternative to increase clinicians participating in Raman spectroscopy projects.

Raman spectroscopy and imaging have been used for *in vitro* cell studies with two main approaches: identifying cancer cells (Raman microscopy or microspectroscopy) and investigation of subcellular processes and/or microenvironment. The first one was successful for differentiating a variety of cell types including neoplastic or normal hematopoietic cells<sup>29</sup>, leukemia cells among other types blood cells<sup>30</sup>, and among oral normal, dysplastic epithelial cells, and oral epithelial cancer <sup>24</sup>. These differences result mostly from analysis of nucleic acids, lipids, carbohydrates, and protein components (such as amide I).

Studies of subcellular processes are focused in the discovery of cellular origins of diseases that can be probed with Raman spectroscopy. Once these origins are clarified, choosing specific diseases to probe using Raman spectroscopy and evaluating its potential for diagnostics becomes easier. One example is the composition of lipid droplets in cell cytoplasm, which is related to atherosclerosis and Hepatitis C infection<sup>31–33</sup>. Cellular microenvironment has been studied by using surface enhanced Raman spectroscopy (SERS) to monitor pH and enzymatic activity in cells <sup>34–38</sup>. Although SERS studies present a very advantageous concept, we have observed in recent years that its application has become increasingly distant from clinical practice, due to the instability of the signal found, difficult manipulation and reproducibility.

Previously reported studies involving *ex vivo* tissues are separated in two main approaches: complementary tool for histopathological analysis and alternative analysis of freshly excised tissues. The first approach aims to help pathologists by providing a chemical analysis after processing and staining the specimen. One alternative is reported by Lutz et. al., who demonstrated a similar performance between SERS immunostaining and conventional

fluorescent immunostaining techniques<sup>39</sup>. The second approach is focused in the identification and quantification of chemical differences in freshly excised tissues. This is a powerful method in terms of reducing the time required to process the sample, since Raman spectroscopy and/or imaging can provide an immediate biochemical analysis of the specimen. However, differences in methodology such as time between the tissue removal and data collection, time when the blood supply is restricted, ambient conditions (such as ambient light, temperature, and humidity), and probe contact pressure hinder the possibility of collecting reproducible and reliable data. *Ex vivo* studies using this approach include the differentiation between benign and cancerous lesions in breast tissue<sup>40</sup>, identification of parathyroid adenomas and hyperplasia in parathyroid tissue<sup>41</sup>, and estimation of higher lipid, protein, collagen, nucleic acid, and glycogen content in normal and cancerous bladder tissues<sup>42</sup>. By using Coherent anti-Stokes Raman scattering (CARS), a good correlation between H&E staining and CARS imaging was reported in *ex vivo* mouse brain tissue<sup>43</sup>, tissue morphological changes could be monitored in endothelial and smooth muscle cells of carotid artery (collagen fibrils and elastin)<sup>44</sup>, spinal tissues and mouse sciatic nerves (demyelination and myelin repair processes in myelin sheaths)<sup>45</sup>.

### Oral clinical applications of Raman spectroscopy

Recent oral clinical applications of Raman spectroscopy can be divided into disease detection<sup>46,47</sup>, determination of surgical margins<sup>48</sup>, and estimation of treatment response<sup>47,49–51</sup>. Disease detection includes studies using point measurements<sup>52–54</sup>, Raman microscopy<sup>55–58</sup>, and Raman mapping<sup>59–63</sup>. Raman microscopy can give access to biomolecular and cellular information in the same image, which may be useful to understand pathological processes involved in the studied diseases. On the other hand, Raman microscopy may be difficult to be translated to the clinic. The advantage of Raman mapping is the spatial resolution that allows border identification and image features extraction. However, most of reported studies use point measurements in order to obtain better spectral quality in shorter times. Disease detection applications are also dependent on the sample that is being probed and will be better discussed in the next section. Assessment of surgical margins can be performed by both point measurements<sup>52</sup> and Raman imaging<sup>61,64,65</sup>. Imaging is more suitable for this particular application and can be better adapted to display the surgical margins for precise lesion removal. However, the implementation of this application includes the development of sophisticated apparatus to provide real-time and user-friendly information to guide the surgeon. Therefore, this implementation may be challenging to be done at low cost, especially when the existing technology available in the market is taken into consideration. Finally, few studies report treatment response prediction, including oral cancer cell monitoring<sup>66</sup>, serum analysis<sup>67</sup>, and *in vivo*<sup>68</sup>. In this case, studies in *ex vivo* and *in vivo* tissues are still required for the next step for proof of concept of Raman spectroscopy for treatment response monitoring.

### Samples for oral disease detection

Oral clinical measurements of Raman spectroscopy can be performed in biofluids, exfoliated cell samples, and *ex vivo* and *in vivo* tissues.

Biofluid analysis has the potential to enable low-cost and distance diagnosis<sup>49</sup>, but its implementation in the clinic is still challenging and may take longer than other approaches. This implementation require standardizing operational (e.g. when the sample is analysed in the clinic workflow), sample collection (e.g. fasting or not fasting), and sample handling (e.g. sample volume, dilutions, and storage) procedures. Taking into account biological variability and patient behaviour aspects (e.g. smoking and hydration) is necessary once the first standardization takes place. Successful studies of biofluids for oral applications were mostly performed in blood<sup>69,70</sup> (especially serum<sup>67,71–74</sup>), saliva<sup>75–78</sup>, and urine<sup>79</sup>. However, even though previous studies report the proof of concept works, further investigation is essential to move forward to large-scale clinical settings.

Analysis of exfoliated oral cells has potential to be suitable for screening and routine clinical applications. On the other hand, clinical translation is dependent on the same standardization procedures discussed for biofluids. In addition, the use of microscopes in the clinic is not common, which means the microscopy market needs further development in order to meet clinicians' requirements before exfoliated cell applications take place. These applications are based on the assumption slight biochemical changes towards the diseased condition can be identified earlier than morphological alterations. Therefore, the sensitivity provided by Raman spectroscopy could be used to enhance diseased tissue features and diagnose the disease in an early stage. As enhanced features are mostly known by molecular biologists and pathologists, the supervision of these professionals is critical for the success of multicentre exfoliated cell studies and clinical translation. Research groups have reported advances in spectral differential analysis for normal, dysplastic, and squamous cell carcinoma cells<sup>56</sup>, and feasibility of spectral acquisition and cell collection<sup>80,81</sup>.

*Ex vivo* studies are easier and quicker to be conducted for validation purposes, as clinical research ethics approval takes shorter for non-interventional studies. Then, more multicentre studies can be carried out and clinical translation may happen earlier than other applications. Conversely, the *ex vivo* tissues do not reproduce the same conditions of *in vivo* tissues and the detection of slight biochemical changes may be more difficult. This may hinder early-stage disease diagnosis. Also, biological variability and handling and storage differences among health institutions must be taken into consideration when analysing *ex vivo* tissues spectra. Reported studies show results for both frozen and fixed tissues to differentiate normal, inflammatory, precancerous, and cancerous tissues<sup>26,46,58,82–86</sup>. Good sensitivity and specificity was reported by Girish et. al. for tissue sections on Ag-TiO<sub>2</sub> nanostructured SERS substrate<sup>49,87</sup>. Initiatives to optimize the data analysis were performed by our group, which evaluate the classification accuracy by multivariate analysis methods<sup>6,7</sup>.

*In vivo* studies represent the most advantageous approach for clinical translation. By assessing the variation of the biochemical



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content in early stage diseases *in vivo*, slight differences can be observed directly in the patient. Therefore, applications in the clinical routine are more realistic and translation is more feasible. Previous studies report efforts to decrease the time of spectral acquisition<sup>47,88</sup>, age-related and tobacco-related<sup>86</sup> or oral anatomical subsite variations<sup>89–91</sup>, and feasibility of detecting changes associated to cancer, precancer, and tissue malignancy<sup>92–97</sup>. The next step requires real-time analysis improvements in the spectra collection, signal and data processing, and data analysis. These improvements can enhance the data quality, automated system responses, and generation of user-friendly reports.

### Interference due to optical processes and implications in signal processing

Currently, there is no technique capable of measuring only Raman scattered photons in biological tissues. In Raman spectroscopy, several optical processes contribute to the captured signal such as fluorescence, Mie and Rayleigh scattering. Mie and Rayleigh scattering can lead to biased changes in the captured signal, since higher energy photons scatter more than the lower energy ones, e.g., blue light scatters more than red light. This affects both incident and collected light, thus making the efficiency of the collected signal depend on the probe geometry (such as positions of light source and detector and area of the detector) and the measured tissue profile. Tissue structures such as striations in collagen fibres and mitochondria membranes increase Rayleigh scattering processes, while organelles and cell surfaces contribute to Mie scattering. This happens because Rayleigh and Mie scattering are generated by refractive index mismatches in structures smaller or larger than the light wavelength. On the other hand fluorescence occurs due to light absorption and subsequent emission of lower energy light by specific biomolecules, as molecules lose energy through vibration before emission. The observed effect in Raman spectra is a broad spectral background present only in biological tissue measurements.

Scattering and fluorescence processes happen together in biological tissues, which hinders the extraction of pure Raman intensity spectra out of intensity measurements. In order to perform this extraction, the research community has used a number of background subtraction algorithms<sup>98,99</sup> based on scattering correction and polynomial subtraction. However, subtraction of arbitrary baselines will not recover the ideal spectrum constituted only by the intensity of Raman photons, since the minimum of the real Raman spectrum may be different than the spectrum after the subtraction of a polynomial (arbitrary baseline), for example. Therefore, prior knowledge about the processes that contribute to the signal background is required in order to recover the true Raman spectrum.

In order to take optical processes contributing to the intensity of the underlying background in tissue Raman spectra, it is possible to use multimodal optical spectroscopy. By using reflectance and fluorescence spectroscopy, it is possible to extract fluorescence, absorption and scattering coefficients of biological tissues. This information can be coupled to Raman systems and taken into account when subtracting the signal background. In this case, clinical

translation would occur only after processing algorithms for measurements with the three optical techniques could be implemented together to give a real-time analysis of the captured Raman signal. This implementation requires computer science experts in programming, integration, and automation. Another requirement for heterogeneous tissues is collecting the three types of spectra from the same spot, which may be challenging in the instrumentation aspect, as it involves integrating the three techniques in the same measurement probe. The same applies to analysis of *ex vivo* samples in a substrate, since the substrate spectrum should be removed by digital dewaxing<sup>100</sup>.

### Instrumentation standards and limitations

Conventional instrumentation for clinical Raman spectroscopy comprises a laser, fibre optic probe, spectrograph, and detector (CCD or spectrometer). Lasers are used to produce light in a specific, stable, and narrow wavelength band, which allows the Raman shifts to be calculated using the spectral distance from the excitation (Rayleigh scattered) band. They produce enough power to generate a significantly high Raman signal, but are limited by the intensity that leads to heating effects that may damage biological tissues. This may lead to time-consuming measurements due to the low probability to produce Raman photons. In addition, the light source wavelength may be absorbed by tissue chromophores (such as oxy and deoxyhaemoglobin, melanin, water, lipid), making 785 nm the choice of most of the systems for medical applications, as this wavelength is positioned in the “optical window”, the spectral region where biological tissue has highest light penetration.

Fibre optic probes are generally used for clinical Raman spectroscopy to optimize the light delivery and collection. These probes allow clinicians to access the region of interest anywhere in the patient body and can be designed for specific applications. This design involves optimum choice of optical fibre material, size, geometry, and surface modifications of the probe. Typically, silica (glass) fibre probes are used thanks to their relative low-cost and possibility to be sterilized. However, glass fibres have a strong Raman signal below  $700\text{ cm}^{-1}$ <sup>28</sup>, which cannot be extracted from the collected measurement signal, in many cases. With regards to the size of the probe, a high collection signal and most of the design configurations are possible for probes with centimetres of diameter. On the other hand, if millimetre-sized probes are required, the options of design and intensity of captured signal may drop<sup>28</sup>. Major limitations for clinical translations rely on the high cost of the probes currently used in research (expensive assembly and optical filters) and artefacts of ambient light, which cannot be totally suppressed by currently used probes. Ambient light is a factor that must be taken into account, since creating a complete dark environment for clinical diagnostics or surgical guidance is not feasible.

### Advances in instrumentation

One of the main advances in instrumentation is the design of fibre optic probes for depth selectivity and fast acquisition time<sup>27,28,101–105</sup>. Depths from hundreds of micrometres until few centimetres can be reached by different fibre probe designs and

techniques (by changing the shape of the probe tip and the distance between the source and detector fibres) and adoption of the spatially offset Raman spectroscopy technique<sup>106–115</sup>.

Advances in integration between instrumentation and data analysis reduced the acquisition times to less than one second and still had potential to give a precise diagnosis<sup>25,116–120</sup>. These systems are very sophisticated, but the equipment can be bulky and expensive, which hinders perspectives of clinical translation. Alternatively, the development of hand-held equipment may diversify the use of expensive instrumentation, since it is more ergonomic and portable<sup>25,119,121</sup>. In this case, the instrumentation specifications tend to be high enough to give a precise diagnosis only if it contains expensive components that allow sufficient spectral resolution and acquisition time. For Raman imaging instrumentation, specifications may need to include spatial resolution and field-of-view, which can add even more costs. Ideally, if the instrumentation needs to be used in a clinical routine, alternatives must be sought to allow clinics/hospitals to afford the equipment. Another relevant advance is fabricating low-cost disposable probes. Potentially cheap probes have been proposed by two research groups<sup>122,123</sup>. One of them used a disposable needle tip with fused silica fibers connected to a reusable probe with expensive components, while the other could be produced without expensive optical filters<sup>28</sup>.

### Spectral pre-processing techniques

In the Raman spectroscopy context, data pre-processing consists in preparing the data to be analysed after spectra collection. It involves background subtraction for obtaining the desired Raman scattered signal, noise suppression, and enhancement of spectral differences based on shape, absolute intensity, and spectral position of Raman bands.

Appropriate background subtraction must be performed in order to recover the true Raman spectrum, i.e., the Raman spectrum without interference of optical processes other than Raman scattering. Background subtraction includes techniques to eliminate the influence of fluorescence and other types of scattering processes, as mentioned in the previous sections. One of the few techniques developed to remove the influence of scattering in Raman spectra is called extended multiplicative scatter correction<sup>124</sup>, which was initially designed to analyse infrared spectra of powder mixtures. On the other hand, a wide range of methods is used for removing the influence of fluorescence. These methods include wavelet transformation, principal component analysis (PCA), penalized least squares, multistage smoothing, first- and second-order differentiation, polynomial subtraction, and frequency filtering<sup>27,125–132</sup>. The latter two approaches are the most used ones. Polynomial subtraction consists in fitting a polynomial of sufficiently high order to describe the smooth fluorescence contribution without eliminate high frequency Raman signals and subsequent subtraction of this polynomial. Frequency-domain filtering consists in eliminating low frequency contributions in the signal after applying a fast Fourier transform. More advanced methods include principal component analysis, which removes the components of high spectral variance by assuming they are associated to the fluorescence background, and wavelet transformation, which relies on the shape of fluorescence

spectrum underlying the Raman signal and on the choice of the decomposition method<sup>27,132–134</sup>. DOI: 10.1039/C8AN01363B

Noise suppression is mandatory in Raman spectra, as the real Raman contribution is weak and most of times hidden in a highly noisy signal. This suppression is especially useful to posterior biochemical characterization based on fitting of the Raman peaks, as it removes high-frequency features that might hinder the right determination of the peak position. It can be performed by applying filters such as Savitzky–Golay, Gaussian, median, and moving average window filters. The order of Savitzky–Golay filters is highly dependent on the system specifications (such as spectral resolution and efficiency of light collection) and measurement conditions (such as short integration time or dark current in the detector) that contribute to the signal noise. Gaussian filters rely on the system spectral resolution and are usually have the full width at half maximum set to half of this resolution<sup>135–139</sup>. Advanced methods to suppress high signal frequency components can use multivariate statistics or analysis such as PCA and genetic algorithms<sup>139–143</sup>. Even though these methods remove noise extremely efficiently, they must be used carefully to not eliminate features of the true Raman spectrum, which has a significantly higher frequency than the background, but lower frequency than the random signal noise. An alternative to estimate the measurement noise is probing the different sources of noise in the used instrumentation. These sources may involve all the equipment components, thus making calibration essential to check for reliable noise suppression. In this calibration, a standard substance with known Raman spectrum should be measured to characterize the system response and checking whether the processed signal matches with the known (calibrated) Raman spectrum.

Enhancement of the spectral differences is highly used for tissue classification purposes after spectral pre-processing. It is typically performed by using difference spectra (subtraction from average spectra of the dataset) and normalizing the Raman spectrum in several ways. The most common types of normalization are normalizations to the area under the spectrum, maximum intensity of the spectrum, and mean scaling of the spectrum based on the intensity of the average spectrum in a particular Raman shift for a specific patient. For interpretation purposes, the first type assumes the same number of photons will be detected in each measurement, including the whole spectrum. On the other hand, the second and third types rely on a constant intensity of the strongest emission of the sample and on a constant emission in a certain wavelength for all the patients.

### Opportunities and challenges in data analysis

Analysis for clinical Raman spectroscopy can be divided in three main approaches: spectral feature extraction, tissue classification for direct diagnosis and biochemical characterization for interpretation of patient state. Feature extraction is based on the idea of finding new aspects that characterize hidden information or divisions in the patient group under study. As an example, one may be looking for grouping patients with benign or cancerous lesions. However, after analysing how patients are grouped upon comparison of certain features, one can see that patients could be separated by age

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instead. This is performed by using unsupervised multivariate analysis methods, i.e., methods to analyse experimental spectra without prior knowledge of the studied subjects. These methods include cluster analysis and component analysis. Cluster analysis is based on grouping sets of subjects. The formed groups can have characteristics assigned to them (such as age, skin colour, and pathology) in order to check whether the groups are compatible with one of these characteristics. If each group obeys a trend in a given characteristic, a potential differentiation of subjects can be performed by using the measurement technique targeted in the study (Raman spectroscopy, in this case). Cluster analysis techniques comprise hierarchical cluster analysis, K-means, fuzzy C means, and Density-Based Spatial Clustering of Applications with Noise<sup>144–153</sup>. Component analysis is based on describing the subjects with new features. Examples of component analysis include PCA, linear discriminant analysis, and vertex component analysis. PCA is the most used method. It calculates features based on independent components (sum of weighted signals for each Raman shift) and order them from the highest to the lowest variance<sup>141,143,153–157</sup>. This makes it suitable for decreasing the complexity of the spectra, i.e., analysing less Raman spectral features. Analysing thousands of values of Raman spectra may be complex and can lead to high computational costs, which means more time is required for data analysis. Therefore, PCA is used to extract the combinations of spectral features for quick data analysis while keeping the diagnosis performance<sup>158</sup>.

Tissue classification is widely used to create models to discriminate/differentiate among several types of biological tissues and predict what the category of a new measured tissue is. It is typically performed by using supervised multivariate analysis methods, i.e., methods that use prior knowledge of the studied subjects to analyse the data (Raman spectra). Classification methods are incredibly powerful and can be used for precise diagnosis of many diseases. However, in order to achieve a reliable tissue classification, three requirements must be met. First, the sample size must be large enough to represent the whole group of studied objects, e.g., oral lesions. When the dataset used to generate the classification model is not sufficiently diverse and large, i.e., if it does not contain the typical heterogeneity of a clinical setting, the model may be too specific for the setting the study was conducted. This was demonstrated by a study that attributed acceptable diagnosis performance by models generated with 2 to 25 subject and good classification in studies involving 75 to 100 patients<sup>159</sup>. Moreover, misclassification can lead patient severe consequences to the patients, thus making significant statistical difference a critical factor for diagnosis. Second, the model must be robust enough to achieve the diagnosis performance with every additional input data. In order to do this, the model should have as many internal and external validations as possible. For internal validation, part of the dataset is used as training set to build the model, while the other part (test set) is used to apply the model and test its accuracy. This can be done repeatedly until all parts of the dataset are used to calibrate the model and test it. Optimum model for each dataset is calculated by using the root-mean squared error of calibration and root-mean-squared error of prediction. Several methods can be employed to estimate this accuracy such as k-fold cross-validation, permutation

testing, and leave-one-out<sup>153,160–162</sup>. After this, an external validation is required for testing the generated model with a second dataset, i.e., a larger dataset of newly measured Raman spectra (independent of the first dataset that was used to generate the model). The external validation is necessary because the first generated model may be biased, since it was optimized to classify data (biological tissues) in the first dataset. Third, the classification must be completely independent on the measurement conditions. This means that a standard of procedure must take place in order to make measurements more uniform and systematic across studies, in order to allow comparison among them and ultimately build a general model that is valid for all the set of instruments of a company, for example. This is important to calibrate Raman spectroscopy systems over time, as they can lose their diagnostic performance and may need maintenance. Tissue classification methods include support vector machines, linear discriminant analysis, quadratic discriminant analysis, partial least squares, logistic regression models, neural networks, decision trees, genetic algorithms, and general optimization techniques.<sup>27,141,143,163–170</sup>

Finally, biochemical characterization is attractive for estimating biomolecules concentrations in tissues, which can provide metabolic information that may be useful in clinical monitoring cases, where many possibilities can happen such as processes of tissue healing and mineralization, treatment prognosis, and progress of bacterial or fungal infection. In these cases, classifying tissues may be hard due to many changes that can occur, which hinders the differentiation among stages. In this case, biochemical information may be useful for the clinicians to understand the patient situation by taking into consideration both this information and the patient history. Biochemical characterization is usually performed by deconvolving Raman spectra peaks into combinations of Gaussian curves. Each curve will be centred in the Raman shift correspondent to the described vibrational mode and the abundance certain biomolecules can be calculated by the area of the Gaussian curve, which indicates the intensity of the deconvolved peak.

### Perspectives of clinical translation



Our team frequently makes clinical diagnosis in our units, and deal with histopathological results regarding biopsies previously performed in our offices. We take a recent clinical case as an example, to illustrate some possible applications of the technique and in what direction we can follow.

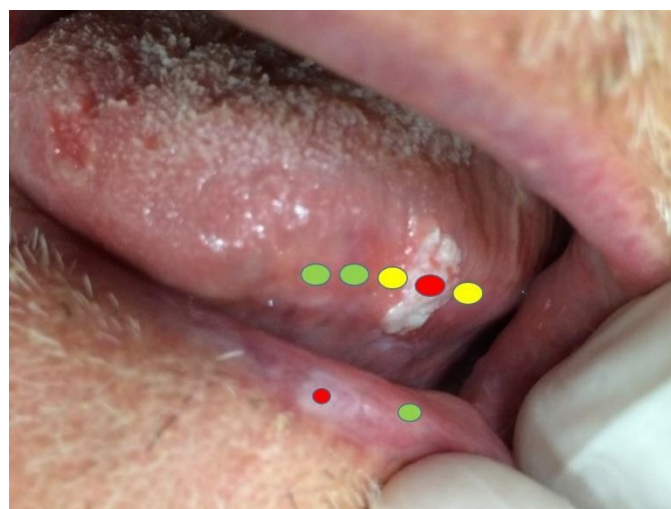


Figure 1. Early oral squamous cell carcinoma of the tongue. Leukoplakia in lip. Using the dots we would like to point out some Raman approaches. The spectrum itself could be translated into colours, as described in the text.

In figure 1, we show a clear example of a patient in whom optical biopsy procedures may assist both for diagnosis and for treatment. The patient has two distinct lesions, one in tongue and one in lip. The hypothetical clinical diagnosis of these lesions is leukoplakia, which is considered a potentially cancerous lesion. This makes the early-stage diagnosis highly recommended.

Let us consider the lesion of the tongue (figure 1), which presented a nodule at the time of diagnosis. This nodule can consequently generate greater severity and is evidence that the lesion can progress towards an epidermoid carcinoma, as it was confirmed in the real case afterwards. In this context, traditional incisional biopsy is the chosen procedure for the final diagnosis, but a portable Raman spectroscopy device could help clinicians to identify features of each area, as shown in the figure 1. A "green" spectrum would represent a normal area, a "yellow" spectrum would represent an area doubtful, and a "red" spectrum would represent a potentially malignant/disease area.

The problem addressed above refers to the use of the technique as diagnostic method. Once the oral lesion is diagnosed as cancer, the procedure that must be performed is the surgical removal with associated safety margin or not to radiotherapy. Then, we can use again the Raman spectroscopy technique for the surgical procedure to guide us in the healthy or pathological safety margin, thus helping the clinician in his/her limitations (e.g. evaluations using naked eye) and providing a database for analysis of oral tissues at molecular level for fundamental applications.

Researchers have engaged into creating new groups and networks to accelerate the clinical implementation of vibrational spectroscopy techniques. Some of these groups include Raman4clinics and Clinical Infrared and Raman Spectroscopy

Network (CLIRSPEC), in which some events are organized to stimulate the international and interdisciplinary community to collaborate and narrow discussions about challenges of clinical translation of Raman spectroscopy.

### What is next?

In terms of instrumentation, Raman spectroscopy systems should include six main features: portability, low-cost, user-friendly interface, capable of providing accurate diagnosis, fast data acquisition and analysis, automated. Portability and low-cost development rely mostly on instrumentation. User-friendly interface and accurate diagnosis require both instrumentation and software aspects. Building an intuitive interface for clinicians involve the implementation of instrumentation features and software guidance based on a deep understanding about clinicians background and work environment. Improvements in instrumentation include buttons at the probe or foot pedals for taking measurements, while software advances may include possibility of modes for real-time signal update and for recording precise spectra (acquisition mode), real-time display of (easy interpretable) clinical data reports, and intuitive interface for changing equipment configurations. Automation and fast data acquisition and analysis are very dependent on software development. A big step in automation is integrating the data acquisition, data processing, and data analysis software, which should process all the information in real-time, including background removal, spectral smoothing, normalization, feature extraction, classification. For fast data acquisition and analysis, optimizing both steps with regards to computational costs is essential to provide a real-time data updates and generation of intuitive reports.

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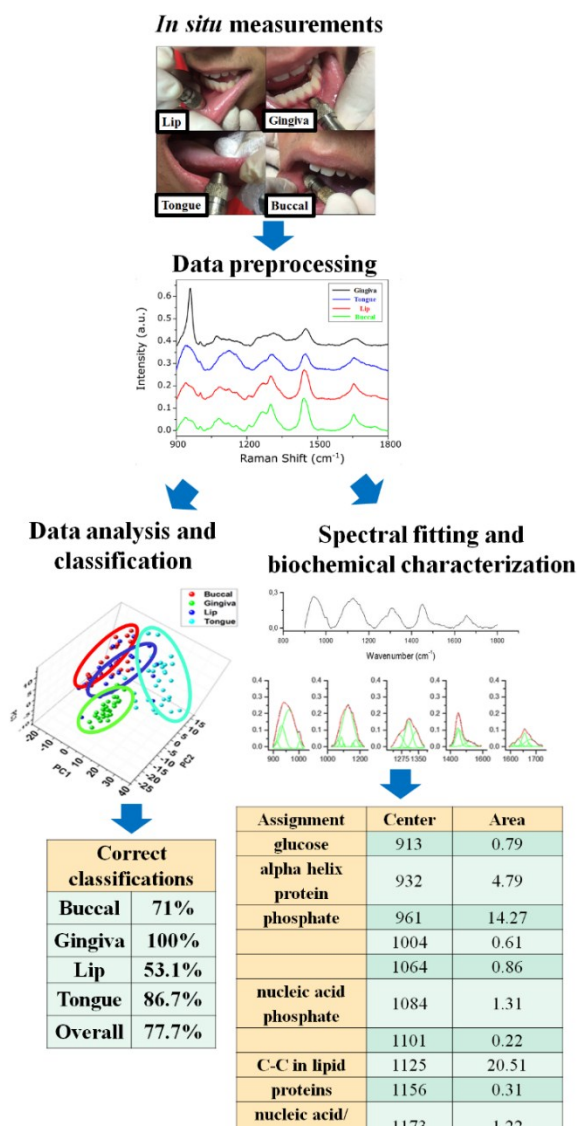


Figure 2. Workflow of the *in vivo* Raman system showing our previous healthy oral subsites *in vivo* measurements. It is important to note that the role procedure should be in "one click".

Finally, with regards to the use of Raman spectroscopy as a daily clinical practice, obviously more studies and *in vivo* tests should be performed, but always with a focus that should have a real application; and it was our objective in the present work to show the community of spectroscopists what is really necessary, starting from a clinical point of view. Thus, we must expand our studies to: 1 - precise identification of potentially cancerous lesions (leukoplakia, erythroplakia, submucous fibrosis and actinic cheilitis), 2 - seek subtle changes in the healthy mucosa of patients at risk (e.g. smokers, or very exposed to solar radiation), changes in which we were not able to evidence in routine clinical examination, 3 - evaluation of infiltration or margin of lesions of squamous cell carcinoma, or even early stage epidermoid carcinoma, 4 - development of robust *in vivo* spectroscopy technique to identify other pathological complications that require early diagnosis (including cancer or not), such as autoimmune diseases, some fungal infections, among others.

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