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Fourier Transform Infrared Imaging Spectroscopy in Biomedicine – Important Things to Consider When Planning a New Experiment

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1. Introduction

Fourier transform infrared imaging spectroscopy (FT-IRIS) offers unique possibilities to collect chemical information from biological samples with high spatial resolution (generally ~10 μm) [1]. The development of FTIR instruments has introduced this technique also for biomedicine. FT-IRIS is a demanding technique that requires a good understanding of the measurement and analysis principles. Application of the technique can be divided into three phases: 1) sample preparation 2) data collection and 3) data analysis. Each of the three steps is crucial for the outcome of the study and significant sources of error may exist in all three steps. Biologists and medical doctors are often not aware of the technical aspects of the measurement principle or the data analysis methods. Therefore, lack of information may impair successful application of this technique, and the full potential of the technique is not achieved.

This book chapter is written for an entry level user without the formal training for spectroscopy or data mining techniques. This chapter addresses important issues which should be considered when the FT-IRIS experiments are designed and data is analysed. We cover briefly: 1) the overall potential of FT-IRIS in qualitative and quantitative biomedical research, 2) essential issues to be taken into account in preparation and measurement of the biological samples and 3) different methods for analysis of acquired spectral data.

2. Planning a new experiment

FT-IRIS studies require careful planning of the study protocol beforehand. The first task is to define the aim of the study and to evaluate whether FT-IRIS is suitable for a planned experiment. FT-IRIS is a diverse technique that can be applied to numerous applications. Existing literature introduces numerous studies varying from simple qualitative applications to highly sophisticated applications of complex neural networks. FT-IR spectroscopy has been applied e.g. to cancer diagnostics [2-7], bone diseases [8-10], osteoarthritis [11-13], neurological diseases [14-16] and atherosclerosis [17,18]. Each and

every study is different and the used study protocols can be transferred only in rare cases directly to a new study. In general, sample preparation has the least variation, and also the measurement conditions can be standardized up to certain point, but the data-analysis has to by designed for each study individually.

2.1 Sample preparation

FT-IRIS studies are carried out either using thin histological sections (transmission measurement) or by using smooth, polished surfaces (reflection measurement). Histological tissue sections can be produced from: 1) fixated samples embedded into paraffin or other resins or 2) application of cryosectioning. Both techniques have their own advantages and limitations, which are discussed next.

2.1.1 Chemically fixed histological samples

Chemical fixatives have been used for decades in histology for preparing thin tissue sections for microscopy. Typical sample preparation involves formalin fixation and decalcification process. Samples are further dehydrated with ascending series of alcohol, and finally lipids are removed with xylene before sample is infiltrated with liquid paraffin [19]. Paraffin embedding is needed to create support for fragile sections in order to be able to cut 3-7 μm-thick histological samples. Embedded samples are easier to cut and, therefore, they have been used most often in histological studies. However, it should be realized that chemical fixatives introduce a few problems in FT-IRIS studies. Fixatives form chemical cross-links to protein structures preventing tissue deformation during the section processing. This is advantageous when only the morphology of the sample is considered, as is the case in traditional histology. However, since chemical fixatives alter the proteins and carbohydrates of the sample, they have potentially negative effects on spectroscopic analysis. It is known that the effect of chemical cross-linking can be seen in IR spectra [20, 21]. This clearly indicates that the fixation has permanently changed the tissue chemical properties. However, chemical fixation is typically done in a standardized manner within one study, and its effects can be assumed to be similar for each sample. Therefore, chemical fixation does not necessarily hinder the use of embedded sections.

Another possible source of error is that residual traces of paraffin are evident in paraffin-embedded sections even after chemical removal of paraffin [22]. Instead of chemical removal of paraffin, it is also possible to subtract paraffin after the measurements by using paraffin spectra [5]. Fortunately, a paraffin spectrum contains only a few narrow peaks. In order to minimize the contribution of paraffin residues to the analysis, one might also simply exclude the paraffin peak areas from the analysis.

In addition, the fact that lipids and part of the solubilized proteins are most likely lost from the sample due to the sample processing must be taken into account. Thus, information arising from these compounds cannot be studied from the fixated samples. Taken together, these limitations should be taken into account when the use of chemically fixed tissue sections are planned in new FT-IRIS studies.

2.1.2 Cryosections

When cryosections are used in FT-IRIS measurements samples are not treated with fixatives prior to sectioning, and it requires only minimal sample preparation. Tissue is prepared and embedded into cryo-embedding medium. Subsequently, sample is frozen with liquid nitrogen and sectioned with a cryotome. However, here one should note that the accuracy of

section thickness of cryotome is not at the level of paraffin or plastic embedded samples. On the other hand, since the method does not require chemical processing of the sample, the sample is maintained close to its original biological form. Embedding media used for cryosectioning often stains the samples, which causes problems for data analysis. After cutting tissue samples with a microtome, sections have to be rinsed with water, but the lack of chemical cross-linking makes the thin sections pliant, and consequently sections wrinkle easily during the preparation. In general, repeated sectioning needs to be done until the good quality section is found for the final measurements.

Advice: Chemical treatment of the sample is not needed with cryosections. Tissue is minimally altered compared to chemical fixation. Soluble proteins and lipids can also be studied when cryosections are used.

Conclusions: Chemical fixatives alter the collected IR data. Different fixatives cause different alterations and therefore sample processing must be standardized between the specimens. Fixative-related alterations are not a problem with simple univariate analysis techniques, but they may cause potential artefacts when sophisticated multivariate techniques are used. Chemical fixation and embedding is also poorly suited for research problems when solubilized proteins or lipids are main interests of the study. Furthermore, a significant section thickness variation caused by a microtome itself exists both with embedded sections and cryosections. This variation has to be kept in mind in quantitative analysis and compensated, if necessary, with repeated measurements or with the reference sample technique [23]. Preparation of the cryosections is fast but the overall time consumption is considerable since the quality of cryosection is inferior to embedded sections.

2.1.3 The effect of the variable section thickness

Section thickness varies significantly between histological sections due to the various uncertainties (e.g. temperature changes during cutting, inadequate embedding media support, microtome-related inaccuracy and sample-related variation). In general, the variation is smaller with embedded samples. Section thickness variation affects directly the measurement results, since according to Beer-Lambert law, absorption is directly related to section thickness. Normalization (e.g. vector normalization) of the spectral data is usually conducted before qualitative multivariate analysis. Therefore, section thickness variation is usually not a problem with qualitative analyses. However, quantitative analysis requires a strict control of the section thickness since the thickness variation is one of the main sources of errors in the FT-IRIS experiments. Variation is particularly harmful in sample-to-sample correlation analysis where FT-IRIS measurements are correlated with the reference technique. The nature of section thickness error is random, and therefore its distribution can be assumed as Gaussian. Consequently, the section thickness error is not as significant when group means of different sample groups are compared. However, it is essential to keep in mind that the error caused by the section thickness variation can be greater than the biological variation itself.

Advice: Evaluate whether the type of experiment you are conducting can be affected by the variable section thickness. If quantitative analysis from FT-IRIS spectra is carried out and compared with reference methods (correlation analysis), a good control of section thickness is essential. On the other hand, qualitative (multivariate) analysis is least affected by the section thickness variation. As a rule of thumb, section thickness artefact can be reduced by averaging multiple measurements from single specimen or by using homogenous reference material cut along with biological tissue, as described by Rieppo et al. (2004) [23].

2.2 Data collection

Type of the experiment sets the requirements for data collection. Acquisition of spectral data has to be optimized for signal quality and the measurement time. Measurement of a single specimen can be very time consuming when high quality data is measured and large areas are covered. A simple univariate data analysis can be carried out with low quality data, but when sophisticated multivariate analysis is carried out, the signal-to-noise ratio has to be adequate. The length of the data collection is determined by four factors: 1) measured spatial area, 2) spectral resolution, 3) spatial resolution and 4) number of averaged scans. Spectral resolution, spatial area and spatial resolution are directly proportional to the data collection time.

2.2.1 Spatial resolution

Modern imaging instruments allow the use of different spatial resolutions. Data collection time increases significantly when spatial resolution is increased due to the decreased signal-to-noise ratio and the increased amount of the measured pixels. Better spatial resolution requires more repeated scans to obtain equal signal-to-noise ratio. It is important to notice that spatial resolution is always limited by the diffraction due to the long wavelength used in mid-IR measurements. Spatial resolution can be approximated to be one-half of the used wavelength. Thus, best spatial resolution is achieved with the shortest wavelength of the measured spectrum (approx. 2.5 µm with 4000 cm^{-1}), and the resolution decreases gradually as the wavelength increases. However, optimal spatial resolution requires synchrotron operated devices due to the poor S/N-levels at diffraction limited resolutions. Consequently, in practice with standard thermal globar IR light sources the resolution is limited to ~7-15 µm depending on the used wavelength (1700-700 cm^{-1}). A true spatial resolution of the FT-IRIS device is defined as the minimum distance where two separate features can be fully separated from each other. Many FT-IRIS devices offer a better pixel resolution by magnification but that is only nominal resolution of the device (limited by sample stage movement or magnification), as actual signal, limited by the physical diffraction, arises from larger area. When planning FT-IRIS investigations one should carefully think whether there is a need for the smallest pixel sizes. For example, increase from 6.25 µm to 25 µm increases the number of measured pixels to 16-fold. Furthermore, if an equal signal-to-noise ratio is wanted, the measurement time is increased even more.

Advice: High spatial resolution tremendously increases the measurement time. A very critical evaluation has to be carried out whether the diffraction limited resolution is needed for carrying the experiment. High quality spectral data is preferential to high spatial resolution in most cases. Better spatial resolution increases the measurement time mainly because the number of repeated scans has to be increased to simultaneously keep signal-to-noise-ratio at an adequate level.

2.2.2 Spectral resolution

Spectral resolution is the accuracy (measured as wavenumbers) at which the spectral data is acquired. Depending on the type of experiment, spectral resolution is typically 4-16 cm^{-1}. Sharp spectral features are only seen with a high spectral resolution. Therefore, it is advantageous to increase the spectral resolution up to a certain point. On the other hand, required measurement time is directly proportional to the spectral resolution. It is important to remember that as the resolution increases, so does the noise. Thus, number of repeated scans has to be increased if the S/N-ratio is kept unchanged, and therefore duration of the measurement is considerably longer. It has been demonstrated that more spectral features

can be seen with 4 cm^{-1} resolution compared to 8 or 16 cm^{-1}. The effect is particularly evident when 2nd derivative spectra are used in the analysis (Figure 1). Most of the reported FT-IRIS studies have been conducted using either 4 or 8 cm^{-1} resolution. Application of the 2 cm^{-1} resolution is not typically used for two main reasons: 1) protein features generally lack very sharp peaks, i.e., additional information is limited, and 2) measurement time becomes impractical due to poor signal-to-noise-ratio.

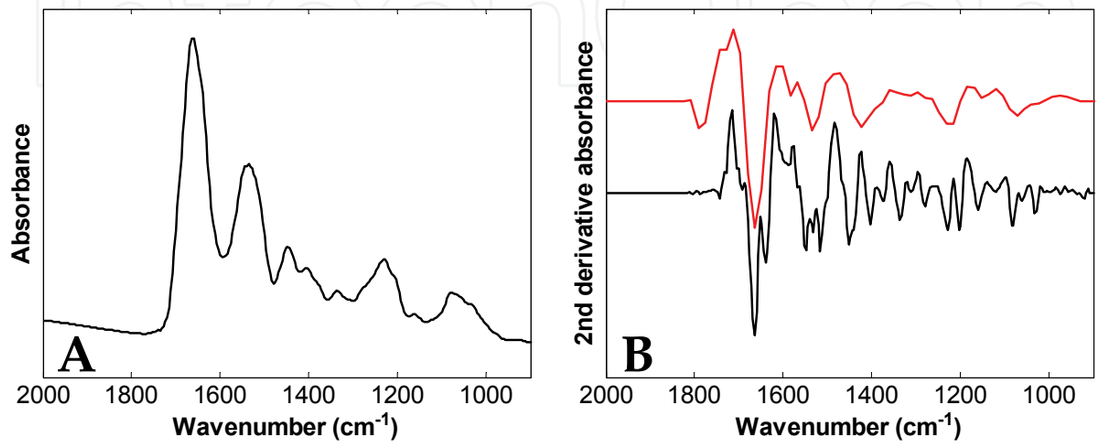


Fig. 1. An IR absorbance spectrum of articular cartilage measured with 4 cm^{-1} spectral resolution (A) and second derivative spectra with 4 cm^{-1} (black) and 16 cm^{-1} (red) spectral resolutions (B)

Advice: Simple univariate analysis can be carried out using spectral resolution of 8-16 cm^{-1}. If second derivative spectra are used for analysis, spectral resolution should be increased to 4-8 cm^{-1} to gain any advantage. From the theoretical point of view, a better spectral resolution reveals more information from the sample, but in practice measurement time is often a limiting factor. Signal-to-noise-ratio gets progressively worse as the spectral resolution increases. Spectral resolution can be considered as the most important parameter when a new FT-IRIS study is planned since it directly affects the subsequent possibilities for data analysis. Thus, data analysis methods (univariate vs. multivariate analysis) required need to be also planned beforehand. If numerous samples are measured, a careful balancing between the optimal spectral resolution, signal-to-noise ratio and measurement time is needed. Pilot studies are often essential to evaluate the spectral resolution needs against the time consumption.

2.2.3 Standardized measurement conditions

IR measurements are significantly affected by the carbon dioxide and water vapour of the atmosphere [24]. Humidity is substantially changed between winter and summer time causing a significant variance to the atmospheric conditions. Altered measurement conditions hinder the data quality when multivariate analyses are carried out. Standardized measurement conditions are, thus, essential for FT-IRIS measurements. Equipment is typically purged with N_2-gas or dried air. For example, water vapour free air can be guided into spectrometer, microscope and sample compartment. Furthermore, thin histological

sections absorb moisture from the environment. Therefore, it is beneficial to store the samples in a desiccator where steady environment can be maintained.
Advice: Each measurement system and sample type has to be separately tested for the measurement stability. Temperature and humidity levels of the laboratory should be kept as constant as possible. Separate purge system can be used to obtain low humidity and carbon dioxide levels in the measurement chamber. It is good to keep in mind that the measurement environment is altered each time the specimen is changed. It is a good idea to measure the duration when steady measurement conditions are reached after the sample compartment has been opened. A regular quality control also ensures that the measurements are consistent with each other regardless of the measurement time.

3. Data analysis – a crucial step to get the answers for your research problem

Data-analysis is the most difficult part of the FT-IRIS experiments. A good quality data set is a prerequisite for successful spectral analysis. Raw spectral data gives only a little information without proper knowledge of spectral pre-processing and analysis. Chemometric methods vary and offer different approaches to gather specific information from the measured data. Analysis has to be designed to match the desired research problems.

3.1 Spectral pre-processing

Spectral pre-processing is an essential procedure prior to the actual spectral analysis. Pre-processing is particularly needed when data from several studies are combined. Data has to be evaluated and filtered so that the non-biological variation between different measurements is minimized. Pre-processing routines include data quality analysis (signal-to-noise-ratio criteria, water vapour limitations, removal of corrupted data), measurement area criteria (definition of the region of interest, removal of unwanted pixels), selection of spectral region (data truncation, masked areas) and baseline corrections. In general, spectral pre-processing should be conducted in a way that do not alter the actual biological information but reduces the variance caused by the measurement itself or by the measurement conditions.
There exists different kind of baseline correction methods. Offset correction is simply conducted by setting, e.g., the minimum value of the spectrum (or alternatively a wavenumber that should not have any absorbance) to zero level. Linear baseline correction is done by fitting a line through two zero-absorbance points. The line is subsequently subtracted from the spectrum. Also polynomial-based fitting is used, but too heavy correction might alter not only the baseline but also the actual spectroscopic data. A more realistic model-based approach has also been used for data pre-processing. In Extended Multiplicative Signal Correction (EMSC), offset error, linear error and second order polynomial error are fitted simultaneously. The model uses a good quality reference spectrum when estimating the baseline errors, which makes EMSC a reliable method for baseline correction [25-28]. Application of second derivative spectra for the analysis eliminates the need for baseline correction as the differentiation removes most significant offset and linear baseline errors [27]. Proper pre-processing is essential, as baseline variations might hide the real biological variation if the baseline errors are not removed.
Advice: Pay attention for the spectral pre-processing before actual data-analysis. Pre-processing steps and demands depend on the data analysis method.

3.2 Univariate methods

Univariate methods are the simplest and most used data analysis techniques in IR spectroscopy. Univariate analyses are usually carried out by using peak height, integrated peak area or peak ratio for quantitative spectroscopic measurements [29]. These methods are hindered by the fact that biological tissues are essentially composed of the same building components regardless of the tissue type. Therefore, univariate methods are not suitable for solving complex research problems. Biological tissues usually lack the distinctive spectral features, which hinders the possibilities of simple univariate analysis. It seems impossible to achieve specificity and sensitivity level of biochemical reference techniques with univariate based parameters in most biological research problems [30]. Univariate analysis is, however, useful if the tissue has major tissue constituents that can be isolated with a single parameter. For example, bone and partially also cartilage, are good examples of tissues where separate components can be measured with reasonable accuracy also with univariate methods [8, 9, 31, 32]. Application of second derivative spectroscopy offers enhanced spectral features that potentially increase the parameter specificity. Second derivative spectroscopy increases significantly the spectral features, but the noise related to measurements is also significantly amplified. This sets higher demands for data quality.

3.3 Multivariate methods

Multivariate methods use more than one variable at a time. Multivariate methods offer means to increase parameter specificity since a larger part of the spectral information is exploited than in univariate analysis. Multivariate methods are suitable both for qualitative and quantitative analysis. The main difference between the univariate and multivariate methods is that the multivariate approach does not require accurate isolation of the differentiating spectral feature before the analysis is conducted. Univariate analysis is dependent on a priori knowledge of the spectral features and therefore the technique has limited efficiency. Multivariate techniques can handle the complete spectral data set with statistical means, and therefore the technique is more potential than user-limited univariate methods. Multivariate approach is often needed when specificity of the IR parameters has to be increased. This is commonly done by multivariate calibration, where a multivariate model is calibrated against some actual reference information. The model can be then used to obtain the same information from the spectroscopic data. Multivariate methods can also be used to separate two or more sample groups by spectral means.

4. How to select appropriate analysis method for different research problems?

A key element for successful use of the FT-IRIS is to be able to define the research question before actual experiments are started. Potential and the limitations of the FT-IRIS technique have to be kept in mind when study protocol is designed. Type of the research question determines the demands for sample harvesting and for type of analysis methods best suited for the particular research problem.

4.1 Quantitative measurement of tissue constituents

Quantitative measurement of different tissue constituents requires a specific parameter for a given compound. This is not a trivial demand to meet and often univariate parameters are not feasible. Multivariate models have a better chance to work since they do not require

fully separated, specific spectral features evident for researcher to be noticed. Univariate parameters are best suited in special situations where spectral differences between tissue components are evident, e.g., bone mineral and matrix content [8-10]. Major tissue constituents can probably be measured using univariate methods with reasonable specificity. However, if the studied compound is present only in small quantities, then only multivariate methods should be considered. Multivariate methods, such as principal component regression (PCR) and partial least squares (PLS) regression, offer more efficient means for quantitative analysis, since they can handle also overlapping spectral data. On the other hand, multivariate analysis needs good understanding of the background of the methods in use. Furthermore, in order to routinely use multivariate techniques to quantificate the composition of different tissue constituents, a comprehensive reference data set needs to be collected. This illustrates the complexity of FT-IRIS spectral analysis.

Example: Univariate vs multivariate analysis of proteoglycan distribution of articular cartilage

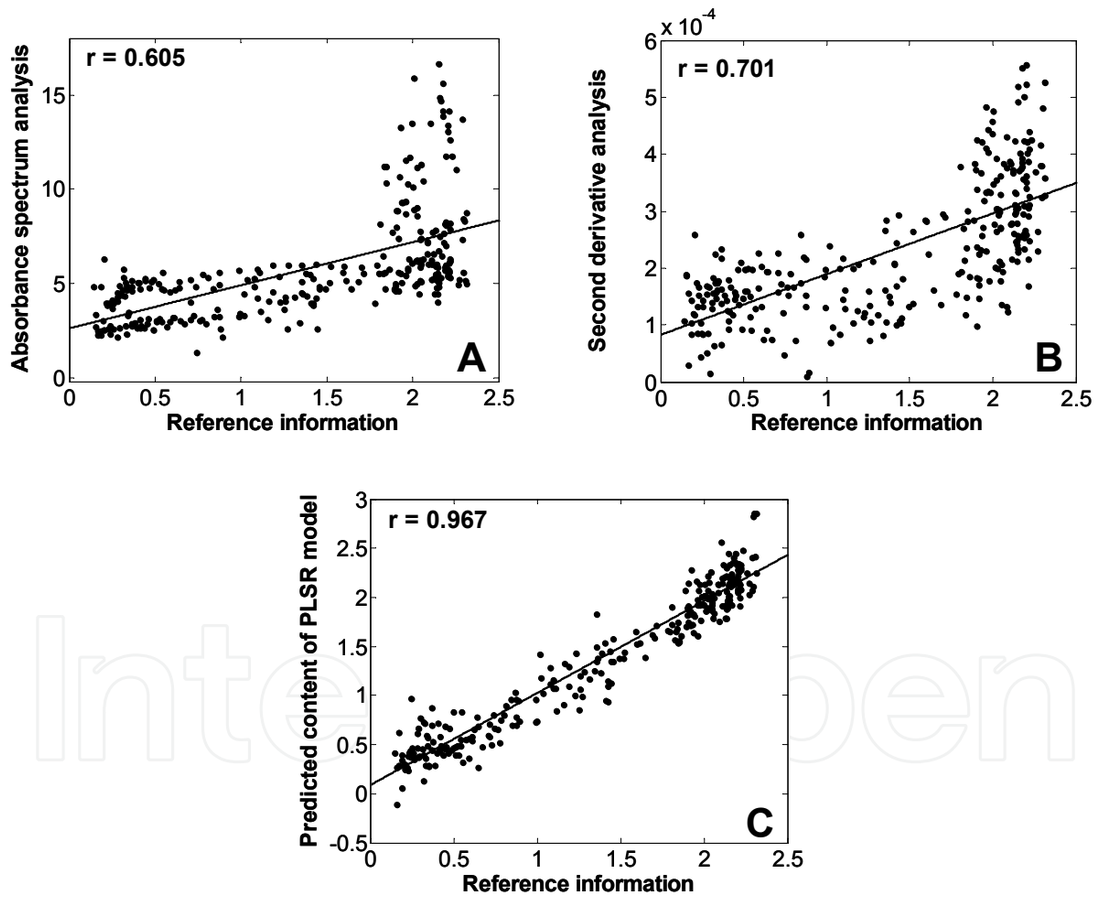


Fig. 2. FT-IRIS analysis of proteglycan content of articular cartilage. Univariate analysis from the absorbance spectrum (A) produces a little worse result than the second derivative analysis (B). However, a multivariate PLS regression model is clearly the most efficient analysis method

As a practical example, let us now consider univariate vs multivariate analysis techniques for determination of proteoglycan distribution in articular cartilage tissue. Univariate based solutions for FT-IRIS analyses of spatial proteglycan content in articular cartilage have been

used in several studies [30-33]. We have demonstrated that specificity for cartilage proteoglycans is significantly increased by taking advantage of the increased spectral separation of the second derivative spectroscopy. Furthermore, we have compared univariate results with the results of the PLS regression model. The PLS regression results were calculated using the whole collected spectral region. The results demonstrated that the PLS regression model is more consistent with the reference technique as compared to univariate methods [34] (Figure 2).

4.2 Qualitative visualization of tissue morphology

Tissue morphology is traditionally investigated through light microscopy of stained thin tissue sections. Morphological features are seen with specific staining patterns of different tissue types. FT-IRIS can produce similar information, but it does not require any staining. Image contrast is created with IR absorption of the tissue. Different tissue types absorb IR energy differently, i.e., their absorption spectra are different. However, it is often difficult to create contrast between different tissue types with univariate techniques. Therefore, if morphological features are one of the main interests of the study, multivariate techniques have to be considered. Application of the neural networks probably gives the most accurate results, but building such a model requires a large data pool and is a very time consuming procedure [3]. A large reference spectra data is collected and the model is trained to recognise spectral features of different tissue types. Nevertheless, neural networks are accurate and fast way to analyze specimens once the neural network is established. Simple multivariate techniques can be used also within a single specimen by using cluster analysis. The aim of cluster analysis methods is to minimize spectral differences within clusters while maximizing differences between clusters. Therefore, tissue types can be classified into their own groups by cluster analysis. Cluster analysis methods, such as K-means clustering or fuzzy c-means clustering, arrange data into desired number of user-determined clusters according to the spectral features [4]. Hierarchical cluster analysis allows unsupervised clustering without pre-determined number of clusters [4].

Clustering methods are often used for isolating the regions of interest from the sample (e.g. cancer area from surrounding tissue). Clustering methods can be used with a limited number of samples but the calculations become very time consuming with large number of spectra or with large number of different samples.

Advice: Morphological information can be gathered with multivariate clustering methods. Data clustering can be done with various multivariate techniques depending on the research application. Clustering methods become essential when large tissue sections are used and only a part of the data is interesting. Proper spectral pre-processing is essential prior to clustering. Any impurities or foreign material, e.g., such as embedding material, are likely to produce a new cluster, which is particularly harmful when fixed number of cluster is used.

4.3 Classification studies

Multivariate techniques are particularly useful when research problem can be simplified into few classes (disease vs. non-disease, tissue types 1,2,3,4 etc). Univariate based parameters have only limited capability for clustering purposes. Multivariate clustering methods are especially useful when different classes are sought (Figure 3). Spectral data can be assigned into subsets according to its spectral features either in unsupervised or supervised manner. In unsupervised techniques, only unlabeled data is used as an input. Clustering is done blind without knowing any additional information of the studied sample.

Data is re-arranged only on the basis of their spectral properties. In supervised clustering, known class information is also included as an input, e.g. disease vs. non-disease. Known information is used to seek spectral features that can be linked to the known information. Once the model is built and verified, the data analysis can be done with great speed. This type of application can be used if the specific feature is looked from the samples.

Advice: Multivariate methods are powerful to find even the smallest chemical differences from the samples. However, analysis methods cannot automatically distinguish artefacts from true differences. Therefore, maintaining stabile measurement conditions and a proper spectral pre-processing is essential for application of multivariate techniques.

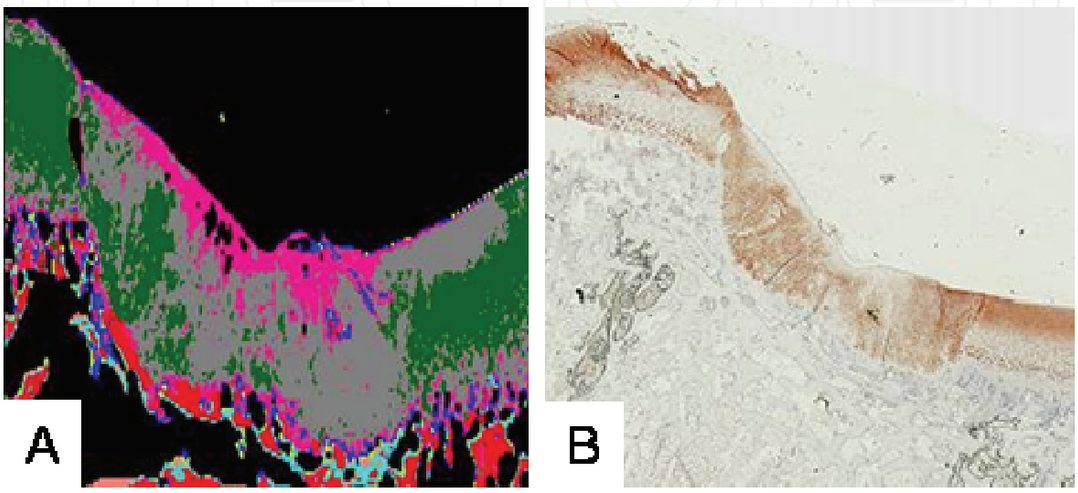


Fig. 3. Pictures of the FT-IRIS k-means clustering map (A) and a histological cartilage section stained with type II collagen antibody (B) that shows a repaired cartilage defect and surrounding intact cartilage. Clustering of the FT-IRIS map is done by mathematical calculation without any user intervention or a priori information of the tissue properties. Grey colour represents repair tissue, pink shows most likely tissue originating from the periosteal flap used in repair surgery and green colour indicates the surrounding intact cartilage

5. Conclusion

Application of FT-IRIS offers new potential for biomedicine. Biologist and medical doctors require training for data mining techniques since the methodology is still relatively new in biomedicine. Multivariate methodology has increasingly been used in spectroscopy, and the research questions are becoming more demanding all the time. Recent progress in biospectroscopy has shown to be fast. From the theoretical point of view, FT-IRIS can be used for the research problems that cannot be answered with traditional imaging techniques. FT-IRIS links the tissue molecular information together with histological imaging. FT-IRIS instruments have rapidly developed and become available for numerous research groups worldwide. Utilization of the modern data mining techniques is likely to further increase the development of the biospectroscopy.

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