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A study examining the effects of tissue processing on human tissue sections using vibrational spectroscopy

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Abstract

The use of vibrational spectroscopy in the detection of cancer is a newly emerging diagnostic field, which has shown great potential to date. Many investigations have been carried out on frozen tissue samples, which by their very nature are hard to obtain. However, histology departments have archives of thousands of tissue samples, preserved and mounted in wax blocks. If this archival material can be shown to yield good Raman and IR spectra capable of differentiating between normal and cancerous tissue, it would improve the diagnostic capabilities of spectroscopy even further. Results from these formalin-fixed paraffin processed (FFPP) tissue sections, will give a better understanding of the effects of processing and could unlock the potential diagnostic capabilities of FFPP sections. This study investigated the effect of freezing, formalin fixation, wax embedding and de-waxing. Spectra were recorded from parallel tissue sections of placenta to examine biochemical changes before, during and after processing with both Raman and IR spectroscopy. FFPP sections were shown to provide good quality Raman and IR spectra but new peaks due to freezing and formalin fixation as well as shifts in the amide bands resulting from changes in protein conformation and possible cross-links were found. Residual wax peaks were observed clearly in the Raman spectra. In the FT-IR spectra a single wax contribution was seen which may contaminate the characteristic CH_3 deformation band in biological tissue. This study has therefore confirmed that FFPP sections have diagnostic potential provided that researchers are aware of the biochemical changes due to tissue processing highlighted by this study.

Keywords: Raman; Fourier transform infrared spectroscopy; Fresh tissue; Frozen sections; Formalin-fixed paraffin processed sections; Tissue processing; Fixation; Wax; Cancer

1. Introduction

In the past number of years many studies have been carried out using vibrational spectroscopy to classify tissue with a view to cancer diagnosis [1]. Both IR and Raman spectroscopy have been employed. Raman spectroscopy is very similar to the more frequently used Fourier transform infrared (FT-IR) spectroscopic technique and both spectroscopic techniques are very complementary. Vibrations that are strong in an infrared spectrum, those involving strong

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dipole moments, are usually weak in a Raman spectrum. Likewise, those non-polar vibrations that give very strong Raman bands usually result in weak infrared signals. For example, hydroxyl or amine stretching vibrations and the vibrations of carbonyl groups, are usually very strong in an FT-IR spectrum and usually weak in a Raman spectrum. However, the stretching vibrations of carbon–carbon double or triple bonds and symmetric vibrations of aromatic groups are very strong in the Raman spectrum. Raman however, has the advantage of minimal interference from water so is a good choice for biological samples with a view to in vivo measurements.

Some of the tissue types examined by various groups include; cervical [2–4], breast [5–7], skin [8–12], lung [13], brain [14,15] bladder [16], esophagus [17–19], colon [20], liver [21,22], thyroid [23] and prostate [24,25]. A variety of different methods of sample preparation have been employed in these studies, such as; fresh, frozen, air-dried, formalin-fixed and de-waxed formalin fixed paraffin preserved (FFPP) tissue sections. The overwhelming majority of tissue studies have been carried out on either frozen tissue or de-waxed FFPP sections [1]. Although considered the gold-standard, frozen tissue is difficult to obtain, deteriorates rapidly and cannot provide retrospective analysis. However, there is a plentiful supply of archival tissue samples, preserved and mounted in wax blocks. Due to the prevalence of these FFPP tissue sections, a better understanding of the effects of processing could unlock the potential diagnostic capabilities of these sections. The sections go through many processing steps before spectroscopic evaluation. If these processing steps result in changes in bands identified as diagnostically significant, this would have implications for tissue classification and could compromise the potential of these FFPP sections in a diagnostic capacity. Previous studies using vibrational spectroscopy have been carried out into the effect of formalin fixation as well as ex vivo handling [26–28]. Huang et al. [26] investigated the effects of formalin fixation on human bronchial tissue using Raman spectroscopy. A decrease in overall Raman intensities was observed and notable formalin peaks were identified at 907, 1041 and 1492 cm^{-1}. Shim and Wilson [27] examined the effect of tissue drying, formalin fixation and snap freezing on normal hamster tissues using Raman spectroscopy. The study found that formalin did not contaminate the Raman spectrum (with the exception of the 1041 cm^{-1} band), however drying/dehydration was found to disrupt protein vibrational modes. FT-IR spectroscopy was used by Pleshko et al. [28] to study the effects of fixation in ethanol and formalin, as well as methacrylate embedding. The study found ethanol fixation of fetal rat bone tissue resulted in changes in the Amide I and II bands (1650 and 1550 cm^{-1}, respectively), a result of alteration of the protein conformation of the tissue.

This study seeks to re-examine the above and extend the study to investigate the effect of xylene, paraffin wax embedding and subsequent de-waxing on human tissue using both Raman and FT-IR spectroscopy. The use of both techniques on the same samples maximises the amount of structural information obtained from the tissue. The steps involved in processing these sections can be summarised as follows: tissue excising, fixation in formalin, tissue dehydration in alcohol, embedding in paraffin wax, microtomy and removal of wax.

This study examined the biochemical structure of normal parenchymal tissue from the placenta at each of these processing steps using both Raman and IR spectroscopy. This tissue was chosen due to its homogenous nature. This minimises the likelihood of recording spectra from different cell types, which would be expected to produce different spectral features. A homogenous tissue ensures that it is only the effects of processing that are being detected, rather than point-to-point inhomogeneities. The effect of tissue freezing was also examined and compared to freshly excised tissue.

2. Experimental

2.1. Raman spectroscopy

An Instruments S.A. Labram Raman spectroscopic confocal microscope was used, with an Argon ion laser operating at a wavelength of 514.5 nm. The Labram imaging system is a fully confocal Raman microscope system, with a motorised XY sample stage for automated Raman imaging. The system includes a stigmatic spectrometer with two motorised gratings, of which the 1800 grooves/mm grating was used. The resolution of the system operating with the 1800 grooves/mm was 1.65 cm^{-1}/pixel. Detection of the scattered light was performed using a Peltier cooled, 16 bit dynamic range CCD detector with 1024×256 pixels. A $50\times$ objective lens was used. The laser power at the sample was measured and found to be about 6.5 ± 0.05 mW, focused to a spot size of 2 μm at the tissue surface. The focal depth and thus the maximum sampling thickness is about 2 μm. The scattered Raman signal was integrated for 60–150 s and measured over a spectral range of 400–1900 cm^{-1} with respect to the excitation frequency. Once acquired each spectrum was baseline corrected, noise subtracted and lightly filtered using a third order linear model to improve clarity.

2.2. FT-IR spectroscopy

A Perkin-Elmer Spectrum GX single-beam, Michelson interferometer-based, Fourier transform infrared spectrometer was used. The spectrometer has a dual level optical module that is sealed and desiccated. The radiation source was provided by a built-in 35 W tungsten halogen illuminator. The medium beam MCT detector covers the range from 5500 to 550 cm^{-1}. MIR and FIR beam splitters and DTGS detector kits allow the range 7000–50 cm^{-1} to be covered. The range was set to 4000–400 cm^{-1} with a