Raman spectroscopy has proven to be a valuable tool for pathology, offering detailed molecular information about the tissue under investigation. Using a Raman microscope tissue sections can be mapped, providing a new means to study the changes in molecular composition of tissue, that are associated with disease, or to characterise crystals or other tissue enclosures. Here we explain the principles of Raman mapping and illustrate its usefulness by a number of applications.

Raman in pathology: mapping of tissue sections

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Routine pathology is gradually moving from the traditional visual examination of stained tissue sections to more objective methods that offer specific molecular information. Apart from genetic methods like in situ hybridisation and comparative genomic hybridisation, MALDI mass spectrometry, and optical techniques, such as fluorescence and Raman spectroscopy can be employed to study the overall molecular structure of tissue sections. Important characteristics of Raman spectroscopy are: its sensitivity to small structural changes, minimal sample preparation, the possibility to perform measurements with high spatial resolution, and the fact that it is a non-destructive technique. Using a Raman microscope biochemical images can be made of tissue sections, cells, and sub-cellular structures, without the need for staining or fixation. This offers a unique means to look at molecular changes associated with growth, development, disease and death of cells and tissues. Here we illustrate the potential of Raman spectroscopic mapping in routine pathology for a number of different tissues.

Figure 1. *Raman microscope consisting of microscope with camera, spectrometer, computer and camera monitor*

Raman mapping microscope

A Leica DM microscope with an automated sample stage was converted to a Raman microscope by adding a hot mirror to the light path, which couples in the laser light and reflects the scattered Raman light to a spectrometer. Tissue sections can be scanned along a two dimensional grid. Of each grid element, a Raman spectrum is recorded, yielding a two dimensional map of Raman spectra. The spectra are then grouped by cluster analysis, and each group is assigned a color. Each grid element, or pixel, is then given the color of the group, to which the spectrum of that element was clustered, resulting in a pseudo color Raman map. Areas in the maps which have the same color have the same or very similar molecular composition.

Discriminating white matter from grey matter

The Raman mapping procedure is demonstrated in figure 2 on a brain tissue section, containing grey and white matter. The red entangled area in figure 2A was scanned using the

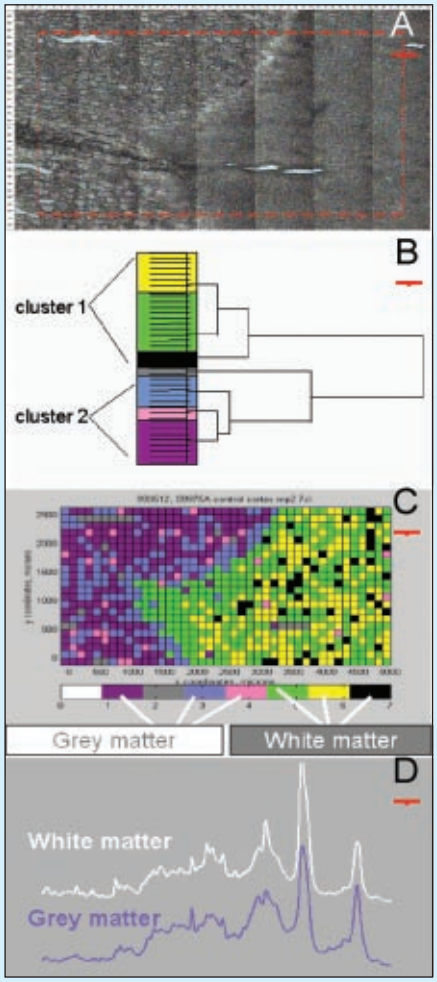

Figure 2. *Illustration of making a Raman map of white and grey matter: 2A: white light image of an unstained brain tissue section 2B: clustering of tissue spectra 2C: Raman (cluster) map 2D: Average spectra of grey and white matter.*

Raman microscope. The resulting spectra were clustered in 7 major clusters (figure 2B), and each spectrum was assigned a color, resulting in the map shown in figure 2C. In the Raman map the outline in the white light image can be recognised, giving the division between grey and white matter, but the map also shows that within the two areas different subclusters can be observed, signifying biochemical heterogeneity. From the map the average spectrum for grey and white matter can be calculated (figure 2D), from which in turn the biochemical differences can be deduced

Chemical characterision of atherosclerotic plaque

Atherosclerosis is one of the main causes of death in the world. It is characterized by the build-up of lipid rich and calcified plaques in the arterial wall. To understand the mechanisms behind the development of plaques and to be able to characterize plaque a detailed insight into their molecular composition and anatomy is of great importance. Figure 3 shows Raman maps of cross sections of a normal human artery (figure 3A) and an atherosclerotic human artery (figure 3B). The blue areas are protein rich, as would be expected for normal arterial wall which is rich in collagen and elastin. The green areas show the postion of adventitial fat (i.e. lipid rich tissue present on the outside of the artery). The red area in the atheros-lecrotic artery indicates the position of a very lipid rich plaque, located inside the arterial wall.

Testicular microlithiasis (aka crystal balls)

Raman can be used to identify the place and type of crystals and other enclosures in tissue as shown in figure 4 which shows the results of the mapping of section of testicular parenchyma with a microlith adjacent to a seminoma. The presence of these crystals was noted before, but the exact place and chemical composition was not known. Note that the microlith (figure 4A, 'M') was lost during staining (figure 4C). The Raman map shows the monolith clearly, and showed another 4 sperate clusters. The averaged spectra of the clusters shown in 4D. The colour of each spectrum matches with the colour of the corresponding cluster. Specific band postions: glycogen at 481 cm-1, hydroxyapatite at 959 cm-1 and lycopene at 1157 cm-1 and 1523 cm-1. The black cluster is basal membrane of the seminiferous tubule (high in collagen). The green and blue cluster are identical except for glycogen content. The yellow cluster shows high carotenoid content (most likely lycopene). The monolith (red cluster) is located within the seminiferous tubule and mainly consists of hydroxy-apatite as can be deduced from the corresponding spectrum in figure 4D. This result was also found in 6 other samples of different patients.

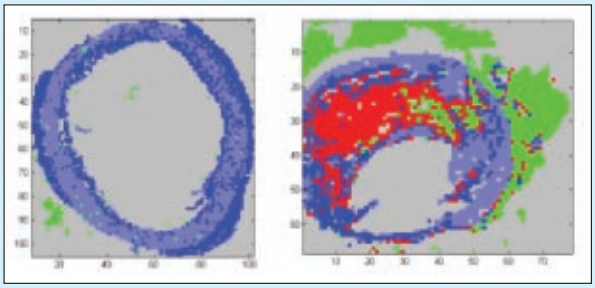

Figure 3. *Raman map of a normal(left) and (atherosclerotic) human artery cross section*

Cluster				
Cholesterol	0%	0%	0%	10%
cholesterol esters	11%	13%	23%	37%
triglycerides	89%	5%	19%	34%
protein	1%	82%	58%	19%

Table 1. *Breakdown of the molecular composition of the different tissue areas in fig 3B.*

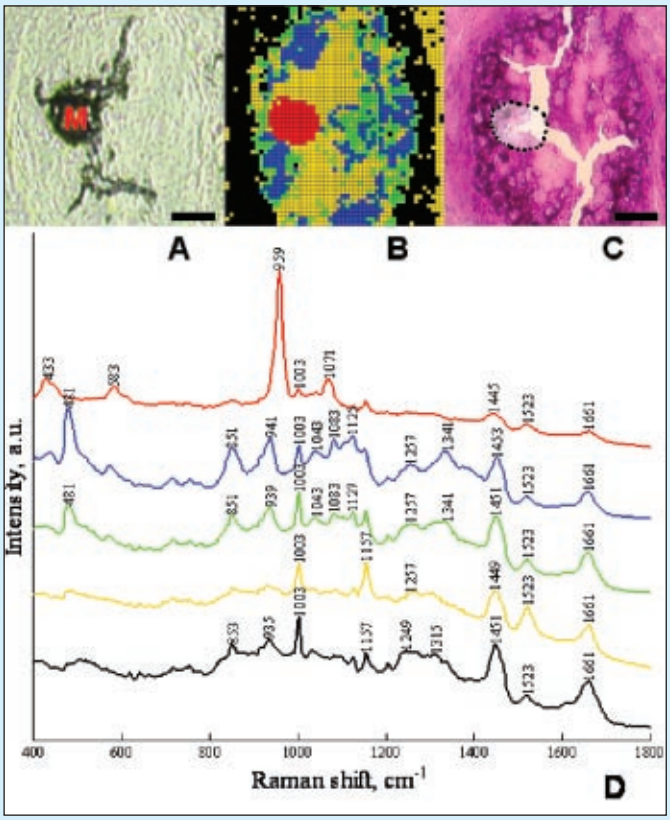

Figure 4. *Characterizing crystalsin testicular tissue. 4A: unstained section of testicular parenchyma with a microlith (M). 4B: Raman map of the section. 4C: stained section. 4D: average spectra of the Raman map of 4B*