Raman microspectroscopy: shining a new light on reproductive medicine

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BACKGROUND: The last 20 years have seen an enormous upsurge in the number of publications reporting findings obtained by Raman spectroscopy, a non-invasive, non-destructive method which uses the inelastic scattering of light to provide a ‘fingerprint’ of the sample’s chemical composition and constituents. Long neglected because of practical difficulties, the technique has been transformed by recent technological advances into a powerful analytical tool capable of opening avenues of investigation that were previously out of the reach of biomedical scientists. Beyond introducing the approach and describing its relative merits and weaknesses, the aim of this review is to provide a spur for discussion of what may become an invaluable tool for biomedical investigations.

METHODS: A comprehensive review of the literature was conducted searching PubMed and Ovid databases using numerous MeSH terms associated with reproductive medicine. Furthermore, the reference lists of all reported literature were explored. The searches were restricted to English language articles published in the last 50 years.

RESULTS: Beginning with simple characterizations of biologically and medically important substances, aided by increasing technological sophistication, the use of Raman spectroscopy in biomedicine has quickly expanded to the investigation of complex biochemical interactions, the assessment of organelles and now the evaluation of living cells and tissue. The first Raman investigations of reproductive organs were primarily oncological in nature; however, the past few years have seen an increase in the application of the technique for the assessment and evaluation of both male and female gametes. In particular, progress has been made in the characterization, identification and localization of sperm nuclear DNA damage.
CONCLUSIONS: The use of Raman spectroscopy has already provided many tantalizing glimpses into the potential that the technique has to answer many of the unresolved issues in investigative and therapeutic reproductive medicine. However, without stringent assessment and the clear representation of the methods’ findings, their true meaning cannot be revealed nor should any conclusions be hastily derived. For the potential of Raman microspectroscopy to be truly realized, the dependability and reliability of the technique and its results can only be ascertained by multidisciplinary collaborations that undertake carefully conducted, controlled and analysed studies.

Key words: Raman / microspectroscopy / DNA / reproductive medicine

Introduction

Something old is new again

Every so often a great deal of excitement and enthusiasm is generated by the potential analytical power afforded by a newly developed instrument and/or method. Such is now the case with Raman spectroscopy, a technique whose principle is not exactly new, having been first described over 80 years ago, but one which has relatively recently undergone a transformation from an interesting but peripheral investigative device used by specialist chemists and physicists to an important tool in the vanguard of biomedical research. The expansion in utility and interest can best be gauged by the meteoric growth over the past 20 years in the number of studies published involving the method (i.e. from 141 in 1992 to over 2300 in 2012). The basis for this newly found fervor is that advances in optics, miniaturization and information technology have made possible the hitherto unrealized capacity of the method to provide a molecular ‘fingerprint’ of a sample in detail, and in a time frame that is feasible for use on biological/medical material. Furthermore, the augmentation of the method’s ability to non-invasively and non-destructively profile chemical structures by the 3D spatial resolution afforded by confocal microscopy (i.e. Raman microspectroscopy) has now made possible the detection of changes in a specific cell’s contents and components as well as its localization in situ, thus paving the way for a myriad of previously impossible investigations on living cells. However, as is the situation with the various methods of the ‘omics’ revolution, the form, complexity and sheer quantity of the data provided by Raman microspectroscopy constitutes a double-edged sword, i.e. opportunity but also challenge. For the time being, the correct implementation of the method and interpretation of its results necessitates a multidisciplinary approach (i.e. involvement of mathematicians, physicists and chemists) as it requires specialized knowledge and skills normally outside those of biomedical scientists and clinicians.

The aim of this review is to introduce the method and its underlying principles, to provide a synopsis of its uses in biomedical and reproductive medicine thus far, to offer some ideas as to its application in the future and also to counsel on how best to validate and verify the technique and its results and thus avoid any potential pitfalls so that it may truly realize its promise.

The Raman effect

Despite being first proposed by Smekal in 1923, the phenomenon of inelastic scattering of parts of a light source by a transparent material bears the name of C.V. Raman, the person who succeeded in experimentally verifying the effect in 1928, an achievement for which he was awarded the Nobel prize in 1930. In broad terms, what was discovered (Fig. 1) was that whereas most photons from an incident light source retain their energy after encountering the atomic shells/bonds of molecules (i.e. elastic or Raleigh scattering), in a small percentage (approximately one in a million), the interaction causes changes in frequency and wavelength (i.e. inelastic or Raman scattering). These changes or shifts, typically in the range of a few hundred to a few thousand wave numbers, vary depending on the atomic mass, quantity of valence electrons and molecular bonds encountered and consequently they are characteristic of the molecular constituents, their arrangement and their state (Ellis et al., 2013).

After an initial flurry, interest in the phenomenon waned for many years as its utility as an analytical approach was severely handicapped by the low-signal generation efficiency of spontaneous Raman scattering, a limitation that could only be compensated by the application of high excitation light intensity and/or very sensitive light detection (Turrell and Corset, 1996). It was not until the 1960s that both of these requirements could be fulfilled. Since then, the introduction of lasers, semiconductor detectors and sensitive amplifiers have seen spontaneous Raman spectroscopy evolve into a standard analytical tool capable of providing chemical fingerprints quickly, without the need for preparatory steps and with negligible fluctuations in temperature due to the laser (i.e. thermal invasion). The breadth of information contained in the profiles was also improved, so that a sample’s composition (by characteristic Raman frequencies), its stress or strain state (by frequency changes of Raman peaks) crystal symmetry and orientation (by polarization of Raman peaks), crystal quality (by the width of Raman peaks) or the amount of included specific ingredients (by measuring the relative intensities of Raman peaks) could all be determined.

Instrumentation and variations on a theme

A typical Raman spectrometer (Fig. 2) comprises a laser, highly transmissive imaging optics (of high numerical aperture), a high-contrast rejection filter for Rayleigh scattered light and a high-resolution spectrometer containing an efficient grating and a sensitive (cooled) charge-coupled camera detector.

Numerous variants of this set-up have been developed that use alternative light sources (i.e. Raman resonance spectroscopy, near infrared (NIR) Raman spectroscopy), multiple power sources (stimulated Raman scattering; coherent anti-Stokes Raman scattering), the incorporation of enhancing surfaces (Surface Enhanced Raman, SERS) or tips (Tip Enhanced Raman) etc. all of which possess different attributes. (For more details on the various types of Raman spectroscopy, we recommend the excellent review by Ellis et al., 2013).

For biomedical purposes, the coupling of the spectrometer with a confocal microscope (i.e. Raman microspectroscopy) provides the most exciting possibilities. Using pin holes or tightly focussed lasers, confocal microscopy eliminates any out of focus light emanating from specimens...
that are thicker than the focal plane thereby improving both the resolution and contrast obtainable by conventional microscopes (Drazba, 2006). When combined with computer driven precision controls, this enhanced clarity allows for an optical ‘slicing’ (i.e. focussing of the scanning to sub-micron areas) through the specimen and thus a reconstruction of any underlying three dimensional structures. With the addition of a motorized stage, the information obtainable by the Raman microspectroscope is further enhanced as multiple, consecutive

![Figure 1](image1.png)

**Figure 1** (A) Schematic of the light contributions from Raman scattering in terms of their intensity versus frequency (both not to scale): When light is shone upon a substance its frequency can either be conserved (i.e. Rayleigh scattering, green peak) giving a strong signal, down-shifted (i.e. a Stokes signal, red) giving a weaker side band or up shifted (i.e. the anti-Stokes signal, blue side band) resulting in the weakest signal. (B) These changes result from the energy differences which occur from excitation by the pump (incident) light (dark green arrow) which causes the targeted molecule to transfer from its original ‘ground’ energy state to a ‘virtual’ state. During this transition, which lasts approximately a femtosecond, energy can either be maintained giving a Raleigh signal, lost by driving the vibration (i.e. a Stokes signal) or gained due to the vibration (i.e. an anti-Stokes signal).

![Figure 2](image2.png)

**Figure 2** Typical Raman spectrometer set-up in backscattering mode: a narrow-band continuous-wave laser (the excitation source) emits a light (green line) that is steered via a dichroic beam splitter through a microscope objective of high numerical aperture to the sample which is fixed onto a precision three-axis translation stage. The Raman scattered light (red line) is collected by the same microscope objective, separated from residual pump light by a (notch) filter then steered into a high-resolution spectrometer which generates a spectrum that is detected by a sensitive charge-coupled camera (CCD).
scannings, each adjacent to each other, can be conducted in all three dimensions. When these spectra are combined and spatially arranged, they constitute a ‘hyperspectral’ representation or map of the various chemical components of each part of the sample. As the non-invasive and non-destructive nature of the analysis is maintained, this technique allows for not only the detailed fingerprinting and assessment of an organ/tissue/cell’s chemical components but also their localization and tracking.

Methods


Results

Uses in biomedicine

Due to the aforementioned technical constraints of the early instruments, for ~50 years following its discovery, the method was the sole preserve of physicists and chemists. It was not until the beginning of the 1970s that the first blossoming in the use of Raman spectroscopy in biological/medical investigations was seen. The type and breadth of studies that were then undertaken reflect the pace of technological advances and the capabilities afforded by the newly developed instruments.

Investigations were initially basic descriptions of the spectra obtained from biologically important components [e.g. haemoglobin (Strekas and Spiro, 1972), cytochrome c (Spiro and Strekas, 1972)]. With greater sensitivity, more complex compounds [e.g. insulin and proinsulin Yu et al. (1972)] and interactions [e.g. sites on DNA for arginine-containing histones Mansy et al., 1976]; DNA with actinomycin D Chinsky and Turpin (1978)] were evaluated. With advances in spatial resolution, examination of structures composed of multiple elements [e.g. erythrocyte membranes Bulkin (1972); viruses Hartman et al. (1973), antibodies Painter and Koenig (1975)] were possible and, by the end of the 1970s, whole organelles [e.g. mitochondria Adar and Erecińska (1978)] were being assessed.

The growing sophistication of the various Raman techniques expanded the scope of studies that were possible, permitting detailed examinations of entire cells and tissue sections to be undertaken. In two early SERS studies, the incorporation of anti-tumour agents into living erythrooleukaemic cells could be localized if the agents were first absorbed onto silver hydrosol (Jennenson et al., 1983; Nabiev et al., 1991). The need for such pre-treatment was circumvented by the use of Raman microspectroscopy which was able to assess the cytoplasm, nucleus and chromosomes of living cells (Puppels et al., 1990). This first protocol, however, was not without its own problems as photochemical reactions initiated by the laser caused degradation of the sample, a fault that was corrected when a more suitable excitation frequency was utilized (Puppels et al., 1991). The ability of the Raman technique to identify and differentiate between tissue types and pathology was recognized in 1992 when, based on spectra obtained by near-infrared Fourier Transform Raman spectroscopy, grey and white matter in rat brain tissue could be distinguished (Mizuno et al., 1992) and the extent of atherosclerosis in human aorta discerned (Baraga et al., 1992).

The ensuing 20 years have seen an almost exponential rise in the number of Raman investigations being undertaken and published. Particularly prominent in oncology, the technique has been introduced into almost every area of biomedical research and although not as yet a routine assessment, the use of Raman profiling is making inroads into clinical practise.

Uses in reproductive medicine

As with most other disciplines, cancer research was the portal by which Raman spectroscopy was first introduced into reproductive medicine (Fig. 3).

Studies in female reproductive medicine

The pioneering work in the gynaecological use of the technique was conducted by Liu et al. (1992) who employed NIR FT Raman to examine human cervical, uterine, endometrial and ovarian tissue. Although the resolution of the spectra obtained was relatively low compared with that achievable by modern instruments, distinctions could still be made between normal/benign and cancerous states based on the intensities of four specific regions of the Raman profile.

Breast

Although breast cancer was one of the earliest malignancies to be evaluated by Raman (Cooper and Theimer, 1980), it was not until the early 1990s that variants of the technique were used to provide meaningful spectra that could identify, characterize and image breast tissue. This began with the broad descriptions of peaks associated with the disappearance of proteins in malignant tissue (Alfano et al., 1991), then included more detailed spectra providing some corroboration that the Raman profiles of benign and malignant lesions were dominated by five structural protein modes, and finally involved an extension of the discriminatory power of the information by the identification of two shifts (1445–1450 and 1650–1667 cm$^{-1}$) which were indicative of abnormal tissue (Frank et al., 1994). These regions were later assigned to lipids and augmented by the identification of a peak specifically associated with oleic acid (Manoharan et al., 1998) which differs in cancerous tissue, a variance that has been substantiated by subsequent studies (Brozek-Pluska et al., 2011; Abramczyk et al., 2012). Beyond breast tissue itself, Raman has been used to assess axillary lymph nodes in breast cancer (Smith et al., 2003), detect nuclear membrane lipid fluctuations in senescent epithelial breast cancer cells (Mariani et al., 2010), identify microcalcifications during stereotactic breast core needle biopsies (Saha et al., 2010) and chart the pathological and biophysical changes associated with silicone implants (Schaeberle et al., 1996; Kidder et al., 1997; Luke et al., 1997).

The Raman evaluation of breast tissue has also been the source of various innovations, such as ‘optical biopsy’ systems (Abramczyk et al., 2012) and the development of various probes from optical fibre (Motz et al., 2004) to subcutaneous (Day and Stone, 2013) and to plasmatic nano-probes capable of detecting single-nucleotide polymorphisms in the breast cancer BRCA1 gene (Wabayele et al., 2010).
Cervix

Mahadevan-Jansen et al. (1998) further advanced the value of the information that could be discerned by Liu et al. (1992) from cervical tissue, when they identified differences in the peaks associated with phospholipids, collagen, DNA and glucose 1′-phosphate. As a consequence, they devised an algorithm which could distinguish between samples with inflammation, metaplasia and low- and high-grade squamous intraepithelial lesions. Extending their work they developed an optical fibre probe which could be used in concert with a colposcope to obtain Raman spectra (Mahadevan-Jansen et al., 1998). Analysis of the in vivo profiles using their in vitro-determined algorithm enabled the identification of a high-grade pre-cancer which was not distinguishable by colposcopy alone. In the ensuing years, the group chronicled variations in normal and diseased tissues and altered facets of their approach in order to increase sensitivity (Vargis et al., 2011a, b). Examining preserved tissue samples, Lyng et al. (2007) identified a similar but not identical series of peaks, the differences most probably being due to the influence of differing types of tissue preservation (i.e. snap frozen versus formalin-fixed paraffin embedded). Regardless, from the profiles obtained, distinctions could be made between normal and carcinoma samples, specifically increases in nucleic acid and protein bands which they attributed to the increased proliferation of the tumour cells. More recently, the method has been used for the early detection of pre-cancers (Durapandian et al., 2011), and to gauge response to radiotherapy (Vidyasagar et al., 2008), as well as the role of cervicitis (Martinho et al., 2008) and the detection of cervical dysplasia (Robichaux-Viehoever et al., 2007).

Ovary

Considering the prevalence of ovarian cancer and the enthusiastic use of Raman in so many oncological investigations, it is surprising how few Raman-based studies have been conducted into the disease. Of those undertaken, the bulk comprise the work of one group who found significant differences between spectral profiles of normal and malignant formalin fixed tissues (Krishna et al., 2005), ascribed these to an excess of DNA and lipids and decreased amount of proteins in cancerous tissue, associations they confirmed by Fourier transform infrared spectroscopy (FTIR), (Krishna et al., 2007) and performed a small pilot study showing the potential clinical usefulness of the approach (Maheedhar et al., 2008).

The insights that Raman spectroscopy can provide into the reproductive capability and function of the ovary remain unexplored, as other than one study charting the presence and location of β-carotene in living cells from bovine corpus lutea (Arikan et al., 2002), no investigations thus far have been published.

Oocyte, embryo

In the first Raman examination of an oocyte, instead of collecting separate single spectra from a specific area, Wood et al. (2008) investigated mouse formalin-fixed metaphase II (MII) oocytes by constructing
univariate maps generated by integrating the area underneath the total profiles as well as those under three specific modes (i.e. amide I mode, CH stretching region and ester carbonyl band). In this way, they identified and localized large lipid deposits (subsequently confirmed by FTIR imaging), regions of high cytoplasmic protein content which suggested the presence of large numbers of mitochondria that were typically distributed, with the number of mitochondria being taken as an indication of oocyte health and viability prior to implantation. Furthermore, they found regions composed mostly of lipids or phospholipids, with small amounts of protein which presumably were complexes of smooth-surfaced endoplasmatic reticulum. A similar mapping strategy was used by Davidson et al. (2012) on paraformaldehyde-fixed material, to localize and identify the biochemical variations within mouse oocytes and embryos at different stages of development. As well as marked changes in protein and lipid distribution, they found changes in the relative concentrations of certain macromolecules, the localization of which was consistent with changes in cytoplasm organization. Interestingly, an asymmetric distribution of phenylalanine was seen in the MI oocytes, a phenomenon they speculated could be the result of oocyte polarization. Comparisons of the Raman profiles of MI oocytes with 2-cell and 4-cell embryos showed a loss of macromolecules from the cytoplasm following fertilization and cleavage. In particular, there was a significant reduction in lipid content between the oocyte and 2-cell stage which the authors speculated could be due to the recruitment of lipid droplets for membrane production during cleavage. A further difference was the presence of saturated lipids close to the cell membrane seen in both 2-cell and 4-cell embryos but absent in the oocytes, suggesting that these constituents may be involved in enlargement of the membrane which is needed to accommodate the expanding surface area of the growing embryo. With the greater degree of detail afforded by higher resolution profiles, Bogliolo et al. (2013) enhanced the peak attributions of the lipids and proteins found in murine MI oocytes and noted that these differed depending upon the age of the mouse from which the oocytes were collected, in vitro culturing and after oxidative attack: Interestingly, similar peaks and attributions were derived from earlier scans of pre-vitellogenic Xenopus laevis oocytes which suggest that the prominence of these constituents is conserved across species (Rusciano et al., 2010). In the same study, the presence and location of β-carotene in the oocytes was determined and found to absent from the nucleus, a result similar to that seen in the bovine corpus luteum (Arikan et al. 2002).

Beyond the evaluation of embryonic stem cells (e.g. cell cycle (Konorov et al., 2013), differentiation (Pascut et al., 2013) etc.) and the aforementioned study by Davidson et al. (2012), few studies have investigated embryos in their entirety and then only in species other than human [e.g. Drosophila (Bhattacharya et al., 2009), zebrafish (Wang et al., 2010)]. The most intriguing is the examination of a fossilized lower Cambrian cetnophore embryo (Chen et al., 2007) which, beyond the palaeo-ontological interest, highlights the flexibility and power of the technique.

Assisted reproduction technology, pregnancy
To date, there is a scarcity of studies conducted using Raman to investigate the procedural elements of assisted reproduction technology (ART), its outcome and pregnancy. The first was a retrospective profiling and comparison of Day 3 spent IVF culture media from Day 3 human embryos that implanted and those that did not (Seli et al., 2007). Distinct differences were found, in particular an increase in the relative amount of -SH and a concomitant decrease in the relative amounts of -CH and -NH in the media of embryos that implanted. These perturbations were confirmed by near-infrared spectroscopy and are consistent with normal embryonic metabolism, specifically that -SH, -CH, -NH and -OH are biomarkers of oxidative stress which have been shown to affect embryo viability. Based on these spectral differences, the authors calculated an index which could predict the viability of individual embryos with high sensitivity and specificity. The same group conducted an analogous prospective study assessing Day 3 and Day 5 culture media which confirmed the strong association of the metabolomic profile and clinical outcome and the high sensitivity, specificity and accuracy of the profiling (Scott et al., 2008). Recently, these findings have been extended by Zhao et al. (2013) who found that a combination of embryo morphology scores and Raman determined that sodium pyruvate and phenylalanine levels in culture medium were indicative of high reproductive potential.

A differing element of ART was investigated by Bogliolo et al. (2012) who constructed Raman maps to study the effect of cryopreservation on ovine oocytes, focussing on the identification of any modifications to the zona pellucida. Conformational changes were observed in the protein secondary structure following vitrification, specifically a significant increase of β-sheet content and an attendant decrease in α-helix content. Furthermore, significant modifications of zona pellucida glycoproteins carbohydrate components were noted, in particular the presence of large amounts of sialic acid and sub-terminal N-acetyl-D-galactosamine, changes which together with the modification of the glycoproteins secondary structure could alter the viscoelastic properties of the zona pellucida and thus hamper spermatozoal penetration.

Implantation, foetal growth, pregnancy and labour have all thus far not been explored using Raman, the only exception being a recent study evaluating whether an advanced form of the technique could be a possible rapid point-of-care diagnostic tool for the detection of early-onset pre-eclampsia (Goodall et al., 2013). Electrochemically enhanced SERS (E-SERS) was able to detect concentrations of uric acid (the earliest indicator of the pre-eclampsia) below 1 mM in a urine ‘simulant’ but how pertinent this approach is clinically is yet to be determined.

Studies in male reproductive medicine
The impetus for the introduction and use of Raman in the investigation of male reproductive function came not from the clinic or the research laboratory but from forensic medicine, the practitioners of which saw in the technique a quick, dependable and reproducible means of identifying semen.

Seminal plasma
Focussing on the most obvious differences in the Raman profile, Virkler and Lednev (2008) distinguished several key constituents that were present in human semen, namely albumin, fructose, lysozyme, lactate and urea. Although assignments of the smaller peaks were not made, the extent of differences in these main components was such that, based on the changes, various bodily fluids could be identified and even semen from different species (i.e. human and canine) differentiated (Virkler and Lednev, 2009).

Our own initial study (Mallidis et al., 2011) found that the spectra of human seminal plasma showed three broad regions of overlaid signals (i.e. 820–850, 1010–1100 and 1220–1350 cm⁻¹) and five particularly prominent peaks (i.e. 714, 955, 1000, 1447 and 1666 cm⁻¹) which we...
broadly assigned as proteins. Some corroboration of the significance of these peaks was provided by Huang et al. (2013) who found that the ratio between the peaks at 1418 cm\(^{-1}\) (lipids, \(\alpha\)-methylene CH\(_2\) scissoring) and 1448 cm\(^{-1}\) (tryptophane, C\(_6\)H\(_2\)C\(_2\) bending mode) could be used to discriminate between seminal plasma from patients with normal and abnormal sperm morphology. Using polarized SERS spectroscopy, the same group later provided further substantiation when they found that changes in some of the more prominent previously identified peaks were indicative of seminal plasma from patients with either normal or abnormal (i.e. low volume, low concentration and/or weak motility) semen parameters (Chen et al., 2012).

Testis

In comparison to other tissues and cells, the use of Raman to investigate testicular function and content has lagged behind, most probably due to the sheer number and variety of cells present and the complexity of the testicular milieu. Some work has now tentatively begun with the first being mappings of microliths located in the seminiferous tubules from men with a variety of testicular pathologies. From these, De Jong et al. (2004) found that regardless of the underlying condition, the microliths were always composed of hydroxyapatite. Interestingly, when glycogen was found surrounding the microliths, the samples were always associated with malignant germ-cell neoplasms. A further step in the Raman characterization of testicular carcinoma was undertaken by Eppelmann et al. (2013) who examined the most utilized model of seminoma (i.e. TCam-2 cells) and found that the culture contained two different types of cells, one of which comprised two subgroups and was similar to some embryonic carcinoma cells. These findings, corroborated by the evaluation of protein expression levels, indicate that most probably seminoma is not the consequence of a simple expansion of homogeneous cells but rather the result of a complex multicellular growth.

Two further studies have been conducted on testicular cells using Raman: one investigated bovine spermatogonia which could differentiate into spermatids in vitro (Xie et al., 2010), the other Sertoli cells obtained from biopsies from patients with differing causes ofazoospermia (Ma et al., 2013). Although potentially interesting, the true worth of these findings is difficult to appraise as the credence of the data is hampered by various experimental limitations (Mallidis et al., 2013).

Sperm

Owing to their relatively simple structure and easily recognizable components, sperm were amongst the first cells to be investigated using Raman spectroscopy. In 1986, Kubasek et al. (1986) evaluated DNA extracted from salmon sperm and found that it had a B-type conformation similar to that of a synthesized model B-DNA oligomer.

Since this initial study, no other Raman investigations of sperm were reported until relatively recently when the results of three different approaches were described (Huser et al., 2009; Meister et al., 2010; Mallidis et al., 2011). All used the micro-spectroscopic form of the technique and, in broad terms, there was agreement in the results. All found distinct spectra in the different regions of the sperm and all identified a large DNA-rich region which comprised the majority of the head. However, there were also substantial discrepancies in the findings, namely Huser et al. (2009) described a specific spectral region of the head near the tail which was not seen in the other assessments and also purported that variations in the ratio of the 785 cm\(^{-1}\)/1442 m\(^{-1}\) peaks were predictive of normal morphology, a relationship that we could not find (Mallidis et al., 2011). In their spectral mapping, Meister et al. (2010) described two ‘neck’ and ‘mid-piece’ regions and speculated that a peak at 751 cm\(^{-1}\) was indicative of the presence of mitochondria, yet neither the regions nor this peak were found in the other analyses (Huser et al., 2009; Mallidis et al., 2011) (Fig. 4). With higher resolution afforded by subsequent analyses, we could localize mitochondria and identify what may be their sheaths; however, no neck region could be seen (Fig. 3). Where there was no disagreement between the studies was the prominence and importance of the peak at 1092 cm\(^{-1}\), the signal attributable to the P\(_2\)O\(_4\) backbone of DNA.

Based on changes in this peak (i.e. a decrease in signal intensity and a concomitant increase in the peak at 1040–1050 cm\(^{-1}\)), we could evaluate nuclear DNA (nDNA) status, identify damage and construct maps showing the area where nDNA fragmentation had occurred (Mallidis et al., 2011). Furthermore, these changes were not influenced by the cause of the nDNA fragmentation, as we were able to distinguish different levels of sperm nDNA damage whether occurring naturally, following ultraviolet irradiation, induced by artificially generated oxidative stress or bacterial infection (Mallidis et al., 2011; Sánchez et al., 2012; Lang et al., 2013). The reliability, reproducibility and accuracy of the results obtained were corroborated using the most widely used and clinically applied method for nDNA fragmentation (i.e. the sperm chromatin structure assay, SCSA\(^\text{®}\)) and an alternative but complementary spectroscopic technique (i.e. FTIR) (Sánchez et al., 2012). The overall spectral profile of the head region and the variation in the nDNA peaks we described have recently been independently confirmed in a study examining sperm that were previously bound to the zona pellucida (Liu et al., 2013).

Beyond information on the status of a single component, the spatial distribution of the differing spectral profiles provided maps from which not only could the known features of a sperm head be readily distinguished but also small anomalies, such as vacuoles (Fig. 3). When these mappings are further processed and more meticulously analysed using differing mathematical methods (e.g. principal component analysis (PCA), spectral angle and k-clustering analyses), hitherto unseen differences can be distinguished. For example in our recent study, examining the effect of uropathogenic Escherichia coli (i.e. the most common causative agent of epididymitis), analysis of the Raman profile verified that infected sperm had nDNA damage and provided a clue as to the possible mechanism of attack (i.e. increased 8-oxoguanosine, the most common DNA lesion resulting from oxidative stress). Macro-mapping clustering analyses of the profile showed changes in both nuclear and mitochondrial DNA distribution and quantity, evidence of protein sloughing and in one instance even the presence of the bacteria themselves.

Prostate

As with other tissues, Raman investigations of the prostate have primarily focussed on the recognition and characterization of cancerous cells. Crow et al. (2003) initially used the technique to identify benign prostatic hypertrophy and three different grades of prostatic adenocarcinoma in vitro. Based on these findings they subsequently evaluated whether a NIR fiberoptic Raman system, which is potentially suitable for in vivo use, could differentiate between in vitro pathologic conditions of the prostate (Crow et al., 2005). As the derived spectra correlated with histologic features they were used to construct a diagnostic algorithm which could differentiate benign samples (i.e. benign prostatic hyperplasia and prostatitis) from malignant samples (prostate cancer), with a high overall accuracy.
With the aim of developing a means of determining the presence of malignant prostatic cancer cells in urine and peripheral fluids, Harvey et al. (2008) developed a Raman optical tweezer system that could trap, analyse and differentiate between live PC-3 (prostatic) and MGH-U1 (bladder) cancer cells. Although their spectra overall resemble those of Crow et al. (2003, 2005) and the differences found were in similar regions, there are a number of notable discrepancies in the profiles. Specifically, the presence of prominent peaks at 1297 cm\(^{-1}\) (lipid) and 1580 cm\(^{-1}\) (protein), signals that were also seen in their subsequent use of the tweezers which examined various formalin-fixed urological cells (Harvey et al., 2009). These two peaks were also prominent in the profiles obtained by Patel and Martin (2010) who, by assessing paraffin-embedded blocks of normal human prostate tissue, identified zone-specific spectral signatures. Based on the plots of PCA scores and their corresponding loadings, they identified peaks which could be used to discriminate between epithelia from the peripheral zone and all others (781 cm\(^{-1}\): cytosine/uracil and 787 cm\(^{-1}\): DNA) and the transition from the central zone (1003 cm\(^{-1}\): phenylalanine and 1459 cm\(^{-1}\): lipids and proteins). A follow-up study by the same group (Patel et al., 2011) used three spectroscopic methods (FTIR spectroscopy, microspectroscopy and Raman) to evaluate prostatic tissue from cohorts of men considered to be at high risk and low risk of the disease. Based on variations in protein secondary structure, they could identify the susceptible patients who were also recognizable by alterations in DNA which were exclusively located in the glandular epithelial layers.

The ability of Raman to recognize which cells would respond to radiotherapy was examined by Matthews et al. (2011) who investigated the profiles of six human tumour cell lines (three derived from prostate, two from breast and one from lung) prior to and after irradiation \textit{in vitro}. They found that the Raman response signatures arose from radiation-induced changes in cellular concentrations of aromatic amino acids, conformational protein structures and certain nucleic acid and...
lipid functional groups. Furthermore, correlation analysis between the radiation-induced PCA components could separate the cell lines into three distinct categories according to their radio-sensitivity and provide clues as to the potential radiation-induced biochemical response mechanisms (i.e. regulated synthesis and degradation of structured proteins and expression of anti-apoptosis factors or other survival signals).

**Reasons for enthusiasm but also caution**

The speed at which technological advances are incorporated into the instrumentation of Raman spectroscopy means that the capabilities and information obtainable by the method are constantly and quickly expanding. Of the many recent developments, micro-sensors that are able to detect subtle differences in tissue have been integrated into surgical instruments for use during robot-assisted surgery (Ashok et al., 2013) whilst others can monitor the metabolism of single cells (Li et al., 2012). The resolution of advanced instruments is now such the examination of microstructures, nano-scale phenomena and even single molecules is possible (Treffler et al., 2012).

For reproductive medicine, these innovations present a glimpse of what types of investigation may soon be possible. Amongst others (Table 1), in situ microprobes able to scan testicular tissue for seminiferous tubules containing sperm during microsurgical extraction procedures (e.g. testicular sperm extraction), can detect foci of cancer and discriminate between metastatic and non-metastatic forms, or can grade oocyte quality whilst still in the follicle. Most tantalizingly, the method may provide the long sought after means of accurately but harmlessly assessing living gametes which, based on the evaluation, can then be selected and used in ART. As already mentioned, for both oocytes (Davidson et al., 2013) and sperm (Mallidis et al., 2011; Sánchez et al., 2012), the preliminary steps towards this goal have already been undertaken; however, the process is not one without considerable obstacles, foremost being that, thus far, the process has been shown to work on sperm that have been air dried or fixed, samples that are unusable for treatment. Despite suggestions that live sperm have been successfully scanned (Kubasek et al., 1986; Liu et al., 2013), until now, no data have been presented to substantiate the claims.

In the case of the oocyte, the sheer size of the cell is such that for an accurate profiling to be obtained the number of scans required is very large. This presents numerous procedural (e.g. the duration of exposure to the laser), analytical (e.g. the complexity of innumerable interrelated and overlaying compounds/reactions) and physiological (e.g. the impact on the oocyte of such long exposure) challenges all of which have not yet been resolved.

Beyond the careful verification of each step which is mandatory for any new procedure, similar to the various ‘omic’ procedures, the complexity of the information obtained by Raman microspectroscopy requires particularly stringent analysis for its correct interpretation and the determination of its true worth. As the manner in which the mathematical analysis is conducted is of major importance, an example of the necessary steps and the degree of detail required to allay any concerns is provided (Supplementary data).

**Need for circumspection**

The introduction of Raman microspectroscopy to biomedical science has resulted in an outburst of studies and publications examining a multitude of questions and conditions. The type of evaluations possible, the data obtainable and the potential of the technique constitute a heady mix. However, this enthusiasm must be tempered by a level of discretion. The newer instruments are quick, efficient, reliable and relatively simple to operate but (at present) they are bulky, expensive and require special conditions (temperature control, specific stable tables etc.). The information obtainable is detailed, precise and reproducible; however, the

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complexity, type and sheer amount of the data require specialized handling and analysis in order to avoid making rash statements and reaching hasty conclusions.

These concerns do not in any way negate the promise that the technique holds; on the contrary, they show the scope and power that are yet to be fully understood. Consequently, for the time being at least, the use and progress of Raman spectroscopy is dependent on the contribution of specialists from disparate fields of knowledge, and the thorough interpretation and meticulous validation of the results. Once these are achieved then the true promise of the technique may be realized.

Conclusions
Recent technological innovations have made the previously published out of reach analytical power of Raman spectroscopy available to biomedical science. The possible research, diagnostic and prognostic uses of the technique are manifold however at present it requires the input of a multidisciplinary team of experts, careful interpretation of the data and stringent validation before it can truly fulfill its promise.

Supplementary data
Supplementary data are available at http://humupd.oxfordjournals.org/.

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Authors’ roles
C.M.: conceived, structured and wrote the review. V.S., J.W., F.W., M.B., C. F. and S.S.: all contributed to the writing of sections dealing with their realm of expertise and provided critical appraisal of the overall manuscript.

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References
Davidson B, Murray AA, Electk A, Spears N. Raman micro-spectroscopy can be used to investigate the developmental stage of the mouse oocyte. PLoS One 2013;8:e67972. doi:10.1371/journal.pone.0067972.


