Review:

Raman Spectroscopy for rapid intra-operative margin analysis of surgically excised tumour specimens

Authors: Mr Thomas James Eliot Hubbard [1,2]

Professor Angela Shore[1]

Professor Nicholas Stone [1,2]

Authors Affiliation:

1. Institute of Biomedical and Clinical Science, University of Exeter Medical School, UK
2. Biophysics group, Department of Physics and Astronomy, University of Exeter, EX4 4QL, UK

Corresponding author: Prof Nicholas Stone,

Address: Department of Physics, Stocker Road, University of Exeter, EX4 4QL

E – mail: n.stone@exeter.ac.uk

Abstract

Raman Spectroscopy, a form of vibrational spectroscopy, has the ability to provide sensitive and specific biochemical analysis of tissue. This review article provides an in- depth analysis of the suitability of different Raman Spectroscopy techniques in providing intra-operative margin analysis in a range of solid tumour pathologies.

Surgical excision remains the primary treatment of a number of solid organ cancers. Incomplete excision of a tumour and positive margins on histopathological analysis is associated with a worse prognosis, the need for adjuvant therapies with significant side effects and a resulting financial burden. The provision of intra-operative margin analysis of surgically excised tumour specimens would be beneficial for a number of pathologies, as there are no widely adopted and accurate methods of margin analysis, beyond histopathology. The limitations of Raman spectroscopic studies to date are discussed and future work necessary to enable translation to clinical use is identified. We conclude that, although there remain a number of challenges in translating current techniques into a clinically effective tool, studies so far demonstrate that Raman Spectroscopy has the attributes to successfully perform highly accurate intra-operative margin analysis in a clinically relevant environment.

Introduction

New cancer cases continue to rise annually worldwide and are the second leading cause of mortality after heart disease, and account for 28% of all UK deaths [1] and 23% of all US deaths [2] and poses a major societal and financial cost to public healthcare systems that is predicted to continue to rise [3]. Surgical excision of the primary tumour remains the primary treatment for many solid organ tumours [4].

The aim of cancer surgery is to remove the smallest amount of tissue necessary to minimise tissue trauma and collateral structural damage, whilst excising the entirety of the diseased tissue [4]. This requires the affected tissue to be excised with a rim of normal tissue with an adequate ~~or a~~ 'margin'. The amount of margin, or distance from the cancerous tissue to the edge of the specimen, required to be termed 'clear' is different for each pathology. If there is cancerous tissue within the defined distance from the resected surface, it is a ~~Having a margin that has cancerous tissue in it, or a~~ 'positive' margin and ~~is defined as~~ an inadequate resection, ~~and~~ which increases the risk of recurrence [5-7] (see Figure 1). A positive margin not only affects prognosis, but also future management, meaning the need for further operations or adjuvant therapies with significant side effects. The cost of a positive surgical margin to the patient, in terms of increased treatment burden, further anxiety and additional side effects is difficult to quantify but the effect on the financial resources of the healthcare provider is undoubtedly significant [8, 9]. A method to assess the margins of the excised specimen intra-operatively to allow further tissue to be taken at the time of the initial procedure, if possible or necessary would be efficient, could reduce the risk of residual cancer at the end of the operation and improve patient care.

Formatted: Not Highlight

Formatted: Not Highlight

Comment [HT1]: Careful with definint he word margin.
Re-define with clarity here and check throughout. E.g. not 'having a margin', but 'having a resected surface where there is an inadequate margin.

Formatted: Not Highlight

Current methods of margin analysis

The 'gold standard' method for analysis of resection margins of surgically excised specimens is currently histopathology. Histopathology analysis of prepared, *ex vivo*, tissue is conducted with light microscopy by trained physicians and is able to provide a detailed analysis of the excised specimen and the biochemical characteristics of the tumour, which contributes to clinical management decisions. However, the 'gold standard' of histopathology is prone to errors although it is likely underreported; one study found an error rate of up to 11% in cancer diagnosis [10], there is variability between pathologists in the reporting of the tumour

grade [11, 12], and even variability in the final diagnosis [13]. This is even more apparent when diagnosing early or pre-cancers, where histopathology can have of the order of nearly 50% inter-observer agreement[13, 14].

Histopathology analysis of surgical margins may also be subject to errors. Even small specimens of around 2 cm across would require an impractical number of histological sections if the entire resected ~~margin~~ surface was to be examined for adequate margins, and so margin assessment should be seen as a representative sampling procedure [15]. The process also requires a number of steps which introduce sampling error - orientation by the surgeon, fixation to preserve the specimen, labelling the specimen, transportation to the pathology department, re-orientation by the pathologist, slicing the specimen, dehydration, embedding, sectioning, staining, representative sampling and then subjective assessment by histopathologists [16-18]. In addition to this, the lack of real-time reporting delays treatment decision making meaning histopathology is an imperfect technique for the reporting of margins. This precludes any removal of further, possibly cancerous, tissue without the need for a second, often more difficult, operation. In many pathologies, the risk of a second operation outweighs the benefits of the risk of recurrence, and so in cases of a positive resection margin, potentially cancerous tissue remains [19, 20]. It is possible that the concept of requiring a defined margin for surgically excised specimens is a result of the current inability to check the entire surface margin, and that if a technique could accurately assess a specimen, smaller, or no, margin distances would be required.

Formatted: Not Highlight

Formatted: Not Highlight

Methods of Intra-operative Margin Analysis (IMA)

Formatted: Not Highlight

Recognising these limitations of the traditional model, there ~~has been a rapid increase in the~~is a large body of research investigating methods of IMA. Current and prospective methods of IMA are generally based on *ex vivo* analysis of the excised specimen, as it is the most

practical way of avoiding surgical artefacts such as blood contamination and the space constraints of *in vivo* analysis. ~~Although it could be postulated that *in vivo* analysis of a tumour margin bed or lymph node could be advantageous, the reality of a contaminated field due to other structures and blood mean that *ex vivo* analysis of the excised specimen is the most realistic way this can be achieved.~~ The number of methods being investigated is vast, what follows is an overview of more established techniques that have had routine clinical application, though not necessarily widespread adoption.

A commonly used method in cancer surgery is frozen section analysis. The specimen is transferred to the pathology department, frozen and sections of interest taken for analysis by a pathologist. It is a technically difficult procedure, requiring a histopathologist to be available on demand, a turnaround time that can impede surgical workflow, the processing can damage specimens which require further histopathological examination and it is expensive [21]. Frozen section is most successfully used in Moh's micrographic surgery in the treatment of Basal Cell Carcinoma (BCC) of the skin, where the entire resected surface is examined in horizontal sections intra-operatively and the surgeon continues to excise tissue until the margin is clear. Recurrence rates are as low as 1-3% even in recurrent and complex disease [22]. However due to cost and time issues it is reserved for cosmetically sensitive areas and recurrent BCC. Frozen section is used widely in head and neck cancers [23] however there is a significant number of false negatives [23] and it is unreliable for eradicating positive final margins [24]. The technique has also been used for IMA in breast cancer excision (where there is reported sensitivity 70-90% and specificity 80-90%[25, 26]) and radical prostatectomy (with a poor sensitivity of 42%[27]), but has not been widely adopted due to clinical and cost-effectiveness concerns [28, 29]. ~~[27] In Breast cancer surgery the technique has been reported as having sensitivity between 70 - 90% and specificity of 80 ->90%.~~ [22, 23] ~~however it has not~~

Formatted: Not Highlight

Comment [HT2]: Re word.

Formatted: Not Highlight

Formatted: Not Highlight

~~been adopted as standard care as it may not be cost-effective [25]. In the diagnosis of margins for radical prostatectomy, in one study the sensitivity was only 42% [24], and a subsequent meta-analysis concluded that the sensitivity and specificity of frozen section meant the technique was of limited clinical value [24, 26]. In head and neck cancers frozen sectioning is widely used to determine tumour bed margins, however the precise method varies between surgeons [28]. The concordance of frozen section analysis with final histopathological diagnosis is reasonable but there can be a significant number of false negatives [27] and it is unreliable for eradicating positive final margins [28].~~

Intraoperative imprint cytology has shown promise in Breast surgery. A slide is pressed onto the lumpectomy ~~margin~~ resected surface and analysed by a pathologist for malignant cells, which can be reported within the time frame of an operation. Issues identified with the technique are that slide preparation can affect the outcome, it is less accurate in lobular carcinoma[30] and in tissue that has been subject to previous radiotherapy [31], and it reports only on the resected surface, not the entire margin depth. ~~In a~~A meta-analysis of eleven studies ~~it has shown good~~revealed a pooled sensitivity of 91% and specificity 95% [32]~~,~~. h~~H~~owever, ~~despite this, in~~in clinical trials the need for delayed re-excision remained disappointingly high ~~one study the intra-operative excision rate was high at 38%, and the delayed re-excision rate after formal histopathological analysis remained at 13%~~ [30] suggesting this may not translate into improved clinical practice. ~~Issues identified with the technique are that slide preparation can affect the outcome, the technique is less accurate in lobular carcinoma [31] and in tissue that has been subject to previous radiotherapy [32], and, as a surface technique, it only reports on involved margins, not close margins.~~

Comment [HT3]: unclear

~~Touch imprint cytology has been used to assess the staple lines of resected specimens in pulmonary excision for non-small cell lung cancer to inform the extent of resection [33] and for~~

~~prognostic information [34], and has been shown to have 83% accuracy in assessing the margins of oral squamous cell carcinoma resection specimens [35], but has not been utilised in intraoperative decision making for these pathologies.~~

Intra-operative imaging to assess margins has been used in various pathologies.

In breast surgery the most common adjunct in the UK to analyse margins is an intraoperative specimen X – ray to determine how close radio-opaque lesions are to the edge of the sample. It ~~was~~ is used by 96% of UK units ~~who responded to one survey~~ [33], as ~~this~~ it is readily available and requires no additional equipment. However, ~~for margin analysis it was shown~~ in a recent meta-analysis of nine studies to have a pooled sensitivity of ~~0.~~53% and specificity of ~~0.~~84% [32].

Intraoperative ultrasound has also been used to guide lumpectomy in breast conserving surgery, but margin assessment was associated with a high false positive and false negative rate [34] and poor sensitivity [35]~~. However, in one study the outcomes appeared favourable, as although there was a high false positive rate of 76%, there was a low second operation re-excision rate of 4%, with an acceptable total specimen weight~~[36]. In brain lesions, intraoperative US has been seen as useful to guide excision, however there is a high degree of user variability and has been associated with a high rate of false positives possibly due to blood artefact~~s such as blood~~ which ~~also appears as~~is also hyperechoic [36].

Magnetic Resonance Imaging (MRI) is used extensively in the planning of brain cancer surgery and ~~is used~~ intra-operatively to guide excision. In a randomised controlled trial, with a small population of 58 patients, the intra-operative use of MRI was associated with complete tumour resection [37]. However, an issue is ‘brain-shift’ where loss of cerebrospinal fluid and oedema changes the anatomy and so reduces the accuracy of neuronavigation [38] and a Cochrane review concluded that further studies into efficacy were needed [39].

The sheer number of techniques proposed for IMA demonstrates that no single method has proved to be ~~accurate, quick, reproducible, available or cost~~ effective enough to be accepted into routine clinical practice. ~~The ideal method for analysing excised tissue would be sensitive, specific, not alter the specimen (to allow subsequent histopathological analysis), recordable to allow accountability, give a definitive answer which is easily understood without the need for specialist training, and processes the sample without delaying surgery. The tool to deliver the analysis should be portable to allow use in multiple locations, robust to withstand everyday use, easily sterilised and not interfere with the theatre environment / procedure [40].~~

To address this gap in surgical care, n~~N~~ovel optical techniques show promise as they can provide sensitive and specific biochemical information at a molecular level in a rapid and non- destructive manner. A number of microscopy techniques, such as Microscopy with UV surface excitation (MUSE), and light sheet microscopy, show the ability to differentiate between cancerous and non-cancerous tissue but studies so far have been limited in sample size and to physically small samples due to speed of analysis, which limits the conclusions as to clinical relevance [40] [41]. Other optical techniques have struggled to succesfully translate promising laboratory work into the clinical environment, such as o~~O~~ptical coherence tomography (OCT) which was found to have reduced effectiveness when it encountered cauterised tissue and blood [42], and a clinical trial in breast specimens demonstrated it only identified 63% of those with a positive margin[43]. Diffuse optical spectroscopy has the potential to provide excellent sensitivity and specificity in cancer diagnosis[44], but so far this has not been realised in the analysis of margins[45]. ~~,~~Raman Spectroscopy (RS) is a technique of vibrational spectroscopy that has gained particular momentum as it can provide detailed biochemical information within clinically relevant times and has been succesfully used in the

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

surgical environment in human tissue in a range of pathologies. RS has the potential to change the paradigm of oncological surgery and provide IMA; an idealised surgical workflow of this is suggested in ~~with a rapidly increasing volume of literature of its clinical application to margin analysis, which is the subject of this review~~ (see Figure 2).

Raman spectroscopy

RS ~~was first [redacted] by C.V. Raman in 1928 for which he was subsequently awarded the Nobel Prize [41]. It~~ is a mode of vibrational spectroscopy measuring the inelastic photon scattering after it has interacted with a molecule [46]. ~~To acquire this information,~~ An incident source of photons (commonly a laser to provide a intense monochromatic light) is directed at the molecule(s) to be analysed. The majority of interactions result in a transient molecular vibration at the incident photon's frequency and a re-emission of a photon of equal frequency to the incident photon. A small fraction of interactions lead to inelastic scattering, resulting in differences in energy between the incident photon and the scattered photon. This can be a gain or loss of energy, it is dependent on the inherent vibrational modes of movement a molecule has which is a product of its atoms and bonds, resulting in each molecule producing a unique Raman spectrum, or 'fingerprint'[47].

The light collection system splits the light into its constituent frequency and provides the information on the intensity at each shift in energy (or frequency) from the laser line. The fingerprint spectrum of a tissue can be analysed and compared to established reference spectra to elucidate its biochemical composition; such as nucleic acids, proteins, lipids and carbohydrates, or the relative differences between spectra can be used to differentiate between tissues [48, 49]. Although highly specific, Raman scattering is considered weak, with only one Raman scattered photon in every 10^6- 10^8 of total scattered photons, which ~~has~~ initially limited its clinical application. However, recent advances in instrumentation, such as

Formatted: Not Highlight

Comment [HT4]: Shorten the whole section to a brief overview of Raman? It is 6 pages as it stands. Half it? Could just have basic RS here, then describe the different techniques briefly e.g. SERS as it appears in the main body. Probably avoids repetition

Formatted: Not Highlight

filters to reject the elastically scattered photons, CCD cameras and cheaper, portable laser sources are enabling this challenge to be overcome. The molecular specificity of the Raman spectrum is holding great promise in medical diagnostics [50], and. a

A variety of RS techniques have been developed in response to different needs. What follows is a summary of the RS techniques that have been explored in clinically relavant scenarios.

Raman micro-spectroscopy

This technique combines Raman spectroscopes with optical microscopes, allowing for analysis of sub millimetre specimens, such as histological slides [51]. The tissue is scanned using raster scanning (point by point) and then uni- or multivariate analysis is performed to analyse the resulting Raman spectrum. This is point scanning, which iswhich is time consuming as each point measurement can cover as little as 300-500 nm across and so processing a sample can potentially take, many hours. Line scanning (changing the size of the incident beam to a line across the sample), and multi-focal Raman microspectroscopy (dividing the incident laser into several beams to measure multiple Raman spectra simultaneously) can reduce the analysis time to more clinically relevant times [52-54]. Wide-field global imaging techniques, where the whole sample is illuminated and all spectra associated with a particular wavelength are collected can also decrease analysis times, but flat field illumination can be difficult and this reduces the laser power per pixel, unless a very high power laser is utilised [55].

Formatted: Not Highlight

A further method to increase the speed of analysis is to reduce the number of Raman spectra taken for measurement by highlighting targeted areas for intensive raster scanning, in

selective scanning Raman microscopy [56]. This can be performed by predictive algorithm, where spectra are taken at two random points on the sample, and the difference between the spectra informs an algorithm to predict where to take the next measurement [56]. Another method is to use another, less specific, but highly sensitive optical technique such as auto-fluorescence to rapidly assess the sample and determine where to take Raman spectra [57]. This allows a substantial reduction in the number of Raman spectra taken, with a similar diagnostic yield, and in a shorter time period. ~~These techniques have been assessed in melanoma excision surgery, both with a substantial reduction in spectra taken, sample processing in less than an hour and with excellent sensitivity and specificity~~ [52]~~.~~

Surface Enhanced Raman Spectroscopy (SERS)

SERS is a method to enhance the inherently weak Raman scattering by using receptor targeted metallic nanoparticles combined with bright Raman reporter molecules as biomarkers. Nanoparticles bind to the tissues of interest in a specific configuration and ratio that gives a unique spectrum. These nanoparticles give an intense signal due to their brightness and specificity, and so a large area can be rapidly scanned to give a reliably sensitive and specific Raman Spectrum [58]. However, the obvious disadvantage lies in the time needed for pre-processing of samples with nanoparticles, and the potential risk of toxicity of metallic nanoparticles that may limit its *in vivo* use [59]. The inherent heterogeneity of tumour biochemistry and receptor expression both within and between patients can affect the accuracy of these techniques [60].

~~SERS with topically applied nanoparticles have been used in the diagnosis of Breast pathology and validated against flow cytometry and immunohistochemistry [55] before being used on 57 *ex vivo* fresh tissue samples, with a 89% sensitivity and 92% specificity of identifying cancerous tissue within 15 minutes [56]. The use of this technique to guide the~~

Formatted: Not Highlight

Comment [HT5]: This statement is not correct, the technique has been applied to basal cell carcinoma, not melanoma, and the correct reference here should be:

Biomed Opt Express. 2014 Dec 10;6(1):98-111. doi: 10.1364/BOE.6.000098. eCollection 2015 Jan 1. Optimization of multimodal spectral imaging for assessment of resection margins during Mohs micrographic surgery for basal cell carcinoma.

~~assessment of margins in lumpectomy samples, and the logistical difficulties inherent in processing samples intra-operatively, has yet to be assessed.~~

Spatially Offset Raman Spectroscopy (SORS)

~~The previous techniques are restricted to analysing the surface of the cut tissue, as illumination and collection take place in the same location.~~ SORS can provide biochemical information at depth below the surface. ~~In many pathologies such as head and neck cancer [24], breast cancer [55], melanoma [56], rectal cancer [57], anal cancer [58] and cervical cancer [59]; a cancer within millimetres of a margin is associated with an increased risk of recurrence and may prompt further tissue excision, and therefore information below the cut surface is required to inform intra-operative decision making.~~ SORS illuminates at a central point then collects scattered data at a distance from this central point, the light having travelled through varying depths of tissue. In using multi-variate analysis of the resultant spectra, the offset between illumination and collection is accounted for, and a depth profile of the tissue with tissue information can be gained [61]. An essential component of this technique is that it ameliorates interference by the often- stronger Raman scattering and fluorescence from the tissue surface to be able to analyse the tissue below. The configuration of the laser illumination centrally with an annular arrangement of collection fibres 2 – 3 mm has allowed for analysis 1 – 4 mm below skin [62].

~~SORS has been used in *in vivo* rat models to assess the incorporation of bone allografts in rat tibia [62], and in the healing process after burn injury [63]. In our labs we have various demonstrations of *in vivo* SORS systems able to measure skin layers and bone composition in 1 second [64]. Although not direct clinical applications, it demonstrates the potential of SORS to provide non-invasive transcutaneous information.~~

~~Keller *et al* used a SORS probe that was initially validated in phantom models prior to the detection of breast cancer to assess margins in *ex vivo* breast cancer specimens [65], the limitations of which are discussed later.~~

Transmission Raman Spectroscopy (TRS) is an extreme version of SORS, where incident beam and collecting camera are opposite one another, to allow analysis through the sample to gain clinically relevant information at depths of up to 40 mm [63].

High Wave Number (HWVN) Raman Spectroscopy

~~RS can be measured with fibre optic probes which opens the way to numerous clinical applications and can be used within already available commercial devices such as a hypodermic needle [58] core needle biopsy system [59] and endoscopes [60] (see Figure 3.).~~

Field Code Changed

The majority of biomedical RS uses Near Infra-Red (NIR) light to acquire spectra within the 'Finger Print' (FP) region of 400-1800 cm^{-1} which has been shown to provide extensive detail of the tissue biochemistry. However, when this laser light illuminates the fused-silica fibre required for fibre optic probes it generates an intense background signal [64]. Using the HWVN region of 2400-3800 cm^{-1} ~~to gain data from a fibre optic~~ can allow Raman spectra to be collected without this background interference [64]. ~~RS can be measured with fibre optic probes which~~This opens the way to numerous clinical applications and can be used within ~~already available~~ commercially available devices such as a hypodermic needle [65] core needle biopsy system [66] and endoscopes [67] (~~see~~ Figure 3~~.~~). Thise advantage ~~gained in the small size of the probe and flexibility~~ must be weighed against the disadvantage of using ~~spectra in the~~ HWVN region spectra, which is less specific and may have limited diagnostic capabilities compared to data ~~gained~~ from the FP region [68].

Field Code Changed

Coherent anti – Stokes Raman scattering (CARS) and stimulated Raman spectroscopy (SRS)

CARS and SRS are non- linear processes, where the observed effect is not linear to the incident laser power, as it is a result of multiple overlapping photons. It can therefore generate a signal intensity greater than coherent Raman. By probing specific, narrow spectra with high intensity, specific molecular information can be gained rapidly [69]. A CARS image is the result of the interactions of three photons, the pump, probe and Stokes photon. The targeted molecule groups will vibrate coherently if the difference in energy between the pump and the Stokes photon equals the energy of a molecular vibration [70]. The probe photon then interacts with this oscillation and an anti-Stokes photon is produced at a new frequency which is detected and produces a CARS image. The advantages of CARS relate to its sensitivity to CH molecules making it particularly effective at studying lipid and fat distributions, which can been performed at video rate of 100 ns per pixel [70]. However it is a near surface technique and interpretation is made difficult by the presence of a non-resonant background, causing spectral distortion and artefact [69, 71].

A SRS signal is generated when the molecular vibration is equal to the difference in frequency between the pump and Stokes photon. The change in intensity of these beams as a result is measured. Its use was previously limited by slow acquisition times and its reliance on back-scattering meant it was inherently weak. However, new techniques have overcome these issues and allowed rapid acquisition of spectra to give detailed biochemical information [71]. Further, SRS is non-resonant background free, can be performed with ambient light present and its ability to provide high-speed imaging has been used in clinical tissue diagnosis [72].

~~The ability of these techniques to differentiate lipid content has shown particular promise in the diagnosis and intra-operative management of brain tumours [72, 73]~~

Spectroscopic Data Analysis

Regardless of the method of Raman spectroscopy utilised to gain spectra, the raw spectra require careful analysis to elucidate clinically relevant information. Differences between tissue spectra, although highly specific and holding detailed chemical information, can be subtle. In the setting of ~~intraoperative margin analysis~~IMA, data analysis is focused on providing a binary outcome of whether the margin is ~~clear~~ adequate or not.

In general, there will be a 'training set', which are spectra assigned to known tissue correlations i.e. a spectrum taken from tissue which has a histopathological diagnosis (as demonstrated in Figure 4). These are used to create a model, which is then tested on a 'validation set', where the diagnostic accuracy can be assessed. This training – validation can be run multiple times with a Leave One Out Cross Validation (LOOCV) protocol to provide a measure of independence in the performance measures [73].

The types of mathematical models used to create spectral classifications are numerous, and expanding. A simple technique is Direct Peak Analysis – where individual spectral features are analysed e.g. the areas under an individual Raman band can be compared [74]. Principal Component Analysis (PCA) and Cluster analysis are unsupervised techniques, that do not require assignation of spectral peaks, but identify where in the spectrum the greatest variance between data lies and classifies data according to these groups. Linear Discriminant Analysis (LDA) is a supervised technique that is commonly used to distinguish differences in the classes identified by PCA to increase accuracy. Increasingly complex models have been developed such as Support Vector Machines (SVM) and Random Forest Classifiers [75] and can improve diagnostic accuracy[76], however they can be more difficult to apply and interpret. The machine learning classifier used to analyse spectral data is an important part of system development. The balance between simplicity and speed of simpler techniques versus the complexity and improved accuracy of more recent models needs to be considered when developing a Raman system suitable for performing intraoperative margin analysis in the clinical environment.

~~ab~~

Comment [HT6]: Brief comparison of Raman with other spectroscopic techniques. Or this could have happened earlier

The use of RS to determine the surgical margin

Breast Cancer

Formatted: Not Highlight

Formatted: Not Highlight

Breast cancer holds great potential for intra-operative use of RS, with a number of groups reporting a variety of advanced techniques to assess the margins of lumpectomy specimens.

Background

For the majority of patients with early primary breast cancer, Breast Conserving Surgery (BCS) (with adjuvant radiotherapy) offers an alternative treatment to traditional Mastectomy, with equivocal survival rates and improved patient satisfaction (Fisher, 1985). In Breast conserving surgery, the cancer is removed from the breast, termed a lumpectomy. The method of assessing margins are currently mainly by surgeon palpation, if it is a palpable lump, or radiographically with an intraoperative X - ray. Neither are a reliable way of assessing the specimen for involved or close margins [77], and as a result, the rate of close or involved margins is high [33].

The definition of a positive margin is most commonly described as 'ink on the tumour' [78, 79], the definition of a 'close' margin is much more debated, however in the UK it is defined as < 2mm, however, the resultant practice as to whether a re-excision of margins is recommended, or indeed undertaken, is much more variable [33]. A positive margin is associated with a 2 fold increase risk of local recurrence, despite adjuvant therapies [80]. Therefore, a positive margin, and more variably, a close margin usually necessitates a re-excision of margins. This is a further operation where the operation site is re-opened and the tumour bed examined, the surgeon then takes a further 1 cm rim of breast tissue at the ~~margin~~ site that was reported as involved. The number of cases requiring re-excision is high, with a UK average of 17% and some units reporting up to 38-41% [30, 33]. The steps involved in this re-excision are numerous and complex and the sheer volume is a significant financial burden.

RS ability to differentiate normal from abnormal breast tissue

There is a large body of evidence confirming the ability of RS to differentiate between normal, benign and malignant changes. A meta-analysis included 9 studies and concluded that using RS *in vitro* in breast tissue to diagnose breast cancer gave a pooled sensitivity of

0.92 and specificity of 0.97 [81]. However, there was a marked heterogeneity between study techniques, and so studies need to be considered individually.

In 2005, Haka examined lumpectomy and mastectomy tissue from 58 patients that had been snap frozen then thawed. They used Raman micro-imaging to gain a sampled volume of $1mm^3$, and determined a total of 130 spectra, which they used in a leave-one-out cross-validation analysis. With this model they got a 94% sensitivity, 96% specificity and an overall accuracy of 86% for detecting infiltrating carcinoma. Fibroadenomas appeared to count for this diagnostic uncertainty; in 2 instances the Raman diagnosis was fibroadenoma, and the histopathological diagnosis was infiltrating carcinoma. The reason may have been the sole differentiation between the two pathologies in the diagnostic algorithm was fat content [82].

In a paper to compare the spectroscopic techniques of fluorescence, diffuse reflectance, combined fluorescence and diffuse reflectance and RS, in the diagnosis of Breast cancer, Majumder *et al.* found RS to be the most effective [83]. They used 74 frozen – thawed specimens to measure 293 spectra with point RS. They found distinct peaks associated with connective tissue proteins and fatty acids discriminated well between normal tissue, fibroadenoma, invasive ductal carcinoma and DCIS being able to classify 99% of spectra correctly. This was done in laboratory conditions, and the area sampled was not reported, but can be presumed to be small.

Barman *et al* developed a single step Support Vector Model algorithm using point RS to diagnose breast lesions from 33 patients undergoing Vacuum assisted biopsy, specifically in those with microcalcifications [84]. They achieved an overall accuracy of 82% of diagnosis

with excellent Positive Predictive Value (PPV) (the probability of a positive result being a true positive) of 100% and Negative Predictive Value (NPV) (the probability of a negative result being a true negative) of 95% for breast cancer. However, this was performed in physically small biopsy samples, with a selective population of tissue all with calcifications present, which are known to produce relatively intense Raman spectra [85], and thus may be unrepresentative of all breast cancers.

Han *et al* used a confocal Raman system to look at freezing microtome sections of breast tissue [86]. They defined the peak assignments of Raman spectra associated with breast tissue and found that the relative intensity of the C = O peaks increased with increasing grade of malignancy. They took 475 spectra from 39 patients and identified that there was little inter-subject variation in the spectra. They used a Support Vector Model for their classification model and achieved an overall accuracy of 74%.

An issue with these techniques is that they are surface techniques, so if there was cancerous tissue more than a few hundred microns from the surface this would not be detected, so 'close' margins (which usually require excision) of up to 2 mm would go undetected. Spatially offset Raman spectroscopy (SORS) can resolve this and Keller *et al* developed a SORS probe that obtained spectra at a depth of 2 mm [87]. In 35 samples of frozen- thawed tissue they attained sensitivity and specificity of >94%. They assessed the margins compared to histopathology as simply 'positive' or 'negative' rather than gaining specific tissue diagnosis. The sampling size of the probe, nor the method of location of tissue sampling is described, but the authors recognize that assessing the ~~margins of an~~ entire specimen in a clinically relevant time is a limitation of the technique.

These studies were all done on micro or point spectroscopy, sampling very small areas of breast tissue to obtain Raman spectra. For application to IMA, it is necessary for a large area to be analysed, rapidly and so complimentary techniques have been applied to enable this. Kong *et al* used auto-fluorescence imaging to inform selective -sampling Raman microscopy to provide an accurate diagnosis within a clinically relevant time frame [57]. Tissue samples cut from blocks that were frozen-thawed were used and sensitivity and specificity of >90% were achieved. An example of these microspectroscopy mapping results and assignation of spectra to tissue is shown in Figure 4. This study only differentiated between ductal carcinoma and normal tissue, and in other studies, it is DCIS and fibroadenomas that negatively affect the overall accuracy of the analysis. Once again, the breast tissue samples were small (5 X 5 mm^2), and so the conclusion of analysis within a clinically relevant time frame was extrapolated. How the technique would be applied to a whole specimen without cutting the sample is also unexplored.

Intra-operative use of Raman spectroscopy

Based on these promising initial results, the same groups went on to use RS in the clinical setting with the potential to give an intra-operative diagnosis.

Haka *et al* used their previously validated technique in freshly excised tissue from 28 patients measuring 220 spectra [88]. Tissue with Ductal carcinoma *in situ* was excluded, as the validating set had not encountered this pathology which is an important exclusion for intra-operative use, as DCIS is associated with a higher rate of re-excision of margins [89]. It also excluded patients having undergone neo-adjuvant chemotherapy, which is increasingly common, and those patients with calcifications. Once again, fibroadenoma proved a diagnostic challenge, and the positive predictive value of 36% can be considered poor, with an overall accuracy of 86%. Although the authors felt that the high NPV of 99% was the

main clinically relevant outcome, with such a low PPV in clinical use this would lead to a high volume of breast tissue unnecessarily being excised. The tissue area sampled is not mentioned, meaning the relevance of the technique to assessing an entire sample for margin adequacy is difficult to assess.

Despite these limitations, this was performed adjacent to the operating room, in a light box, and analysis was performed in 30 minutes, recreating conditions necessary for intra-operative use of the technique.

Wang *et al* used SERS with nanoparticles to assess 57 freshly excised lumpectomy specimens and processed samples within 15 minutes [90]. Each specimen was topically stained with Raman active nanoparticles that were functionalised with antibodies to target HER2, mER, EGFR and CD44 and then raster-scanned to acquire spectra for the entire resected surface~~margin~~ on the exposed glandular tissue. It was possible to differentiate between normal, benign changes and invasive carcinoma tissue, and the overall sensitivity for breast carcinoma detection was 89% with 92% specificity, with the accuracy for the specific biomarkers slightly less than this. This technique is not affected by haemoglobin, surgical dyes or diathermy increasing the clinical relevance, however, it is limited by the sensitivity and specificity of not only the functionalised nanoparticles ability to bind to the molecules of interest, but also the accuracy of the Raman signal of the nanoparticles. Another limitation is that, as a surface technique, 'close' margins are not detected.

Using a method of selective scanning, Shipp *et al* performed analysis on freshly excised lumpectomy samples from 51 patients, and analysed one resected surface~~margin~~ of each specimen which was identified as being most likely to be positive by a member of the team [91]. They used multimodal spectral histopathology (MSH), obtaining autofluorescence images,

which was highly sensitive but non-specific, to inform targeted Raman measurement points in identified 'segments' to reach a final diagnosis. The diagnostic algorithm was validated using a training set based on mastectomy samples which included tissue containing fibroadenoma, fibrocystic change, DCIS and invasive carcinoma. MSH in the lumpectomy samples was highly sensitive, identifying all the margins that contained residual cancer even as small as 1 X 1 mm^2, and so was 100% sensitive, with around 80% specificity. They analysed a single ~~margin~~ resected surface of up to 4 X 6.5 cm^2 in 12-24 minutes, which was achievable as MSH reduced the number of Raman spectra required by 100 – 200 fold. Although this study shows significant improvement in the speed of analysis and the diagnostic accuracy, there are some limitations in the way the lumpectomy specimens were subsequently examined, and that only one ~~margin~~ resected surface could be examined in a clinically relevant time.

Skin Cancer

Background

Skin cancer is the most common cancer diagnosed and its diagnosis and treatment represents a huge burden on the health economy [92]. Basal Cell Carcinoma has the highest incidence and is predicted to rise. It is a slow growing tumour, that rarely metastasises, but local invasion leads to local tissue destruction and deformity. Surgery remains a treatment of BCC, the tumour can be excised by standard surgical excision where the lesion is excised with the aim of obtaining margins of 3 – 5 mm. A positive, or close margin has been reported in up to 7% of cases and is associated with a high recurrence rate of up to 27% [93] and requires further treatment or re-excision [94]. Mohs' micrographic surgery is ~~another technique where the complete surgical margins are examined in horizontal sections intra-operatively and the surgeon continues to excise tissue until the margin is clear.~~a current technique used for IMA

but i~~I~~t is time consuming, ~~and~~ expensive and ~~is~~ only recommended in high risk cases ~~such as large tumours in cosmetically sensitive sites, certain histological subtypes or recurrent BCC~~ [94, 95].

Melanoma is less prevalent than non-melanotic cancer but incidence is rapidly rising, it has metastatic potential and late presentation is associated with a very poor prognosis [96]. The management of melanoma is based on surgical excision, with margins of 0.5 – 2 cm required based on the stage of disease, and re-excision performed if there are involved margins[97]. A margin narrower than this is an inadequate resection which increases the risk of recurrence associated with a poor prognosis, and may possibly be associated with worse survival [98]. For both melanoma and nonmelanoma skin cancer, there is a need for IMA to improve patient outcome.

RS ability to differentiate normal from abnormal skin tissue

Confocal RS ~~has been~~was first used to differentiate~~identify~~ BCC from normal skin ~~non-melanotic skin cancers~~in 2002, where Raman maps from a small sample of 15 specimens were taken and compared to surrounding normal tissue which yielded sensitivity and specificity of over 90% in a logistic regression model[99]. ~~Nijssen acknowledged~~Acknowledging the practical limitations of ~~previous studies in~~ using confocal RS, the group ~~acquiring spectra from the FP region of 400 – 1800 cm $^{-1}$~~, used a handheld fibre optic probe and gained spectra using HWVN RS in the region of 2500 – 3800 cm $^{-1}$ to avoid background signal from silica[100]. A number of readings from each of 19 biopsies taken from the centre of confirmed BCC's were analysed, which demonstrated large and consistent differences between the spectra from BCC and normal tissue, particularly that collagen contains discriminative information at this wavelength, with a 100% diagnosis of BCC.

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

However, gaining spectra at this wavenumber region took longer due to suboptimal signal-to-noise ratio, which may limit its clinical use, and there was a small study size.

Formatted: Highlight

~~[101].~~ These studies looked solely at BCC, whereas non-melanotic skin cancer also includes squamous cell carcinoma. Lieber *et al* analysed~~Measurements were taken from~~ 21 suspected non melanotic skin cancers and took measurements with confocal RS from within the tumour and compared to normal skin adjacent (1 cm distant) from the tumour ~~margin~~ edge at a depth of 20 µm[101]. They achieved good sensitivity of 100% for determining the cancerous lesion and specificity of 91%, with squamous cell carcinoma lesions accounting for the diagnostic uncertainty. The sample population of 19 patients was small and although marked differences in Raman spectra were observed for each pathology this was after subtracting the matched normal reading. There is marked inter-subject variability in the Raman spectra of normal skin, and without a matched normal reading the diagnostic accuracy is likely to have been affected. The same group went on to perform measurements at varying depths on fresh-frozen samples from 39 patients with no skin pathology, BCC, SCC or melanoma and achieved 100% diagnostic accuracy at the surface which decreased towards depths of 100 µm [102].

Comment [HT7]: First confocal Raman on non-melanoma was Discriminating basal cell carcinoma from its surrounding tissue by Raman spectroscopy.
Nijssen A, Bakker Schut TC, Heule F, Caspers PJ, Hayes DP, Neumann MH, Puppels GJ.
J Invest Dermatol. 2002 Jul;119(1):64-9.

This should be cited definitely.

~~Nijssen acknowledged the limitations of previous studies in acquiring spectra from the FP region of 400 – 1800 cm^{-1}, and gained spectra using HWVN RS in the region of 2500 – 3800 cm^{-1}[102]. A number of readings from each of 19 biopsies taken from the centre of confirmed BCC's were analysed, which demonstrated large and consistent differences between the spectra from BCC and normal tissue, particularly that collagen contains discriminative information at this wavelength, with a 100% diagnosis of BCC. However, gaining spectra at~~

~~this wavenumber region took longer due to suboptimal signal-to-noise ratio, which may limit its clinical use, and there was a small study size.~~

Schleusener *et al* recruited 104 patients scheduled for excision of a suspicious lesion and used a fibre coupled probe *in vivo* with direct skin contact to sample 3 measurements on each lesion and the mean used to inform the spectra [103]. The heterogeneity of the lesions led to poor accuracy in determining non-melanotic skin cancer from normal skin cancer. The major differences in skin variability between body site also contributed to the results that achieved an accuracy of 78% in discriminating BCC and SCC from normal skin. For melanotic lesions the lesion inhomogeneity was insignificant, and they achieved a balanced accuracy of 91% of differentiating melanoma from normal pigmented nevi.

In a~~A~~ large~~er~~ study, Lui *et al* investigated all ~~skin cancers~~suspicious skin lesions *in vivo*, both potential non-melanoma and melanomas, in 848 patients and acquired 1022 spectra [104]. Spectra from the *in vivo* ~~suspicious~~ lesion were taken, and compared with ~~then~~ spectra from normal appearing skin 5 cm from the tumour edge ~~were taken for comparison~~. The aim was to detect which lesions required invasive biopsy to histologically confirm malignancy which was achieved with 90% sensitivity and 64% specificity. The strength of this study was its clinical relevance – measurements were gained within 1 second, with a handheld probe, and was concerned with the relevant task of differentiating cancerous lesions from benign lesions, rather than from normal skin like other studies. However, the results were ultimately disappointing, with poor specificity. This may have been due to the heterogenous group of benign lesions to compare against (which didn't necessarily have a confirmed histopathological diagnosis), and a relatively small number of malignant melanomas (n=44)

Formatted: Not Highlight

which may have underpowered the diagnostic algorithm. ~~The final diagnosis was confirmed by clinical evaluation or histological diagnosis if a biopsy was taken.~~

The same group used a similar approach with a probe measuring a diameter of 3.5mm at a depth of 1mm to validate the previous diagnostic algorithm on a new cohort of 127 cases, where they achieved similar sensitivity and specificity in cancer versus non cancer diagnosis to the previous study[105]. It is noted that, setting the sensitivity level to 95%, only 8 of 9 melanoma cases were correctly classified as a cancer, and the specificity was generally poor at 30 – 46% depending on the sensitivity level. The results are perhaps unsurprising as the diagnostic algorithm had previously had poor accuracy at melanoma diagnosis.

In a meta -analysis to investigate the accuracy of RS for differentiating cancer from normal tissue, 12 studies using different methods of RS were included, then analysed according to *ex vivo* and *in vivo* studies and types of skin cancer. 10 of the studies investigated discrimination of BCC, and *in vivo*, the pooled sensitivity for discriminating BCC from normal tissue samples was 69% and specificity 85%, compared to *ex vivo* sensitivity of 99% and specificity 96% [106]. This suggests the use of RS to examine *ex vivo*, resected cancer samples, for margin assessment may be a highly accurate technique.

There are a number of studies investigating mixed methods of combining RS with other techniques to improve performance. Combining autofluorescence with RS, with six spectroscopic criteria, 79 *ex vivo* patient samples were analysed and cancerous tissue was classified with 97% accuracy [107]. Another group used CARS, second harmonic generation and two- photon excited fluorescence imaging to analyse 140 *ex vivo* skin samples in a

multimodal approach that allowed large -area scans and the identification of dermal layering, which may assist in diagnosis of cancerous lesions [108].

Intra -operative use of Raman spectroscopy

Kong *et al* used a method of measuring tissue autofluorescence to determine the sampling points for RS [109], a technique alluded to in the previous section [110]. This MSH technique was used to analyse samples from 20 patients treated with Mohs' microscopic surgery for BCC, half were BCC positive. Analysing tissue samples of up to 1 X 1 cm^2 the sensitivity and specificity was 95% and 94% respectively for the detection of BCC within a time of under 60 minutes. The same group has now reported a fully-automated prototype instrument based on this technique that allows assessment ~~measurement~~ of skin surgical resections of up to a 2 X 2 cm^2 area which detects residual tumour at the ~~margin~~ surface of the resected sample [111]. This prototype will be used to validate their previous work with a larger population of patients.

Brain cancer

Background

Gliomas are tumours of the neuroglia graded by histopathological features that account for the majority of malignant brain tumours in adults. They have varying prognoses, but the commonest, glioblastoma multiforme accounts for 55% of all gliomas and has a 5 year prognosis of 5% with almost inevitable recurrence after treatment [112]. Surgical resection is generally recommended as part of initial management for both histological diagnosis and to remove as much of the tumour as possible, if it is safe to do so [113]. A major challenge of resectional surgery is achieving adequate margins, particularly as excessive tissue excision can lead to major neurological morbidity for the patient. With current imaging techniques of

neurosurgical microscopes or intraoperative MRI, even in cases of an apparently complete resection with 'clear' surgical margins, the vast majority of recurrences occur at the site of resection ~~margin~~, suggesting current techniques of assessing intraoperative resection are inadequate [114].

Improving the intra-operative assessment of surgical resection margins could improve adequacy of tumour resection, and thus recurrence rates for glioblastoma.

RS ability to differentiate normal from abnormal brain tissue

Formatted: Not Highlight

The majority of initial diagnostic work has been performed in mouse models of brain tumours. RS has been used to analyse tissue from mouse models of glioblastoma *ex vivo* and is able differentiate between normal tissue (white and grey matter) and malignant tissue with 100% accuracy [115] and has been used to examine the tumour margins in mice *in vivo*, where RS identified tumour undetected by bright field microscopy [116]. Uckerman *et al* used CARS to probe the C-H molecular vibration, thus imaging the lipid content of samples [117]. A mouse model of glioblastoma was analysed *ex vivo* then the same technique used in human glioblastoma tissue to confirm the findings. They found malignant tissue was identified by a reduction in lower CARS signal intensity which was related to a lower content of total lipids in tumour tissue than normal tissue. This was at a cellular level and so tumour borders could be discerned precisely, the technique could gain images at 20 Hz, representing clinically relevant time for intra-operative use.

Two studies using induced glioma formation in mice models have reported the use of systemically injected gold nanoparticles preferentially up- taken by tumour to inform SERS guided tumour resection [118, 119]. The nanoparticles are hypothesised to cross the Blood Brain Barrier via low-density lipoprotein-receptor -related protein 1, an active transport endothelial

receptor that carries exogenous substrates across the BBB [120]. The activation of the nanoparticles is then reliant on the acidic tumour environment, which results in a specific signal at the tumour site [118]. A handheld Raman probe was used in both cases to demonstrate the delineated tumour margin and guide tumour excision. Although they show promise in mice models at assisting in obtaining clear excision margins, the translation of using a systemic agent in humans for diagnostic purposes only is likely to be complex and remains un-investigated.

In human tissue, Raman microspectroscopy has been used to differentiate normal brain tissue, necrosis and brain metastasis and achieved accuracy of >95% [121]. Kalkanis *et al* used *ex vivo* human tissue from 17 donors to create histological slides from frozen samples. Within homogenous areas of normal, necrotic and glioblastoma areas a diagnostic accuracy of over 97% was achieved in the validation group. However non-diagnostic areas, heterogenous areas and those with freeze artefact were excluded which limited sample size, and limits validity of application *in vivo* where heterogenous areas are likely [122].

Another clinically relevant study by Ji *et al* used Stimulated Raman Scattering (SRS) to examine tissue from 19 patients with CNS malignancy [72]. They produced two- colour images based on the Raman intensity ratios which displayed whether the structure was lipid or protein-rich. Using biopsy samples, they asked pathologists to compare these SRS images with standard H+E pathology images and achieved excellent diagnostic concordance. This was carried out in standard lighting conditions. However, they acknowledge the areas sampled were much smaller than a true tumour bed, and SRS can only sample at a depth of 100 μm. Furthermore, this approach is only likely to work when normal tissues are mostly lipid rich and the cancer or disease leads to a change in protein rich tissues.

Another group using SRS to analyse histology slides achieved similar results in differentiating between normal brain tissue and tissue containing a lesion. They used this to develop a machine learning process that was able to predict an automated diagnosis of tumour subtype with 90% accuracy [123].

Intra -operative use of Raman spectroscopy

A recent study by Bury *et al* analysed 29 fresh brain tissue samples that had been excised during surgery within a clinically relevant time[124]. Using SERS, the samples were processed with gold nanoparticles and Raman spectra obtained, the tissue then underwent routine histopathological analysis. There were a number of diagnoses within the small sample size meaning it is likely to be statistically underpowered. Despite this they gained sensitivity and specificity above 75% in diagnosis of normal, glial and metastatic brain tumours, with meningiomas proving a diagnostic challenge with poorer accuracy. Results were comparable to currently used methods of IMA and superior diagnostic accuracy is needed for clinical adoption. However, this could be overcome by increasing sample size and measurements were taken in real time in a laboratory linked to the operating theatre via air-tube, which is an innovative solution to the often encountered problem of space, and demonstrates successful clinical application.

In 2015 Jermyn *et al* reported the use of a handheld spectroscopy device that used a Raman fibre optic to perform sub-millimetre single point measurements of 0.2 mm^2 *in vivo* in humans [125]. 161 MRI guided intra-operative measurements were taken from 17 patients with gliomas, and a biopsy taken at the corresponding site for correlation of Raman spectra with H+E pathological diagnosis. They found specimens with cancer cells had a difference in the lipid bands, a higher nucleic acid content, and an increase in the band associated with the

Formatted: Not Highlight

breathing mode of phenylalanine in proteins. Tissue with cancer cells present were distinguished from normal brain tissue with an accuracy of 92%, which was significantly better than the operating surgeons' visual analysis with a bright field microscope. This was performed intra-operatively with a small, hand held probe and measurements took less than 1 second. The limitations related to the restricted field of view offered by the small area sampled by the probe, and the false negatives in the Raman analysis were due to the system needing > 15% cancer cell burden to be accurate.

The same group then went on to integrate intrinsic fluorescence spectroscopy, diffuse reflectance spectroscopy and RS into one system to analyse biopsies taken from 15 patients with brain tumours of any type in a similar study design [126]. Using this multimodal approach they achieved sensitivity of 100% and specificity of 93% in differentiating between normal brain tissue and tissue with cancer cells.

This group have recently developed a probe incorporated within a commercially available biopsy system to allow Raman measurements to be taken without disrupting surgical workflow [66]. It used HWVN RS to collect data mainly from lipids and proteins. It was successful at detecting normal brain tissue and dense cancer tissue but could not differentiate between normal brain tissue and tissue infiltrated with cancer- which is likely to represent the specimens with inadequate tumour resection margins ~~of tumours~~.

Head and Neck Cancer

Background

Head and neck squamous cell carcinoma (HNSCC) represent the main oncological burden of head and neck oncology. Resection remains the mainstay of treatment for the majority of HNSCC locations [127]. Complete resection of the tumour is the goal of surgical treatment, as a positive margin doubles the risk of local recurrence compared to those with a negative

margin [128]. Despite this goal, a significant proportion (30-65%) of HNSCC resections have positive resection margins [129]. A pathologically involved or close margin affects further management which is often the use of adjuvant therapy such as chemotherapy and/or radiotherapy. Re-resection can be considered, but only if anatomical location allows and after associated morbidity is considered [20, 130]. A common definition of a close margin is <5mm for HNSCC [20, 131]. The Intra-operative technique for margin analysis has been frozen section which has been specimen or tumour – bed based, with variability in the way it is utilised, with no standard method adopted [132]. However, there has been no convincing evidence that this reduces the positive margin rate or improves outcome [24, 133].

RS ability to differentiate normal from abnormal head and neck tissue

The first report of RS to differentiate between normal and malignant larynx tissue was from Stone *et al* [49]. Raman microspectroscopy was used on biopsies from 19 patients to differentiate homogenous samples of normal tissue from dysplastic and squamous cell carcinoma tissue. Diagnostic peak height ratios were used rather than absolute spectral peaks to inform a diagnostic prediction model that demonstrated sensitivity and specificity of >90% for the diagnosis of squamous cell carcinoma.

Using frozen – thawed biopsy samples of vocal cord lesions, Lau *et al* analysed areas of 3.5 mm within 5 seconds. There was heterogeneity of tissue type within measured samples which may have accounted for the reduced diagnostic sensitivity of 69% of detecting carcinoma [134].

Lin *et al* developed a miniaturized RS fibre optic probe that was inserted down a working channel of nasendoscopy instrument to take measurements of suspicious laryngeal lesions in 39 patients [135]. The probe was put in contact with the lesion for < 1 sec prior to taking biopsies for histopathological analysis. They used the HWVN range (2800–3020 cm^{-1}) to obtain 94 spectra and identified spectral peaks that successfully differentiated normal and

malignant tissue. In a similar experimental design in 60 patients, the same group used a combination of FP and HWVN RS to acquire over 2000 spectra and compared this to histopathological biopsy. They gained spectra rapidly in < 1 second, and the combined spectra yielded an overall diagnostic accuracy of 91.1% [136]. The same group then acquired spectra from 90 patients with nasopharyngeal cancer and used PCA and LDA with a leave-one-out cross validation method to achieve a diagnostic accuracy of 93.1% [137].

In the detection of oral carcinoma, Cals *et al* took histological sections from 11 samples of oral SCC with surrounding normal tissue, and histopathological evaluation then selected the regions for RS measurements [138]. Raman mapping with an automated confocal Raman microscope took point measurements at 5 μm steps to determine spectral differences between oral SCC and squamous epithelium, connective tissue, gland, muscle, adipose tissue and nerve. They achieved excellent distinction between SCC and healthy tissues with >97% accuracy. They went on to develop a two-step classification model using a similar experimental method for validation in 19 samples and achieved diagnostic accuracy of 91% to differentiate tumour vs non-tumourous tissue [139].

In vivo detection of oral lesions was performed by Guze *et al* with a handheld probe in real time [140]. The probe, which had disposable plastic sleeves, was used to diagnose lesions within 5 minutes and the procedure was tolerated well by the 18 patients who had a previously known histological diagnosis of the oral lesion. They were able to differentiate between pre-malignant and malignant tissue versus normal or benign lesions with 100% sensitivity and 77% specificity.

The difference in water content between normal tissue and SCC has been used by one group as a marker to identify cancerous tissues in the head and neck. Using a confocal Raman microscope with HWVN RS at 2500 - 4000 cm^{-1} they used freshly excised oral SCC samples from 14 patients to take up to 30 spectra from each sample within 30 minutes and subsequently compared them to histopathological evaluation. They found the intensity of the OH-stretching vibration increased in SCC more than normal tissue, along with the water concentration being significantly higher in the SCC containing tissue. They concluded that water concentration could be determined with HWVN RS and was a useful diagnostic marker of SCC tumour [74]. The group then used freshly resected oral SCC specimen sections containing both tumour and normal tissue to analyse how water concentration changes with distance from the tumour. Using a confocal Raman microscope at the same wavelength they then obtained over 3000 Raman spectra to determine that mean water concentration within the tumour was 76% and decreased further away from the tumour down to 54% when > 4mm from the tumour in healthy tissue[130]. A similar design was used in 26 mandibulectomy specimens and it was also found that water concentration is high within tumour (mean of 77%) and decreases with distance from tumour to a mean of 44% in healthy tissue. These spectra were then used to develop a classification model for diagnosing SCC in bone in a training set and in the validation set achieved a sensitivity of 95% and specificity of 87% in tumour detection [141]. They showed good diagnostic accuracy within clinically relevant times of less than 30 minutes. However, there are limitations to clinical applicability; the specimens had to be handled in a particular way to avoid desiccation, and these studies used cut specimens which may have different water properties to an uncut specimen. A flat surface was also necessary to achieve adequate contact with the Raman probe, which may not be achievable with a clinical specimen.

The data demonstrates a large inter-subject variation in water content in healthy tissue of 17% in bone and 24% in mucosal tissue which suggests pathological tissue measurements would always need to be compared with concurrent 'normal' tissue readings [141]. The calculation of water content for these measurements were based on a protein model and ignored the contribution of lipids to the measured spectral band. Although this is acknowledged and in oral mucosal tissue any high lipid signals were always associated with healthy tissue, this may not be the case with other tissues and so the ability to apply this methodology to other pathologies may be limited [141].

~~Prostate Cancer~~

~~*Background*~~

~~The management of prostate cancer is based on a risk stratification composed of stage of disease, grade of disease by Gleason score and Prostate Specific Antigen reading. Radical prostatectomy is the surgical treatment of choice in men with intermediate or high risk prostate cancer [143]. The balance of the oncological goal of achieving a complete resection with negative resection margins is countered by the functional goal of preserving the neurovascular bundle that controls erectile and continence functions and urethral length [19]. There is a wide variation in the incidence of positive surgical margins reported in the literature (10-42%) [144, 145], a positive resection margin being defined as tumour at the inked edge of tissue [146]. A positive resection margin is associated with an increased risk of biochemical and local recurrence and is an indication for additional treatment such as radiotherapy, which have functional side effects [144, 147]. The extent of residual disease or extra-prostatic invasion can be difficult to discern intra-operatively and so IMA could improve oncological outcomes of patients undergoing radical prostatectomy.~~

Comment [HT8]: Could get rid of this as a separate section and put in 'other pathologies'

RS ability to differentiate normal from abnormal prostate tissue

The first study to differentiate benign prostatic hypertrophic (BPH) tissue using RS was performed in snap frozen core biopsy tissue, sampling around 30 cells in each spectrum. They evaluated the ability to differentiate between BPH and differing grades of prostate cancer, and demonstrated the best sensitivity (94%) and specificity (100%) in diagnosing high grade prostate cancer with a Gleason score >7 [148]. The biochemical basis for the differences observed were later elucidated by comparing a prostate tissue with a range of normal, benign changes and malignant prostate changes to known cell and tissue constituents. A relative increase in DNA and changes in collagen composition were notable features of malignancy [149].

Aubertin *et al* analysed freshly excised specimens from radical prostatectomy of 32 patients with histologically confirmed prostate cancer [150]. A section of the resected specimen thought most likely to contain cancer was taken and analysed at multiple points using a hand-held probe, gaining readings from the FP region of the spectrum and matched them to histopathological diagnosis. The exclusion criteria resulted in almost a quarter of spectra taken being disregarded for analysis, sample sizes for some grades of cancer were small, and only a small proportion of readings were from cancerous tissue (149 of 776), affecting interpretation of results. However, they achieved a sensitivity of 87% and specificity of 86% in differentiating benign from malignant tissue.

The same group evaluated the diagnostic capabilities of RS by combining FP and HWVN RS in a separate study [74]. Here they analysed sections from 18 prostate specimens to gain 477 spectra from both regions and tested with Leave-One-Patient-Out-Cross-Validation (LOPOCV), resulting in an overall accuracy of 88%.

~~*Intra -operative use of Raman spectroscopy*~~

~~On the background of these diagnostic capabilities, the group have successfully integrated a RS probe into the arm of a robotic system. In a proof of principle study they analysed, *in vivo*, tissue adjacent to the recently excised prostate, which demonstrates the potential for clinical application in RS to prostate cancer may not be *ex vivo* analysis of the excised tissue like most of the other reviewed pathologies, but *in vivo* surgical guidance to ensure there is no residual disease[151].~~

Other pathologies

There has been investigation into RS in the diagnosis of other solid tumours such as ovary [142], lung [143, 144] and thyroid [145], but with little further exploration to the application of this technology to improving the adequacy of surgical excision margins. In some solid tumour pathologies, the use of RS *in vivo* for the detection of cancer for identification of residual tumour and ensuring adequacy of resection is another method of improving surgical oncological outcomes.

In prostate disease, histological studies have differentiated between benign prostatic hyperplasia, prostate cancer and normal prostate tissue with a sensitivity of 94% and sepecificity of 100%[146], which was seen to be due to increases in DNA and collagen changes in malignancy[147]. In freshly excised tissue both the Fingerprint and HWVN region has been used with a hand held probe to get diagnostic accuracy of over 85%[73, 148]. The same group have succesfully integrated a RS probe into the arm of a robotic operating system and demonstrated the possibility of *in vivo* surgical guidance to ensure there is no residual disease [149].

There has also been substantial research into the detection of early malignant change in the cervix, aided by the well- defined nature of the disease and ability to gain measurements

without excision of tissue. Multiple studies have demonstrated the ability of RS to differentiate between colposcopically normal and abnormal areas of cervical tissue to a clinically relevant degree of accuracy [150-152]. This may be useful in improving early, accurate diagnosis to guide targeted treatment and ensure complete resection of any cervical pre-cancers.

The ability of RS probes to be incorporated into fibre optics has significant benefit to the potential for use as a surgical adjunct. In bladder cancer fibre optic RS probes have been shown to be able to differentiate normal bladder and bladder cancer with an accuracy of 84% in pathological samples [153]. Another group developed a fibre optic probe used *in vivo* to gain measurements at sites within the bladder of 32 patients with suspected bladder cancer that were subsequently biopsied and then matched with the definitive histopathological diagnosis. These measurements took place alongside fluorescence cystoscopy, a technique already in use to improve bladder cancer detection compared to simple white light cystoscopy. This clinically relevant technique obtained a sensitivity of 85% and specificity of 79% [154].

The introduction of RS enabled fibre optic probes down working ports in endoscopic instruments holds promise for early diagnosis of oesophageal, gastric and colorectal pathology, with *ex vivo* and *in vivo* studies demonstrating consistently good diagnostic accuracy and clinical relevance [155-158]. The utility of this in achieving adequate surgical margins has not been investigated but given the increased risk of recurrence associated with involved circumferential resection margins in GI cancers [159-161], further work should be considered.

Translating ~~Assessment of~~ Raman ~~as~~ to the clinical environment ~~a tool~~ for IMA

Comment [HT9]: Change this section. To more of a 'how to translate to a clinical environment section.'
Brief overview of the pros and cons of Raman as a tool for IMA, then more detail on how to overcome issues.
Reviewer 2 suggests:
-Description of data management routines via chemometrics
-How transferable and robust would the technique be
-Inter-use/inter-instrument differences
-How to handle massive data flow whilst used by clinical staff?
-Strategy for implementation in theatre

Formatted: Not Highlight

The ideal method for ~~analysing excised tissue~~providing IMA would be sensitive, specific, not alter the specimen (to allow subsequent histopathological analysis), recordable to allow accountability, give a definitive answer which is easily understood without the need for specialist training, and processes the sample without delaying surgery. The tool to deliver the analysis should be portable to allow use in multiple locations, robust to withstand everyday use, easily sterilised and not interfere with the theatre environment / procedure [162].

In many ways RS meets these criteria. The ability of RS to differentiate between cancer and normal tissue in a non destructive manner has been established and appears reliable in a range of pathologies. The practical advantages of Raman as a tool for IMA are the relatively cheap equipment (£10-30K) is also small and transportable allowing for easy insertion into operating theatres. RS systems have been developed that have taken measurements within the confines and limitations of the clinical environment, overcoming the obstacles of theatre lighting, handling the specimen and the need for a disposable/re-sterilisable component [126, 163, 164], demonstrating its ability to perform in the operating theatre.

Despite these promising advances, RS is still not used in routine clinical practice, suggesting limitations to the technique for providing IMA. For effective translation and widespread adoption, Raman systems must be cost-effective. The advances in detector technology and lower cost lasers resulting in cheaper Raman systems is addressing some of the historical short falls in the technique, though it is now the detectors and cameras that account for the greatest expense [165], systems able to make use of high-quality but mass produced CMOS cameras used in mobile phones may be expected to reduce costs significantly.

The time taken to analyse samples remains an issue, however innovative techniques such as selective scanning microscopy and SERS has reduced this time and studies presented in this

Comment [SN10]: Which is most important? Sensitivity?

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

review analysed samples in a clinically relevant time frame of 15 – 60 minutes, which is within acceptable and clinically relevant limits [87, 90, 91, 110, 166]. The fact that sample analysis can take place within the operating theatre obviously saves significant time compared to techniques that require the specimen leaving theatres, such as frozen section analysis. ~~though newer techniques beyond spontaneous RS such as selective scanning microscopy and SERS has reduced this time.~~

Thus far, studies have required large data sets with complex and potentially lengthy chemometrics to provide accurate diagnostic information. Generally academic teams have been gaining spectra for a training set to construct a diagnostic algorithm. This process requires a significant amount of data processing and handling with large volumes of data and computing power to 'train' the diagnostic algorithm, which would be well beyond the capabilities of routine clinical staff. However, these are preliminary studies, where the diagnostic algorithm is being constructed and tested, but once the diagnostic algorithm has been refined~~constructed~~, robustly tested and ~~accepted~~validated, the data produced from a single specimen for analysis would not be overwhelming. ~~The number of measurements required per specimen and the processing power and memory required would obviously be dependent on the system, but is very likely to be an affordable cost.~~ The speed of running new measurements through a pre-constructed diagnostic algorithm is in the order of milliseconds.

~~?description of data management routines via chemometrics~~

~~? how to handle massive data flow and used by clinical staff~~

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

When planning for the translation of a technology to the clinical environment, the focus must be on the end -user, which for IMA will be surgeons, as they will ultimately determine whether the technique is adopted. When using new technologies there can be difficulties with inter-user variability- in one study assessing IMA in breast specimens using a bio-impedance spectroscopy probe, the results were negatively impacted by the surgeons incorrectly following the probe protocol or incorrectly interpretating the results [167]. Inter-user variability may prove particularly problematic for hand held probe systems, where data can be rejected or inaccurate due to incorrect probe positioning [87, 103]. However, there are a range of other systems such as an automated tissue processing machine that uses cassettes[111], or automated 3D scanner [166] that may reduce this potential for user error.

Not only must the measurements be taken by surgeons, but a~~A~~ clear and definitive interpretation of data is required in order to translate to clinical use. It cannot be expected that surgeons should be ~~able~~required to understand and interpret raw Raman spectra to inform the procedure. ~~So far to the authors knowledge, studies in the clinical environment have been in a controlled theatre environment with members of the investigating team present to interpret Raman spectra.~~ Examples of systems that provide an indicator of the Raman IMA result to the surgeon ~~Some systems have been developed to provide an indicator of the Raman IMA result to the surgeon, to advance the technique to provide a more clinician friendly output and therefore more clinically relevant outcome. Examples of this~~ is ~~the~~ the system ~~work by Clemens *et al* in developing a system~~ capable of emitting a sound to indicate abnormal tissues [168] and ~~work by Thomas *et al.* on t~~the automated 3D scanner ~~development of a~~ 'Marginbot' which has the potential to analyse a specimen and provide a colour coded interpretation of the Raman spectra for the surgeon mapped onto the 3D image of the specimen (see Figure 5.B) [166].

Field Code Changed

So it can be seen that despite the inevitable challenges in translating from bench to bedside (or theatre-side), ~~These techniques~~there are solutions that enable clinical Raman systems ~~could~~to provide easily interpretable ~~assessment~~IMA of surgically excised tissue to aid intra-operative decision making.

~~The technical and logistical aspects of delivering IMA with a RS system need to be considered,~~ Figure 5 ~~demonstrates~~ provides ~~some~~ examples of ~~potential~~ existing ~~methods for using~~ Raman probes that have the potential to provide ~~for~~ IMA, demonstrating the beginnings of successful translation into the clinical environment. Indeed, there are a number of companies developing commercially suitable Raman systems showing a move away from the laboratory and towards larger scale use by clinicians [165, 169].

~~A major advantage of Raman is the equipment required is relatively cheap (£10-30K), small and is transportable. Indeed, there are a number of companies developing commercially suitable Raman systems.~~[165] ~~Some groups have already developed systems that have taken measurements within the confines and limitations of the clinical environment, overcoming the obstacles of theatre lighting, handling the specimen and the need for a disposable/re-serializable component~~ [124, 166, 167], ~~demonstrating its ability to perform in the operating theatre. Most of the clinical studies presented in this paper are aware of the need to analyse samples in a clinically relevant time frame and this is reported as 15 – 60 minutes depending on the technique~~ [88, 91, 92, 109, 168].

~~Despite the advanced stage of some of the Raman systems in use, RS is still not used in routine clinical practice, suggesting limitations to the technique.~~ ~~The time taken to analyse samples remains an issue, though newer techniques beyond spontaneous RS such as selective scanning microscopy and SERS has reduced this time.~~

Comment [HT11]: Make more of this figure – take about how clinical systems are already overcoming the challenges of the surgical environment and logistical difficulties.

Comment [HT12]: Move this to above, when comparing Raman with other optical techniques

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

~~The advances in detector technology and lower cost lasers resulting in cheaper Raman systems is also addressing some of the historical short falls in the technique, though it is now the detectors and cameras that account for the greatest expense [165], systems able to make use of high-quality but mass produced CMOS cameras used in mobile phones may be expected to reduce costs significantly.~~

~~A clear and definitive interpretation of data is required in order to translate to clinical use. It cannot be expected that surgeons should be able to understand and interpret raw Raman spectra to inform the procedure. So far to the authors knowledge, studies in the clinical environment have been in a controlled theatre environment with members of the investigating team present to interpret Raman spectra. Some systems have been developed to provide an indicator of the Raman IMA result to the surgeon, to advance the technique to provide a more clinician friendly output and therefore more clinically relevant outcome. Examples of this is the work by Clemens *et al* in developing a system capable of emitting a sound to indicate abnormal tissues [169] and work by Thomas *et al.* on the development of a 'Marginbot' which has the potential to analyse a specimen and provide a colour-coded interpretation of the Raman spectra for the surgeon mapped onto the 3D image of the specimen [168]. These techniques could provide easily interpretable assessment of surgically excised tissue to aid intra-operative decision making.~~

Field Code Changed

Conclusions

This review has outlined the importance of the optimal management of surgical margins for oncological excised specimens, current methods of IMA and a review of the existing literature relevant to the use of RS in IMA in a number of solid organ tumour pathologies. It must be noted that the majority of RS studies remain in the realms of laboratory work, or

'processing labs' adjacent to theatres with the work performed by members of academic units. Protocols have not evolved to the point of being able to be used by non-experts, which is crucial to its expansion into the clinical arena. Other disadvantages of RS is the time taken for spectral acquisition, though this is being addressed by multimodal techniques [91], using the HWVN spectra [141] or automation of specimen processing [166]. Ultimately, larger scale clinical studies are required to demonstrate the diagnostic accuracy of the technique, and subsequent improvement in patient outcomes. As part of this, probes suitable for regular clinical use will have to be developed and go through the relevant regulatory processes, and inevitable cost-effectiveness evaluation. The focus on translation of RS to the clinical environment must persist. In an increasingly competitive market of emerging disruptive technologies, future studies must focus not only on improvement of outcomes compared to established techniques of IMA, but also show relevance amongst novel technologies and techniques.

Despite these hurdles, RS has the ability to provide detailed biochemical information of surgical margins with excellent diagnostic accuracy in a range of solid tumour pathologies. Further studies are necessary for the translation of this technology to a clinically relevant environment and demonstrate improved patient outcomes. RS techniques have the potential to provide intra-operative margin analysis of surgically excised solid tumours.

Disclosure/Conflict of Interest:

TH, AS and NS have no conflicts of interest to declare.

References:

1. O. o. N. S. (UK), Cancer registration statistics, England:2016, https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancerregistrationstatisticsengland/final2016, (accessed 21/01/2019, 2019).
2. R. L. Siegel, K. D. Miller and A. Jemal, *CA Cancer J Clin*, 2017, **67**, 7-30.
3. A. B. Mariotto, Y. K. Robin, Y. Shao, E. J. Feuer and M. L. Brown, *J Natl Cancer Inst*, 2011, **103**, 117-128.
4. M. A. Potter, in *Principles and Practice of Surgery*, ed. R. W. P. O. James Garden, Elsevier, Edinburgh, 7th edn., 2018, ch. 6, pp. 86-97.
5. I. Gage, S. J. Schnitt, A. J. Nixon, B. Silver, A. Recht, S. L. Troyan, T. Eberlein, S. M. Love, R. Gelman, J. R. Harris and J. L. Connolly, *Cancer*, 1996, **78**, 1921-1928.
6. Y. Neuzillet, M. Soulie, S. Larre, M. Roupret, G. Defortescu, T. Murez, G. Pignot, A. Descazeaud, J. J. Patard, P. Bigot, L. Salomon, P. Colin, J. Rigaud, C. Bastide, X. Durand, A. Valeri, F. Kleinclauss, F. Bruyere and C. Pfister, *BJU Int*, 2013, **111**, 1253-1260.
7. M. Partridge, S.-R. Li, S. Pateromichelakis, R. Francis, E. Phillips, X. H. Huang, F. Tesfa-Selase and J. D. Langdon, *Clin Cancer Res*, 2000, **6**, 2718-2725.
8. Y. Grant, R. Al-Khudairi, E. St John, A. Carter, M. Barschkett, D. Cunningham, R. Al-Mufti, K. Hogben, D. J. Hadjiminas, P. Thiruchelvam and D. Leff, *Eur J Surg Oncol*, 2018, **44**, 891.
9. J. Yu, L. C. Elmore, A. E. Cyr, R. L. Aft, W. E. Gillanders and J. A. Margenthaler, *J Am Coll Surg*, 2017, **225**, 294-301.
10. S. S. Raab, D. M. Grzybicki, J. E. Janosky, R. J. Zarbo, F. A. Meier, C. Jensen and S. J. Geyer, *Cancer*, 2005, **104**, 2205-2213.
11. J. H. F. Frierson, R. A. Wolber, K. W. Berean, D. W. Franquemont, M. J. Gaffey, J. C. Boyd and D. C. Wilbur, *Am J Clin Pathol*, 1995, **103**, 195-198.
12. P. Robbins, S. Pinder, N. De Klerk, H. Dawkins, J. Harvey, G. Sterrett, I. Ellis and C. Elston, *Hum Pathol*, 1995, **26**, 873-879.
13. J. G. Elmore, G. M. Longton, P. A. Carney, B. M. Geller, T. Onega, A. N. Tosteson, H. D. Nelson, M. S. Pepe, K. H. Allison and S. J. Schnitt, *JAMA*, 2015, **313**, 1122-1132.
14. J. G. Elmore, H. D. Nelson, M. S. Pepe, G. M. Longton, A. N. Tosteson, B. Geller, T. Onega, P. A. Carney, S. L. Jackson and K. H. Allison, *Annals of internal medicine*, 2016, **164**, 649-655.
15. D. E. Wazer, R. K. Schmidt-Ullrich, C. H. Schmid, R. Ruthazer, B. Kramer, H. Safaii and R. Graham, *Int J Radiat Oncol Biol Phys*, 1997, **38**, 291-299.
16. T. Upile, C. Fisher, W. Jerjes, M. El Maaytah, A. Searle, D. Archer, L. Michaels, P. Rhys-Evans, C. Hopper, D. Howard and A. Wright, *Oral Oncol*, 2007, **43**, 321-326.
17. J. A. Woolgar and A. Triantafyllou, *Oral Oncol*, 2009, **45**, 361-385.
18. M. A. Makary, J. Epstein, P. J. Pronovost, E. A. Millman, E. C. Hartmann and J. A. Freischlag, *Surgery*, 2007, **141**, 450-455.
19. J. L. Silberstein and J. A. Eastham, *Indian J Urol*, 2014, **30**, 423-428.
20. M. Alicandri-Ciufelli, M. Bonali, A. Piccinini, L. Marra, A. Ghidini, E. M. Cunsolo, A. Maiorana, L. Presutti and P. F. Conte, *Eur Arch Otorhinolaryngol*, 2013, **270**, 2603-2609.
21. J. M. Jorns, S. Daignault, M. S. Sabel and A. J. Wu, *Am J Clin Pathol*, 2014, **142**, 601-608.

22. N. W. Smeets, G. A. Krekels, J. U. Ostertag, B. A. Essers, C. D. Dirksen, F. H. Nieman and H. M. Neumann, *The Lancet*, 2004, **364**, 1766-1772.
23. E. Du, T. J. Ow, Y. T. Lo, A. Gersten, B. A. Schiff, A. B. Tassler and R. V. Smith, *Laryngoscope*, 2016, **126**, 1768-1775.
24. K. Thomas Robbins, A. Triantafyllou, C. Suarez, F. Lopez, J. L. Hunt, P. Strojan, M. D. Williams, B. J. M. Braakhuis, R. de Bree, M. L. Hinni, L. P. Kowalski, A. Rinaldo, J. P. Rodrigo, V. Vander Poorten, I. J. Nixon, R. P. Takes, C. E. Silver and A. Ferlito, *Auris Nasus Larynx*, 2018, DOI: 10.1016/j.anl.2018.08.011.
25. W. P. Weber, S. Engelberger, C. T. Viehl, R. Zanetti-Dallenbach, S. Kuster, S. Dirnhofer, D. Wruk, D. Oertli and W. R. Marti, *World J Surg*, 2008, **32**, 2599.
26. T. Olson, J. Harter, A. MuÒoz, D. Mahvi and T. M. Breslin, *Ann Surg Oncol*, 2007, **14**, 2953-2960.
27. T. Tsuboi, M. Ohori, K. Kuroiwa, V. E. Reuter, M. W. Kattan, J. A. Eastham and P. T. Scardino, *Urology*, 2005, **66**, 1287-1291.
28. J. B. Osborn, G. L. Keeney, J. W. Jakub, A. C. Degnim and J. C. Boughey, *Ann Surg Oncol*, 2011, **18**, 3204.
29. A. L. Nunez, G. A. Giannico, F. Mukhtar, V. Dailey, R. El-Galley and O. Hameed, *Ann Diagn Pathol*, 2016, **24**, 11-18.
30. F. D'Halluin, P. Tas, S. Rouquette, C. Bendavid, F. Foucher, H. Meshba, J. Blanchot, O. Coué and J. Levêque, *The Breast*, 2009, **18**, 248-253.
31. K. Simiyoshi, T. Nohara, M. Iwamoto, S. Tanaka, K. Kimura, Y. Takahashi, Y. Kurisu, M. Tsuji and N. Tanigawa, *Exp Ther Med*, 2010, **1**, 641-645.
32. E. R. St John, R. Al-Khudairi, H. Ashrafian, T. Athanasiou, Z. Takats, D. J. Hadjiminas, A. Darzi and D. R. Leff, *Ann Surg*, 2017, **265**, 300-310.
33. S. S. Tang, S. Kaptanis, J. B. Haddow, G. Mondani, B. Elsberger, M. K. Tasoulis, C. Obondo, N. Johns, W. Ismail, A. Syed, P. Kissias, M. Venn, S. Sundaramoorthy, G. Irwin, A. S. Sami, D. Elfadl, A. Baggaley, D. D. Remoundos, F. Langlands, P. Charalampoudis, Z. Barber, W. L. S. Hamilton-Burke, A. Khan, C. Sirianni, L. A. G. Merker, S. Saha, R. A. Lane, S. Chopra, S. Dupre, A. T. Manning, E. R. St John, A. Musbahi, N. Dlamini, C. L. McArdle, C. Wright, J. O. Murphy, R. Aggarwal, M. Dordea, K. Bosch, D. Egbeare, H. Osman, S. Tayeh, F. Razi, J. Iqbal, S. F. C. Ledwidge, V. Albert and Y. Masannat, *Eur J Cancer*, 2017, **84**, 315-324.
34. B. Mesurolle, M. El-Khoury, D. Hori, J.-P. Phancao, S. Kary, E. Kao and D. Fleiszer, *AJR Am J Roentgenol*, 2006, **186**, 1014-1024.
35. V. Londero, C. Zuiani, M. Panozzo, A. Linda, R. Girometti and M. Bazzocchi, *The Breast*, 2010, **19**, 532-537.
36. R. Sastry, W. L. Bi, S. Pieper, S. Frisken, T. Kapur, W. Wells III and A. J. Golby, *J Neuroimaging*, 2017, **27**, 5-15.
37. C. Senft, A. Bink, K. Franz, H. Vatter, T. Gasser and V. Seifert, *Lancet Oncol*, 2011, **12**, 997-1003.
38. N. Sanai and M. S. Berger, *Nat Rev Clin Oncol*, 2018, **15**, 112.
39. D. G. Barone, T. A. Lawrie and M. G. Hart, *Cochrane Database Syst Rev*, 2014.
40. A. K. Glaser, N. P. Reder, Y. Chen, E. F. McCarty, C. Yin, L. Wei, Y. Wang, L. D. True and J. T. Liu, *Nat Biomed Eng*, 2017, **1**, 0084.
41. F. Fereidouni, Z. T. Harmany, M. Tian, A. Todd, J. A. Kintner, J. D. McPherson, A. D. Borowsky, J. Bishop, M. Lechpammer and S. G. Demos, *Nat Biomed Eng*, 2017, **1**, 957.

42. F. T. Nguyen, A. M. Zysk, E. J. Chaney, J. G. Kotynek, U. J. Oliphant, F. J. Bellafiore, K. M. Rowland, P. A. Johnson and S. A. Boppart, *Cancer Res*, 2009, **69**, 8790-8796.
43. A. M. Zysk, K. Chen, E. Gabrielson, L. Tafra, E. A. M. Gonzalez, J. K. Canner, E. B. Schneider, A. J. Cittadine, P. S. Carney and S. A. Boppart, *Ann Surg Oncol*, 2015, **22**, 3356-3362.
44. R. Nachabé, B. H. Hendriks, G. W. Lucassen, M. van der Voort, D. J. Evers, E. J. Rutgers, M.-J. V. Peeters, J. A. Van der Hage, H. S. Oldenburg and T. J. Ruers, *J Biomed Opt*, 2011, **16**, 087010.
45. L. G. Wilke, J. Q. Brown, T. M. Bydlon, S. A. Kennedy, L. M. Richards, M. K. Junker, J. Gallagher, W. T. Barry, J. Geradts and N. Ramanujam, *The American Journal of Surgery*, 2009, **198**, 566-574.
46. C. V. Raman and K. S. Krishnan, *Nature*, 1928, **121**, 501.
47. G. D. Ewen Smith, *Modern Raman Spectroscopy*, John Wiley & Sons Ltd, Chichester, 1st edn., 2005.
48. Z. Movasaghi, S. Rehman and I. U. Rehman, *Appl Spectrosc Rev*, 2007, **42**, 493-541.
49. N. Stone, P. Stavroulaki, C. Kendall, M. Birchall and H. Barr, *Laryngoscope*, 2000, **110**, 1756-1763.
50. B. Chase, *J Appl Spectrosc*, 1994, **48**, 14A-19A.
51. M. Delhaye and P. Dhamelincourt, *J Raman Spectrosc*, 1975, **3**, 33-43.
52. C. J. De Grauw, C. Otto and J. Greve, *Appl Spectrosc*, 1997, **51**, 1607-1612.
53. J. Hutchings, C. Kendall, B. Smith, N. Shepherd, H. Barr and N. Stone, *J Biophotonics*, 2009, **2**, 91-103.
54. F. Sinjab, K. Kong, G. Gibson, S. Varma, H. Williams, M. Padgett and I. Notingher, *Biomed Opt Express*, 2016, **7**, 2993-3006.
55. S. Schlücker, M. D. Schaeberle, S. W. Huffman and I. W. Levin, *Anal Chem*, 2003, **75**, 4312-4318.
56. C. J. Rowlands, S. Varma, W. Perkins, I. Leach, H. Williams and I. Notingher, *J Biophotonics*, 2012, **5**, 220-229.
57. K. Kong, F. Zaabar, E. Rakha, I. Ellis, A. Koloydenko and I. Notingher, *Phys Med Biol*, 2014, **59**, 6141-6152.
58. S. Y. Leigh, M. Som and J. T. C. Liu, *PLoS ONE*, 2013, **8**, e62084.
59. S. Laing, L. E. Jamieson, K. Faulds and D. Graham, *Nat Rev Chem*, 2017, **1**, 0060.
60. M. V. Yezhelyev, A. Al-Hajj, C. Morris, A. I. Marcus, T. Liu, M. Lewis, C. Cohen, P. Zrazhevskiy, J. W. Simons and A. Rogatko, *Adv Mater*, 2007, **19**, 3146-3151.
61. P. Matousek, I. P. Clark, E. R. C. Draper, M. D. Morris, A. E. Goodship, N. Everall, M. Towrie, W. F. Finney and A. W. Parker, *Appl Spectrosc*, 2005, **59**, 393-400.
62. M. V. Schulmerich, K. Dooley, M. D. Morris, T. M. Vanasse and S. A. Goldstein, 2006.
63. A. Ghita, P. Matousek and N. Stone, *J Biophotonics*, 2018, **11**, e201600260.
64. L. F. Santos, R. Wolthuis, S. Koljenović, R. M. Almeida and G. J. Puppels, *Anal Chem*, 2005, **77**, 6747-6752.
65. I. E. I. Petterson, J. C. Day, L. M. Fullwood, B. Gardner and N. Stone, *Anal Bioanal Chem*, 2015, **407**, 8311-8320.
66. J. Desroches, M. Jermyn, M. Pinto, F. Picot, M. A. Tremblay, S. Obaid, E. Marple, K. Urmey, D. Trudel, G. Soulez, M. C. Guiot, B. C. Wilson, K. Petrecca and F. Leblond, *Sci Rep*, 2018, **8**, 1792.
67. J. Day, R. Bennett, B. Smith, C. Kendall, J. Hutchings, G. Meaden, C. Born, S. Yu and N. Stone, *Phys Med Biol*, 2009, **54**, 7077.

68. S. Pahlow, K. Weber, J. Popp, B. R. Wood, K. Kochan, A. Ruther, D. Perez-Guaita, P. Heraud, N. Stone, A. Dudgeon, B. Gardner, R. Reddy, D. Mayerich and R. Bhargava, *Appl Spectrosc*, 2018, **72**, 52-84.
69. C. H. Camp Jr and M. T. Cicerone, *Nat Photonics*, 2015, **9**, 295-305.
70. C. L. Evans, E. O. Potma, M. Puoris' haag, D. Côté, C. P. Lin and X. S. Xie, *Proc Natl Acad Sci U S A*, 2005, **102**, 16807-16812.
71. B. G. Saar, C. W. Freudiger, J. Reichman, C. M. Stanley, G. R. Holtom and X. S. Xie, *Science*, 2010, **330**, 1368-1370.
72. M. Ji, S. Lewis, S. Camelo-Piragua, S. H. Ramkissoon, M. Snuderl, S. Venneti, A. Fisher-Hubbard, M. Garrard, D. Fu, A. C. Wang, J. A. Heth, C. O. Maher, N. Sanai, T. D. Johnson, C. W. Freudiger, O. Sagher, X. S. Xie and D. A. Orringer, *Sci Transl Med*, 2015, **7**, 309ra163-309ra163.
73. K. Aubertin, J. Desroches, M. Jermyn, V. Q. Trinh, F. Saad, D. Trudel and F. Leblond, *Biomed Opt Express*, 2018, **9**.
74. E. Barroso, R. Smits, T. Bakker Schut, I. Ten Hove, J. Hardillo, E. Wolvius, R. Baatenburg de Jong, S. Koljenovic and G. Puppels, *Anal Chem*, 2015, **87**, 2419-2426.
75. V. Svetnik, A. Liaw, C. Tong, J. C. Culberson, R. P. Sheridan and B. P. Feuston, *Journal of chemical information and computer sciences*, 2003, **43**, 1947-1958.
76. N. C. Dingari, I. Barman, A. K. Myakalwar, S. P. Tewari and M. Kumar Gundawar, *Anal Chem*, 2012, **84**, 2686-2694.
77. G. Ciccarelli, M. Di Virgilio, S. Menna, L. Garretti, A. Ala, R. Giani, R. Bussone, G. Canavese and E. Berardengo, *Radiol Med*, 2007, **112**, 366-376.
78. NICE, Early and locally advanced breast cancer: diagnosis and management [NG101], https://www.nice.org.uk/guidance/ng101, (accessed 24.01.2019).
79. M. S. Moran, S. J. Schnitt, A. E. Giuliano, J. R. Harris, S. A. Khan, J. Horton, S. Klimberg, M. Chavez-MacGregor, G. Freedman and N. Houssami, *Ann Surg Oncol*, 2014, **21**, 704-716.
80. N. Houssami, P. Macaskill, M. Luke Marinovich and M. Morrow, *Ann Surg Oncol*, 2014, **21**, 717-730.
81. K. Deng, C. Zhu, X. Ma, H. Jia, Z. Wei, Y. Xiao and J. Xu, *PLoS One*, 2016, **11**, e0159860.
82. A. S. Haka, K. E. Shafer-Peltier, M. Fitzmaurice, J. Crowe, R. R. Dasari and M. S. Feld, *Proc Natl Acad Sci U S A*, 2005, **102**, 12371-12376.
83. S. K. Majumder, M. D. Keller, F. I. Boulos, M. C. Kelley and A. Mahadevan-Jansen, *J Biomed Opt*, 2008, **13**, 054009.
84. I. Barman, N. C. Dingari, A. Saha, S. McGee, L. H. Galindo, W. Liu, D. Plecha, N. Klein, R. R. Dasari and M. Fitzmaurice, *Cancer Res*, 2013, **73**, 3206-3215.
85. R. Baker, K. Rogers, N. Shepherd and N. Stone, *Br J Cancer*, 2010, **103**, 1034.
86. B. Han, Y. Du, T. Fu, Z. Fan, S. Xu, C. Hu, L. Bi, T. Gao, H. Zhang and W. Xu, *Appl Spectrosc*, 2017, **71**, 300-307.
87. M. D. Keller, E. Vargis, N. de Matos Granja, R. H. Wilson, M. A. Mycek, M. C. Kelley and A. Mahadevan-Jansen, *J Biomed Opt*, 2011, **16**, 077006.
88. A. S. Haka, Z. Volynskaya, J. A. Gardecki, J. Nazemi, R. Shenk, N. Wang, R. R. Dasari, M. Fitzmaurice and M. S. Feld, *J Biomed Opt*, 2009, **14**, 054023.
89. L. Langhans, M. Jensen, M. M. Talman, I. Vejborg, N. Kroman and T. F. Tvedskov, *JAMA Surg*, 2017, **152**, 378-384.

90. Y. W. Wang, N. P. Reder, S. Kang, A. K. Glaser, Q. Yang, M. A. Wall, S. H. Javid, S. M. Dintzis and J. T. C. Liu, *Cancer Res*, 2017, **77**, 4506-4516.
91. D. W. Shipp, E. A. Rakha, A. A. Koloydenko, R. D. Macmillan, I. O. Ellis and I. Notingher, *Breast Cancer Res*, 2018, **20**.
92. G. P. Guy Jr, S. R. Machlin, D. U. Ekwueme and K. R. Yabroff, *Am J Prev Med*, 2015, **48**, 183-187.
93. Y. Gulleth, N. Goldberg, R. P. Silverman and B. R. Gastman, *Plast Reconstr Surg*, 2010, **126**, 1222-1231.
94. N. Telfer, G. Colver and C. Morton, *Br J Dermatol*, 2008, **159**, 35-48.
95. K. Mosterd, G. A. M. Krekels, F. H. M. Nieman, J. U. Ostertag, B. A. B. Essers, C. D. Dirksen, P. M. Steijlen, A. Vermeulen, H. A. M. Neumann and N. W. J. Kelleners-Smeets, *Lancet Oncol*, 2008, **9**, 1149-1156.
96. V. Nikolaou and A. Stratigos, *Br J Dermatol*, 2014, **170**, 11-19.
97. NICE, Melanoma: assessment and management [NG14], https://www.nice.org.uk/guidance/ng14, (accessed 24.01.2019).
98. M. J. Sladden, C. Balch, D. A. Barzilai, D. Berg, A. Freiman, T. Handiside, S. Hollis, M. B. Lens and J. F. Thompson, *Cochrane Database Syst Rev*, 2009, **4**, CD004835.
99. T. C. B. Schut, P. J. Caspers, G. J. Puppels, A. Nijssen, F. Heule, M. H. Neumann and D. P. Hayes, *Journal of Investigative Dermatology*, 2002, **119**, 64-69.
100. A. Nijssen, K. Maquelin, L. F. Santos, P. J. Caspers, T. C. B. Schut, J. C. den Hollander, M. H. Neumann and G. J. Puppels, *J Biomed Opt*, 2007, **12**, 034004.
101. C. A. Lieber, S. K. Majumder, D. L. Ellis, D. D. Billheimer and A. Mahadevan-Jansen, *Lasers Surg Med*, 2008, **40**, 461-467.
102. C. A. Lieber, S. K. Majumder, D. D. Billheimer, D. L. Ellis and A. Mahadevan-Jansen, *J Biomed Opt*, 2009, **13**, 024013.
103. J. Schleusener, P. Gluszczynska, C. Reble, I. Gersonde, J. Helfmann, J. W. Fluhr, J. Lademann, J. Rowert-Huber, A. Patzelt and M. C. Meinke, *Exp Dermatol*, 2015, **24**, 767-772.
104. H. Lui, J. Zhao, D. I. McLean and H. Zeng, *Cancer Res*, 2012, DOI: 10.1158/0008-5472.CAN-11-4061 %J Cancer Research.
105. J. Zhao, H. Lui, S. Kalia and H. Zeng, *Anal Bioanal Chem*, 2015, **407**, 8373-8379.
106. J. Zhang, Y. Fan, Y. Song and J. Xu, *Medicine (Baltimore)*, 2018, **97**, e12022.
107. I. A. Bratchenko, D. N. Artemyev, O. O. Myakinin, Y. A. Khristoforova, A. A. Moryatov, S. V. Kozlov and V. P. Zakharov, *J Biomed Opt*, 2017, **22**, 027005.
108. S. Heuke, N. Vogler, T. Meyer, D. Akimov, F. Kluschke, H. J. Röwert-Huber, J. Lademann, B. Dietzek and J. Popp, *Br J Dermatol*, 2013, **169**, 794-803.
109. K. Kong, C. J. Rowlands, S. Varma, W. Perkins, I. H. Leach, A. A. Koloydenko, H. C. Williams and I. Notingher, *Proc Natl Acad Sci U S A*, 2013, **110**, 15189-15194.
110. K. Kong, C. J. Rowlands, S. Varma, W. Perkins, I. H. Leach, A. A. Koloydenko, A. Pitiot, H. C. Williams and I. Notingher, *J Mol Struct*, 2014, **1073**, 58-65.
111. R. Boitor, K. Kong, D. Shipp, S. Varma, A. Koloydenko, K. Kulkarni, S. Elsheikh, T. B. Schut, P. Caspers and G. Puppels, *Biomed Opt Express*, 2017, **8**, 5749-5766.
112. M. L. Goodenberger and R. B. Jenkins, *Cancer Genet*, 2012, **205**, 613-621.
113. NICE, Brain Tumours (primary) and brain metastases in adults [NG99], www.nice.org.uk/guidance/ng99/chapter/recommendations#management-of-glioma, (accessed 24.01.2019).

Field Code Changed

114. K. Petrecca, M.-C. Guiot, V. Panet-Raymond and L. Souhami, *J Neurooncol*, 2013, **111**, 19-23.
115. A. Beljebbar, S. Dukic, N. Amharref and M. Manfait, *Anal Bioanal Chem*, 2010, **398**, 477-487.
116. M. Ji, D. A. Orringer, C. W. Freudiger, S. Ramkissoon, X. Liu, D. Lau, A. J. Golby, I. Norton, M. Hayashi and N. Y. Agar, *Sci Transl Med*, 2013, **5**, 201ra119-201ra119.
117. O. Uckermann, R. Galli, S. Tamosaityte, E. Leipnitz, K. D. Geiger, G. Schackert, E. Koch, G. Steiner and M. Kirsch, *PLoS One*, 2014, **9**, e107115.
118. X. Gao, Q. Yue, Z. Liu, M. Ke, X. Zhou, S. Li, J. Zhang, R. Zhang, L. Chen and Y. Mao, *Adv Mater*, 2017, **29**, 1603917.
119. H. Karabeber, R. Huang, P. Iacono, J. M. Samii, K. Pitter, E. C. Holland and M. F. Kircher, *Acs Nano*, 2014, **8**, 9755-9766.
120. M. Demeule, J. C. Currie, Y. Bertrand, C. Ché, T. Nguyen, A. Régina, R. Gabathuler, J. P. Castaigne and R. Béliveau, *J Neurochem*, 2008, **106**, 1534-1544.
121. N. Bergner, T. Bocklitz, B. F. M. Romeike, R. Reichart, R. Kalff, C. Krafft and J. Popp, *Chemometr Intell Lab Syst*, 2012, **117**, 224-232.
122. S. N. Kalkanis, R. E. Kast, M. L. Rosenblum, T. Mikkelsen, S. M. Yurgelevic, K. M. Nelson, A. Raghunathan, L. M. Poisson and G. W. Auner, *J Neurooncol*, 2014, **116**, 477-485.
123. D. A. Orringer, B. Pandian, Y. S. Niknafs, T. C. Hollon, J. Boyle, S. Lewis, M. Garrard, S. L. Hervey-Jumper, H. J. Garton and C. O. Maher, *Nat Biomed Eng*, 2017, **1**, 0027.
124. D. Bury, C. L. Morais, K. M. Ashton, T. P. Dawson and F. L. Martin, *Biosensors*, 2019, **9**, 49.
125. M. Jermyn, K. Mok, J. Mercier, J. Desroches, J. Pichette, K. Saint-Arnaud, L. Bernstein, M.-C. Guiot, K. Petrecca and F. Leblond, *Sci Transl Med*, 2015, **7**, 274ra219-274ra219.
126. M. Jermyn, J. Mercier, K. Aubertin, J. Desroches, K. Urmey, J. Karamchandiani, E. Marple, M. C. Guiot, F. Leblond and K. Petrecca, *Cancer Res*, 2017, **77**, 3942-3950.
127. NICE, Cancer of the upper aerodigestive tract: assessment and management in people aged 16 and over [NG36], www.nice.org, (accessed 24.01.2019).
128. T. R. Loree and E. W. Strong, *Am J Surg*, 1990, **160**, 410-414.
129. R. W. Smits, S. Koljenović, J. A. Hardillo, I. ten Hove, C. A. Meeuwis, A. Sewnaik, E. A. Dronkers, T. C. Bakker Schut, T. P. Langeveld and J. Molenaar, *Head Neck*, 2016, **38**, E2197-E2203.
130. E. M. Barroso, R. W. Smits, C. G. van Lanschot, P. J. Caspers, I. Ten Hove, H. Mast, A. Sewnaik, J. A. Hardillo, C. A. Meeuwis, R. Verdijk, V. Noordhoek Hegt, R. J. Baatenburg de Jong, E. B. Wolvius, T. C. Bakker Schut, S. Koljenovic and G. J. Puppels, *Cancer Res*, 2016, **76**, 5945-5953.
131. D. T. Helliwell and D. J. Woolgar, Standards and datasets for reporting cancers Dataset for histopathology reporting of mucosal malignancies of the oral cavity, https://www.rcpath.org/uploads/assets/uploaded/e485236e-6d2a-4109-96fe1da9d5e140f9.pdf, (accessed 24.01.2019).
132. J. D. Meier, D. A. Oliver and M. A. Varvares, *Head Neck*, 2005, **27**, 952-958.
133. K. A. Pathak, R. W. Nason, C. Penner, N. R. Viallet, D. Sutherland and P. D. Kerr, *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 2009, **107**, 235-239.
134. D. P. Lau, Z. Huang, H. Lui, D. W. Anderson, K. Berean, M. D. Morrison, L. Shen and H. Zeng, *Lasers Surg Med*, 2005, **37**, 192-200.
135. K. Lin, D. L. P. Cheng and Z. Huang, *Biosens Bioelectron*, 2012, **35**, 213-217.

Field Code Changed

136. K. Lin, W. Zheng, C. M. Lim and Z. Huang, *Biomed Opt Express*, 2016, **7**, 3705-3715.
137. K. Lin, W. Zheng, C. M. Lim and Z. Huang, *Theranostics*, 2017, **7**, 3517.
138. F. L. J. Cals, T. C. Bakker Schut, J. A. Hardillo, R. J. Baatenburg de Jong, S. Koljenović and G. J. Puppels, *Lab Invest*, 2015, **95**, 1186.
139. F. L. J. Cals, S. Koljenović, J. A. Hardillo, R. J. Baatenburg de Jong, T. C. Bakker Schut and G. J. Puppels, *Oral Oncol*, 2016, **60**, 41-47.
140. K. Guze, H. C. Pawluk, M. Short, H. Zeng, J. Lorch, C. Norris and S. Sonis, *Head Neck*, 2015, **37**, 511-517.
141. E. M. Barroso, I. ten Hove, T. C. Bakker Schut, H. Mast, C. G. F. van Lanschot, R. W. H. Smits, P. J. Caspers, R. Verdijk, V. Noordhoek Hegt, R. J. Baatenburg de Jong, E. B. Wolvius, G. J. Puppels and S. Koljenović, *Eur J Cancer*, 2018, **92**, 77-87.
142. K. Maheedhar, R. A. Bhat, R. Malini, N. Prathima, P. Keerthi, P. Kushtagi and C. M. Krishna, *Photomed Laser Surg*, 2008, **26**, 83-90.
143. H. C. McGregor, M. A. Short, A. McWilliams, T. Shaipanich, D. N. Ionescu, J. Zhao, W. Wang, G. Chen, S. Lam and H. Zeng, *J Biophotonics*, 2017, **10**, 98-110.
144. L. Gao, Z. Wang, F. Li, A. A. Hammoudi, M. J. Thrall, P. T. Cagle and S. T. Wong, *Arch Pathol Lab Med*, 2012, **136**, 1502-1510.
145. C. S. Teixeira, R. A. Bitar, H. S. Martinho, A. B. Santos, M. A. Kulcsar, C. U. Friguglietti, R. B. da Costa, E. Â. Arisawa and A. A. Martin, *Analyst*, 2009, **134**, 2361-2370.
146. P. Crow, N. Stone, C. A. Kendall, J. S. Uff, J. A. M. Farmer, H. Barr and M. P. J. Wright, *Br J Cancer*, 2003, **89**, 106-108.
147. N. Stone, M. C. Hart Prieto, P. Crow, J. Uff and A. W. Ritchie, *Anal Bioanal Chem*, 2007, **387**, 1657-1668.
148. K. Aubertin, M. Birlea, M. C. Vladoiu, A. A. Grosset, V. Q. Trinh, D. Trudel, M. Latour, R. Albadine, F. Leblond, P. Baksic, F. Saad, J. Desroches, K. St. ?Arnaud and M. Jermyn, *BJU Int*, 2018, **122**, 326-336.
149. M. Pinto, K. C. Zorn, J.-P. Tremblay, J. Desroches, F. Dallaire, K. Aubertin, E. Marple, C. Kent, F. Leblond and D. Trudel, *J Biomed Opt*, 2019, **24**, 025001.
150. A. Robichaux-Viehoever, E. Kanter, H. Shappell, D. Billheimer, H. Jones, 3rd and A. Mahadevan-Jansen, *Appl Spectrosc*, 2007, **61**, 986-993.
151. R. S. Shaikh, T. K. Dora, S. Chopra, A. Maheshwari, D. K. Kedar, R. Bharat and C. M. Krishna, *J Biomed Opt*, 2014, **19**, 087001.
152. S. Duraipandian, W. Zheng, J. Ng, J. J. Low, A. Ilancheran and Z. Huang, *J Biomed Opt*, 2013, **18**, 067007.
153. P. Crow, A. Molckovsky, N. Stone, J. Uff, B. Wilson and L. M. WongKeeSong, *Urology*, 2005, **65**, 1126-1130.
154. R. O. Draga, M. C. Grimbergen, P. L. Vijverberg, C. F. van Swol, T. G. Jonges, J. A. Kummer and J. L. Ruud Bosch, *Anal Chem*, 2010, **82**, 5993-5999.
155. C. Kendall, J. Day, J. Hutchings, B. Smith, N. Shepherd, H. Barr and N. Stone, *Analyst*, 2010, **135**, 3038-3041.
156. S. Teh, W. Zheng, K. Ho, M. Teh, K. Yeoh and Z. Huang, *Br J Surg*, 2010, **97**, 550-557.
157. J. Wang, K. Lin, W. Zheng, K. Y. Ho, M. Teh, K. G. Yeoh and Z. Huang, *Sci Rep*, 2015, **5**, 12957.
158. M. S. Bergholt, K. Lin, J. Wang, W. Zheng, H. Xu, Q. Huang, J. l. Ren, K. Y. Ho, M. Teh and S. Srivastava, *J Biophotonics*, 2016, **9**, 333-342.
159. G. Gravante, D. Hemingway, J. A. Stephenson, D. Sharpe, A. Osman, M. Haines, V. Pirjamali, R. Sorge, J. M. Yeung and M. Norwood, *J Surg Oncol*, 2016, **114**, 642-648.

160. S. Dexter, H. Sue-Ling, M. McMahon, P. Quirke, N. Mapstone and I. Martin, *Gut*, 2001, **48**, 667-670.
161. L. M. Quinn, A. C. Hollis, J. Hodson, M. A. Elshafie, M. T. Hallissey, J. L. Whiting and E. A. Griffiths, *Eur J Surg Oncol*, 2018.
162. I. Pence and A. Mahadevan-Jansen, *Chem Soc Rev*, 2016, **45**, 1958-1979.
163. A. S. Haka, Z. Volynskaya, J. A. Gardecki, J. Nazemi, J. Lyons, D. Hicks, M. Fitzmaurice, R. R. Dasari, J. P. Crowe and M. S. Feld, *Cancer Res*, 2006, **66**, 3317-3322.
164. J. D. Horsnell, C. Kendall and N. Stone, *Lasers Med Sci*, 2016, **31**, 1143-1149.
165. I. P. Santos, E. M. Barroso, T. C. Bakker Schut, P. J. Caspers, C. G. F. van Lanschot, D. H. Choi, M. F. van der Kamp, R. W. H. Smits, R. van Doorn, R. M. Verdijk, V. Noordhoek Hegt, J. H. von der Thusen, C. H. M. van Deurzen, L. B. Koppert, G. van Leenders, P. C. Ewing-Graham, H. C. van Doorn, C. M. F. Dirven, M. B. Busstra, J. Hardillo, A. Sewnaik, I. Ten Hove, H. Mast, D. A. Monserez, C. Meeuwis, T. Nijsten, E. B. Wolvius, R. J. Baatenburg de Jong, G. J. Puppels and S. Koljenovic, *Analyst*, 2017, **142**, 3025-3047.
166. G. Thomas, T. Q. Nguyen, I. J. Pence, B. Caldwell, M. E. O'Connor, J. Giltnane, M. E. Sanders, A. Grau, I. Meszoely, M. Hooks, M. C. Kelley and A. Mahadevan-Jansen, *Sci Rep*, 2017, **7**, 13548.
167. J. M. Dixon, L. Renshaw, O. Young, D. Kulkarni, T. Saleem, M. Sarfaty, R. Sreenivasan, C. Kusnick, J. Thomas and L. J. Williams, *Eur J Surg Oncol*, 2016, **42**, 1834-1840.
168. R. Stables, G. Clemens, H. J. Butler, K. M. Ashton, A. Brodbelt, T. P. Dawson, L. M. Fullwood, M. D. Jenkinson and M. J. Baker, *Analyst*, 2017, **142**, 98-109.
169. C. Fink and H. Haenssle, *Skin Research and Technology*, 2017, **23**, 261-271.
170. J. C. C. Day and N. Stone, *Appl Spectrosc*, 2013, **67**, 349-354.
171. O. Stevens, I. E. Iping Petterson, J. C. Day and N. Stone, *Chem Soc Rev*, 2016, **45**, 1919-1934.
172. J. Horsnell, P. Stonelake, J. Christie-Brown, G. Shetty, J. Hutchings, C. Kendall and N. Stone, *Analyst*, 2010, **135**, 3042-3047.

Figure 1. A graphic to illustrate the concept of tumour margins. A. A surgically excised specimen with an ~~'clear'~~adequate ~~-~~margin~~s~~ along the resected surface; the cancerous tissue is in the centre of the surgically excised specimen, with a rim of normal tissue surrounding it. The distance of what defines an 'adequate' margin varies between pathologies. B. A surgically excised specimen with an inadequate or 'positive' margin; the cancerous tissue is at the edge of the specimen. This could mean there is further cancerous tissue in the patient that has not been excised.

Figure 2. A graphical representation of the ideal paradigm for the ~~-~~surgical workflow of intraoperative margin analysis (IMA) by Raman Spectroscopy. This would allow the surgeon to remove all cancerous tissue at the initial operation, thus improving patient outcome.

Figure 3. Examples of fibre optic probes capable of Raman Spectroscopy measurement in a clinical setting for cancer diagnosis, or application to *in vivo* surgical guidance to provide IMA, images Authors own. A. A fibre optic probe is incorporated into a standard 5ml syringe with a 15cm long, 20 gauge needle and so is capable of subcutaneous measurements (in this example in a Turkey leg) as detailed in reference [170]. B. A miniature confocal Raman packaged probe with a GRIN lens objective for endoscopic use. A detailed review on fibre optics for clinical use of Raman Spectroscopy is found in reference[171]

Figure 4. Example of assigning Raman spectra to tissue structures and ductal carcinoma of breast tissue to inform the diagnostic algorithm. (a) + (b) invasive ductal carcinoma (IDC), (c) normal breast tissue. Red arrows show focus of IDC, green arrow tumour surrounding inflammatory stroma, blue arrows lobules and ducts, black arrows, stroma and orange, fat (Reproduced from reference [57] K. Kong, F. Zaabar, E. Rakha, I. Ellis, A. Koloydenko and I. Towards intra-operative diagnosis of tumours during breast conserving surgery by selective-sampling Raman micro-spectroscopy. https://doi.org/10.1088/0031-9155/59/20/6141, under a Creative Commons Attribution 3.0 licence).

Figure 5. Examples of ~~potential methods for using~~ Raman probe systems that could be used~~s~~ for ~~intraoperative margin analysis~~IMA using Raman spectroscopy. A. Ex vivo ~~Raman spectroscopy~~RS analysis of a specimen, where the specimen is placed on top of a probe to enable a surface to be analysed. This example uses an axillary lymph node (reproduced from reference [172], with permission from the Royal Society of Chemistry, and the authors) B. Design of an automated 3D margin scanner prototype (Marginbot), where the specimen is placed on a stage and automated movement of the specimen and the Raman probe (depicted by arrows) is required to assess the specimen margins (reproduced from reference [166] G. Thomas, T. Q. Nguyen, I. J. Pence, B. Caldwell, M. E. O'Connor, J. Giltnane, M. E. Sanders, A. Grau, I. Meszoely, M. Hooks, M. C. Kelley and A. Mahadevan-Jansen, Evaluating feasibility of an automated 3-dimensional scanner using Raman spectroscopy for intraoperative breast margin assessment, https://doi.org/10.1038/s41598-017-13237-y, under a creative commons attribution 4.0 International License) C. Handheld probe (Emvision, LLC) for use *in vivo*, in this example to interrogate brain tissue during surgery with the potential to assist in gaining clear margins in the excised specimen. The schematic diagram illustrates the excitation of different molecular species that produces a Raman spectra. From M. Jermyn, K. Mok, J. Mercier, J. Desroches, J. Pichette, K. Saint-Arnaud, L. Bernstein, M.-C. Guiot, K. Petrecca and F. Leblond, *Sci Transl Med*, 2015, **7**, 274ra219-274ra219 [125] . Reprinted with permission from AAAS.

Comment [HT13]: With the potential to assist in gaining clear margins in the excised specimen.

Formatted: Font: Italic