Raman spectroscopy – A potential new method for the intra-operative assessment of axillary lymph nodes

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ABSTRACT

Sentinel Lymph Node Biopsy has become the standard surgical procedure for the sampling of axillary lymph nodes in breast cancer. Intra-operative node assessment of these nodes would allow definitive axillary surgery to take place immediately with associated benefits for patient management. Our experimental study aims to demonstrate that a Raman spectroscopy probe system could overcome many of the disadvantages of current intra-operative methods.

59 axillary lymph nodes, 43 negative and 16 positive from 58 patients undergoing breast surgery at our district general hospital were mapped using Raman micro-spectroscopy. These maps were then used to model the effect of using a Raman spectroscopic probe by selecting 5 and 10 probe points across the mapped images and evaluating the impact on disease detection.

Results demonstrated sensitivities of up to 81% and specificities of up to 97% when differentiating between positive and negative lymph nodes, dependent on the number of probe points included. The results would have concurred with histopathology assessment in 89% and 91% of cases in the 5 and 10 point models respectively. Using Raman spectroscopy in this way could allow lymph node assessment within a time-frame suitable for intra-operative use.

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Introduction

Each year approximately 45,000 patients are diagnosed with invasive breast cancer in the UK alone and this accounts for 1 in 6 of all cancer deaths in women.1 Surgical treatment for the disease includes resection of both the tumour itself and in cases where the disease has metastasised to the axillary lymph nodes, excision of these nodes. Further adjuvant...
treatment is then offered, dependent on the type and stage of each patient’s disease.

The assessment of axillary lymph nodes traditionally involved either an axillary clearance or a 4 node axillary sampling procedure. The introduction of Sentinel Lymph Node Biopsy (SLNB), a technique first pioneered in penile cancer and malignant melanoma has reduced the invasiveness and the morbidity associated with lymph node assessment. SLNB has now become the standard surgical procedure for the sampling of axillary lymph nodes in breast cancer. This technique was developed on the principle that malignant cells that have invaded the lymphatic vessels will first reach specific, sentinel, lymph nodes. Assessment of these sentinel nodes determines whether lymphatic spread has occurred without the need for further more invasive sampling. 25–30% of patients undergoing an SLNB will have a positive result. If these nodes are assessed postoperatively a subsequent axillary lymph node clearance will be required at a separate date. This results in a significant level of psychological stress for the patient and a delay in adjuvant treatment. It also has an impact on healthcare resources as a second operation and hospital admission are required.

A number of techniques have been used to assess lymph nodes intra-operatively and to allow for immediate axillary node clearance if required. The use of frozen section analysis, touch imprint cytology and molecular assays have all been reported in this context.

Frozen section analysis is an established technique that is used in a wide range of intra-operative situations. It has been reported as having a sensitivity of between 57 and 87% and specificities of greater than 99%. The effect of freeze artefacts, processing time and the need for immediate access to an experienced histopathologist represent significant disadvantages for this method of assessment.

Touch Imprint Cytology (TIC) is a simple and rapid method of preparing a cytological specimen for analysis. It involves bisecting the node and pressing the two surfaces onto a slide which are then reviewed by a histopathologist. Although a meta analysis of 31 studies reported a sensitivity of 63% and a specificity of 99% the need for immediate histopathological input has prevented its universal uptake.

Recently molecular assays using either reverse transcriptase polymerase chain reactions (RT-PCR) or reverse transcriptase loop-mediated isothermal amplifications (RT-LAMP) have been developed to detect metastasis by measuring the concentration of tumour specific mRNA markers. The two markers CK-19 (used in both techniques) and mammaglobin (used in the RT-PCR only) are expressed at high levels in cells of breast origin but are absent or at a low level in normal axillary nodes. Studies analysing the efficacy of these methods to differentiate between positive and negative nodes have reported sensitivities of between 88 and 98% and specificities of greater than 93% for the RT-PCR technique. Sensitivities of 95% and specificities of up to 97% have been reported with the RT-LAMP technique.

Raman spectroscopy, first described in the late 1920s, is an analytical method which relies on the inelastic scattering of light. Incident light interacts with the tissue under investigation and transfers energy that alters the vibrational mode of molecules within it. Subsequently light is emitted from the tissue with a frequency that is dependent on changes in the vibrational mode of the matter. The frequency of the emitted light can be measured and as the vibrational energy levels are unique for every molecule Raman spectra are chemically specific. Raman spectroscopy has previously been demonstrated to have potential for in vivo use as the excitation wavelengths are non-destructive to the tissues under interrogation.

Raman spectroscopy has already been investigated as a tool to aid the diagnosis of breast disease. Work has focused on the breast tumour itself, either at the stage of initial diagnosis or during its excision. It has been shown that Raman spectroscopy could be used to assess tumour margins following a wide local excision or potentially reduce the likelihood of a non diagnostic biopsy. The use of deep Raman techniques such as spatially offset Raman spectroscopy (SORS) techniques could also be used to complement mammography by allowing further assessment of breast calcifications without tissue removal.

Raman spectroscopic mapping techniques have previously been used to interrogate sentinel lymph nodes, demonstrating a sensitivity of 91% and a specificity of 93% for the differentiation of negative and positive nodes. Analysis of mean spectra from the positive and negative groups suggest that positive nodes have increased DNA and tyrosine contributions and reduced levels of collagen contributions when compared to the negative nodes. Despite the results achieved in this study the time taken to acquire the number of spectra required to produce precise mapped images, 12–120 h, rendered this technique inappropriate for the use in an intra-operative setting. We believe that Raman spectroscopy can overcome many of the disadvantages of current intra-operative assessment methods. Using this technique a classification model would assess the recorded spectra and output a diagnostic result independent of immediate pathology review. Further it is non-destructive, allowing for post operative review, and as we will show for the first time in this paper, can achieve sensitivities and specificities comparable to current techniques in a timeframe that is suitable for intra-operative use.

Methods and analysis

59 axillary lymph nodes were collected from 58 patients during axillary lymph node sampling or axillary lymph node dissection. In one patient a lymph node was collected during both the
initial sampling and subsequent clearance procedure. At the
time of tissue sampling sentinel lymph node biopsy was not
being performed routinely within our institution. Informed
consent was given by all patients included in this study prior to
the procedure being performed and all protocols had been
approved by our local research ethics committee.

The node was dissected free from axillary fat immediately
after identification and resection. It was hand cut into two
approximate halves using a disposable scalpel and one half of
the tissue was sent for routine histopathological analysis
whilst the other half was placed in a labelled cryovial and snap
frozen in liquid nitrogen. The frozen section was transported
to our research department where 7 μm sections were cut
using a freezing microtome. Contiguous sections were cut for
both spectral and histopathological analysis. The research
half of the node was categorised according to the pathological
analysis of the section cut within the research laboratory. 42
of the 59 nodes were reported histopathologically as negative
and 17 were reported as positive for metastasis.

Prior to analysis the tissue was defrosted at the ambient
laboratory temperature of 22 °C for 30 min. Raman spectro-
scopic mapping analysis was performed using a Renishaw
Raman System 1000® spectrometer that incorporated an
a 830 nm diode laser. Spectra were collected in an automated
spatial scanning mode in a raster pattern with a step size of
100 μm in both the X and Y direction. At each step 3 accumu-
lations of 10 s were taken. The spot size was approximately
2 μm × 10 μm. Each map took between 12 and 120 h to
collate from the probe models.

All spectral data were energy sensitivity corrected, using
a fluorescent green glass standard, and normalised using
standard Matlab® mathematical software. To mimic the use of
a probe, models were created that selected mean spectra from
5 to 10 equally spaced positions across the mapped images.
Each probe point represented a tissue volume of 560 μm³. Six
and 12 nodes were excluded from each model respectively, as
the area originally mapped was too small to allow for
adequate spacing of the probe points. 53 nodes (36 negative
and 17 positive) and 265 spectra were included in the 10 point
model whilst 47 nodes (31 negative and 16 positive) and 470
spectra were included in the 10 point model (Fig. 1; Table 1).

As we move towards clinical implementation of Raman
spectroscopy the importance of robust methods of spectral
analysis becomes more paramount. Linear discriminant
analysis has been the technique that has been predominantly
used for the interpretation of Raman spectra. It has recently
been shown that support vector machines (SVM) offer
advantages over this methodology. We therefore utilised
radial basis function (RBF) SVM models to classify the data
collated from the probe models.

Using RBF-SVM an assessment of the diagnostic capability
each model was made following leave one node out cross
validation. Sensitivity and specificity were calculated both on
an individual spectra and whole node basis. The node was
said to be positive if greater than 50% of the spectra for that
node were classified as positive and negative if greater than
50% of the spectra were classified as negative.

Table 1 – Details of nodes used in each probe model.

<table>
<thead>
<tr>
<th>Probe model</th>
<th>5 Point</th>
<th>10 Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of eligible nodes</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>Number of positive nodes</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Number of negative nodes</td>
<td>36</td>
<td>31</td>
</tr>
<tr>
<td>Total spectra analysed</td>
<td>265</td>
<td>470</td>
</tr>
</tbody>
</table>

Results

5 point probe analysis

Cross validated assessment of the node based on spectra from
5 points, achieved a sensitivity of 71% and a specificity of 97%
(Table 2).

Analysis of results from the 5 point probe model demonstra-
tes that the Raman based system would have agreed with
the histopathological classification in 47 of the 53 cases (88%).
If used in a clinical setting the Raman system would have
correctly identified 12 of 17 positive nodes and 35 of 36 nega-
tive nodes. Consequently there were 5 false negative results
and 1 false positive result (Fig. 2).

10 point probe analysis

Cross validated assessment of the node based on spectral
information from 10 points achieved a sensitivity of 81% and
a specificity of 97% (Table 3).

Using the 10 probe model the Raman system would have
concorded with the histopathology classification in 43 of 47
patients (91%). 13 of the 16 positive nodes and 30 of the 31
negative nodes were correctly identified. There was one false
positive and 3 false negative results in this series (Fig. 3).

Discussion

Intra-operative assessment of axillary lymph nodes is fav-
oured by the majority of patients and can also confer

Table 2 – Results for nodes in the 5 point probe model.

<table>
<thead>
<tr>
<th>Raman Classification</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>36</td>
</tr>
</tbody>
</table>

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significant benefits on health resources by reducing the need for a second operation. It has been estimated that the cost saving per patient would be £1368 for each patient who underwent an axillary clearance following intra-operative node assessment. The data presented in this preliminary study confirm the potential for the use of a Raman spectroscopy probe to assess axillary lymph nodes in the operative setting. Our Raman spectroscopy probe models concurred with post operative histopathology assessment in 89–91% of cases. The sensitivity and specificity of our models, 71% and 97% in the 5 point model and 81% and 97% in the 10 point model are at least comparable to other current methods of intra-operative analysis.

As we move towards the use of Raman spectroscopy as a diagnostic adjunct we must recognise the clinical consequences of false negative and false positive results. In this clinical situation the avoidance of false positive results is paramount. The consequence of such an occurrence would see a patient with negative nodes undergoing an ultimately unnecessary axillary clearance. In our study there was one node which was classified as a false positive in both the 5 and 10 probe point groups. One method of avoiding such a scenario would be to optimise the classification system to ensure 100% specificity. It is very likely that this would have a detrimental effect on the sensitivity of the test and this will be explored further in larger on-going studies.

The clinical consequences of a false negative result whilst less severe are nonetheless important to consider. In this situation the patient would go on to require a second operation following the post operative histopathological analysis of her nodes. Patients would have to be warned of this possibility when they were consented for their primary operation. A potential way of reducing the numbers of false negatives would be to increase the volume of tissue sampled by the probe. We have demonstrated that by increasing the total volume sampled from 2800 µm$^3$ to 5600 µm$^3$ in the two probe models that our sensitivity increased from 71 to 81%. Early metastatic change within a lymph node may only be seen in small areas of the node. Its is anticipated that the use of alternative probes with larger tissue measurement areas would be more likely to acquire spectral information from these areas and provide further improvements in the sensitivity of the technique without increasing the total time for sampling. Current work is exploring the use of a probe that collects spectral information from volumes of at least 340,000 µm$^3$.

Ideally the time taken for any method of node assessment should not increase the length of the operative procedure. The reported time taken for current intra-operative techniques is as much as 40 min. The estimated time taken to acquire the spectra in the 5 and 10 point probe models was 9 and 18 min respectively. It is recognised that this does not include preparation time, but as we move from laboratory based assessment to the theatre setting it is envisaged that spectra would be obtained from fresh samples, unlike the frozen samples in this study. The only sample preparation, required for the use of probe systems, would be the removal of surrounding fat and bisection of the node. This is not likely to add significantly to the time taken to output a result and would allow for the assessment of the node whilst the surgeon proceeds with the tumour excision.

### Conclusions

We consider that the results presented here support the potential use of Raman spectroscopy probe systems for the intra-operative assessment of axillary lymph nodes. Sensitivities and specificities are at least comparable to current techniques. Utilising a probe system, rather than mapping techniques could allow node assessment to be performed within a time-frame, that is consistent with other methods and that would not increase the overall operative time. As a non-destructive technique it would allow for post operative assessment to confirm intra-operative results.

Further on-going studies within our department are assessing the use of probes with a larger sampling volume and with equipment that would be within the price range of surgical departments (£10,000-£15,000). Clinical implementation in the theatre setting during an SLNB will first require studies to repeat these results in the presence of blue dye and radiolabelled colloid, commonly used in the procedure, as well as methods to overcome the effects of the operating environment.

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