Label-free identification and characterization of living human primary and secondary tumour cells

Dimitrios Tsikritis, Susanna Richmond, Patrick Stewart, Alistair Elfick and Andrew Downes

We used three label-free minimally invasive methods to characterize individual cells derived from primary and secondary tumours from the same patient, and of the same type – colorectal. Raman spectroscopy distinguished cells by their biochemical ‘fingerprint’ in a vibrational spectrum with 100% accuracy, and revealed that the primary cell line contains more lipids and alpha-helix proteins, whereas the secondary cell line contains more porphyrins and beta-sheet proteins. Stimulated Raman scattering (SRS) microscopy distinguished cells in chemically-specific images of CH₂ bonds which revealed lipid droplets in secondary tumour cells. Atomic force microscopy (AFM) was used to distinguish cells with 80% accuracy by measuring their elasticity – secondary tumour cells (SW620) are around 3 times softer than primary ones (SW480). As well as characterizing the physical and biochemical differences between cell lines in vitro, these techniques offer three novel methods which could potentially be used for diagnosis – to assign a tumour as primary or secondary.

1. Introduction

Metastasis – the spreading of cancer from one organ to another – results in secondary tumours which are responsible for 90% of deaths from cancer.¹ To reduce death rates, it is essential to understand metastasis and characterize primary and secondary tumours. Primary tumours possess different genetic profiles²,³ from secondary tumours, so this is used to clinically diagnose whether a tumour is primary or secondary in nature.

Receptor biomarkers are specific to a cancer type,⁴ and these biomarkers of a secondary tumour should remain unchanged from the primary source. Alternatively, some biomarkers may be specific to metastasis.⁵ So, in future it may be possible to distinguish primary and secondary tumours with MRI or CT contrast agents, such as nanoparticles coated with such antibodies.

However, there is currently no label-free method to distinguish primary from secondary tumours. Label-free methods which are real time would improve treatment over genetic tests, as they would be performed during surgery in vivo or on excised tissue. As well as being directly relevant to optimum patient treatment, such label-free methods may also offer an improvement in understanding the mechanism of metastasis.

Raman spectroscopy⁶ is a form of optical spectroscopy which does not require labels like fluorophores. A well-defined laser frequency excites vibrational bonds in biomolecules (such as C–C, C–H, C==O). A small proportion of the laser light is absorbed by the molecule, then re-emitted at a lower frequency. The frequency shift of the light is equal to the frequency of the molecular vibration. When the scattered light is passed through a spectrometer, a spectrum of peaks occurs at characteristic frequency shifts which correspond to the vibrational bonds. This won C.V. Raman the Nobel prize in 1930. The technique is applicable to individual living cells⁷,⁸ by focussing the laser within a microscope, and the Raman spectrum can be considered as a biochemical fingerprint of the cell. Raman spectroscopy has been successfully applied to cancer cells and tissues to distinguish between cancerous and healthy tissue or cells.⁹–¹³

Raman spectroscopy has also been applied to the study of metastasis in several ways. Firstly, cells or tissue from a primary tumour are compared with a metastatic tumour spread to a different organ.¹⁶ Secondly, unrelated cell lines which cause metastasis are compared with those which do not.¹⁷,¹⁸ Thirdly, different patients with either a primary or secondary tumour have been compared.¹⁹Fourthly, primary tumours were compared between patients who went on to develop metastasis with those who did not.²⁰ Finally, Raman spectroscopy has distinguished between benign, primary
2. Materials and methods

2.1 Cell culture

SW480 and SW620 cell lines were acquired from the ATCC and have been cultured for less than 6 months. They were both cultured in Dulbecco’s Modified Eagle Medium (Gibco 41966), supplemented with 10% Foetal Calf Serum (Gibco 16140) without antibiotics. Cells were plated onto glass-bottom dishes (WPI Fluorodish FD35) for stimulated Raman scattering (SRS) microscopy and atomic force microscopy (AFM), and onto 0.15–0.18 mm thick quartz substrates (SPI supplies 1019T-AB) for Raman spectroscopy. For SRS fresh media was added, but for AFM and Raman spectroscopy the media was replaced by Phosphate Buffer Saline which maintained cell viability during measurements.

Oil red O (Sigma O0625) was dissolved in isopropanol, filtered and diluted to 60% working solution with distilled water. Cells were fixed with 10% formaldehyde (Sigma 47608) for 20 minutes at room temperature and washed twice with PBS. Lipid droplets were stained by adding Oil red O working solution for 15 minutes at room temperature, followed by 2 washing steps with distilled water.

2.2 Atomic force microscopy (AFM)

We used an AFM (Bruker Bioscope II) on an inverted microscope, with a 20× phase contrast objective lens to view cells and the AFM cantilever tip. A soft cantilever (Bruker MLCT) was mounted with a 20× phase contrast objective lens to view cells and the AFM cantilever tip. Living adherent cells were located and the tip apex positioned above the centre of the nucleus. A cantilever stiffness, k, this enables measurement of the repulsive force, F, experienced by the cantilever tip by calibrating according to the equation $F = kZ_c$, where $Z_c$ is the upward deflection of the cantilever tip. Living adherent cells were located and the tip apex positioned above the centre of the nucleus. A ‘force–distance’ curve was then measured by indenting until the force reaches a given trigger value to avoid damage. The ‘distance’ is the movement of the fixed end of the cantilever, $Z_t$. The indentation depth of the tip apex into the cell, $\delta = Z_1 - Z_3$, where $Z_1$ was known and $Z_2$ was deduced using $F = kZ_2$.

The cell Elasticity ($E$), or Young’s Modulus, is extracted using the Hertz model for a spherical-ended indenter, $F = 4/(3[1 - \nu^2])ER\delta^{3/2}$ where $R$ is the AFM tip radius (taken as 20 nm for these probes), and $\nu$ is the Poisson’s ratio – a measure of compressibility – taken as 0.37. The Poisson’s ratio of living cells was measured as 0.36–0.38, and 0.37. AFM data was fitted to this Hertz model using the open source software, AtomicJ. Both indentation and retraction Force–distance curves were acquired sequentially for the same cell.

2.3 Raman spectroscopy

We used a Renishaw InVia Raman microspectroscopy system, which focuses laser light of wavelength 785 nm into a spot of...
We employed a home-built multi-photon microscopy system adapted for SRS microscopy. This adaptation consists of the addition of an amplitude modulator (New Focus 4103-01) at 5 MHz to the Stokes beam, and a lock-in amplifier (Stanford Research SR844) to the detection of transmitted pump beam.

We used 30 mW of each beam, measured at the sample, delivered by a water-immersion multi-photon objective lens of numerical aperture 1.05 (Olympus XLPlan N). The resolution of the system was previously measured as 250 nm FWHM laterally and 1.1 µm FWHM axially. Stacks of 10 images were acquired at 1 µm separation, and converted into a 2-dimensional image by $Z$-projection at maximum intensity to observe all the lipid droplets throughout the cell. Images had a 61 µs pixel dwell time, consisted of $512 \times 512$ pixels, and were averaged 10 times to enhance quality.

## 3. Results and discussion

### 3.1 Atomic force microscopy (AFM)

Elasticity values were deduced for a total number of 56 primary cells (SW480) and 45 secondary cells (SW620), and are plotted in a histogram in Fig. 1. We found the mean values and standard deviations of elasticity to be $1.39 \pm 0.71$ kPa (SW480 retrace) and $0.834 \pm 1.43$ kPa (SW620 retrace), using the retraction part of the force–distance curve. Median values of $1.26$ kPa (SW480 retrace) and $0.406$ kPa (SW620 retrace) remove the outliers to offer more representative values for the cell types, and show that the secondary cells are three times softer than primary cells. We performed Welch’s $t$-test on these elasticity values to determine the likelihood of the null hypothesis, that both cell types have the same elasticity. This was rejected and was found to be statistically significant [$p = 0.0197$]. SW480 cells were previously measured to have an elasticity of $0.47$ kPa, using a tipless cantilever and a model of the cell as an ellipsoid to extract the elasticity, but no comparison was made with SW620 cells. Uncertainty in the AFM cantilever’s tip radius $R_s$, nominally taken as 20 nm from manufacturer’s data for the Hertz model, will result in values which are not absolutely accurate, but relatively accurate when using the same probe – as was the case in this study. For absolute values of elasticity, a calibration sample is required. 46 of 56 measurements on primary cells have an elasticity greater than a threshold of 0.70 kPa, and 35 of 45 secondary cells have an elasticity below this threshold. This corresponds to an accuracy of 80% for discriminating cell type by elasticity.

These results compare favourably with previous AFM measurements which show secondary tumours have lower elasticities (i.e. are softer) than primary tumours from different
mice, and that cells with a high metastatic potential have lower average elasticities than cells with low metastatic potential. However, we are the first to compare primary and secondary cells from the same organ, or from the same patient. We are also the first to give a quantitative measure of the accuracy of discriminating cell types by this AFM mechanical measurement.

### 3.2 Raman spectroscopy

Raman spectra were acquired on 16 individual cells of each type, SW480 and SW620. The average spectrum for each type, plotted in Fig. 2, shows significant changes. The difference spectrum (SW620 minus SW480) highlights the biochemical differences between the two cell types. These biochemical differences are presented in Table 1, according to assignment of the peaks in the Raman spectrum to classes of biomolecule. Broadly speaking, these suggest that the SW620 cells contain a higher proportion of porphyrins and beta-sheet proteins than SW480 cells, and SW480s contain a higher proportion of lipids and alpha-helix proteins than SW620s. The increased concentration of porphyrins in tumours was first revealed in 1923 and these molecules are also autofluorescent in the red part of the spectrum so can be detected by imaging. We also compared unnormalised spectra, which showed similar spectral differences and essentially the same biochemical differences as those presented in Fig. 2 and Table 1. Ratios of normalised or unnormalised spectra also demonstrated the same positive and negative peaks as those in Fig. 2 and Table 1.

Principal Component Analysis (PCA) was used to see how well individual cells could be distinguished by Raman spectroscopy. PCA reduces the differences in spectra between all 32 cells in the study, to just two variables. In Fig. 3, each spectrum from an individual cell is plotted as a separate data point. It is clear that all the cells could be identified clearly as SW480 or SW620 just by noting their position on the plot. When an unknown cell is probed by Raman spectroscopy, it can be plotted onto the same figure and easily characterized as SW480 or SW620, with an accuracy approaching 100%. When we randomly selected 11 of each cell type to create a training set, the remaining 10 cells were used as a test set and were all correctly identified when superimposed onto the training set PCA plot, with the resulting plot appearing very similar to Fig. 3.

The only other Raman study to compare metastatic and non-metastatic cell lines was performed over the range 2820–3030 cm$^{-1}$, which is populated only by C–H bonds, and only produced partial discrimination using PCA.

#### Table 1 Assignment of vibrational modes in the difference Raman spectrum in Fig. 2

<table>
<thead>
<tr>
<th>Raman frequency (wavenumbers, cm$^{-1}$)</th>
<th>Biomolecules more abundant in SW480</th>
</tr>
</thead>
<tbody>
<tr>
<td>621</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>642</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>854</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>1002</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>1031</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>1445</td>
<td>Lipids</td>
</tr>
<tr>
<td>1660</td>
<td>$\alpha$-helix proteins</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Raman frequency (wavenumbers, cm$^{-1}$)</th>
<th>Biomolecules more abundant in SW620</th>
</tr>
</thead>
<tbody>
<tr>
<td>752</td>
<td>Porphyrins</td>
</tr>
<tr>
<td>1520</td>
<td>Porphyrins</td>
</tr>
<tr>
<td>1551</td>
<td>Porphyrins</td>
</tr>
<tr>
<td>1622</td>
<td>Porphyrins</td>
</tr>
<tr>
<td>1676</td>
<td>$\beta$-sheet proteins</td>
</tr>
</tbody>
</table>
3.3 Stimulated Raman scattering (SRS) microscopy

We performed SRS microscopy to image the concentration of CH$_2$ groups, and imaged three types of sample in Fig. 4. As well as the purely SW480 and SW620 samples, we also imaged a mixture of these cell types plated the previous day. Images of CH$_3$ bonds are dominated by lipids due to the high proportion of these groups in lipids, compared to other biomolecules. The SW480s are usually larger than the SW620s, and can be seen in the mixed sample both due to their size and the far smaller number of lipid droplets.

Combining the results of Raman spectroscopy and SRS microscopy, we deduced that although the SW480 cells have a higher proportion of lipids than SW620 cells, there are more lipid droplets – which are predominantly triglycerides$^{51}$ – in the SW620 cells. This is supported by a previous observation that there is a dramatic increase in the amount of triglycerides in SW620 compared to SW480 cells.$^{52}$ The only Raman study of metastatic and non-metastatic cell lines$^{17}$ was inconclusive about the amount and type of lipids in metastatic cells, but suggested a higher likelihood of lipid droplets in stained metastatic cells. The relative abundances of lipid droplets in both cell types is confirmed by Oil red O staining of fixed cells in Fig. 5.

4. Conclusion

Three techniques were presented for analysis of living human primary and secondary tumour cells. All techniques are label-free, non-destructive and applied to individual cells. Crucially, SW480 and SW620 cell lines are from the same patient and the same organ. This removed all unwanted variations and revealing only the primary or secondary nature of the cells. All other comparisons either involve different organs, different patients, or unrelated cell lines.

A good deal of physical and biochemical information was revealed about the differences between primary & secondary cells. This assumes that the differences observed for one patient, are also observed for other patients, and that differences between cells in vitro are observed in tissue samples. Further work is required to prove this. Secondary (metastatic) cells are softer, are smaller, have far more porphyrins and lipid droplets, but have a lower proportion of lipids and structural proteins than primary tumour cells.

We also distinguished between cell types using these three analytical methods, enabling all three techniques as methods for diagnosis in humans of primary or secondary tumour. All techniques can be applied to excised tissue from surgery, to

Fig. 4 Stimulated Raman Scattering (SRS) images, acquired at a frequency of 2930 cm$^{-1}$ (specific to the CH$_2$ anti-symmetric stretch, dominated by lipids). All three images are maximum intensity Z-projections, from volume stacks of size 200 × 200 × 10 µm into two-dimensional images of size 200 × 200 µm.

Fig. 5 Transmitted light images of primary (SW480, left) and secondary (SW620, right) tumour cells stained with the lipophilic dye, Oil red O. Red staining confirms the presence of high levels of lipid droplets in the secondary tumour cells. Image sizes are 120 × 90 µm.
biopsy or fine needle aspirates, or modified to application in vivo during surgery – without the requirement to fix, slice, treat or stain the sample. SRS microscopy and Raman spectroscopy can also be used in vivo beneath the surface of tissue.

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References


