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ARTICLE TYPE

Label-free identification and characterization of murine hair follicle stem cells located in thin tissue sections with Raman micro-spectroscopy

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Stem cells offer tremendous opportunities for regenerative medicine. Over the past decade considerable research has taken place to identify and characterize the differentiation states of stem cells in culture. Raman micro-spectroscopy has emerged as an ideal technology since it is fast, nondestructive, and does not require potentially toxic dyes. Raman spectroscopy systems can also be incorporated into confocal microscope imaging systems allowing spectra to be obtained from below the tissue surface. Thus there is significant potential for monitoring stem cells in living tissue. Stem cells that reside in hair follicles are suitable for testing this possibility since they are close to the skin surface, and typically clustered around the bulge area. One of the first steps needed would be to obtain Raman micro-spectra from stem cells located in thin sections of tissue, and then see whether these spectra are clearly different from those of the surrounding differentiated cells. To facilitate this test, standard 5 µm thick sections of murine skin tissue were stained to identify the location of hair follicle stem cells and their progeny. Raman spectra were then obtained from adjacent cells in a subsequent unstained 10 µm thick section. The spectra revealed significant differences in peak intensities associated with nucleic acids, proteins, lipids and amino acids. Statistical analyses of the Raman micro-spectra identified stem cells with 98% sensitivity and 94% specificity, as compared with a CD34 immunostaining gold standard. Furthermore analyses of the spectral variance indicated differences in cellular dynamics between the two cell groups. This study shows that Raman micro-spectroscopy has a potential role in identifying adult follicle stem cells, laying the groundwork for future applications of hair follicle stem cells and other somatic stem cells *in situ*.

Introduction

The accurate characterization of stem cells is an important first step towards understanding their full potential in regenerative medicine.¹ There are two main aspects to this characterization. First, physical attributes need to be identified to reliably separate stem cells from their progeny, and second, there needs to be careful monitoring of the differentiation processes themselves. Molecular biology techniques for identifying and characterizing stem cells and their progeny in a cell culture include RT-PCR, and microarray methods such as RNA hybridization and immunocytochemistry.² Although these methods can achieve good results, they are very time consuming and destructive.

More recently researchers have turned to Raman micro-spectroscopy for carrying out these tasks because it delivers information-rich spectra with a high spatial resolution that can be analyzed in real time without damaging cells or altering their function.³⁻¹⁰ The intensity of individual Raman peaks can be correlated to some extent with the relative abundance of different biomolecules, and these in turn relate to differences between cells, and temporal changes that occur in a single cell. Although broad peaks within the complex Raman spectra of cells are not

generally specific to individual biomolecules, changes in a spectrum as a whole can be correlated with biomolecular changes using multivariate statistics. Once a particular spectral pattern, or range of patterns, is identified with a particular cell type, an algorithm can be trained to rapidly identify these patterns within a collection of cells where the differentiation states are unknown.

Most of the Raman spectroscopy work conducted to date on stem cells has been on cultured human embryonic stem cells (hESCs). Very little work has been done on non-destructive methods for identifying and monitoring the regenerative behaviour of endogenous somatic stem cells present in living tissue. Localizing and observing stem cells in their normal environment would offer large improvements in knowledge of the underlying mechanisms, laying the groundwork to precisely analyze and manipulate the controlling molecular pathways.¹¹

Although somatic stem cells exist in a wide range of tissue,^{7,9,12} localizing and monitoring them with Raman spectroscopy is extremely challenging. One possible exception is the somatic stem cells that are present in the hair follicle bulge of most mammals.¹³ Generally cell lineages within skin are quite complex, with stem cell populations in a number of spatial locations,¹⁴ however the high density of stem cells in the bulge

1 makes localization much easier. Furthermore these cells are at
2 depth from the skin surface that can be reached by a combined
3 confocal microscopy imaging and Raman system thereby
4 allowing spectra to be obtained *in situ* from living cells.^{15,16} Apart
5 from learning more about stem cell behaviour, an *in situ* study of
6 hair follicle stem cells will help develop therapeutic treatments
7 for a variety of hair disorders and diseases such as alopecia as
8 well as help find new ways to promote wound healing.¹⁷⁻²⁰

9 In a first step to realizing these potentials, the methodology
10 and the results obtained from using Raman micro-spectroscopy to
11 characterize hair follicle stem cells in 10 μm thick murine tissue
12 sections are presented. This work was carried out in accordance
13 with Directive 86/609/EEC for animal experiments. For this
14 initial study, fixed samples were used rather than fresh ones
15 because this enabled their manipulation over an extended period
16 of time to optimize the Raman signal without sample degradation.
17 It was found that putative follicular stem cells could be reliably
18 identified with Raman micro-spectroscopy with high sensitivity
19 and specificity. The differences in the spectra from the different
20 cell groups were correlated with changes in the relative content of
21 nucleic acids, proteins, and lipids. Furthermore, the within, and
22 between, group spectral variances demonstrated varying cellular
23 dynamics for differentiated cells.

25 Materials and Methods

27 Preparing Mouse Hair Tissue

29 Skin and hair tissue were obtained from the back of a C3H/HeJ
30 female mouse, fixed with formalin, embedded in paraffin, and cut
31 into serial sequences of 3 sections. The sections were dewaxed by
32 washing in xylene for 3 minutes, then in a graduated series of
33 alcohols from 70 to 100% for 3 minutes and finally in distilled
34 water.²¹ The first section in the sequence was cut 10 μm thick for
35 Raman measurements to ensure it contained a large percentage of
36 whole, or nearly whole, cells. This section was mounted on a
37 transparent barium fluoride (BaF_2) substrate to avoid the large
38 fluorescence and Raman backgrounds emitted from standard
39 glass slides during laser excitation,²² and was not stained. The
40 second and third sections in the sequence were mounted on
41 standard glass slides, and stained for orientation purposes. These
42 sections were cut 5 μm to increase the chance that parts of the
43 same hair follicle would be seen in all three slides of the
44 sequence. The section adjacent to the Raman section was stained
45 with CD34 (BD Pharmingen, San Diego, CA, USA) as this is
46 recognized as the most accurate marker for stem cells.²³⁻²⁵ The
47 other orientation slide was stained with hematoxylin and eosin
48 (H&E) to highlight nucleic and protein features. Altogether a
49 total of eight sets of three sections were prepared this way.

51 Raman Instrumentation

52 White light illuminated visible images and Raman micro-spectra
53 were obtained by coupling an argon ion laser (Spectra Physics,
54 Santa Clara, USA), with a LabRam system (Jobin Yvon Inc.,
55 Edison, NJ, USA), see figure 1. The LabRam system consisted
56 of a sample imaging video camera and a high-throughput grating
57 spectrograph integrated with an Olympus BX40 microscope
58 (Olympus Optical Corp., Melville, NY, USA). The output from
59 the laser was tuned to 515 nm and focused onto the sample with

either a 50x Olympus (N.A. 0.9) objective or a 100x (N.A. 0.8)
long-working-distance objective. The diameters of the excitation
spots were estimated to be 0.5 and 1.0 μm at 50% of the peak
intensity for the 100x and 50x objectives respectively. The
maximum excitation power at the sample was about 30 mW. The
scattered light was collected by the same objective and focused
onto the 150 μm entrance slit of the spectrograph that contained
diffraction gratings of 600 g/mm and 1800 g/mm. A CCD array,
cooled to approximately -60°C for lower noise, measured the
dispersed light. Sample excitation and data acquisition were
computer controlled with LabRam's proprietary software.^{26,27}

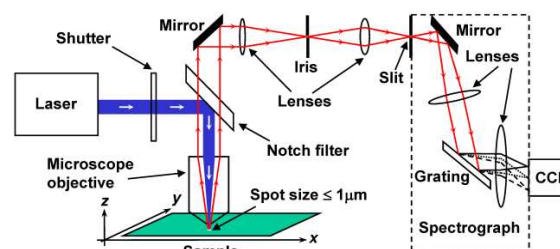


Fig.1 Schematic of the LabRam system used for recording Raman micro-spectra. Not shown in this figure are the additional components required to record white-light images of the sample for selecting different sites, and to focus the laser beam onto them.

The frequency dispersion of the Raman system was calibrated by measuring the spectrum of a cyclohexane standard. The spectral resolution of the system was about 2 cm^{-1} for the 1800 g/mm grating and 8 cm^{-1} for the 600 g/mm grating. A motorized X-Y stage moved the samples with calibrated steps of 0.5 or 1.0 μm so that an automated sequence of micro-spectra could be taken over an entire cell. The depth-of-field was limited to approximately 10 μm for the 50x objective and 5 μm for the 100x objective by setting the aperture of an iris placed in the light path to a diameter of 200 μm .

Obtaining Raman Micro-Spectra

The tissue sections were examined to find parts of the same hair follicle present in all 3 sections of a serial sequence. First the H&E stained slide was viewed with a conventional bench top microscope to clearly identify a follicle (figure 2A). The same follicle was then located in the CD34 stained section and checked for positive identification of putative stem cells (figure 2B). The corresponding follicle was thus identified in the unstained section from LabRam video images of the section under white light illumination (figure 2C). Based on location, and morphological features, a putative stem cell, or a putative differentiated cell, within the selected hair follicle was chosen. Test Raman micro-spectra were obtained over different spectral ranges using different combinations of focussing objective, grating and integration time. Spectra obtained from 300 to 1850 cm^{-1} using the 100x objective, the 1800 g/mm grating and a 5 second integration were selected for the study as these were of good quality, rich in peaks, and without any obvious signs of photo-damage to the cells. A total of 30-50 spectra were obtained per cell (depending on cell size) at locations distributed uniformly over the cell. The acquisition was automated by programming the motorized X-Y stage to move to each location within the cell.

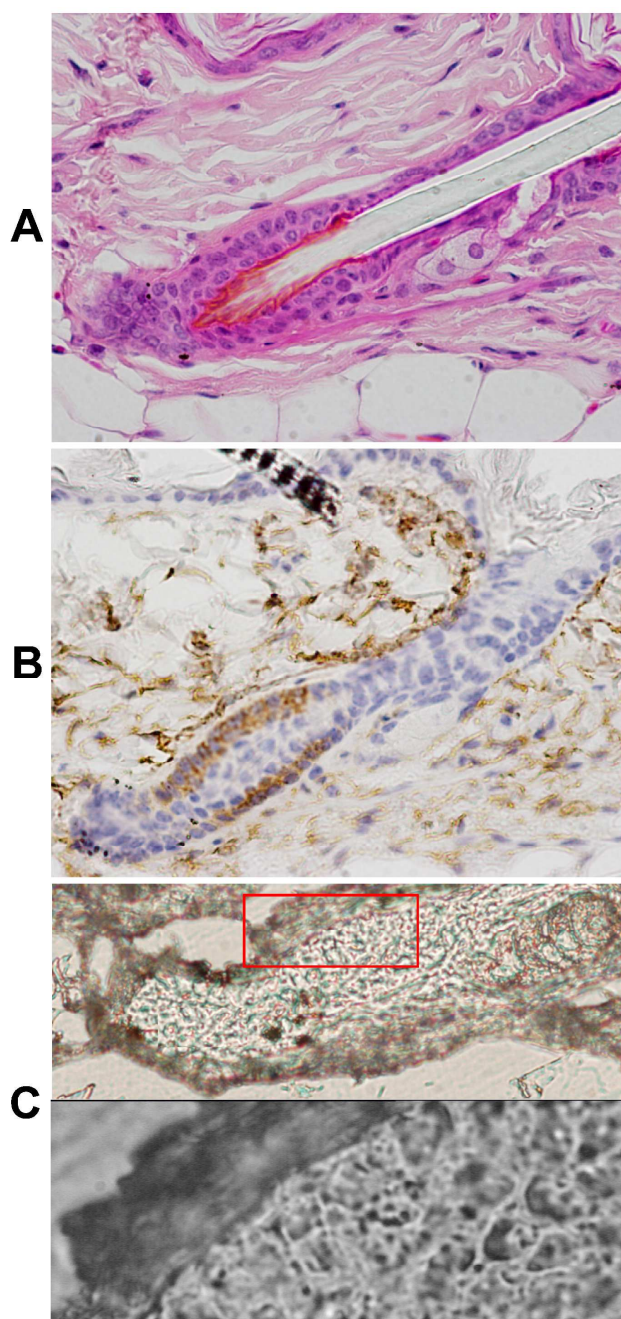


Fig.2 Images of the same hair follicle: **A)** H&E stained section (30x); **B)** CD34 stained section (30x) showing brown colored cells in the bulge; and **C)** two views of the unstained section, upper (30x), and the lower is a high power (100x) view of the bulge region (red box) showing clear cellular features.

Statistical Analysis

Raman micro-spectra from the same cell were baseline corrected, averaged, smoothed with a 13 point Savitzky-Golay algorithm²⁸ and normalized by the area under the whole curve from 300 to 1850 cm^{-1} . Multivariate statistical analyses were then used to compare spectra from different cells. All the averaged cell micro-spectra were first reduced into principal components²⁹ with the STATISTICA software package (StatSoft, Inc. Tulsa, OK, USA). Student t-tests and Mann Whitney U tests were used on the PCs

to determine those most significant at distinguishing between stem and differentiated cells. Only PCs that accounted for 0.1 % or more of the variance were used. Linear discriminant analyses (LDA) were used on the PCs with a leave-one-out cross validation procedure to determine the utility of the Raman micro-spectra for identifying different cell types.^{30,31} Although in general the more PCs used in the LDA model the better the results, the maximum number used was restricted to slightly more than one third (16 in total) of the smallest number of cases to avoid over fitting the data. Standard F tests were used to analyze the within and between group variance.

Raman Micro-Spectra of Reference Samples

As a comparison, Raman micro-spectra were obtained from two commercially available reference samples: DNA and RNA. The DNA was purified from a human placenta, and the RNA purified from baker's yeast (Sigma-Aldrich Canada Limited Oakville, Ontario, Canada, their reference numbers D4642, and R6750 respectively). These reference materials were directly measured neat as supplied. For each reference material, a small quantity was placed on a BaF₂ slide and a Raman micro-spectrum obtained and processed in the same way as the cellular micro-spectra described above.

Results

Spectra

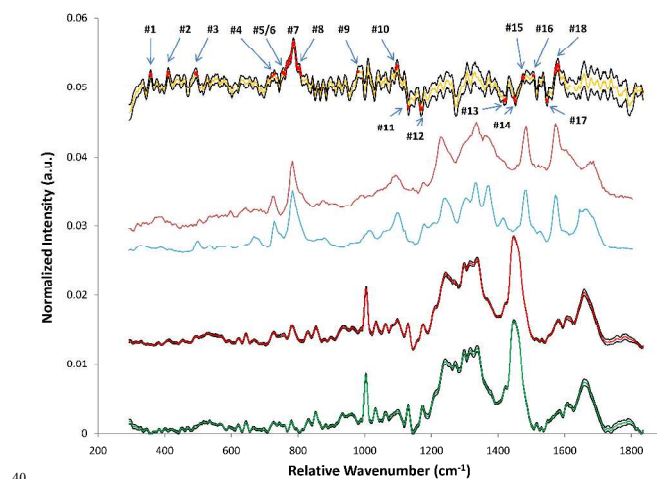


Fig.3 Average Raman spectra of stem cells and differentiated cells. Spectra have been arbitrarily offset on the intensity scale for clarity. Differentiated cells (bottom, green), and stem cells (second from bottom, red) and their standard error envelopes (black). Raman spectra of the nucleic acid standards are: third from bottom, cyan (DNA), and fourth from bottom, brown (RNA). The top spectrum (yellow) is the difference between the average stem cell and differentiated cell spectra (scaled by 4x for clarity) and bounded by its standard error envelopes (black). The dashed line represents zero difference. The red dots in the difference spectrum are regions of significant difference ($\geq 95\%$ confidence).

Figure 3 shows the average Raman micro-spectra obtained from 54 stem cells and 46 differentiated cells. Significant differences between the average intensities of the two cell groups occur at 18 spectral positions corresponding to Raman peaks or shoulders (labelled #1 to #18 in figure 3). A number of these positions coincide with peaks in the micro-spectra obtained from the nucleic acid standards, and many are in agreement with PO₂

stretching modes found by others^{32,33} for nucleic acids in stem cells (table 1). However, not all peaks from the standards match significant differences in the cell micro-spectra. For example there is clear correspondence at 492 (#3), 722/9 (#4), 786 (#7), 1097 (#10) and 1578 cm⁻¹ (#18) but not for strong nucleic acid peaks at 1230, 1330 and 1368 cm⁻¹. Differences at other positions in the cell micro-spectra can be assigned to changes in lipids, amino acids and proteins typically found in tissue.³⁴

Table 1 Assignment of Raman peaks or shoulders in the spectra that are significantly ($\geq 95\%$ confidence) between stem cells and differentiated cells.

Peak #	Position	t-test	F-test	Assignment
1	358 cm ⁻¹	2.1*10 ⁻²	1.5*10 ⁻²	Uncertain
2	409 cm ⁻¹	1.6*10 ⁻²	1.8*10 ⁻²	Phosphatidylinositol
3	492 cm ⁻¹	2.1*10 ⁻²	0.5*10 ⁻²	RNA/DNA, Glycogen
4a ^a	722 cm ⁻¹	3.6*10 ⁻²	2.0*10 ⁻²	Adenine
4b ^a	729 cm ⁻¹			
5	755 cm ⁻¹	2.0*10 ⁻²	5.1*10 ⁻⁴	Phosphatidylinositol
6	772 cm ⁻¹	1.1*10 ⁻⁶	2.8*10 ⁻³	Pyrimidine ring
7	786 cm ⁻¹	2.7*10 ⁻¹⁰	9.3*10 ⁻²	Cytosine/Uracil rings
8	803 cm ⁻¹	5.2*10 ⁻⁴	3.0*10 ⁻¹	Uracil ring
9	981 cm ⁻¹	4.3*10 ⁻²	1.4*10 ⁻¹	Ribose RNA
10	1097 cm ⁻¹	1.8*10 ⁻²	6.0*10 ⁻²	RNA/DNA
11	1131 cm ⁻¹	4.6*10 ⁻²	1.9*10 ⁻⁵	Palmitic acid
12	1169 cm ⁻¹	1.1*10 ⁻²	1.6*10 ⁻⁶	Cytosine, Tyrosine
13	1421 cm ⁻¹	2.8*10 ⁻²	4.7*10 ⁻²	Adenine, Guanine
14	1451 cm ⁻¹	1.1*10 ⁻²	8.9*10 ⁻²	CH ₂ and CH ₃
15	1475 cm ⁻¹	1.2*10 ⁻²	9.3*10 ⁻²	Uncertain
16	1506 cm ⁻¹	2.9*10 ⁻²	9.1*10 ⁻¹	Uncertain
17	1546 cm ⁻¹	3.5*10 ⁻²	9.0*10 ⁻³	Tryptophan
18	1579 cm ⁻¹	0.6*10 ⁻²	4.9*10 ⁻¹	Adenine, Guanine C-N

^a Unable to resolve peaks 4a and 4b.

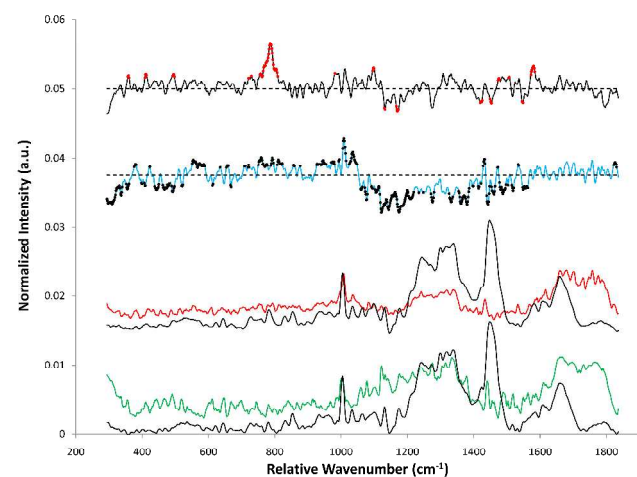


Fig.4 Standard deviations in the Raman micro-spectra for differentiated cells (bottom, green) and stem cells (second from bottom, red) superimposed on the mean spectra of the group (black curves). Standard deviations have been scaled to match the intensity of the phenylalanine peak at 1003 cm⁻¹. Spectra have been arbitrarily offset on the intensity scale for clarity. The top two spectra show the difference in standard deviations (cyan), and the difference in average intensities (black) for comparison (scaled by 4x for clarity) where the dashed lines are at zero intensity. The highlighted regions are regions of significant difference ($\geq 95\%$ confidence) using F and t tests on the between group variances and between group intensities respectively.

Analyses of the within group and between group variance is shown in figure 4 where it is apparent that the standard deviation of each group did not follow the same intensity profile as the

average of the micro-spectra from which they were derived. This indicates that some constituents within each group of cells have a greater variance than others.⁸ Figure 4 also shows the results of an F test performed on the variances from the two different cell groups. There were significant differences at many wavenumbers. Peaks at 359, 410, 495 and 1097 cm⁻¹ have significantly increased intensities in stem cells, yet the standard deviations were significantly greater in differentiated cells. Furthermore many points in the average micro-spectra have insignificant intensity changes between groups, but significant differences in variance. Specifically, between 1100 and 1400 cm⁻¹ the differentiated cell micro-spectra have a significantly larger variance. Interestingly, although the intensity of the Raman peaks at 722/9, 786 1505, and 1578 cm⁻¹ are significantly greater for stem cells than differentiated cells, there were no significant differences in variance at these points.

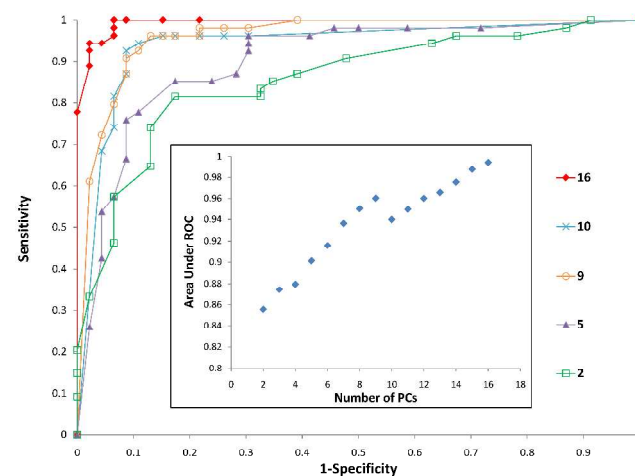


Fig.5 Receiver operator characteristic (ROC) curves obtained from LDA analyses with leave-one-out cross validation of the spectra with different number of principal components. **Inset:** area under the ROC curves calculated using PCs from 2 through 16.

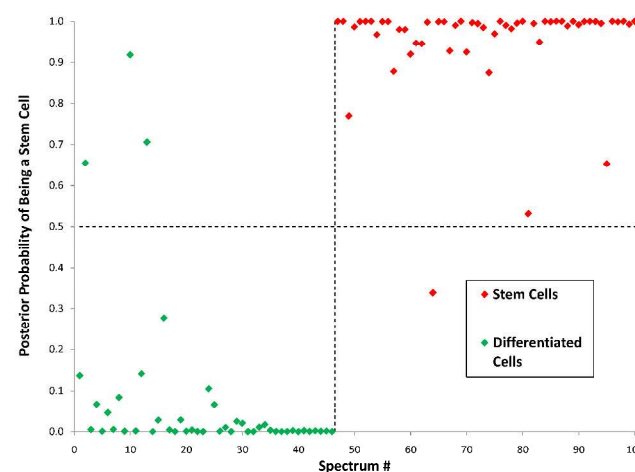


Fig.6 Posterior probability plot of a cell being a stem cell derived from LDA with leave-one-out cross validation and a 16 PC model.

Stem Cell Identification

Over ninety percent of the total variance could be accounted for by the first 16 PCs, and these factors account for 0.1% or more of the variance individually. The LDA with leave-one-out cross validation was performed 15 times, each time increasing the

number of PCs used in the model by one from 2 through to a maximum of 16. Figure 5 shows the receiver operating characteristic (ROC) curves generated for some of these LDA runs, and the inset to figure 5 shows how the area under the curve changed for all 15 runs. As the number of PCs used increased from 2 to 9 the area under the curve also increased. At 10 PCs there was a drop in the area under the curve, and then a steady increase until the maximum number of PCs was reached. The leave-one-out posterior probability that each measured cell is a stem cell is shown in figure 6 where 16 PCs were used in the LDA model. From this a sensitivity of 98% (95% CI, 90-100%) and a specificity of 94% (95% CI, 82-99%) for identifying stem cells were obtained. Decreasing the number of PCs used in the model to 9 decreased the sensitivity and specificity to 96% (95% CI, 87-100%) and 87% (95% CI, 74-95%) respectively.

Discussion

Figure 3 shows that clear, information-rich, Raman micro-spectra can be obtained in 2-4 minutes from the cells surrounding a hair follicle within 10 μm thick tissue sections. The wavenumber positions of the peaks agree well with what others have found for cellular material with overlapping Raman signatures from nucleic acids, amino acids, lipids, and proteins. There were statistically significant differences in the average Raman micro-spectra from the two groups of cells measured: those highly likely to be hair follicle stem cells and those that are not. Although the average Raman spectrum for each cell was normalized by the area under the whole curve to correct for total intensity variations, this process preserves to a large degree the correlation between peak heights and biochemical abundance. This is because increases in individual peaks of a complex spectrum are usually offset by decreases in others, and any net change is small compared to the total area under the curve. Thus differences in Raman peak heights between stem and non-stem cells are indicative of changes in the biomolecules from which they arise. The differences observed seemed to be largely related to higher intensities for peaks associated with nucleic acids for stem cells and lower intensities for peaks associated with lipids, proteins and amino acids. This is consistent with stem cell biology which is known to have a relatively greater nucleic acid concentration, but fewer lipids, amino acids, and proteins than typically found in differentiated cells.^{6,8,32,33}

The standard deviations computed for the stem and differentiated cell groups reveal significant differences in variance. Differentiated cells had greater amounts of variance in their Raman micro-spectra that cannot be accounted for by differences in average intensity. Specifically peaks at 359, 410, 495 and 1097 cm^{-1} were affected as well as multiple points in the range from 1100 to 1400 cm^{-1} . These wavenumber regions are dominated by protein, amino acid and lipid signatures and may reflect different cellular dynamics of differentiated cells as was found by others.⁸ In contrast there were no significant differences in variance for the Raman peaks at 729, 786 1505, and 1578 cm^{-1} between the two cell groups despite a significantly higher intensity for those peaks in stem cells. This indicates that the variances in nucleic acid concentrations are similar for each cell group. It is worth noting that there were no Raman peaks that

were both significantly more intense in differentiated cells than stem cells and with a small variance. This would explain why combinations of simple peak ratios (not shown) had limited discriminatory utility for classifying a cell. This is in contrast to their successful use in discriminating between hESCs and their progeny.⁸ Presumably our micro-spectra have greater within group variances which precludes the use of simple peak ratios as an accurate cell discriminator.

The alternative cell discrimination method used here of reducing Raman micro-spectra into principal components followed by a cross validated LDA showed that putative hair follicle stem cells could be identified with 98% sensitivity and 94% specificity. Four cells were misclassified when using 16 factors, which may be partly due to the labelling of cells based solely on the staining pattern of a sequential 5 μm thick section. CD34 staining of the same section after Raman measurements would reduce this uncertainty, although not totally eliminate it since the success of CD34 for identifying stem cells is unlikely to be 100%.¹² However, it was not technically possible in this present work to quantify any improvement with post measurement staining because the tissue sections detached very easily from the BaF₂ substrates making it difficult to reprocess these samples. The fact that differentiated cells have a larger variability will also increase the probability of misclassification. In addition there is the possibility that some cells were sectioned into two parts each with a different normalized Raman micro-spectrum than the whole. Other likely sources of uncorrelated variance are varying contributions to the micro-spectra from the extracellular matrix either adjacent to the cells or above or below them within the 10 μm thick section. It may be possible in future work to obtain some reduction in Raman from non cellular sources by sectioning the tissue in a 5, 10, 5 μm sequence as this would ensure the unstained section was completely within the hair follicle.

Conclusions

It has been shown that murine hair follicle stem cells can be identified in 10 μm thick tissue sections using Raman spectroscopy. It was also shown that information on cell dynamics can be derived by examination of the spectral variances. There were several areas where the experimental methodology could potentially be improved. First, the results were based on a relatively small number of samples containing one particular type of stem cell. Although the differences in the spectra between the two cell groups were clear and were consistent with previous findings, a greater number of samples would improve the accuracy. Whether other types of stem cells can be differentiated from their progeny within tissue sections remains to be tested. Second, although there were no obvious peaks due to fixing or paraffin wax, the results may not be identical to those obtained with fresh samples. Third, average spectra were obtained for a cell in around 3 minutes at relatively high excitation powers. Although there were no obvious signs of photo damage during spectral acquisition, it is acknowledged that longer wavelengths would allow higher excitation powers to be used³³ or longer integration times, to improve the quality of the spectra. Furthermore longer wavelengths will penetrate deeper into tissue thus facilitating the acquisition of spectra below the

tissue surface which would be a prerequisite for identifying hair follicle stem cells *in vivo*. There may also be some advantage in studying the CH stretching region (2800 to 3050 cm^{-1}) of stem cells. Although this region is only comprised of a few Raman peaks, they are usually more intense and have been used successfully to characterize tissue and cells.³⁵ Fourth, post Raman measurement staining was not used to identify stem cells which may have contributed to some of the cell misclassifications, and increased the within cell group variance. Despite these limitations, the potential for identifying hair follicle stem cells *in situ* is evident, and once refined, this approach could have potential benefits for the development of new therapeutic approaches.

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