Assessing Dysplasia of Bronchiole Biopsies with FPA-FTIR imaging and ATR-FTIR spectroscopy

Liberty Foreman

Report Summary

This report has three sections which are outlined below:

1. Signal:Noise Ratio Requirements for Discrimination of NDBE and HGD IR Signatures

The ATR-FTIR spectral region of 1090-1036 cm^{-1} has previously shown differences between Non-dysplastic Barrett's oesophagus (NDBE) and High Grade Dysplasia (HGD) biopsies. The signal:noise ratio was calculated for ATR-FTIR in this region and subsequently used to estimate the current signal: noise ratio for FPA-FTIR microscopy.

From the calculations we can conclude that the signal: noise in the 1090-1036 cm^{-1} region is 1.0 ($\pm$0.5 s.d.) when using a 96x96 FPA detector with 128 scans at 4 cm^{-1} resolution, with a CaF_2 pseudo hemisphere and 64x64 co-added and averaged pixels. This resolution is currently at the limit of detection for dysplasia-related spectral changes. However, it could be possible to detect tissue type changes.

2. FPA-FTIR microscopy of a Bronchiole Biopsy

Analyses of the stitched FPA-FTIR image of the normal to cancer graduated bronchiole biopsy show that different lung tissue types can be distinguished when analysing 64x64 co-added and averaged pixels. Distinctions can be made using the peak height of the second derivative trough at 1556 cm^{-1}, but more effectively the two cell types can be separated into two groups by hierarchical clustering of the 1575-1450 cm^{-1} region. However disease state cannot be distinguished with only 64x64 binned pixels. If larger areas of each disease state are manually selected and their averages compared, differences between normal, mild dysplasia, severe dysplasia and cancer can be seen in regions 1300-1250 cm^{-1} and 1150-1070 cm^{-1}. Where 1070 cm^{-1} is the spectral cutoff due to noise exceeding the signal with the parameters used in this particular experiment.

3. ATR-FTIR of Bronchiole Biopsies

Eighty fresh lung biopsies were recently collected and measured using ATR-FTIR spectroscopy. After separating the four histologically classified groups (normal, LGD, HGD and cancer) into their sidedness using information from FPA-FTIR microscopy data, disease stage differences can be noted in both the surface of the biopsy, the epithelium, and the underlying side of the biopsy, the lamina propria. In the analyses of the epithelium catagory the most prominent differences occurred in the lipid region at 1738 cm^{-1}, and also between regions 1300-1250 cm^{-1} and 1100-950 cm^{-1}. In the analyses of the lamina propria catagory, cancerous tissue has a larger lipid band at 1738 cm^{-1} than the other classes of lung biopsies, the 1190-1140 cm^{-1} region also shows a possible transition from healthy to cancerous biopsies.

These initial results are encouraging with only 80 biopsies. Additional data should further resolve these spectral differences.

1 Signal:Noise Ratio Requirements for Discrimination of NDBE and HGD IR Signatures

1.1 Calculation of single element ATR-FTIR signal:noise ratio

In order to detect the subtle differences between NDBE and HGD IR signatures, the noise has to be significantly lower than the discriminatory signal(s).

The single element ATR-FTIR difference spectra of the of 36 averaged NDBE surface biopsies and 31 averaged HGD surface biopsies (approximately 5 mm^3 in size), together with their original spectrum, is shown in Figure 1. It is most likely that the largest difference (1635 cm^{-1} shown in Figure 1b.) is due to differences in water contributions, and not related to differences in the disease state of the tissue. Therefore, the next most significant difference between the two tissue types is at 1090-1036 cm^{-1}. This typically has an absorbance difference of 0.0015 (Figure 1 b) in relation to the amide II signal intensity of 0.02 in their normalised absolute spectra (Figure 1 a). Therefore, the difference signal: amide II ratio is 0.075. More generally, un-normalised biopsy amide II signal intensities average around 0.07 (Figure 2 a), so that the expected difference between NDBE and HGD at 1090-1036 cm^{-1} would be 0.005. The underlying thermal noise in this region, based on 500 co-added interferograms of both the sample and the background at 4 cm^{-1} resolution using a SensIR 3-reflection silicon prism is approx. 3.6 x 10^{-5} when calculated from a moving average using a 54 cm^{-1} window (Figure 2 b). A window of this size was chosen as this is the width from 1090-1036 cm^{-1}. Therefore, the signal:noise ratio at 1036 cm^{-1} was approx. 150.

This does not take into account other factors that might distort the baseline and decrease this ratio. However, it should also be noted that the single element system is averaging the spectra of many individual cells, and there will inevitably be mixtures of cell types in such samples. Hence, the distinguishing factor may appear as a greater percentage of the amide II band in single cell IR spectra.

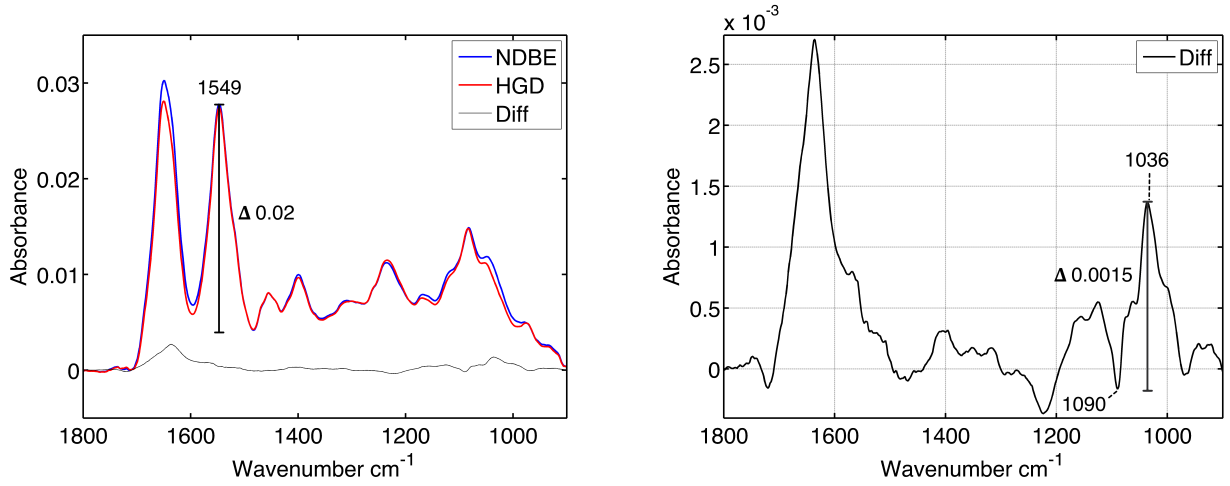

Figure 1: *a) Absolute ATR-FTIR spectra of 36 averaged surface NDBE biopsies, 31 averaged surface HGD biopsies, both normalised to the amide II area, with water and water vapour subtracted. In black is their difference spectrum (NDBE-HGD). b) The NDBE-HGD difference spectrum.*

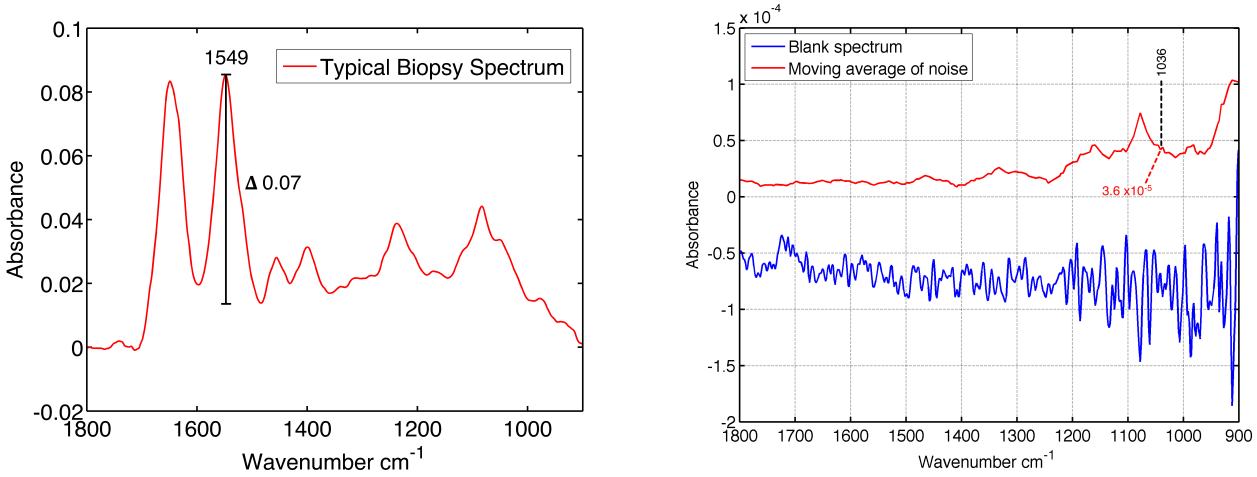

Figure 2: *a) A typical biopsy spectrum recorded on an ATR-FTIR spectrometer, with water and water vapour subtracted (500 co added scans) b) In blue, an absolute spectrum of a blank sample from 1800-900 cm^{-1} (500 co-added scans) and in red is the moving average of the peak heights of the noise using a 54 cm^{-1} window.*

1.2 Calculation of FPA-FTIR Microscopy of Dysplastic Lung Biopsy signal:noise ratios

To determine the signal: noise ratio requirement of FPA-FTIR microscopy to discriminate dysplastic from normal regions, a deparaffinised, 8 µm dysplastic lung biopsy section was used. A 96x96 element Focal Plane Array (FPA) detector was used for the measurements, this gave a projected pixel size of 2.7 µm^2. Spectra of each element were calculated from 128 co-added interferograms at a 4 cm^{-1} resolution. The transmission image was obtained using a lens on top of the CaF_2 window to create a pseudo hemisphere in order to focus the IR light, and reduce light scattering.

The amide II signals varied between 0.05 and 0.3. Using the mean amide II signal of 0.1, the expected difference signal between dysplastic and normal tissue at 1090-1036 cm^{-1} is therefore estimated at 0.0075 absorbance units when based on the calculations in the above section 1.1 - Calculation of single element ATR-FTIR signal:noise ratio. The underlying thermal noise across the FPA detector varied significantly, this is most likely due to varying intensity of the power spectrum. However this would need to be confirmed by analysis of the power spectrum. The noise level was improved by the co-addition and averaging blocks of 64 adjacent pixels, in 8x8 matrices. Table 1 shows the standard deviation, mean and range of the amide II heights and the signal:noise ratios of thermal noise from the 1090-1036 cm^{-1} region of blank regions of the image that have also been co-adding and averaged spectra from blocks of 64 pixels. Figure 3b shows examples of baseline spectra from such binned blocks of pixels that were selected from regions of the image where no sample was present. The signal:noise ratio of 1.0 ($\pm$0.5 s.d.) determined for binned 8x8 pixel blocks, is at the limit for detection of the dysplasia-related discriminatory band difference at 1090-1036 cm^{-1}. It should be noted that this signal: noise ratio calculation is an estimate as it assumes that the signal between ATR-FTIR and transmission are directly comparable. In ATR-FTIR spectroscopy a signal at ratio of 1000cm^{-1}:1600 cm^{-1} would be higher than the same ratio in transmission mode because of the ramping effect in ATR due to depth of penetration verses frequency.

Table 1: *The average signal:noise ratio of the graduated dysplastic lung biopsy FTIR image from 1090-1036cm^{-1}. All signal: noise ratio calculations were carried out on blank regions of the image after co-addition and averaging of blocks of 64 adjacent pixels.*

Mean amide II height	0.1 abs units
Lowest amide II height	0.05 abs units
Highest amide II height	0.3 abs units
Mean signal:noise (assuming 0.1 amide II height)	1.0
Standard deviation of signal:noise	$\pm$ 0.5
Lowest signal:noise	0.2
Highest signal:noise	3.8

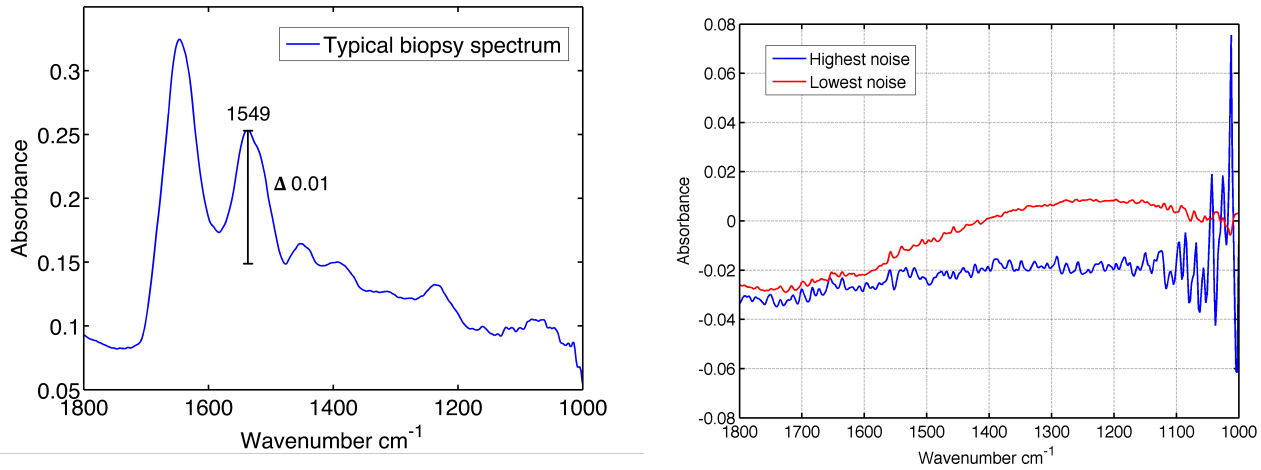

Figure 3: *a) A typical biopsy spectrum recorded with FTIR microscope fitted with a 96x96 FPA detector (64 co-added spectrum at 128 scans each) b) Absolute spectra showing the range of highest and lowest noise around 1036cm-1 in blank samples (64 co-added spectrum at 128 scans each)*

2 FPA-FTIR microscopy of a Bronchiole Biopsy

2.1 Measurement parameters

In order to distinguish the tissue tissue type and disease stages of the bronchiole biopsy, the same spectral image was used as in the section above. To summarise; a deparaffinised, 8 µm dysplastic lung biopsy section was used. A 96x96 element Focal Plane Array (FPA) detector was used for the measurements, this gave a projected pixel size of 2.7 μm^2. Spectra of each element were calculated from 128 co-added interferograms at a 4 cm^{-1} resolution. The transmission image was obtained using a lens on top of the CaF_2 window to create a pseudo hemisphere in order to focus the IR light, and reduce light scattering.

2.2 Assessing tissue types

In order to make comparisons between the different disease states of a fresh biopsy with ATR-FTIR spectroscopy, the sidedness of the biopsy first needs to be determined. In order to improve the signal: noise ratio blocks of 64 adjacent pixels, in 8x8 matrix, were co-added and averaged.

Hierarchical clustering analysis (HCA) of the 1575-1450 cm^{-1} region shows the separation of the epithelium and lamina propria (Figure 4b). Figure 4c shows the average of the extracted spectra, after their conversion to their second derivative forms, from these clusters. The epithelium spectrum (blue) has a second derivative peak at 1538 cm^{-1} which is absent from the spectrum of the lamina propria (red). This feature, that distinguishes tissue type, is present in ATR-FTIR spectra of fresh bronchiole biopsies and oesophageal biopsies.

These analyses of the 1354-1186 cm^{-1} and 1141-1070 cm^{-1} spectral regions highlight the importance of separating tissue type before analysing them further to assess disease state because these regions of the spectra show greater differences between tissue types than between disease states.

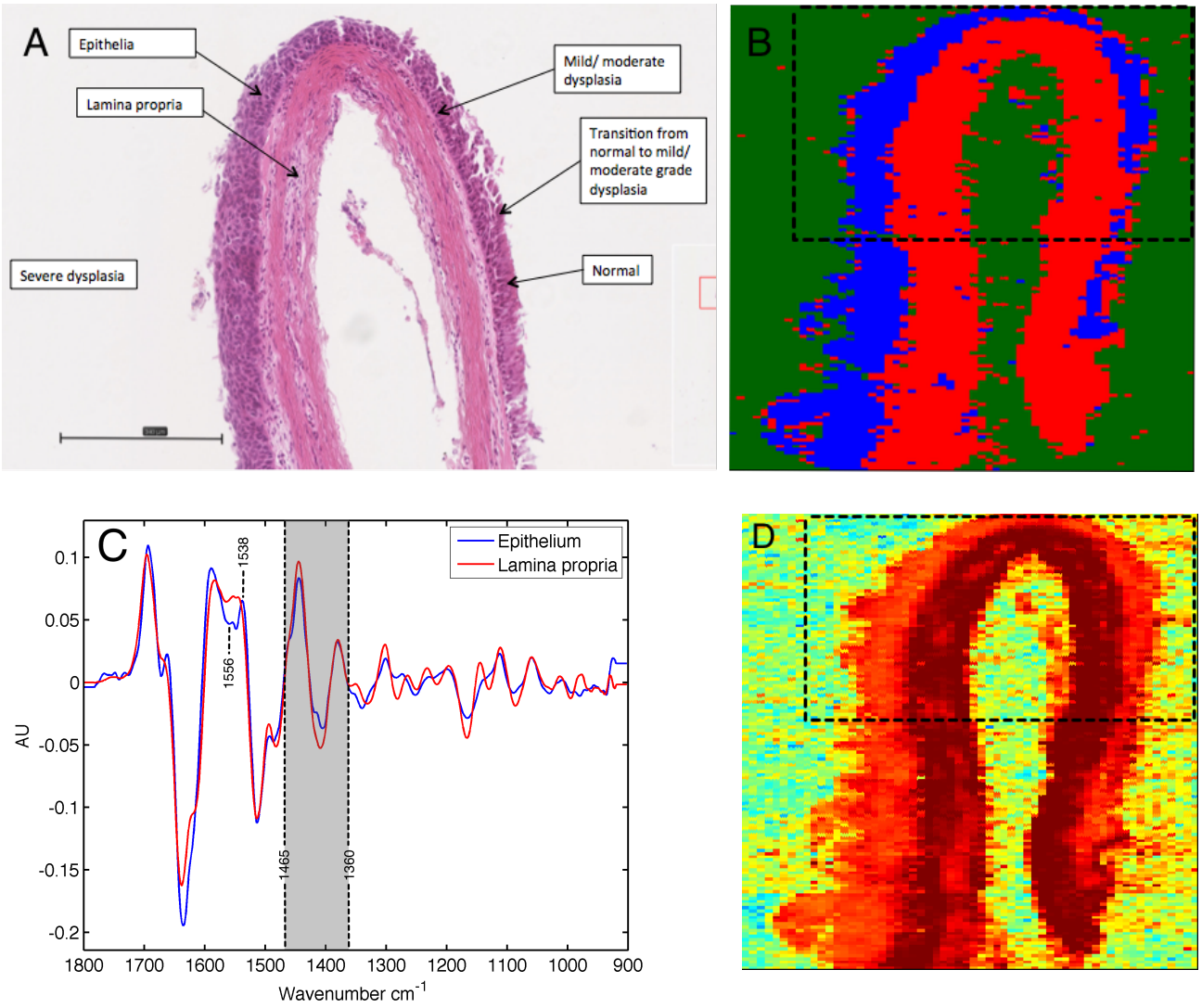

Figure 4: *a) H&E section of the bronchiole sample. b) Epithelial cells (red), lamina propria tissue (blue) and blank (green) separated using HCA of the 1575-1450 cm^{-1} region of the spectra. All spectra were normalised to the second derivative amide II area before analysing. The dashed box indicates the area shown in the H&E section. c) Extracted spectra showing the average of the epithelium and lamina propria, where the grey area shows the region in which paraffin is known to contribute. d) Heat map that shows epithelium (light red) and lamina propria (dark red) separated on the second derivative 1556 cm^{-1} trough height to zero.*

2.3 Assessing dysplasia

Although the signal:noise ratio after the co-addition and averaging in blocks of 64 pixels was high enough to distinguish tissue types, it was not enough to resolve the smaller differences expected between different disease states within the same tissue type. Instead, regions which were known to be either normal, mild dysplasia, severe dysplasia or cancer from the H&E stain, and which were known to be epithelia from the HCA, were manually selected using Cytospec^{TM} software from the regions indicated in Figure 5a. Figure 5b and c show the spectral changes between these selected regions. The second derivative trough between 1295 and 1265 cm^{-1} was the most significant difference between the disease states of the epithelial tissue. The averaged normal region has the largest trough at 1282 cm^{-1} (Figure 5c), mild dysplasia shows a smaller trough with the peak at 1281 cm^{-1}, severe dysplasia shifts to 1283 cm^{-1} with a smaller trough still, and finally cancer which has almost no peak at all. The normal averaged spectrum in Figure 5 contained 72 averaged spectrum from the 64 co-added FTIR image in the normal group, 53 in mild dysplasia, 106 in severe dysplasia and 96 in the cancerous group. Therefore, a total of total of 4608 from the original resolution were averaged in the normal, 3392 in mild, 6784 in severe dysplasia and 6144 in the cancerous group.

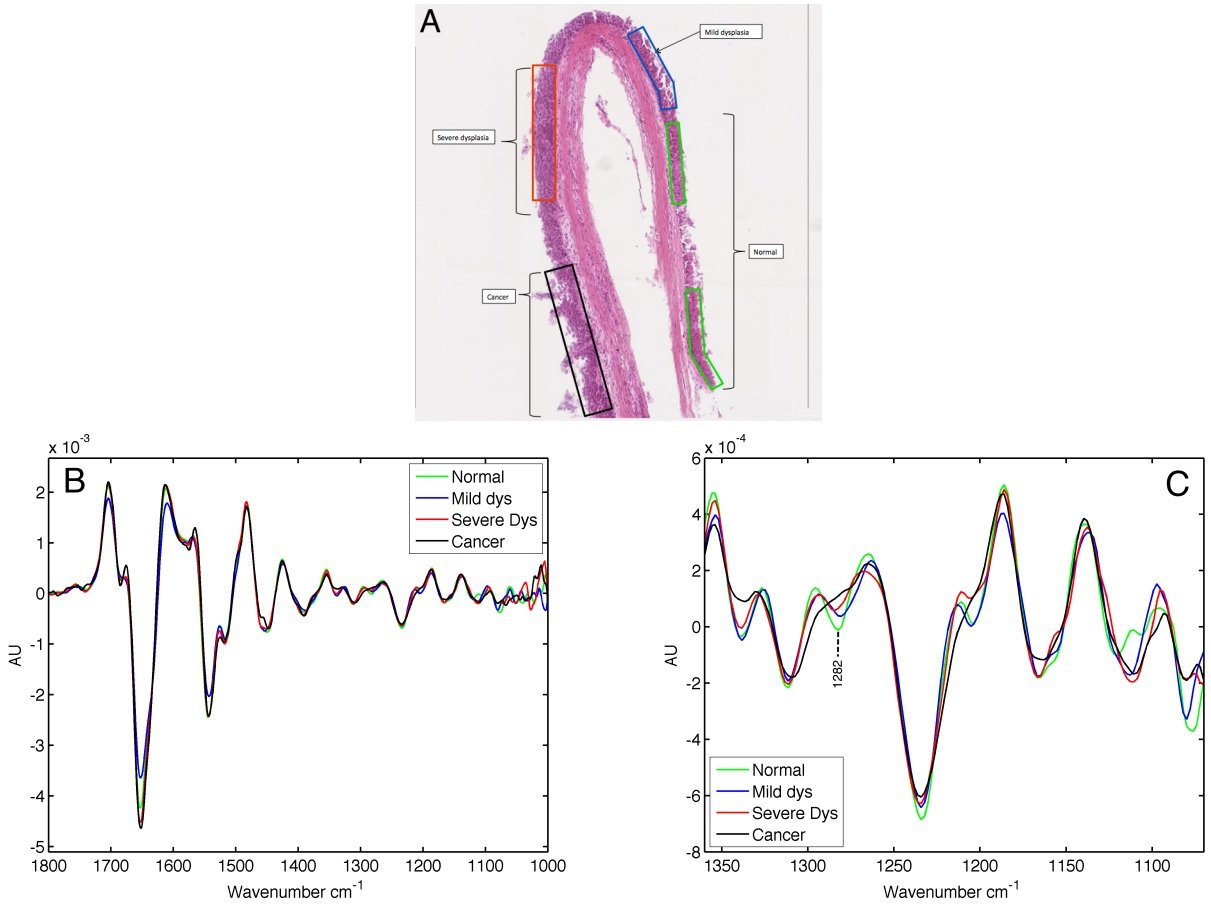

Figure 5: *a) H&E stained bronchiole section. The histology is indicated by arrows and the coloured boxes show the regions from which the spectra were manually selected. b) FPA-FTIR microspectroscopic data in the region of 1800-1000 cm^{-1}, from the regions of normal, mild, severe and cancerous epithelium regions of the biopsy indicated above. c) Identical spectra from the 1350-1050 cm^{-1} region, showing the main differences between the disease states. Spectra were converted to second derivative with 17 pt SG smoothing and normalised to the amide II area. There were 72 normal, 53 mild dysplasia, 106 severe dysplasia, 96 cancerous spectra averaged from the 64 co-added FPA-FTIR image in each group.*

3 ATR-FTIR of Bronchiole Biopsies

3.1 Data collection

Biopsies were placed in individual wells of a custom made device, which controls the humidity and temperature (4˚C) of the biopsy during transportation. Samples were placed individually onto the prism and ATR-FTIR spectra were recorded at room temperature with a Bruker ISF 66/S spectrometer fitted with a liquid nitrogen-cooled MCT-A detector and equipped with an Attenuated Total Reflection (SensIR 3 mm silicon crystal; 3 internal reflections). 500 co-added interferograms at 4 cm^{-1} resolution were averaged before Fourier Transformation. In all cases, a background spectra were calculated from 1000 co-added interferograms. In each case, an attempt was made to turn the biopsies onto the reverse side in order to record second spectrum was recorded. Data was collected using OPUS 6.5 software.

All bronchiole biopsies analysed with ATR-FTIR were subsequently stored in 4% formaldehyde and processed for an official diagnoses by histopathology. The biopsies were categorised into the following classes by a UCLH expert histopathologist: Healthy, LGD, HGD and cancerous, the numbers of patients, biopsies and spectra in each diagnosis can be seen in

Table 2: *Bronchial biopsies collected to date. Table updated on 13.05.14.*

	Patients	Biopsies	Spectra
Normal	21	42	81
LGD	4	7	11
HGD	9	13	26
Cancer	4	14	28
TOTAL	28	80	153

3.2 Separating healthy ATR-FTIR bronchiole biopsies into their sidedness

Based on the analysis of ATR-FTIR data from Barrett's biopsies and the lung cell physiology, different predominant cell types are expected on the two main biopsy surfaces: epithelial cells on the natural surface and lamina propria on the excised side of the biopsy. From previous analyses using FPA-FTIR microscopy, it was determined that the spectral differences between tissue type were larger than the more subtle spectral differences between the disease state. Therefore it is important to separate the spectra into their tissue types before comparing spectral disease state changes.

It is important to note that when placed on the ATR-FTIR prism, one of the biopsy surfaces should be in contact with the prism. However, in some instances when the biopsy sample is small and not in a clear flattened disc shape, it is possible that the biopsy is oriented such that both sides of the biopsy are in contact with the prism surface. In addition, the tissue types present are also dependent on the size of the biopsy as if a biopsy. If the biopsy taken is smaller than the depth of the epithelium layer, both sides of the biopsy will be epithelium. Therefore, the sidedness of the biopsy cannot be pre-determined using ATR-FTIR alone.

Hierarchical clustering (Ward's algorithm) of the 1585-1527 cm^{-1} region, which is known exhibit differences between cell types, was used to separate the healthy spectra into classes. There were three main classes whose spectra are shown inFigure 6. The red spectrum was assigned epithelium as in the FPA-FTIR analyses of a bronchiole biopsy, spectra containing a second derivative peak at 1538 cm^{-1} originate from epithelial cells. FPA-FTIR spectra that did not contain a second derivative peak in this 1538 cm^{-1} region originated from lamina propria tissue, hence the assignment of lamia propria to the blue spectrum in Figure 6. The green spectrum has been labelled 'Mixed', this is because it is expected to be a combination of the two cell types. To support this hypothesis, the average of the sum of the epithelium and lamina propria spectra is shown in black. The average is almost identical to the mixed spectrum in green, supporting the hypothesis that the spectra in the mixed group are representative of a mixture of the epithelial cells and lamina.

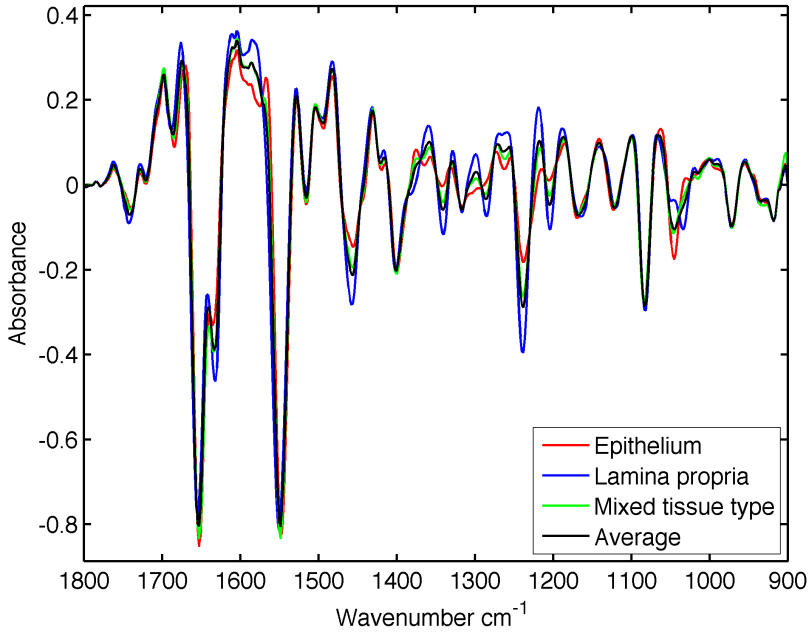

Figure 6: *Three main clusters after HCA of the 1585-1537 cm^{-1} region normalised to amide II area. The average of the epithelial signatures (red) and the lamina propria (blue) is shown in black, whilst the biopsies showing a mixed cell physiology IR signature is shown in green.*

Table 3: *a) Distribution of normal, LGD, HGD and cancerous spectra across the possible IR signatures of epithelium, lamina propria and mixed tissue types b) Ideally each biopsy should contain one spectra from the epithelium and one from the lamina propria tissue type. This table shows whether the two spectra from either side of the biopsy resemble IR signatures from the same tissue type, different tissue types, or whether the biopsy only had one spectrum. The assignment of ATR-FTIR tissue type is based on data from the FPA-FTIR image data.*

Table a)	Tissue type spectra			TOTAL
Diagnoses	Epithelia	Lamina propria	mixed	
Healthy	41	16	24	**81**
LGD	5	3	3	**11**
HGD	8	12	6	**26**
Cancer	11	17	N/A	**28**

Table b)		Number of biopsies			
Biopsy side one	Biopsy side two	Healthy	LGD	HGD	Cancer
Epithelium	Lamina propria	6	0	0	3
Epithelium	Mixed	14	1	4	N/A
Lamina propria	Mixed	6	1	2	N/A
Epithelium		9	1	2	4
Lamina propria		2	1	2	7
Mixed		2	0	3	N/A
Biopsies with single spectra		3	3	0	0
TOTAL		**42**	**7**	**13**	**14**

After successfully separating the healthy biopsies into their sidedness, the protocol was repeated for the LGD, HGD and cancer biopsies. Table 3a shows the distribution of spectra across the three possible tissue types, epithelium, lamina propria or mixed. Table 3b indicates whether the two spectra from a single biopsy contained one spectrum from either side of the biopsy.

Ideally a biopsy would have one spectrum from the epithelium and one from the lamia propria. However due to the size of the biopsy and orientation difficulties this is unlikely to occur with every biopsy. Additional biopsies would be needed

to make any conclusions about the distribution of spectra for LGD, HGD and cancer. However, for the healthy biopsies a total of 22 healthy biopsies showed signatures containing two cell types, and only 2 of the biopsies showed spectra from the underlying lamina propria side of the biopsy. Small forceps were used, producing biopsies with a size of ~1 mm^3, with biopsies this size or smaller, it is possible that only the epithelial cell type is present, explaining why 9 of the biopsies only contained epithelial cells. It is also possible, although unlikely, that due to the nature of epithelial tissue, and the sometimes unavoidable harsh treatment when removing a biopsy, the the epithelial layer is removed from the biopsy, explaining why only 2 of the biopsies had a lamina propria signature.

3.3 Assessing the ATR-FTIR differences in disease stages of the lung

After separating the spectra into the sidedness of the biopsies, comparisons of disease state were made between the epithelium group and the lamina propria group separately.

Comparisons of the epithelia

As shown, ATR-FTIR can distinguish cell type. In order to make accurate distinctions between disease states, the same cell type must be compared. Figure 7 shows the second derivative differences between normal, LGD, HGD and cancer disease states of the epithelium, or surface layer of the biopsies. The main differences between the disease states occur between 1130 and 900 cm^{-1}, where the main contributing factors are DNA/RNA and glycogen/glycoproteins. An additional region which can be used to distinguish normal epithelial tissue is the peak at 1273 cm^{-1}, this peak is larger than the other diseased epithelial signatures. In contrast, cancer epithelial tissue has a larger peak at 1255 and trough 1738 cm^{-1}, where the latter region is known to contain contributions from the lipid C=O bond.

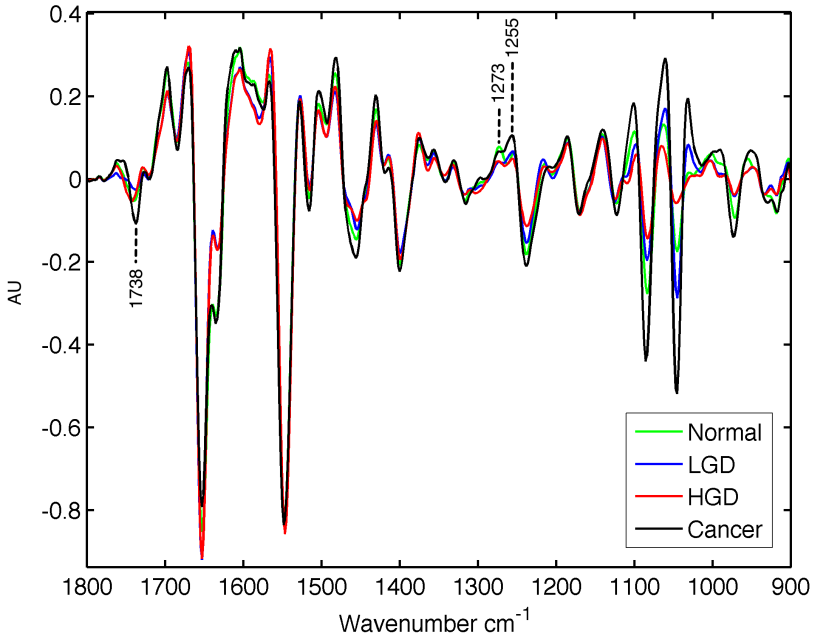

Figure 7: *Average second derivative ATR-FTIR spectra from 41 normal epithelia (green) spectra (18 patients, 32 biopsies), 5 LGD epithelia (blue) spectra (3 patients, 4 biopsies), 8 HGD epithelia (red) spectra, (3 patients, 6 biopsies), and 11 cancer epithelial (black) spectra (3 patients, 7 biopsies). All spectra have been normalised to the amide II area in the second derivative, with condensed water and water vapor subtracted in their absolute form.*

Comparisons of the lamina propria

Bronchial dysplasia develops in the epithelial cells, therefore minimal change in the lamina propria tissue is expected. However, Figure 8 shows that there was significant difference between normal, LGD, HGD and cancer lamina propria tissue, although it must be noted that the number of spectra, patients and biopsies included is small. Normal lamina propria shows a second derivative C=O lipid trough at 1743 cm^{-1}, where as LGD, HGD and cancer have a shifted peak at 1738

cm^{-1}, where cancer had a significantly larger trough. At 1360 cm^{-1} there appears to be a transition from normal to cancer with a decrease in the 1360 cm^{-1} second derivative peak and a total of 2 cm^{-1} shift to a lower frequency. The region between 1163-1171 cm^{-1} suggests there is a change in component composition. Normal lamina propria has a single broad peak at 1163 cm^{-1}, where as LGD and HGD have a combination of bands at 1163 and 1171 cm^{-1}, and cancer has a much larger 1171 cm^{-1} trough, this can be seen in Figure 8b.

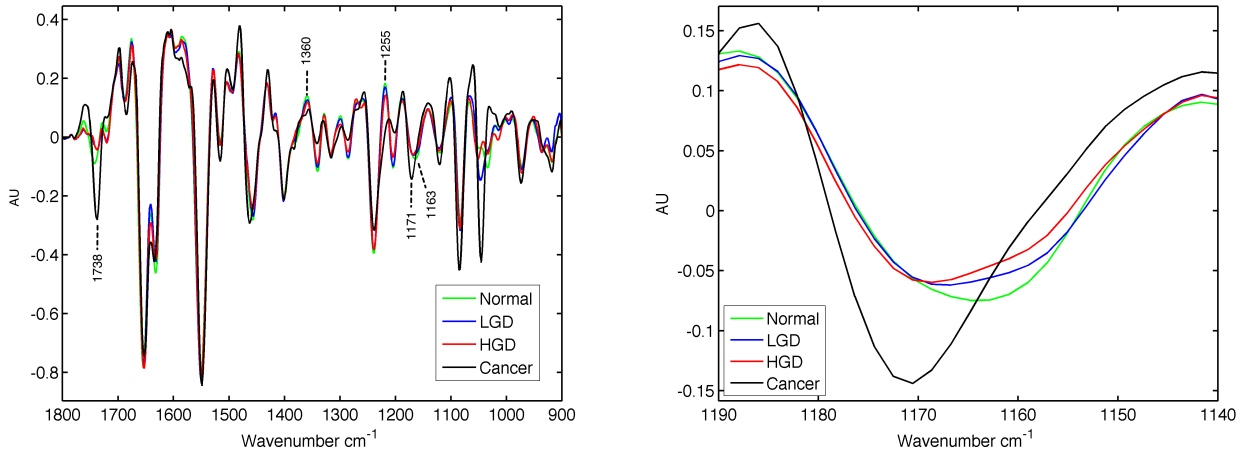

Figure 8: *a) Average second derivative ATR-FTIR spectra from 16 normal lamina propria (green) spectra (12 patients, 14 biopsies), 2 LGD lamina propria (blue) spectra (2 patients, 3 biopsies), 6 HGD lamina propria (red) spectra, (4 patients, 4 biopsies), and 17 cancer epithelial (black) spectra (3 patients, 10 biopsies). All spectra have been normalised to the amide II area in the second derivative, with condensed water and water vapor subtracted in their absolute form. b) 1190-1140 cm^{-1} region showing a potential change in component composition.*

4 Conclusions

With the current FPA-FTIR imaging parameters, (96x96 element FPA detector, 128 co-added interferrograms, 4 cm^{-1} resolution, with the CaF_2 pseudo hemisphere) we can distinguish the epithelium and lamina propria tissue types of the bronchiole biopsy. When regions of interest are selected from the image, it is possible to observe changes between, normal, mild and severe dysplasia and cancer. It would be interesting to analyse the same bronchiole biopsy image without the lens, in order to assess whether signal:noise can be improved between 1100-950 cm^{-1}. Additionally it would be interesting to see if the same imaging methods were applied to the Barrett's samples, could the disease state differences observed in the ATR-FTIR spectroscopic data be confirmed.

When the tissue type changes between the epithelium and the lamina propria at 1556 cm^{-1} are used to separate the ATR-FTIR spectra into their respective sidedness, changes between normal, LGD, HGD and cancer can be observed. Statistical discrimination between the disease classes cannot be accurately categorised due to the low number of biopsies in the LGD, HGD and cancerous classes.