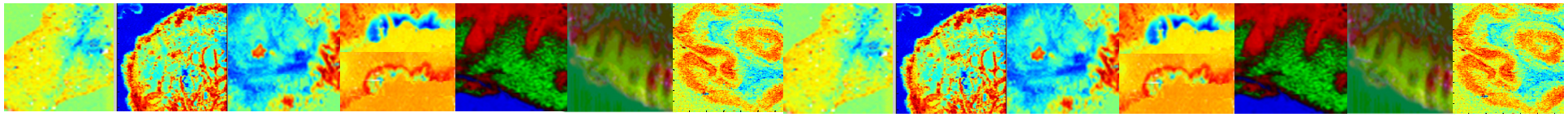


High resolution mid-infrared spectral pathological imaging

Prof Nick Stone

Professor of Biomedical Imaging and Biosensing
NHS Consultant Clinical Scientist – GHs / RD&E

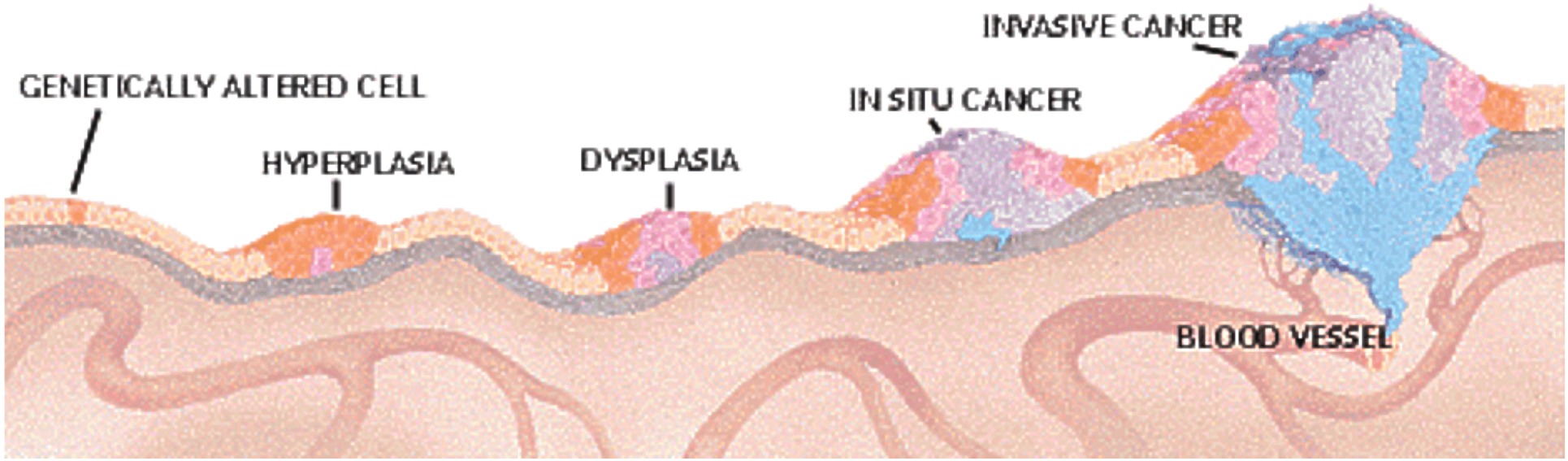
Gloucestershire Hospitals NHS
NHS Foundation Trust

LabHuman

UNIVERSITY OF EXETER

Early diagnosis is vital

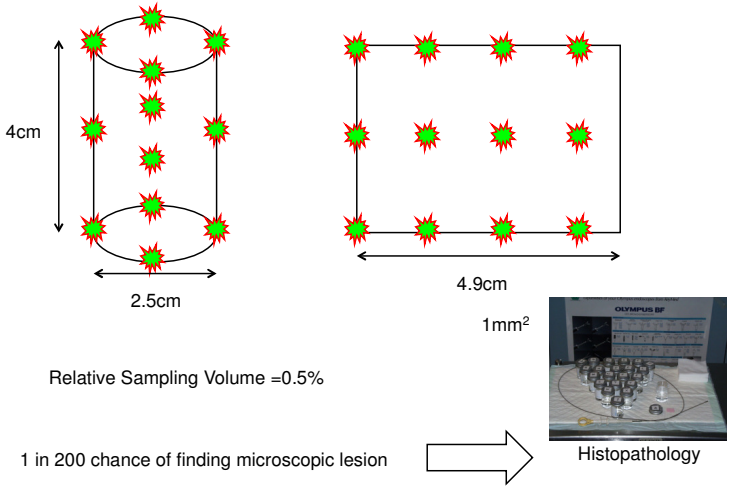
1 in 3 will get Cancer
1 in 4 will die from Cancer

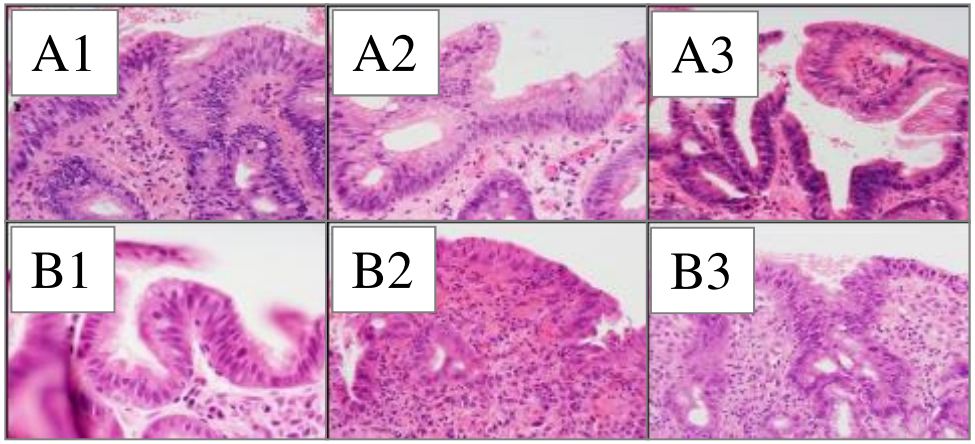


Primary requirement for successful treatment of any malignancy is early detection.

Survival can be 10% at 5 years for advanced disease.

Histopathology – 50% agreement LGD/HGD





	A1	A2	A3	B1	B2	B3
Path 1	No dys	No dys	Indef	Indef	Indef	Indef
Path 2	Indef	Indef	LGD	Indef	Indef	Indef
Path 3	LGD	LGD	LGD	LGD	LGD	Indef
Path 4	No dys	No dys	Indef	Indef	Indef	Indef

HEALTH

Pigeons trained to spot signs of cancer

By John von Radowitz

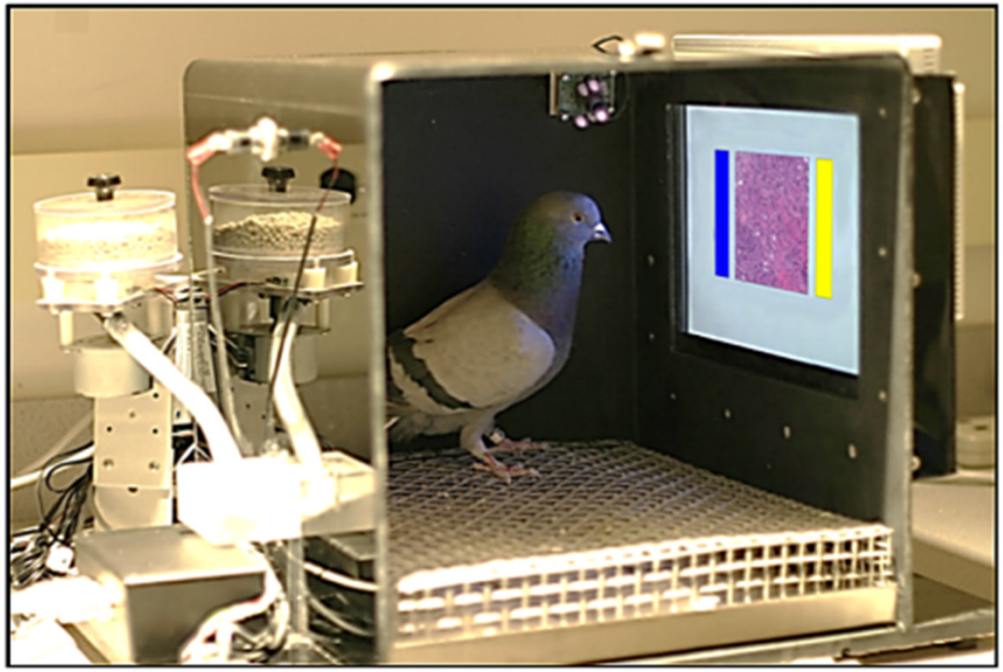
Pigeons are as good as humans at spotting signs of breast cancer in biopsy samples and mammogram scans, according to remarkable new research.

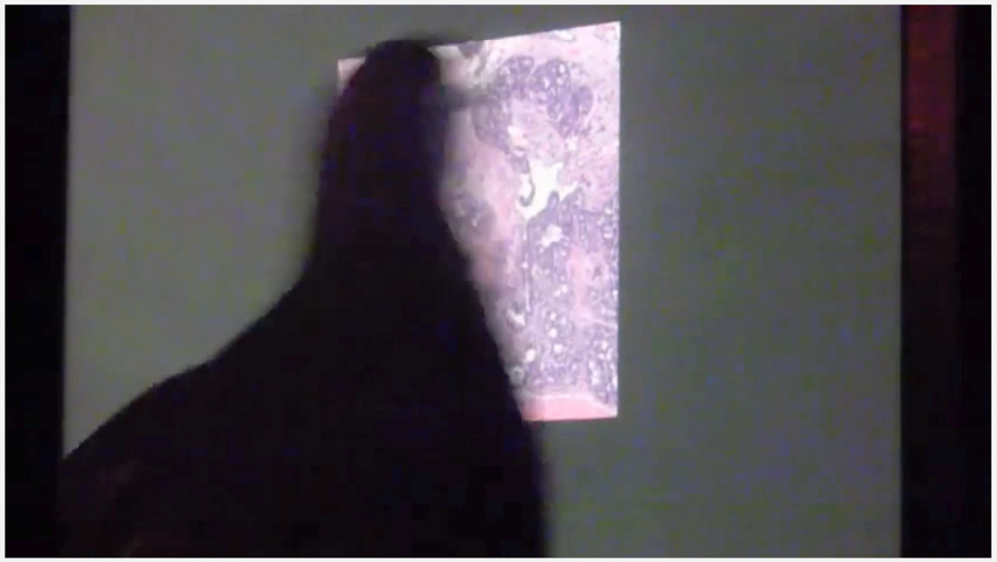
In a new experiment, the birds were taught how to recognise microscope slides and mammogram scan images and could distiguish between be-

nign and malignant tissue.

Eight pigeons took part in the experiment, with findings published in online journal Public Library of Science One.

Lead researcher Professor Richard Levenson, from the University of California, said that pigeons' accuracy "increased from 50 per cent correct to nearly 85 per cent by days 13 to 15".





Pigeons (Columba livia) as Trainable Observers of Pathology and Radiology Breast Cancer Images

Victor Navarro

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14,812

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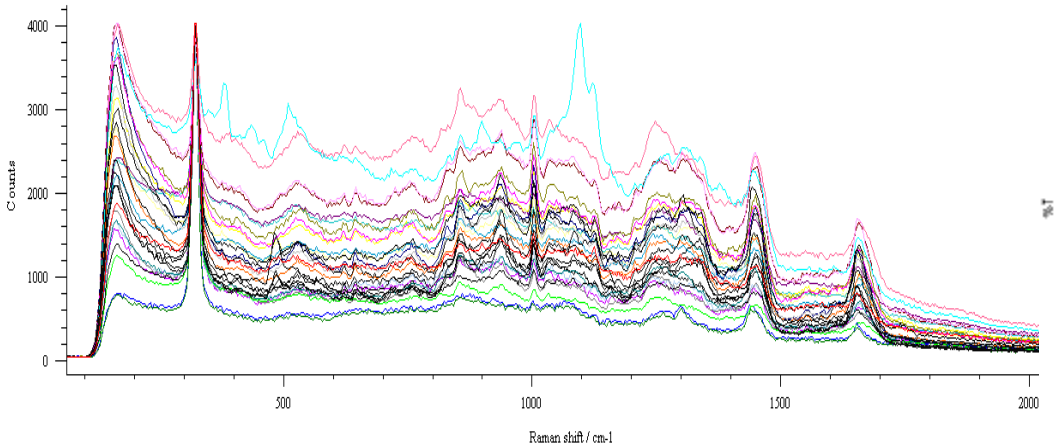
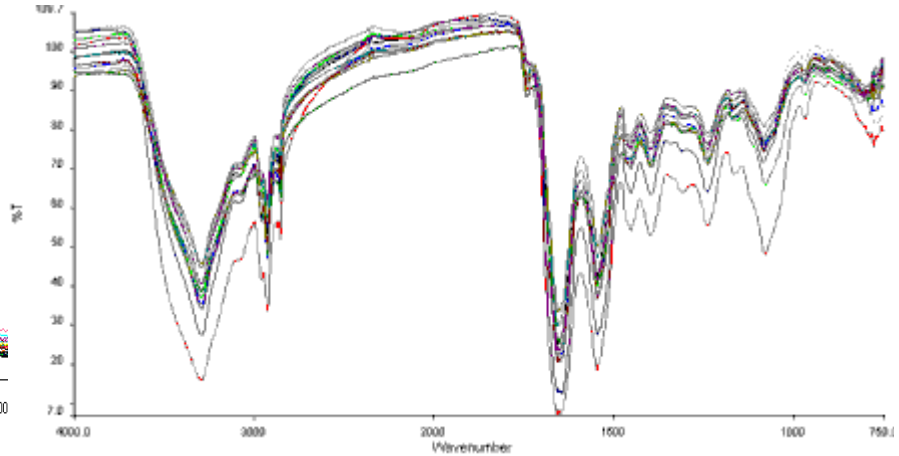
What is vibrational spectroscopy?

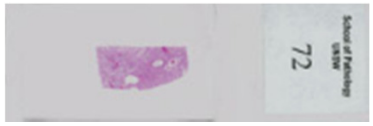
Symmetrical stretching | Antisymmetrical stretching | Scissoring | Rocking | Wagging | Twisting





Sample Measurements (Point Map and Image Spectra)

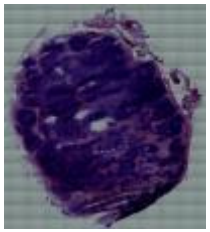
On paired lymph node study IR and Raman both provide >90% agreement with histopathology



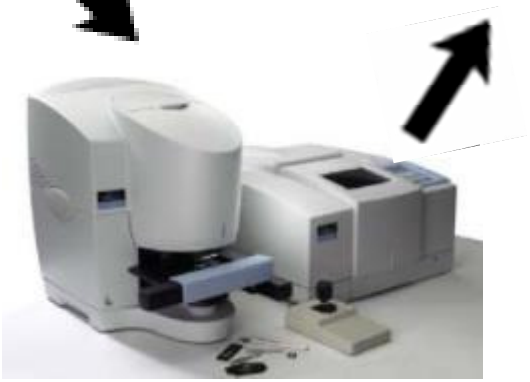
15µm frozen tissue sections

UNSTAINED SECTION

H&E STAINED SECTION

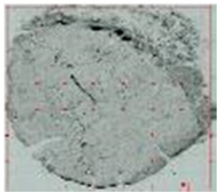


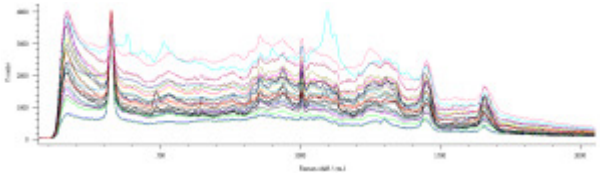
RAMAN MICROSPECTROMETER
(Near Infrared - 830nm laser)
[~60-2000cm^{-1} wavenumber Raman shifts]
Monochromatic

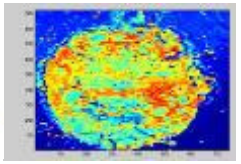


FTIR MICROSPECTROMETER
(Mid-infrared - 4000-700 cm^{-1})
Polychromatic)

- 80/100x obj
- ~3.75cm^{-1} spectral resolution
- 20s / 1 accumulation
- 100-200µm spatial steps (x and y)
- 24-48 hour maps (3500-9500 spectra)
- 10-56MB maps

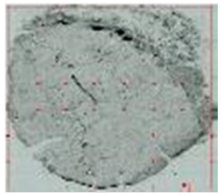


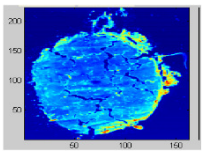




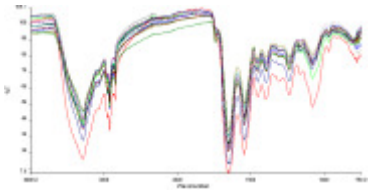
Raman Hyperspectral map

- 1 (pt) and 4 (maps) cm^{-1} spectral resolution
- 6.25 / 25µm spatial steps (x and y)
- 64 co-scans
- 2-6 hour maps (60,000-150,000 spectra)
- 300-800MB maps





FTIR Hyperspectral map



Vibrational Spectroscopy 60 (2012) 43–49

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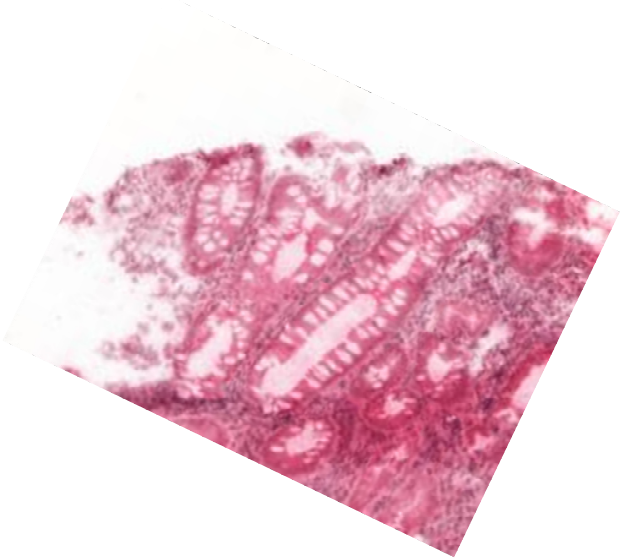
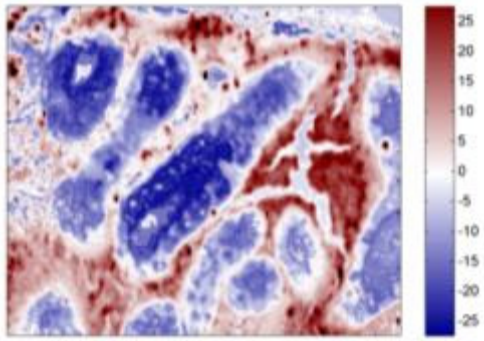
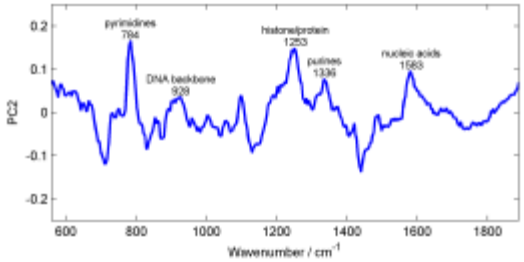
journal homepage: www.elsevier.com/locate/vibspec

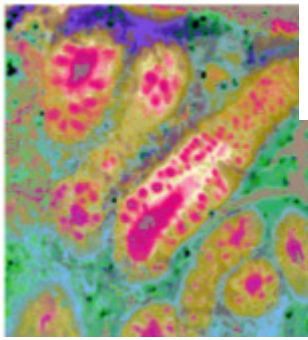


Histological imaging of a human colon polyp sample using Raman spectroscopy and self organising maps

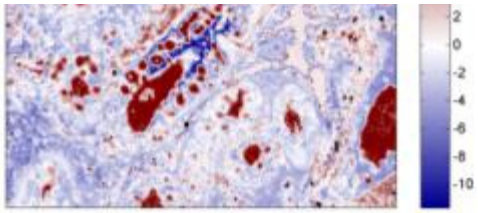
Gavin Rhys Lloyd[a], James Wood[a,c], Catherine Kendall[a,b], Tim Cook[c], Neil Shepherd[b,d], Nick Stone[a,b,*]

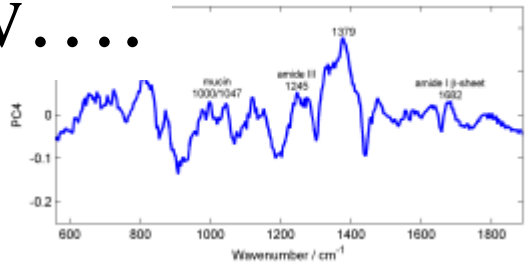


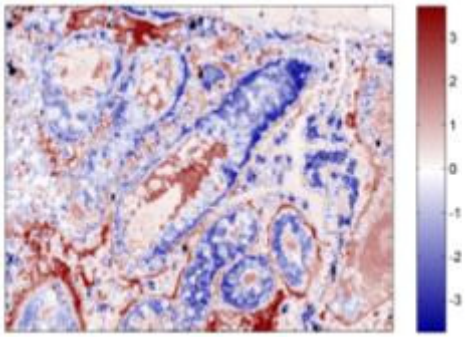


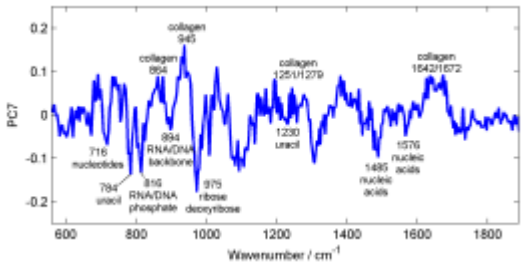


Probably too slow….







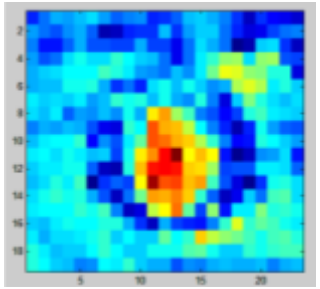


Raman
- Colon data
- SOM fed PCA

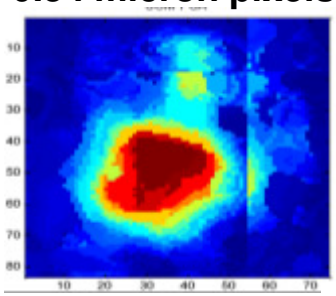
Novel Microscope Technology - imaging smaller imaging faster

FTIR-Diamond Synchrotron, UK

10 micron pixels



0.54 micron pixels



FTIR/DFIR-Benchtop instrument

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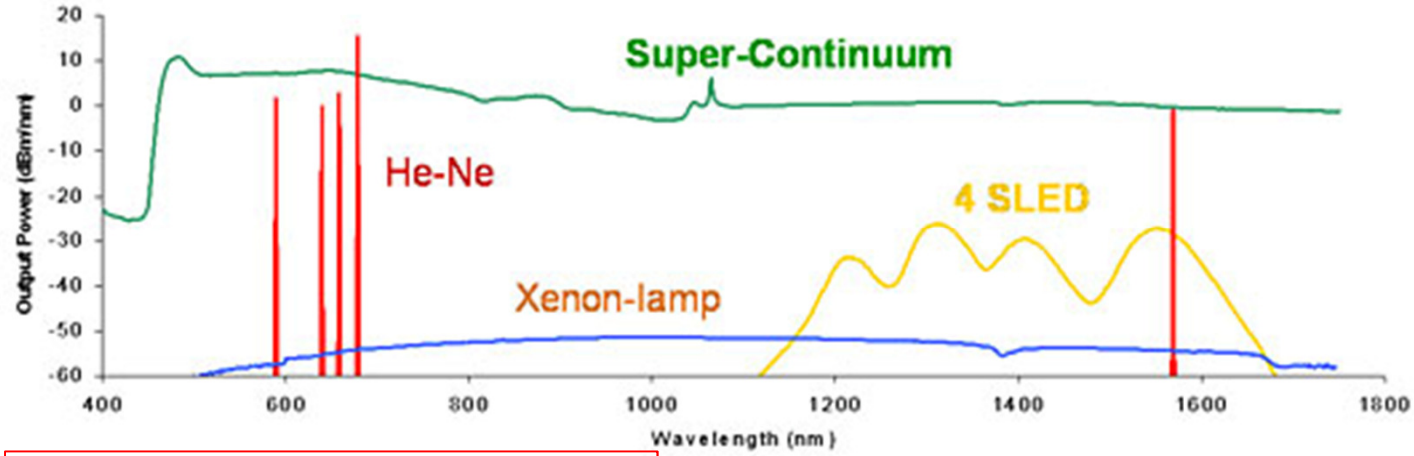
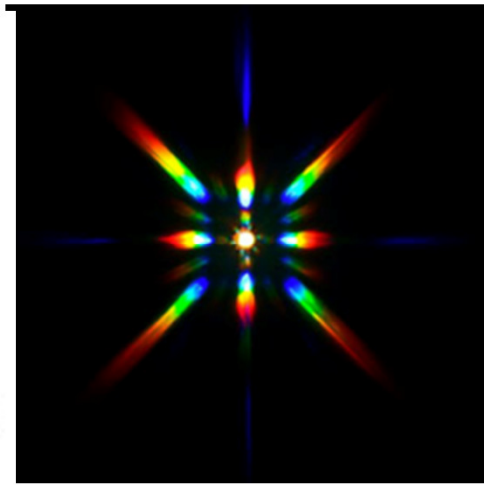
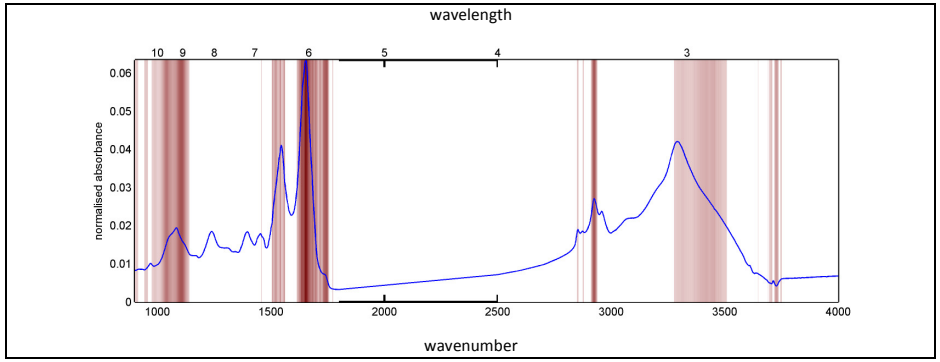
SEVENTH FRAMEWORK PROGRAMME

www.minerva-project.eu

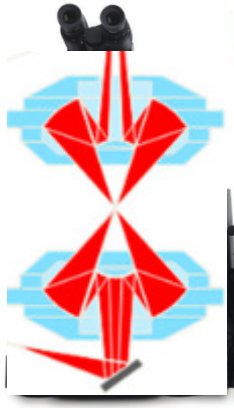


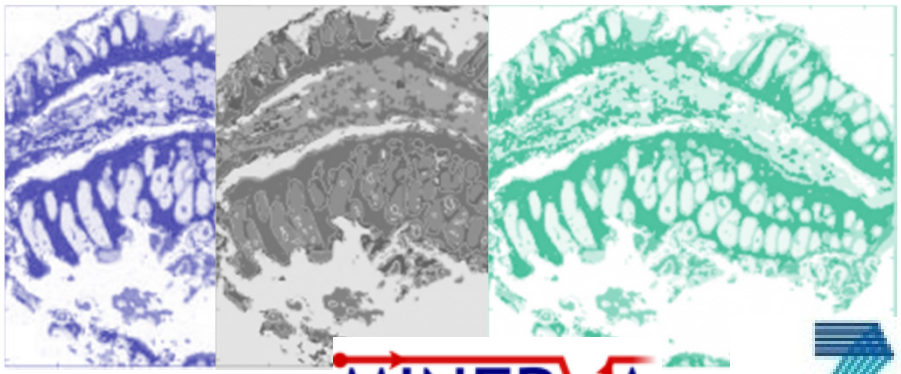


Tunable Mid-IR supercontinuum source



High NA Optics





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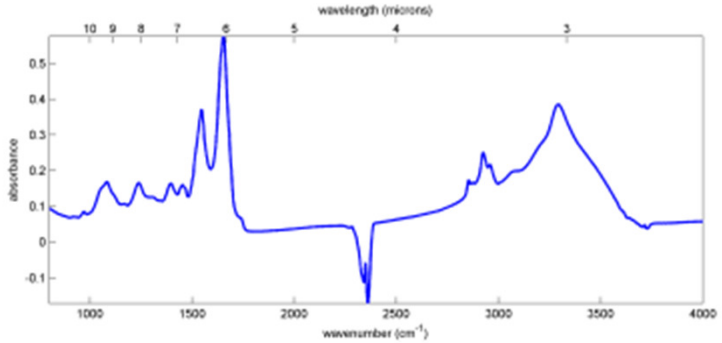
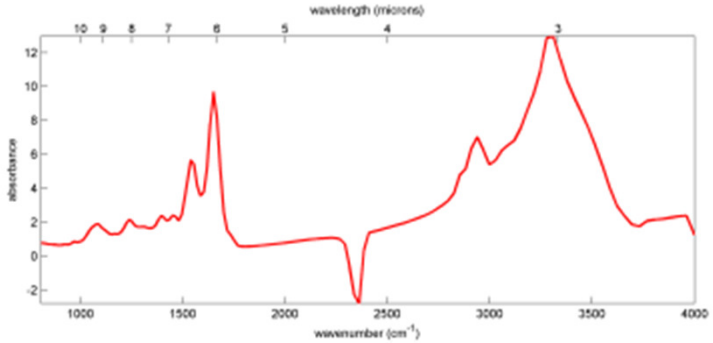
SEVENTH FRAMEWORK PROGRAMME

Agilent 620 FTIR microscope coupled with an Agilent 670 FRIR spectrometer for FTIR microscopic imaging

Re-examination of existing data

- **How might the AOTF parameters affect classification performance?**
  - **Gaussian profile centred on wavelength**
  - **FWHM = 1% of wavelength**
  - **Sampling at 1% wavelength**





Frozen section colon model: Effect of spectral range

5-group

wn range	wn spacing	n datapoints	Overall CC	Average SN	Average SP	Average PA	Average MS
2500-4000	1%	48	74%	65%	90%	60%	64%
900-1800	1%	69	83%	74%	93%	79%	77%
800-4000	1%	154	80%	68%	91%	64%	70%
900-1800 + 2500-4000	1%	129	82%	73%	93%	74%	72%

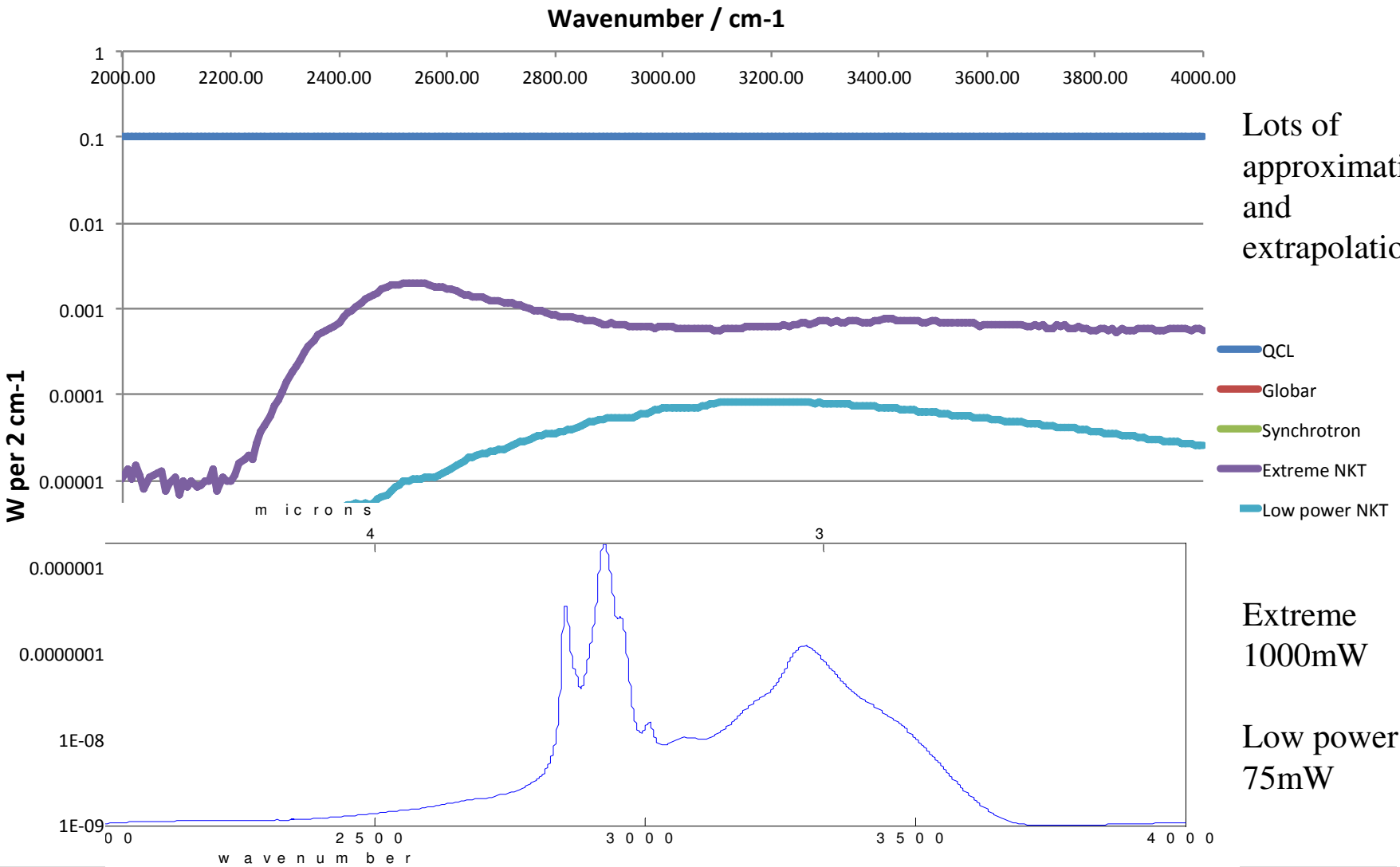
2-group

wn range	wn spacing	n datapoints	Overall CC	Average SN	Average SP	Average PA	Average MS
2500-4000	1%	48	90%	85%	85%	88%	85%
900-1800	1%	69	98%	93%	93%	96%	90%
800-4000	1%	154	94%	88%	88%	90%	83%
900-1800 + 2500-4000	1%	129	95%	88%	88%	91%	84%

Conclusions

- 900-1800 cm-1 gives the best performance (CC, SN and SP) overall.
- 2500-4000 cm-1 gives the worst performance overall.
- Performance and model stability decrease rapidly when wavenumber spacing exceeds 32 wavenumbers.
- 800-4000 cm-1 appears least affected by increased wavenumber spacing.
- Furthermore the addition of the 2500-4000 cm-1 region to the 900-1800 cm-1, although it doesn't increase performance, it doesn't significantly reduce it, however the inclusion of the region between 1800 and 2500 cm-1 wavenumbers clearly introduces spectral features which do not correlate with disease condition.

Source W / 2cm^{-1} through 10µm pinhole – CH region



Lots of approximations and extrapolations!

Extreme 1000mW

Low power 75mW

Optimal regions for model performance Variable Importance for Projection (VIP)

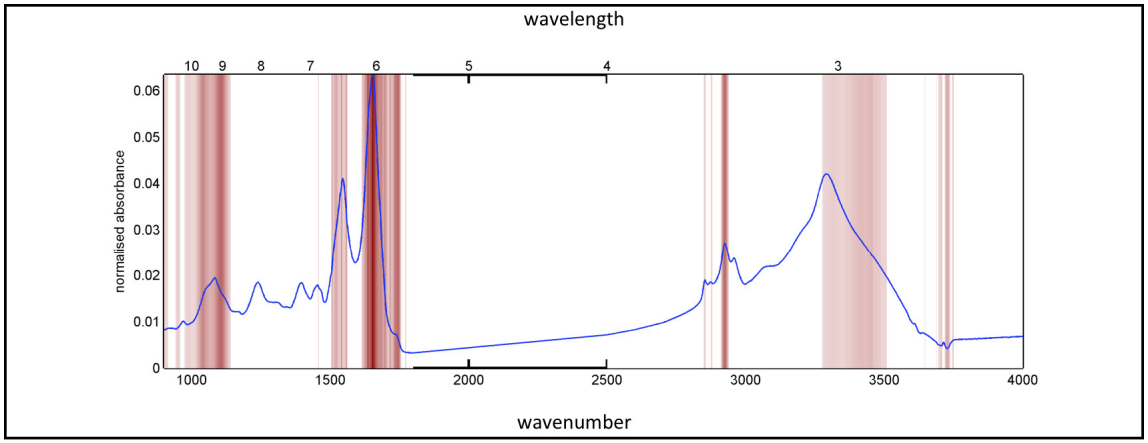


Figure 1 – Mean spectrum of colon dataset for the two supercontinuum laser wavlength regions combined. Shaded regions indicate those identified by VIP as significant.

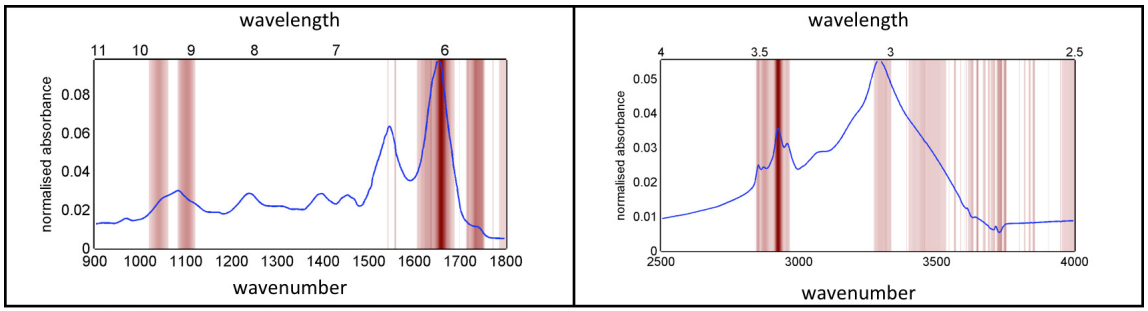


Figure 1 – Mean spectrum of colon dataset for the two supercontinuum laser wavelength regions. Shaded regions indicate those identified by VIP as significant.

nature photonics
LETTERS
PUBLISHED ONLINE: 14 SEPTEMBER 2014 | DOI: 10.1038/NPHOTON.2014.213

Are SC light sources good alternatives to QCLs?

Mid-infrared supercontinuum covering the 1.4–13.3 μm molecular fingerprint region using ultra-high NA chalcogenide step-index fibre

Christian Rosenberg Petersen[1]*, Uffe Møller[1], Irnis Kubat[1], Binbin Zhou[1], Sune Dupont[2], Jacob Ramsay[2], Trevor Benson[3], Slawomir Sujecki[3], Nabil Abdel-Moneim[3], Zhuoqi Tang[3], David Furniss[3], Angela Seddon[3] and Ole Bang[1,4]

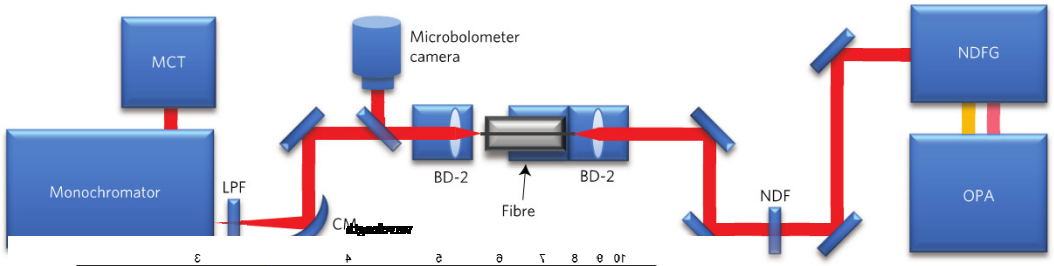
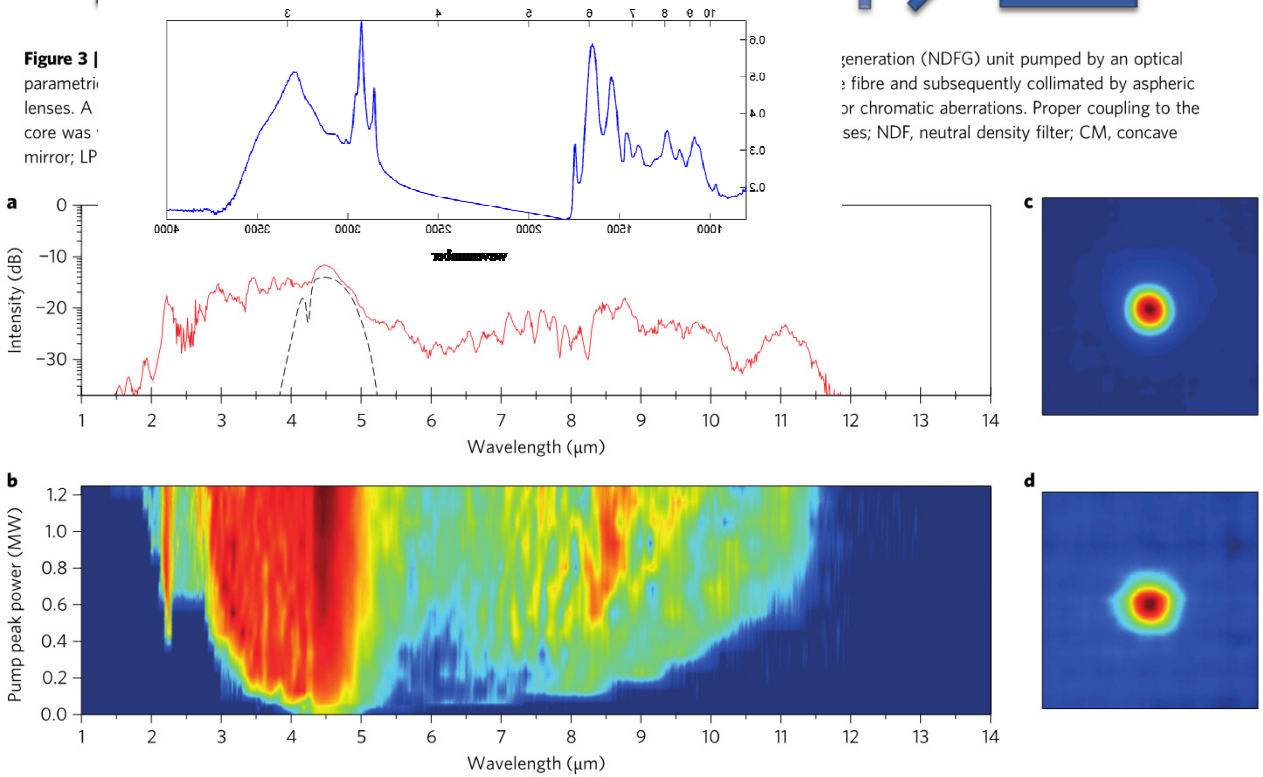


Figure 3 | parametri… generation (NDFG) unit pumped by an optical lenses. A … fibre and subsequently collimated by aspheric core was … or chromatic aberrations. Proper coupling to the mirror; LP… ses; NDF, neutral density filter; CM, concave



SEVENTH FRAMEWORK PROGRAMME
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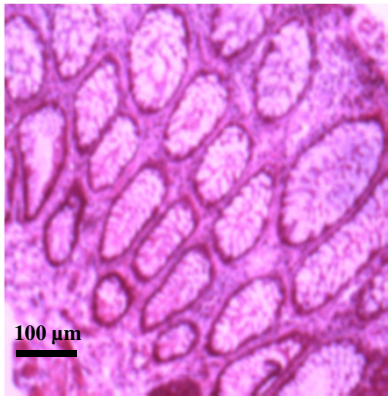
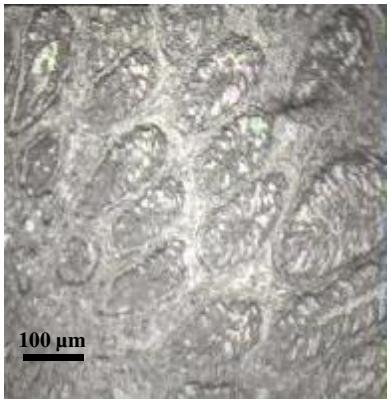
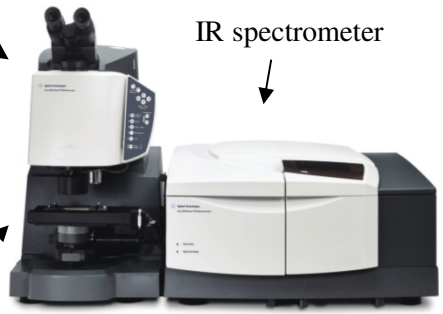
Infrared spectral imaging of tissues - Methodology

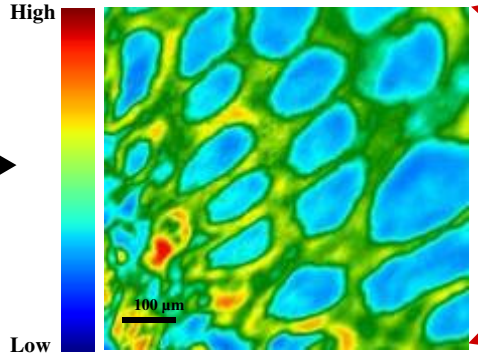


HE stained tissue section
of normal colonic mucosa

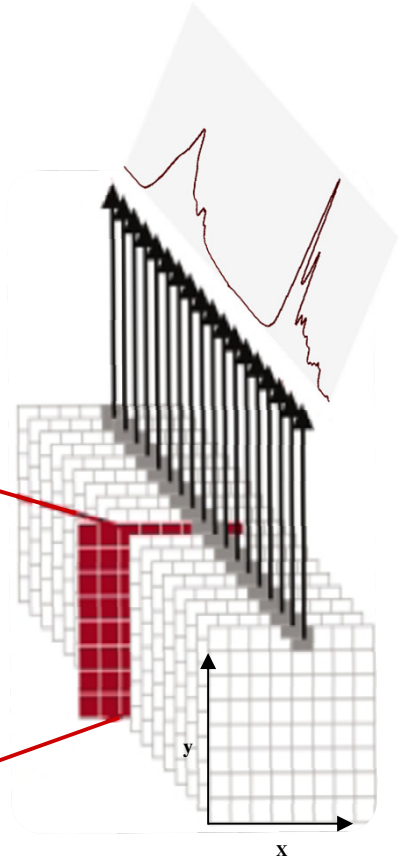


Adjacent unstained tissue
section (7 µm thick)





IR spectral image
(Amide I peak)

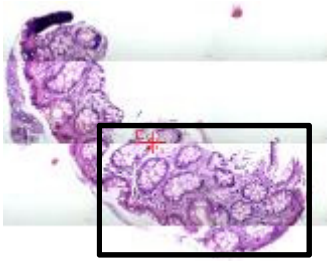


Spectral Hypercube

Agilent FTIR imaging system

Comparison of different de-paraffinization methods

H&E stained



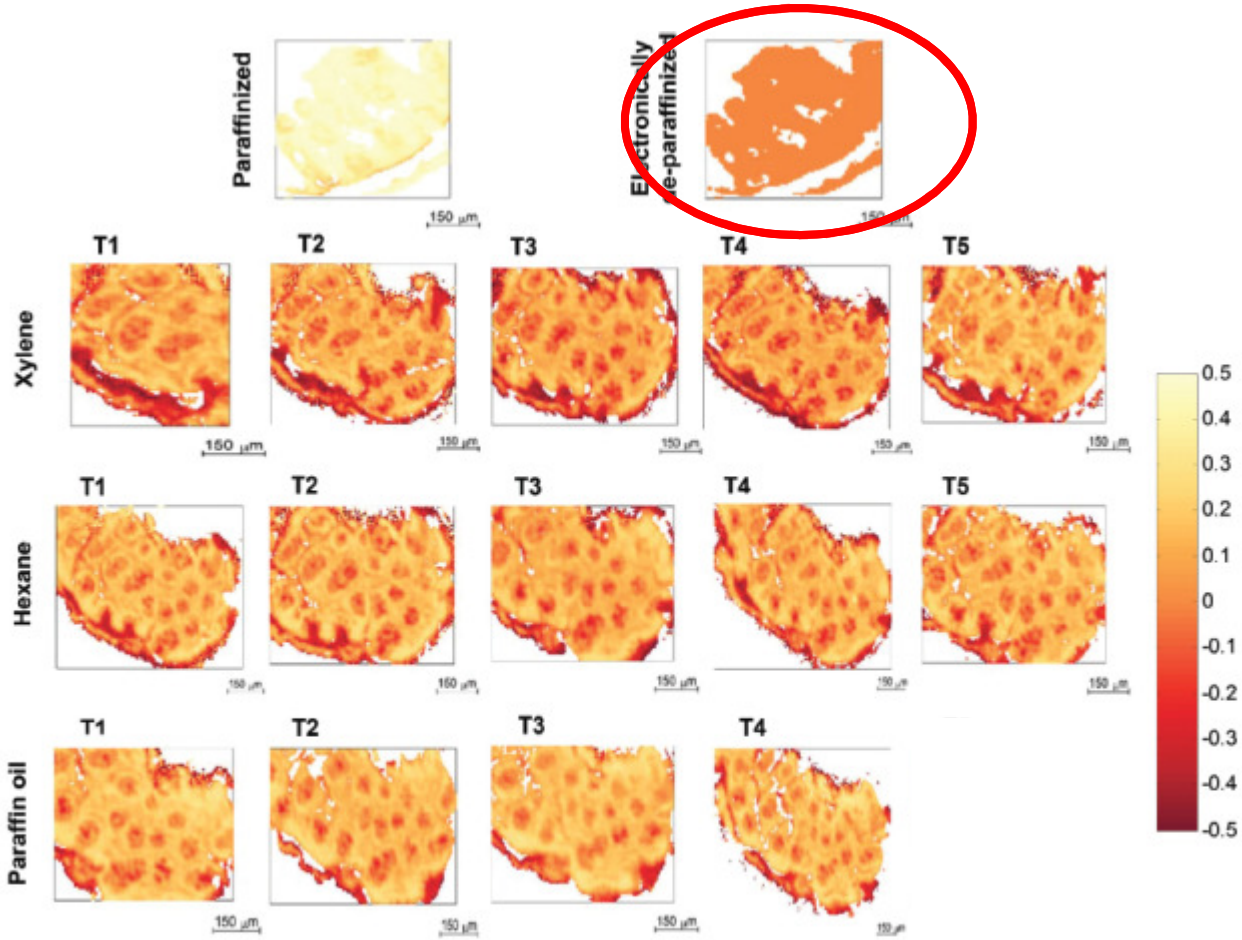
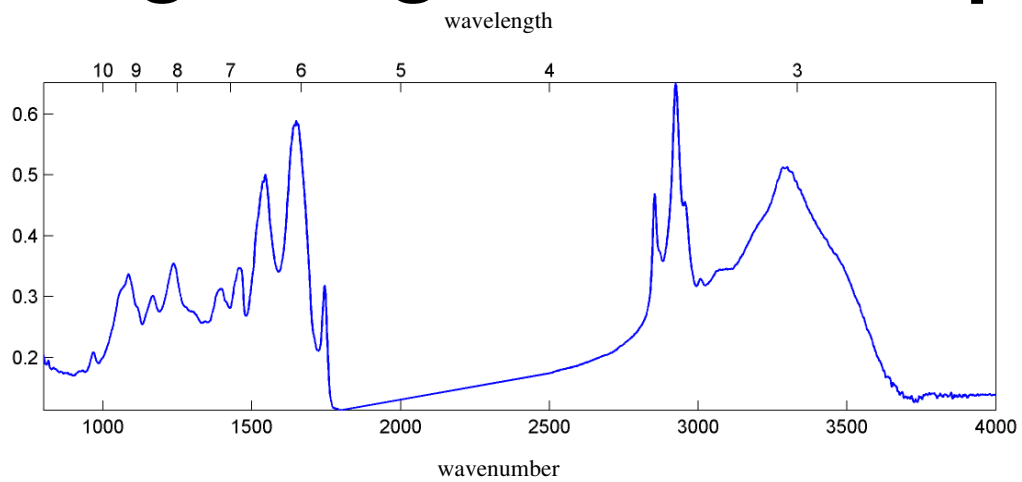
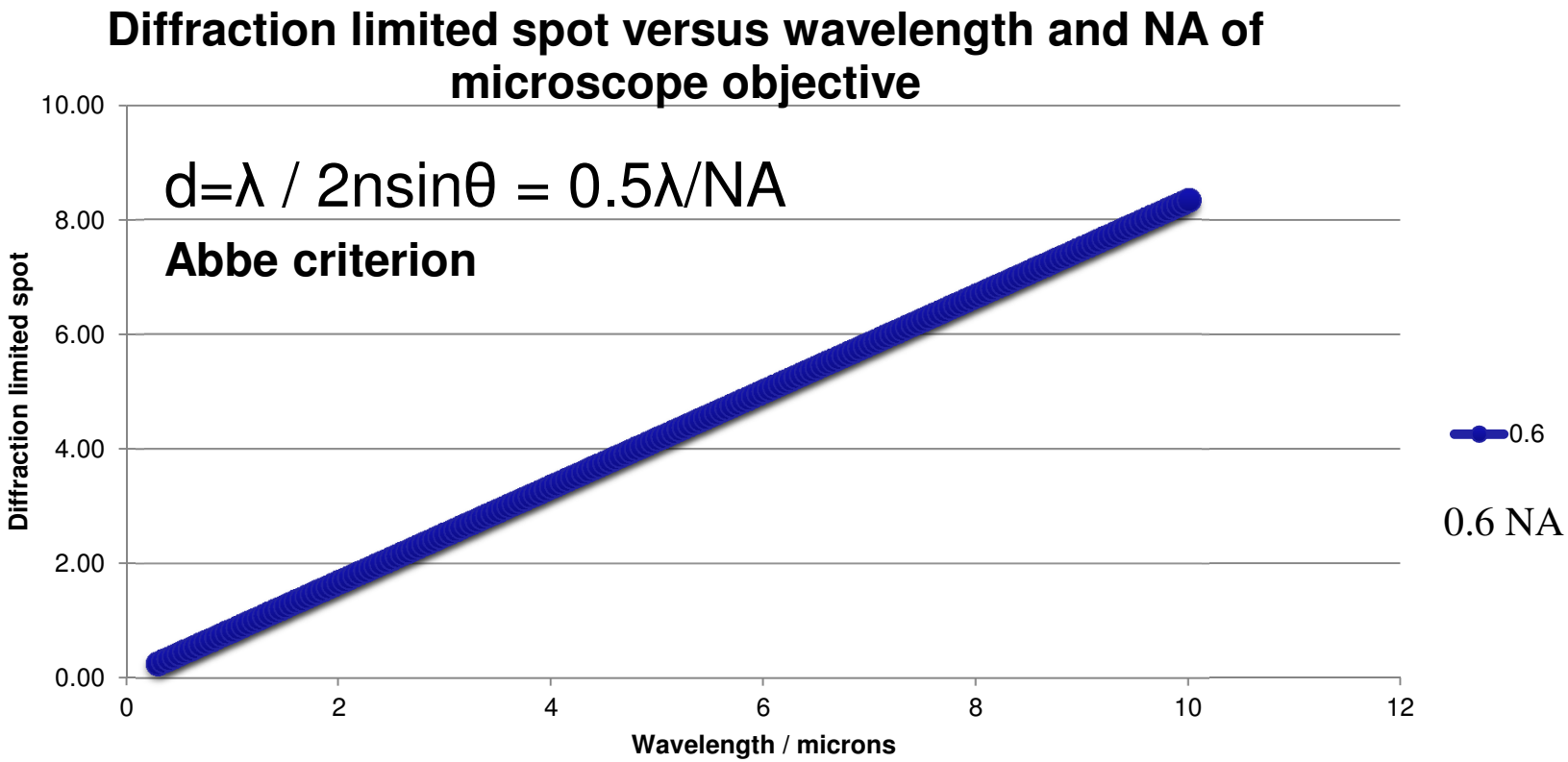


Fig. 3 Intensity maps of the paraffin fit to all the de-paraffinization methods with their respective time points (T1 to T5). First row: pure paraffinized tissue without any de-paraffinization and electronic de-paraffinization; second row: xylene de-paraffinization; third row: hexane de-paraffinization; fourth row: paraffin oil de-paraffinization. All the methods show differential retention of paraffin across all time points.

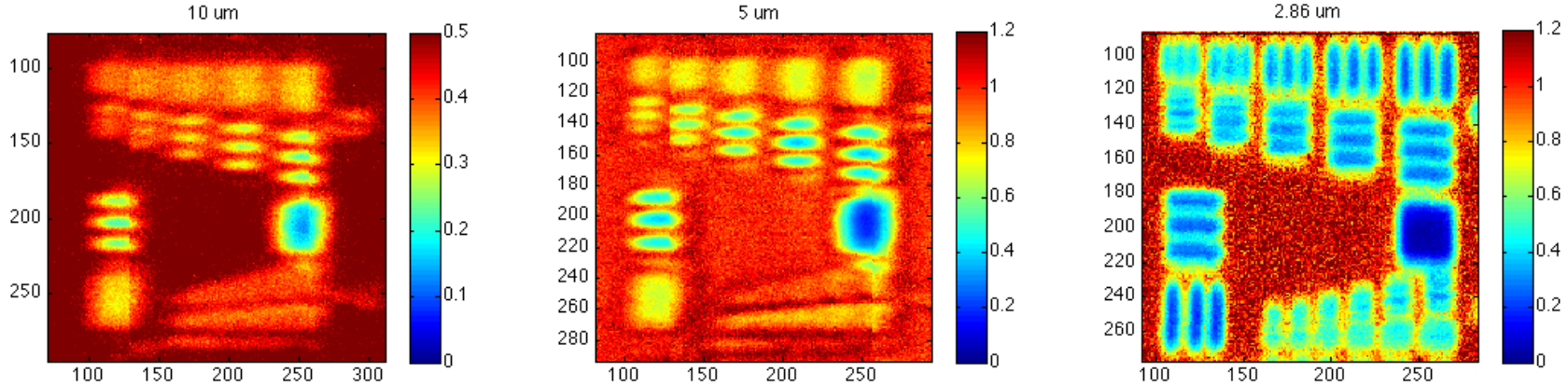
J Nallala, G Lloyd and N Stone, Analyst, 2015

Can high magnification help?

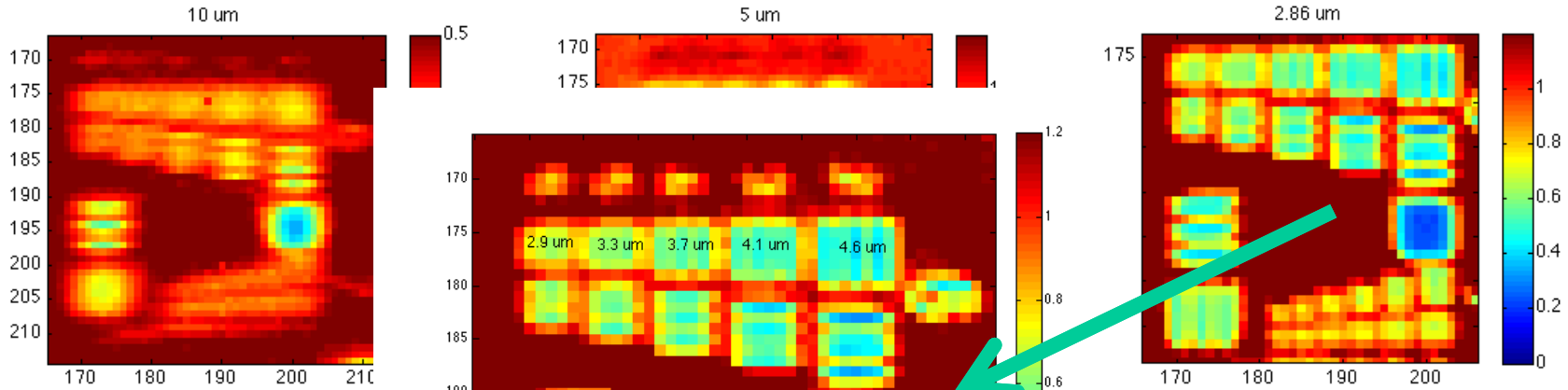


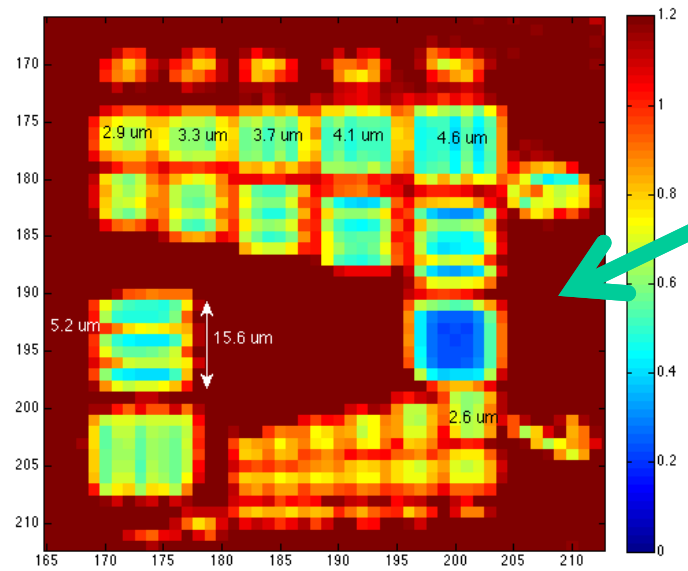


High Magnification 1.1 micron pixels



Low Magnification 5.5 micron pixels



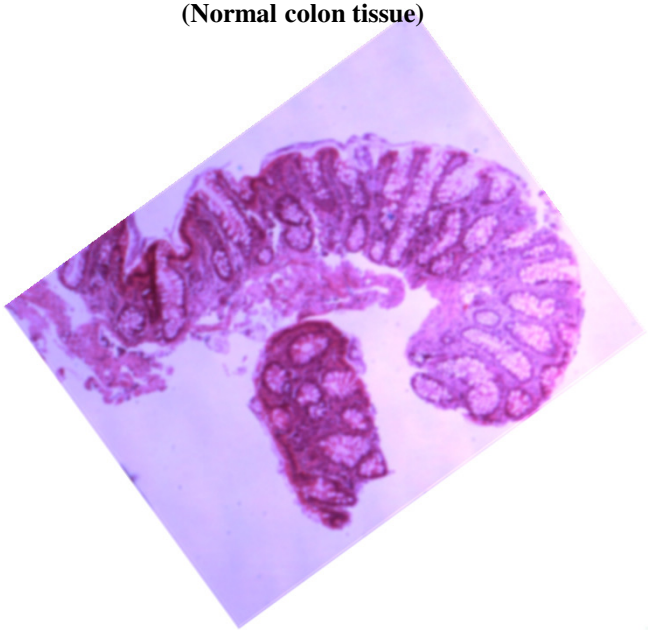


Agilent system

Novel Microscope Technology – High-resolution IR imaging

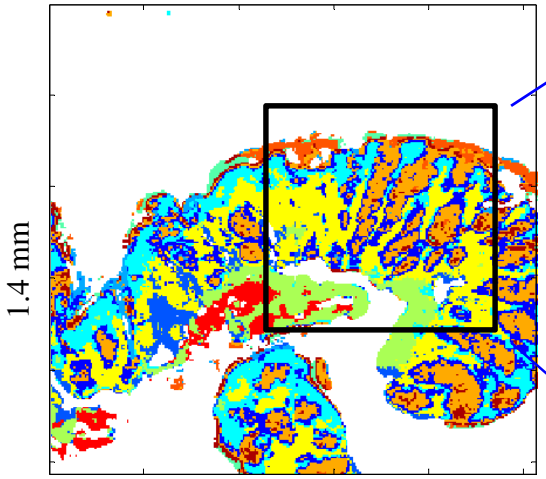
Histological image

(Normal colon tissue)



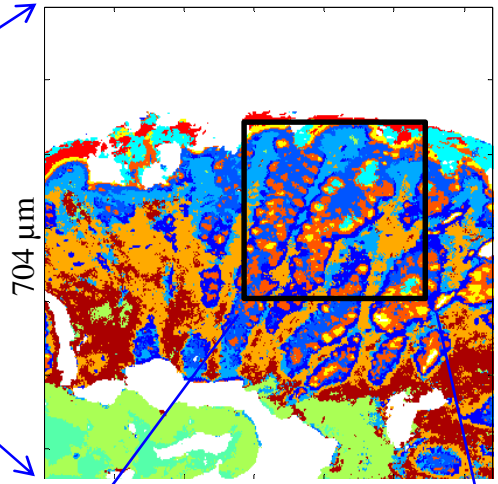
IR based cluster image

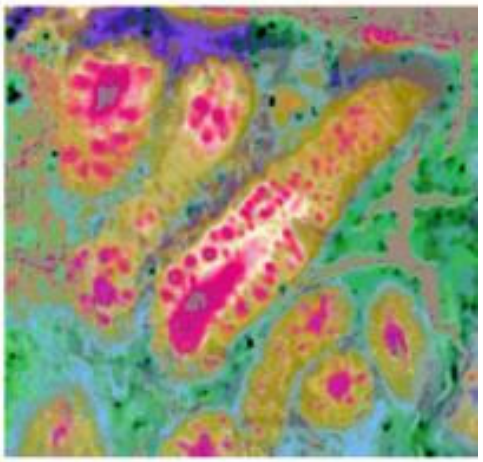
Conventional resolution (5.5x5.5 µm^2 pixel size)



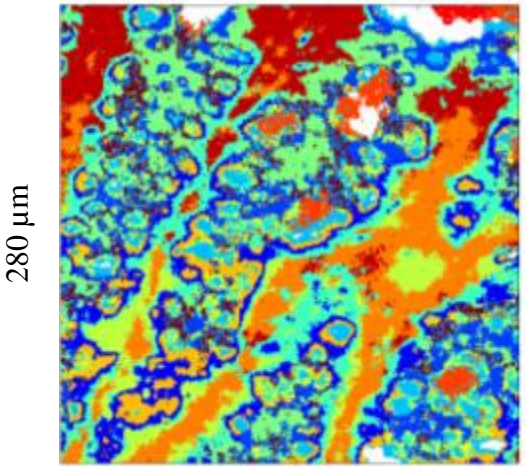
IR based cluster image

High-resolution (1.1x1.1 µm^2 pixel size)





c.f. Raman



Experimental details

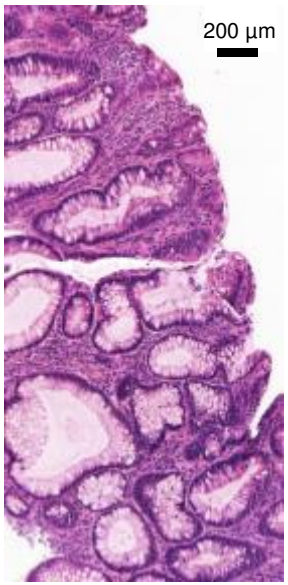
Imaging parameters:
Spectral resolution: 4 cm^{-1}
Pixel size: 5.5 µm and 1.1 µm
Scans per pixel: 128
Range: 900-4000 cm^{-1}

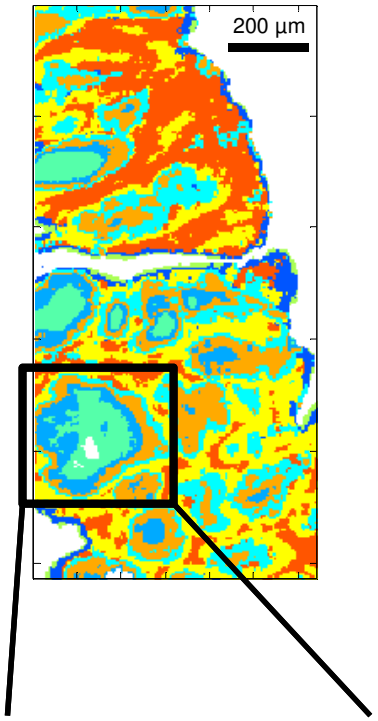
Spectral pre-processing:
Background correction, EMSC (baseline, normalization and electronic de-paraffinization)

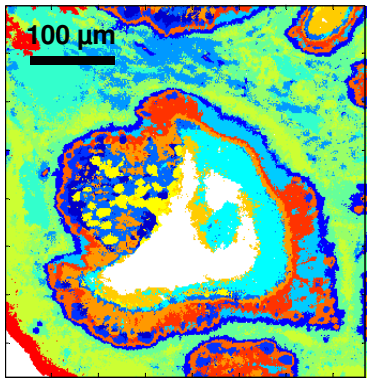
Multivariate statistical analysis:
K-means clustering

Nallala et al, Analyst, November 2015 (DOI: 10.1039/C5AN01871D)

Colonic tubular adenoma







Standard magnification
(7X; 5.5x5.5 µm^2 pixel size)

High magnification
(36X; 1.1x1.1 µm^2 pixel size)

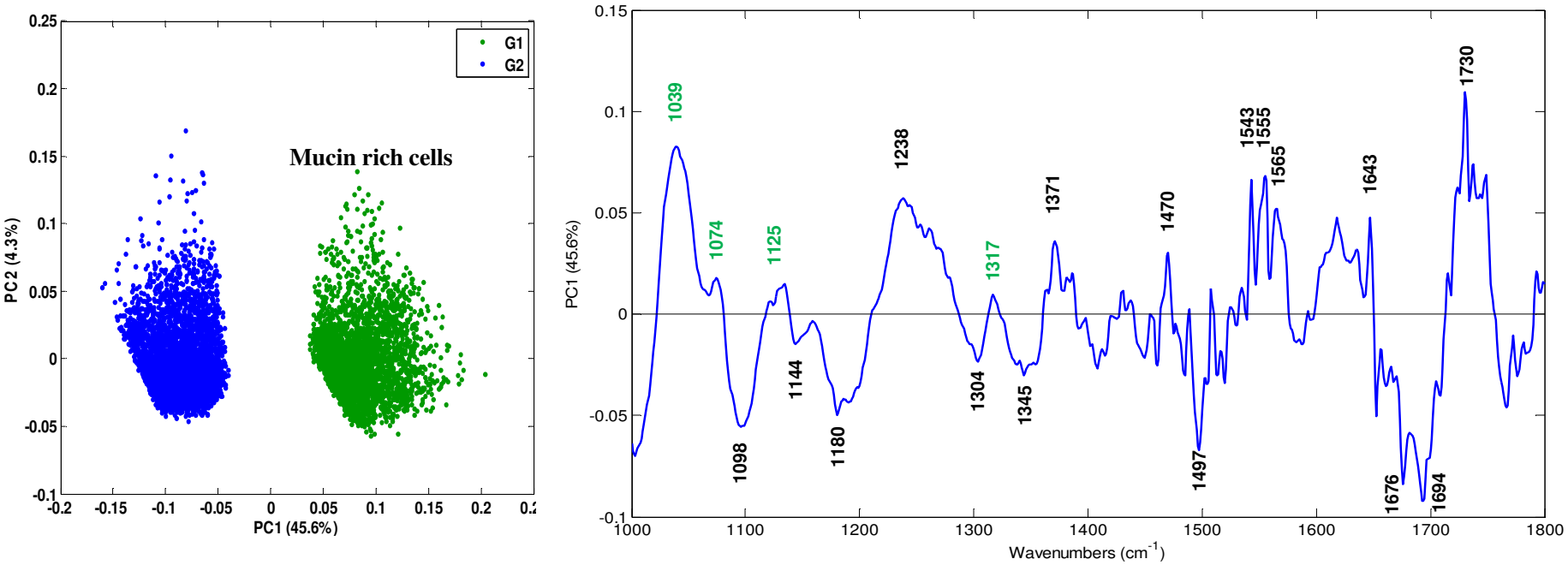
Imaging parameters:
Spectral resolution: 4 cm^{-1}
Pixel size: 5.5 and 1.1 µm^2
Scans/pixel: 64
Spectral range:1000-3900 cm^{-1}

Spectral pre-processing:
EMSC based corrections (baseline, normalization and mathematical de-paraffinization)

Multivariate statistical analysis:
K-means cluster analysis

J. Nallala, G. Lloyd, N. Shepherd, N. Stone; Analyst,141, 2016

Differentiating cell types: Principal component analysis (PCA)



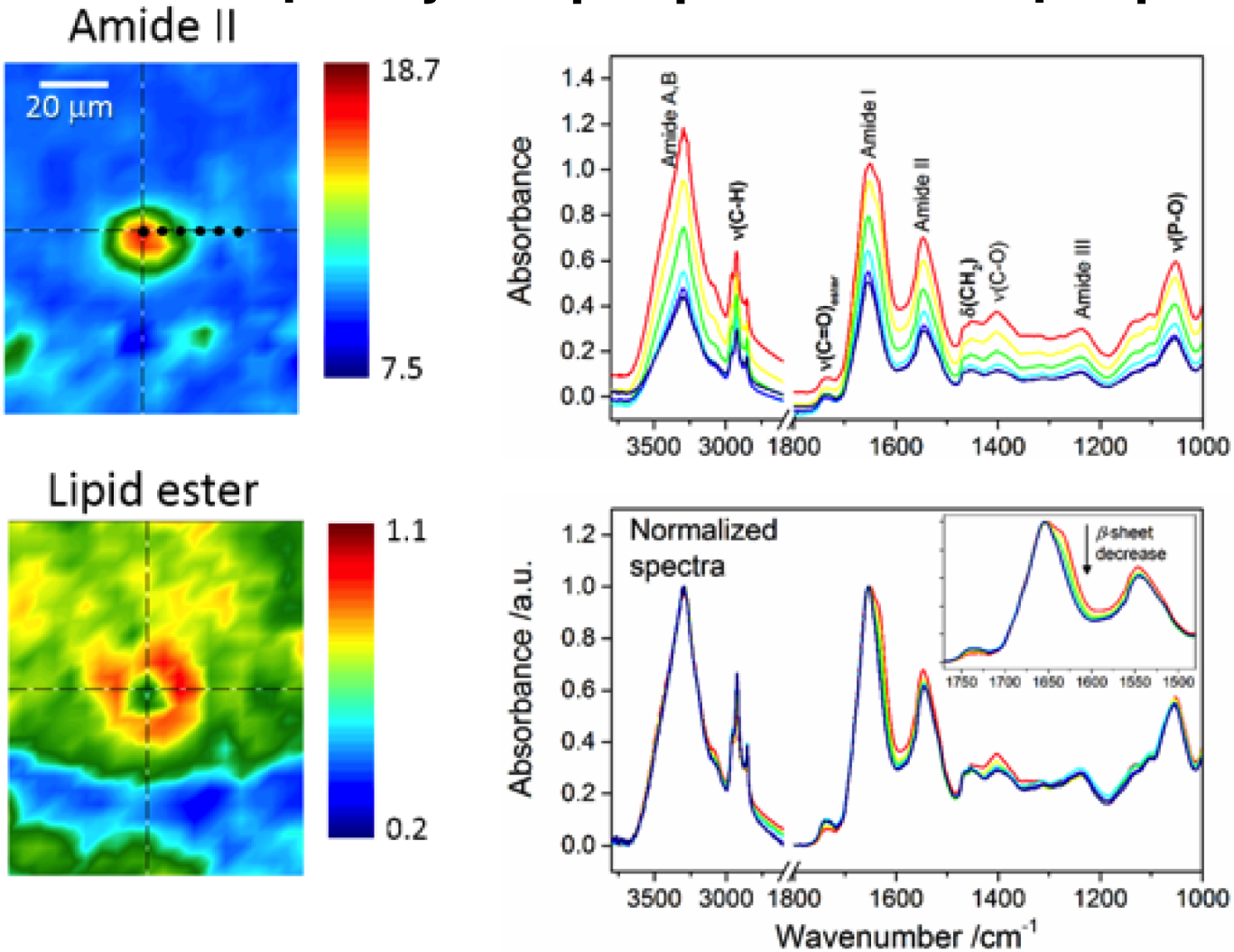
Principal component analysis (PCA) showing the score plot (left) and the loading (right) for the clusters 12 (G1) and 3 (G2). For the score plot, PC1 shows a clear separation with the highest explained variance. PC1 loading is plotted on the right which shows predominant mucin features (highlighted in green) separated from the other histological features.

High-resolution FTIR imaging of colon tissues for elucidation of individual cellular and histopathological features.

Jayakrupakar Nallala, Gavin Rhys Lloyd, Neil Shepherd and Nick Stone

Analyst, November 2015 **(DOI: 10.1039/C5AN01871D)**

Alzheimer's β-amyloid plaques with 5.5 µm pixels



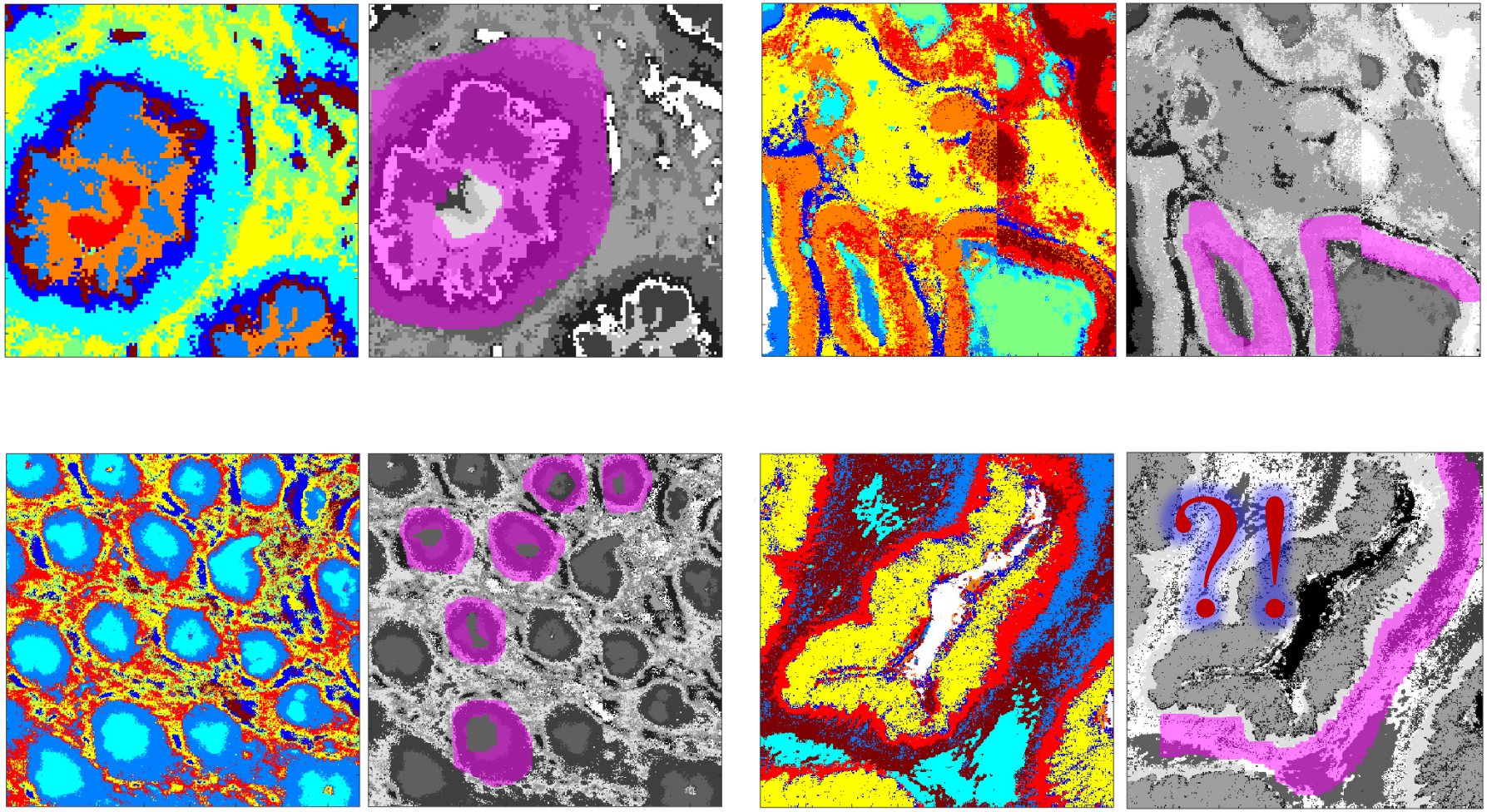
Agilent Technologies Cary 670 FTIR spectrometer coupled to a Cary 620 FTIR microscope with a 0.62 NA, 15× Cassegrain objective, and a liquid nitrogen-cooled focal plane array (FPA) detector. The detector has 16384 pixels arranged in a 128×128 array. The area imaged by micro-transmission through the 15× objective corresponds to 704×704 µm2.

Pathology type	Non-tumoral	Dysplastic	Hyperplastic	Cancer	Total
No. of samples	31	29	14	26	100
Status				T1-T3	

- **FTIR imaging**
  - Standard mode (5.5x5.5 μm^2)
  - High-magnification mode (1.1x1.1 μm^2)
  - Total 200 FTIR images
- **Pre-processing**
  - EMSC based mathematical de-paraffinization
  - Baseline correction
  - Normalization
- **Multivariate analysis**
  - K-means cluster analysis
  - Pathology attribution
  - PCA-LDA (LOSOCV) based discrimination

- **15,000,000 spectra retained !**
- **50 GB data cube !**

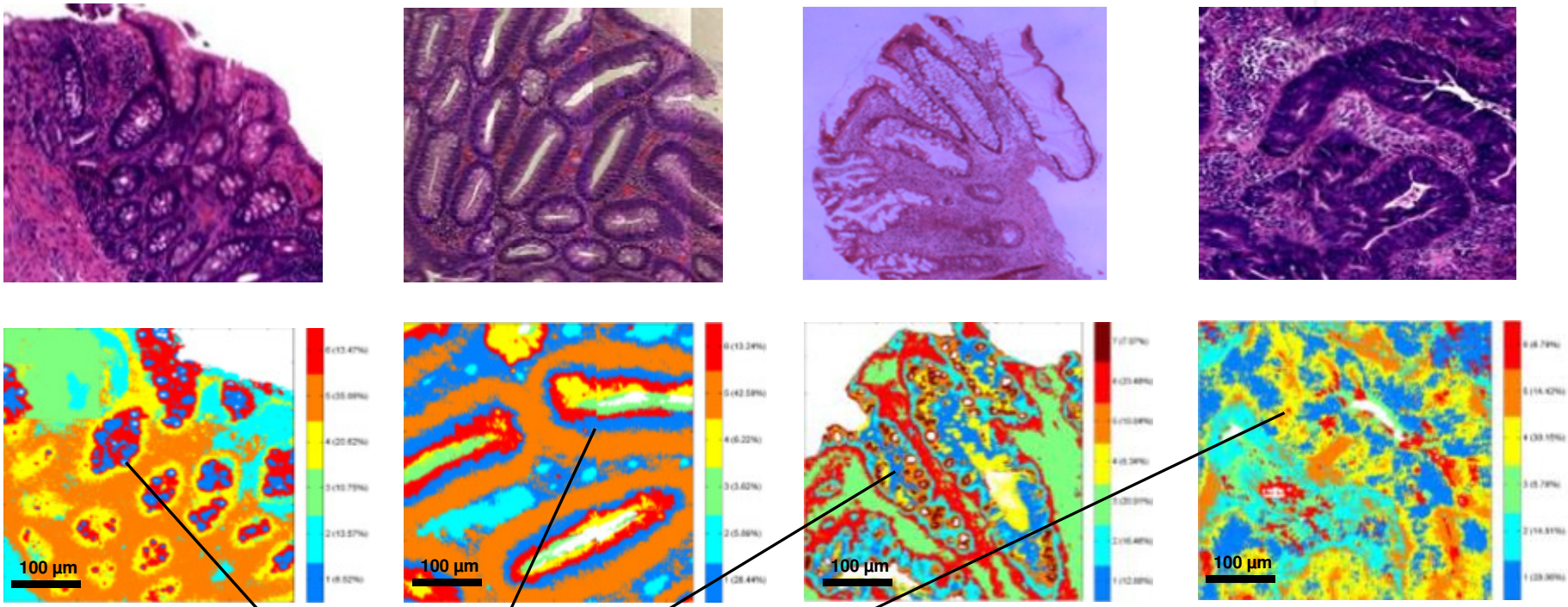
Clustering vs Manual

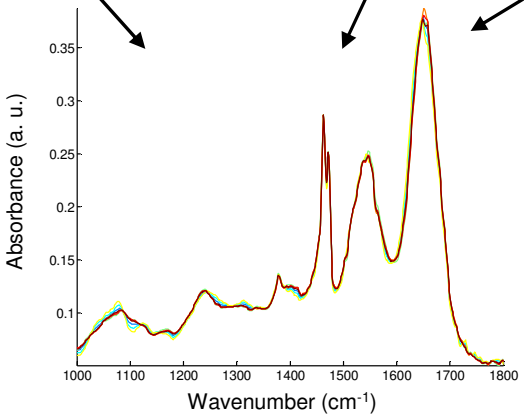


- **64 images; 5 pathologies**
- **Paraffin corrected**
- **K-means segmentation**
- **Manual segmentation (surgical registrar)**

Segmentation of histopathological features

Non-tumoral | **Dysplastic** | **Hyperplastic** | **Cancer**



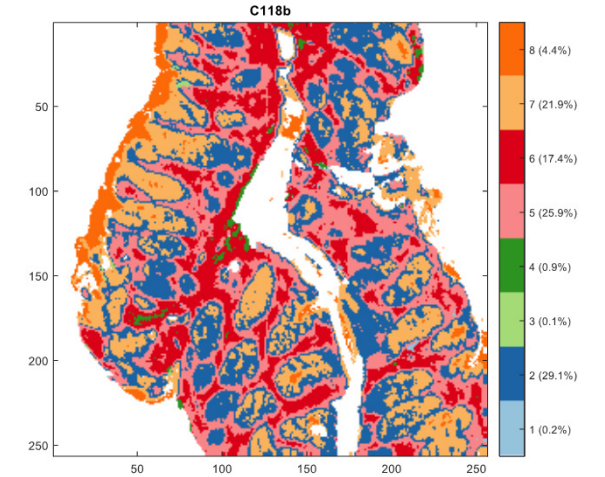


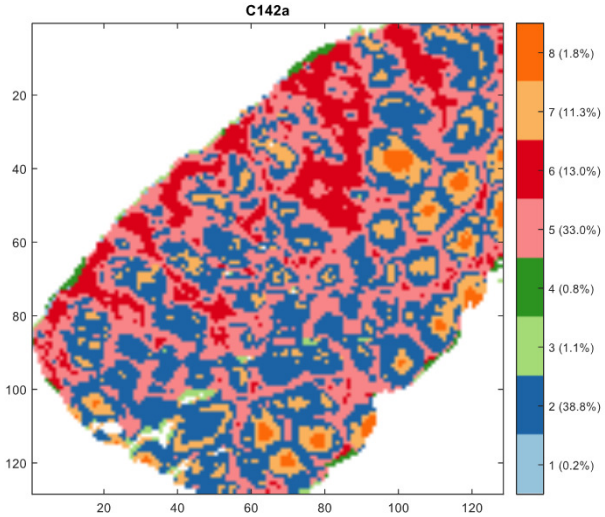
PCA-LDA
leave one sample out cross-validation
(per spectrum)

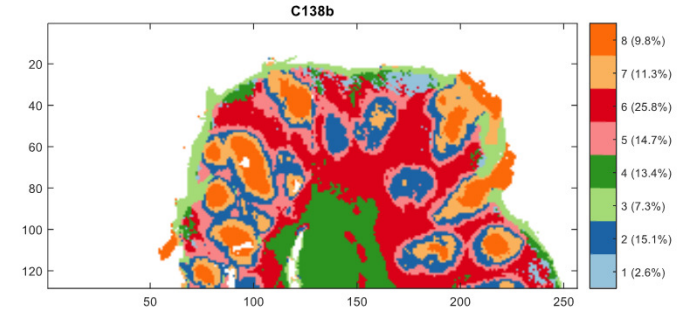
MINERVA 317803

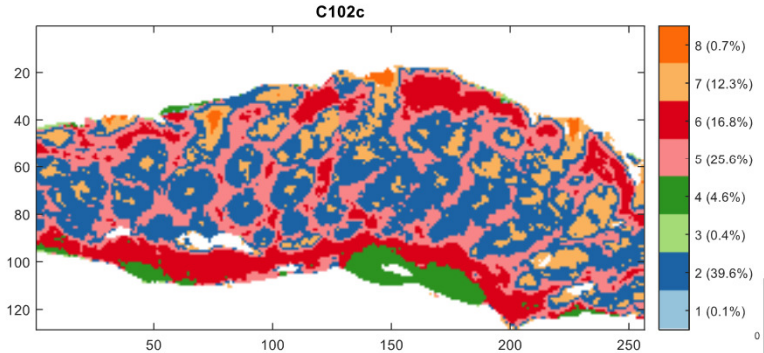
WP7

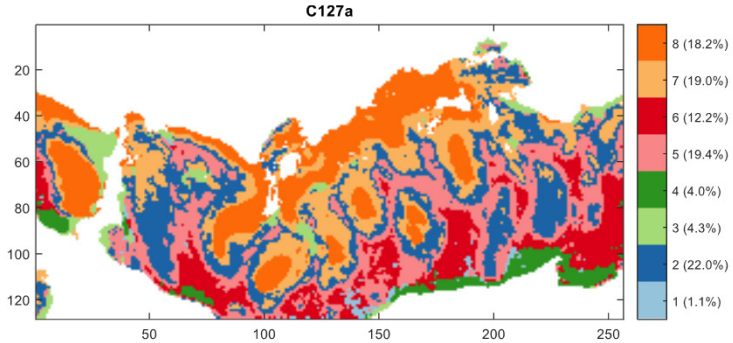
Common K-Means Clustering

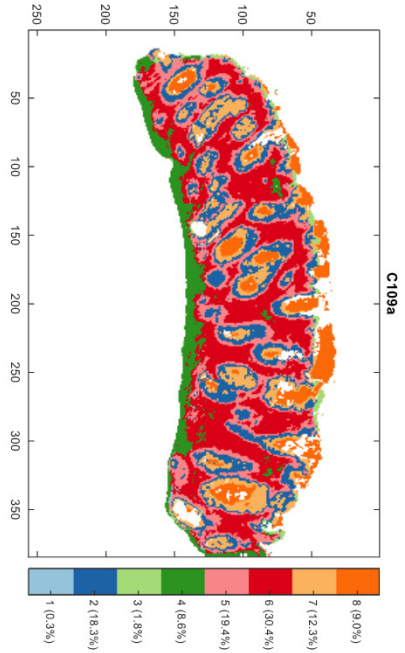


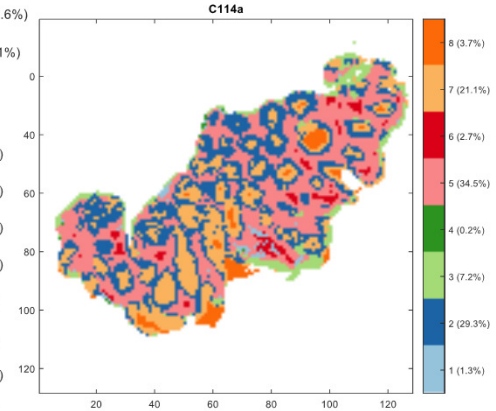












Gloucestershire Hospitals NHS
NHS Foundation Trust

LabHuman

UNIVERSITY OF EXETER

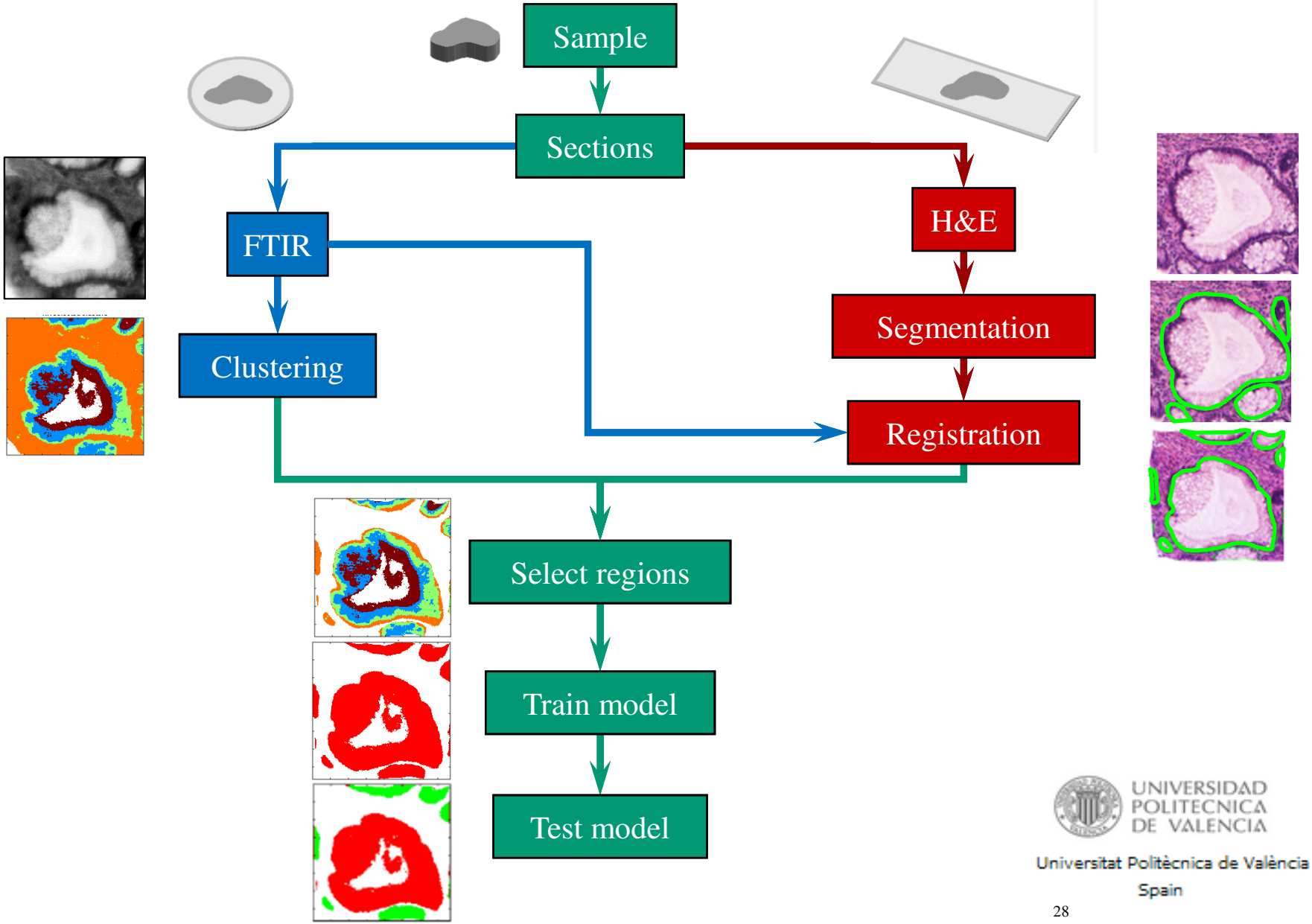
Segmentation using common K-means

Standard magnification

26 samples

6 classes
Epithelium
Stroma

Work flow for spectral histopathology



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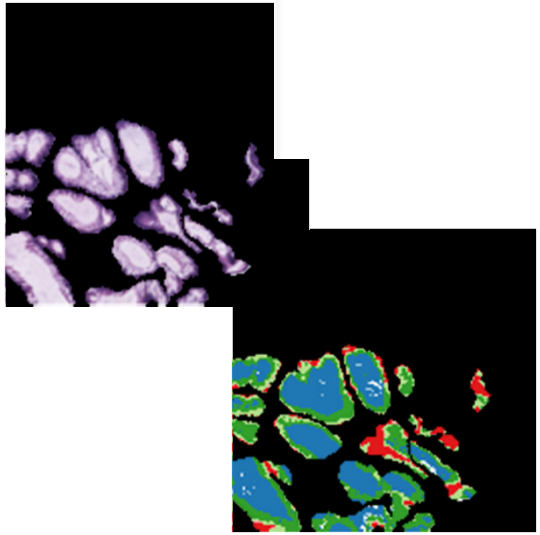
Universitat Politècnica de València

Spain

Results so far….

- PCA-LDA
  - Leave-one-map-out cross-validation
  - 2 groups (Normal vs Cancer)
- Standard magnification (5.5x5.5 μm^2)



	SN	SP	No. Spectra	No. samples
Standard k-means only (NM)	81%	82%	500K	57
Standard k-means only (HM)	80%	83%	3M	57
Common k-means only (NM)	84%	86%	260K	26
Segmentation + registration only (HE-IR)	79%	83%	770K	25
Segmentation + registration + K-means (HE-IR-KM)	90%	93%	81K	24

Discriminating cancerous tissues from non-cancerous

2 group model (N=57)
500K spectra NM / 3M spectra HM

Normal magnification	High magnification
SN = 81%	SN = 80%
SP = 82%	SP = 83%
t = x mins	t = 25x mins

- **The jury is out regarding high versus standard mag for discrimination – work on going**
- **Discrimination reduces with the addition of intermediate groups**
- **In the process of building large models with many patients / samples**

Raman: Pixel size versus classification performance

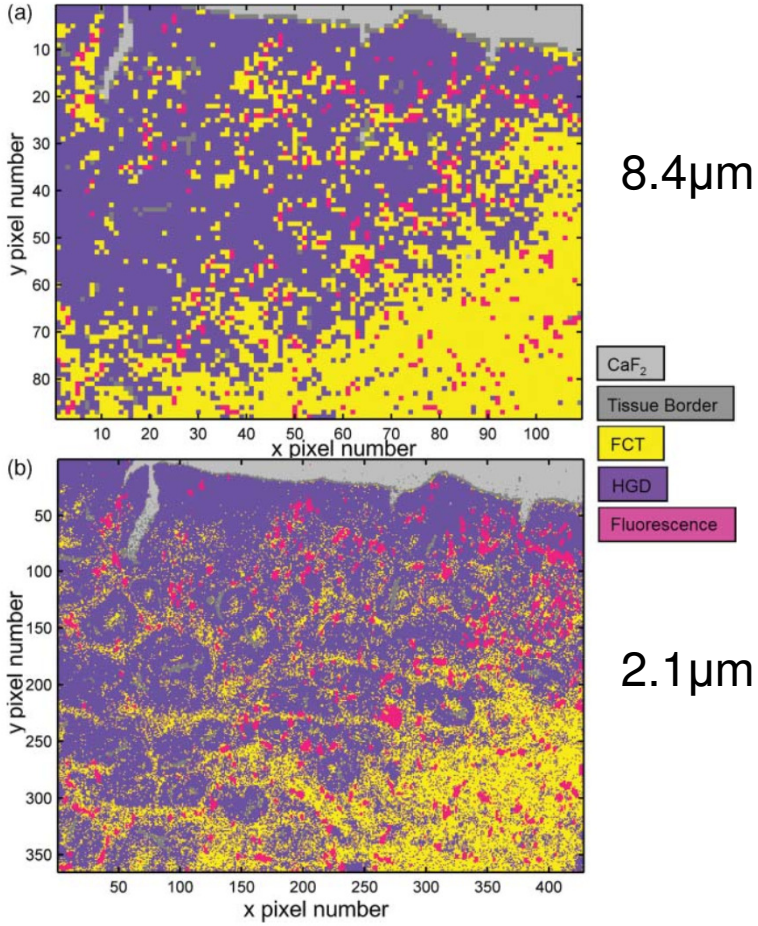


Fig. 3 Performance of the LDA tissue classification model (generated using sample 1: (a) 8.4-μm pixel size and (b) 2.1-μm pixel size. There is a subtle discontinuity (at x pixel number 210) in the 2.1-μm map, since the large map area was generated by joining two smaller maps together.

Table 1 Classification performance of the training dataset of the PCA-fed LDA model (8.4- and 2.1-μm pixel sizes, 15-s acquisition time) measured on sample 1.

		CaF_2	TB	FCT	HGD	Fl	Correctly classified (%)
8.4 μm	CaF_2	374	7	0	0	0	98.2
	TB	14	150	24	1	0	79.4
	FCT	0	0	990	4	6	99.0
	HGD	0	3	196	3420	43	93.4
	Fl	0	2	0	0	165	98.8
2.1 μm	CaF_2	5541	543	0	0	0	91.1
	TB	187	1352	11	0	0	87.2
	FCT	0	1	5399	0	0	100.0
	HGD	0	125	3951	65,813	714	93.2
	Fl	0	4	2	0	4067	99.9

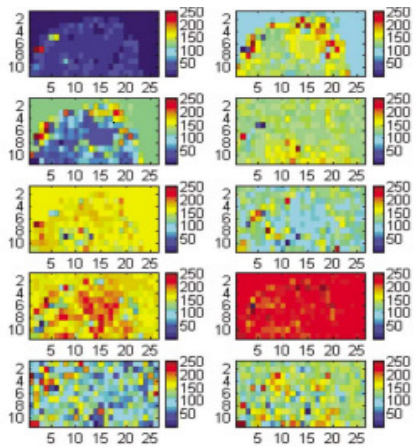
TB = Tissue border…

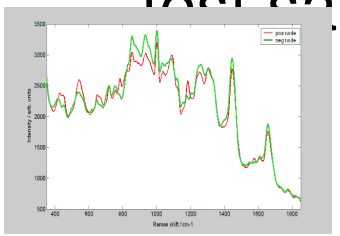
BIOSPEC Biomedical Spectroscopy Labs

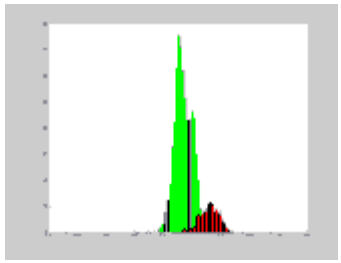
Journal of Biomedical Optics 15(6), 000000 (November/December 2010)

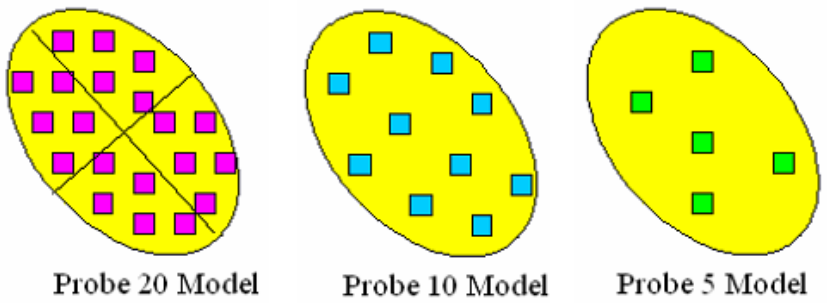
UNIVERSITY OF EXETER Biomedical Physics

53 Axillary nodes - LDA Probe models to test sampling options - LONO-CV







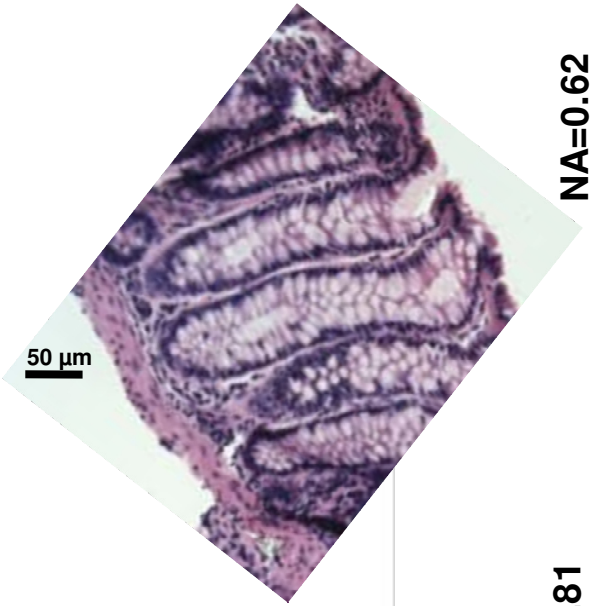


	All Spectra		20 Point	10 Point	5 Point
Sensitivity (%)	86		86	82	82
Specificity (%)	93		90	93	91

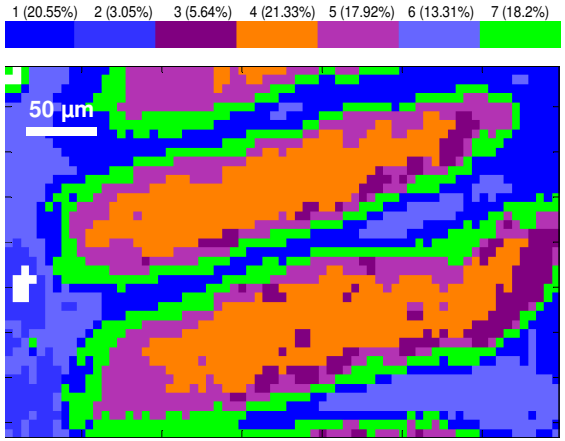
Smith et al., *Technology in Cancer Research and Treatment*, 2003.

Comparison of different NA's and magnifications

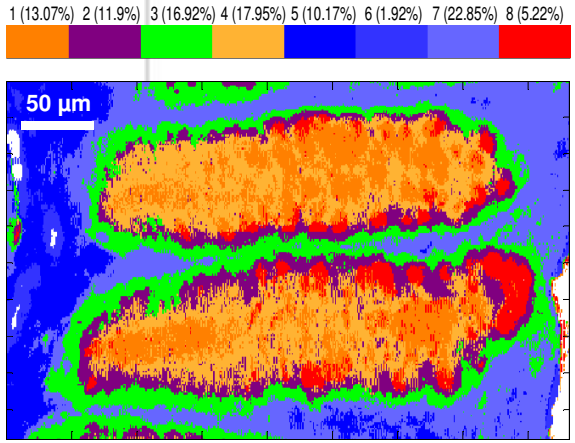
HE reference image



A. Conventional magnification (7X)
(Pixel size: 5.5 x 5.5 µm²)

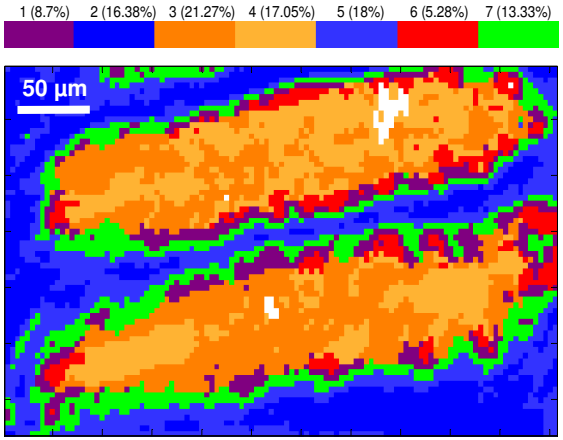


B. High magnification (36X)
(Pixel size: 1.1 x 1.1 µm²)

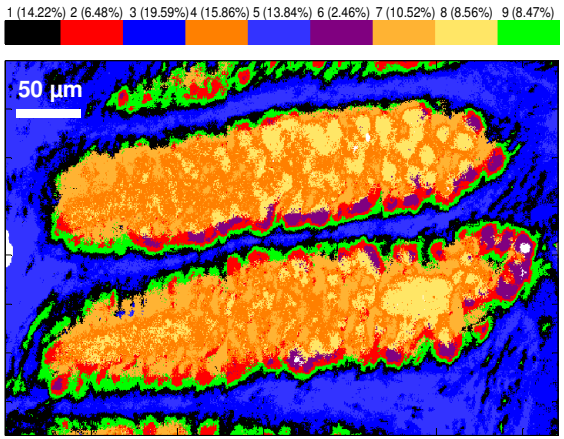


NA=0.62

C. Conventional magnification (12X)
(Pixel size: 3.3 x 3.3 µm²)

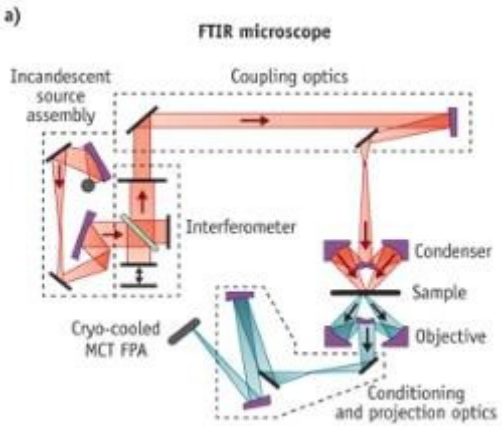


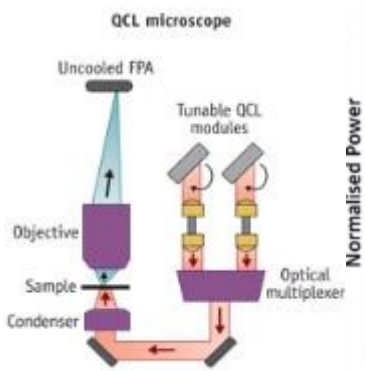
D. High magnification (63X)
(Pixel size: 0.66 x 0.66 µm²)

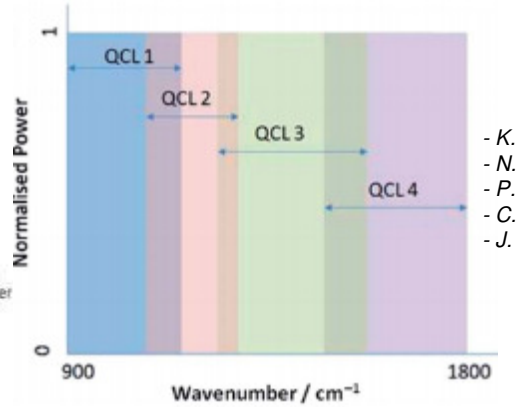


NA=0.81

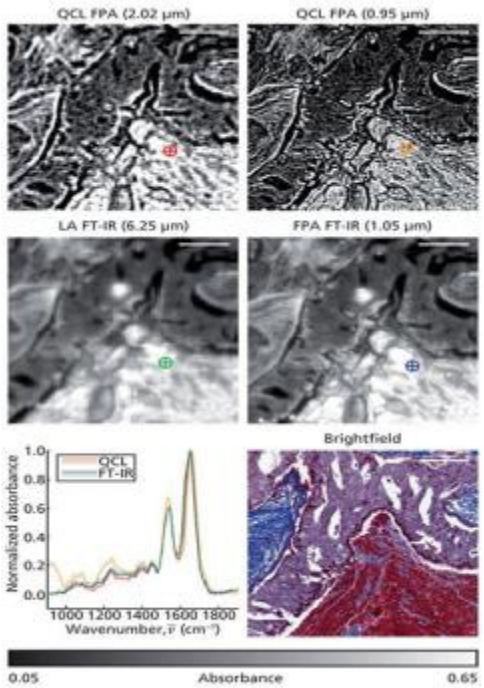
Recent advances – discrete frequency imaging



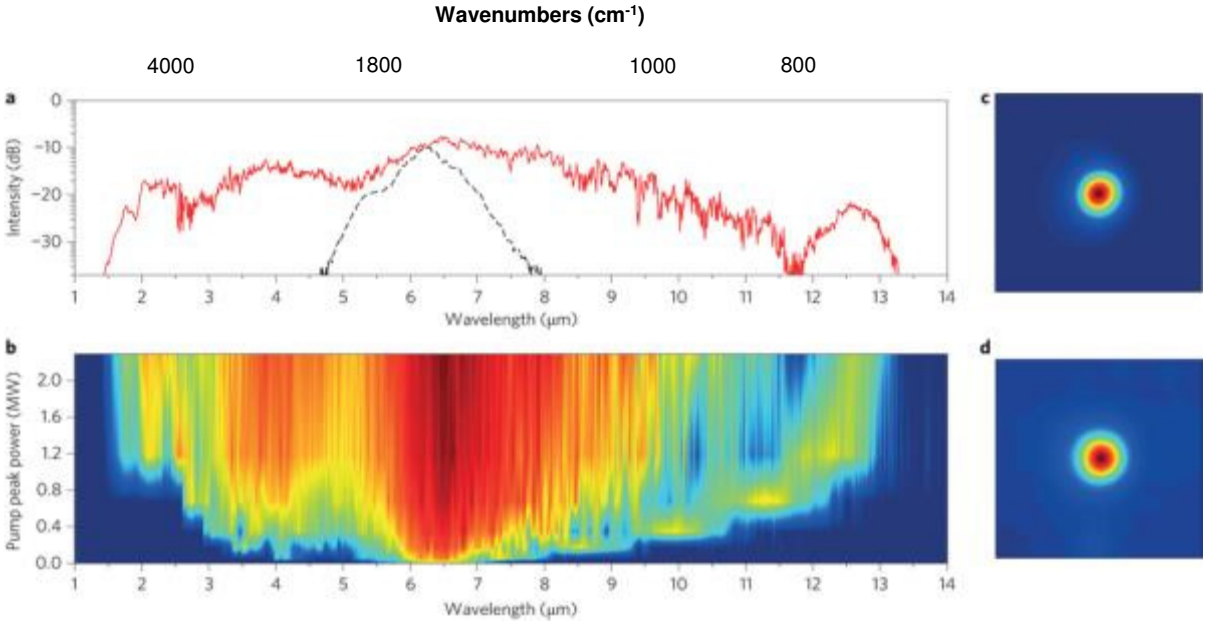




- K. Yeh, R. Bhargava, et al, ***Anal. Chem., 2014***
- N. Kröger, W. Petrich, et al, ***J. Biomed. Opt., 2014***
- P. Bassan, P. Gardner, et al, ***Analyst, 2014***
- C. Hughes, M. Baker et al, ***Scientific reports, 2016***
- J. Rowlette, B. Bird, et al ***BioOptics World, 2014***

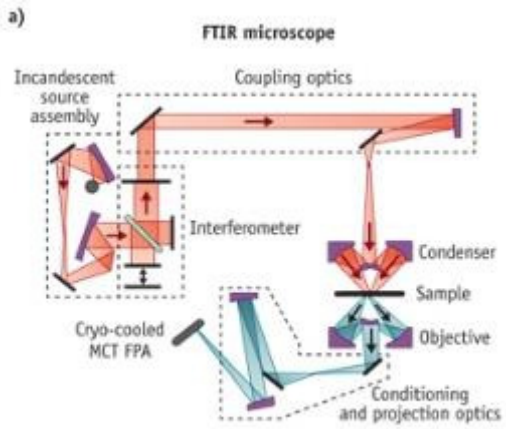


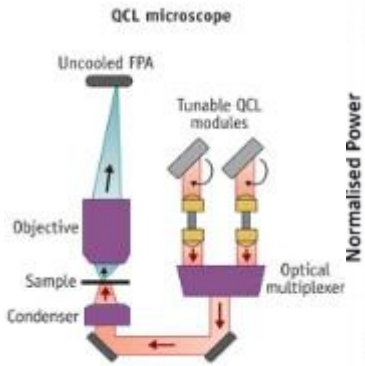
R. Bhargava, et al, Anal. Chem., 2014

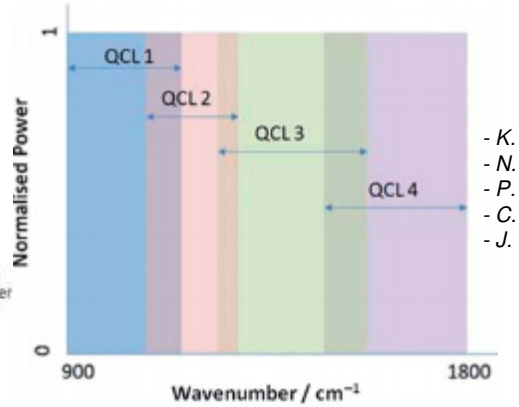


Christian Petersen, et al, Nat. Photonics, 2014

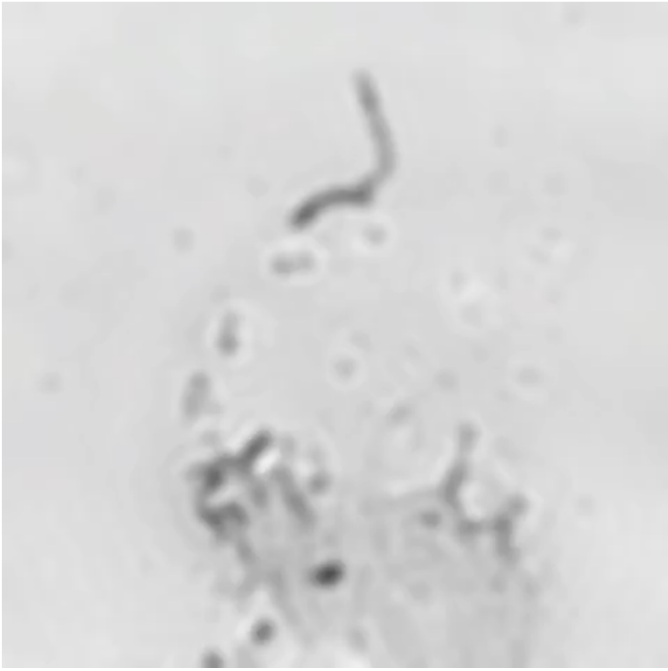
Recent advances – discrete frequency imaging

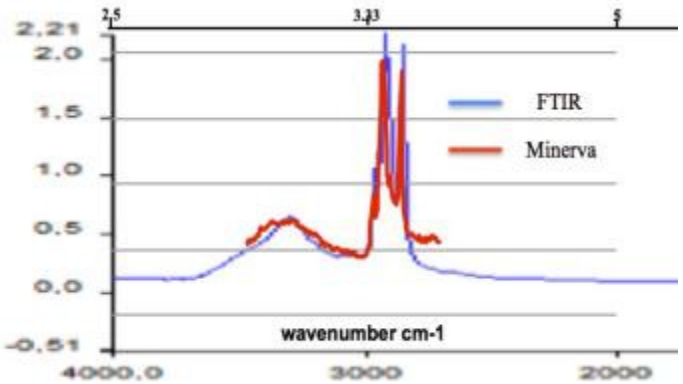


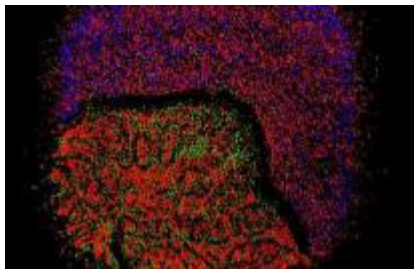




- *K. Yeh, R. Bhargava, et al, **Anal. Chem., 2014***
- *N. Kröger, W. Petrich, et al, **J. Biomed. Opt., 2014***
- *P. Bassan, P. Gardner, et al, **Analyst, 2014***
- *C. Hughes, M. Baker et al, **Scientific reports, 2016***
- *J. Rowlette, B. Bird, et al **BioOptics World, 2014***







- Supercontinuum source (2 to 4.5 µm)
  0.5 milliwatt/nm

- Acousto-optic tuneable filter (5 cm^{-1} spacing)

- Mid-IR camera (500x600 pixels)

- 160 frequencies, Transmission mode

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Quantum cascade laser–based hyperspectral imaging of biological tissue

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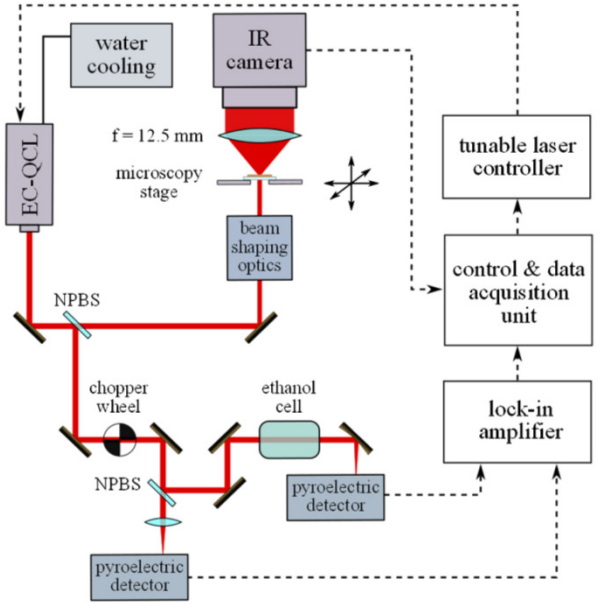
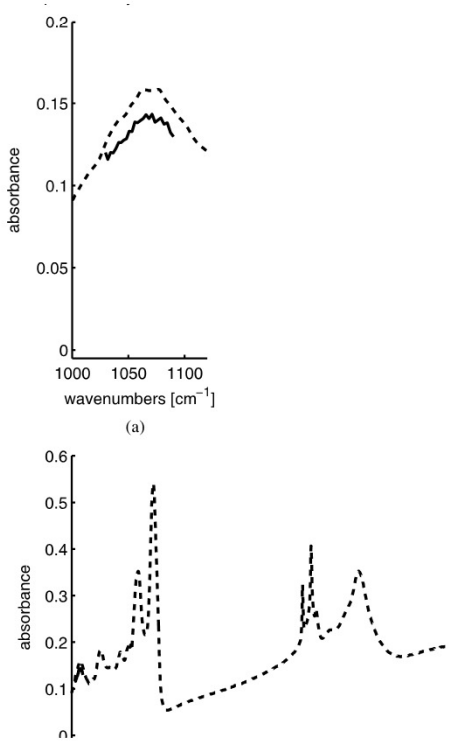
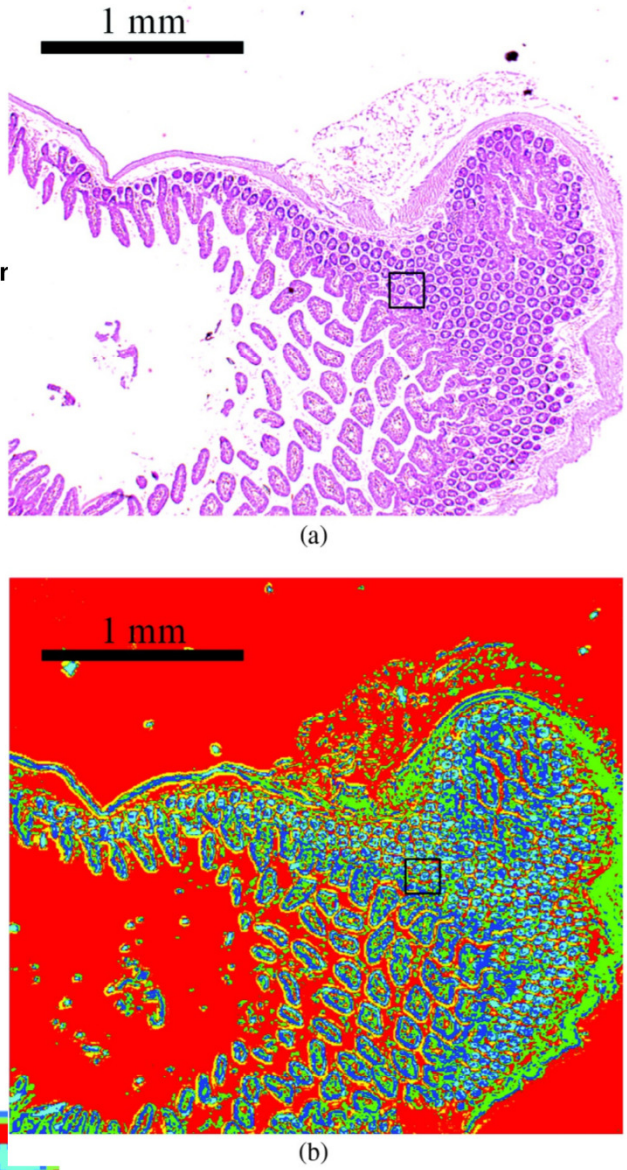


Fig. 1 Experimental setup: Infrared radiation is emitted from the external cavity quantum cascade laser (QCL). The beam is split by a nonpolarizing beam-splitter for simultaneous power- and wavelength-monitoring as well as sample illumination. NPBS, nonpolariz-





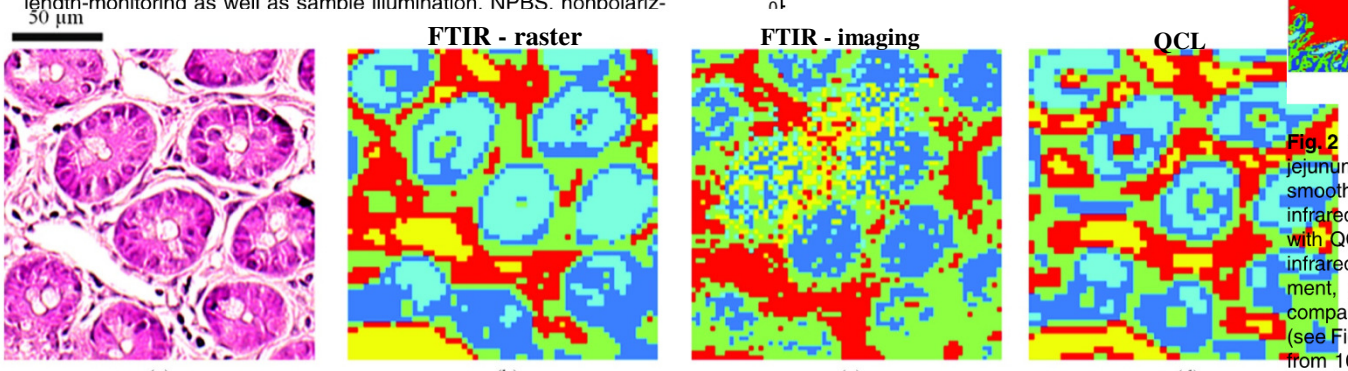


Fig. 2 (a) Image of haematoxylin and eosin-stained section of mouse jejunum. The center of the slice shows villi surrounded by crypts and smooth muscle tissue of the lamina muscularis. (b) Corresponding infrared label image of the adjacent unstained tissue slice, measured with QCL-based imaging. The total acquisition time of the complete infrared image of 3.1×2.8 mm^2, including the reference measurement, amounted to 5 min. The box indicates the area used for comparing the QCL-based imaging with other infrared methods (see Fig. 3). The clustering was performed in the spectroscopic region from 1030 to 1090 cm^{-1}.

Preliminary conclusions and future plans

- Spectral discrimination between tissue-type works well in comparison to the gold-standard

  Epithelial tissue, connective tissue., etc

- Higher magnification is providing greater understanding of biochemical changes associated with pathological cells and tissues

-Discrimination between pathologies is promising for the two-group model:

  Normal and Cancerous

- Discrimination capabilities reduces with the addition of intermediate groups

  Adenomatous and hyperplastic

- Efforts underway to test more discriminant models to separate different pathologies of colon tissues

- Key discrete frequencies for discrimination to be identified and targeted

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MINERVA improved medical diagnostics

SEVENTH FRAMEWORK PROGRAMME

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Gooch & Housego
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NKT Photonics the power of light
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3	LISA Laser Products OHG	D
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12	Gloucestershire Hospitals NHS Foundation Trust	UK
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