QCL imaging of tissues and biofluids
MALDI imaging of fingermarks
Assessment of therapeutic protein candidates
FT-IR spectroscopy of pollen
Quantum cascade laser-based mid-infrared spectrochemical imaging of tissues and biofluids

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Introduction
Mid-infrared spectroscopic imaging is a rapidly emerging technique in biomedical research and clinical diagnostics that takes advantage of the unique molecular fingerprint of cells, tissue and biofluids to provide a rich biochemical image without the need for staining. Spectroscopic analysis allows for the objective classification of biological material at a molecular level. This "label-free" molecular imaging technique has been applied to histology, cytology, surgical pathology, microbiology and stem cell research, and can be used to detect subtle changes to the genome, proteome and metabolome.1-3 The new wealth of biochemical information made available by this technique has the distinct potential to improve cancer patient outcome through the identification of earlier stages of disease, drug resistance, new disease states and high-risk populations.4 However, despite the maturity of this science, instrumentation that provide increased sample throughput, improved image quality, a small footprint, low maintenance and require minimal spectral expertise are essential for clinical translation.

The latest generation of Fourier transform infrared (FT-IR) spectrometers, that incorporate large liquid nitrogen cooled focal plane array (FPA) detectors within an infrared microscope system, have no doubt accelerated the development of the field. An FPA detector is a 2D device that is sensitive to the infrared region of the electromagnetic spectrum and consists of an array of photon-sensitive pixels on the focal-plane of a lens.5 Significant improvements in data acquisition, processing and classification times, in part due to the increased field of view (FOV) of these FPA based devices, have enabled spectroscopic investigations that now include clinically relevant patient populations.5 Nevertheless, despite these marked improvements when compared to linear detector array (LDA) or point detector based systems, data collection times from tissue micro-array (TMA) cores or whole tissue sections are still of the order of hours or days.6 The problem can be characterised in part by the trade-off that must be made between spatial and spectral resolution, and the signal-to-noise ratio required to provide robust spectral classification. Utilising a low magnification objective increases the FOV and signal-to-noise ratio of the recorded data, thereby allowing larger areas of the sample to be imaged more rapidly. However, smaller spatial features within the sample cannot be resolved, so consequently there is a need for high definition, diffraction-limited, spatial resolution for the identification and classification of early stages of disease. However, without the photon throughput available from a synchrotron source, and the requirement of high magnification reflective optics that offer a much reduced FOV, acquisition times can dramatically increase to acquire images of adequate size and signal-to-noise ratio. Furthermore, the multiplex advantage exploited by traditional Fourier transform based systems can be computationally prohibitive, requiring large amounts of readout data to be processed before subsequent reduction to only a few key wavelengths or spectral biomarkers used for classification. This has led to a debate within the spectroscopic community as to whether all spectral frequencies need to be collected once a set of specific spectral biomarkers have been identified, and thus instead explore more efficient non-interferometric methods of data collection.

Quantum cascade laser based mid-infrared imaging
Recently, broadly tunable mid-infrared quantum cascade lasers (QCLs) have been successfully integrated within a microscope for spectrochemical imaging across the molecular fingerprint region.8 The main components of this type of microscope include multiple QCL modules, an optical multiplexer, a condenser, a switchable objective, an automated stage and an FPA detector system. No longer requiring an interferometer, simpler instrument architecture can be achieved allowing a reduced footprint; approximately a third of high-end commercial FT-IR bench and microscope systems.8 The high brightness of these broadly tunable QCL sources has
also enabled the use of large format (480 × 480 pixels) uncooled micro-
obolometer detector systems, removing the need for a cryogenically cooled
mercury cadmium telluride (MCT) based detector system. Of particular note has
been the development of purpose designed high numerical aperture (NA),
achromatic, wide-field and refractive-
based infrared objectives. The unique
combination of a broadly tunable laser
source, refractive-based objectives opti-
mised for coherent light and a large
format detector system has enabled
high-definition diffraction-limited resolu-
tion, without a trade-off in signal-to-noise
and field of view, as associated with
FT-IR microscopes with their extended
global thermal light sources. Utilising a
tunable laser as a source also provides
new modalities of data collection not
previously available. Real-time discrete
frequency spectrochemical imaging at
30 frames per second is a modality that
can provide a number of unique appli-
cations, allowing the user to quickly
screen large samples, moving back and
forth between a handful of impor-
tant frequencies that enhance chemi-
ical contrast of the sample and help
segment areas of disease. This type
of modality would suit intra-operative
tumour screening of frozen biopsy tissue
or multiplexed chemotyping of cells or
biofluids. Another unique modality is the
ability to perform a sparse frequency
data collection, whereby a target set of
discrete frequencies can be collected.
During the learning phase of a spectral
diagnostic, it is clearly wise to acquire
data from the full spectral range in order
to mine the spectral patterns between
class types. However, once the learning
phase is complete, and the most diag-
nostic spectral features have been iden-
tified, a targeted list of only a few key
wavelengths needs to be collected by
the instrument, thereby greatly improving
acquisition time and sample throughput.

**High-resolution mid-infrared
spectrochemical imaging of tissue**

The current method for diagnosis of
cancer is histopathology. This method
requires a trained pathologist, often a
specialist (e.g. neopathologist), to inter-
pret morphometric changes in cellular
and tissue architecture to render a diag-
nosis. The technique has a long diag-
nostic window and can be prohibitively
subjective.5 Limitations with this diagnos-
tic modality have led to an interest in the
development of spectroscopic analytical
techniques for the diagnosis of cancer.10

All mid-infrared based microscope
systems are inevitably limited by a trade-
off between spatial resolution, signal-to
noise ratio, field of view and acquisi-
tion time. Traditionally with a conven-
tional bench-top FT-IR-based system, to
achieve true diffraction-limited resolu-
tion, high magnification objectives with
the largest possible NA are required that
provide a much reduced field of view
and extended sample dwell times to
achieve an acceptable signal-to-noise
ratio for tissue classification purposes.11

Previous rigorous analysis indicates
that it is also imperative to provide an
effective pixel spacing of ~λ/4 assum-
ing the best commercially available NA of
~0.65. However, these calculations
were based on models of a circular
objective, whereas a classical Cassegrain
objective is annular. Nevertheless, when
combining high magnification objectives
on the order of 36× and above, with a
128 × 128 pixels FPA detector system, the
best FOV achievable is on the order of
150 µm × 150 µm with a pixel resolution
of 1.1 µm. Given these limitations the
application of high resolution imaging
for spectral tissue classification has been
limited, with a preference to perform lower
resolution imaging with a larger
FOV in order to increase sample through-
put. More recently, however, the applica-
tion of high resolution imaging for clinical
diagnostics has shown clear advantages
for the identification of small tissue struc-
tures that are essential for detecting early
forms of disease.12 Traditional histochem-
ical and immunohistochemical staining,
despite their drawbacks of inter-observer
variability of morphological interpre-
tation, are still very reliable for gross
tumour identification and subtyping,
providing a difficult technology to disrupt
with an infrared spectral diagnostic.
However, mid-infrared spectral imaging
is more likely to make a real and bene-
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and a pixel size of 1.36 µm for each tile. Data was acquired from 900 cm⁻¹ to 1800 cm⁻¹ with a spectral resolution of 4 cm⁻¹ and took 2.1 h. The FT-IR microscope utilised a 15× magnification objective with a NA of ~0.65 to provide a FOV of 700 × 700 µm and a pixel size of 5.5 µm for each tile. Data was acquired from 950 cm⁻¹ to 3800 cm⁻¹ with a spectral resolution of 4 cm⁻¹, 1× zero filling factor, Blackman Harris apodisation and took 2.25 h. All spectroscopic data was acquired in transmission geometry.

The higher-resolution data acquired using the QCL microscope system clearly provide images with superior clarity and allow fine tissue features to be visualised. By enabling small but important tissue features to be resolved, spectrochemical information can be unlocked to not only identify early and rare forms of diseases, but also isolated primary and metastatic tumour cells, providing the potential to uncover previously unavailable information for the clinical sphere. In addition, once a subset of diagnostic discriminatory frequencies has been identified (see biofluid section below), acquisition times can be markedly reduced since a QCL-based system can, as mentioned above, be tuned to only acquire data from frequencies of interest, and therefore allow more clinically relevant timescales to be achieved. This technology has a huge potential to help enable spectrochemical histopathology and drive forward the technique being applied in surgical pathology for highly important intra-operative decisions.

**Wide-field mid-infrared spectrochemical imaging of biofluids**

Biofluids (e.g. urine and serum) provide an easily accessible, relatively non-invasive sample for analysis, the collection of which can be performed worldwide and in the field. The application of mid-infrared spectroscopy to characterise and classify human biofluids is a rapidly emerging field with a multitude of researchers now providing strong evidence that both primary and metastatic cancers and other autoimmune diseases can be identified and robustly classified. The success is due in part

**Figure 1.** Spectral images of the 1650 cm⁻¹ band intensity across medulloblastoma (A and B) and glioblastoma (C, D, E and F) tissue cores (approximately 1.5 mm diameter and 5 µm thickness) imaged using a Spero QCL microscope (A, C and E) and a 128 × 128 FPA microscope system (B, D and F). E and F are zoomed images of the areas highlighted by the white boxes on C and D of the glioblastoma tissue core. Spectra from the areas highlighted in yellow on C and D are shown for the QCL microscope (G) and for the FT-IR microscope 128 × 128 system (H).
by the application of a holistic approach, whereby the entire biochemical make-up of the biofluids is scrutinised, rather than a small subset of biomarkers that can often result in misleading interpretations.\textsuperscript{13} Research to date has been performed using a variety of different techniques, namely attenuated total reflection (ATR) and conventional imaging of dried biofluids spots.\textsuperscript{15–17} However, a viable spectroscopic method for high throughput multiplexed screening of large patient populations is still lacking. One route toward this goal could be the rapid infrared imaging of biofluid spots that have been prepared in a patient orientated grid map. For example, the x-dimension of such a grid could describe a patient’s biofluid profile from different organs or extracted biochemical components therein, and the y-dimension different patients. When utilising a sessile drop technique, circular films on the order of 1–2 mm in diameter are formed when using 0.5 µL of fluid; the smallest reliable measurement using handheld pipettes. The dried spots can describe variable topology, but provide what is more commonly known as the coffee stain effect, whereby a thick ring is formed on the surrounding edges of the spot. Within this ring the data recorded is often compromised since the absorbance intensities measured for protein bands can often be beyond the linear range of the detector response and show

![Figure 2.](image-url)

Figure 2. (A) Mosaic image of serum spots acquired using 14 discrete frequencies from non-cancer, brain cancer, lung cancer, breast cancer and skin cancer patients; (B) baseline-corrected and vector normalised serum spectra extracted from the centre of the non-cancer and brain cancer serum spots; (C) mean spectra from each non-cancer (black) and brain cancer (blue) serum spot; (D) PCA scores plot of brain cancer serum spectra (blue) and non-cancer serum spectra (black) and (E) peak centroid distribution plot of the Amide I and Amide II bands for the brain cancer (blue) and non-cancer (black) serum spectra.
saturation. However, within the middle of the spot, a large area can be actively extracted for spectral analysis. What is also inherently different with dried biofluids, and somewhat advantageous over tissue or cell analysis, is the relatively homogenous sample morphology that in turn provides spectral data relatively free of adverse scattering. Spectral profiles show little, if any, broad baseline oscillation allowing robust classification algorithms to be constructed without rigorous scattering correction.

By use of the low magnification refractive-based objective of the QCL microscope system, which has a NA of 0.15 and a magnification of 4×, a FOV of 2 × 2 mm can be achieved. This objective allows an entire dried biofluid spot to be examined in a single camera shot, with a sample-referred pixel size of 4.25 µm and a spatial resolution on the order of 25 µm at 1650 cm⁻¹. Given the homogenous nature of the sample deposition, spatial resolution is not an absolute necessity for this type of analysis, and the wide-field imaging capability allows for a more rapid assessment of the entire dried spot. Since these types of samples do not provide adverse scattering profiles they are also ideal candidates for a sparse frequency data collection protocol, whereby a reduced number of discrete wavelengths are targeted rather than collecting a more time prohibitive full spectrum.

Figure 2 displays spectrochemical imaging analysis of a spotted human serum sample prepared onto a CaF₂ substrate. Each serum spot was deposited manually using a micro-pipette and 0.5 µL of sample per patient, to form a grid of 2 × 5 spots. The columns of the grid describe patients diagnosed as having a normal, brain, lung, breast and skin cancer diagnoses, respectively. Each deposited serum spot was ca 2 mm in diameter. The entire grid of serum spots was subsequently analysed using the wide-field 4× objective of the QCL microscope system using a discrete frequency collection protocol. Intensity values at 14 discrete wavenumbers (1000, 1030, 1080, 1482, 1520, 1546, 1570, 1600, 1630, 1654, 1686, 1726, 1734 and 1770 cm⁻¹), associated with the peak maxima and shoulders of absorption bands associated with lipid, protein, nucleic acid and carbohydrate macromolecules, were recorded from a total of 24 frames in a total time of 50 min (ca 2 min per tile); these absorption bands have previously been shown to be salient discriminating bands when comparing ATR/FT-IR recorded spectra from cancerous and non-cancerous blood serum spots.¹⁶,¹⁷ The image in Figure 2A was constructed by plotting the recorded intensity value at 1654 cm⁻¹ and allows the serum morphology to be easily visualised, often highlighting the coffee ring like shape of the serum deposits’ outer rims; spatial areas of the image mosaic highlighted as red correspond to high absorption at 1654 cm⁻¹ and spatial areas of the image mosaic highlighted as blue correspond to no absorption or very little absorption at the 1654 cm⁻¹. By use of a quality test that probes sample absorbance, a threshold criterion was utilised to extract pixels that were contained within the central part of the serum spots and did not describe saturation of the Amide I and Amide II protein specific bands. The remainder of the multivariate data (chemometric) analysis was performed on 11 discrete frequencies (1482, 1520, 1546, 1570, 1600, 1630, 1654, 1686, 1726, 1734 and 1770 cm⁻¹), that predominantly describe protein and lipid profiles, in order to assess their ability to segment healthy from diseased patients. Figure 2B displays all extracted spectra after baseline correction and vector normalisation. Figure 2C conversely displays the average spectra calculated for the two normal (black) and two brain cancer (blue) serum spots. Even at this very basic mean spectrum level, spectral differences between these groups can be visualised. A more comprehensive chemometric analysis using all of the spectra extracted from the normal and brain cancer serum spots was performed using both principal component analysis (PCA), as shown in Figure 2D, and peak centroid analysis, as shown in Figure 2E. PCA analysis was performed using all 11 discrete frequencies of the data, whereas the peak centroid analysis was performed using the nine frequencies associated with the Amide I and Amide II bands alone. Each dot in the figures represents a single pixel spectrum, whereby the black colour denotes a normal diagnosis (3242 spectra), and the blue colour brain cancer (1899 spectra).

These preliminary results (shown in Figure 2) clearly show that through recording data at a reduced number of wavelengths the structural shape of the Amide I and II absorption bands can still be maintained. Thus, band shifts in frequency and band structural shape changes due to disease state can still be captured from the recorded data. Therefore, the results highlight the time advantage that can be made when adopting a sparse frequency collection paradigm for diagnostic applications, with no resulting penalty in the accuracy of data classification. Salient features for the differentiation of non-cancer and brain cancer serum spots can be easily extracted with PCA and then targeted by a sparse frequency data collection, achieving robust classification using the shift in peak centroid of the Amide I and Amide II bands alone. Previous investigations of the serum samples used in this study, whereby spectral data was collected using a traditional FT-IR based system from the entire mid-IR window (4000–400 cm⁻¹), identified the very same peaks and features as being implicated in the discrimination of disease states.¹⁶,¹⁷ However, such data sets comprised intensity values from over 900 wavelength dimensions, the overwhelming majority of which are not important for classification purposes, and can necessitate computationally expensive Fourier transformation and pertinent wavelength extraction. The use of a tunable laser-based microscope system has conversely allowed the focused acquisition of 14 pertinent mid-infrared spectral features required for classification, representing a step change in spectral image collection speed, within a clinically relevant time scale. Further studies are ongoing and include the utilisation of the nucleic acid and glycation associated discrete frequencies to segment multiple primary and metastatic cancers, which also utilise state of the art piezo-electric jetting devices for sample deposition.
Conclusions
The advent and application of QCL technology to the mid-infrared spectroscopic microscopic evaluation of biomedi-
cal samples is likely to rapidly expand over the next few years. The successful
coupling of a tunable laser source with
refractive based optics and large room
temperature FPA cameras has opened a
new door for practical spectral pathology.

As evidenced in this contribution, spec-
troscopic images with a fidelity and de-
finite relevant to clinical needs are now
viable, and multiplexed imaging anal-
ysis can be performed using discrete
frequency targeting protocols.

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