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Multicolor Discrete Frequency Infrared Spectroscopic Imaging

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Abstract

Advancement of discrete frequency infrared (DFIR) spectroscopic microscopes in image quality and data throughput are critical to their use for analytical measurements. Here, we report the development and characterization of a point scanning instrument with minimal aberrations and capable of diffraction-limited performance across all fingerprint region wavelengths over arbitrarily large samples. The performance of this system is compared to commercial state of the art Fourier transform infrared (FT-IR) imaging systems. We show that for large samples or smaller set of discrete frequencies, point scanning far exceeds (~10–100 fold) comparable data acquired with FT-IR instruments. Further we show improvements in image quality using refractive lenses that show significantly improved contrast across the spatial frequency bandwidth. Finally, we introduce the ability to image two tunable frequencies simultaneously using a single detector by means of demodulation to further speed up data acquisition and reduce the impact of scattering. Together, the advancements provide significantly better spectral quality and spatial fidelity than current state of the art imaging systems while promising to make spectral scanning even faster.

Graphical Abstract

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Infrared (IR) spectroscopic imaging has undergone several innovations in recent years that have advanced it as an analytical technique.^{1–3} This is a nondestructive approach that directly images the distribution of chemical composition of samples in the absence of exogenous contrast agents. Instead, contrast is derived directly from molecular structures which absorb optical frequencies that are resonant with the vibrational modes of the comprised functional groups.^{4,5} Each peak in the absorption spectrum corresponds to a characteristic which together can be used for the identification and classification of spatially- and spectrally complex samples. While IR vibrational frequencies span the mid-IR spectral range (800–4000 cm^{-1}), the fingerprint region (800–1800 cm^{-1}) has traditionally been critical for biological and polymeric sample identifications.^{6,7} The acquisition of a limited spectral range is often a feasible route to reduce data acquisition time while maintaining the analytical capabilities of IR spectroscopy and imaging. Recent studies have shown that the ability to obtain actionable information about a sample does not require the measurement of the continuous spectral range,^{8,9} a restriction fundamental to Fourier transform (FT) technology, the historical state-of-the-art. A discrete subset of spectral positions, typically less than 30 bands, is often sufficient for histopathological tissue imaging, for example. This idea has led to the development of discrete frequency infrared microscopy (DFIR) systems, predominantly using quantum cascade lasers (QCL) where 50-fold gains in speed have been reported by only measuring the spectral frequencies required. Furthermore, with substantially higher source power and a narrower spectral range of lasers, refractive optics can be used for better image formation. This study focuses on design of instruments under these conditions and how laser-based DFIR imaging may be enhanced even further in performance.

Imaging instruments were first designed in widefield configuration with fixed frequency sources¹⁰ followed by broadly tunable systems^{11–15} as lasers became more prevalent. IR light from a QCL is condensed onto the sample and the residual signal after attenuation is imaged using an uncooled bolometer camera or cooled mercury–cadmium–telluride (MCT) focal plane array. In this configuration, the narrow spectral bandwidth of the coherent laser, multiple reflections within the optical train, stray light and scattering from the sample result in speckle that degrades image quality. Since the analytical quantity of interest is absorbance, a ratio of the recorded signal from a sample to a reference signal is measured. The ratio is a measure of total beam attenuation, which includes contributions from both absorption and scattering.^{16,17} Since scattering is wavenumber and morphology dependent, errors arise in correct estimation of absorbance. In FT-IR imaging, the scattering

contribution is largely subtracted from recorded attenuation by using a nearby nonabsorbing region to estimate the slowly varying scattering. Other correction methods also work well, primarily because the incoherence of the thermal source does not result in additions of the scattered electric fields but of intensities. For a coherent source, scattered field additions manifest in the formation of speckle patterns and cannot simply be subtracted out due to redistribution of light in the spatial plane. Measuring the sample again at identical location but with a nonabsorbing frequency tens or hundreds of wavenumbers away alleviates the nonuniformity of laser illumination but is not effective in alleviating speckle patterns. Hence, several additional techniques have been proposed to mitigate speckle effects. Rotating diffusers rapidly vary the speckle pattern while integrating the signal over time,^{11,14,18} spectral averaging integrates the fluctuating speckle patterns as the laser tuner sweeps,¹³ and time delayed integration averages speckle pattern by shifting the sample.¹⁹ Regardless of their individual efficacy for specific samples, none of these methods provide a general solution to scattering induced effects. They also increase experimental times, which reduce the multichannel detection advantage of wide-field imaging.

In contrast, exceptional image quality has been reported with scanning systems that map the sample point by point.^{20–22} These scanning systems typically have higher imaging quality simply due to the ability to condense light with substantially higher intensities per illuminated area but are also immune to many of the drawbacks observed for widefield systems. First, only a single point is illuminated and detected at a time; hence, recorded intensity is affected by coherent addition of scattered light only from a very small (single pixel) area. Without the need to average laser speckle over time, spectra or space, absorbance at single locations can be recorded in much shorter time. Second, high laser powers are not required, as covering large areas and a flat-field illumination is no longer required. With these advantages, the image quality and signal-to-noise ratio (SNR) of these systems facilitates the development of analytical capabilities not previously possible with DFIR systems. Finally, point illumination enables the development of instruments that measures both transmitted light and novel means to measure absorption, for example, through photoexpansion of the sample using devices such as nanoscale cantilevers²³ with corrections,^{24–26} acoustic transducers,²⁷ and visible light probes.^{28,29}

A major drawback of point scanning systems, however, is a loss of speed in acquiring images compared to widefield systems. Since each point must be measured individually, the advantages of higher fidelity data must sufficiently offset the inefficiency of scanning. There are several designs of point scanning systems that build up the image by raster scanning a point across the sample as fast as possible. In the visible spectrum, laser scanning confocal microscopes commonly incorporate mirror galvanometers and resonant scanners. However, the optical design of these involves scan lenses, tube lenses, and infinity-corrected objectives each containing many refractive elements. This is difficult in the mid-infrared spectrum as chromatic and field corrections are more complicated and expensive when recording a spectral range 20-fold larger while being restricted to a very limited selection of IR compatible materials with viable dispersion characteristics. Thus, far, IR scanning systems have relied on on-axis scanning by either sweeping the lens³⁰ or sweeping the sample.²¹ Here, we describe a point scanning system that operates by sweeping the sample. After optimizing the system, we further demonstrate a new technique that can increase data

acquisition rates using simultaneous illumination at dual frequencies. This “multi-color” scanning approach is then described and evaluated.

DESIGN AND OPTIMIZATION OF A POINT SCANNING, QCL-BASED DFIR MICROSCOPY SYSTEM

The designed system is shown in Figure 1A and is based on with earlier versions reported in previous studies.^{20,21} It is powered by an array of QCL modules that together have a tuning range that spans the mid-IR fingerprint region (block engineering). Dual apertures (**A_I** and **A_{DET}**) equidistant from the sample on the illumination and detection paths are aligned such that they have identical focal points in the sample plane. Half the beam is split (**BS**) to the sample and half blocked (**BB**) and focused onto the sample and detector using a refractive lens (**OBJ**) and a reflective off-axis parabolic mirror (**OAP**) respectively. The design of the microscope allows for epi-illumination of the sample with an interchangeable selection of 3 objectives with numeric apertures 0.56, 0.71, and 0.85 (LightPath Technologies), which spans the range of NAs reported in most previous IR imaging studies. The transflected signal is detected by a cryogenic photovoltaic mercury cadmium telluride (**PV MCT**) detector (MCT-13–0.5PV, InfraRed Associates) without preamplification or biasing, and demodulated using a lock-in amplifier (MFLI, Zurich Instruments) referenced to the QCL’s pulse repetition frequency. The magnitude (**R**) of the signal’s in-phase and quadrature components is used to form the image as the stage scans the sample. A standard white-light epi-illuminated inverted microscope (not illustrated) is built underneath the microscopy stage and used for targeting selecting the area for IR scanning as well as additional features later described.

We have designed several subroutines that carefully increase the fidelity of the acquired signal but operate in the background as diagrammed by Figure 1B. First, the power stability of each wavenumber is a function of the laser’s repetition rate (**F_{REF}**) and pulse width (**PW**). This is not consistent even within the tuning range of each of the four lasers. Before scanning an image, these parameters are either (in priority) overridden by the user, directly selected from a precalibrated table of optimal settings, automatically estimated using the nearest entry in the table, or restored to default which will result in a loss of SNR of undetermined magnitude. The laser’s intensity (**V_{AC}**) and pointing angle (**θ_{XY}**) are also unique so we adjust the sensitivity of the lock-in amplifier and galvanometer angle corrections, respectively. Chromatic dispersion tables, simulated with Code V (Synopsys), are used to estimate the jump in focal position (**Z**) before running a quick short-range autofocus subroutine. The magnification of mapping systems is arbitrarily adjustable allowing us to optimize each image by wavenumber (ν). The pixel size ($\Delta X \leq d_{XY}/2$) is adjusted proportional to the lateral point spread function ($d_{XY} \cong 1.22/\nu NA$). To prevent aliasing due to excessively low sampling rate and to minimize noise in the signal, the lock-in time constant ($\tau = C^{-1}t_{\Delta X}$) is set relative to the minimum pixel dwell time ($t_{\Delta X}$), where C is a parameter specific to the lock-in amplifier that has a low-pass filter order that reaches steady state in C_τ .

The axial tolerance of the scanner's ability to trace the sample substrate is coupled to the axial point spread function ($d_z \cong 2d_{xy}$). Any deviations as the sample moves out of focus will result primarily in a loss of power. Vibrations manifest as image striping, while a slanted sample causes vignetting. This becomes problematic since absorbance measurements are referenced to the original laser power, often taken at a single point with ~100% reflectance on the background. Clearly, a single background measurement is not enough when the power varies by position. We implement two methods to address this problem. The first performs a ~9-point autofocus across the sample and calculates a best-fit plane. Manual sample leveling improves results, minimizing the required travel range of the z -axis, but even a carefully adjusted sample is still not flat. The scanner corrects for residual misalignment by tracing the sample in 3 dimensions, keeping parallel with the substrate regardless to how it is mounted. Second, following the mechanical corrections, we reference to an interpolated background plane instead of a single background value.

Once these initialization procedures are completed, the instrument is designed to scan the sample line by line. For discussion purposes, we assume the instrument scans row by row, but technically, it is not required that we scan horizontally or vertically. Since the stage coordinates are adjusted to align to the sample plane, any affine transformation matrix is possible, including rotations that scan diagonally. Scan areas do not need to be rectangles either. This framework can improve efficiency by fitting the scan pattern to the arbitrary sample, thereby minimizing the amount of empty space that is wastefully mapped point by point. This feature is also an advantage over widefield imaging and, for sparse samples like those in cytology, may render point scanning a better option. When scanning each row, the acquisition is triggered based on distance traveled, as shown in Figure 1B. Instead of scanning and acquiring data at a fixed rate, this encoding minimizes errors because of acceleration, deceleration, or other velocity instabilities. A consequence is that pixel dwell time is not uniform across the scan direction; pixel dwell time is shortest when the stage is at maximum velocity near the center of the sample. Noise remains consistent throughout since the lock-in amplifier's time constant is dependent on the shortest pixel dwell time. The steady stage value of each pixel should be measured C_τ after each trigger and error in the delay creates an offset between the forward and backward scans.

Image alignment procedures to first align the rows, and then to align the frequency bands is necessary, as show in Figure 1C. Row alignment is GPU accelerated and performed in MATLAB where the odd and even lines are nonrigidly registered only in the x -direction to correct for residual local distortions. To reduce memory requirements, we may bin the data prior to registration and correspondingly scale the calculated displacement fields afterward. Once each band is constructed, we must also align the bands with each other to account for sample drift. This is tracked using the IR images or more accurately by using the white light images from the inverted brightfield microscope if imaging on a transparent substrate. The displacement is calculated by detecting corners using the features from accelerated segment test (FAST) algorithm, using the fast retina key point (FREAK) algorithm to match the key points between successive images, and finally estimating the geometric transform. Inaccurate band alignment results in corrupted spectra. A point spectrum extracted from a multispectral cube acquired from a system that measures spatial before spectral information

must be averaged within a window of surrounding pixels representing the area of uncertainty due to sample drift. In the opposing case, for instance all FT-IR instruments and some QCL DF-IR instruments, which acquire all spectral information at a given location before moving the sample, the averaging is unnecessary. Errors due to sample drift appear as misaligned image tiles and this type of error is less impactful for most chemical imaging applications.

One of the disadvantages of discrete frequency systems is the necessity of prior knowledge of the sample to optimally select useful spectral features for imaging. To acquire full spectra when needed, a flexibility in design is built-in to our system by enabling use as a rapid scanning point spectrometer. This capability is analogous to the ability of FT-IR imaging systems to provide a single spectrum. Along with a standard white-light epi-illuminated inverted microscope (not illustrated) built underneath the microscopy stage in Figure 1A for targeting the spectrometer, we rapidly collected representative spectra of key locations on the sample. The control scheme of the spectrometer shown in Figure 2A is essentially identical to an FT-IR spectrometer where spectral information is computationally derived from the physical position of a moving reference mirror encoded with a copropagating reference laser. In QCL spectrometer design, the spectral tuning of an external cavity (EC) QCL is relative to the rotation of a grating (Gr) that directs a specific frequency into the quantum cascade (QC) gain medium where the wavenumber is tracked by a TTL signal at every 0.5 cm^{-1} . Spectral resolution and SNR do not depend on acquisition time as they do for an FT-IR spectrometer.³¹ The raw data acquired here is 0.5 cm^{-1} spaced, but the data is typically binned to 4 cm^{-1} for better SNR. The small piezoelectric-driven grating can be oscillated quickly. We acquire fingerprint spectra at 4 cm^{-1} resolution every 200 ms and cocoverage 10 iterations to obtain the diagnostic PMMA thin film spectrum shown in Figure 2B at 0.5 Hz. These specifications are adjustable according to the experiment and viable spectra can be acquired at up to 10 Hz.

METHODS

All FT-IR reference images and spectra are acquired using a Cary 620-IR imaging microscope with a 680-IR FT-IR spectrometer (Agilent Technologies). This microscope is equipped with a liquid nitrogen cooled 128×128 pixel focal plane array (FPA) detector and a 0.62 NA reflective Schwarzschild objective with 36X total magnification resulting in pixel sizes of $1.1\text{ }\mu\text{m}$. Images are collected with 16 coadditions at 4 cm^{-1} resolution, processed with a Blackman-Harris apodization function, and then ratioed to a 128 coaddition background. QCL-equipped spectrometer data is acquired as described with each configuration in this study. Average FT-IR point spectra were collected using the $250\text{ }\mu\text{m}$ single element detector in approximately 2 s with 16 coadditions at 4 cm^{-1} resolution with an asymmetric single sided interferogram sampling speed of 75 kHz and a reference laser sampling interval of 2.

Chrome on glass USAF 1951 resolution test targets (II–VI Max Levy) are used for resolution testing since glass has broadband IR absorbance and the thin chrome plating does not introduce scattering artifacts. PMMA film was spun coat on low-emissivity microscope slides (Kevley Technologies) at $1\text{ }\mu\text{m}$ thickness and used for spectrometer calibration. SU-8 photoresist USAF 1951 resolution test targets were prepared on low-e microscope slides at 5

μm thickness, a common biological sample thickness, and used for to test multicolor imaging.

RESULTS AND DISCUSSION

The key performance metric in design of any instrument is the SNR. The analytical signal (absorbance) is determined by the sample in IR spectroscopy and noise is the metric for comparison. Two sources of noise must be examined in imaging. The first is the variation across the field of view, while the second is the spectral noise at any point. The spatial noise includes a component arising from spectral noise (at every pixel) and variations in the image (arising from illumination, focusing, drift, or other sources). In addition, each system has its unique spatial noise contributions. In point scanning systems, abrupt motion of the microscope stage can vibrate the sample and appear as low frequency directional fluctuations in absorbance. Similarly, sensor nonuniformity of the staring array provides a spatial noise for widefield FT-IR imaging systems. Hence, it is instructive to examine spatial noise first. The noise characteristics of the DFIR scanning system are compared to a leading, commercially available FT-IR imaging spectrometer in Figure 3A. Noise in each case is calculated as the root-mean-square (RMS) of each band for similar fields of view. As anticipated, the noise across the FT-IR image is devoid of spectral features (other than increased noise due to water vapor lines) The noise in DFIR imaging data is influenced by several factors but mostly dependent on the design of the composite QCL (consisting of four individual modules). We observe variable noise that corresponds to changes in the emission spectrum of each module of the composite source. Noise control for the DFIR microscope is determined by adjusting scan speeds to affect pixel dwell times, while the lock-in time constant and filter orders are set such that their settling time resides within the minimum dwell time of any pixel. Consequently, for each pixel, we ensure that the number of laser pulses recorded is the same, regardless of dwell time at any specific position with respect to the range of stage motion. These controls ensure that the SNR achieved is both maximized and consistent across the image. Optionally, if we decrease the stage velocity, pixel dwell times increase causing the system to select longer filter time constants thereby improving SNR further without needing repeated coaveraged scans.

It is notable that noise in the QCL system here is approximately 1 order of magnitude lower as the advantage of a low noise sensor and higher source intensity overcomes the multichannel and multiplex advantages of the FT-system. Spectral noise reduction algorithms, such as the minimum noise fraction (MNF) method, can reduce the FT-IR RMS noise by less than an order of magnitude,¹³ which presents an opportunity to increase data quality using the white noise properties of the FT spectra. However, it should be noted that the noise reduced data is an estimate arising from a model of noise and the native precision of the data does not increase with mathematical methods. Similarly, DFIR systems' speed can be increased significantly by only acquiring data needed for particular applications. While the native recorded SNR of the QCL system will be higher, conversely, spectral noise rejection methods are typically unusable for discrete frequency data sets with a small number of recorded bands.

While overall noise in an imaging data set is our primary metric, it is also instructive to estimate the noise and spectral reproducibility of the instrument using single pixel spectra. Here, we compare spectra by examining the 100% spectral lines acquired on a reflective surface, as shown in Figure 3B. These spectral lines are extracted from the hyperspectral data set acquired with DFIR and FT-IR instruments at typical experimental settings described previously in the methods. The comparisons of spectral and spatial noise show that the DF instrument is capable of lower noise performance, often in much less time. The DF system benefits from real-time coaddition as previously noted, whereas the high performance of the FT-IR spectrometer, despite an orders of magnitude weaker source, attests to the effectiveness of spectral multiplexing of the continuous spectrum (Fellgett's advantage). This advantage has allowed FT-IR spectroscopy to dominate all other forms of spectral acquisition in the mid-IR range for nearly five decades.

While the above comparison is valid for imaging, it is also instructive to examine the performance of the QCL-DFIR spectrometer against the performance of a single point FT-IR spectrometer. We acquire 100% lines using a point detector, similar to that used in our QCL instrument, on the FT-IR imaging system. To compare with the point scanning system, we restrict the microscope's physical aperture to decreasing sizes and calculate the noise levels. Figure 3C shows the noise in recorded 100% lines as the aperture is closed from 100 μm ; in all cases, the data are acquired by signal averaging for 2 s to keep the total measurement time approximately equal to the DFIR spectrometer. As the aperture is reduced to 10 μm , noise rises and eventually light throughput is too low for measurements at diffraction-limited pixel sizes. For larger aperture sizes, noise decreases and stabilizes for larger aperture sizes once the detector area is filled. A comparison of the 100% lines reveals that the performance of the $\sim 3\text{ }\mu\text{m}$ focal spot of the QCL system is matched by 30–60 μm aperture sizes in the FT-IR system across the spectral bandwidth. For localized measurements, FPA measurements are advantageous³² as each pixel has effectively a virtual aperture and there is no loss of intensity, though the minimum sampling area is defined by the point spread function (PSF) of the system optics, not the magnification. Each of these physically smaller (39-fold) individual elements on the FPA have lower readout noise, which is dominant in low light conditions. Additional coadditions (256) can reduce the noise such that the averaged measurement is competitive with QCL noise levels. In the ideal conditions at maximum aperture, the performance of a highly stable FT-IR thermal source illuminating a single element detector is unmatched.

The advantages of QCL systems are only in measurements with resolutions under $\sim 60\text{ }\mu\text{m}$ because a coherent source can be condensed to a much smaller focal point with enough power remaining to effectively illuminate a detector. The minimum focal spot achievable with an incoherent thermal source depends on the magnification of the system and the size of the source, which is comparatively large. Further winnowing is necessary to restrict the measurement area which also restricts the available light and consequently degrades the SNR. Thus, on both spectral and spatial measures, we can conclude that the performance of an FT-IR imaging system for a full spectrum at a given pixel has been exceeded when small areas (smaller than $50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$) are measured per pixel. This is essentially the microscopy regime and the improved performance of QCL-based microscopes represents is

now better than comparable FT-IR imaging. This is a major milestone in IR spectral analyses, wherein an appropriately utilized, high laser power has overcome the dominant multiplex and multichannel advantages of FT-IR imaging. While the spectral range of QCLs cannot yet cover the full range of FT-IR instruments, laser innovations are continuing to bridge the gap.

It is also interesting to compare the imaging rates between scanning systems that record data while in motion versus array-based microscopes that record multipixel fields of view (FOV) which are stitched together as a mosaic. The imaging time of an FPA equipped system (eq 1) to measure a specific area at a defined data quality (SNR) is linearly dependent on the total pixels in the image $n_X \times n_Y$ scaled by that in the FPA FOV (n_{FPA}) and the time it takes to acquire each interferogram (t_{INT}), which is a function of the inverse of the spectral resolution ($\Delta\nu$). SNR normalization increases the number of coadditions (n_C) for each FOV. For a scanning system, however, the scaling is not as straightforward (eq 2). Efficiency is maximized at peak stage velocity. One option is to accelerate to peak stage velocity as quickly as possible and to hold that velocity constant for the largest fraction of time. These abrupt dynamics tend to degrade SNR, increase instability due to overshoot, and lengthen settling time. Instead, the motion of the scanning stage follows a third order trajectory³³ to sweep the sample in s-curves minimizing unwanted vibrations. The time to traverse each line (t_{LINE}) is dependent on its length; the number of pixels (n_X) nor the size of pixel (ΔX) alone do not affect scan times. Instead, scan times for each band is primarily scaled by the number of rows (n_Y). For small images, the fixed time (t_{INIT}) to refocus the image for chromatic correction, as well as other initialization procedures, described previously per frequency become dominant resulting in diminishing improvements. Lastly, the imaging time is scaled by the number of discrete frequencies bands selected (n_ν).

$$t_{\text{FTIR}} \propto n_C \left[\frac{n_X n_Y}{n_{\text{FPA}}} t_{\text{INT}} (\Delta\nu^{-1}) \right] \quad (1)$$

$$t_{\text{QCL}} \propto n_\nu \left[n_Y t_{\text{LINE}} \left(n_X \Delta X, \frac{dX}{dt}, \frac{d^2 X}{dt^2}, \frac{d^3 X}{dt^3} \right) + t_{\text{INIT}} \right] \quad (2)$$

It is notable that high aspect ratios (n_X/n_Y) are more efficient for the scanning QCL system, whereas this value has no impact with the FT-IR system. The fast and slow axes (simplified as x and y , respectively) do not need to align with the microscopy stage axes and an appropriate rotation of the sample can improve efficiency. To compare the performance of these two approaches as simulated in Figure 4A, we assume the most favorable scenario for the widefield systems—we use square images from 0.1 μm up to 20 mm per side for comparisons.

The minimum scan time for an image is given by a single QCL band or a single FT-IR coaddition. The upper bound is represented by hyperspectral QCL data with 250 bands at 4 cm^{-1} spacing covering the fingerprint spectral region or 256 FT-IR coadditions, which brings the SNR in line with that of the QCL. Under typical experimental parameters, however, we acquire between 10 and 30 QCL bands as this information is typically what is required in DFIR approaches to obtain information about biological samples. Similarly, a typical parameter for FT-IR imaging systems is 16 coadditions to achieve a reasonable SNR for tissue imaging, which is then augmented by postprocessing noise reduction. We do not include time required for this postacquisition noise rejection in our comparisons, though it can be substantial.

As we compare these systems and their current limits of performance, we emphasize that no system is globally preferred in all scenarios. A particular system may be the preferred one depending on specific experimental conditions; we illustrate this situation using three scenarios next.

Case 1: Small Samples below $300\text{ }\mu\text{m}$.

For an image the size of a single FOV, the FT-IR system simply sweeps the interferometer ($n_{\text{C}}t_{\text{INT}}$), whereas a scanning system must sweep the image line by line ($n_{\text{L}}n_{\text{Y}}t_{\text{LINE}}$). While we can reduce n_{C} and (n_{L}) if appropriate, the inefficiency in turning the stage and rescanning each line (n_{Y}) makes QCL scanning impractical. Thus, for small areas, it is best to employ FT-IR imaging with a staring camera. The lower times to scan with the FT-IR system holds up to the crossover point (A_1) shown in Figure 4A. Under normal conditions, where we would need to acquire a reasonable set of discrete frequency bands, it is preferable to perform this experiment using FT-IR at 16 coadditions or even 256 coadditions, where the SNR between the systems is equivalent.

Case 2: Hyperspectral Acquisition of Samples up to 5 mm.

The spectral multiplexing advantage of FT-IR interferometers remains up to moderately sized images (Figure 4A, A_2). Both systems take roughly a day to image this area, however the QCL system is limited to the fingerprint spectral region. Here we are assuming an experiment that draws no advantage from the ability of discrete band selection. This is often the case for completely unknown samples in which discovery of new information is targeted. Once prior information becomes available and the spectral set is reducible to smaller bandwidths, the speed advantage of DF-IR scanning grows to an order of magnitude.

Case 3: Large Samples.

The advantage of QCL DFIR systems is most significant when being used to screen large areas. For instance, 20 mm samples such as tissue surgical sections or microarrays, can be imaged in a day with a typical band set, whereas this task would take an FT-IR system upward of 1000 h while still obtaining lower SNR. A means to quantitatively compare performance is to normalize ratings by noise (σ) and the number of pixels (n) required to cover a fixed area as follows:

$$R_{2:1} = \frac{n_2 t_1 \left(\frac{\sigma_1}{\sigma_2} \right)^2}{n_1 t_2} \quad (3)$$

These relations are shown in Figure 4B, where the QCL point scanning time is compared for different numbers of bands against FT-IR imaging with the dotted line indicating parity. On a per pixel basis when SNR is equivalent, the FT-IR system can surpass the QCL system only when hyperspectral data is required, and even so only for small images. In all other cases, the QCL is faster and its advantage climbs rapidly as SNR required increases or spectral bands needed decrease. It is also instructive to compare the point scanning system against a widefield QCL based system previously reported using the same FPA detector as used in wide-field FT-IR imaging. In Figure 4C, performance of a wide-field QCL DF-IR system¹³ is compared to the point system, where it is evident that array detectors are faster up to midsized images (C_1). These metrics, however, do not account for image quality where minimization of artifacts and aberrations allow for laser-based point scanning techniques to be dominant.

The comparison of wide-field and point DFIR systems demonstrates that the QCL point scanning, DFIR approach can be a candidate of choice for scanning large areas at limited spectral bands. Hence, we sought to optimize image quality and speed up spectral acquisition. First, we evaluate the image resolving performance of our designed system against the state-of-the-art commercial FT-IR imaging system by measuring the contrast of an USAF 1951 resolution test target consisting of groups of progressively smaller features. The features of these negative chrome on glass targets broadly absorb IR frequencies while the background is highly reflective. These targets allow us to determine the resolution within the absorbance image across all spectral positions. From these measurements, in Figure 5A, we observe that the resolving power of the QCL system ($1 \mu\text{m}$ pixels) far exceeds that of a FTIR system ($1.1 \mu\text{m}$ pixels). Refractive optics focus all the light incident on the lens aperture to a single point. Since this is a stage-scanning system, the focal point is always on axis with minimal field-induced aberrations. The performance, which is proportional to wavenumber, is described by the modulation transfer function (MTF) curves that indicate how accurately a system reproduces contrast with increasing spatial frequency (Figure 5B top). The use of refractive materials, however, has well-known drawbacks of not being able to simultaneously focus across a wide spectral range. Since the developed laser imaging spectrometer is a DF system, chromatic focal shifts are easily correctable mechanically, while losses due to attenuation of the material and limits of the antireflection coating are mitigated by higher source power.

None of these corrections are feasible with FT-IR systems; since the broad spectral range is simultaneously acquired, they are designed with reflective optics.³⁴ A reflective Schwarzschild objective focuses light without introducing chromatic aberrations using two reflective surfaces. Unlike a refractive singlet, these reflective objectives are corrected for third-order aberrations making them viable for wide-field imaging. However, there is a trade-off between resolution and light throughput due to the large hole in the primary reflector. A larger obscuration allows for increased light throughput up to a limit but reduces

the inner NA of the objective. This obscuration acts as a Fourier filter reducing the contrast of the lower spatial frequencies as evident by the stepped drop (Figure 5B bottom). Additionally, in epi-illumination configuration, a mirror is rotated above the objective to direct the light to the sample and consequently blocks half of the light returning to the detector. Unlike a refractive beam splitter that evenly attenuates the beam but introduces other chromatic issues, the mirror completely blocks half the angular aperture but only in the vertical direction. The horizontal direction remains unaffected, thereby causing significant asymmetric blurring in the images. For trans-illuminated images we expect the vertical MTF to match the horizontal MTF.

From these MTF measurements, we can calculate the optimal pixel size as half the resolution where the contrast falls below the 26% specification of the Rayleigh criterion which is considered reasonable for common microscopy applications. As shown in Figure 5C, the pixel size is inversely proportional to the wavenumber and often considered to be no larger than the highest utilized band to prevent aliasing by under-sampling. Due to the large wavenumber range that spans the mid-infrared fingerprint region, considerable oversampling is inevitable. When pixel sizes are kept consistent, halving the wavenumber would result in 4-fold undersized pixels, increased scan times, and excess data processing requirements. Fourier transform systems especially have low data efficiency as they cannot decouple magnification from spectral information. Even camera-based discrete frequency systems cannot easily change magnification without additional optics. Only DF systems that employ point scanning for imaging formation allow for arbitrary pixel sizes that can be precisely adjusted to the optimal value for each wavenumber. The pixel sampling is triggered by the stage encoder to any multiple of encoder ticks, which is spaced 50 nm in this hardware. Instead of acquiring a data cube where each wavenumber slice has an equal number of pixels, we can accelerate this by avoiding over sampling and imaging the sample area always at appropriate resolution. Of course, such an approach would need a careful interpolation of the data to ensure ease of data processing and assuring that spectral fidelity from each location is maintained as well as noting that the localized region from which each analytical result is derived changes with wavenumber. The same happens currently in any imaging system, of course, given the changing point spread function. The result above not only demonstrates that this varying pixel size is a possibility but also provides the optimal size, which can help optimize designs of the future that employ optimal spatial sampling to speed up data acquisition.

Another approach to speed data collection is to employ multiplexing.^{35–37} Current QCL imaging systems do not address the loss of spectral multiplexing inherently advantageous when using FT systems. Despite modern configurations commonly consisting of four individual QCL lasers per microscope to span the fingerprint region, only one laser is transmitted at a time. Each discrete band in the set is measured sequentially while the other 3 lasers remain idle representing a clear inefficiency. IR detectors simply measure the intensity of irradiance without any information regarding the incident wavelength. Therefore, when using slower detectors that measure the average power of the laser, only one laser can be active at once. While it may be possible to use multiple detectors and a series of cutoff filters, such a system would have cross-talk relative to the quality factor of the filter and, therefore, have limited practicality.

We present a detection scheme that demodulates multiple incident beams to allow multiple spectral bands to be acquired simultaneously on a single detector as shown in Figure 6A. Two essentially identical but separate laser assemblies, each with 4 laser tuners, are colinearly aligned with a beam splitter. This arrangement eventually can be replaced with a single laser system with completely independent tuners, but as of this study, no commercial system with this capability is available. The combined beam is passed through the QCL microscope as for a single frequency but with the lasers emitting at slightly different repetition rates. A digital lock-in amplifier equipped with multiple demodulator units, each locked to respective reference frequencies, separate the signals from each laser. The first consequence is that the data acquisition is further sped up by a factor of 2. The same approach may be adopted for even further multiplexing with emerging multicolor QCL lasers.³⁸

While measuring multiple gas or liquid species has been shown to be possible, one unique application of multiplexing is to actively remove scattering effects in the recorded data that arise from refractive index mismatches at domain boundaries. We have imaged SU-8 photoresist bars that are 7 μm wide and 5 μm thick. These are relatively small structures that are roughly the same size scale as the PSF. A key band set that is reduced from and representative of the full spectrum typically contains absorbing and baseline pairs that can be imaged simultaneously. As can be seen when imaging the photoresist at an absorbing wavenumber (Figure 6B), edge scattering is clearly visible and reduces the image contrast by approximately half according to the vertical line profile. At a nonabsorbing wavenumber, which could be used for baseline correction in a continuous spectrum, only scattering-induced features are present in the attenuated beam. These are well-known to arise from refractive index mismatches at boundaries (Figure 6C). Using the dual color system here, we record only the differential signal (Figure 6D) that is the scattering corrected absorbance normally used for analysis. As shown by the line profile from Figure 6D, the edge scattering contributions are clearly observed and duly subtracted in real time. For any sample, the precise wavenumbers for absorption bands and nonabsorbing spectral regions can be quickly determined using the rapid scanning spectrometer described in Figure 2. Simultaneous multicolor imaging, while common across other microscopy modalities, has yet to be broadly implemented for mid-infrared discrete frequency microspectroscopy. When 4-tuner QCL laser systems that are truly independent become available, no additional design changes are expected to pair with existing 4-demodulator lock-in amplifiers. But as development of multichannel QCL chips consisting of larger arrays become more prevalent, this may exceed capabilities feasible with lock-in amplifiers. Nevertheless, the ability to compress large numbers of frequency bands into a single receiver is well-known in the telecom industry and many of these techniques can be implemented in future iterations of discrete frequency microscopy systems as IR laser technology continues to advance.

CONCLUSIONS

In this study, we describe and characterize a DFIR point scanning spectrometer and compare it to state-of-the-art commercial FT-IR spectroscopic imaging systems. The separation of magnification from optics or sensors, and arbitrarily controllable by software, allows us to optimize imaging parameters to prevent oversampling, minimize scan times, and reduce data

overhead. Consequently, the performance of the system reported here exceeds that of previous QCL imaging or mapping systems as well as a leading FT-IR imaging system. We demonstrate the experimental parameter space where the performance of the DFIR system exceeds that of FT-IR wide-field imaging whereas the spectral quality exceeds that of any point or wide-field FT-IR imaging spectrometer. Finally, we show marked improvements in spatial image quality with the new optical design. Further advancement through multicolor mapping demonstrates potential to provide corrected absorbance images or multiplexed chemical information. These extensions in performance exceed current state of the art in both spectral and spatial data quality and provide exciting new opportunities for advancing IR imaging technology.

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Figure 1.

Schematic of a QCL-based point scanning microscope depicting the (A) optical and electronic components, (B) signals diagram, and (C) software postprocessing.

Figure 2. Schematic of a QCL-based rapid scanning point spectrometer depicting the (A) control scheme and (B) a standardized diagnostic spectrum.

Figure 3.

(A) Spatial root-mean-square (RMS) noise, calculated using spatial variance of recorded data at each band in a hyperspectral image acquired using the developed DFIR point scanning and commercial, widefield FT-IR imaging systems. (B) 100% spectral lines from a single pixel under typical experimental conditions. (C) RMS noise of the 100% line with different aperture settings in a commercial single point FT-IR microscope and its comparison with the point scanning DFIR system.

Figure 4.

(A) Estimated imaging time for a square sample area linearly scaled by the number of frequency bands or coadds for the DFIR and FT-IR systems, respectively. (B) The SNR-normalized pixel rate ratio of the QCL system to the FT-IR system. (C) The SNR normalized pixel rate ratio of the QCL point scanning system to a QCL widefield imaging system.

Figure 5. Spatial resolution characteristics of a QCL microscope (top row) and a FT-IR imaging spectrometer (bottom row). (A) Absorbance images of USAF 1951 chrome on glass negative resolution test target images of groups 6 and 7. (B) Modulation transfer function (MTF) curves showing the reflectance contrast decay with increasing spatial frequency per wavenumber measured from a slant-edge. (C) Ideal pixel size measurements per wavenumber with a fitted trendline.

Figure 6.

(A) Schematic of a DFIR microscope with a simultaneous multicolor acquisition scheme. In this setup, each color illuminates the sample with a unique repetition rate. The recorded signal is demodulated at the respective reference frequencies by a multichannel lock-in amplifier. SU-8 photoresist bars on low-e reflective glass acquired at (B) an absorbing wavenumber and (C) a nonabsorbing wavenumber. (D) The difference image cancels scattering contributions resulting in a corrected absorbance image.