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High-resolution Fourier-transform infrared chemical imaging with multiple synchrotron beams

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Abstract

Conventional Fourier-transform infrared (FTIR) microspectroscopic systems are limited by an inevitable trade-off between spatial resolution, acquisition time, signal-to-noise ratio (SNR) and sample coverage. We present an FTIR imaging approach that substantially extends current capabilities by combining multiple synchrotron beams with wide-field detection. This advance allows truly diffraction-limited high-resolution imaging over the entire mid-infrared spectrum with high chemical sensitivity and fast acquisition speed while maintaining high-quality SNR.

Stains and labels to enhance contrast in microscopy have been used for many years, leading to many important discoveries. However, their use is often time-consuming and cumbersome, can perturb the function of drugs or small metabolites or may be cytotoxic. In contrast, label-free chemical imaging requires no artificial modification of biomolecules or additional sample preparation and permits a comprehensive characterization of heterogeneous materials¹. Chemical imaging is generating considerable interest for biomedical analysis as dyes or stains are not required for contrast and substantial chemical and structural information can be extracted without prior knowledge of molecular epitopes or manual interpretation. Vibrational spectroscopic techniques, including both mid-infrared absorption and Raman scattering-based imaging, permit molecular analyses without perturbation. Spontaneous Raman scattering relies on a very weak effect and therefore involves a trade-off between measurement time and sensitivity, potentially leading to photoinduced sample damage. Emerging instrumentation² involving nonlinear Raman

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AUTHOR CONTRIBUTIONS

M.J.N., R.R. and C.J.H. designed research; M.J.N., M.J.W. and E.C.M. performed research; M.J.W., A.K.-B., V.M. and R.B. contributed prostate samples; M.J.N., M.J.W., E.C.M., R.B. and C.J.H. analyzed data; and M.J.N., R.B. and C.J.H. wrote the paper.

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contrast has considerably extended imaging capabilities beyond these traditional trade-offs, and exciting work is underway to carefully match lasers and reject spurious backgrounds (for example, in coherent anti-Stokes Raman scattering) and in extending wavelength coverage and speed (for example, in stimulated Raman scattering). Conversely, the strong mid-infrared absorption contrast makes infrared spectroscopy and microscopy a straightforward, nondestructive, label-free chemical contrast modality with broad applications^{1,3} ranging from the analysis of graphene-based materials, pharmaceuticals, volcanic rocks and biominerals to applications in forensics and art conservation, among others. Infrared spectroscopic tools are particularly interesting for applications in biomedical fields such as marine biology, cancer research, stem cells (for example, to delineate cell mechanisms or lineage), real-time monitoring of live cells, Alzheimer's disease, Malaria parasites and more³ (Online Methods).

Infrared instrumentation, however, has stagnated mostly owing to spectral-spatial trade-offs. Commonly, low-brightness thermal sources and synchrotron sources are used for Fourier-transform infrared (FTIR) microspectroscopy. Synchrotron sources yield stable, broadband and high-brightness radiation, making them excellent for FTIR microspectroscopy, but the flux of conventional single-beam beamlines is limited by the relatively small horizontal collection angle and the resulting comparatively small source étendue makes them challenging to use with wide-field imaging characterized by a relatively large acceptance or étendue (Supplementary Note 1). Here we used multiple synchrotron beams with a wide-field detection scheme. This allowed us to acquire truly diffraction-limited, high-spatial-resolution infrared images of high spectral quality with outstanding speed, considerably extending the potential of infrared microscopy.

For an optical system permitting diffraction-limited imaging, spatial resolution is defined as the capacity to separate two adjacent (point-like) objects. To achieve the highest (diffraction-limited) resolution, an objective with the largest possible numerical aperture (NA) should be used, and the instrument's signal-to-noise ratio (SNR)^{4,5} should be optimized. Also, it is indispensable to match the image pixilation to the NA of the objective using the appropriate spatial sampling or pixel size. Too-large pixels inevitably lead to resolution loss, whereas smaller pixels do not improve the resolution further. A detailed analysis⁴ (Online Methods) shows that, assuming the largest commercially available NA of ~ 0.65 , diffraction-limited resolution over the entire mid-infrared spectrum can only be achieved with an effective pixel spacing not larger than $\sim \lambda/4$ or $\sim 0.6 \mu\text{m}$ for the shortest wavelength of interest ($\lambda = 2.5 \mu\text{m}$).

One approach to infrared microscopy uses a single element detector and confocal-like apertures to localize light incident on the sample. In this configuration, pixel size is given by the raster-scanning step size⁴. Apertures of dimension a only deliver diffraction-limited resolution⁶ when $\lambda \geq a$. For $\lambda < a$, diffraction-limited resolution⁶ is not attained, whereas for longer wavelengths the throughput decays rapidly. This trade-off between resolution and throughput (or SNR) is particularly penalizing for infrared microspectroscopy because of the broad bandwidth. In practice, reasonable SNR limits the smallest aperture for the illumination at the sample plane to $\sim 10 \mu\text{m} \times 10 \mu\text{m}$ for a thermal source⁶ and, in a few demonstrations⁷, down to $\sim 3 \mu\text{m} \times 3 \mu\text{m}$ for synchrotron sources. The small aperture transmissivity of only a few percent makes point-by-point sampling systems very inefficient because of the dual need for signal averaging to obtain high SNR and rastering a small pixel size to acquire data, leading to exceedingly long acquisition times. These trade-offs make sequential point sampling impractical for micrometer-scale aperture sizes and sub-micrometer-scale raster step sizes (necessary for correct spatial sampling⁴) to achieve diffraction-limited maps. For example, it takes 2–4 h to acquire an area of only $30 \mu\text{m} \times 30 \mu\text{m}$ as a fully diffraction-limited map at a state-of-the-art third-generation synchrotron⁷

equipped with a conventional confocal system. Lengthy collection times, in most practical cases, lead experimenters to choose larger aperture and step sizes, thereby compromising the achievable spatial resolution. In contrast, our system can cover this area in under a minute without compromising the spatial sampling required for diffraction-limited resolution.

We based our approach on the more recent strategy of wide-field imaging using multichannel focal plane array (FPA) detectors^{8–10}, in which no lossy apertures are used. This increases spatial coverage and imaging speed greatly, but the SNR using a thermal source limits pixel sizes to $\sim 5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ at the sample plane. Achieving a pixel size ~ 100 times smaller to correctly sample the diffraction-limited illumination is very ineffective, resulting in a ~ 100 -fold lower SNR (Supplementary Fig. 1) and thus in a $\sim 10^4$ -fold longer scanning time⁸. Hence, to our knowledge there are no reports of a true diffraction-limited FTIR imaging system with a thermal source.

In 2006 independent groups^{11–13} pioneered the coupling of a synchrotron beam with an FPA detector, which is not obvious because wide-field illumination seems incompatible with a small, low-emittance synchrotron beam. These groups demonstrated that, with a single synchrotron beam, a local region of the FPA can be illuminated, and that this region yielded increased SNR compared to thermal sources. This inhomogeneous illumination, however, means that either a relatively small FPA (and thus sample area) must be used or that the acquisition time must be increased to compensate the inhomogeneous illumination. This coverage-SNR trade-off has hampered the use of synchrotron-based technology: only one recent publication¹⁴ uses a single synchrotron beam with an FPA.

Here we present an infrared imaging system specifically designed and optimized to overcome these limitations by coupling multiple low-emittance synchrotron beams with a large FPA detector. We extracted a large fan of radiation from a dedicated bending magnet, split it into 12 beams and subsequently rearranged these into a 3×4 matrix beam bundle to illuminate a large field of view in the sample plane (Fig. 1). We engineered the matrix to achieve homogeneous illumination over areas of up to $52\text{ }\mu\text{m} \times 52\text{ }\mu\text{m}$ ($96\text{ pixels} \times 96\text{ pixels}$; Fig. 1b and Supplementary Fig. 2) with each pixel corresponding to $0.54\text{ }\mu\text{m} \times 0.54\text{ }\mu\text{m}$ at the sample plane. This pixel size, ~ 100 times smaller than conventional thermal or synchrotron systems, is smaller than the maximum pixel size allowed for correct spatial sampling (over-sampling) so that diffraction-limited images even at the smallest wavelength of interest ($2.5\text{ }\mu\text{m}$) are possible (Online Methods). Although we designed this system explicitly for acquisition in transmission mode, it also yields equivalent quality images in reflection mode (Supplementary Figs. 3 and 4).

To test this approach, we compared data from the same prostate tissue using various state-of-the-art infrared imaging systems (Fig. 2 and Supplementary Fig. 1). None of the other instruments provided diffraction-limited resolution at all wavelengths (Fig. 2a–c). Raster-scanning the area shown in Figure 2a–d ($\sim 280\text{ }\mu\text{m} \times 310\text{ }\mu\text{m}$) at diffraction-limited resolution using a synchrotron-based dual-aperture microscope would require over 11 d. In contrast, using our technique we recorded the same area (Fig. 2d) in ~ 30 min (16 scans). The spectral quality was essentially identical (Fig. 2f) to that of the best commercial systems, despite the ~ 100 -fold pixel area reduction. This pixel size provided the additional spatial detail (Fig. 2) necessary for infrared imaging to become competitive with optical microscopy in biomedical applications. In another example, wide-field multibeam synchrotron imaging revealed lymphocytes (diameter, $\sim 2\text{--}7\text{ }\mu\text{m}$) and other tissue features that were clearly visible in hematoxylin and eosin-stained images (the clinical gold standard for diagnosis; Fig. 3a–c). The same visualizations were impossible using conventional table-top infrared systems (Fig. 3d,e). The contrast in these images can be used to color-code

images into constituent cell types¹⁵; hence the capability of our technique opens up the possibility of subcellular classification.

Furthermore, pixel localization also improved spectral purity of data extracted from images. The hematoxylin and eosin contrast was well-reproduced with our technique using simple absorption features, and epithelial and stromal regions were clearly delineated without staining (Fig. 3f). The additional detail in synchrotron wide-field images allowed relatively limited cross-contamination of spectra from both intralobular stromal and epithelial regions. Although we expected these characteristic spectra to be different, the limited pixel size of the thermal source systems demonstrated substantial overlap, but the multibeam synchrotron system provided distinct spectra (Fig. 3g). Using our technique, we also classified an infrared image of prostate tissue into constituent cell types (Fig. 3h). Although it is well-known that the basement membrane lies at the interface of epithelial and stromal cells and is critical in diagnosing lethal cancer, the basement membrane is not discernable in images from thermal systems. We classified infrared tissue images into cell types¹⁵, and identified the interface between the epithelial and stromal cells (Fig. 3h). Thermal source spectra from these regions were an average of epithelial and stromal pixels, whereas interface spectra extracted from the synchrotron image were distinct from both contributions (Fig. 3i), which, with the higher collagen triplet absorption, was suggestive of the basement membrane. Additional investigations are in progress.

To validate the optical capability of our system, we recorded images of a 1951 US Air Force test target⁵ (Supplementary Figs. 3a,b and 4). We used line profiles⁵ (Supplementary Fig. 3e–h) to determine the contrast for each pattern, quantitatively confirming that our system reached and exceeded (Supplementary Note 2) the Rayleigh resolution criterion and delivered diffraction-limited images over the entire mid-infrared bandwidth. Furthermore, spatial oversampling at all wavelengths and high SNR, as offered by our system, are a prerequisite^{12,13} for developing computational resolution enhancement techniques. We implemented a spatial deconvolution algorithm (Supplementary Note 3) based on (wavelength-dependent) measured point-spread functions (Supplementary Figs. 5 and 6). The increased contrast and resolution of the deconvolved US Air Force target sample images were apparent in the line profiles (Supplementary Fig. 3c–h). Furthermore, measurements of $\sim 1\ \mu\text{m}$ polystyrene beads confirmed that our system reached a spectral limit of detection of $6 \pm 1\ \text{fmol}$ (mass, $600 \pm 100\ \text{fg}$; and volume, $0.6 \pm 0.1\ \text{fl}$) in a single $0.54\ \mu\text{m} \times 0.54\ \mu\text{m}$ pixel (Supplementary Fig. 7). We estimated that this limit is about two orders of magnitude finer than that of present instrumentation¹⁶.

The use of multiple synchrotron beams enabled us to achieve a homogeneously high SNR over a large FPA area, which improved sample coverage and acquisition speed compared to conventional thermal or synchrotron-based systems and enabled high diffraction-limited spatial resolution over the entire mid-infrared spectrum. The improvement in acquisition time opens the way to real-time noninvasive and label-free live-cell imaging. We hope that our technique spurs the community to develop appropriate optical designs for table-top instruments and provides a rationale for laser-based systems and other multibeam synchrotron-based imaging beamlines.

ONLINE METHODS

Requirements for diffraction-limited resolution

Mid-infrared spectroscopy and microscopy has very broad applications in many scientific fields, ranging from fundamental and applied research to engineering and biology^{15–29}. Infrared microspectroscopy in particular can contribute to the biomedical sciences because

of its noninvasive spatially resolved chemical specificity. Here we describe the requirements to obtain diffraction-limited spatial resolution with a mid-infrared microscope.

Spatial resolution can be quantified, for example, by the Rayleigh⁵ criterion as $d = 0.61 \lambda / \text{NA}$, in which d is the minimum distance between two adjacent (point-like) objects that are just resolved (the factor 0.61 is strictly valid only for lenses without obscuration and smaller for Schwarzschild optics; see Supplementary Note 2). But achievable spatial resolution is not only dependent on the wavelength and the NA of the objective via the Rayleigh criterion but also on the pixel size, that is, the objective's magnification and the SNR of the imaging system⁴. To observe diffraction-limited performance, a spatial sampling of at least ~ 8 pixels⁴ per Airy pattern is required to achieve sufficient contrast. Smaller pixel sizes (oversampling) do not improve the resolution, which is then limited by diffraction, whereas larger pixels unavoidably deteriorate contrast and thus resolution (undersampling). For the smallest wavelength ($2.5 \mu\text{m}$) using an NA of 0.65, we need a pixel size not larger than $1.22 \times 2.5 \mu\text{m} / 0.65 / 8 = 0.59 \mu\text{m}$. Even the less restrictive Nyquist theorem yields a maximum pixel size of $1 / (2.3 f_{\text{cutoff}}) = 0.84 \mu\text{m}$ (usually 2.3 is used instead of the theoretical 2 suggested by Nyquist to account for factors such as noise in real optical systems³⁰), where $f_{\text{cutoff}} = 2 \text{NA} / \lambda$ is the spatial cutoff frequency, equivalent to the Sparrow frequency^{5,31}. In summary, this means that the NA of an objective alone is not enough to provide the resolution promised by the Rayleigh criterion, but its magnification also has to match. In the case of an objective with an NA of 0.65 (approximately the largest commercially available NA, giving the best possible spatial resolution), it needs at least a magnification of $40 \mu\text{m} / 0.59 \mu\text{m} = 68$ (assuming a typical FPA pixel size of $40 \mu\text{m} \times 40 \mu\text{m}$). We used a $74\times$ objective (NA = 0.65) in our setup, leading to a pixel size of $0.54 \mu\text{m} \times 0.54 \mu\text{m}$ (slight oversampling). In addition this high spatial sampling offers the advantage that subdiffraction objects can be localized (but of course, not resolved) with an accuracy better than the diffraction limit³².

Instrument design

Synchrotron storage rings are excellent light sources for aperture-based infrared microspectroscopy³³ as the small horizontal and vertical emittance (source étendue) of conventional single-beam beamlines and the relatively small acceptance (detector system étendue) of the microscopy system can be closely matched (Supplementary Table 1). Increasing the photon flux by extracting a larger horizontal angle from a bending magnet, however, is not beneficial because the additional photons cannot be coupled efficiently to the small acceptance of such microscopy systems. For wide-field microscopes without throughput-restricting apertures, in contrast, single beams from conventional beamlines have limited flux owing to their relatively small emittance, making it challenging to match the relatively large acceptance of a multichannel FPA imaging instrument. The instrument described here substantially increased the horizontal collection angle to match the large acceptance of a wide-field imaging system to fully exploit the source brightness. It is located at the Synchrotron Radiation Center in Stoughton, Wisconsin, USA, which already houses a conventional aperture-based infrared microscope. This synchrotron facility encourages scientists to apply for peer-reviewed access to beamtime and/or initiate a collaboration with the authors of this work. Applications are accepted for review every six months and rapid requests for initial experiments are handled more frequently (http://www.src.wisc.edu/users/new_users.html).

We extracted $320 \text{ mrad} \times 27 \text{ mrad}$ of infrared radiation from a dedicated bending magnet and split this fan of radiation into twelve beams with a set of twelve toroidal mirrors (M1; Fig. 1), which refocused each beam (magnification of 1). Each beam exited an ultrahigh vacuum chamber via one of twelve flat mirrors (M2; Fig. 1) through one of twelve ZnSe

windows (Fig. 1) into a nitrogen-purged area. Next, twelve parabolic mirrors (M3; Fig. 1) collimated the beams, followed by twelve stacked small flat mirrors (M4; Fig. 1) that rearranged the beams into a 3×4 matrix. We used a subsequent piezo-driven optical feedback system (feedback system is not shown) to stabilize the beam bundle, reduce vibration effects and increase the SNR. Next, we sent the bundle through a Vertex 70 (Bruker) spectrometer (Fig. 1), which was coupled to a Hyperion 3000 (Bruker) infrared and visible light microscope. There, the slightly defocused beam bundle illuminated the sample area through a $15\times$ or $20\times$ Schwarzschild condenser (Fig. 1) to spread out each beam so that the beams overlap spatially to provide quasi-homogeneous illumination at the sample. Finally, a $74\times$ objective (Ealing) imaged the sample onto a $128 \text{ pixel} \times 128 \text{ pixel}$ FPA (Santa Barbara Focalplane), so that each pixel had an effective geometrical area at the sample plane of $0.54 \text{ } \mu\text{m} \times 0.54 \text{ } \mu\text{m}$ (Fig. 1). Additional design details of the imaging system have been reported elsewhere³⁴. In contrast to other implementations of thermal or synchrotron sources, our multibeam system allowed us to simultaneously uniformly illuminate an order of magnitude more pixels ($96 \text{ pixels} \times 96 \text{ pixels}$; Fig. 1b) and used an objective with a substantially higher NA of 0.65 with a correctly matched⁴ pixel size ($0.54 \text{ } \mu\text{m} \times 0.54 \text{ } \mu\text{m}$) to maintain full high diffraction-limited resolution over the mid-infrared spectrum at a high SNR. We used a condenser with an NA of ~ 0.6 to match the NA of the objective. Owing to its higher NA, this objective delivered 38% and 23% higher spatial resolution (according to the Rayleigh criterion) compared to previous studies (for example, the $15\times$ objective with $\text{NA} = 0.4$ and pixel size = $2.7 \text{ } \mu\text{m} \times 2.7 \text{ } \mu\text{m}$ or $36\times$ objective with $\text{NA} = 0.5$ and pixel size = $1.1 \text{ } \mu\text{m} \times 1.1 \text{ } \mu\text{m}$)^{11,14}. Furthermore, owing to the multibeam design, a high synchrotron storage ring current was not mandatory to obtain high SNR. The $\sim 270 \text{ mA}$ current of our storage ring was sufficient to achieve similar SNR (Fig. 2d,f) leading to shorter acquisition times compared to those reported in previous publications¹⁴. The present design can cover more than double the sample area in equivalent or shorter times with better spatial resolution as compared to single synchrotron beam systems.

Synchrotron sources may have coherent properties, for example, synchrotrons with pulse lengths shorter than tens of femtoseconds in the far infrared. The present source, however, had nanosecond pulses, and we designed the path lengths for the twelve beams to never temporally overlap on the sample or detector plane. Hence, temporal coherence did not have an impact on the imaging quality of the images produced by the microscope. Experimentally we observed no spectral evidence of spatial or temporal coherence effects, nor any impact on image quality or resolution, as can be seen, for example, by the correspondence between the thermal and synchrotron spectral data.

Experimental details, data processing, and samples

We conducted conventional thermal source-based imaging on two commercial systems: Stingray (Varian; Fig. 2c) using an FPA detector and Spotlight 400 (PerkinElmer; Figs. 2a,b and 3d,e and Supplementary Fig. 1a) equipped with a single element and a 16-pixel linear array detector. We acquired the synchrotron point-by-point scanning image (Supplementary Fig. 1b) on a Continuum (Thermo Nicolet) dual-aperture microscope connected to beamline 031, and we collected the remaining images with the multibeam synchrotron system connected to a Hyperion 3000 (Bruker) microscope at beamline 021, both at the Synchrotron Radiation Center. The Varian, PerkinElmer and Thermo Nicolet measurements used a Happ-Genzel, the Bruker measurements a Norton-Beer (medium) apodization. We baseline-corrected the images in Figures 2 and 3 (including spectra), Supplementary Figures 1 and 7; all other infrared images as well as spectra show raw data. We did not use post-acquisition smoothing or filtering. The infrared data were analyzed and images were created with software packages IRidys (in-house development) and ENVI (ITT VIS).

The prostate cancer sample (Gleason grade 6) with epithelial cells (Fig. 2 and Supplementary Fig. 1) was a viable tumor without necrosis, in a cribriforming pattern and had some strands of stroma crossing through it. A second prostate cancer sample, which was also Gleason grade 6 for comparison (Fig. 3a–e), had chronic inflammation (mostly mononuclear cell infiltration of macrophages and lymphocytes) and contained two glands, a small vessel with a muscular wall and capillaries (with blood). The tissue shown in Figure 3f was a normal human breast tissue core including the terminal ductal lobular unit (TDLU) region and the tissue shown in Figure 3h contained two benign prostate glands from a cancerous prostate tissue core (Gleason grade 6). Tissues used here were from anonymized samples from individuals and involved secondary analysis as approved by the University of Illinois at Urbana-Champaign Institutional Review Board, protocol 06684. We fixed all biomedical samples in 4% para-formaldehyde, embedded them in paraffin, sectioned them at a thickness of 4 μm , mounted them on a BaF₂ infrared transparent window and deparaffinized them with hexane for 48 h before measurement. In transmission mode sample thickness can affect the obtainable spatial resolution. Using a simple geometric model we estimated that the sample thickness should not be above $\sim 3\text{--}4\text{ }\mu\text{m}$ to achieve full diffraction-limited resolution.

We purchased the apertures (Supplementary Figs. 5 and 6) from National Aperture, Inc., the high-resolution US Air Force (USAF) test target (Supplementary Fig. 3) from Edmund Optics Inc. and the polystyrene beads (Supplementary Fig. 7) from Polysciences, Inc. We diluted the polystyrene bead suspension with water, dispensed it on an ultrathin formvar film substrate and then air-dried it.

We recorded images of polystyrene beads with a diameter of ~ 1 and $2\text{ }\mu\text{m}$ (acquisition time, ~ 5 min) to examine spectral limits of detection per pixel. We detected the 6 ± 1 fmol or 3.4×10^9 ($\pm 0.7 \times 10^9$; s.d.) CH₂ groups contained in a $1\text{ }\mu\text{m}$ polystyrene bead (mass, 600 ± 100 fg; volume, 0.6 ± 0.1 fl) in a single $0.54\text{ }\mu\text{m} \times 0.54\text{ }\mu\text{m}$ pixel using the International Union of Pure and Applied Chemistry (IUPAC) detection limit criterion (Supplementary Fig. 7). We estimated this to be ~ 100 -fold better than with current instrumentation¹⁶ and this compared favorably with the lowest detection limit reported³⁵ using destructive methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

FTIR imaging with a multibeam synchrotron source. **(a)** Schematic of the experimental setup. M1–M4 are mirror sets. **(b)** A full 128×128 pixel FPA image with 12 overlapping beams illuminating an area of $\sim 50 \mu\text{m} \times 50 \mu\text{m}$. Scale bar, $40 \mu\text{m}$. **(c)** A visible-light photograph of the 12 beams projected on a screen in the beam path (dashed box in **a**). Scale bar, $\sim 1.5 \text{ cm}$. We display the beams as one beam from then on in the schematics. Each beam exhibits a shadow cast by a cooling tube upstream, which is not shown in **a**. **(d)** Long-exposure photograph showing the combination of the 12 individual beams into the beam bundle by mirrors M3 and M4. Scale bar, $\sim 20 \text{ cm}$.

Figure 2.

Chemical images from various FTIR systems. **(a–d)** The same cancerous prostate tissue section (area, $\sim 280 \mu\text{m} \times 310 \mu\text{m}$) measured with different instruments, using the integrated absorbance of the CH-stretching region ($2,800\text{--}3,000 \text{ cm}^{-1}$), without dyes or stains. We processed all images identically (baseline correction only) and used the same color scale (color bar in **a**; AU, absorbance units). Scale bars, $100 \mu\text{m}$ and in insets, $10 \mu\text{m}$. Images acquired with a conventional table-top system (PerkinElmer Spotlight) equipped with a thermal source in raster-scanning mode ($10 \mu\text{m} \times 10 \mu\text{m}$; **a**) and linear array mode ($6.25 \mu\text{m} \times 6.25 \mu\text{m}$; **b**), with an FTIR imaging system (Varian Stingray) equipped with a $64 \text{ pixel} \times 64 \text{ pixel}$ FPA ($5.5 \times 5.5 \mu\text{m}$ per pixel at the sample plane; **c**) and with our multibeam synchrotron-based imaging system (pixel size, $0.54 \mu\text{m} \times 0.54 \mu\text{m}$; **d**). **(e)** Hematoxylin and eosin (H&E)-stained prostate tissue (diameter, 0.75 mm). Scale bar, $100 \mu\text{m}$. Dashed box specifies the corresponding area of a serial, unstained section from which we generated images in **a–d**. **(f)** Typical unprocessed spectra from a single pixel acquired with each instrument (crosshairs in **a–d** indicate corresponding pixel positions in the infrared images).

Figure 3.

High-resolution multibeam synchrotron FTIR imaging. (a) Hematoxylin and eosin (H&E)-stained image of cancerous prostate tissue with chronic inflammation obtained using visible light microscopy. (b,c) Multibeam synchrotron absorbance images obtained from an unstained serial section of the sample shown in a. Spatial detail in images from the new system is highlighted for lymphocytes (blue arrow) and red blood cells (red arrow). (d) Image of the same unstained section imaged with a conventional table-top system (PerkinElmer Spotlight, linear array mode). (e) Expanded views of the boxed area in b showing the typical appearance of lymphocytes in H&E stained samples (top), the new system (bottom left) and a conventional table-top instrument (bottom right). (f) H&E-stained visible light image (top), asymmetric CH-stretching ($2,840\text{ cm}^{-1}$, center) and collagen-specific ($1,245\text{ cm}^{-1}$, bottom) infrared images of an unstained section of normal breast tissue (terminal ductal lobular unit region). Epithelial (green arrow) and intralobular stromal regions (magenta arrow) are highlighted. (g) Spectra of epithelial and stromal cells recorded with a multibeam synchrotron versus a thermal source. (h) Absorbance image ($2,840\text{ cm}^{-1}$; top) of an unstained cancerous prostate tissue showing two benign prostate glands. Inset, potential presence of basement membrane at the interface between stroma and epithelium is marked (arrows). Image (bottom) showing epithelial (green) and stromal (magenta) cells classified using previous algorithms. (i) Average spectra from epithelial, stromal (two each):

one closer to the interface, one farther away), and interface pixels identified manually from data obtained using two different instruments. AU, absorbance units. Scale bars, 50 μm .