High-speed coherent Raman fingerprint imaging of biological tissues

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An imaging platform based on broadband coherent anti-Stokes Raman scattering has been developed that provides an advantageous combination of speed, sensitivity and spectral breadth. The system utilizes a configuration of laser sources that probes the entire biologically relevant Raman window (500–3,500 cm⁻¹) with high resolution (<10 cm⁻¹). It strongly and efficiently stimulates Raman transitions within the typically weak ‘fingerprint’ region using intrapulse three-colour excitation, and utilizes the non-resonant background to heterodyne-amplify weak Raman signals. We demonstrate high-speed chemical imaging in two- and three-dimensional views of healthy murine liver and pancreas tissues as well as interfaces between xenograft brain tumours and the surrounding healthy brain matter.

Raman spectroscopy is a powerful label-free technique for analysing the chemical species within biological samples. It has been extensively applied to a variety of tissue types and pathologies, providing a high level of sensitivity and specificity. In these and similar studies, multiple peaks within the weakly scattering Raman fingerprint region (<1,800 cm⁻¹) are used to discriminate subtly different states of cells and tissues. Until now, fingerprint spectra of sufficient quality for such studies have only been available with collection times ranging from 0.2 to 30 s (refs 4–8), seriously limiting the use of Raman spectroscopy in high-resolution scattering systems such as neat liquids and polymer films. Coherent Raman imaging (CRI) techniques have been developed that coherently populate selected vibrational states of molecules through their nonlinear response to multiple pulsed laser fields.

Narrowband CRI techniques, such as coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS), are capable of video-rate imaging of single Raman bands, but suffer from limitations in breadth and speed of laser tuning rates. CARS, specifically, is also limited by a non-resonant background (NRB) that distorts the Raman signal through coherent mixing and seriously limits Raman peak identification without scanning over a wide spectral range. The net effect limits narrowband CARS to species with a high oscillator density and uniquely isolated Raman peaks, essentially preventing access to the chemically rich fingerprint region.

Multiplex techniques, such as multiplex SRS and broadband CARS (BCARS), provide an alternative stimulation profile in which multiple Raman transitions are probed simultaneously. Multiplex SRS is free of NRB, but it is currently limited by small bandwidths, pulse shaping rates, coarse spectral resolution and competing nonlinear phenomena. The BCARS signal is accompanied by an NRB, but, because it is a spectroscopic technique, it can be performed in a manner such that the NRB is used as a heterodyne amplifier for weak Raman peaks, and NRB-induced spectral distortions are removed numerically.

In this Article we present a BCARS system that efficiently stimulates Raman transitions, especially within the weak fingerprint region, using intrapulse three-colour excitation, and exploits the strong NRB to amplify the inherently weak fingerprint signal. The combination of these two features allows us to record spectra one to two orders of magnitude faster than previously possible and with high spectral clarity, paving the way towards CRI integration into widespread biological and clinical use.

System design

Figure 1 presents a schematic of the BCARS system, which uses tailored co-seeded fibre lasers to generate a narrowband flat-top probe (770 nm; ~16 mW, 3.4 ps pulses on-sample) and a supercontinuum (SC: ~900 nm to 1,350 nm; ~9.5 mW, ~16 fs pulses on-sample) with negligible jitter, in a laser design similar to that of Selmi and colleagues. This particular configuration provides an independent, robust probe source for high-resolution spectra (~10 cm⁻¹); it stimulates the fingerprint region using intrapulse three-colour excitation, which is particularly strong, efficient and maximal at the lowest energy levels, and stimulates higher-energy transitions using two-colour excitation, thus accessing the entire biologically relevant Raman window (500–3,500 cm⁻¹). We avoid NRB-reduction schemes so as to maximally generate the resonant signal and the non-resonant signal for heterodyne amplification.

The developed system uses two different excitation methods. These mechanisms operate simultaneously as they are simply different permutations of the same two pulses, but their properties differ
significantly. To illustrate this, we begin with an expression for the frequency-domain CARS signal intensity, $I_{\text{CARS}}(\omega)$:

$$I_{\text{CARS}}(\omega) \propto \left| \chi^{(3)}(\omega) \left| E_p(\omega) \ast E_p(\omega) \right| \right|^2$$

(1)

where $\omega$ is frequency, $\chi^{(3)}$ is the third-order nonlinear susceptibility, $E_p$, $E_s$ and $E_{pr}$ are the pump, Stokes and probe fields, respectively, and $\ast$ and $\ast$ are the cross-correlation and convolution operators, respectively. The term in square brackets is the frequency-domain coherence generation profile, which will maximize at the frequency difference between the peaks of the pump and Stokes fields. Assuming real, Gaussian fields, the integrated spectral intensity over all frequencies is given as

$$\langle I_{\text{CARS}} \rangle \propto P_p P_s P_{pr} \frac{\sigma_p^2 \sigma_s \sigma_{pr}}{\sqrt{\sigma_p^2 + \sigma_s^2 + \sigma_{pr}^2}}$$

(2)

where $P_p$, $P_s$ and $P_{pr}$ are respectively the pump, Stokes and probe spectrally integrated modulus-squared field (proportional to the average power), such that $P = \langle |E|^2 \rangle = |E_0|^2 \sqrt{\sigma}$, where $E_0$ is the field envelope amplitude with 1/e half-width $\sigma$.

Under two-colour (2C) excitation (Fig. 1b), used in BCARS/MCARS systems with degenerate pump and probe sources\textsuperscript{18-22} ($P_{p, pr} \equiv P = P_{pr}; \sigma_p \equiv \sigma_p = \sigma_{pr}$), the BCARS signal resolution is provided by the narrowband pump–probe source, and the spectral breadth is provided by the Stokes source. Thus, from equation (2),

$$\langle I_{2C} \rangle \propto P_p^2 P_s P_{pr} \frac{\sigma_p^2 \sigma_s \sigma_{pr}}{\sqrt{2 \sigma_p^2 + \sigma_s^2}} \approx P_p^2 P_s \sigma_{p, pr} \sigma_{p, pr}$$

(3)

For intrapulse three-colour (3C) excitation (Fig. 1c) in which the probe is independent and the SC provides the pump and Stokes photons ($P_{p,s} \equiv P_p = P_s; \sigma_p = \sigma_s$):

$$\langle I_{3C} \rangle \propto P_p^2 P_s P_{pr} \frac{\sigma_p^2 \sigma_s \sigma_{pr}}{\sqrt{2 \sigma_p^2 + \sigma_s^2}} \approx P_p^2 P_s \sigma_{s, pr} \sigma_{s, pr}$$

(4)

We note two important differences between these coherence generation mechanisms. One is that the two-colour mechanism has a peak excitation profile at the difference frequency between the narrowband and SC pulses (near 2,800 cm$^{-1}$ for our system), whereas the intrapulse three-colour mechanism has a peak excitation frequency at 0 cm$^{-1}$, because the pump and Stokes fields are degenerate. Thus, the former excites the CH/OH stretch region, which typically presents an intrinsically stronger response, whereas the latter excites the fingerprint region, with the weaker intrinsic response. The other important difference between these mechanisms is their efficiency over a broad bandwidth. With two-colour excitation, as
described in equation (3), the total CARS signal is independent of the Stokes source bandwidth $\sigma_s$. Thus, with increasing $\sigma_s$, the total integrated CARS signal remains constant, but the signal at each spectral increment will decrease. In contrast, as described in equation (4), the total three-colour CARS signal rises with increasing bandwidth $\sigma_{p,S}$. Importantly, the signal at each spectral increment also increases with increasing $\sigma_{p,S}$. From this comparison, one can appreciate that the three-colour mechanism is much more efficient than the two-colour mechanism for the present system. We can quantify the relative efficiency as $\langle I_{3C} \rangle / \langle I_{2C} \rangle \propto \sigma_S / \sigma_{p,S} \approx 100$. Accordingly, this

Figure 2 | CRI of murine liver tissue. a, Spectral image of a portal triad within murine liver tissue with the nuclei in blue, collagen in orange and protein content in green. A, portal artery; B, bile duct; V, portal vein; Ep, epithelial cell; En, endothelial cell. b, SHG image highlighting the fibrous collagen network. c, SHG spectrum for a single pixel. d–f, Spectral images of individual vibrational modes represented by the colour channels at 785 cm$^{-1}$ (d); 855 cm$^{-1}$ (e); 1,004 cm$^{-1}$ (f). g, Single-pixel spectra from the nucleus (DNA), collagen fibre, arterial wall and a lipid droplet. h–l, Additional spectral channels that provide histochemical contrast: 1,302 cm$^{-1}$ (h); 1,665 cm$^{-1}$ (i); 2,884 cm$^{-1}$ (j); 3,228 cm$^{-1}$ (k); elastin (l), 1,126 and 1,030 cm$^{-1}$ but not 677, 817 and 1,302 cm$^{-1}$. Scale bars, 20 $\mu$m.
system provides strong and efficient excitation where it is most needed within the fingerprint region. A more thorough treatment of these topics is presented in Supplementary Section ‘2-colour and 3-colour excitation methods’.

Utilizing three-colour generation is necessary, but not sufficient, to achieve the required signal levels within the fingerprint region. CARS imaging with three-colour excitation was first reported more than 10 years ago\(^3\), but until now has been limited to fingerprint imaging of only strongly scattering systems such as neat liquids and polymer films\(^30,31\). To best take advantage of the strong three-colour stimulation requires full utilization of the NRB. Without the heterodyne amplification provided by the NRB, our signal-to-noise ratio (SNR) at high-speed acquisition would be less than 1 for most Raman fingerprint peaks. As previously described, the NRB limits the vibrational sensitivity and specificity of narrowband CARS techniques\(^9,24\). However, it acts as a robust local oscillator for heterodyne amplification of the resonant signal when spectral phase retrieval is applied numerically after the signal is collected\(^{25,26}\). This amplification can bring the weaker Raman peaks above the noise floor, increasing their effective SNR by over an order of magnitude (Supplementary Section ‘Nonresonant background as heterodyne amplifier’).

The spectra generated by this combination of two-colour and three-colour excitation are collected with a spectrometer equipped with a thermoelectrically cooled charge-coupled device (CCD) camera that affords acquisition times down to 3.5 ms per spectrum. Our spectrometer detection range is sufficiently broad (\(>250\) nm) to acquire the signal from BCARS, as well as other nonlinear processes such as second-harmonic generation (SHG) and two-photon excited fluorescence (TPEF), providing an additional layer of information for BCARS spectral interpretation. Figure 1d shows a raw BCARS spectrum of 99% glycerol (acquisition time, 3.5 ms; SNR, \(15–23\) dB), which shows the intense three-colour response in the range \(\sim 425–2,000\) cm\(^{-1}\), which dwarfs the two-colour response of \(\sim 2,000–3,600\) cm\(^{-1}\). Although the raw BCARS spectrum is distorted due to coherent mixing between the resonant CARS signal and the NRB\(^9\), Fig. 1e demonstrates the use of a time-domain Kramers–Kronig (TDKK) transform to retrieve the imaginary component of the nonlinear susceptibility\(^25\), \(\text{Im}\{\chi^{(3)}\}\) (convolved with the probe source spectral profile), which is proportional to the (spontaneous) Raman response of the molecule. See Supplementary Section ‘Spontaneous and coherent Raman spectroscopy of glycerol’, which demonstrates the significant speed enhancement. We use the TDKK for its speed advantage over competing techniques\(^27\). To examine the detection limit of the BCARS system and demonstrate its molecular response linearity, we recorded spectra from a methanol–water dilution series (time-averaged over 1 s; ref. 10). As shown in Fig. 1f, the response of the retrieved \(\text{Im}\{\chi^{(3)}\}\) is linear with respect to methanol concentration (starting from 1 mol l\(^{-1}\); zoomed-in for clarity), and the detection limit of the system was determined to be \(<23\) mmol l\(^{-1}\) using the C–O stretch peak at \(\sim 1,037\) cm\(^{-1}\) and \(<8\) mmol l\(^{-1}\) using the C–H stretch peak at \(\sim 2,839\) cm\(^{-1}\), which matches closely with similar SRS measurements\(^10\).

**Tissue imaging**

To date, histological analysis of tissues using CRI has relied on limited spectral information, primarily in the strong CH/OH stretch region of the Raman spectrum (\(\sim 2,700–3,500\) cm\(^{-1}\))\(^{12,17,28,33}\). With these limitations and the complexity of tissue specimens, spectrally identifying even such common features as nuclei are non-trivial tasks. To demonstrate the sensitivity of the present CRI system using molecular fingerprint signatures, we imaged murine liver tissue sections. Figure 2a presents a pseudocolour image of liver tissue near a portal triad (hepatic artery, hepatic portal vein and bile duct), which was collected with 3.5 ms dwell times over a \(200\) \(\mu\)m \(\times\) \(200\) \(\mu\)m area (300 pixels \(\times\) 300 pixels). This image shows nuclei contrasted in blue, based on the Raman band at \(\sim 785\) cm\(^{-1}\), which emanates from DNA/RNA pyrimidine ring breathing and the phosphodiester stretch\(^34\). For further chemical...
Figure 4 | Histopathology using broadband CRI. a, Brightfield image of xenograft glioblastoma in mouse brain, with the tumour hard boundary outlined (black, dashed line). The cyan dashed box indicates a region of interest (ROI). Scale bar, 2 mm. b, Phase contrast micrograph of BCARS ROIs with boxes and associated subfigure labels. Scale bar, 200 μm. c, Pseudocolour BCARS image of tumour and normal brain tissue, with nuclei highlighted in blue, lipid content in red and red blood cells in green. d, BCARS image and axial scan with nuclei highlighted in blue and lipid content in red. e, BCARS image with nuclei highlighted in blue, lipid content in red and CH3 stretch in green. NB, normal brain; T, tumour cells; RBC, red blood cells; L, lipid bodies; WM, white matter. f, Single-pixel spectra. g, Spectrally segmented image of internuclear (blue) and extranuclear (red) tumoural spaces. h, Histogram analysis of phenylalanine content. i, Mean spectra from within a tumour mass. c-e, Scale bars, 20 μm.

contrast or specificity, one could use other nucleotide peaks at 668, 678, 728, 750, 829, 1,093, 1,488 or 1,580 cm⁻¹. Additionally, the peak at 830 cm⁻¹ could be used to gauge the amount of DNA in the β-conformation relative to the total genetic content, thereby providing information about the functional state of the cells. As a general protein contrast, the ring-breathing contribution of phenylalanine at 1,004 cm⁻¹ is presented in green. The collagen is highlighted in red using the 855 cm⁻¹ C–C stretch from the pyrrolidine ring of proline (the C–C stretch at 938 cm⁻¹ also provides similar contrast). Previous CRI investigations of tissue have incorporated SHG and TPEF imaging to identify collagen and elastin, respectively, as shown in Fig. 2b, with examples of spectra in Fig. 2c. It should be noted, however, that SHG and TPEF provide uncertain chemical specificity, as other biologically relevant molecular species are also known to generate a response. Additionally, we note that Raman spectroscopy and SHG present differing contrasts for collagen, as Raman (and by extension, BCARS) is sensitive to molecular structure, but SHG is sensitive to supermolecular crystalline structure.

With this level of spatial resolution and chemical contrast, several hepatic structures are identifiable by their histology: the hepatic artery (with its circular protein-rich, collagen-poor band—probably smooth muscle—surrounding a thin endothelial layer and lumen), the bile ducts (lined by tightly packed cuboidal epithelial cells) and the relatively large portal vein (with its sparse—due to micrometre sample preparation—endothelial layer). One can also see the connective tissue septa (primarily collagen) that enmesh the portal triad.

Although the pseudocolour image in Fig. 2a is limited to three colours, which are presented in high-contrast greyscale in Fig. 2d–f, one can identify significant spectral complexity in the sample, as illustrated by the single-pixel spectra in Fig. 2g. Using isolated peaks, one could create dozens of unique images based on vibrational susceptibilities, such as those shown in Fig. 2h–k: 1,302 cm⁻¹ (CH₂ deformation), 1,665 cm⁻¹ (amide I/C = C stretch), 2,884 cm⁻¹ (CH₂ stretch), 3,228 cm⁻¹ (O–H stretch), respectively. Additionally, a multivariate analysis of contributions from several peaks—their locations, intensities and shapes—

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presents significant avenues of chemical contrast. For example, Fig. 21 highlights elastin by segmenting the chemical species that have vibrations at 1,126 and 1,030 cm⁻¹ but lack vibrations at 677, 817 and 1,302 cm⁻¹, which isolates elastin from collagen and other proteins, lipids and nucleotides. Similarities and differences between the BCARS image and the TPEF image in Fig. 2b, indicate that although elastin is the most abundant fluorescent molecule, multiple chemical species contribute to the TPEF signal.

Beyond histochemical imaging in two dimensions, non-linear excitation in CARS makes it an intrinsically sectioning microscopy, affording the generation of ‘z-stack’ images in three dimensions. Narrowband CARS and SRS have demonstrated this capability, but three-dimensional microspectroscopy with BCARS or spontaneous Raman has been uncommon due to their long acquisition times. Figure 3a is a BCARS image of murine pancreas, with the nuclei highlighted in blue (785 cm⁻¹), collagen in red (855 cm⁻¹), and a general contrast for lipids and protein in green (1,665 cm⁻¹; lipids, C = C stretch; proteins, amide I). This image shows a single plane from a ten-stack collection with each plane covering 150 µm × 100 µm (0.667 µm lateral, 1 µm axial step size; <2 min per image). Two reconstructed axial planes are also shown. This image shows an interlobular exocrine duct surrounded by epithelial cells, the edge of a larger interlobular exocrine duct (as identified by the columnar epithelial cells), a collagen matrix and acinar cells (and the lumen separating the acini). Figure 3b presents the reconstructed three-dimensional image, which more clearly shows the shape, size and orientation of the individual cells and tissue constituents. Figure 3c shows single-pixel spectra from the nucleus of an epithelial cell, collagen and from the cytosol of an acinar cell.

For histopathological analysis, spontaneous Raman and infrared micro/spectroscopy have demonstrated adequate chemical specificity and sensitivity to delineate a variety of neoplasms, but require long integration times and have a coarse spatial resolution, which may limit accurate tumour-boundary identification and early-stage tumour detection. Conversely, CRI techniques have demonstrated high-speed, high-spatial-resolution imaging of normal and diseased brain tissue, but with contrast limited to single or few Raman peaks. We present images of orthotopic xenograft brain tumours (see Methods for more detail) within a murine brain. Figure 4a shows a brightfield image of a brain slice (10 µm nominal thickness) with an identifiable tumour mass, from which we imaged several areas (Fig. 4b shows a close-up polarization micrograph of the specific imaging sites). Figure 4c shows a CRI image with nuclei in blue (730 cm⁻¹), lipid content in red (2,850 cm⁻¹) and red blood cells in green (1,548 cm⁻¹ + 1,565 cm⁻¹; C-C stretch from haemoglobin). This image clearly shows the large tumour mass and a projection of neoplastic cells within healthy tissue. Additionally, smaller tumour bodies are identifiable by their high density of distorted nuclei with high nuclear-to-cytoplasmic ratio. Figure 4d shows several small extensions of the main tumour mass invading healthy brain matter. The mesh-like cytoplasmic ratio. Figure 4d shows several small extensions of the main tumour mass invading healthy brain matter. The mesh-like cytoplasmic ratio.
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**Author contributions**

C.H.C. performed all experiments, analyzed all data and drafted the manuscript. M.T.C. and C.H.C. designed all experiments and constructed the final manuscript. M.T.C. and Y.J.L. conceptualized the complementary two/three-colour excitation scheme. C.H.C. constructed the instrument, modified the laser system and developed the high-speed acquisition and processing software. C.H.C., Y.J.L., C.M.H. and M.T.C. developed the signal-processing methodology and protocols. M.T.C. developed the Kramers–Kronig transform and C.H.C. developed the parallelized, high-speed implementation. A.R.H.W., J.M.H., J.N.R. and J.D.L. provided materials and/or the tumour sections and provided histopathology insights and direction. J.M.H. assisted in performing the tumour section study, as well as contributing to the text of the manuscript. A.R.H.W., J.M.H. and C.H.C. collected the spontaneous Raman spectra of glycerol and C.H.C. performed the analysis. C.H.C. developed the presented mathematical framework of CARS generation and associated efficiencies with two/three-colour stimulation. M.T.C. supervised the study.

**Competing financial interests**

The authors declare no competing financial interests.

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**Supplementary information**

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.T.C.