

Advanced vibrational microscopes for life science

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Providing molecular fingerprint information, vibrational spectroscopic imaging opens a new window to decipher the function of biomolecules in living systems. While classic vibrational microscopes based on spontaneous Raman scattering or mid-infrared absorption offer rich insights into sample composition, they have very small cross sections or poor spatial resolution. Nonlinear vibrational microscopy, based on coherent Raman scattering or optical photothermal detection of vibrational absorption, overcomes these barriers and enables high-speed and high-sensitivity imaging of chemical bonds in live cells and tissues. Here, we introduce various modalities, including their principles, strengths, weaknesses and data mining methods to the life sciences community. We further provide a guide for prospective users and an outlook on future technological advances.

Optical microscopy has been a foundational tool in life science since Van Leeuwenhoek's observation of microorganisms in the 17th century using his single-lens microscope. The invention of phase contrast microscope in the 1930s further allowed the observation of structures such as chromosomes inside live cells. For imaging biomolecules, fluorescence microscopy is currently the tool of choice, aided by the development of dyes, fluorescent proteins and advanced modalities such as super-resolution microscopy. However, the fluorescent reporters are often larger than the target molecules including small proteins, fatty acids, amino acids, carbohydrates and cholesterol. Thus, labeling inevitably perturbs the functions of small biomolecules. Click chemistry has been developed to partially address this concern, wherein the molecules of interest are first tagged with an azide or alkyne causing minimal perturbation to biochemistry and are then conjugated to a dye through a click reaction for visualization under a fluorescence microscope. Yet, the click-chemistry approach only allows one snapshot of the system, prohibiting dynamic imaging. Additionally, the click reaction efficiency can be heterogeneous in a cellular environment.

One elegant way to address these limitations is using chemical bond vibrations as contrast. Vibrational microscopy can be pursued in a label-free manner by targeting intrinsic bonds within biomolecules or in a click-free manner by direct imaging of the azide or alkyne bonds. In

the spectral domain, fluorescence is broad (~50 nm), limiting the number of fluorophores to be used, and is often affected by photobleaching. In contrast, vibrational lines are much narrower (~1 nm), allowing for highly multiplexed imaging without photobleaching. These unique advantages have made vibrational imaging a highly desirable goal for scientists. However, it is technically very challenging since signals such as spontaneous Raman scattering from a chemical bond are many orders of magnitude weaker than the fluorescence emission from a dye. Mid-infrared absorption offers a much larger cross section, but the spatial resolution of an infrared light microscope is too poor to resolve features inside a cell, and strong water absorption impairs infrared spectroscopic imaging of living systems. Advanced vibrational microscopes, which include coherent Raman scattering (CRS) microscopy, vibrational photothermal (VIP) microscopy, vibrational photoacoustic imaging and up-conversion of mid-infrared absorption (Fig. 1), are overcoming these daunting barriers, enabling high-speed, high-sensitivity imaging of specific chemical bonds in live cells and organisms.

In this Review, we provide an overview of the development of these advanced vibrational microscopes with a focus on CRS (Box 1) and VIP (Box 2) microscopy. We describe the principles, instrumentation, advantages and limitations of each modality. We further discuss the role of data science in vibrational imaging, illustrate multi-scale

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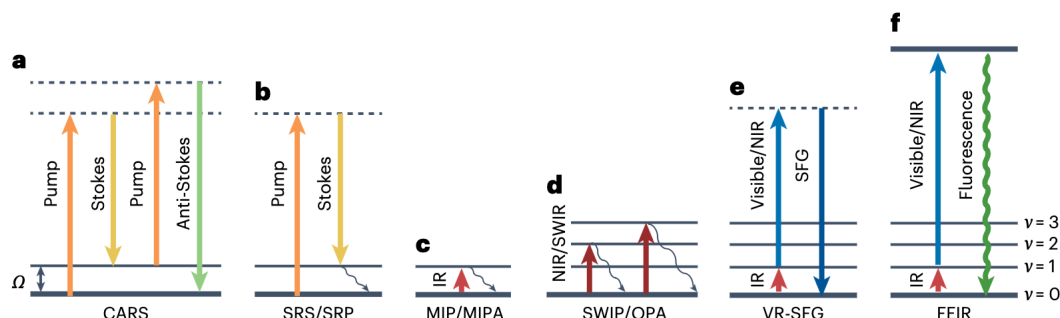


Fig. 1 | Energy diagrams for advanced vibrational microscopy. a–f, Energy diagrams. CARS, coherent anti-Stokes Raman scattering. SRS, stimulated Raman scattering. SRP, stimulated Raman photothermal. MIP, mid-infrared photothermal. MIPA, mid-infrared photoacoustic. SWIP, shortwave infrared

photothermal. OPA, overtone photoacoustic. VR-SFG, vibration-resonant sum frequency generation. FEIR, fluorescence-encoded infrared. IR, infrared. NIR, near infrared. SWIR, shortwave infrared. Ω , chemical bond vibrational frequency. v , vibrational energy level.

life science applications from single viruses to humans, offer a user guide on modality selection and sample preparation, and present an outlook for the field.

CRS microscope

CARS microscope

The coherent anti-Stokes Raman scattering (CARS) microscope was reported by Duncan et al. in 1982 (Fig. 2a)¹, in which a non-collinear scheme was adopted to meet the phase-matching condition required in CARS spectroscopy. In 1999, Zumbusch et al. reported a collinear CARS microscope (Fig. 2b)². With a high numerical aperture (NA) objective lens, the phase-matching conditions are relaxed due to the large cone of wave vectors and the short interaction length within the focal volume. Additionally, with near-infrared (NIR) lasers, the linear absorption-induced photodamage is minimized and the nonresonant background is reduced compared to visible lasers. The collinear beam geometry and NIR excitation were widely adopted and revived the CARS microscopy field. The development of advanced CARS modalities (Supplementary Table 1) is summarized below.

In 2001, Cheng et al. improved the spectral resolution and signal-to-background ratio using a NIR picosecond laser as the excitation source (Fig. 2c)³. In CARS, the nonresonant signal is quadratically dependent on the pulse spectral width, while the resonant CARS signal reaches a plateau when the spectral width of a laser pulse exceeds the Raman line width in the condensed phase. As a result, laser pulses of a few picoseconds, corresponding to a spectral width of about 10 cm^{-1} , are ideal for CARS imaging and this concept has been adopted by the field since then. In the meantime, epi-detected CARS microscopy (Fig. 2c) was reported as a means of imaging nanoscale objects in a bulk medium³. For small scatterers or thin samples with a size much smaller than the excitation wavelengths, the phase-matching condition is satisfied in both forward and backward detections. Therefore, the same signal intensity is expected in both forward-detected and epi-detected CARS. On the other hand, the nonresonant background from the bulk solvent builds up coherently and predominantly goes forward, allowing small objects to be epi-detected with high contrast. For thick samples, epi-detected CARS arises from back-scattering of forward signals⁴. Recently, Heuke and Rigneault reported coherent Stokes Raman scattering microscopy as a potential means of enhancing the backward-to-forward signal ratio⁵.

Although picosecond lasers allow high-speed CARS imaging of a specific Raman band, single-color CARS images are insufficient to identify the chemical composition of a sample. With picosecond lasers, a CARS spectrum can be acquired by slow wavelength tuning. Integration of a narrowband picosecond laser with a broadband femtosecond laser was applied to enable acquisition of a CARS spectrum at each pixel. In 2002, Cheng et al.⁶ and Wurfel et al.⁷ reported multiplex CARS (Fig. 2d) by combining a narrowband and a broadband laser

BOX 1

Spontaneous Raman versus coherent Raman scattering

In spontaneous Raman scattering, a single laser beam (pump) interacts with molecules to generate both Stokes and anti-Stokes Raman scattering. In CRS, including CARS (Fig. 1a) and SRS (Fig. 1b), two laser beams, one at pump frequency (ω_p) and the other at Stokes frequency (ω_s), drive the vibration of a chemical bond. The light-matter interactions produce a signal at the anti-Stokes frequency ($\omega_p - \omega_s + \omega_p$) and the Stokes frequency. Meanwhile, the pump beam experiences an intensity loss and the Stokes beam experiences an intensity gain, referred to as stimulated Raman loss and stimulated Raman gain, respectively. CARS, stimulated Raman loss and stimulated Raman gain can be implemented in the single-color mode using picosecond laser pulses, or in the multiplex mode with a narrowband and a broadband laser source.

Intuitively, CARS and SRS gain a large signal level by interfering with a local oscillator. The CARS field interferes with the nonresonant background, whereas SRS interferes with one of the input laser fields. Notably, the local oscillator carries noise. At the shot noise limit, the signal grows faster than the noise with increasing laser power and builds sufficient SNR on the microsecond timescale. Therefore, CARS and SRS outperform spontaneous Raman for imaging a specific Raman mode with microsecond dwell time.

When the molecular concentration is at the micromolar to nanomolar level, down to the single molecule, the very limited SRS or CARS photons are buried in noise. Raman imaging is also ineffective under these conditions due to the low photon count. Nevertheless, spontaneous Raman can accumulate sufficient photons to build the SNR with second-level integration. In this sense, CARS, SRS and Raman spectroscopy are complementary techniques. CARS and SRS are suitable for fast imaging of molecules with relatively high local concentrations, often at millimolar levels, such as lipid, myelin, proteins and nanoparticles. Spontaneous Raman scattering is suitable for slow, whole-spectrum measurements at a point of interest.

to acquire a CARS spectrum covering a few hundred wavenumbers with a spectrometer. The spectral resolution was determined by the narrow-bandwidth picosecond laser and the covered range of the spectra window was determined by the femtosecond laser. In 2004,

BOX 2

Photothermal versus photoacoustic imaging of chemical bonds

Vibrational transitions are quantized and the lifetime of a vibrationally excited state is a few picoseconds. Thus, a nanosecond mid-infrared pulse, a nanosecond SWIR pulse, or a train of NIR pump and Stokes pulses, could repetitively excite a chemical bond, with all absorbed energy converted to heat via efficient vibrational relaxation. The heat diffusion then follows Newton's law of heating and cooling on the nanosecond to microsecond timescale. Since vibrational excitation and relaxation are on the much shorter picosecond timescale, the generated heat accumulates and induces a substantial temperature rise, a thermal lens of a medium or a thermal expansion of a particle, which can be measured through fluorescence, optical phase or Rayleigh scattering detection. This mechanism makes VIP microscopy an extremely sensitive method for chemical bond imaging. The concept of photothermal imaging based on the change in refractive index (the mirage effect)¹⁴⁵ was proposed in 1983. Initial effort on mid-infrared photothermal imaging based on a quantum cascade laser was reported in 2012; however, with 1–2- μm spatial resolution¹⁴⁶. A 2016 high-performance MIP microscope⁶² achieved submicrometer spatial resolution and micromolar sensitivity. This work triggered rapid technical developments^{147,148} and commercialization of optical photothermal infrared microscopy into a mIRage system by Photothermal Spectroscopy Corporation¹⁴⁹. Advanced MIP schemes achieving 100-nm resolution were reported recently^{150,151}.

Notably, the transient heat further expands the specimen and generates an ultrasound wave via the photoacoustic effect. Transducer-based NIR and MIPA imaging of lipid, glucose and protein has been demonstrated^{89,93,95}. Both the photothermal and photoacoustic effects modulate the refractive index and can be optically probed. A recent work shows that the optical photothermal signal is ~63 times larger than the photoacoustic signal under the same excitation⁸⁷. The low efficiency of thermal energy to ultrasound wave conversion and the difference between thermo-optic and elasto-optic coefficients account for this difference. On the other hand, unlike the photothermal signal, which is highly confined to the excitation focus, the photoacoustic wave experiences much less tissue scattering than the optical wave and can propagate to a far-field transducer. In this scenario, the two modalities are complementary for chemical imaging. The photothermal effect allows highly sensitive detection of nanoscale objects or low-concentration molecules via a pump-probe scheme, while the photoacoustic effect allows deep-tissue imaging of abundant chemical bonds, such as lipids deposited inside an arterial wall⁸⁹.

broadband CARS was developed by Kee et al. to cover a spectral window of more than 2,500 cm^{-1} with a laser of broader bandwidth⁸. In 2014, Cicerone and coworkers developed three-color broadband CARS that allowed the acquisition of fingerprint CARS spectra with a pixel dwell time of a few milliseconds⁹.

Multiplex CARS was also achieved in the time domain. In 2002, Volkmer et al. developed time-resolved CARS that used two femtosecond lasers to excite multiple vibration modes and a third femtosecond laser to probe the signal¹⁰. The CARS spectrum was reconstructed by

Fourier transform. In 2004, spectral focusing was introduced to femtosecond laser-excited CARS, in which the femtosecond pulses were temporally chirped into picosecond pulses with optical gratings¹¹. A CARS spectrum was generated by tuning the delay between the pump and Stokes pulses. In 2006, Ogilvie et al. reported Fourier-transform CARS (Fig. 2f) to achieve a 1,500 cm^{-1} spectral window using ultra-short pulses¹². Ideguchi et al. harnessed laser frequency combs for high-speed CARS spectroscopy and spectral imaging¹³. By a fast delay scanner, Goda and coworkers applied Fourier-transform CARS to high-throughput single-cell analysis^{14,15}.

Most CARS microscopes are built by scanning the sample or the tightly focused laser beams. Laser-scan CARS reached video-rate imaging speed in 2005 (ref. 4). To further improve the imaging speed and throughput, widefield CARS (Fig. 2e) was developed by Heinrich et al.¹⁶. A dark-field counterpropagating excitation with nanosecond pulsed lasers was used to satisfy the phase-matching conditions. Since then, different excitation schemes for widefield CARS have been adopted, including non-phase-matching illumination¹⁷, dynamic speckle illumination¹⁸, CARS holography¹⁹, collinear non-phase-matching illumination²⁰, phase imaging²¹, surface-enhanced²² and random illumination²³. In particular, widefield surface-enhanced CARS allowed rapid acquisition of Raman spectra with 10 cm^{-1} spectral resolution and achieved an imaging speed of 120 frames per second²².

CARS imaging is affected by a nonresonant background that arises from an electronic contribution to the third-order susceptibility. Such background limits the sensitivity of detecting low-concentration molecules and disperses the CARS spectrum. Persistent efforts have been made to minimize the background, as detailed in the Supplementary Information.

SRS microscope

The stimulated Raman scattering (SRS) microscope reported in 2007 by Ploetz et al.²⁴ is based on multiplex detection of signals generated by high-energy pulses, whereas the low-repetition-rate laser limited the signal-to-noise ratio (SNR). A high-speed SRS microscope using MHz modulation was reported in 2008 by Freudiger et al.²⁵ and independently in 2009 by two other groups^{26,27}. In the standard configuration of an SRS microscope (Fig. 3a), two spatially and temporally overlapped ultrafast lasers are tightly focused on a sample by a high-NA objective. Because of SRS, a modulation transfer from the intensity-modulated laser to the unmodulated laser occurs in the focus. The MHz-frequency modulation brings the SRS signal out of the low-frequency noise, which enables high-sensitivity and high-speed detection. The transmitted light is collected by a high-NA condenser to minimize a cross-phase modulation background and then spectrally filtered to remove the modulated laser. After converting the light-intensity to electrical signal by a photodetector, a demodulator, typically a lock-in amplifier, is used to obtain the SRS signal. Based on this basic configuration, various advanced SRS instrumentations have been developed to address the need for faster speed, higher sensitivity, super resolution, extended spectral range, volumetric imaging and clinical translation. Theoretically, SRS and CARS share the same level of SNR²⁷.

Targeting video-rate imaging, an epi-detected SRS microscope was demonstrated by incorporating resonant scanners and placing the detector directly in front of the objective lens²⁸. The high-speed epi-detection enabled in vivo imaging without motion artifacts, whereas the difficulty in building the specialized detector blocked the wide use of this method. Spatially multiplexed SRS further pushed the frame rate to 2 kHz with an arrayed detector²⁹, allowing real-time imaging of chemical kinetics. The challenges of this method are the alignment complexity and the low power density per channel.

Pursuing high-speed spectral acquisition, a spectrally broadband femtosecond laser was used for spectral focusing or spectral multiplexing. Spectral focusing is a technique to improve the spectral resolution of coherent Raman by chirping the spectrally broadband lasers

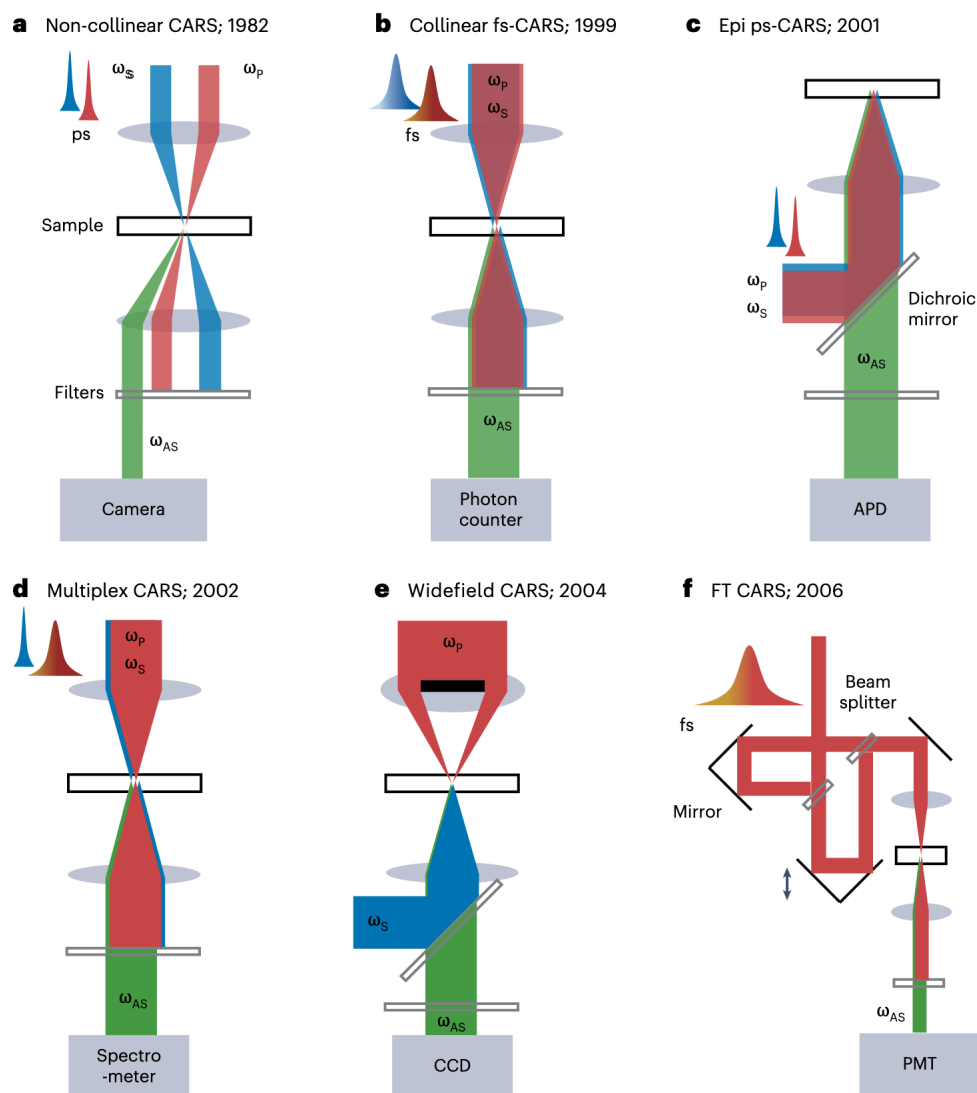


Fig. 2 | Various modalities developed for CARS imaging. **a–f**, CARS imaging modalities. ps, picosecond. fs, femtosecond. ω_p , frequency of pump laser. ω_s , frequency of Stokes laser. ω_{AS} , frequency of anti-Stokes signal. APD, avalanche photodiode. CCD, charge-coupled device. FT, Fourier transform. PMT, photomultiplier tube.

(Fig. 3b)^{30,31}. As the laser pulses are parallelly stretched in the temporal–spectral domain, the instantaneous frequency difference between the two pulses is constant and an SRS spectrum can be acquired by tuning the temporal delay. Delaying scanning with a motorized delay stage is the most straightforward approach, but it is slow. Galvo scanner-based³², polygon mirror-based³³ and acousto-optic-based³⁴ delay lines realized kilohertz spectral acquisition at the expense of alignment complication. Spectral multiplexing is another approach to achieve high-speed spectrum acquisition, where a spectrally broadband femtosecond laser is combined with a narrowband picosecond laser for simultaneous excitation of multiple vibration bands. The unmixing of the spectral channels is realized by a spectrometer with an arrayed detector^{24,35} (Fig. 3c) or by time-domain encoding and decoding with a single-pixel detector^{35,36}. The shared disadvantage of spectral focusing and spectral multiplexing is the limited spectral range, which is determined by the laser bandwidth. This problem can be addressed by using a laser with a larger bandwidth³¹. However, the instability and insufficient output power of these lasers remain a concern. Other efforts have been made to develop a fast wavelength tuning laser for high-speed spectral scanning. A pulse pair-resolved wavelength-switchable laser has been reported for ultrafast multicolor SRS imaging on flow cytometry³⁷. The shortcoming is the

limited number of spectral channels. On the laser side, high-speed swept-source techniques using a Fourier-domain mode-locked laser³⁸ or galvo/resonant scanner-based wavelength sweeping³⁹ were developed for high-speed spectral acquisition. A fast-tuning fiber laser outputting picosecond pulses has been developed for multi-window SRS imaging⁴⁰.

Toward higher detection sensitivity and higher resolution, pre-resonance SRS reported in 2017 achieved sub-micromolar sensitivity of engineered Raman tags⁴¹. SRS excited fluorescence further stepped into the single-molecule regime by integrating highly sensitive fluorescence detection⁴². Combining SRS excited fluorescence with stimulated emission depletion allows for high-resolution vibrational imaging with 180-nm lateral resolution⁴³. Inspired by reversible saturable optical fluorescence transition nanoscopy, vibrational photoswitchable probes⁴⁴ have been developed to advance super-resolution SRS imaging^{45,46}. Plasmon-enhanced SRS offers another approach to reach single-molecule imaging sensitivity⁴⁷. The major issue of these methods is the requirement of a specific exogenous label with NIR pre-resonance or plasmonic resonance. Alternatively, SRS microscopy using visible excitation wavelengths achieved pre-resonance sensitivity enhancement of endogenous molecules by two orders of magnitude⁴⁸. The usage of short-wavelength laser also benefits the spatial resolution,

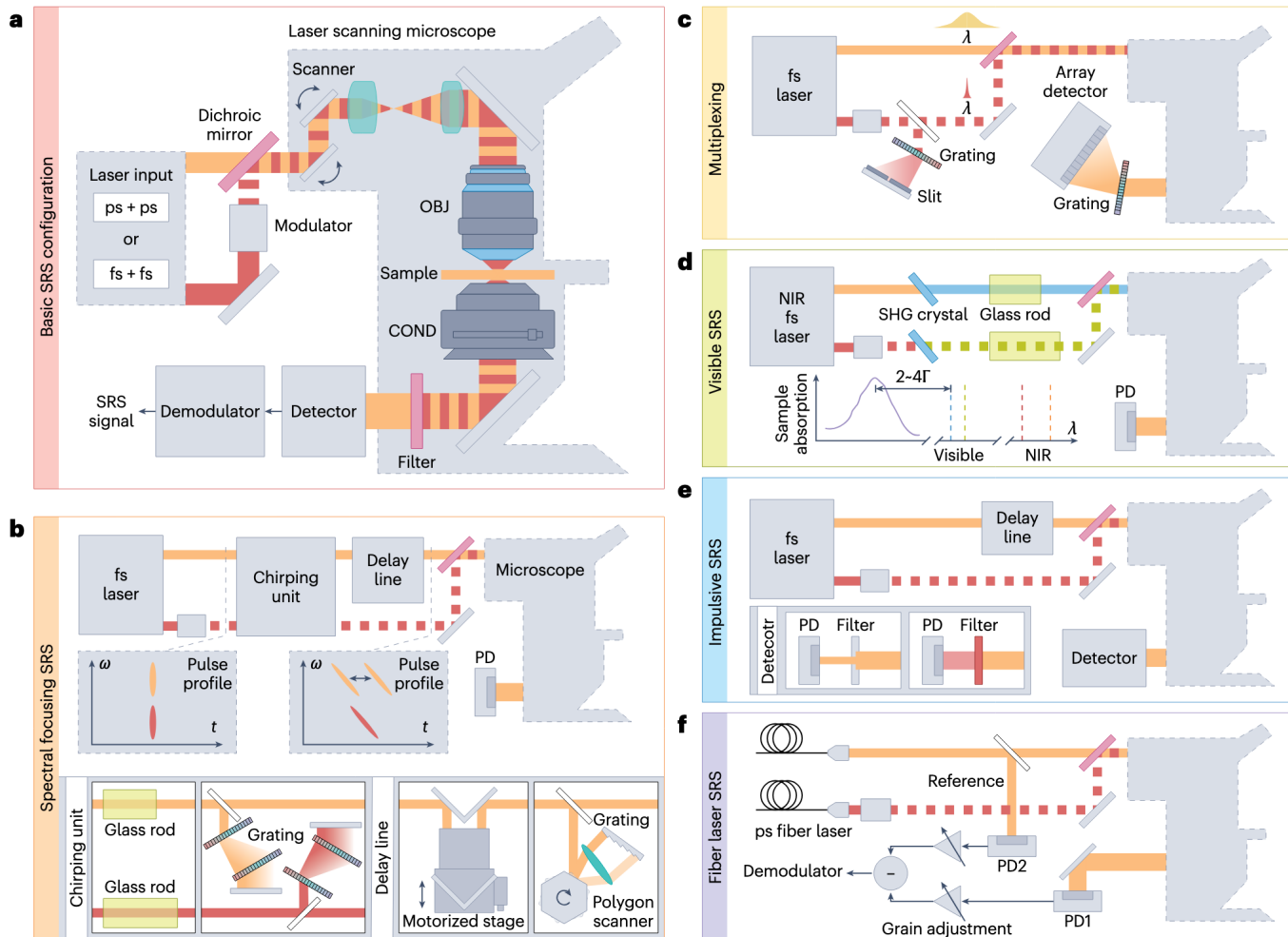


Fig. 3 | Various modalities developed for SRS imaging. a–f, SRS imaging modalities. OBJ, objective lens. COND, condenser lens. PD, photodiode. SHG, second-harmonic generation.

enabling 130-nm lateral resolution⁴⁹. As shown in Fig. 3d, the visible SRS system is built by adding second-harmonic generation crystals to a spectral focusing SRS setup. The downside is the increased photodamage induced by the visible light. Such photodamage can be mitigated by extensive pulse chirping.

Low-wavenumber detection is difficult for standard spectral-domain SRS because the two excitation laser wavelengths are too close to be separated. Impulsive SRS allows for low-wavenumber detection of crystals by detecting the evolution of an impulsively excited vibration wave packet in the time domain with a probe pulse^{50,51}. In an impulsive SRS microscope (Fig. 3e), a vibrational wave packet is excited by the pump pulse and the sample refractive index is modulated at the vibration frequencies. Such refractive index modulation is sensed through probing the spectral shift or the deflection of a probe laser. The spectral shift or the deflection is converted into laser intensity change through a filter or an aperture, respectively. The temporal profile of the vibration is acquired by scanning the delay between the pump and probe pulses. Impulsive SRS imaging on biological samples has not been achieved due to the limited detection sensitivity.

A few volumetric SRS imaging techniques have been developed toward a comprehensive understanding of a three-dimensional (3D) biological system. Utilizing a self-reconstructing Bessel beam, SRS tomography improved the imaging depth by twofold in the scattering environment^{52,53}. By encoding the Bessel beam axially, fast depth scanning can be achieved without mechanical movement⁵³. The disadvantages of Bessel beam SRS tomography are the system complexity

and the low Bessel beam generation efficiency. Another method is SRS optical coherence tomography (OCT), where spectral-domain OCT was combined with spectral-multiplexed SRS to enable simultaneously multiplexed spatial and spectral imaging⁵⁴. However, this leads to a trade-off between axial resolution and spectral resolution in SRS-OCT. For a non-tomography method, remote-focusing SRS integrating a deformable mirror into a standard SRS system was developed for fast axial scanning and aberration correction⁵⁵, yet within a small axial scan range.

Efforts have been made to translate laboratory-based SRS systems to clinical and industrial applications using fiber lasers as robust, compact laser sources^{38,40,56,57}. To suppress the high-intensity noise of the fiber laser, balanced detection techniques were implemented (Fig. 3f) to remove the noise in the signal by subtracting a reference with the same noise. According to the reference-generating method, balanced detection schemes used in SRS can be categorized into collinear balanced detection⁵⁶, in-line balanced detection⁵⁸ and auto-balanced detection⁵⁷. Toward clinical use, an intraoperative SRS system has been built in a cart and used in human study⁵⁹. A proof-of-concept handheld SRS system with a compact objective lens along with an integration of a miniaturized meta-lens into SRS were reported⁶⁰.

Data science in CRS imaging

Data science methods used in CRS imaging could be broadly categorized into two major groups as reviewed recently⁶¹. The first category encompasses spectral unmixing techniques, which serve

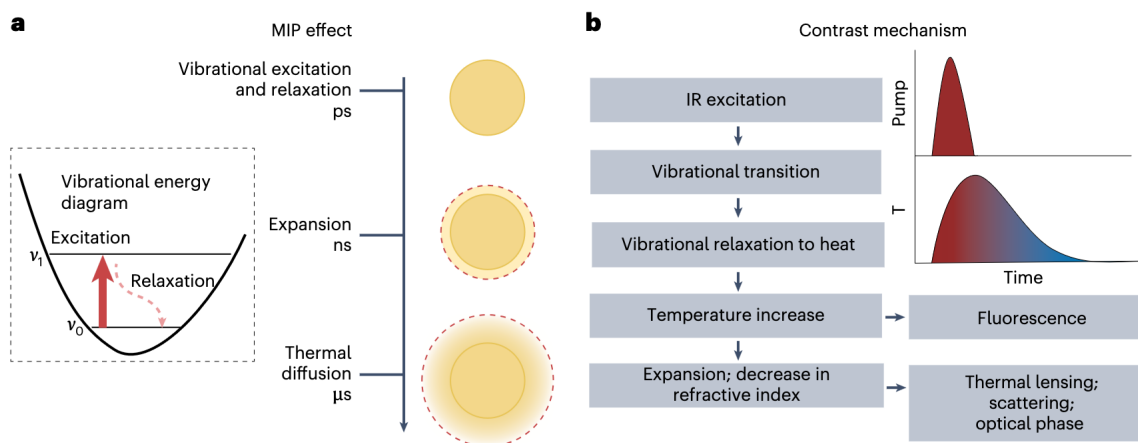


Fig. 4 | Contrast mechanisms of MIP imaging. **a**, The MIP effect occurs over three distinct timescales: vibrational relaxation on the picosecond scale, sample expansion on the nanosecond scale and thermal diffusion on the microsecond scale. **b**, MIP signal can be detected through optical fluorescence, diffraction, scattering or phase measurement.

as the cornerstone for deciphering chemical information embedded within a hyperspectral or multicolor CRS image. Hyperspectral CRS imaging incorporates spatial dimension with the spectral domain to uncover the chemical composition of a complex system. The resulting abundant data require advanced data mining tools, as summarized in Supplementary Table 2. The second category, driven by booming computing power, comprises various machine learning methods (Supplementary Table 3) that break the design space trade-offs imposed by the physical limits of CRS instrumentation. Details can be found in the Supplementary Information.

VIP microscope

MIP microscope

MIP contrast mechanisms. Mid-infrared photothermal (MIP) microscopy enables super-resolution infrared imaging via a pump-probe technique where the chemical contrast arises from vibrational absorption-induced thermal effects (Fig. 1c). In an MIP microscope, a pulsed mid-infrared beam excites molecules on the picosecond timescale (Fig. 4a). Following vibrational relaxation, the molecules quickly return to the ground state, with infrared photon energy transforming into heat, leading to a local temperature rise within the object. The generated heat then dissipates within nanoseconds to microseconds. Following thermal expansion (Δr) on the nanosecond timescale, there is a subsequent change in the refractive index (Δn) of the object. Photothermal-induced alterations in size and refractive index modulate the amplitude, phase and angular distribution of scattered probe light, while the temperature rise can affect the fluorescence emission of thermosensitive dyes. Thus, MIP can exploit multiple contrast mechanisms for chemical imaging, where the visible probe beam measures photothermal-modulated lensing, scattering, phase or fluorescence signals (Fig. 4b).

For solutions, thermal lensing is the predominant contrast mechanism. When molecules in a solution are excited by the infrared beam, localized heating causes a change in the refractive index, thus forming a thermal lens. The thermal lens modulates the propagation of the probe beam and generates MIP contrast⁶². For particle absorbers, heating by the infrared beam leads to particle expansion and refractive index reduction, thus altering the scattering intensity^{63,64}. For nanosized objects, interferometric scattering-based MIP can be applied, where the interference with a reference field amplifies the scattering from the nanosized object^{65,66}. In transparent samples, the probe beam propagates through the sample, and a phase image can be acquired via the interference with a reference beam. The photothermal effect-induced refractive index change alters the optical path length of the probe beam. By measuring the transient change in phase shift induced by

vibrational absorption, the MIP effect brings chemical information into a phase image^{67–70}. Other than thermal lensing, scattering and phase, fluorescence serves as an effective sensor of the local temperature rise, with many fluorescent probes exhibiting modulation of over 1% per Kelvin. Fluorescence-detected MIP (F-MIP) microscopy has been independently reported by the Cheng⁷¹ and Simpson⁷² groups, and commercialized into a fluorescence-detected photothermal infrared microscope by Photothermal Spectroscopy Corporation. F-MIP offers a 100-fold enhancement in modulation depth over scattering-based MIP typically on the scale of 10^{-4} per Kelvin, attributed to the small thermal expansion and thermo-optic coefficient of the samples. Moreover, since photothermal signals only originate from fluorescent chromophores, F-MIP effectively captures the target of interest. Additionally, fluorescence emission is incoherent and distinct from the excitation wavelength, ensuring interference-free imaging.

Representative implementations. MIP microscopy is implemented using two major modalities: scanning and widefield. Quantum cascade lasers with nanojoule pulse energy and MHz repetition rate are suitable for point-scanning MIP. The initial sample-scanning MIP system adopted a co-propagation geometry⁶², which combines a mid-infrared pump and visible probe focused onto a diffraction-limited spot (Fig. 5a(i)). Images are generated by collecting signals in a point-by-point manner using a lateral translation stage. In co-propagating MIP, the mid-infrared and visible beams are combined using a dichroic mirror and are directed to a reflective objective. The probe beam is focused to provide a comparable size to the pump, thus matching the thermal lens size of liquid specimens or diffuse objects. This makes it suitable for detecting dissolved molecules in bulk solution or diffused molecules in biological samples. The sample-scanning speed is limited by the slow operation of the scanning stage and the lock-in integration time, resulting in millisecond pixel dwell times that are insufficient for capturing dynamic processes in living samples. Video-rate laser-scanning MIP imaging was recently developed to overcome this limitation by using galvo mirrors for synchronized scanning of the pump and probe beams, achieving a line rate of over 2 kHz through single-pulse photothermal detection per pixel⁷³.

In co-propagation geometry, spatial resolution is restricted by the NA of the reflective objective, typically around 0.5–0.8. The Hartland Group⁶³ demonstrated a counterpropagating MIP geometry (Fig. 5a(ii)), where a high-NA refractive objective, such as a water/oil immersion objective (NA = 1.2–1.5), can be used to boost the collection efficiency of scattered visible photons. The tightly focused probe beam further enables the visualization of nanoscale intracellular features with a spatial resolution of 300 nm⁷⁴.

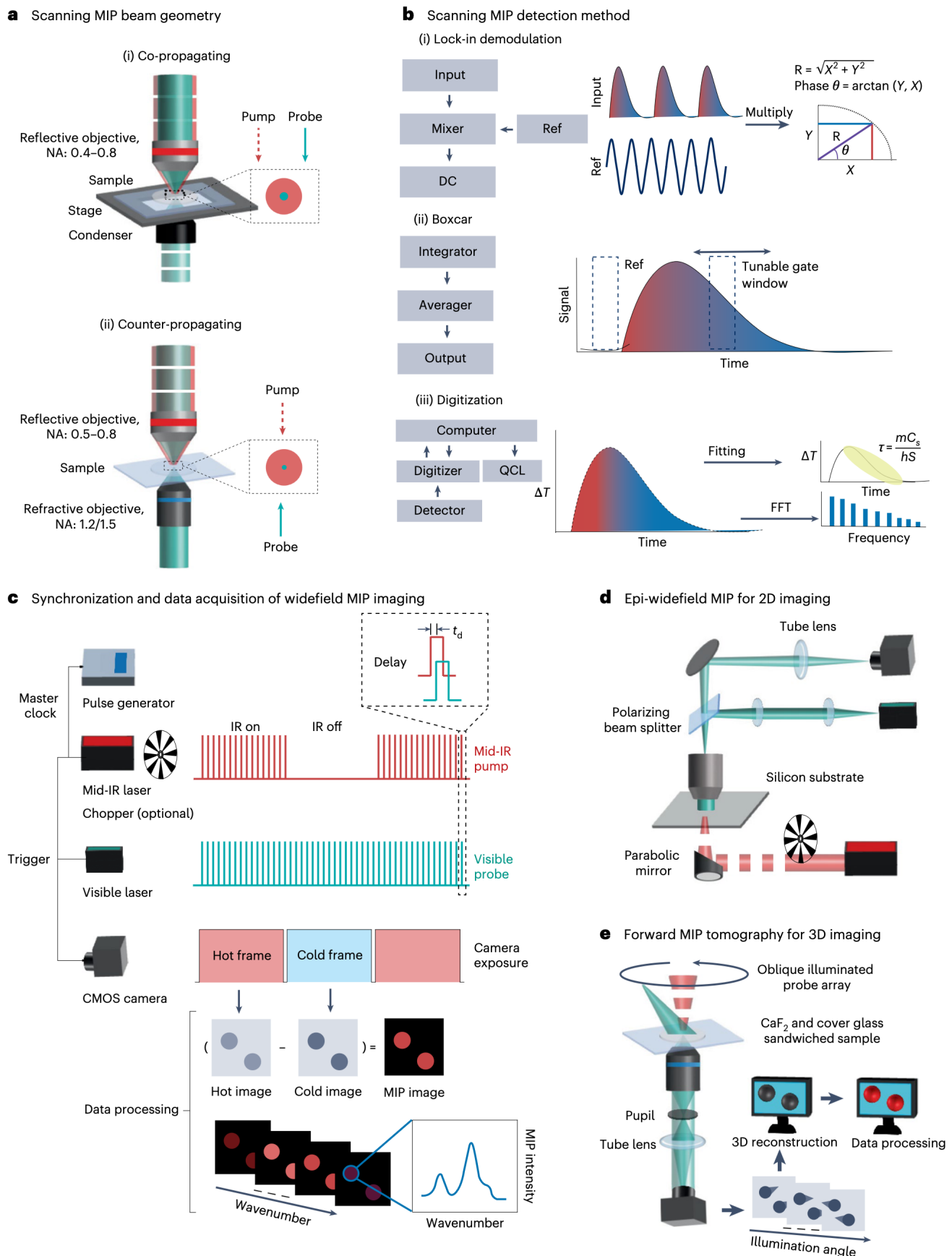


Fig. 5 | MIP imaging implementations. a–e, Scanning (a and b), widefield (c and d) and tomography (e). 2D, two-dimensional. CMOS, complementary metal-oxide-semiconductor. FFT, fast Fourier transform. QCL, quantum cascade laser.

Three detection methods were developed for scanning MIP microscopy: lock-in detection, boxcar detection and signal digitization. Lock-in detection, which is most widely used in photothermal microscopy⁷⁵, involves mixing a reference signal with the modulated photothermal signal, which then goes through a low-pass filter. Lock-in outputs an in-phase component, X , and an out-of-phase component, Y , giving the amplitude and phase of the signal via the R and θ components, respectively (Fig. 5b(i)). This method allows for weak signal to be extracted at the fundamental frequency of the reference component⁷⁶. However, single-frequency lock-in detection methods loses temporal information past the first harmonic due to demodulation at the fundamental frequency⁷⁷. This makes it challenging to separate the photothermal signal from the solvent background in a complex environment⁷⁷.

In boxcar detection, the photothermal signal within a specific time-gated window is retrieved, enabling for a high SNR by rejecting all signal and noise outside this window. The photothermal signals within the specific gate window from multiple inputs are integrated and averaged together to produce the boxcar output (Fig. 5b(ii)). Boxcar detection is also advantageous because it involves minimal post-processing of data⁷⁸. Boxcar detection by the Sander group allowed for nanosecond-scale temporal resolution to elucidate the difference in temporal dynamics of axon bundles and water background⁷⁸. Notably, having a single gate window does not allow for real-time recording of thermal dynamics.

In signal digitization (Fig. 5b(iii)), the heating and cooling dynamics induced by each individual infrared pulse are recorded, followed by digital processing. Therefore, the complete dynamics per pixel are recorded in a single measurement. Using a MHz digitizer, Yin et al. revealed the difference in thermal decays by exponentially fitting the temporal data of lipid droplets and water background⁷⁷. This allowed for water-background suppression by utilizing higher-order harmonics after converting the data to the frequency domain via fast Fourier transform⁷⁷. While this method is able to acquire a full snapshot of the dynamics, it acquires a large amount of data, which makes real-time display difficult.

In widefield MIP microscopy^{79,80}, both the infrared and visible beams are arranged to illuminate a large area to enable high-throughput measurements. Infrared optical parametric oscillators with microjoule pulse energy and a kHz pulse repetition rate are suitable for widefield illumination. By using a virtual lock-in camera technique, widefield MIP integrates a mid-infrared pump, a visible probe, a complementary

metal-oxide-semiconductor camera, and a pulse generator for synchronization and data acquisition (Fig. 5c). The pulse generator produces the master clock signal and triggers the mid-infrared laser, probe beam and complementary metal-oxide-semiconductor camera externally. This ensures synchronization of the mid-infrared pulses and visible probe pulses with precisely controlled delays, as well as sequential exposure of the camera to each hot (infrared-on) and cold (infrared-off) frame. The MIP image is then created by subtracting the cold frame from the hot frame. In instances where the mid-infrared cannot be externally triggered, an optical chopper is used to modulate the pump pulses. Similarly to scanning MIP, widefield MIP microscopy has also reached video-rate imaging speed⁸¹.

To enhance sensitivity, widefield MIP operated in epi mode utilizes interferometric scattering to amplify weak scattering signals, enabling fingerprint detection of single viruses at a speed of 635 frames per second⁶⁶. In an epi-detected widefield MIP setup (Fig. 5d), the sample is placed on a silicon wafer substrate to reflect forward-scattered visible photons, serving as a reference field for interferometric scattering measurements. The infrared beam is weakly focused onto the sample plane from the bottom of the silicon substrate via an off-axis gold parabolic mirror, with an illumination area around 50 μm in diameter. Simultaneously, the probe beam illuminates the sample from above through a 50/50 polarizing beam splitter, a quarter-wave plate and a high-NA air objective. The reflected visible photons are collected using the same objective and then projected onto the camera with a tube lens.

By integrating widefield infrared illumination with tomography, such as OCT, intensity diffraction tomography and optical diffraction tomography, MIP tomography enables 3D chemical analysis and volumetric chemical phase imaging of thick tissues, live cells and worms^{82,83}. A forward-detected MIP tomography system is illustrated in Fig. 5e. The probe beam illuminates the sample at various incident angles by a ring laser array. For each oblique illumination, wavefront distortion is captured through interference imaging or intensity-based phase retrieval. Using 3D reconstruction algorithms, tomographic refractive index images are recovered by the collected wavefront data at all illumination angles. Finally, the MIP tomography image is generated by subtracting the cold from the hot refractive index images.

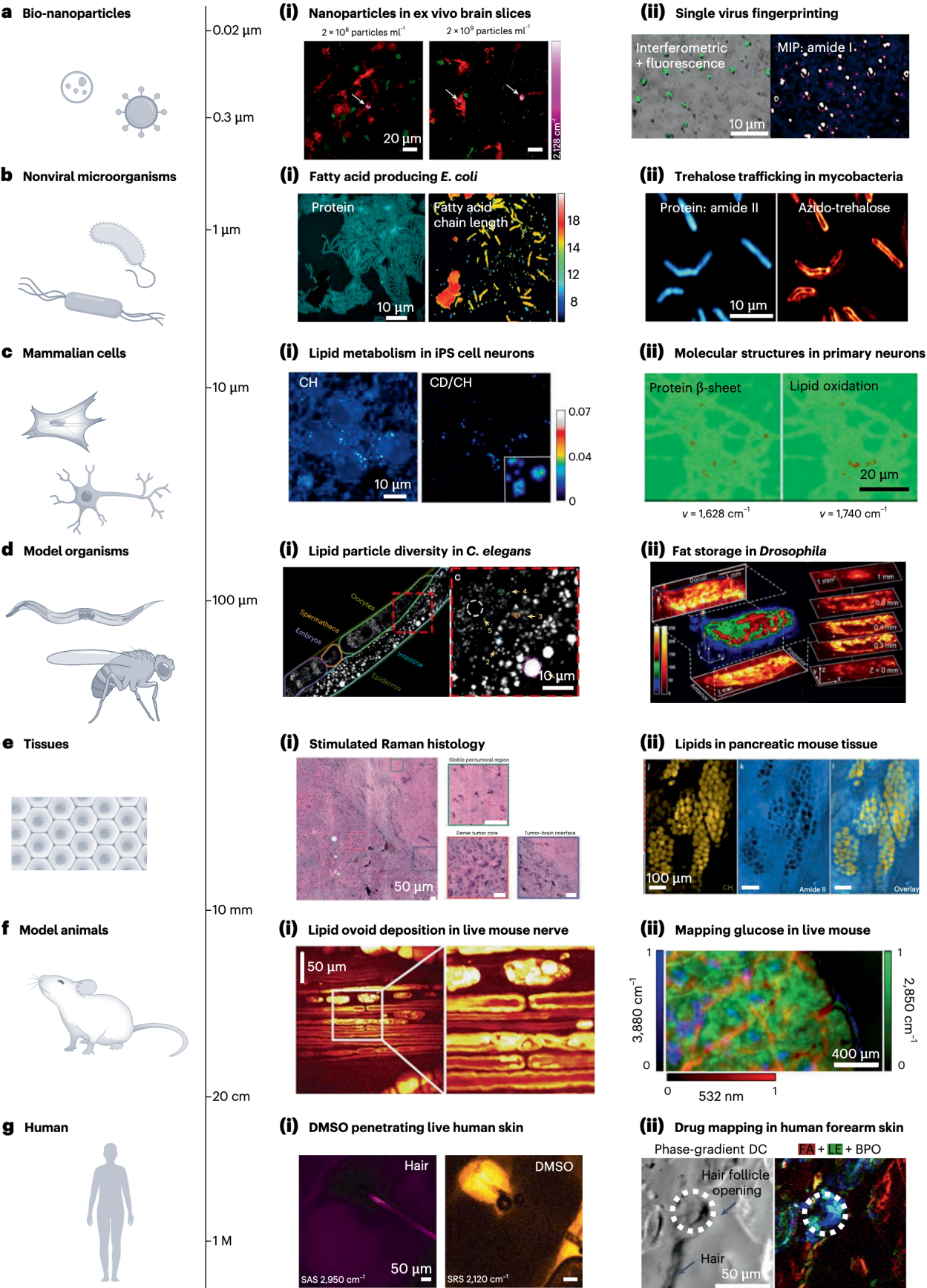
Other modalities of VIP microscopy

Recently developed stimulated Raman photothermal (SRP) and short-wave infrared photothermal (SWIP) microscopy expand the VIP imaging family. In these modalities, the absorber within a sample absorbs energy

Fig. 6 | Broad applications of advanced vibrational microscopy to life sciences.

a–g. The left column represents various biological models, and the middle and right columns show representative applications for each model based on Raman scattering and infrared absorption, respectively. **a.** Bio-nanoparticles (20 nm to 300 nm), including viruses and nanomedicines. **(i)** SRS image of alkyne-labeled polymeric nanoparticles (magenta) in ex vivo mouse cortical brain slices. Scale bar, 20 μm . **(ii)** Widefield interferometric defocus-enhanced MIP imaging allows high-throughput fingerprinting of single viruses, with interferometric and fluorescence imaging as references. Scale bar, 10 μm . **b.** Nonviral microorganisms (0.5 μm to 10 μm), including bacteria and fungi. **(i)** SRS imaging for saturated fatty acid chain length prediction in fatty acid-producing *E. coli* grown on an agarose pad by analyzing the CH_2/CH_3 ratio. Scale bar, 10 μm . **(ii)** Click-free MIP imaging of trehalose incorporation into the mycobacterial membrane using an azido photothermal probe, with proteins distributed throughout the cell body. Scale bar, 10 μm . **c.** Mammalian cells (10 μm to 100 μm). **(i)** SRS images of C–D/C–H bond ratios in V337M induced pluripotent stem (iPS) cell neurons at 72 h after D_2O labeling, showing lipid metabolic dynamics. Scale bar, 10 μm . **(ii)** MIP imaging of β -sheet structures ($1,628\text{ cm}^{-1}$) and lipid oxidation ($1,740\text{ cm}^{-1}$) in cultured APP-KO neurons after incubation with 42-residue human amyloid- β . Scale bar, 20 μm . **d.** Model organisms (50 μm to 10 mm): *C. elegans* and *Drosophila melanogaster*. **(i)** Coherent Raman imaging of a lipid intensity map in a wild-type *C. elegans* adult, with region of interest no. 5 highlighting the oocyte nucleus, a region that is lipid poor¹²⁶. Scale bar, 10 μm . **(ii)** In vivo

3D vibrational photoacoustic imaging of fat bodies in a third-instar *D. melanogaster* larva, with longitudinal images highlighting lipid storage¹²⁹. Scale bar, 1 mm. **e.** Tissues (100 μm to 10 mm). **(i)** Stimulated Raman histology of a brain–tumor interface from an individual with glioblastoma, showing a hypercellular tumor and reactive astrocytes in peritumoral regions. Scale bar, 50 μm . **(ii)** Mid-infrared optoacoustic imaging of freshly excised pancreatic mouse tissue of 4-mm thickness, showing a lipid map at $2,850\text{ cm}^{-1}$ and a protein map at $1,550\text{ cm}^{-1}$. Scale bar, 100 μm . **f.** Model animals (10 cm to 20 cm). **(i)** In vivo SRS imaging of progressive lipid ovoid deposition in live mouse sciatic nerves. Scale bar, 50 μm . **(ii)** Merged mid-infrared/visible in vivo optoacoustic images for noninvasive glucose monitoring in the mouse ear. Scale bar, 400 μm . **g.** Human skin (1.5 m to 1.8 m). **(i)** SRS image at $2,950\text{ cm}^{-1}$ showing proteins vibrations in hair on the living human forearm skin surface and at $2,120\text{ cm}^{-1}$ showing d6-DMSO penetration into the skin. Scale bar, 50 μm . **(ii)** In vivo MIP imaging of endogenous lipids and administered drug content on human skin. The phase-gradient DC (direct current) image shows the stratum corneum, a hair and the hair follicle opening. FA, fatty acid; LE, lipid ester; BPO, benzoyl peroxide. Scale bar, 50 μm . Credit: images adapted under a Creative Commons license CC BY 4.0 from: **a(i)**, ref. 106; **a(ii)**, ref. 66; **b(i)**, ref. 108; **b(ii)**, ref. 107; **c(ii)**, ref. 124; **f(i)**, ref. 136; **f(ii)**, ref. 94; or adapted with permission from: **c(i)**, ref. 123, Elsevier; **d(i)**, ref. 128, Springer Nature America; **d(ii)**, ref. 89, American Physical Society; **e(i)**, ref. 130, Springer Nature America; **e(ii)**, ref. 93, Springer Nature America; **g(i)**, ref. 28, AAAS; **g(ii)**, ref. 138.



from the excitation laser and the energy is quickly relaxed into heat. This thermal effect alters the refractive index, creating a thermal lens that alters the propagation of the probe laser. An aperture is placed before the detector to turn the alternation in propagation into a probe intensity change.

Specifically, SRP microscopy⁸⁴ harnesses stimulated Raman as a two-photon vibrational absorption process (Fig. 1b). The thermal lensing mechanism making SRP more effective than SRS for detecting subtle vibrational transitions, particularly in thermally enhancing media like glycerol and other tissue-clearing agents. For dimethyl sulfoxide (DMSO), SRP achieved up to 500 times higher modulation depth compared to SRS⁸⁴. This technique has been applied to biological samples, including viruses, mammalian cells and brain tissues. SRP offers several advantages over SRS. First, it is resistant to noise in the pump or Stokes lasers, permitting the use of noisy ultrafast sources like optical parametric amplifiers or fiber lasers. Second, it relaxes the high-NA light collection condition, allowing flexible sample handling with a long-working distance lens. On the other hand, SRP can be affected by non-vibrational absorption background. Such background can be minimized through advanced modulation schemes, like delay modulation.

Beyond the fundamental vibrational transitions, overtone transitions (Fig. 1d)—higher-order harmonics of molecular vibrations—occur within the shortwave infrared (SWIR) window (1,000–2,500 nm) and provide vibrational contrast^{85,86}. Utilizing these overtone transitions, SWIP and overtone photothermal microscopy have been developed to leverage the advantages of the SWIR window^{87,88}. This spectral region offers reduced scattering compared to SRS and lower water absorption than MIP techniques, making it ideal for deep-tissue chemical imaging^{89,90}. The pump-probe photothermal detection scheme used by SWIP offers another advantage of decoupling absorption from scattering, enabling precise quantitative chemical analysis in a scattering sample. Notably, SWIP achieves millimeter-deep vibrational imaging with micrometer-level lateral resolution. With a 1,725-nm excitation laser targeting the first overtone of C–H vibration and a 1,310-nm probe laser, SWIP can resolve intracellular lipid distributions in tumor spheroids and myelin structures in thick tissues, bridging the gap between high-resolution and penetration depth in bond-selective imaging⁸⁷. This capability makes SWIP suitable for applications such as live organoid imaging.

Vibrational photoacoustic imaging

Photoacoustic detection of vibrational transitions allows deep-tissue chemical imaging. In photoacoustic imaging, the molecules absorb the pulsed excitation laser and convert the energy into heat. A small part of the thermal energy is converted into acoustic waves through thermal expansion and contraction⁹¹. The low tissue scattering of acoustic waves enables deep-tissue imaging capability. Vibrational photoacoustic imaging can be categorized into mid-infrared photoacoustic (MIPA; Fig. 1c), overtone photoacoustic (OPA) and stimulated Raman photoacoustic microscopy.

MIPA imaging of biological samples was reported in 2017, for non-invasive skin glucose monitoring⁹². In 2019, Pleitez et al. reported an improved MIPA microscopy and achieved live-cell imaging with 5.3- μ m lateral resolution and micromolar sensitivity⁹³. High-sensitivity blood glucose sensing was achieved by combining MIPA with visible photoacoustic microscopy⁹⁴. Ultraviolet-localized photoacoustic-detected MIP microscopy was developed to push the resolution limit⁹⁵. The tight optical focus and the low water absorption of ultraviolet light enabled water-background-suppressed imaging with a lateral resolution of 260 nm.

Overtone absorptions are based on the high-order harmonics of the fundamental modes of molecular vibrations. OPA imaging of biological tissue was demonstrated around 2011 to detect lipid⁸⁹ and recently water content in tissues⁹⁶. Benefiting from the fact that the

wavelength corresponding to overtone absorption falls in the SWIR window—which has lower water absorption than the mid-infrared window and lower scattering than the visible window—OPA allows deep-tissue chemical imaging and has garnered interest in clinical applications. Fiber-based OPA catheters were fabricated for intravascular lipid-laden plaque detection^{97,98}. A mobile OPA system was developed and used in the hospital setting for human breast cancer margin assessment⁹⁹.

Stimulated Raman photoacoustic microscopy¹⁰⁰ was reported in 2010 using a femtosecond laser as the excitation source and chloroform in a capillary tube as the test bed. Although the feasibility of stimulated Raman photoacoustic imaging was shown, its application to bio-samples remains challenging due to the weak signal.

Up-conversion of mid-infrared absorption

Up-conversion of mid-infrared absorption refers to methods that encode, within the same molecule, mid-infrared absorption in an optical emission at shorter wavelengths. The up-conversion of mid-infrared absorption can be realized through vibration-resonant sum frequency generation (VR-SFG) or fluorescence-encoded infrared (FEIR) spectroscopy.

The energy diagram of VR-SFG is shown in Fig. 1e. The signal is generated when a mid-infrared photon and a visible/NIR photon are simultaneously absorbed by the sample. In this way, VR-SFG combines the vibrational contrast from mid-infrared absorption with the sensitivity to non-centrosymmetric media from SFG. Application of VR-SFG to bio-sample imaging was reported in 2011. By using a high-repetition rate laser and a collinear excitation geometry, this work enhanced the signal level and achieved rapid imaging of collagen¹⁰¹. VR-SFG based on the second-order SFG is only sensitive to non-centrosymmetric molecules. To break this limitation, third-order SFG was developed for vibration-sensitive detection of lipid and water¹⁰².

FEIR (Fig. 1f) utilizes a double-resonance scheme of mid-infrared excitation and NIR/visible up-conversion. Taking advantage of the high fluorescence quantum yield, FEIR can achieve single-molecule detection sensitivity¹⁰³. Based on this principle, bond-selective fluorescence-detected infrared-excited imaging was demonstrated to visualize various intracellular targets¹⁰⁴. This method, also called infrared-encoded spontaneous emission, could benefit fluorescence imaging by distinguishing spectrally overlapping fluorophores with vibrational information¹⁰⁵. This capability has the potential to enable super-multiplexed fluorescence imaging.

Life science applications and user guide

Applications from single bio-nanoparticles to humans

Figure 6 illustrates the broad applications of advanced chemical microscopy, underscoring its versatility in addressing biological questions across a broad range of length scales. As summarized below, these techniques were applied to a diverse set of biological models, including single bio-nanoparticles, nonviral microorganisms, mammalian cells, model organisms, tissue slices, live animals and human skin.

At the nanoscale (Fig. 6a), bio-nanoparticles like viruses and nanomedicines (20 nm to 300 nm) can be visualized using SRS and MIP microscopy. These methods enable single-particle imaging and tracking of nanoparticles in complex biological environments, including crossing the blood–brain barrier. Using SRS, Vanden-Hehir et al. visualized both deuterium-labeled and alkyne-labeled polymeric nanoparticles within primary rat microglia and in ex vivo cortical mouse brain tissue (Fig. 6a(i))¹⁰⁶. Independently, MIP microscopy facilitated single-virus fingerprinting by detecting viral proteins and bases in nucleic acids, allowing identification of single DNA and RNA viruses (Fig. 6a(ii))⁶⁶. These methods open new doors for drug delivery research and understanding of viral content and behavior.

For nonviral microorganisms such as bacteria and fungi (0.5 μ m to 10 μ m; Fig. 6b), SRS and MIP microscopy have enabled the study of biological structure and function^{64,107}, metabolic activities^{107–109}

and metabolite distributions^{110,111}. In particular, these techniques are crucial for investigating single-cell metabolic responses to antibiotics and antifungals¹¹², offering a powerful platform for rapid antimicrobial and antifungal susceptibility testing¹¹³. For example, longitudinal hyperspectral SRS imaging has been applied to visualize free fatty acids in metabolically engineered *Escherichia coli* across multiple cell cycles¹⁰⁸ (Fig. 6b(i)), allowing for compositional analysis of fatty acid chain length and unsaturation in live cells. Additionally, click-free MIP imaging has been performed to track trehalose uptake in single live mycobacteria via an azido photothermal probe¹⁰⁷ (Fig. 6b(ii)), allowing direct visualization of the trehalose recycling pathway from cytoplasmic uptake to membrane localization. These findings are critical for understanding microbial metabolism and have potential in infectious disease diagnosis and new antibiotics development.

At the mammalian cell level (Fig. 6c), advanced vibrational imaging has enabled comprehensive studies of cellular chemical composition¹¹⁴ and metabolism¹¹⁵, particularly in mammalian cells ranging from 10 μm to 100 μm in size. Techniques like SRS and MIP have been widely used to detect and monitor the metabolism of key biomolecules such as lipids^{115–117}, nucleic acids^{118,119}, proteins^{120,121} and carbohydrates^{107,122}. For example, SRS imaging has been extensively used to investigate cellular lipid metabolism¹²³ (Fig. 6c(i)), revealing key insights into processes like lipid storage and mobilization, and its role in neuronal health and disease. Additionally, label-free MIP imaging exhibits high sensitivity to protein quantities and secondary structures, making it effective for studying protein aggregation and lipid oxidation (Fig. 6c(ii)), especially in relation to β -sheet structures associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease^{124,125}. These advanced vibrational imaging techniques are invaluable for elucidating cellular metabolism and dynamics, offering insights into the roles of biomolecules in health and disease.

As a widely used model organism in life science research, *Caenorhabditis elegans* (~50 μm in diameter) is an elegant test bed for vibrational imaging applications (Fig. 6d)^{73,126–128} due to its simple structure, transparency and ease of genetic manipulation. Leveraging the label-free lipid quantification capabilities of SRS microscopy, researchers have explored genes regulating fat metabolism in worms under physiological conditions with RNA interference¹²⁶. By integrating hyperspectral SRS imaging in the fingerprint window, *k*-means clustering and multivariate curve resolution analysis, Wang et al. achieved quantitative mapping of fat distribution, fat unsaturation, lipid oxidation and cholesterol storage throughout *C. elegans*¹²⁷. Chen et al. used broadband CARS imaging to study protein content and lipid dynamics in live worms, uncovering that intestinal fat is diverse and mainly allocated to offspring development, while epidermal fat, with higher triglyceride content, is more stable and functions as an energy reserve (Fig. 6d(i))¹²⁸. *Drosophila*^{123,129} and zebrafish embryos are also extensively used as model organisms. Using *Drosophila* as a model, the Shi group investigated metabolic activities in cells and tissues during aging through deuterated water (D_2O)-probed stimulated Raman scattering. They examined lipid metabolism in fat bodies, focusing on the effects of different diets and insulin signaling pathways¹²⁹. In neurons, studies on tauopathy revealed that *Drosophila* overexpressing tau exhibit impaired brain metabolic dynamics and accumulate lipid droplets with slow lipid turnover¹²³. OPA microscopy, which has a penetration depth of a few millimeters, has allowed for whole-body lipid analysis in *Drosophila* (Fig. 6d(ii))⁸⁹.

Both sliced and intact ex vivo tissues (100 μm to 10 mm) have been widely used in nonlinear vibrational imaging (Fig. 6e). Virtual histology by label-free SRS images at the C–H region could mimic the hematoxylin and eosin staining for rapid intraoperative diagnosis, in which the CH_3 for protein is used to identify the cell nuclei and the CH_2 for lipid gives a contrast as the cellular body and extracellular matrix (Fig. 6e(i))¹³⁰. With the aid of convolutional neural networks, a rapid pathological diagnosis could be completed in less than 2.5 min, while

conventional diagnosis takes about 30 min. Meanwhile, mid-infrared optoacoustic microscopy has been applied for biomolecule imaging in freshly excised pancreatic mouse tissue (Fig. 6e(ii))⁹³. Both the C–H region and the fingerprint region were targeted to map the distribution of lipid and protein. The optoacoustic process converts mid-infrared vibrational absorption into a positive contrast with negligible photo-damage and high sensitivity. SRS has also been used to image amyloid plaques in Alzheimer's disease¹³¹ and glioblastoma xenografts^{132,133} in humans. With millimeter-scale penetration depth, OPA imaging has been used for atherosclerosis detection in ex vivo blood vessels¹³⁴ and spinal cord white matter loss assessment¹³⁵.

In vivo vibrational imaging of small animals (5 cm to 20 cm) has been demonstrated (Fig. 6f). For example, SRS was used to monitor the cell cycle in mouse skin¹¹⁹ as well as peripheral nerve degeneration in amyotrophic lateral sclerosis (ALS; Fig. 6f(i))¹³⁶. With high spatial resolution and chemical specificity, small lipid ovoid deposition derived from myelinating cells could be detected quantitatively, even in the early stage of ALS. A long-term observation of live animals during ALS progression was conducted using SRS imaging, where the number of lipid ovoids was related to ALS progression. A depth-gated mid-infrared optoacoustic sensor was developed to minimize interference caused by the skin and allow depth-selective localization of glucose readings in the blood-rich skin of mice (Fig. 6f(ii))⁹⁴. In this work, a 20-ns pulsed mid-infrared laser at 1,080 cm^{-1} was used to target the glucose, and the optoacoustic readout was colocalized with the blood vessel optoacoustic imaging, which was targeted by a 3-ns 532-nm pulsed laser. In vivo intravascular photoacoustic imaging was conducted to detect lipid plaque in the abdominal aorta of a live rabbit¹³⁷. In vivo CARS has been conducted to image the sebaceous glands, corneocytes and adipocytes in the skin of a live mouse⁴.

Nonlinear vibrational imaging has also been applied to humans, with two examples given in Fig. 6g. SRS imaging was adopted to monitor deuterated DMSO penetration of human skin at video-rate imaging speed (Fig. 6g(i))²⁸. DMSO is a commonly used polar aprotic solvent. Imaging the penetration of DMSO or other solvents in human skin can potentially reflect the drug distribution during transdermal delivery. With video-rate imaging speed, motion artifacts could be minimized when recording short-lived events. Similarly, studies on DMSO accumulation in human skin have been conducted^{55,60}. With oblique detection, MIP microscopy can not only image intrinsic biomolecules such as proteins, fatty acids and lipid esters, but also visualize the penetration of benzoyl peroxide in live human skin (Fig. 6g(ii))¹³⁸. The mapped distribution of active components offers valuable feedback for hydrophobic or hydrophilic modification of drugs.

Overall, the illustrated applications highlight the potential of advanced vibrational microscopy in life science research. These techniques offer chemical bond specificity, submicrometer spatial resolution, and up to video-rate speed enabling the tracking of dynamic processes, to researchers working across diverse systems, from bio-nanoparticles to whole organisms.

Bridging biomolecules to vibrational imaging modalities

Infrared absorption and Raman scattering spectroscopy are complementary in the analysis of biomolecules including lipids, proteins, carbohydrates and nucleic acids. Each type of biomolecule exhibits unique vibrational modes that can be detected through infrared or Raman. To serve as a user guide for bridging applications with vibrational imaging modalities, Supplementary Table 4 provides an overview of chemical bonds commonly used for nonlinear vibrational imaging of different biomolecules, along with their infrared and Raman transition strengths. The triple-bond tags, including infrared-preferred azides^{107,116}, Raman-preferred alkynes¹³⁹ and nitriles used in both modalities^{74,140}, are not included in the table. Details of vibrational imaging modalities for specific biomolecules can be found in the Supplementary Information.

Sample preparation for vibrational imaging

The overall criterion for achieving good image quality is to maximize the laser delivery efficiency to the sample, optimize the signal detection, and minimize the photodamage. General rules should be followed unless special cases arise. For coherent Raman imaging, due to high laser energy at the focal spot, measurements should be performed with a glass coverslip to minimize photon absorption from the polymer dish. For MIP imaging, given the strong absorption of the mid-infrared light of most materials, a high transmission window, such as CaF_2 , should be used on the side of the mid-infrared excitation. Additionally, a minimal amount of water should be kept between the sample and the coverslip to minimize water absorption. For transducer-based photoacoustic imaging, coupling medium such as water should be used to minimize acoustic impedance mismatch. Detailed sample preparation guidelines for different imaging modalities can be found in the Supplementary Information.

Discussion and outlook

Vibrational microscopy bridges the gap between phase contrast and fluorescence microscopy, and is emerging as a major branch of the optical microscopy family. Although this Review focuses on nonlinear vibrational microscopes, we emphasize that linear vibrational imaging modalities are equally important. Commercial confocal Raman microscopy and Fourier-transform infrared microscopy are widely used for single-cell spectroscopic analysis and large-area chemical mapping of tissues. For example, confocal Raman microspectroscopy revealed the spatial distribution of fumarate, a metabolite within the tricarboxylic acid cycle, in cells and tissues¹⁴¹. In this work, the number of pixels was limited by the slow spectral acquisition speed on the second level. Similarly, Fourier-transform infrared microspectroscopy enabled metabolic profiling of single-cell responses to drug treatments¹⁴², whereas its poor spatial resolution prohibits intracellular visualization and applicability to live-cell imaging. With much improved speed and spatial resolution, CRS microscopy and MIP (also called O-PTIR) microscopy overcome such limitations. For comparison, broadband CARS, covering the entire spectral window, allows spectral acquisition at the speed of a few milliseconds per pixel⁹. Similarly, MIP imaging of azide photothermal probes allows mapping of carbohydrate trafficking in live cells with submicrometer spatial resolution¹⁰⁷.

Both CRS microscopy and VIP microscopy were initially developed as label-free imaging technologies. These methods have found broad use in the study of human specimens and functional materials, where label-free techniques are essential. In live-cell and tissue studies, vibrational tags such as the C–D bond for Raman imaging and azide for infrared imaging enable the visualization of specific biomolecules, including fatty acids, amino acids or carbohydrates. Unlike fluorescent tags, the volume of a chemical bond is one million times smaller, thus inducing minimal perturbation to chemistry inside the cells. This strategy has been used in bio-orthogonal fluorescence imaging, where azide-tagged biomolecules undergo a click reaction to conjugate dyes for visualizing the final state. By vibrational microscopy, one can perform click-chemistry-free imaging of azide-tagged molecules in real time. For example, aided by an azido photothermal probe, MIP microscopy allowed real-time imaging of azido-trehalose trafficking in live cells¹⁰⁷. Notably, while MIP was initially proposed as a dye-free technique, it was revealed that fluorescence is a more sensitive probe of temperature than scattering^{71,72}. Thus, fluorescence can be used as both a guide and a sensor in photothermal imaging of specific organelles inside a cell. In F-MIP, photothermal spectroscopy gives the chemical content and/or protein secondary structure information, adding new information to fluorescence imaging.

Thus far, the cost of the laser system has been a bottleneck for broader use of coherent Raman microscopy. We envision that, with the development of fast-tuning fiber lasers, this issue will be addressed in

the near future. Notably, MIP microscopy is broadly used. The product of MIP microscopy, mIRage, is now used worldwide for various applications from detection of protein secondary structure in Alzheimer's disease^{124,125} to mapping of nanoplastics in the environment¹⁴³. Two factors contributed to this entrepreneur success. First, the quantum cascade laser is much more compact and cost effective than the ultrafast lasers needed for CARS/SRS microscopy. Second, the operation of MIP microscopy is easier compared to CARS/SRS and AFM-IR, which allows routine use by non-experts.

The limited detection sensitivity, compared to fluorescence microscopy, remains a challenge facing the chemical imaging field. Raman scattering and infrared absorption are complementary to each other, with Raman scattering being sensitive to C–H, C–D, C=C and C≡C bonds, and infrared absorption being sensitive to C=O, C–O, N–O, S=O and P=O vibrations in the fingerprint window, as well as azide and nitrile in the silent window. Innovations are urgently needed to push the sensitivity to micromolar and nanomolar levels for both Raman and infrared-based imaging technologies. Such high sensitivity will be the cornerstone for the development of vibrational nanoscopy. A promising approach is vibrational modulation of fluorescence via up-conversion, which offers single-molecule sensitivity^{42,103–105}. Coherent Raman microscopy with high-energy laser pulses and VIP microscopy with ultrasensitive thermal reporters are alternative solutions. Together with advanced instrument development, the sensitivity and specificity of vibrational imaging has been greatly advanced by vibrational probes, including stable isotope probes and small-size Raman and infrared tags (see the Review by Wei Min et al. in the same issue¹⁴⁴).

More transformative applications are needed for vibrational imaging to gain wider acceptance among the life science community. Thus far, most applications are focused on lipid bodies and protein aggregates due to their abundance of specific chemical bonds. A broader range of applications beyond the capabilities of fluorescence microscopy is to be demonstrated. Examples include click-chemistry-free mapping of specific biomolecules like carbohydrates, amino acids or proteins, imaging of low-concentration drug molecules, and mapping the metabolism in single cells and/or specific organelles.

Finally, joint efforts by academic researchers and the commercial sector are key to disseminating nonlinear vibrational microscopes to end users globally. Currently, coherent Raman microscopes are available from Leica (Germany), VibroniX (China), Invenio (USA), Lightcore Technologies (France), CRI Chemometric Imaging (United Kingdom and Italy) and Refined Lasers (Germany). MIP microscopes are available through Photothermal Spectroscopy Corporation (USA). Lower-cost microscopes and transformative applications promise to expand the life science market.

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Author contributions

J.-X.C. drafted the outline, introduction, outlook and two boxes. Y.Y. drafted the CARS section. H.N. drafted the SRS and SWIP sections. J.A. drafted the data science section. R.B. and Q.X. drafted the MIP section. X.G. and H.N. drafted the SRP and SWIP section. Q.X., J.A. and Y.Y. drafted the applications and user guide section. J.A., Q.X. and Y.Y. contributed to manuscript revision.

Competing interests

J.-X.C. declares competing interests with VibroniX and Photothermal Spectroscopy, which did not fund this work. The remaining authors declare no competing interests.

Additional information

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