Coherent Raman scattering microscopy for chemical imaging of biological systems

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Abstract
Coherent Raman scattering (CRS) processes, including both the coherent anti-Stokes Raman scattering and stimulated Raman scattering, have been utilized in state-of-the-art microscopy platforms for chemical imaging of biological samples. The key advantage of CRS microscopy over fluorescence microscopy is label-free, which is an attractive characteristic for modern biological and medical sciences. Besides, CRS has other advantages such as higher selectivity to metabolites, no photobleaching, and narrow peak width. These features have brought fast-growing attention to CRS microscopy in biological research. In this review article, we will first briefly introduce the history of CRS microscopy, and then explain the theoretical background of the CRS processes in detail using the classical approach. Next, we will cover major instrumentation techniques of CRS microscopy. Finally, we will enumerate examples of recent applications of CRS imaging in biological and medical sciences.

1. Introduction
Optical microscopy is one of the major biotechnologies for modern biological science. Fluorescent labeling and signal detection, due to the high chemical selectivity and sensitivity, is the most widely adopted optical microscopy technique [1]. Recently developed super-resolution fluorescence microscopy tools can break the optical diffraction limit and reveal unprecedented details of biomolecules at a resolution of a few tens of nanometers [2–5]. Light-sheet fluorescent microscopy has enabled volumetric imaging of biological samples at high speeds [6–8]. However, with the continuous growth of biological science, there are conditions that imaging methods beyond fluorescence are desired. First, fluorescent microscopy typically requires effective delivery of fluorescent labels, which, in some cases, can be challenging. The fluorescent labels might also bind to unwanted molecules due to nonspecific binding. Second, fluorescent molecules are usually exogenous and therefore might perturb biological functions in living samples. This might hamper the accurate measurement of certain biological processes. Third, small molecules, especially metabolites, are difficult to label specifically by fluorescent approaches without significantly altering their bio-functions. Fourth, fluorescent emission spectra are broad. This feature indicates that only a few detection channels are typically available for fluorescent microscopes. It is extremely challenging to increase the number of parallel signal channels and design fluorescent probes for simultaneous imaging of multiple targets. Fifth, fluorescent molecules experience photobleaching, especially over a long exposure time. For any biological research that these abovementioned limitations would be problematic, it is preferable to apply other imaging techniques that circumvent the issues while maintaining the chemical specificity. One of the most attractive imaging modalities to fulfill these requirements is Raman microscopy.

Raman scattering was theoretically predicted by Adolf Smekal in 1923 [9] and discovered by C.V. Raman in 1928 [10]. It describes an inelastic scattering of photons by matter, where photons exchange energy with molecular vibrations. The photons that lose energy to molecular vibrations are shifted to longer wavelengths and are known as the Stokes shifts, while the photons that gain vibrational energy are shifted to shorter wavelengths and are referred to as anti-Stokes shifts (figure 1(A)). The vibrational transitions of chemical
bonds generate narrow Raman scattering peaks which can be used as fingerprints to identify chemical species. Modern Raman microspectroscopy typically involves a narrowband laser source for excitation, a microscope for sample loading, and a spectrometer-detector combination for spectra graph acquisition [11]. Raman microscopes have been used to image chemical distributions in materials and biological samples. Compared to mid-infrared absorption spectroscopy which uses mid-infrared light sources, the visible excitation wavelength used in Raman allows for better beam penetration in water and glass media, and higher spatial resolution for imaging. One of the major drawbacks of Raman spectroscopy is the weak signal level and the corresponding long signal integration time. Typically, the Raman spectral acquisition time for a femtoliter biological sample is on the level of sub-seconds to seconds, resulting in hours of image acquisition time. Although imaging modalities such as line-scan and widefield Raman microscopy have been invented to improve the imaging speed [12, 13], spontaneous Raman imaging is still orders of magnitude slower than fluorescence imaging. Enhancing Raman signals is an effective way to improve the imaging speed. Surface-enhanced Raman scattering has demonstrated $10^8$–$11$ times enhancement for Raman signals [14–16], but requires rough metal surfaces or nanoparticles to generate Raman ‘hot spots’. The metal nanoparticle delivery and perturbations to biological samples create new challenges for biological imaging.

In the past two decades, a new field has emerged at the forefront of bioimaging by utilizing two synchronized laser pulses to enhance the Raman scattering signal coherently, known as the coherent Raman scattering (CRS) microscopy [17–21]. This imaging approach offers millions of times of signal enhancement compared to spontaneous Raman scattering while avoiding the use of metal nanoparticles, making Raman microscopy as fast as fluorescence microscopy, and thus gives new insights into cell biology and new opportunities for diagnosis.

**Figure 1.** Energy diagrams of optical processes related to spontaneous Raman scattering, CARS, and SRS. (A) Stokes and anti-Stokes processes in spontaneous Raman scattering. (B) CARS (left), virtual-state-mediated four-wave-mixing (FWM) (middle), and electronic-state-mediated FWM (right). (C) Left and middle panels are diagrams of stimulated Raman loss (SRL) and stimulated Raman gain (SRG) processes, respectively, from the wave mixing point of view. The right panel is the SRS process from the energy conversion point of view. The straight arrows indicate coherent transitions while the wave arrows represent incoherent or spontaneous transitions. The notations $g$, $v$, and $e$ indicate the ground state, vibrational excitation state, and electronic excitation state, respectively.
CRS effects are nonlinear optical phenomena and are only observed when the input laser pulses have high intensity. The two most widely used CRS processes for microscopy are coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS). Both CARS and SRS were observed in the early 1960s shortly after the invention of the laser [22, 23]. CARS was first developed as a spectroscopy technology for combustion analysis. Not until 1982 was CARS first applied to a microscope by Duncan and co-workers [24]. Modern CARS microscopes were pioneered by Xie and co-workers in 1999 using a 250 kHz high-repetition-rate ultrafast laser in a collinear and tight-focusing geometry [17]. Over two decades, many imaging techniques based on CARS have been developed for biomedical imaging. During the early years of CARS microscopy, one of the major technological focuses was to suppress or separate the nonresonant background which distorts the Raman spectrum. Many strategies have been invented to address this challenge, such as polarization-resolved CARS [25], epi-detection CARS [26], time-resolved CARS [27], frequency-modulated CARS [28], heterodyne CARS [29, 30], and spectral phase retrieval [31]. Another trend in CARS microscopy was to improve the chemical selectivity by hyperspectral imaging. Multiple hyperspectral CARS imaging schemes have been developed based on narrowband or broadband laser pulses [32–40].

The first SRS microscope based on a 1 kHz low-repetition rate laser was reported in 2007 [41]. Soon, high-speed SRS microscopy pumped by a 76 MHz high-repetition-rate laser was demonstrated and triggered the revolution of CRS microscopy [19]. Key advantages of SRS over CARS include the absence of nonresonant background, the nondistorted Raman spectra, the linear signal-concentration dependence, and full operation under ambient light [42, 43]. These key features quickly caught the attention of imaging scientists and chemists and gradually transitioned SRS into a more favorable microscopy technology over CARS for biological imaging. Compared to CARS, SRS typically requires a laser modulation-demodulation scheme to detect the energy transfer between the pump and Stokes beams. It can be easily modified from a CARS microscope by adding a few optical components [44, 45]. One of the major technological directions in SRS microscopy is, similar to CARS, to improve chemical selectivity by hyperspectral imaging. Many hyperspectral imaging schemes have been invented for SRS using picosecond or femtosecond pulses [46–51].

In this review, we first briefly go over the theoretical background of both CARS and SRS. Next, we discuss the basic optical instrumentation of CRS microscopes. Then, we introduce some applications of CRS for biological and medical imaging.

2. The theoretical background of CRS

CRS processes can be described using both classical and quantum theories. Although full quantum descriptions of both molecular systems and photons provide the most accurate interpretation of coherent Raman processes, they require a more advanced physics background which is beyond the expertise of many researchers in chemistry and engineering. The classical approach, on the other hand, is easier to understand and gives sufficient explanation to the majority of signal properties of CRS responses. Therefore, in this review, we briefly walk through the classical theory for both CARS and SRS. More information on the quantum descriptions can be found in other references [52–54].

Figure 1 illustrates the energy diagrams of spontaneous Raman scattering, CARS, four-wave-mixing (FWM)-related nonresonant responses, and SRS. Both spontaneous Raman scattering and SRS are non-parametric processes involving energy transfer between photons and molecular systems. CARS, on the other hand, is a parametric process in which photon energy is conserved. CARS is a resonance-enhanced FWM process that is usually accompanied by nonresonant responses from both virtual- and electronic-states-mediated FWM (figure 1(B)). Although the SRS process is non-parametric, the evolutions of both the pump and Stokes waves can be derived from four-wave nonlinear interactions similar to CARS (figure 1(C)).

2.1. Nonlinear optical responses

CRS are nonlinear optical processes that involve interactions of high-intensity laser pulses with molecular systems. One of the key differences of nonlinear compared to linear optical responses is the nonlinear polarization of molecules. The optical-field-induced molecular polarization can be expressed as [55]

\[
P(t) = P^{(1)}(t) + P^{(2)}(t) + P^{(3)}(t) + \ldots = \varepsilon_0 \left[ \chi^{(1)} E(t) + \chi^{(2)} E^2(t) + \chi^{(3)} E^3(t) + \ldots \right],
\]

where \( P^{(1)}(t), P^{(2)}(t), \) and \( P^{(3)}(t) \) are the linear, the second-order nonlinear, and the third-order nonlinear polarizations, respectively, induced by the input optical field. \( \chi^{(1)} \) is the linear susceptibility, while \( \chi^{(2)} \) and \( \chi^{(3)} \) are the second- and third-order nonlinear susceptibilities of the sample, respectively. The constant \( \varepsilon_0 \) is the vacuum permittivity and \( E(t) \) is the input optical electric field.
From equation (1), we can see that when the input electric field is high, nonlinear responses can be comparable or even surpass the linear response. These extreme conditions usually happen in nonlinear optical crystals. In biological samples, especially in a small volume or at a low concentration, nonlinear responses are usually orders of magnitude lower than the linear responses. However, they can still be detected and give unique information to understand biomolecules. Next, we will derive the CARS and SRS responses of molecules using the classical theory.

2.2. Harmonic oscillation model for molecular vibrations

From the classical point of view, chemical bonds can be modeled as oscillating dipoles driven by optical electric fields. The motion function of a driven harmonic oscillator can be expressed as [55]

$$\frac{d^2r(t)}{dt^2} + 2\gamma \frac{dr(t)}{dt} + \omega^2 r(t) = \frac{F(t)}{m},$$

(2)

where $r(t)$ is the deviation of inter-nuclear distance from the equilibrium $r_0$, $\omega$ is the resonance frequency, $\gamma$ is the damping constant, $m$ is the mass, and $F(t)$ is the driving force that satisfies

$$F(t) = E(t) \cdot \alpha(t).$$

(3)

Here, $E(t)$ is the input electric field and $\alpha(t)$ is the polarizability of the harmonic oscillator. To illustrate the nonlinear optical response of a molecule, we want to derive the expression of the driving force that arises from the nonlinear polarizability of the molecule. We assume the electric field is a plane wave $E(t, z)$ propagating along the $z$-direction. The optical polarizability of a molecule is not a constant but is a function of $r(t)$. The total polarizability can then be written as

$$\alpha(t) = \alpha_0 + \left( \frac{\partial \alpha}{\partial r} \right) r(t).$$

(4)

Here, $\alpha(t)$ has a linear part and a nonlinear part. $\alpha_0$ is the linear polarizability at the equilibrium position. The dipole moment of this molecule is $p(z, t) = \varepsilon_0 \alpha(t) E(z, t)$. Let us assume the electric field is a sinusoidal plane wave $E(z, t) = E \cos(kz - \omega t) = E e^{i(kz - \omega t)} + c.c.$, where $E$ is the amplitude of the field and c.c. denotes complex conjugates. The average energy required to establish the oscillating dipole in an optical cycle ($\varphi = kz - \omega t$ from 0 to 2$\pi$) is

$$W = \frac{1}{2\pi} \int_0^{2\pi} F(\varphi) \cdot d\varphi = \frac{1}{2\pi} \int_0^{2\pi} q E(\varphi) \cdot d\varphi = \int_0^{2\pi} p(\varphi) \cdot E(\varphi) d\varphi$$

$$= \varepsilon_0 \alpha(t) \int_0^{2\pi} E^2(\varphi) d\varphi = \varepsilon_0 \alpha(t) E^2 \int_0^{2\pi} \cos^2(\varphi) d\varphi = \frac{1}{2} \varepsilon_0 \alpha(t) E^2.$$  

The driving force can then be expressed as

$$F(z, t) = \frac{\partial W}{\partial r} = \frac{\varepsilon_0}{2} \frac{\partial \alpha(t)}{\partial r} E^2.$$  

(5)

The CARS and SRS processes involve interactions of two input fields, the pump $\omega_p$, and the Stokes $\omega_s$. These two fields can be combined and expressed as

$$E(z, t) = E_p e^{i(kz - \omega_p t)} + E_s e^{i(kz - \omega_s t)} + c.c.$$  

(6)

We plug this expression in equation (5), and only look at the term having $k = k_p - k_s$ and $\omega = \omega_p - \omega_s$, we get the expression (appendix A)

$$F_{\omega_p - \omega_s}(z, t) = \varepsilon_0 \frac{\partial \alpha}{\partial r} E_p E_s e^{i[(k_p - k_s)z - (\omega_p - \omega_s)t]} = \varepsilon_0 \frac{\partial \alpha}{\partial r} E_p E_s e^{i(kz - \omega t)}.$$  

(7)

This portion of the driving force is directly related to CARS and SRS processes. If we insert this expression into equation (2), we get

$$\frac{d^2r}{dt^2} + 2\gamma \frac{dr}{dt} + \omega^2 r = \frac{\varepsilon_0}{m} \frac{\partial \alpha}{\partial r} E_p E_s e^{i(kz - \omega t)}.$$  

(8)

The solution of this equation has the form

$$r(\omega, t) = r(\omega) e^{i(kz - \omega t)} + c.c.$$  

(9)
We then get the solution \( r(\omega) \) by plugging this expression in equation (8)

\[
    r(\omega) = \frac{(\varepsilon_0 / m) \cdot (\partial \alpha / \partial r) E_p E_s^*}{\omega^2 - \omega_s^2 - 2k\omega_3}
\]

### 2.3. Signals of CARS

From Maxwell’s equation, and assuming a plane wave propagating along the z-direction, we can obtain a nonlinear optical wave equation (see appendix B)

\[
    \frac{\partial^2 E(z)}{\partial z^2} + \frac{\omega_0^2 \varepsilon_0 E(z)}{c^2} = -\frac{\omega_0^2}{\varepsilon_0 c^2} P_{NL}(z).
\]

Here, \( P_{NL}(z) \) is the nonlinear polarization of the sample; \( \varepsilon_0, c, \) and \( \omega \) are vacuum permittivity, speed of light, and input light frequency, respectively. \( \varepsilon_r = 1 + \chi \) is the relative permittivity of the sample. If we know the expression of \( P_{NL}(z) \) for any nonlinear optical process, we can then solve this equation and derive the evolution of the optical wave, i.e. the nonlinear optical signal. The polarization and dipole moment have a correlation \( P(z, t) = N \cdot p(z, t) \), where \( N \) is the number of oscillating dipoles. The nonlinear polarization is contributed by the nonlinear driving field from the input electric field. Using \( p(z, t) = \varepsilon_0 \alpha(t) E(z, t) \) and equations (4) and (6), we get the expression

\[
P_{NL}(z) = \varepsilon_0 N \left( \frac{\partial \alpha}{\partial r} \right) r(\omega, t) E(z, t)
\]

\[
= \varepsilon_0 N \left[ \frac{\partial \alpha}{\partial r} \right] \left[ r(\omega) e^{i(\omega_s t + \omega_0 t)} + c.c. \right] E_p e^{i(k_0 z - \omega_0 t)} + E_s e^{i(k_0 z - \omega_s t)} + c.c.
\]

The CARS signal is at the frequency \( \omega_s = \omega_p + \omega_p - \omega_0 + \omega_0 = 2\omega_p - \omega_s \). The term in the expression of \( P_{NL}(z) \) having this frequency gives the expression of (appendix C)

\[
P_{NL}(z) = 3\varepsilon_0 \chi_{(3)}^R |E_p|^2 E_s^* e^{i(2k_0 - k_0)z},
\]

where \( \chi_{(3)}^R = \frac{(\varepsilon_0 c / m)_0 \cdot (\partial \alpha / \partial r)^2}{\omega^2 - (\omega_p - \omega_0)^2} \). \( \chi_{(3)}^R \) is the third-order nonlinear susceptibility.

We insert the expression of \( P_{NL}(z) \) into equation (11) and get

\[
    \frac{\partial^2 E(z)}{\partial z^2} + \frac{\omega_0^2 \varepsilon_0 E(z)}{c^2} = -\frac{3\chi_{(3)}^R \omega_0^2}{c^2} |E_p|^2 E_s^* e^{i(2k_0 - k_0)z},
\]

where \( E_s = E_s e^{i\omega_0 z} \). This equation can be further simplified to (appendix D)

\[
    \frac{dE_s}{dz} = \alpha_{as} E_s^* e^{i\Delta k z},
\]

where \( \alpha_{as} = \frac{3\chi_{(3)}^R |E_p|^2}{2\varepsilon_0 c^2} \). \( \Delta k = 2k_0 - k_0 - k_{as} \).

Assuming the interaction length is \( l \). Solving this equation gives (appendix E)

\[
    I_{as} = \frac{3\chi_{(3)}^R |E_p|^2}{2\varepsilon_0 c^2 l_{as}} \left| \chi_{(3)}^R \right|^2 P^2 I_s \cdot \bar{P} \sin^2(\Delta k \cdot l/2)
\]

Assuming that \( \Delta k \) is small, we can simplify the expression.

\[
    \Delta k \to 0, \quad I_{as} \propto \left| \chi_{(3)}^R \right|^2 P^2 I_s.
\]

We can see from this solution that the CARS signal is affected by the \( \Delta k \), which is minimized in phase-matching conditions. Perfect phase-matching conditions may not be satisfied in the collinear imaging geometry and biological samples. The phase mismatch determines the coherence length difference in the forward- and epi-detected CARS. Forward CARS usually has a much longer coherence length compared to epi-CARS (appendix F). Therefore, forward CARS typically has a much stronger signal than epi-CARS.

However, for samples with low concentration, forward CARS might show a much higher nonresonant background from the medium. The third-order nonlinear susceptibility of the sample is proportional to the molecular number density. Therefore, the resonant CARS signal is proportional to the square of the sample number density.
concentration. Besides, the CARS signal is inversely proportional to the square of the anti-Stokes wavelength. Using shorter wavelength lasers for CARS can enhance signal generation.

From equation (13) we see that the third-order nonlinear optical susceptibility has real and imaginary parts. The absolute value square of $\chi^{(3)}$ gives distorted Lorentzian shapes, which represent archetypal CARS spectra.

2.4. Signals of SRS

The SRS signal is the energy transfer between the pump and Stokes beams. The stimulated Raman gain (SRG) of the Stokes beam and the stimulated Raman loss (SRL) of the pump beam provide similar information. If we detect the SRG, we need to look at the frequency $\omega_3$. In the expression (12), the term related to this frequency can be simplified to (appendix G)

$$P^{NL}_s(z) = 3\varepsilon_0\chi_R^{(3)}|E_p|^2E_s e^{i\alpha z},$$

(18)

where $\chi_R^{(3)} = \frac{(Ne_0/3\varepsilon_0)(\partial^2\alpha/\partial r^2)}{\omega_3 - (\omega_p - \omega_s)^2 + i(\omega_p - \omega_s)\gamma}.$

Near Raman resonance, $\omega_p - \omega_s \approx \omega_s$, the third-order nonlinear susceptibility can be simplified to

$$\chi_R^{(3)} = \frac{(Ne_0/6m\omega_s) \cdot (\partial^2\alpha/\partial r^2)}{\omega_3 - (\omega_p - \omega_s)^2 + i(\omega_p - \omega_s)\gamma},$$

(19)

Using this $P^{NL}_s(\omega, z)$ expression and equation (11), we can get the solution as the Stokes wave evolution function (appendix H)

$$\frac{dE_s}{dz} = \alpha_s E_s$$

(20)

where $\alpha_s = \frac{3\omega_3}{2m^2\chi_R^{(3)}|E_p|^2}$.

We see from the procedures of solving this wave equation that the phase terms from both sides of the equation are canceled (appendix H), and the SRS signal does not have a phase factor. This means that SRS does not require phase matching for signal generation. The SRS is an energy dissipation process and thus does not involve phase matching which is needed for parametric processes.

The solution to equation (20) satisfies $E_s = E_{s0} e^{\alpha_s z}$, in which $E_{s0}$ is the electric field at time 0. Typically, for biological imaging, $\alpha_s \cdot z \to 0$. We can simplify this solution using Taylor expansion and get $E_s \approx E_{s0}(1 + \alpha_s z)$.

The SRS signal is the intensity change between $I_s$ and $I_{s0}$. Therefore, we get (appendix I)

$$I_{SRS} = I_s - I_{s0} = \frac{12\pi}{\varepsilon_0 n_p n_s \lambda_s} \text{Im}(\chi_R^{(3)}) I_{s0} I_p |I|$$

$$\propto -\text{Im}(\chi_R^{(3)}) I_{s0} I_p |I|.$$  

(21)

The imaginary part of $\chi_R^{(3)}$ is negative. Therefore, the Stokes signal experiences growth after SRS interactions, which is known as the SRG. From this expression, we can see that, unlike CARS for which the signal has a quadratic dependence on the molecular density, the SRS signal is linearly dependent on the sample concentration. The imaginary part $\chi^{(3)}$ is purely Lorentzian. Therefore, the SRS spectra have similar profiles as the corresponding spontaneous Raman spectra. An example of the comparison of SRS, CARS, and spontaneous Raman scattering can be found in the figure 1(F) of [19]. The SRS signal is inversely proportional to the Stokes wavelength, and hence shorter wavelength Stokes pulses give stronger SRS signals.

From the classical theory, we can derive most of the key properties of CARS and SRS needed to explain Raman spectra in microscopy applications. Nonetheless, semi-classical or full quantum descriptions of CARS and SRS give better descriptions of light–matter interactions in CRS. These theoretical approaches can be found in other literatures [52–54].

3. Instrumentation of CRS microscopy

3.1. Basic instrumentation of CRS microscopy

The most commonly used scheme for CRS imaging is based on laser scanning collinearly combined and tightly focused pump and Stokes beams [17, 44, 56]. Both CARS and SRS are third-order nonlinear optical processes and thus require high energy density to generate signals. Tight focusing of pulsed laser beams to a size of hundreds of nanometers satisfies this requirement and thus has been generally adopted. Laser
scanning allows for high-speed imaging with tightly focused beams. High-repetition rate (>1 MHz) picosecond or femtosecond lasers are commonly used for CRS microscopy. A lower repetition rate indicates higher pulse energy at the same average power and thus is preferable for the signal generation [57, 58]. However, a low repetition rate might hamper the increase in imaging speed and induce photodamage [57]. The minimum requirement is that there’s at least one laser pulse from both pump and Stokes beams in each image pixel. Picosecond laser sources have narrower bandwidths compared to femtosecond laser sources, and thus are preferable to focus energy onto the excitation of a narrow Raman vibrational transition. However, femtosecond lasers give more frequency components and are more versatile for pulse shaping [49, 51]. Besides, femtosecond lasers are more suitable to integrate other nonlinear optical imaging modalities such as multiphoton fluorescence or harmonic generation with CRS microscopy [59, 60].

Figure 2 outlined key components for CRS microscopes. Ultrafast laser sources with 1–100 MHz repetition rates and nJ level pulse energy are commonly used. Two wavelengths to be used as a pump and Stokes need to be directly available from the laser source, or post-converted. These two pulse trains, one of which is usually wavelength-tunable or broadband, are required to be synchronized. Besides using two separate pulses for pump and Stokes, single-pulse CRS was reported using the frequency components from the same pulse for CRS excitation [61–64]. Frequency conversion optics can alter the pump or/and Stokes pulses for different CRS imaging schemes. They can convert femtosecond pulses to picosecond pulses to enhance spectral resolution [49], or extend wavelength coverage by supercontinuum [35], or produce beam chirping for spectral focusing [36, 51], or can be omitted if no frequency conversion is needed. An optical delay line is required in one of the beams to overlap the two pulses in time at the sample. 2D scanning mirrors scan the laser focus at the sample to create an image. Raster scanning is the most common scanning pattern in CRS microscopy. A lens pair is needed to both expand the laser beams for filling the entrance pupil of the objective lens for best spatial resolution and to conjugate the center of scanning mirrors to the objective back aperture to maximize the field of view during laser scanning. High numerical aperture (NA) objective and condensers are usually used for the best image resolution. Detectors of CRS can be placed in both forward and epi directions. Both upright and inverted microscope configurations can be used to accommodate CRS imaging capabilities [44].

Although CARS and SRS microscopes share the above key components, there are several differences in signal detection. The SRS process is an energy transfer from pump photons to Stokes photons and molecular vibrations. To detect the energy transfer from two laser beams, intensity modulation and demodulation are usually needed. Therefore, an intensity modulation device, typically an acousto-optic modulator or an...
electro-optic modulator is applied to the pump or the Stokes beam. After the SRS process, due to the energy transfer process, the modulation will be transferred to the other unmodulated laser beam. Therefore, for SRS signal detection, direct measurement of the Stokes or pump laser is needed and can saturate most highly sensitive detectors. Photodiode detectors are ideal for SRS due to the high saturation energy. Before the photodiode, an optical filter is needed to block the modulated laser beam from entering the photodiode. A lock-in amplifier is usually needed to demodulate and amplify the signals from the photodiodes at the modulation frequency, although lock-in free SRS detectors were reported [48, 65, 66]. The CARS signals are generated at new anti-Stokes frequencies, and thus can be detected directly using photomultiplier tubes (PMTs) or charge-coupled-devices (CCDs) with correct filter selections.

Besides, the differences in optical properties of CARS and SRS impact the optics used for signal delivery. First, SRS detection can be affected by several other nonlinear optical processes, one of which depends on the use of optics is cross-phase modulation (CPM). The CPM, in which one pulse will change the optical properties of the material and hence alter the transmission divergence of the other beam, usually accompanies the SRS. CPM can interfere with the SRS signal once the signal collection optics do not have enough NA to completely collect the slightly expanded laser beam induces by the CPM [45]. Therefore, to avoid the impact of the CPM in SRS imaging, typically the condenser needs to have a higher NA than the objective lens, which is not required for CARS imaging. Second, the SRS signal only follows the pump and Stokes beams that propagate in the transmission direction, while the CARS signal can be generated in both forward and epi directions. This indicates that for nearly transparent samples, negligible signals from SRS will be detected in the epi direction. For thick and opaque samples, SRS can be detected in the epi directions since the sample can scatter back the pump and Stokes photons [50, 67]. To separate the input and back-scattered pump or Stokes beams in epi-SRS, polarization optics which involve a quarter-wave plate and a polarization beam splitter might be used [68, 69]. Although CARS signals can be generated in both directions, the epi-CARS usually has a weaker signal due to the much shorter coherence length compared to the forward-CARS (appendix F). The separation of CARS signals in the epi direction only requires a dichroic beam splitter. It was shown that epi-CARS can more effectively suppress the nonresonant background, also due to the shorter coherence length [26].

3.2. Single-color, multi-color, and hyperspectral CRS microscopy

From the vibrational frequency compositions, CRS imaging schemes can be classified into single-color, multi-color, and hyperspectral. Single-color CRS microscopy is the first developed scheme and the simplest CRS configuration [17]. It only excites a single Raman transition by using narrowband pump and Stokes pulses. Since the laser energy is more focused to excite a single vibrational mode, single-color CRS typically has the highest signal and is preferable for high-speed imaging when the input average power is fixed. However, compared to other schemes, it is the least informative one in chemical compositions.

For complex chemical mixtures in a dynamic environment, it is necessary to simultaneously target multiple Raman bands for chemical content separation. Using broadband pulses and pulse shaping, narrowband spectral windows can be selected for parallel excitation of several Raman transitions. The Xie group used grating-based pulse shaping and selected three pump frequencies for simultaneous imaging of mixed polymer particles at 2900 cm\(^{-1}\), 2950 cm\(^{-1}\), and 3054 cm\(^{-1}\), and separated lipid and protein from biological samples using two frequencies at 2845 cm\(^{-1}\) and 2940 cm\(^{-1}\) [70]. Adding another spectral window at 2967 cm\(^{-1}\), three-color multiplex SRS was shown to separate DNA from lipid and protein compositions in live cells and intact tissue samples [71]. Multicolor SRS was also achieved by adding optical amplifiers to existing laser systems [72], applying a rapidly tunable optical parametric oscillator [73], utilizing a fast wavelength-tunable fiber laser [74], and employing dual-phase signal detection [75]. Multi-color SRS imaging of intact tissue samples highlighted the potential for label-free histopathology [76–80].

Multi-color CRS microscopy can differentiate chemicals when their signature Raman peaks are well-separated in the spectral domain. In many cases, biological samples are composed of molecules with highly overlapped Raman transitions, and even multi-color CRS microscopy has difficulty in distinguishing the chemical compositions. To further improve the chemical selectivity, hyperspectral CRS microscopy, in which a Raman spectrum is acquired at each image pixel, was developed in multiple forms. The simplest way to perform hyperspectral CRS imaging is to continuously tune the frequency of one of the laser beams while collecting CRS images using a narrowband single-color setup [81] (figure 3(A)). The frequency-dependent 2D images then create a three-dimensional (3D) image stack. Spectral information from each pixel can be reconstructed thereafter. The two narrowband pulses can also be converted from broadband femtosecond pulses for wavelength scanning [49]. Another way is to use two broadband beams and spectral focusing to acquire the image stack [33, 51]. In spectral focusing, the broadband beams are chirped in space and time so that frequency compositions from two pulses are reduced at each overlapping time window. Scanning the optical delay between the two chirped pulses step-by-step using a translational stage or other devices equals
scanning the frequency difference, and thus a hyperspectral stack can be created. Although technically simple, the \( x-y-\lambda \) hyperspectral acquisition scheme has a major drawback. Any sample movement or dynamics during the image stack acquisition can distort the spectrum. Therefore, the \( x-y-\lambda \) acquisition is not suitable to study highly dynamic living samples.

Alternatively, the \( \lambda-x-y \) hyperspectral imaging methods, also known as the multiplex CRS, largely avoid spectral artifacts induced by sample dynamics \([32, 35, 48, 82-84]\) (figure 3(B)). In \( \lambda-x-y \) hyperspectral imaging, the spectrum from each pixel is collected before the laser focus move to the next image pixel. For CARS, the \( \lambda-x-y \) scheme is typically achieved by using a narrowband and a broadband laser pulse for excitation \([35, 39]\). A CARS spectrum can be directly generated in a wide spectral range and then detected by a CCD array. For SRS, this \( \lambda-x-y \) scheme is more difficult to implement due to the lack of lock-in-integrated photodiode array detectors. Specially designed photodiode arrays that can acquire a Raman spectrum in microseconds for multiplex SRS have been reported \([48, 84-86]\). Besides, using high-speed scanning methods such as resonant mirrors \([87]\) or polygonal mirrors \([69]\), together with either spectral focusing or grating-based frequency tuning, can scan the frequency components within a single image pixel, offering high-speed multiplex CRS capabilities. High-speed hyperspectral imaging can also be achieved through an \( x-\lambda-y \) scheme by resonantly scanning one image axis together with galvo mirror-controlled wavelength selection \([83]\) (figure 3(C)). 3D sparsely sampled hyperspectral SRS microscopy was reported by Lin et al, in which a 20% fill-rate was enough to reconstruct decent hyperspectral images similar to the 100% fill-rate \([88]\). In general, spontaneous Raman spectroscopy can cover the entire vibrational frequency range from 10 to 4000 cm\(^{-1}\). Broadband CARS was also shown to cover a spectral window over 3000 cm\(^{-1}\) \([39]\). Hyperspectral SRS, however, currently has a much narrower spectral window around 200–300 cm\(^{-1}\) despite the window can be tuned to cover almost any spectral region \([48, 49, 51]\). There’s always a trade-off between the spectral resolution and signal intensity for hyperspectral CRS imaging. A higher spectral resolution is preferred when distinguishing chemicals with very close Raman peaks. However, for samples having far separated spectral peaks, a lower spectral resolution might be a better choice to reach a higher sensitivity.

3.3. Other configurations of CRS microscopy

Over the past two decades, many research groups have reported numerous designs of CRS microscopes with advanced functions and capabilities. Due to the page limit, it is impossible to summarize all efforts here. Readers can refer to other review articles for broader coverage \([42-45, 59, 89-94]\). Besides the commonly used CRS imaging configurations discussed above, there are other CRS microscopy designs. For example, single-pulse CRS using a broadband laser pulse was reported by multiple groups \([61, 62, 95-97]\). Fourier-transform of an interferometric autocorrelated related signal can be integrated with single-pulse CARS for the spectral generation \([61, 95-97]\). Fourier-transform has also been used to extract Raman signals for SRS microscopy \([69, 98]\). Wide-field CARS microscopes were also reported with field-of-views around 20–30 \( \mu m^2 \), enabling high-speed CARS imaging \([99-102]\). Widefield SRS microscopy is still challenging due to the lack of lock-in-integrated high-performance photodiode 2D arrays for signal detection. Frequency modulation CARS and SRS were reported to suppress non-Raman-active backgrounds \([28, 103]\). A
double-modulation scheme was invented to effectively suppress CPM, transient absorption, and photothermal background contributions while enhancing the SRS signals [104]. Instead of using tightly focused Gaussian excitation beams, Bessel beams were used for high-speed volumetric imaging of biological samples through SRS projection microscopy and tomography [105]. The Bessel beam can greatly extend the excitation volume, thus giving a higher chemical quantification speed for a 3D structure [105].

Many groups have tried different routes to improve the spatial resolution of coherent Raman microscopy. Methods such as near field scanning [106–108], structured illumination [109–111], Bessel beam excitation [112], photonic nanojets [113], and periphery CARS signal depletion [114, 115] were reported. Recently, it was shown that higher-order nonlinear optical responses enabled higher resolution CARS images below the diffraction limit of excitation lasers [116]. These super-resolution CARS approaches either require special metal tips or microparticles for imaging or need sophisticated engineering of excitation laser beams. For all the far-field CARS super-resolution methods, the resolution enhancement is still much less than superresolution fluorescence microscopy. Another way to achieve higher spatial resolution is to use shorter wavelength lasers for excitation [117]. Using visible rather than near IR laser pulses, SRS microscopy has reached a spatial resolution around 110 nm [118] (figure 4). From the CARS and SRS signal expressions derived in equations (16) and (21), we found that shorter excitation wavelengths also give better CRS signals. However, shorter wavelength pulses might increase the risk of photodamage of biological samples through one- and/or two-photon absorption (TPA).

Figure 4. Visible SRS microscopy. (A) Experimental schematic of a visible SRS microscope. HWP: half-wave plate; AOM: acousto-optical modulator; BF: bandpass filter; DM: dichroic mirror; SU: scanning unit; OS: objective scanner; OB: objective; OC: oil condenser; PD: photodiode; PMT: photomultiplier tube; PM-SM: polarization-maintaining single-mode fiber. (B) Energy diagram of near-IR and visible excitations for SRS. (C) and (D), near IR and visible SRS images of U2OS cells, respectively. (E) The magnified square area in panel (D). (F) The intensity profile along the selected line in panel (E). The red curve is the Gaussian fitting of the intensity profile, showing a full-width-half-maximum of 113 nm. Scale bars are 5 µm. Reproduced from [118]. CC BY 4.0.
Instead of using conventional microscope frames, CRS has also been coupled into compact handheld devices and miniature fiber-optic microendoscopic probes. The fiber optic probes can be scanned by piezo scanners in spiral or Lissajous patterns for image formation [119, 120]. Strong nonlinear optical signals generated by pump and Stokes pulses in silica fibers were one of the major hurdles for CRS endoscopes. In early designs based on single-mode fibers (SMFs), picosecond laser pulses were preferable compared to femtosecond laser pulses due to the relatively lower pulse peak power for less nonlinear signals from fibers [121]. Photonic crystal fibers (PCFs) also showed better delivery of both pump and Stokes laser pulses than SMFs [122]. In particular, hollow-core PCFs can suppress nonlinear signals from the fiber and maintain high signal collection efficiency [123]. A fiber-delivered free-space probe has been developed to maximize CRS signal collection [124]. Although the SRS signal is mostly propagating forward, backscattered photons allow for imaging of biological samples using SRS endoscopic prototypes [67]. In recent years, several groups have made attractive breakthroughs for both CRS and SRS endoscopes. In 2018, the Regnault group demonstrated a CARS endoscope by scanning a hollow-core Kagome-lattice double-clad fiber (figure 5). They inserted and sealed a 30 µm silica microsphere at the end of the fiber tip as the microlens for beam focusing (figures 5(B) and (C)). These two designs optimized the pulse delivery, signal collection, and spatial resolution for the CARS endoscope [125]. The Hashimoto group reported a rigid CARS endoscope probe which is composed of separate parts for easy assembly and used the device to image rat sciatic nerves [126]. Later, a deep-learning-based denoising method was applied for noise reduction, thus improving the imaging speed of this CARS endoscope [127]. Besides, Trägårdh et al showcased the possibility of using a cost-effective graded-index multimode fiber for CARS endoscopy. The laser foci on the sample can be scanned by changing the patterns of a spatial light modulator in the optical path [128].

CRS has been integrated with flow cytometry settings for single-particle analysis. Compared to fluorescence-based flow cytometry, Raman flow cytometry is label-free and potentially allows for more detection channels. However, spontaneous Raman flow cytometry has low throughput due to the weak signal level and long signal integration time [129]. Both narrow and broadband CRS spectroscopy have been incorporated into microfluidic flow chambers for single-cell analysis [130–132]. In these early developments, the broadband CRS spectra were acquired at speeds of 100–1500 spectra per second, giving a throughput of a hundred cells per second or less [131, 132]. Recently, high-speed Fourier-transform CRS flow cytometry was reported with a high throughput of up to 2000 events per second [133]. The cell stream was focused using an acoustofluidic-focusing microfluidic chip. High-speed single-cell analyses of astaxanthin production and photosynthetic dynamics of Haematococcus lacustris were performed [133]. An SRS flow cytometry technique based on multiplex spectral acquisition was also developed with the capability to acquire spectra at 200 kHz and analyze 11000 particles per second [85]. Recently, Suzuki et al has reported an SRS imaging flow cytometry platform that can collect high-resolution multicolor SRS images in a microfluidic flow setting at a throughput of ≈140 cells per second [134], and highlighted the potential of SRS...
imaging for high-speed and high-content single-cell analysis. Besides, by integrating SRS imaging techniques with cell sorting devices, the Goda group has successfully achieved single-cell sorting at a speed of \( \sim 100 \) cells per second using various vibrational signatures [135] (figure 6). They demonstrated SRS-image-activated sorting of rare cells with unique chemical compositions in a large population.

Besides technological innovations in CRS microscopy, efforts have been put into improving the data analysis of hyperspectral images. For CARS, due to the spectral distortion caused by the nonresonant background and the nonlinear dependence of signal intensity with molecular concentration, the chemical composition analysis is relatively complicated. Kramers–Kronig relation [31] and maximum entropy method [136] have been used to separate resonant and non-resonant portions from CARS spectra. Commonly used composition analysis methods for hyperspectral CARS include principal component analysis [137, 138], classical least-squares analysis [139], singular value decomposition (SVD) analysis [140, 141], hierarchical cluster analysis [142], and multivariate curve resolution (MCR) analysis [143]. Among these methods, the MCR analysis, which requires initial guess or input of spectra from pure compounds, is capable of decomposite CARS spectra to individual chemical species with quantitative concentration values. Masia et al developed an algorithm based on the SVD and Kramers–Kronig relations to factorize susceptibilities and concentrations of chemical components in an unsupervised manner, known as the FSC method [38]. For hyperspectral SRS microscopy, many spectral domain analysis approaches can be applied similarly. Both the MCR [49, 87, 144] and the modified FSC methods have been used for SRS spectral analysis [145]. Another commonly adopted image segmentation method for hyperspectral SRS is spectral phasor which has low computational complexity and separates different image pixels based on Fourier transform and phase value clustering [146, 147].

To improve CRS imaging sensitivity and image quality, several denoising methods have been developed. For example, Liao et al used a total variation minimization algorithm to improve the signal-to-noise ratio of SRS up to 57 times [148]. Zong et al applied a block-matching and 4D filtering algorithm to push the sensitivity of the surface-enhanced SRS imaging to the single-molecule level [68]. The Fu group deployed deep-learning to improve the signal-to-noise ratio of SRS microscopy to reduce the excitation laser power requirement and increase the imaging depth [149, 150].

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**Figure 6.** Raman-image-activated cell sorting system. (A) Illustration of the SRS-imaging-based cell sorting system. (B) Scatter plot of differentiated and undifferentiated 3T3-L1 cells in the spatial distribution of intracellular lipid droplets and the lipid intensity per cell-area. These features are used for sorting the differentiated 3T3-L1 cells. The insets show representative SRS images of the cells. The sorted and unsorted events are \( n = 108 \) and \( n = 11068 \), respectively. (C) Scatter plot of *Chlamydomonas* sp. mutant cells in cell area and lipid density. Highly lipid-rich but rare *Chlamydomonas* sp. mutants (indicated by the yellow region) were sorted. The inset shows representative SRS images of cells. The sorted and unsorted events are \( n = 26 \) and \( n = 7786 \), respectively. Scale bars are 10 \( \mu \)m. Reproduced from [135]. CC BY 4.0.
3.4. Multimodal nonlinear optical microscopy on the CRS platform

It is straightforward to incorporate other nonlinear optical imaging modalities into CRS microscopes [151–154]. The second-harmonic generation (SHG) modality can be easily integrated with a CRS microscope by adding a PMT and correct filters. Multiphoton excitation fluorescence (MPEF) modalities can be added similarly. Both SHG and MPEF only require a single excitation wavelength, which can be chosen from either the pump or the Stokes beam. However, for these modalities, femtosecond pulses are much more effective for signal generation than picosecond pulses, due to the higher peak power. Besides, MPEF can also be excited by the absorption of photons from both the pump and Stokes beams. These nondegenerate multiphoton absorption processes can even dominate the MPEF signals when the total add-up photon energy better matches the absorption peaks of fluorophores. The existence of both pump and Stokes pulses might also generate sum-frequency generation (SFG) signals from certain biological structures [155]. This SFG signal can overlap with fluorescence signals from many dyes and intrinsic fluorophores. Therefore, the selection of spectral windows for clean fluorescence signals may need additional attention.

The intensity modulation and lock-in demodulation scheme used for SRS microscopy is typically adopted for other pump-probe optical detections. Therefore, an SRS microscope is intrinsically a pump-probe microscope that can measure the impacts of one laser beam on the other due to different light-matter interactions. Optical processes related to pump-probe detections include TPA, excited-state absorption (ESA), stimulated emission (SE), SRS, ground-state depletion (GSD), CPM, and photothermal effect (PTE) [59, 156–158]. Contributions from these optical phenomena can mingle together and complicate the imaging contrasts. Assuming we detect the SRL which is measured as the pump beam intensity decrease, other processes that also reduce the pump beam intensity are TPA, ESA, SE, CPM, and PTE, while the process that likely increases the pump beam is GSD. We have mentioned earlier that to avoid the impact of CPM, condensers with higher NA than the objective lenses are needed. This optical arrangement can also to some extent reduce the effect of PTE. For most biomolecules, the vibrational excitation population is negligible compared to the ground state population in normal conditions. Therefore, the effect of GSD is typically ignorable. The major contributions that can interfere with SRS signals are usually TPA, ESA, and SE. For biomolecules with lower electronic transition energy such as heme proteins, pigments, and intrinsic fluorophores, signals from all TPA, ESA, and SE, and even other processes can be detected with an SRS microscope configuration. To separate these processes from SRS, one can detune the optical frequency and mismatch the pump and probe beams with any Raman transition. Since the vibrational peaks are narrow and much more sensitive to frequency than other processes, slight detuning from the vibrational peaks can largely eliminate the SRS contribution while maintaining similar responses for others. Alternatively, a dual-modulation method to separate the SRS response from others was developed [104]. A common way to obtain molecular contrasts from these transient absorption processes is to fix the excitation wavelength and tune the time delay between the pump and probe beams. The transient absorption signal decay curves can be used to distinguish specific molecules through quantitative fitting. Transient absorption modalities were used similarly to SRS for biomolecular imaging. The transient absorption signal decay rates can be used to identify biomolecules such as heme [159], glycated hemoglobin [160], and hemozoin [161], and to quantify the blood oxygen levels [162]. Besides, pump-probe microscopy can also provide unique molecular contrasts such as eumelanin and pheomelanin [163, 164] for diagnostic purposes.

4. Imaging biomolecules in living biological samples by CRS microscopy

CRS microscopy has been used to explore many biological systems to better understand cell metabolism, drug responses, and to produce new contrasts for diagnosis. We do not intent to thoroughly summarize all publications in this field. Instead, we want to give some examples so that the readers would have a general idea of what biological insights CRS imaging can offer.

4.1. The studies on cell metabolism

The most widely studied biomolecules in CRS microscopy are lipids due to the strong Raman signal arising from the dense packing of CH2 moieties. CRS microscopy has been used to explore the lipid metabolism of living mammalian cells. Lipid droplets (LDs), which are mostly composed of triglyceride and cholesteryl esters, were one of the major targets. LDs were first used to evaluate the performance of CARS or SRS microscopes in the early years [19, 165]. Soon, researchers in the CRS field started to explore LDs for more biological insights. For example, Nan et al have observed the formation of large LDs from small LDs during adipocyte differentiation [166]. Le et al have found insulin-signaling-difference-induced variation in LD formation for clonal cells [167]. Parr et al studied LD remodeling during lipolysis and growth in adipocytes by time-lapse CARS imaging [168]. Hyperspectral CRS microscopy added new knowledge to the lipid configuration for clonal cells [167].
metabolism in LDs. Di Napoli et al quantified the uptake of saturated and unsaturated fatty acids by human adipose-derived stem cells cultured in various conditions [37, 169].

Besides LD contents and amounts, CRS microscopes were also deployed to understand LD movements and dynamics. Nan et al first monitored LD movement in live mouse adrenal cortical cells using CARS and separated subdiffusion and active transport of LDs [170]. Jüngst et al performed a video-rate recording of LD movement using CARS to quantify the trafficking features [171]. In a later work, they studied the LD fusion dynamics in adipocytes [172]. Zhang et al applied SRS microscopy to quantify the LD maximum displacement and speed and found that these parameters reveal changes in lipid metabolism in living cancer cells [173]. Recently, a systematic study of LD dynamics associated with cancer cell metabolic changes in stress conditions was reported [174]. Figure 7(A) shows the changes in LD dynamics during apoptosis of MIA PaCa2 pancreatic cancer cells. The basis of this method is that the LDs under synthesis and degradation processes have different dynamic properties. Huang et al discovered LD redistribution and migration to cancer cell protrusions in stress conditions [86]. This metabolic reprogramming was shown to help the cancer cells to survive in stress conditions.

Both CARS and SRS microscopes have the sensitivity to image single lipid bilayers [177, 178]. Besides, it was shown that C–H vibrational signals from SRS imaging are indicators of membrane potentials of erythrocyte ghosts and live neurons [178–180]. A recent study demonstrated using hyperspectral CARS
and SRS microscopies to separate lipid domains on supported lipid bilayers formed by mixing dioleoylphosphatidylcholine, sphingomyelin, and cholesterol molecules [145].

CRS spectroscopy and microscopy can discover important biomarkers for cancer aggressiveness, which helped to identify new targets for cancer treatment. Yue et al found that the loss of tumor suppressor gene PTEN in prostate cancer cells activates the PI3K-ATK pathway and results in the accumulation of cholesterol ester in cellular LDs [181]. The acetyl-CoA acetyltransferase, which is responsible for the conversion of cholesterol to cholesteryl ester, was found as a new target for the treatment of prostate cancer [181]. Lee et al discovered that the suppression of prostate cancer metastasis by cholesteryl esterification inhibition is through the Wnt/β-catenin pathway [182]. This ACAT1 target was also generalized for pancreatic, lung, and colon cancers [183–185]. Li et al has found an increase in lipid desaturation in ovarian cancer stem cells, and targeted lipid desaturase for selective removal of these cancer stem cells [186]. Recently, multiplex CARS microscopy helped researchers find that BDNF-induced TrkB activation is responsible for the formation of LDs in colorectal cancer cells [187]. Du et al studied the correlation between lipid unsaturation and metastasis of melanoma cells and discovered that the mutation of BRAF leads to a high level of mono-unsaturation lipids in cells [175]. Inhibition of Δ9 lipid desaturase induced the formation of intracellular phase-separated solid membranes (figure 7(B)) and led to apoptosis of BRAF mutant melanoma cells [175].

Jasensky et al applied hyperspectral CARS microscopy and SVD-based spectral analysis to quantify LDs in oocytes from different species and the LD amount change during oocyte growth and development [141]. Bradly et al used CARS imaging to analyze LDs in living mouse oocytes and embryos up to the blastocyst stage and showed that different developmental stages have different LD amounts and fatty acids saturation degrees [188]. For direct imaging of living organisms, SRS microscopy has been applied to perform RNAi screening of genes regulating fat accumulation in Caenorhabditis elegans (C. elegans) [189]. Wang et al employed hyperspectral SRS microscopy to quantitatively map fat distribution, fatty acid unsaturation, lipid oxidation, and cholesterol storage in living C. elegans, and discovered the storage of cholesterol in lysosome-related organelles in intestinal cells [144]. A later study by Chen et al found cytoplasmic storage of retinoids in C. elegans which is positively correlated with fat storage in worms and helped slow down fat loss during starvation [190]. Dou et al studied early Drosophila embryo development by SRS microscopy and used a velocity-jump mathematical model to link the LD motion with distributions [191].

Besides lipids, CRS microscopy can image many other biomolecules and pharmaceutical compounds in biological systems. Diffusion of dimethyl sulfoxide and retinoic acids were followed by in vivo SRS imaging [19]. Chemotherapy drug enrichment in lysosomes and chloroquine-induced release were discovered by hyperspectral SRS microscopy, which helped to explain the synergistic effect of chloroquine for the treatment of chronic myeloid leukemia with tyrosine-kinase inhibitor drugs [192]. Lu et al reported the label-free DNA imaging with SRS microscopy targeting the 2967 cm⁻¹ Raman transition [71]. Hyperspectral CARS, which can cover a broader spectral range, can simultaneously separate nucleus, collagen, arterial wall, and LDs in biological tissues [39]. Moreover, CARS was shown to not only image water molecules between phospholipid bilayers but also derive the molecular orientations [193].

Researchers in plant science have deployed CRS microscopy to answer questions related to plant metabolism. Zeng et al used CARS microscopy to visualize the lignin content reduction in cell walls of lignin-downregulated plants [194]. Using the lignin peak at 1600 cm⁻¹ and the cellulose peak at 1100 cm⁻¹, Saar et al performed high-resolution real-time imaging of delignification reaction in corn stover by SRS microscopy [195]. Hyperspectral SRS provides more spectral information to selectively map the aromatic ring of lignin, aldehyde, and alcohol groups in plant cell walls [196]. Real-time reduction of aldehyde groups into alcohol groups in cell walls by sodium borohydride was observed [196]. Hyperspectral SRS also allows differentiating zip-lignin from native lignin in plant cell walls [197].

CRS microscopy has also found applications in microbiology. Hong et al demonstrated in situ detection of single Escherichia coli in milk and urine by hyperspectral CARS microscopy [143]. CRS microscopy offers new ways to rapidly detect antimicrobial-resistant microorganisms. Karanja et al showed that lipogenesis between fluconazole-susceptible and -resistant Candida albicans are quite different, with the latter more enhanced [198]. Hong et al found that vancomycin-resistant Enterococcus faecalis have much higher metabolic rates of deuterated glucose than vancomycin-susceptible ones [199]. Recently, Zhang et al showed that many antibiotic-treated bacterial species have much-reduced metabolism of D₂O compared to their antibiotic-susceptible counterparts [176] (figure 7(C)). These characteristics of small-molecule metabolic differences give rapid ways for antimicrobial susceptibility tests at a single bacteria level. Bae et al used Raman-tagged antibiotics with an SRS microscope to monitor the pharmacokinetics of antibiotics in biofilms formed by Staphylococcus aureus and discovered limited drug penetration [200]. Jaeger et al studied lipid metabolism in oleginuous microalgae and revealed LD formation and enlargement during nitrogen starvation [201]. Wakisaka et al performed video-rate metabolite imaging of live Euglena gracilis by high-speed multi-color SRS microscopy [202]. Using different spectral signatures of lipids, paramylon, chlorophyll, and
protein/nucleic acids, multicolor SRS can separate these chemical species without labeling. The results also unveiled increases in lipid and paramylon and the decrease in chlorophyll during nitrogen starvation [202].

4.2. Chemical insights for diagnosis
At the tissue level, CARS imaging has been employed to study lipid membrane structures and orientations in the myelin sheath in mouse spinal cords [203] and brains [204]. CARS was proven to be a powerful tool to monitor demyelination and remyelination in the injured spinal cord [205] and to evaluate spinal cord repair [206]. Degeneration of peripheral nerves in amyotrophic lateral sclerosis has been visualized by SRS microscopy [207]. Besides imaging lipids, amyloid plaques in Alzheimer’s disease can also be detected by SRS due to the slight vibrational frequency difference between sheet and helical structures of peptides [208]. CRS was also utilized to image cholesterol accumulation in atherosclerotic artery tissues from mice [209, 210] and humans [211]. Multimodal imaging platforms integrating CRS and other imaging modalities have been developed by multiple groups for the diagnosis of cancer [151, 152, 212–215]. These nonlinear optical imaging modalities provide unprecedented contrasts beyond conventional hematoxylin and eosin (H&E) stain to separate cancerous tissue from normal tissue. These contrasts arise from different structural and chemical changes caused by cancer transitions. More importantly, direct imaging from fresh biopsy circumvents the need for sample sectioning and staining and therefore provides a rapid way to improve conventional histology. Although multimodal imaging generates unique physical and chemical contrasts from intact biopsy tissues, interpretation of these image contrasts is still preliminary and requires special knowledge in biochemistry and physiology which is beyond conventional histology. Another route is to use new optical microscopy technologies to generate label-free histological images comparable to conventional H&E stain. Two-color SRS imaging has offered promising results in this direction. Freudiger et al demonstrated label-free SRS histology using the lipid and protein contrasts and added two-photon absorption contrasts for hemoglobin [76]. Ji et al reported rapid two-color SRS imaging for the detection of brain tumors from in vitro mouse brain slices and in vivo human tumor xenografts in mice [77]. In a later study, they showed that SRS can reveal human brain tumor infiltration using fresh surgical samples from 22 neurosurgical patients [216]. A strip image-stitching and two-color SRS imaging platform was developed for large-scale SRS histology [217]. Adding SHG contrasts to SRS images would give collagen distribution information for better diagnostics. Zhang et al showcased rapid histology of laryngeal squamous cell carcinoma by SRS microscopy assisted by deep-learning [80] (figure 8). Sarri et al used SHG and SRS contrasts for instantaneous label-free gastro-intestinal histology for cancer detection [218]. Compact fiber laser systems were also developed for portable SRS histology platforms [219]. Fiber lasers typically have strong laser intensity noise in the MHz frequency range. Consequently, balance detection is usually needed to improve the signal-to-noise-ratio for SRS [219, 220]. Orringer et al operated a portable SRS histology system in the operating room and examined unprocessed specimens from 101 neurological patients [78]. They also leveraged two-color SRS images to create virtual H&E stain slides for diagnostic of brain tumor [78]. This work highlighted the potential of stain-free SRS histology for rapid diagnosis in medical settings.

4.3. Raman tags for better sensitivity and selectivity
To enhance the sensitivity and chemical selectivity, Raman tags are commonly used in CRS microscopy. Raman tags are usually small chemical moieties having strong or/and unique Raman transition signals. Compared to fluorescence tags, Raman tags are usually much smaller in size, thus making it more suitable to label small molecules such as metabolites. Besides, the vibrational transitions are much narrower than electronic transitions, making Raman tags having sharper peaks than fluorescent tags. Raman tags were originally used for spontaneous Raman microscopy [222, 223]. In recent years, along with the continuous advancement of CRS microscopy, more versatile Raman tags have been developed. One of the most widely used Raman tags is alkyne. It has a strong Raman transition at ~2125 cm⁻¹ which is inside the Raman silent region. Therefore, alkyne-based Raman tags are widely used for pulse-chase studies of biochemical in living organisms. The Min group has attached alkyne to various molecules for SRS imaging of DNA, RNA, protein, and lipids [224]. They also edited the vibrational spectral palette by using both ¹³C and ¹²C combinations for the alkynes [225]. Hong et al also demonstrated selective labeling and SRS imaging of thymidine analog, fatty acid, homoproparglycin, and peracetylated N-(4-pentynoyl)mannosamine by alkyne tags [226]. A glucose analog with an alkyne tag was introduced for the glucose uptake study of cancer cells [227].

Besides alkyne, other Raman active molecular moieties were developed as Raman tags. Lee et al reported sensitive SRS imaging of cholesterol in live cells and C. elegans using phenyl-diyne-tagged cholesterol molecules [228]. Wei et al developed 24 Raman dyes which have different vibrational frequencies based on structurally modified nitrile and alkyne [221]. The sharp and narrow Raman transitions allow for supermultiplex optical imaging (figure 9). The Min group also synthesized a class of 20 polyyne-based Raman dyes for supermultiplex SRS imaging [229]. The combination of these Raman probes in polymer
microparticles creates optical barcoding potentially useful beyond imaging. Recently, Tian et al designed polydiacetylene-based Raman tags which give up to $10^4$ enhancement of alkyne Raman signal for targeted live cell imaging [230]. Zhang et al demonstrated a method using a small phenyl-ring-enhanced Raman tag with a single unnatural amino acid to genetically target specific proteins for SRS imaging [231] (figure 10).

Isotope labeling is also a commonly used method for CRS microscopy. Deuterated compounds typically have Raman signals in the Raman silent region. Deuterated glucose has been used to understand lipid de novo synthesis in various cancer cells [232]. Deuterated amino acids were applied to visualize de novo protein synthesis in cells and tissues [233, 234]. Deuterated choline allows for SRS imaging of these small molecules in mammalian cell lines, primary neurons, and C. elegans [235]. Other isotopes were also picked to improve the chemical selective of SRS microscopy. For example, using $^{13}$C-phenylalanine as a marker, Shen et al demonstrated time-dependent protein degradation by using $^{12}$C/$^{12}$C + $^{13}$C ratio maps [236].

5. Summary and outlook

In modern biomedical sciences, there’s a general interest in the label-free understanding of biological compositions. CRS microscopy not only offers label-free advantages but also provides unique chemical information for scientists to understand biological functions. Compared to conventional spectroscopic imaging techniques such as infrared and spontaneous Raman, CRS microscopy has much higher spatial resolution and faster imaging speed. Over the past 20 years, numerous innovations in the field have transitioned CRS microscopy into powerful and mature platforms used by biologists for answering various questions in cell metabolism, cancer research, neuroscience, diagnosis, and more. There are plenty of review papers in this field focusing on either the fundamentals of CRS [21, 43, 89, 154, 237, 238], the technological innovations [18, 44, 45, 59, 91], and the biological applications [90, 94, 239–250]. In this review article, we provide a detailed theoretical introduction of CRS from the classical point of view, which would help...
Figure 9. Manhattan Raman scattering (MARS) dyes for Super-multiplex vibrational imaging. (A) Design principles and structures for a three-dimensional library of electronic pre-resonance (epr)-SRS nitrile (left) and alkyne (right) MARS dyes. (B) Two sets of MARS palettes and their normalized epr-SRS spectra. The upper panel shows 12 MARS dyes, whose vibrational frequencies (in cm$^{-1}$) are indicated by numbers after solid lines, and are solid-underlined in (A). The lower panel shows 10 MARS dyes, whose vibrational frequencies (in cm$^{-1}$) are indicated by numbers after dashed lines, and are dash-underlined in (A). (C) Sixteen-color live-cell imaging with eight MARS dyes, the four commercial vibrational dyes in the fingerprint, and four additional fluorescent dyes (indicated by asterisks). (D), (E) Eight-colour epr-SRS imaging of DNA replication and protein synthesis in hippocampal neuronal cultures (D) and organotypic cerebellar brain slices (E). Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature. [221] (2017).

Figure 10. Incorporation of a Raman tag via genetic code expansion. (A) Schematic representation of the amber-unnatural amino acid-Raman tag system for genetically targeted protein imaging. AA: amino acid; RE: release factor. (B) Click chemistry to link 1 with azide-ended Raman tag 2. (C) Fluorescence and hyperspectral SRS imaging of target Histone3.3-EGFP protein in a HeLa cell, targeted EGFP-Sec61β protein in a HeLa cell, and Htt74Q-EGFP protein in Huntington diseased cells. Reproduced with permission from [231].

beginners in the field understand the ‘math’ related to CRS. A thorough comprehension of the theoretical background would lay a great foundation for the CRS experimental work. Besides, we covered the majority of CRS microscopy instrumentations and reviewed the most recent applications of CRS imaging in biological sciences. We hope the readers can find key information for setting up a CRS microscope and be fascinated by the unprecedented insights that CRS imaging has brought to us.

A major bottleneck for current CRS microscopes is the relatively low sensitivity compared to fluorescence. Although many groups have continued to break the sensitivity record, CRS imaging, which mostly relies on intrinsic biomolecules, has not reached a comparable sensitivity with fluorescence microscopy. Recently reported Raman probes combined with stimulated Raman excited fluorescence imaging can detect Raman active molecules at 500 nM concentration [251]. Using nanoparticle enhancement, CARS and SRS were reported to detect single molecules [68, 252]. However, these methods
either require specially designed conjugated molecules or metal nanoparticles. Direct imaging of intrinsic biomolecules using CRS had never reached a similar sensitivity as fluorescence imaging. Surmounting this barrier would greatly expand the territories of CRS in biological science.

Another challenge that currently limits CRS availability to broader users is the cost of the equipment. To obtain desirable sensitivity and contrast, CRS microscopes are using expensive ultrafast laser systems to generate both pump and Stokes pulses. Although the CRS microscope has been commercialized, reduced laser cost would potentially give birth to more affordable CRS microscopes for biologists. Several breakthroughs have been demonstrated along this path and give promises to the next generation CRS microscopy [219, 253–256].

Further advancements in the CRS microscopy field will not weaken the role of fluorescence microscopy as the major optical imaging modality in biology. Instead, the CRS modalities will thrive along with fluorescence to offer biologists more complete pictures of biological systems.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Appendix A

The expression of the two input electric fields is \( E(z, t) = E_p e^{i(k_p z - \omega_p t)} + E_s e^{i(k_s z - \omega_s t)} + c.c. \). Insert this expression into \( F(z, t) = \frac{\partial b}{\partial t} = \frac{\varepsilon_0}{2} \frac{\partial \alpha(t)}{\partial \tau} E^2 \), we get

\[
F(z, t) = \frac{\varepsilon_0}{2} \frac{\partial \alpha(t)}{\partial \tau} E^2 \left\{ E_p^2 e^{i(k_p z - \omega_p t)} \right. + 2E_p E_s e^{i(k_s z - \omega_s t)} + \left. E_s^2 e^{i(k_s z - \omega_s t)} \right. + 2E_p E_s e^{i(k_p z - \omega_p t)} + \ldots
\]

The term having the frequency \( \omega = \omega_p - \omega_s \) is \( \varepsilon_0 \frac{\partial \alpha(t)}{\partial \tau} E_p E_s e^{i[(k_p - k_s)z - (\omega_p - \omega_s) t]} \). This term is directly related to CRS responses.

Appendix B

In Maxwell’s equation,

\[
\begin{align*}
\nabla \cdot D &= \rho \\
\nabla \cdot B &= 0 \\
\n\nabla \times E &= -\frac{\partial B}{\partial t} \\
\n\nabla \times H &= \frac{\partial D}{\partial t} + J
\end{align*}
\]

\( E, J, \) and \( D \) are the optical electric field, electric current density, and the optical electric displacement field, respectively. \( H \) and \( B \) are the two forms of the optical magnetic field. \( \rho \) is the electric charge density. \( B = \mu_0 H, D = \varepsilon_0 E + P \), in which \( P \) is the polarization density, \( \mu_0 \) and \( \varepsilon_0 \) are the vacuum permeability and permittivity, respectively. Typically, biomolecular systems have no free charge and current. Therefore, \( \rho = J = 0 \). To derive the wave equation, we can apply \( \nabla \times \) to both sides of the third expression and get

\[
\nabla \times (\nabla \times E) = -\nabla \times \frac{\partial B}{\partial t} = -\mu_0 \nabla \times \frac{\partial H}{\partial t} = -\mu_0 \frac{\partial^2 D}{\partial t^2}.
\]

On the left-hand side, \( \nabla \times (\nabla \times E) = \nabla (\nabla \cdot E) - \nabla E \). For typical optical electric field, \( \nabla (\nabla \cdot E) \) is much smaller than \( \nabla E \). Therefore, we can simplify the equation to

\[
\nabla^2 E = \mu_0 \frac{\partial^2 D}{\partial t^2}.
\]

If we separate the nonlinear polarization from the linear polarization, we get \( P = P^{(1)} + P^{NL} \). Since \( D = \varepsilon_0 E + P \), we can write \( D = \varepsilon_0 E + P^{(1)} + P^{NL} = \varepsilon_0 (1 + \chi) E + P^{NL} = \varepsilon_0 \varepsilon_r E + P^{NL} \).
Using this expression, we can get
\[ \nabla^2 E = \mu_0 \varepsilon_0 c \frac{\partial^2 E}{\partial t^2} = \mu_0 \frac{\partial^2 P^{\text{NL}}}{\partial t^2}. \]

The speed of light has the definition \( c = \sqrt{\frac{1}{\varepsilon_0 \mu_0}} \), we can then simplify this equation to
\[ \nabla^2 E = e_r \frac{\partial^2 E}{c^2} = \frac{1}{\varepsilon_0 c^2} \frac{\partial^2 P^{\text{NL}}}{\partial t^2}. \]

The electric field is a function of time and space. If we take the time factor out, using the expression
\[ A(r, t) = A(r) e^{-i\omega t} + \text{c.c.}, \]
where \( A \) applies to \( E, D, \) and \( P^{\text{NL}} \). We have
\[ \frac{\partial^2 E(r)}{\partial t^2} = -\omega^2 E(r), \quad \text{and} \quad \frac{\partial^2 P(r)}{\partial t^2} = -\omega^2 P(r). \]

Using these two equations, we get
\[ \nabla^2 E(r) + \frac{\omega^2 \varepsilon_r E(r)}{c^2} = -\frac{\omega^2}{\varepsilon_0 c^2} P^{\text{NL}}(r). \]

For a plane wave propagating only along the z-direction, it is simplified to
\[ \frac{\partial^2 E(z)}{\partial z^2} + \frac{\omega^2 \varepsilon_r E(z)}{c^2} = -\frac{\omega^2}{\varepsilon_0 c^2} P^{\text{NL}}(z). \]

**Appendix C**

\[ P^{\text{NL}}(z, t) = \varepsilon_0 n^2 \left[ \frac{\partial \alpha}{\partial r} \right] \left[ r(\omega) e^{i(kz - \omega t)} + \text{c.c.} \right] \left[ E_p e^{i(kz - \omega t)} + E_s e^{-i(kz - \omega t)} + \text{c.c.} \right]. \]

We take only the term with a frequency \( \omega_s = 2\omega_p - \omega_s \), which is
\[ P_{as}^{\text{NL}}(z, t) = \varepsilon_0 n^2 \left[ \frac{\partial \alpha}{\partial r} \right] \left[ r(\omega) e^{i(kz - \omega t)} e^{i(2\omega_p - \omega_s) t} \right] = \varepsilon_0 n^2 \left[ \frac{\partial \alpha}{\partial r} \right] \left[ r(\omega) E_p e^{i(2k_p - k_s) z} e^{i(2\omega_p - \omega_s) t} \right]. \]

If we write \( P_{as}^{\text{NL}}(z, t) = P_{as}^{\text{NL}}(z) e^{-i(2\omega_p - \omega_s) t} \), then
\[ P_{as}^{\text{NL}}(z) = \left( \frac{N \varepsilon_0^2 / m}{\omega_p^2 - \omega^2 + 2i\omega \gamma} \right)^2 \left| E_p \right|^2 E_s^* e^{i(2k_p - k_s) z}. \]

Since \( \omega = \omega_p - \omega_s = -(\omega_s - \omega_p) \), we can then define \( P_{as}^{\text{NL}}(z) = 3 \varepsilon_0 \chi_{R,as}^{(3)} \left| E_p \right|^2 E_s^* e^{i(2k_p - k_s) z} \), where
\[ \chi_{R,as}^{(3)} = \left( \frac{N \varepsilon_0^2 / 3m}{\omega_p^2 - \omega_s^2 + 2i(\omega_p - \omega_s) \gamma} \right)^2. \]

**Appendix D**

Equation (11) has the form
\[ \frac{\partial E(z)}{\partial z^2} + \frac{\omega^2 \varepsilon_r E(z)}{c^2} = -\frac{\omega^2}{\varepsilon_0 c^2} P^{\text{NL}}(z). \]

The nonlinear polarization related to the anti-Stokes frequency is
\[ P_{as}^{\text{NL}}(z) = 3 \varepsilon_0 \chi_{R}^{(3)} \left| E_p \right|^2 E_s^* e^{i(2k_p - k_s) z}. \]

From \( E_{as}(z) = E_{as} e^{i\delta z} \), we get
\[
\frac{\partial E_{as}(z)}{\partial z} = \frac{\partial E_{as}}{\partial z} e^{ik_{as}z} + i k_{as} E_{as} e^{ik_{as}z}.
\]

\[
\frac{\partial^2 E_{as}(z)}{\partial z^2} = \frac{\partial^2 E_{as}}{\partial z^2} e^{ik_{as}z} + i k_{as} \frac{\partial E_{as}}{\partial z} e^{ik_{as}z} + i k_{as} \frac{\partial E_{as}}{\partial z} e^{ik_{as}z} - k_{as}^2 E_{as} e^{ik_{as}z}.
\]

Note that \(k_{as}^2 = \frac{\omega^2}{c^2} \epsilon_r\).

Therefore, we can simplify equation (11) to

\[
\frac{\partial^2 E_{as}}{\partial z^2} e^{ik_{as}z} + 2i k_{as} \frac{\partial E_{as}}{\partial z} e^{ik_{as}z} = - \frac{3 \chi_R^{(3)} \omega^2}{c^2} |E_p|^2 E_s^* e^{i(2k_p - k_{as})z}.
\]

For typical laser optic fields used in microscopy \(\frac{\partial E_{as}}{\partial z} \ll 2i k_{as} \frac{\partial E_{as}}{\partial z}\). We can then further simplify this equation to

\[
\frac{\partial E_{as}}{\partial z} = \frac{3 \chi_R^{(3)} \omega^2}{2k_{as} c^2} |E_p|^2 E_s^* e^{i(2k_p - k - k_{as})z} - \frac{3 \chi_R^{(3)} \omega^2}{2n_{as} \epsilon} |E_p|^2 E_s^* e^{i(2k_p - k - k_{as})z}.
\]

If we define \(\alpha_{as} = \frac{3 \chi_R^{(3)} \omega^2}{2n_{as} \epsilon} |E_p|^2\), and \(\Delta k = 2k_p - k - k_{as}\), then

\[
\frac{dE_{as}}{dz} = \alpha_{as} E_s^* e^{i\Delta k z}.
\]

**Appendix E**

To solve equation \(\frac{dE_{as}}{dz} = \alpha_{as} E_s^* e^{i\Delta k z}\), we integrate on both sides from 0 to \(l\), and get

\[
E_{as} = \alpha_{as} E_s^* \int_0^l e^{i\Delta k z} = \alpha_{as} E_s^* \cdot \frac{e^{i\Delta k \cdot l} - 1}{i\Delta k}.
\]

Since \(\alpha_{as} = \frac{3 \chi_R^{(3)} \omega^2}{2n_{as} \epsilon} |E_p|^2\),

\[
E_{as} = \frac{3 \chi_R^{(3)} \omega^2}{2n_{as} \epsilon} |E_p|^2 \frac{E_s^*}{\epsilon} \cdot \frac{e^{i\Delta k \cdot l} - 1}{i\Delta k}.
\]

The optical intensity and electric field have a correlation \(I = \frac{\alpha_{as} \epsilon}{2} |E|^2\). We then get

\[
I_{as} = \left| \frac{cn_{as} \epsilon_0}{2} |E_{as}|^2 \right| = \left| \frac{cn_{as} \epsilon_0}{2} \right| \left| \frac{3 \pi i}{n_{as} \lambda_{as}} \right|^2 |\chi_R^{(3)}|^2 |E_p|^4 |E_s|^2 \left| \frac{e^{i\Delta k \cdot l} - 1}{i\Delta k} \right|^2
\]

\[
= \left\{ \frac{cn_{as} \epsilon_0}{2} \right\} \left| \frac{3 \pi i}{n_{as} \lambda_{as}} \right|^2 |\chi_R^{(3)}|^2 \left( \frac{2}{cn_p \epsilon_0} \right)^2 \left| \frac{2}{cn_p \epsilon_0} \right|^2 \left| \frac{cn_{as} \epsilon_0}{2} \right| \left( \frac{2}{cn_p \epsilon_0} \right)^2 |I_p|^2 |I_s|^2 \sin^2(\Delta k \cdot l/2)
\]

\[
= \left\{ \frac{36 \pi^2}{c^2 n_{as} n_p n_s \lambda_{as}^2} \right\} |\chi_R^{(3)}|^2 \left| \frac{2}{cn_p \epsilon_0} \right|^2 \left| \frac{cn_{as} \epsilon_0}{2} \right| \left( \frac{2}{cn_p \epsilon_0} \right)^2 |I_p|^2 |I_s|^2 \sin^2(\Delta k \cdot l/2).
\]

Therefore,

\[
I_{CARS} = b_{CARS} |\chi_R^{(3)}|^2 \left| \frac{2}{cn_p \epsilon_0} \right|^2 |I_p|^2 |I_s|^2 \sin^2(\Delta k \cdot l/2),
\]

where \(b_{CARS}\) is a constant at a certain wavelength. If we only consider the relative intensity, then

\[
I_{as} \propto |\chi_R^{(3)}|^2 \left| \frac{2}{cn_p \epsilon_0} \right|^2 |I_p|^2 |I_s|^2 \sin^2(\Delta k \cdot l/2).
\]

The simplification from \(\left| \frac{e^{i\Delta k \cdot l} - 1}{i\Delta k} \right|^2\) to \(\sin^2(\Delta k \cdot l/2)\) is
Assuming the pump beam is at 789 nm and the Stokes beam is at 1040 nm, CARS signal is then generated at 636 nm. If the sample for measurement is polyurethane, the refractive indices at these wavelengths are:

\[ n_p = 1.5786 \text{ (789 nm)}, \quad n_s = 1.5719 \text{ (1040 nm)}, \quad n_{as} = 1.5873 \text{ (636 nm)} \] (see http://refractiveindex.info).

In the transmission direction

\[ \Delta k = 2k_p - k_s - k_{as} = 2\pi \left( \frac{2n_p}{\lambda_p} - \frac{n_s}{\lambda_s} - \frac{n_{as}}{\lambda_{as}} \right) \]

\[ = 2\pi \left( \frac{2 \times 1.5786}{789} - \frac{1.5719}{1040} - \frac{1.5873}{636} \right) \approx -0.00035664 \text{ nm}^{-1}. \]

The CARS coherence length is \( l = |2/\Delta k| \approx 56 \mu \text{m} \).

In the epi direction, the anti-Stokes wavevector is opposite from the pump and Stokes wavevectors. Therefore,

\[ \Delta k = 2k_p - k_i + k_{as} = 2\pi \left( \frac{2n_p}{\lambda_p} - \frac{n_i}{\lambda_i} + \frac{n_{as}}{\lambda_{as}} \right) \]

\[ = 2\pi \left( \frac{2 \times 1.5786}{789} - \frac{1.5719}{1040} + \frac{1.5873}{636} \right) \approx 0.031327 \text{ nm}^{-1}. \]

The CARS coherence length is \( l = |2/\Delta k| \approx 64 \text{ nm} \).

Here, we can see that the coherence length of CARS is much shorter in the epi direction, due to the reversed sign of \( k_{as} \).

### Appendix G

\[ P_{NL}^{s}(z, t) = \varepsilon_0 N \left( \frac{\partial \alpha}{\partial r} \right) \left[ r(\omega) e^{i(kz - \omega t)} + c.c. \right] E_p \varepsilon_p e^{i(kz - \omega_p t)} + E_s \varepsilon_s e^{i(kz - \omega_s t)} + c.c. \]

We only consider the term with a frequency \( \omega_s \) for SRG, which is

\[ P_{NL}^{s}(z, t) = \varepsilon_0 N \left( \frac{\partial \alpha}{\partial r} \right) \left[ r^*(\omega) e^{-i(kz - \omega t)} E_p \varepsilon_p e^{i(kz - \omega_p t)} \right] = \varepsilon_0 N \left( \frac{\partial \alpha}{\partial r} \right) r^*(\omega) E_p \varepsilon_p e^{i(kz - \omega_p t)}. \]

If we write \( P_{NL}^{s}(z, t) = P_{NL}^{s}(z) e^{-i\omega_s t} \), then

\[ P_{NL}^{s}(z) = \left( \frac{N \varepsilon_0^2 / m}{\omega_p^2 - \omega^2 + 2i\omega\gamma} \right) \left| E_p \right|^2 \varepsilon_p e^{ikz}. \]

Since \( \omega = \omega_p - \omega_s \), we can then define \( P_{NL}^{s}(z) = 3\varepsilon_0 \chi_3^{(3)} \left| E_p \right|^2 E_s e^{ikz} \), where

\[ \chi_3^{(3)} = \frac{(N \varepsilon_0^2 / 3m) \left( \partial \alpha / \partial r \right)^2}{\omega_p^2 - (\omega_p - \omega_s)^2 + 2i(\omega_p - \omega_s)\gamma}. \]

At near resonance condition, \( (\omega_p - \omega_s) \rightarrow \omega_p \).
\[ \frac{1}{\omega^2_p - (\omega_p - \omega_s)^2 + 2i(\omega_p - \omega_s) \gamma} = \frac{1}{2(\omega_p - \omega_s)} \left( \frac{\omega^2 - (\omega_p - \omega_s)^2}{\omega^2 - (\omega_p - \omega_s)^2 + i\gamma} \right) \]

\[ \approx \frac{1}{2(\omega_p - \omega_s)} \left( \frac{\gamma \omega^2}{2(\omega_p - \omega_s)} \right) + i\gamma \]

\[ = \frac{1}{2(\omega_p - \omega_s)} \left( \frac{\gamma \omega^2}{2(\omega_p - \omega_s)} \right) + i\gamma. \]

Therefore, \( \chi^{(3)}_{R,s} = \frac{(N\varepsilon_0/3m)(\partial\alpha/\partial r)^2}{\omega^2 - (\omega_p - \omega_s)^2 + 2i(\omega_p - \omega_s) \gamma} \) can be simplified to

\[ \chi^{(3)}_{R,s} = \frac{(N\varepsilon_0/6m\omega^2_s)(\partial\alpha/\partial r)^2}{\omega^2 - (\omega_p - \omega_s)^2 + i\gamma}. \]

Appendix H

Equation (11) has the form

\[ \frac{\partial^2 E(z)}{\partial z^2} + \omega^2 c^2 E(z) = -\frac{\omega^2}{c^2} P^{NL}(z). \]

The nonlinear polarization related to the Stokes frequency is

\[ P^{NL}(z) = 3\varepsilon_0 \chi^{(3)}_{R} |E_p|^2 E_i e^{ikz}. \]

From \( E_i(z) = E_i e^{ikz} \), we get

\[ \frac{\partial E_i(z)}{\partial z} = \frac{\partial E_i}{\partial z} e^{ikz} + i\gamma \frac{\partial E_i}{\partial z} e^{ikz}, \]

\[ \frac{\partial^2 E_i(t)}{\partial z^2} = \frac{\partial^2 E_i}{\partial z^2} e^{ikz} + i\gamma \frac{\partial^2 E_i}{\partial z^2} e^{ikz} + i\gamma \frac{\partial E_i}{\partial z} e^{ikz} - k_i^2 E_i e^{ikz}, \]

\[ = \frac{\partial^2 E_i}{\partial z^2} e^{ikz} + 2i\gamma \frac{\partial E_i}{\partial z} e^{ikz} - k_i^2 E_i e^{ikz}. \]

Note that \( k_i^2 = \frac{\omega^2}{c^2} \varepsilon_i \).

Therefore, we can simplify equation (11) to

\[ \left( \frac{\partial^2 E_i}{\partial z^2} + 2i\gamma \frac{\partial E_i}{\partial z} \right) e^{ikz} = -\frac{3\varepsilon_0 \chi^{(3)}_{R} \omega^2_s}{\epsilon^2} |E_p|^2 E_i e^{ikz}, \]

which can be further reformulated to

\[ \frac{\partial^2 E_i}{\partial z^2} + 2i\gamma \frac{\partial E_i}{\partial z} = -\frac{3\varepsilon_0 \chi^{(3)}_{R} \omega^2_s}{\epsilon^2} |E_p|^2 E_i. \]

From here we see that the phase factors on the left and right-hand sides of the equation cancel each other. This indicates that SRS does not have a phase-matching requirement.

For a typical laser optic field for microscopy, \( \frac{\partial^2 E_i}{\partial z^2} \ll 2i\gamma \frac{\partial E_i}{\partial z} \), we can then simply this equation to

\[ \frac{\partial E_i}{\partial z} = \frac{3\varepsilon_0 \chi^{(3)}_{R} \omega^2_s}{2\epsilon^2} |E_p|^2 E_i = \frac{3\varepsilon_0 \chi^{(3)}_{R} \omega^2_s}{2\epsilon^2} \chi^{(3)}_{R} |E_p|^2 E_i. \]

If we let \( \alpha_s = \frac{3\varepsilon_0 \chi^{(3)}_{R} \omega^2_s}{2\epsilon^2} |E_p|^2 \), then

\[ \frac{dE_i}{dz} = \alpha_s E_i. \]
Appendix I

Solution of the equation $\frac{dE}{dt} = \alpha_s E_s$, has the form $E_t = E_{0t} e^{\alpha_s t}$. Here, $E_{0t}$ is the Stokes field intensity at time 0.

When $\alpha_s z \to 0$, we take the first term of Taylor expansion, and get $E_t \approx E_{0t}(1 + \alpha_s z)$.

$$I_s = \frac{c n_s c_0}{2} |E_s|^2 = \frac{c n_s c_0}{2} |E_{0t}(1 + \alpha_s z)|^2.$$  

Here, $\alpha_s = \frac{h c}{2 n_c \chi_R^{(3)} |E_p|^2}$ is a complex number. When $\alpha_s z \to 0$,

$$I_s = \frac{c n_s c_0}{2} |E_{0t}|^2 \left[ 1 + \text{Re}(\alpha_s z) + i \text{Im}(\alpha_s z) \right]^2$$

$$= \frac{c n_s c_0}{2} |E_{0t}|^2 \left[ (1 + \text{Re}(\alpha_s z))^2 + (\text{Im}(\alpha_s z))^2 \right]$$

$$= \frac{c n_s c_0}{2} |E_{0t}|^2 \left[ 1 + 2 \text{Re}(\alpha_s z) + (\text{Re}(\alpha_s z))^2 + (\text{Im}(\alpha_s z))^2 \right]$$

$$\approx \frac{c n_s c_0}{2} |E_{0t}|^2 \left[ 1 + 2 \text{Re}(\alpha_s z) \right].$$

If we use the expression of $\alpha_s$, we get

$$I_s \approx \frac{c n_s c_0}{2} |E_{0t}|^2 \left[ 1 + 2 \text{Re} \left( \frac{3 h c}{2 n_c \chi_R^{(3)} |E_p|^2} z \right) \right]$$

$$= I_{0t} \left[ 1 - \text{Im} \left( \frac{6 \pi}{n_s \lambda_s \chi_R^{(3)} |E_p|^2} z \right) \right] = I_{0t} \left[ 1 - \text{Im} \left( \frac{6 \pi}{n_s \lambda_s \chi_R^{(3)} |E_p|^2} z \right) \left( \frac{2 I_p}{c v_0 n_p} \right) \right].$$

The signal of SRS is measured as the Stokes signal change, then

$$\Delta I_s = I_s - I_{0t}$$

$$= I_{0t} \left[ 1 - \text{Im} \left( \frac{6 \pi}{n_s \lambda_s \chi_R^{(3)} |E_p|^2} z \right) \left( \frac{2 I_p}{c v_0 n_p} \right) \right] - I_{0t}$$

$$= - \frac{12 \pi}{c v_0 n_p n_s \lambda_s} \text{Im}(\chi_R^{(3)}) I_{0t} I_p \cdot z.$$

In most SRS microscopes, the SRS signal generation will not cause significant intensity change of input lasers, then $I_{0t} \approx I_s$. If we integrate signal from 0 to $l$, we then get

$$I_{SRS} = |\Delta I_s| = -b_{SRS} \text{Im}(\chi_R^{(3)}) I_{0t} I_p \cdot l.$$  

Here $b_{SRS}$ is a constant at certain wavelength. The imaginary part of $\chi_R^{(3)}$ is negative, and thus Stokes signal will increase after the SRS interaction. If we only consider the relative intensity,

$$I_{SRS} \propto -\text{Im}(\chi_R^{(3)}) I_{0t} I_p \cdot l.$$

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