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Chemically Specific Imaging Through Stimulated Raman Photoexcitation and Ultrasound Detection: Minireview

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Abstract

A powerful combination of chemically specific Raman excitation and deep tissue ultrasound imaging holds the promise to attain spatially resolved distribution of chemical compounds inside the scattering medium. In this report, an attempt is made to evaluate the recent achievements and possible challenges with an eye on potential clinical applications.

Introduction

An optical imaging modality that is capable of achieving noninvasive, deep-tissue, microscopic-resolution, and molecularly-specific imaging may revolutionize both biomedical research and clinical care. It has the potential to provide the fundamental understanding of the complexity, diversity, and progression of diseases in animal models in vivo and to provide the earliest possible diagnosis of diseases in patients.

The grand challenge of biomedical optical imaging is how to reach a large penetration depth while preserving the molecular specificity and spatial resolution.

The simplicity, availability, and non-invasiveness of optical methods are attractive features for fundamental physiological studies in small animals, disease diagnosis, image-guided surgery, and post-treatment recovery management in patients. Various optical techniques have been previously explored.

Optical scattering imaging is a powerful method to study precancerous tissues.^[1,2] It relies on the wavelength- and size-dependent Mie scattering from cells and organelles to quantify their size, which is considered a good measure of structural characterization of cells and organelles.^[3] Optical scattering imaging allows a fair assessment of the epithelium cells and can be further combined with polarization^[4] and fluorescence imaging.^[5] However, deep tissue imaging has yet not been reported and molecular specificity was not a primary target of this imaging technique.^[3]

Optical coherence tomography (OCT),^[6,7] has advanced considerably in recent years and currently allows high-resolution,^[8] real-time,^[9] structural tissue imaging in extended depth (~1 mm).^[10] Recently, several groups, most notably, Boppart's and Izatt's, attempted to enhance the chemical specificity of OCT by introducing special labels^[11,12] or by relying on naturally existing structural^[13] or molecular^[14,15] contrast mechanisms.

Fluorescence spectroscopy, which is considered to be one of the most powerful and the most sensitive spectroscopic tools for biological microscopy,^[16,17] allows specially engineered labels to be selectively attached to a specific molecule of interest. Fluorescence spectroscopy permits single-molecule sensitivity^[18] and nanoscopic resolution.^[19] It becomes the technique of choice if the photobleaching and phototoxicity of the labelling molecules can be tolerated. For tissue imaging (see, for example, an excellent review on the applications of fluorescence spectroscopy for cancer tissue imaging by Ramanujam et al.^[20] and instrumentation review article by Feld's group^[21]), the major endogenous contrast comes from NAD⁺ and NADH, which are shown to be strongly associated with cellular metabolism^[22] and have slightly different fluorescence spectra. However, imaging both of these molecules needs near-UV excitation that is strongly absorbed and scattered by tissue, which limits the imaging depth of fluorescence spectroscopy. Multiphoton excitation partially resolves this problem, but the chemical specificity, due to overlapping fluorescence spectra, still remains an issue.

Vibrational spectroscopy has always been considered the most chemically specific optical spectroscopy and has a long history of successful applications for biomedical imaging (see^[23] and references therein). Infrared spectroscopy, which relies on optical absorption in the mid-infrared spectral region, has a strong signal,^[24] but its penetration depth is limited only to the epitaxial layer of a tissue. In comparison, Raman spectroscopy allows much deeper penetration as the near-infrared light is used for the excitation of Raman transitions. By using either optical Kerr-gating^[25,26] or spatially off-set spectroscopy^[27] the information from deep (up to several millimetres) tissue can be attained; however, this information can hardly be used for imaging purposes, because it takes a long time (several minutes) to acquire a single Raman spectrum^[28] and it is also integrated over a fairly large volume (~1 mm³^[29]).

A promising alternative to Raman spectroscopy is nonlinear Raman spectroscopy, which is often referred to as coherent anti-Stokes Raman spectroscopy (CARS). By using near-infrared excitation and by tightly focussing light radiation inside a tissue, spatial distribution of specific molecules can be acquired in real time.^[30–32] However, deep tissue imaging of nonlinear Raman micro-spectroscopy can hardly be envisioned unless a microscopic objective is factually inserted into a tissue on the desired depth, as was shown by Cheng et al.^[33] This greatly diminishes the highly desired non-invasiveness. The most recent development on nonlinear Raman microscopy was reported by Xie's group,^[34] where stimulated Raman scattering (SRS) was used for microscopic imaging. While this newly proposed technique avoids some of the problems associated with CARS spectroscopy such as the need for phase-matching and the non-resonant background, SRS imaging is only possible up to the depth of 100 μm ,^[34] which is limited by the strong optical scattering in biological tissue.

The major weakness of pure high-resolution optical imaging is the limited ability of attaining information in deep tissue. Most of the existing optical microscopic technologies rely on the ballistic (unscattered) photons to provide their spatial resolutions. Once photons experience multiple scattering (elastic scattering), they lose the information of their origins and, thus, reduce the spatial resolution of imaging. Because biological tissue usually has strong optical (elastic) scattering, photons will be multiply scattered within a very short

distance. The imaging depth for existing high-resolution optical imaging is often limited to less than one optical transport-mean-free-path (~ 1 mm) in tissue.^[35] Alternatively, one can rely on nonlinear optical interactions to limit the signal's origin only to the focal volume of the beam. However, strong light scattering spreads the ultrashort (femtosecond) pulse both in spatial and temporal domains,^[36] reducing the spatial resolution and diminishing the signal.

To solve this problem, photoacoustic (PA) imaging has been developed. The underlying physics of the PA effect has been known for over 100 years since Bell's famous experiment: the incident light irradiation excites molecules through linear optical absorption; absorbed energy is transferred into heat; and a temperature rise promotes a local tissue expansion, which generates ultrasonic waves that can be detected by transducers set outside. Since the ultrasonic scattering is two orders of magnitude weaker than that of optical scattering, the information about the source of the acoustic wave can be easily attained in deep tissue.^[37,38] The depth of PA imaging is ultimately limited by the penetration depth of diffuse photons and can be as large as several centimetres^[39,40] for a proper optical excitation spectral range. Spatial resolution of photoacoustic imaging in scattering medium is primarily determined by the wavelength of detected ultrasound waves.^[41] The imaging contrast comes from linear optical energy deposition and concentrations of endogenous pigments such as melanin and haemoglobin can be exploited. There are ongoing efforts to develop a class of functionalized optical absorbing agents such as gold nanoshells,^[42] carbon nanotubes,^[43] and polymer-based nanoprobe^[44] to achieve certain molecular specificity in PA imaging, but those attempts drastically diminish the noninvasive nature of this modality.

To address the issue with a chemically specific contrast, we have proposed and demonstrated photoacoustic imaging based on stimulated Raman photoexcitation.^[45–47] Recently, the same approach has been extended for non-invasive cell cytometry.^[49]

An alternative approach is based on using molecular overtone absorption to excite the photoacoustic signal.^[50] This approach has shown the potential of detecting lipid-rich tissues^[51]; however, broad spectral features of overtone absorption and overlapping bands, make it difficult to attain the chemically-specific information unless some prior information is available.

In this report, we will provide an assessment of this new spectroscopic imaging modality, overview recent experimental results, critically evaluate the forthcoming challenges, and outline the possible applications.

Methods and Approach

Ultrasound Detection of Stimulated Raman Scattering

The proposed stimulated Raman photoacoustics (SRPA) combines the deep penetrating capability of PA imaging with the molecular specificity of nonlinear Raman spectroscopy to achieve label-free molecular imaging in deep tissue. Two laser beams of different frequencies, the pump (ω_p) and the Stokes (ω_s) (Fig. 1), are employed to irradiate a tissue. When the wavelength difference of those lasers is tuned into resonance with the vibrational frequency (Ω) of a specific molecule, stimulated Raman scattering occurs and the energy of the pump wave is efficiently converted into the Stokes wave and molecular vibrations.^[46] When the excited molecule relaxes to its ground vibrational state, it releases heat. If the laser irradiation is limited to a short period of time (picosecond (10^{-12} s) to nanosecond (10^{-9} s)), the released heat leads to a local thermoelastic expansion and induces PA waves in the same manner as it is described above. Compared with the PA signal that is generated from linear optical absorption, the vibrational-absorption-based (stimulated Raman) PA signal carries

information only from the pre-selected molecules, and its amplitude is anticipated to be directly related to the local molecular concentration (within a resolvable volume). SRPA detects such molecularly-specific PA signals to achieve molecular imaging in deep tissue.

The SRPA method, as a spectroscopic method, was first proposed by Nechaev and Ponomarev 30 years ago.^[52] Over the years, this technique was used for gas diagnostics and has demonstrated sound success, showing a good signal-to-noise ratio (SNR), selectivity, and accuracy. However, this technique, despite its promotion in the late 1970s by Bell's Laboratory group,^[53] did not get enough attention. Being limited to gas phase diagnostics^[54,55] while demonstrating the sensitivity and the SNR comparable or better than nonlinear Raman spectroscopies,^[56] it has not been used for detecting molecules in a condensed matter and for imaging.

The first and the most important question is the signal strength. Previously, we have done estimations of the local heating owing to stimulated Raman excitation; however, we believe it is important to compare those measurements with optically detected stimulated Raman scattering. The first thing we notice is that the energy efficiency of SRPA is rather small, for every two energetic photons used (ω_p and ω_s), only a small portion of energy, $\hbar\Omega$, is going into photoelastic deformation. The second problem is associated with the ultrasound detection itself. When both the optical and photoacoustic signals are monitored simultaneously, the sensitivity of photoacoustic detection is somewhat similar to the one of a joulemeter, i.e. of the order of nanojoules.^[47] This should be compared with single-photon counting devices, such as photomultiplier tubes and avalanche photodiodes, which can routinely attain attojoule (10^{-18} J) sensitivity when used for optical detection. Clearly, there is a nine-orders-of-magnitude gap of sensitivity, which needs to be bridged for comparable detection. In a particular example of a commercially available ultrasound transducer (needle hydrophone HNC1500 and AH-2010-20-025, Onda, Inc.),^[47] its typical sensitivity is 10 kPa and the bandwidth 10 MHz.

One way to improve the sensitivity of PA detection is to match the bandwidth of the ultrasound wave to the one of the transducer. To achieve high-efficiency stimulated Raman photoexcitation, one has to employ picoseconds laser pulses, which generate very high-frequency ultrasound. While hyper-sound (GHz and higher) waves do not reach the detector owing to their significant absorption by the tissue, detectors with the broadband frequency response extending beyond 50MHz would greatly benefit the detection of those signals.^[57] While GHz ultrasound detection is possible,^[58] those transducers are based on a resonator, which also serves as a filter for a selective band of ultrasound frequencies.^[59] Alternatively, one can improve the detection system by using optical spectroscopy based on ultrasensitive detection by means of microring resonators,^[60] interferometers,^[61] photonic crystal structures^[62] or surface plasmon resonance sensors.^[63] With the current state-of-the-art technology, reaching 30-Pa detection level^[60] using broadband ultrasound detection, it is a three-order of magnitude sensitivity improvement with respect to our previous measurements.

Increasing the SRPA signal is, generally, difficult: the potential tissue damage imposes the upper limit for high intensity laser radiation that is safe to use for imaging.^[64-66] However, the amplitude of a photoacoustic wave can still be increased by combining the excitation power of multiple picoseconds pulses, where each of the pulses is satisfying the safety standards. If all of those pulses arrive into the focal volume before the acoustic wave has time to travel outside this volume (for a typical size of the focal volume of, 15 μm and the speed of sound $1.5 \times 10^3 \text{ m s}^{-1}$, this transit time is 10 ns), then the amplitude of the PA wave will be amplified by the number of pulses used in the sequence.^[67,68] Within a temporal window of 10 ns, one can squeeze as many as 1000 picosecond laser pulses,

increasing the amplitude of the PA wave by three orders of magnitude without increasing the intensity of the laser beam.

The above mentioned techniques for improving the sensitivity of the PA detection can be combined together for the overall six orders of magnitude increase of the overall signal. This is still three orders of magnitude away from the ideal single-photon detection sensitivity, but the signal strength should be strong enough for real time photoacoustic imaging, since only limited scanning is necessary for 3D photoacoustic imaging.^[69] Provided such improvements, we anticipate that for clear medium a 6-orders-of-magnitude sensitivity enhancement can be achieved making possible detection of micromolar concentrations.

Laser System for SRPA Imaging

The complexity of stimulated Raman scattering imaging comes from the necessity of having two synchronized light sources exciting a vibrational transition of a given molecule. Traditionally, it is achieved by having an optical parametric or dye amplifier, which allows tunability of one beam with respect to another. However, such an approach is often associated with a difficulty in controlling the bandwidth of the amplified pulse and in adjusting the wavelength difference between two beams to match the vibrational frequency of the desired molecular transition. To deal with this problem, we note that the most important applications of stimulated Raman photoacoustic imaging, most likely, will be based on utilization of strong Raman transitions, such as CH₂ vibrations in lipids (at around 2850 cm⁻¹) and phosphate (PO₄) vibration in bones and microcalcifications (at around 960 cm⁻¹). To attain those frequencies, one can utilize stimulated Raman process in a medium having a vibrational frequency that is the same or close to the frequency of the desired molecular transition.^[70] For example, to achieve selective excitation of lipids, one can use mineral oil, a widely available substance, whose high-frequency vibrational spectrum provides a good match for a typical lipid. Phosphate vibration can be matched fairly well with a salt solution (see, for example, Fig. 2, where stimulated Raman scattering in sodium acetate and magnesium sulfate is compared with the spontaneous Raman spectrum of a mineralized tissue), which can be prepared at a very high concentration. With a wide spread of high peak power picoseconds laser sources, affordable solutions in the form of compact and energetic diode pumped lasers (see, for example, Passat Ltd) become available, paving the road for inexpensive SRPA imaging devices.

The Effect of Light Scattering

Light scattering negatively affects the SRPA signal in several ways. First, light scattering reduces the intensity of light at the high depth of a scattering tissue. While the use of longer excitation wavelengths allows chemically specific imaging at the depth of 1–2 mm,^[71–73] the further penetration of light into tissue is associated with great reduction of the delivered energy. Figure 3 illustrates this concept, where the light transmission is plotted as a function of wavelength for different parts of the body. The vertical scale shows the absolute value of the light transmission which comes to a multimode fiber (0.5 mm in diameter) and clearly indicates the magnitude of the problem. Considering the nonlinear dependence of SRPA signal, the problem of getting enough light to the sample might become even worse.

There are two possible solutions to the problem: one is to greatly improve the sensitivity of the ultrasound detection and the other is to provide a large area illumination of the body, thus, effectively increasing the signal. However, the major complication is coming from the increasing contribution of the nonspecific background. To illustrate the point, we compare the PA signals from the same solution of chloroform^[46] with two beams (pump and Stokes) simultaneously irradiating the target and with one beam being blocked (see Fig. 4). In the first case, the signal is much stronger, but, if the incident intensity goes down and the

interaction path through a surrounding medium, which provides the origin for the non-specific absorption background, increases due to multiple scattering, the non-specific PA signal due to linear absorption in tissue will dominate the signal.

To deal with this background, we used the fact that SRPA signal originates from the simultaneous excitation by the pump and Stokes pulses, i.e. when they are separated in time through a physical time delay, the SRPA signal vanishes.^[46,48] By using the PA collected at large time delays as background signal, which can be removed from the PA signal collected at the optimal time delay for SRPA excitation either through lock-in detection or digital subtraction, the substantial reduction of the background signal was achieved^[46,48] leading to, essentially, background-free SRPA detection.

Applications

The niche application for SRPA imaging is detection and characterization of chemical species, which are present in relatively large quantities deep in the tissue. The overall signal is proportional to the local concentration and the Raman cross-section of a selected molecule. There are many examples of such systems, such as lipids and lipid droplets,^[74] which owing to their hydrophobic interactions are naturally assembled together, and whose Raman C–H stretching band is particularly strong,^[75] cholesterol^[76] and protein^[77] crystals, microcalcifications^[78] and bones.^[79] In all the above examples, medically relevant chemical compounds can be identified by the appearance of strong and narrow Raman lines.

Conclusion

Photoacoustic imaging based on stimulated Raman excitation contrast is a novel optical imaging modality, which holds the promise of chemically specific non-invasive imaging in deep tissue. With the fast advancement of ultrasfast optical tools and ultrasound detection technology, it should be possible to visualize the distribution of different chemical compounds in tissues. Thus, SRPA can become an invaluable non-invasive imaging tool for fundamental physiological studies in small animals, disease diagnosis, image-guided surgery, and post-treatment recovery management in patients.

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Fig. 1. On the left: schematic diagram of stimulated Raman photoacoustic imaging. On the right: molecular energy diagram illustrating the process of energy transfer via stimulated Raman scattering.

Fig. 2. Stimulated Raman signal in sodium acetate and magnesium sulfate as compared with the Raman spectrum of bovine bone (Bone, day 1) exposed to acetic solution for 3 days (Bone, day 4).

Fig. 3. Tissue transmission in the near-IR spectral region. Signal is detected through a 0.5-mm diameter fibre; the approximate thickness of the finger is 1.2 cm, hand – 2.6 cm, and wrist – 4.3 cm.

Fig. 4. Photoacoustic signal due to stimulated Raman excitation (circles; line shows a quadratic power dependence) and to linear absorption (triangles; line shows a linear power dependence) in chloroform.