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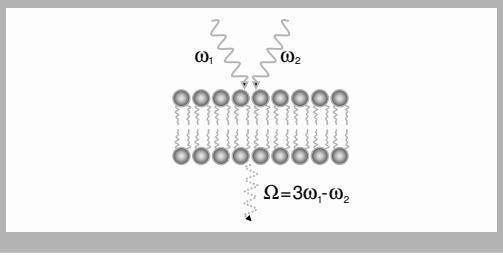
Abstract: We propose a novel nonlinear optical spectroscopic technique H-CARS (Hyper Coherent Anti-Stokes Raman Spectroscopy) to allow imaging of biological structures in real time.

Schematic diagram illustrating spectroscopic imaging of cellular membranes using H-CARS microscopy



Nonlinear optical microscopy of cellular structures

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1. Introduction

Non-invasive microscopic imaging of biological systems remains a key problem in understanding the relationship between structure and function on the cellular and molecular levels. One of the most sophisticated structures, which is essential for life, is the plasma membrane. It enables a homeostatic or dynamic relationship between the cell and its environment. While the fluid mosaic model of membrane organization is widely accepted, new methods of studying membrane function, particularly in normal living cells would be extremely valuable [1].

A big problem in understanding the functional properties of membranes is the lack of tools to study them *in vivo*. Optical methods can be a noninvasive and relatively simple way to obtain structural information. However, cellular membranes, are very thin structures (on the order of 10 nm) and are hard to image using conventional optical spectroscopic techniques. A typical spot size of a focused beam is $\sim$0.5 μm in diameter, and background from neighboring structures is strong enough to wash out all the features associated with the membrane itself. The small changes that accompany normal membrane function, cannot be monitored by many methods. Recently near field optical microscopy has been used because it approaches the lateral resolution required for membrane imaging [2–3], but the necessity of positioning the probe within 100 nm of the membrane to obtain sufficient resolution, limits the application of this technique to single-cell measurements.

Raman spectroscopy can play an important role in chemical and functional analysis of complex structures [4–7]. The advantages of Raman spectroscopy are that it is a remote, non-invasive technique, which uses "internal" molecular markers, i.e. the frequency of molecular vibrations to provide information on molecular structure and its modification. The disadvantages of Raman imaging are in its extremely low signal level (on the order of 10^{-11}) and fluorescence background, which masks the weak signal. A possible nonlinear optical alternative, Coherent anti-Stokes Raman Scattering (CARS) solves the problem of signal level and fluorescence background (it is blue-shifted with respect to the excitation wavelength), but creates another problem, non-resonant background [8], which is a signal coming from **everything** in the focal volume of the laser beam. Given the relatively low concentration of molecules in the entire excitation volume, the problem can only be solved in very specific cases [8–12].

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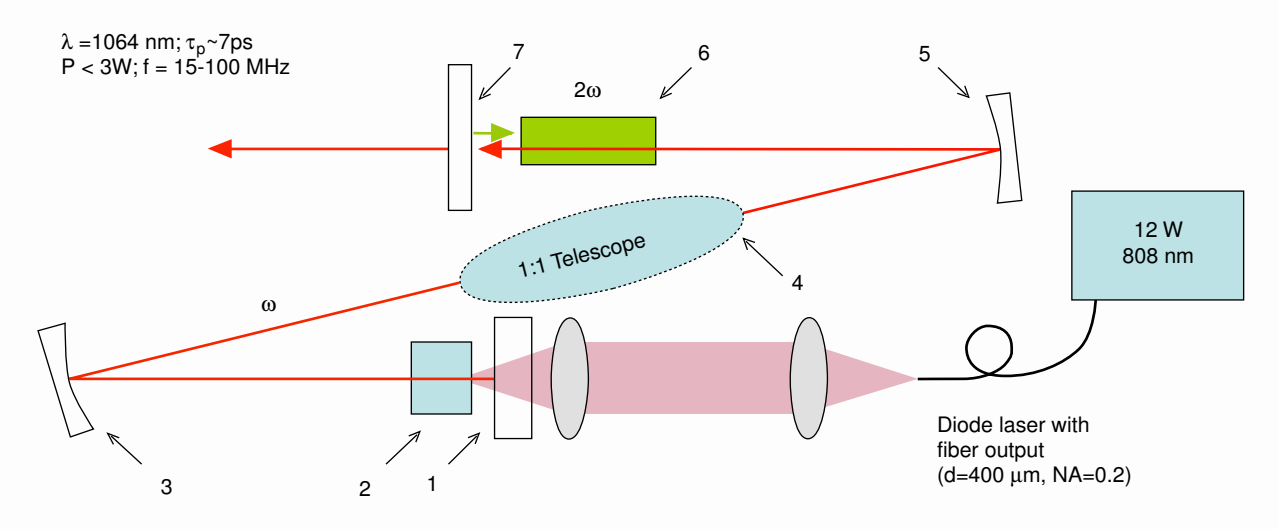

Figure 1 Laser set-up for H-CARS microscopy. 1 – front mirror (HR@1064 nm, HT@808 nm; 2 – 5-mm c-axis Nd:YVO_4 laser crystal; 3 – 50 cm concave mirro HR@1064 nm, 4 – telescopic optics, 5 – 15 cm cc HR@1064 nm, 6 – 12-mm long LBO (Type I for second harmonic generation of 1064 nm), 7 – HR@532 nm, R=80% @1064 nm

The molecular structure of membranes makes it is very hard to design markers that can be used to monitor function. Dye molecules used for fluorescence marking are usually rather large, compared to membrane thickness. Since they are attached to the membrane, the dye molecules can modify membrane function and could potentially stimulate photo-induced modification, bleaching and photo-toxicity. There are still some areas, where appropriate use of dyes may provide significant and important information about the dynamics of membranes and their properties [13–14], however additional methods involving direct measurement of membrane activity would be very helpful.

There is a definite need to develop an optical technique, which can be as informative and non-invasive as Raman spectroscopy, but selectively images the membrane. In this manuscript, we outline the basic idea of truly background-free nonlinear Raman imaging of cellular membranes and describe a prototype instrument capable of non-invasive microscopic imaging of membrane structures under physiological conditions.

2. Theoretical background

Membranes are on the order of 10 nm thick. A focused laser beam has a transverse diameter of about 500 nm, or 50 times greater than the thickness of a membrane. Employing Raman spectroscopy to get information from a single membrane means that the resultant spectrum is buried in background signals coming from an area 125,000 times larger than the membrane. It is rather difficult, if not impossible to get useful information in such experimental conditions. There have been attempts to use nonlinear Raman spectroscopy in the form of Coherent Anti-Stokes Raman Scattering (CARS) to increase the signal, improve spatial resolution and provide discrimination against fluorescence signals (CARS signal is blue-shifted with respect to the excitation wavelength, while luminescence is red-shifted) [15, 16]. However, CARS microscopy, despite receiving significant attention in the last year, suffers from a significant drawback; namely non-resonant background.

In this research, we propose a completely new optical spectroscopic technique, which solves the problems of nonlinear Raman spectroscopy and provides additional discrimination against background signals. We call this technique Hyper-CARS, or H-CARS. In H-CARS microscopy we will detect signal at the frequency

$$\omega_{signal} = \omega_1 + \omega_1 + (\omega_1 - \omega_2) = 3\omega_1 - \omega_2, \qquad (1)$$

where ω_1 and ω_2 are the excitation frequencies, with $\omega_1 > \omega_2$. This is a fourth- order nonlinear optical process, allowed, as any other **even** process, only in media without inversion symmetry. This fact has recently been exploited in nonlinear optical microscopy of membranes using another even order process, second harmonic generation [17–19]. A unique membrane structure gives rise to a coherent second-harmonic signal, which than can be measured and used for characterization of the membrane potential [18, 19]. The difference in our approach is that we not only try to locate membranes, but we also try to get information (a vibrational spectrum) about their properties. When $(\omega_1 - \omega_2)$ is in resonance with the molecular vibration, there is a dramatic increase in the detected signal.

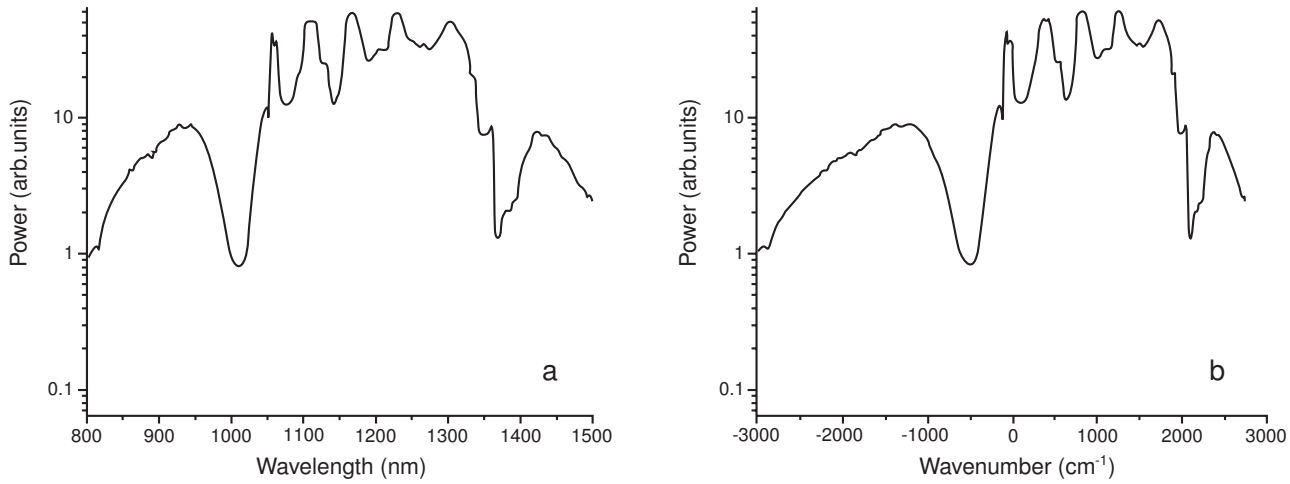

Figure 2 Output spectrum ((a) – as a function of wavelength, (b) – as a function of wavenumber shift with respect to 1064 nm) from a Ge-doped fiber. The total output power is greater than 500 mW

By scanning ω_2 with respect to another frequency (ω_1) we can get the whole vibrational spectrum of molecules composing the cellular membrane under study. In a single measurement we can obtain discrimination from the unordered membrane surroundings and molecular specificity.

3. Practical implementation

3.1. Design considerations

We are trying to study a highly nonlinear optical process. Ultrashort (pico- or femto-second) laser pulses have to be employed to boost the signal level to readily detectable levels. This is also true in all other optical microscopic techniques such as multiphoton fluorescence microscopy, second- and third- harmonic generation microscopy, and CARS microscopy because all rely on nonlinear optical interactions. Over the last decade, the technology for generating ultrashort laser pulses has blossomed. In our approach, we use state of the art techniques to achieve reliable and robust generation of picosecond pulses of two independently tunable frequencies (ω_1 and ω_2).

Raman lines are typically on the order of several wavenumbers. In order to achieve this rather narrow range of spectral resolution it is not necessary to use pulses shorter than several picoseconds. The 1-ps pulse corresponds to approximately 15 cm^{-1} in the spectral domain.

The useful spectral region for ω_1 and ω_2 is limited to the low absorption, low scattering region of a tissue, from 0.8 μm to 1.4 μm [20]. At wavelength longer than 1 μm the probability of damage to living cells is significantly reduced [21–24] due to the higher number of photons required for multiphoton absorption by a molecule.

To get the full Raman spectrum one has to scan ω_2 in a wide range of frequencies (from 900 to 3300 cm^{-1}). It means that the time required to perform these experiments will be dramatically increased if someone wants to record the full spectrum. However, if a broad spectrum is used as a second pump beam, "broad-band" detection is realized in the case of CARS imaging [25]. A spectrometer with an attached CCD camera can simultaneously detect the whole spectrum, thus shortening the acquisition time. Most silicon-based CCD cameras are blind to wavelengths $> 1\mu$m. If both ω_1 and ω_2 are longer than 1 μm, data acquisition also benefits.

3.2. Laser system

From the considerations above, we conclude that a picosecond high repetition laser source is needed, operating at wavelengths $> 1\mu$m, and a temporally synchronized source of broadband radiation.

Recently we have constructed a diode-pumped picosecond Nd:YVO_4 laser that can generate a synchronized broad-band continuum in a specially designed optical fiber [26–27].

We use a 12-W fiber-coupled diode laser (λ = 808 nm (LIMO, Ltd.)) to pump a 5 mm long Nd:YVO_4 crystal to generate 1064 nm radiation. To initiate mode-locking we use a "nonlinear-optical-mirror" self-starting mechanism [28, 29]. Our experimental set-up is sketched in Figure 1. The 12-mm-long LBO crystal is used as a nonlinear optical medium. The second harmonic generated in this crystal on the first pass is then reflected from the back mirror (HR @ 532nm, R=80% at 1064 nm). On the way back the second-harmonic wave can be back converted to a fundamental frequency (under certain conditions) providing positive feedback to initiate mode locking. We use an intracavity telescope to extend the length of the laser cavity. It results

in lowering the repetition rate of the laser pulses and increased energy per pulse promoting nonlinear interactions, and providing better overall stability. Pulses as short as 7-ps are routinely generated. Since the pulse duration depends only on the nonlinear crystal length, no pulse-length fluctuations are allowed in the system leading to a very stable train of pulses at a repetition rate of 15-100 MHz with a total power of up to 3 W. To get a broadband spectrum we use a highly Ge-doped single-mode high-numerical-aperture fiber. We launch approximately 1.5 W (or ∼40 nJ per pulse and 33 MHz repetition rate) into the fiber and observe a broadband continuum output that starts at 800 nm and goes above 1500 nm (our present spectrometer- detector system does not go beyond 1500 nm). It corresponds to spectral shifts with respect to λ_1 = 1064 nm above 2700 cm^{-1}. This spectrum is shown in Figure 2. It is a result of the simultaneous action of high-order Raman processes and self-phase-modulation [26, 28]. Given the losses in the fiber we achieve as much as 500 mW of broad-band spectrum. By using an interference filter we can now select any particular spectral region up to 2000 cm^{-1} in width. Radiation at λ_1 = 1064 nm, and the broadband radiation at λ_2 = 1100-1500 nm are temporally synchronized and provide sufficient energy for microscopic H-CARS imaging.

3.3. Limiting factors in nonlinear Raman imaging

Since proposed H-CARS microscopy is a highly nonlinear technique, it benefits from using intense laser pulses. The resultant signal is proportional to the fourth power of the incident intensity. It is tempting to increase the incident intensity to obtain a higher signal level. However, the laser-induced damage to living cells is always a concern. There are several mechanisms of laser-induced damage. One is pure linear absorption [29], which is minimized by using an excitation wavelength in the low-absorbing region of the spectrum. Multiphoton absorption is also important [21–24], but at wavelengths $> 1\mu$m, this mechanism takes over only at light intensities $> 10^{12}$ W/cm^2, so we can safely focus all our available energy with a high-numerical aperture microscope, and still not stimulate multiphoton absorption. Our group has recently discovered one more mechanism for laser-induced damage. It is based on a combined effect of high peak intensity and high average power of the incident radiation. High intensity radiation results in multiphoton absorption in tissue (in our case [30] it was two-photon absorption in pigmented tissue), which is highly localized in space. If the repetition rate of laser pulses is high enough, so that the absorbed heat does not have time to diffuse, local, thermally induced damage will occur in the focal volume. However, reducing the repetition rate of the incident laser pulses by simply replacing the telescope optics, and fast scanning the excitation volume, we should be able to completely avoid this type of damage.

To estimate the H-CARS signal's level we assume the pulse duration of the incident waves (ω_1 and ω_2) to be τ = *6 ps*, energies of the first (ω_1) and second (ω_2) waves – 60 and 10 nJ, respectively, the laser spot diameter – *d = 1.4 μm*, the membrane's thickness – *t =10 nm*, and the non-resonant fourth-order nonlinear susceptibility $\chi^{(4)}$ = 10^{-21} esu [31]. Simple estimations show $6{\cdot}10^{-16}$ conversion efficiency into the signal wave, or, for a given repetition rate of 20 MHz, approximately 10^4 photons per second, which can be easily detected using a photon counting photomultiplier with a typical noise of about 10-20 counts/second. It is also important that we have not considered any resonant (due to Raman resonances or electronic transitions) enhancement of the signal, i.e. we obtain the lowest estimation for the detectable signal.

3.4. "Roadmap" of nonlinear Raman microscopic imaging

3.4.1. H-CARS spectroscopy of collagen

In order to demonstrate that H-CARS can work on a biological structure, we will first try it using collagen, a simple protein that is common in most tissues. The rationale for choosing collagen is that it forms a well-ordered structure [32–33]. The monomers form a triple helix that assembles into collagen fibrils once they are secreted outside the cell. These fibrils can then assemble into large collagen fibers [34]. Second harmonic microscopy is routinely used to study collagen in living tissues [32–33]. Second harmonic signal is rather strong both in the oriented thin films of collagen prepared on a glass substrate [35] and in natural collagen inside living tissues. While collagen has a relatively weak absorption around 620 nm, it is almost transparent to the second harmonic of 1.064 μm radiation of our laser and to the blue shifted H-CARS signal. The results we obtain from these measurements will serve us as a guide to scale the signal amplitude to more complex oriented cellular structures, such as membranes, and will help to make improvements to our laser and detection system.

3.4.2. H-CARS microscopy of membrane fusion

The cell we propose to study is the large egg or oocyte of the frog *Xenopus laevis.* A mature oocyte that is ready to be fertilized is between 0.5 and 1.0 mm in diameter. At this stage, the egg is arrested, requiring sperm contact to trigger the fertilization reaction and subsequent development. The ultrastructure of the egg shows that the membrane is thrown into small bumps called microvilli [35]. Around the outside of the egg is a protective layer of proteoglycans called the vitelline envelope (Fig. 3). Just beneath the plasma membrane in the cell cytoplasm are many small, membrane-bound vesicles or cortical granules. The egg remains in this state until sperm contacts it. Sperm causes an

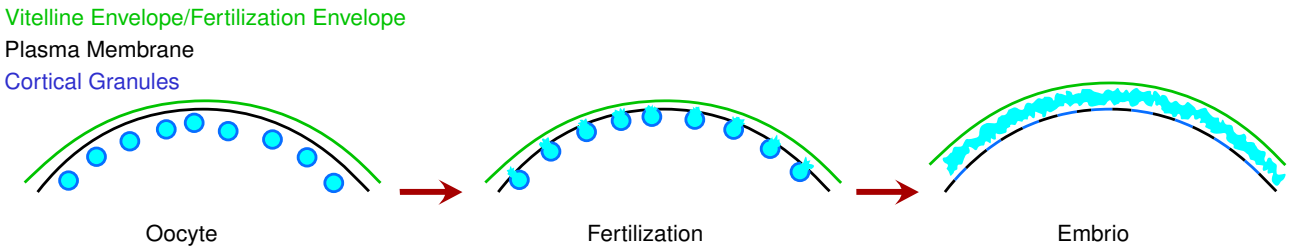

Figure 3 Cartoon showing the membrane fusion events that occur during fertilization. During fusion the cortical granule contents are released and their membranes become incorporated into the plasma membrane

increase in the cytoplasmic concentration of calcium [36], which stimulates the fusion of cortical granules with the plasma membrane and the release of their contents into the space between the plasma membrane and the vitelline envelope [37]. The massive fusion of thousands of cortical granules occurs within a minute of sperm contact.

Newly expressed oocytes can either be fixed or maintained in saline for live imaging with H-CARS. In some preparations the vitelline envelope will be removed before fixation or live imaging. In both cases, fixation will consist of a standard mixture of paraformaldehyde and glutaraldehyde to optimally preserve membrane structure [38]. Because fixation chemically alters the membrane, we expect to detect slight changes between the H-CARS spectra of living and fixed cells. However, cells after fertilization should be quite different from those before fertilization because of the large amount of cortical granule membrane that has been incorporated into the cell membrane. We propose to use fixed oocytes and embryos in our preliminary experiments to test the ability of H-CARS to detect membrane. Thus, experiments will provide us with information on the type of signal we can expect before and after the massive membrane fusion event. The experiments with living embryos will tell us what kinds of signals to expect from living tissue and allow us to optimize the system to test the spatial and temporal sensitivity of H-CARS.

A test of the spatial and temporal response of H-CARS involves live imaging while the cortical granules fuse with the membrane. Cortical granule fusion can be stimulated by sperm contact, but the large numbers of sperm present during imaging could distort the spectrum. Alternatively, the fusion event can be triggered by addition of a calcium ionophore like A23187 [39]. This bacterial protein inserts itself into membranes and selectively allows calcium to enter the cell. Once inside, calcium triggers fusion of the cortical granules with the plasma membrane. The rapid exocytosis of vesicles proceeds in a wave across the egg that takes less than a minute and provides an opportunity for H-CARS to directly measure molecular changes in the membrane that occur continually during this period of time. Unlike measurement of calcium levels or the membrane destaining of FM dyes, which measure events that are linked to exocytosis, H-CARS provides the possibility of measuring them directly.

4. Conclusion

In summary, a novel spectroscopic approach to study cellular membranes *in vivo* is proposed. Simple and relatively inexpensive laser instrumentation is proposed and constructed. The roadmap of practical implementation of proposed technique to real-time microscopic imaging of cellular processes is outlined.

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