

Zurich
Instruments

High-Speed Nonlinear Optical Pump-Probe Microscopy

Application Note

Applications: Coherent Raman Microscopy, CARS, SRS, Absorption Microscopy

Product: HF2LI

Release date: January 2012

Summary

This application note gives an overview of lock-in techniques currently used in coherent Raman microscopy and nonlinear absorption microscopy. All of these enhance the detection sensitivity by using MHz frequency modulation schemes in order to reject low-frequency laser noise and background contributions. For such applications, the HF2LI is a particularly well suited instrument, as it is the only digital lock-in amplifier capable of measurement at 50 MHz. The fastest demodulation filter time-constant is 780 ns and auxiliary outputs operate at a rate of 1 MS/s with an analog bandwidth of 200 kHz. These features make the HF2LI the fastest and most accurate commercially available lock-in amplifier in terms of transient signal detection speed and thus suitable for sensitive high-speed image acquisition using fast frame rates. The practical implementation of the HF2LI in currently used microscopes is described based on published results.

Introduction

In modern microscopy, nonlinear optical pump-probe microscopy emerges as a new field for 3D high resolution imaging. The imaging contrast is based on characteristic spectroscopic properties of certain molecules, i.e. their specific internal energy level structure. This spectroscopic fingerprint is selectively probed by nonlinear light-matter interactions, which yield an image showing

the spatial distribution of the targeted molecule. Depending on the technique, the stimulated energy transitions can be vibrational resonances of molecular bonds in the infrared range, or molecular electronic transitions in the visible range. A typical image with 512 by 512 pixels spans an area of 100 by 100 μm . For high resolution 3D images, consecutive images at distinct focal planes with a typical vertical spacing of 1 μm are recorded.

In comparison with other nonlinear optical imaging techniques, such as fluorescence and incoherent Raman microscopy for example, stimulation and readout of the nonlinear interactions is realized by at least two pulses of well defined properties: a pump and a probe pulse. One advantage of this stimulated readout is that incoherent processes such as spontaneous emission are largely suppressed. A combination of high excitation laser power and sensitive detection thus results in an efficient overall yield of the nonlinear interaction, which then allows for fast image acquisition times. Furthermore, photo damage on the sample caused by the high powered laser is reduced due to the short laser exposure time. In comparison to fluorescence imaging, no particular marker molecules are required in sample preparation.

One important objective in the current research is the reduction of the image acquisition time, which is usually directly related to detection sensitivity. Today's scanning microscope technology already allows for acquisition times at up to 30 frames per second for a 512 by 512 pixel image [1]. On the one hand, such acquisition times allow



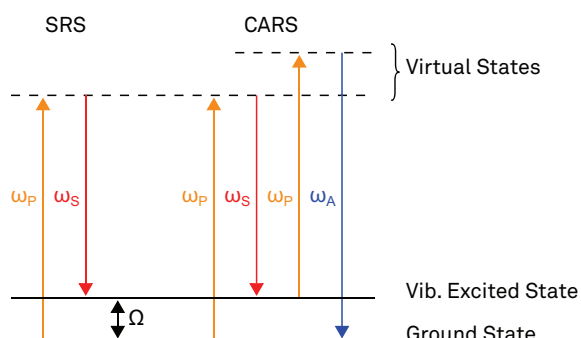


Figure 1. Nonlinear SRS/CARS energy scheme. ω_p : pump pulse, ω_s : Stokes pulse, ω_A : Anti-Stokes pulse, Ω : vibrational resonance frequency.

for fast 3D imaging, on the other hand, 2D images can be recorded at video-rates to study dynamics in complex biosystems on a microscopic scale. With capabilities for 3D imaging within tissues, nonlinear optical pump-probe microscopy is a very promising technique for non-invasive imaging in biomedicine, with the potential to enrich the currently applied fluorescence and magnetic resonance imaging techniques with complementary data.

Table 1. Example wavelengths as used in [2] for C-D and C-H bond vibration

Vibration	C-H bond	C-D bond
Ω	2875 cm^{-1}	2100 cm^{-1}
λ_p	830 nm	830 nm
λ_s	1090 nm	1005 nm
λ_A	670 nm	707 nm

Coherent Raman Microscopy

Coherent Raman microscopy utilizes the vibrational fingerprint for molecule-selective imaging. The two techniques discussed are coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS). An illustration of the corresponding multi-photon process for SRS and CARS is depicted in Figure 1. In both techniques, molecule vibration is coherently excited by simultaneously applying near-infrared Stokes and visible pump pulses with a resonant frequency difference Ω . In order to trigger these multi-photon processes, the Stokes and pump pulses must be synchronized in time and obey a well defined phase relationship.

In SRS, the vibrational excited states are probed by measuring the energy exchange between the laser field and the molecule due to the nonlinear interaction. This exchange manifests in an intensity loss in the probe pulse (stimulated Raman loss) and an intensity gain in the Stokes pulse (stimulated Raman gain). For the measurement, the nonlinear interaction is continuously switched on and off by intensity modulation of the Stokes pulses. The stimulated Raman loss on the pump pulses

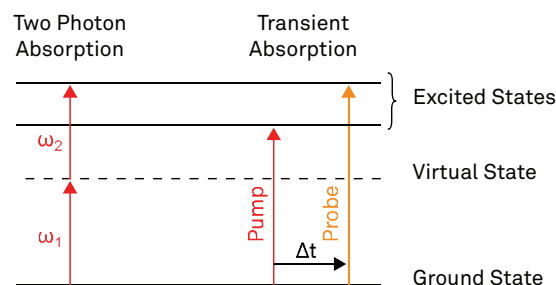


Figure 2. Examples for nonlinear absorption processes.

therefore appears modulated and can be measured with a lock-in amplifier. This detection scheme, known as modulation transfer makes fast imaging possible, an example being the video-rate in-vivo SRS imaging demonstrated by Saar et al [3]. Another possible implementation of modulation transfer in SRS microscopy is to modulate the pump pulse and detect the stimulated Raman gain on the Stokes pulse.

In CARS, the excited vibrational resonances are probed by observing the stimulated anti-Stokes emission. However, a weak background contribution to the anti-Stokes emission, which is not related to any vibrational resonances, limits the sensitivity of direct detection of the anti-Stokes emission. In the review by Day et al [4], two different lock-in schemes for background free CARS imaging with high-sensitivity are mentioned.

The first lock-in technique is frequency modulated CARS (FM-CARS) and implemented for the first time by Ganimanov et al [5]. The vibrational resonance frequency Ω is modulated by frequency modulation of the pump pulses. The demodulated anti-Stokes signal then represents the frequency derivative of the anti-Stokes emission at the excitation frequency. Accordingly, the background contribution is rejected due to its flat frequency response.

The second lock-in technique is interferometric CARS, as introduced by Potma et al [6]. Phase-modulated local oscillator pulses at the anti-Stokes frequency interfere with the anti-Stokes emission of the imaging specimen. Due to the quadrature phase relation between the background and the resonant anti-Stokes contribution, a proper adjustment of the local oscillators optical phase results in a background-free vibrational absorption signal. Based on recent experiments by Jurna et al [7], the phase of the vibrational response is fully resolved for chemical selectivity amongst overlapping resonances from different molecular bonds.

Nonlinear Absorption Microscopy

In nonlinear absorption microscopy the imaging contrast is based on characteristic molecular electronic transitions. The modulation transfer technique is used to

detect the nonlinear interaction by the energy exchange between the molecule and the laser field. A variety of nonlinear absorption examples for imaging can be found in a recent review of Min et al [8]. For two of these, the absorption process is shown in Figure 2.

In Figure 2a, two photons are absorbed by passing a virtual state, provided that the sum of their frequency matches an excited state. This process is thus known as two photon absorption (TPA). The excitation pulses must be synchronized in time and obey a well defined phase relationship. TPA can also be detected if the two photons have the same wavelength, provided that both interacting photons are intensity modulated. In this case, the nonlinear absorption process results in a response at twice the modulation frequency, described by Ye et al [9].

Figure 2b shows one possible process arising in transient absorption (TA) imaging. Both laser frequencies are tuned to an electronic transition and a femtosecond time delay is introduced between the pump and probe pulses. The transient absorption is then the absorption of the probe pulse due to the preceding pump pulse. For the configuration in Figure 2b, the pump pulse excites ground-state electrons. During the time the electrons remain in the excited state, the probe pulse sees a smaller ground-state population than that without the pump pulse. Consequently, the probe pulse is absorbed less in presence of the pump pulse than in absence of the pump pulse. By changing the delay between the pulses, also the decay dynamics of the excited state can be interrogated, as applied in pump-probe spectroscopy to reveal femtosecond electron dynamics.

Example Experiments

Stimulated Raman Scattering Microscopy

In Ji-Xin Cheng's group at the Purdue University, Delong Zhang and Mikhail Slipchenko constructed an SRS imaging setup, which is described in [2]. The parts relevant to modulation transfer SRS experiments and their interconnection are presented in Figure 3. The two tunable excitation pulses (pump and Stokes) are provided by an optical parametric oscillator (OPO) pumped by a femtosecond Ti:Sapphire laser oscillator. Subsequently, the Stokes pulses are intensity modulated at 5.4 MHz by means of an acousto-optic modulator (AOM) and temporally and spatially overlapped with the pump pulses. The 5.4 MHz reference carrier is provided by the lock-in amplifier.

The excitation beams are focused into the sample with a high numerical aperture objective and SRS and CARS signals produced in the sample are collected by another microscope objective in the forward direction. To measure the stimulated Raman loss, the pump pulses are optically filtered from the output pulses, measured by a photodiode and demodulated by the HF2LI. The demodulated signal is sent to the microscope controller via the auxiliary output of the HF2LI.

One novel aspect of the microscope presented in [2] is the use of femtosecond laser pulses, instead of the commonly used picosecond pulses. These short pulses can better excite isolated vibrational resonances due to the

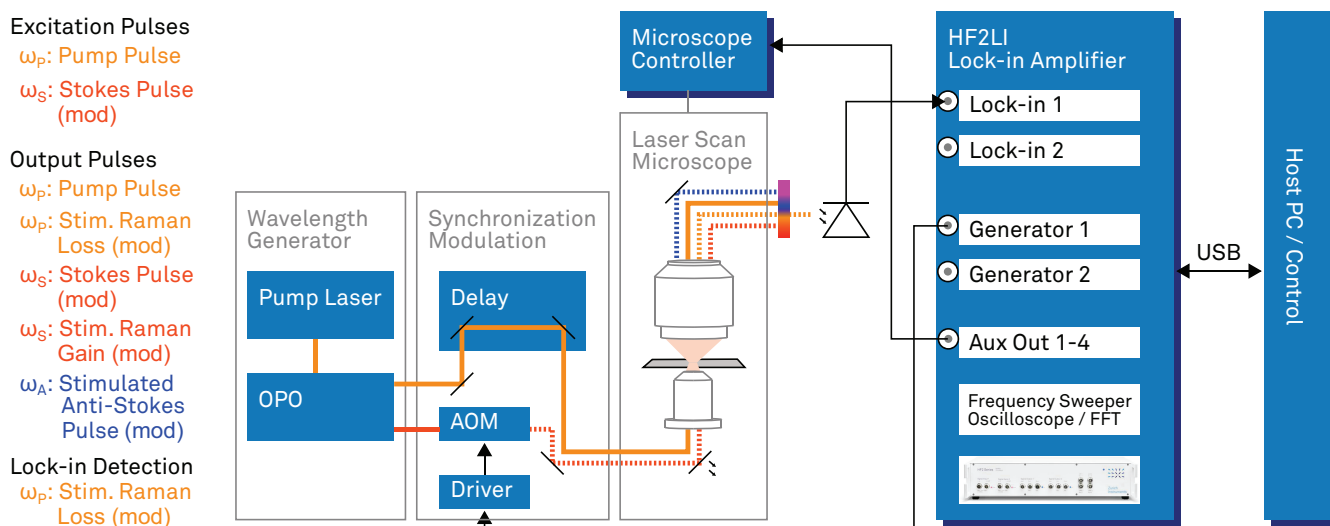


Figure 3. SRS microscope as used by Zhang et al [2] with parts related to backward fluorescence imaging omitted. The list on the left hand side mentions all the involved optical frequencies. **Wavelength Generation:** pump pulses generated with a femtosecond pump laser (repetition rate 80 MHz). Tunable pump (680 - 1080 nm) and Stokes (1000 - 1600 nm) are provided by an optical parametric oscillator (OPO). **Synchronization, Modulation:** time synchronization of the pulse trains with a delay stage and intensity modulation at 5.4 MHz with an acousto optic modulator (AOM). Modulation carrier from HF2LI fed to driver. **Laser Scan Microscope:** microscope with laser steering controlled by the Microscope controller.

increased excitation bandwidth of shorter pulses. Due to the higher peak power associated with femtosecond laser pulses, photodamage on biological samples is critical. The authors solved this problem by balancing the excitation power to the near-infrared Stokes pulses (40 mW), where possible photodamage is smaller than for the visible pump pulses (10 mW). In addition, images are acquired at high-speed with high-sensitivity. The pixel dwell time, which is the exposure time to the laser of one sample pixel, is kept below 4 μ s.

One part of their study focuses on the synthesizing of lipid droplets (LD) containing fatty acids (FA) for energy storage within cells, as previously investigated with Raman microscopy by Slipchenko et al [10]. By imaging C-H bonds at a Raman shift of 2875 cm^{-1} , LDs synthesized from endogenous glucose, which is present within the cell, are identified. By adding deuterated exogenous FA with a unique vibrational response from C-D bonds at a Raman shift of 2100 cm^{-1} , SRS imaging allows for differentiation between endogenous and exogenous FA within LD. Figure 4 demonstrates this principle.

The authors also acquired 3D SRS images of an anesthetized *C. elegans* worm at the C-H vibration. These create a 3D map of the LD distribution inside the worm. In total, 50 frames at different elevations with 512 by 512 pixels are acquired with a pixel dwell time of 2 μ s. By taking into account the repositioning of the beams within each frame, the 150 x 150 x 50 μ m image is acquired in 55 seconds. Furthermore, the SRS microscope is compatible with other imaging modalities such as, for example, two photon fluorescence, which can be detected in the backward direction without the lock-in amplifier.

Transient Absorption Microscopy

In the Potma Group at UC Irvine, Desiré Whitmore and Yong Wang built a nonlinear microscope that performs transient absorption (TA) imaging with an HF2LI for signal acquisition [11]. The related femtosecond pulses with a repetition rate of 80 MHz are listed in Table 2.

Table 2. Components of transient absorption process

Excitation pulses	Output pulses
ω_1 : pump pulse (mod)	ω_1 : pump pulse (mod)
ω_2 : probe pulse	ω_2 : probe pulse
	ω_2 : transient absorption (mod)

The wavelength of the pump pulse is 820 nm, whereas the wavelength of the probe pulse is 943 nm. With respect to Figure 3, pump pulses pass the AOM, whereas the probe pulses pass the delay stage to adjust the interpulse delay. The modulation frequency is 1.5 MHz. The excitation powers are 1 mW for the pump pulse and 0.5 mW for the probe pulse.

Upon interaction with the sample, the transient absorption on the probe pulse is isolated from the output pulses

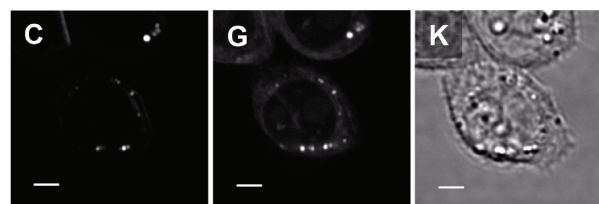


Figure 4. Adapted with permission from [2]. Copyright 2011 American Chemical Society. The figure shows SRS and transmission images of CHO cells incubated for 7 hours in the cell culture medium supplemented with 100 μ M deuterated palmitic acid (exogenous FA with C-D vibration). Figure 4C shows an SRS image of the C-D vibration and indicates the presence of exogenous FA in LD and cell membranes. Figure 4G shows an SRS image at the C-H vibration of the same cells and shows the distribution of endogenous FA in cells. Notably, the location of LD in 4C and 4G indicates that even when cells are supplemented with exogenous FA, intra-cellular LD contain both endogenous and exogenous FA. This suggests that the cell still synthesizes endogenous FA to facilitate the LD formation. The transmission image in Figure 4K lacks any chemical information found in the SRS images. Scale bar: 5 μ m

by optical filtering and demodulation by the HF2LI. The auxiliary outputs of the HF2LI send the signal to the microscope controller.

The wavelength configuration utilized for TA by Desiré Whitmore takes advantage of stimulated emission in metallic structures as imaging contrast. Consequently, one application is the visualization of individual single walled carbon nanotubes (SWNT) with metallic properties. Although carbon nanotubes are expected to provide innovational solutions in many fields of technology, it is still difficult to synthesize nanotubes with specific material properties. In order to select SWNT with specific properties at the end of the fabrication process, carbon nanotubes need to be characterized individually. One such tool is nonlinear optical pump-probe microscopy with the capability of studying fast electron dynamics by TA. As SWNT are very small, the optical response is weak and requires long image acquisition times. Because photodamage limits the maximum pixel dwell time, frame based averaging is applied instead of one single scan with long acquisition time. An example for SWNT imaging with a pixel dwell time of 20 μ s is shown in Figure 5.

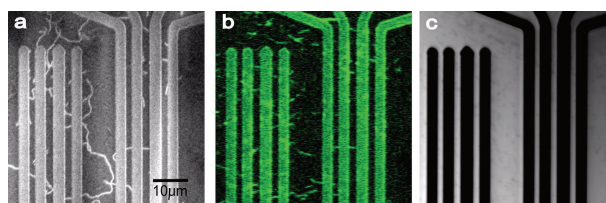


Figure 5. Adapted with permission from [11]. Copyright 2011 Desiré Whitmore. Figure 5a is a scanning electron microscope (SEM) image of synthesized SWNT with Titanium pads for electrical characterization. While the SEM resolves individual SWNT, no information concerning the electronic properties is obtained. Figure 5b shows a TA image of the same region. As the response from SWNT depends on the optical polarization of the laser pulses, only horizontally aligned SWNT are visible. By changing the interpulse delay, the electron dynamics of individual metallic SWNT are studied. The pads are resolved in the TA image due to their metallic properties. The pixel dwell time was 20 μ s and a longer pixel dwell time of 100 μ s resulted in photodamage of the SWNT. Figure 5c shows a transmission image of the same region and does not show any signature from SWNT.

User Benefits

In comparison to any other commercially available instrument, the Zurich Instruments HF2LI lock-in amplifier provides essential features for high-speed nonlinear optical pump-probe microscopy:

- Frequency range up to 50 MHz
- Fastest filter time constant of 780 ns
- Auxiliary outputs sampled at 1 MS/s
- 120 dB dynamic reserve for best-in-class signal-to-noise ratio
- Up to 6 demodulators in one lock-in unit
- Built with the latest integrated components in the market

With typical pulsed laser repetition rates of 80 MHz, the practical modulation frequencies are always below 40 MHz, such that the frequency range of the HF2LI perfectly matches these conditions. For fast scanning speeds, the demodulation bandwidth can be set as high as 200 kHz, by choosing a first order filter with a time constant of 780 ns.

For fast output and acquisition of the demodulated signals, the auxiliary outputs of the HF2LI are updated every microsecond with time equidistant samples avoiding unnecessary distortions. Aliasing on these outputs is suppressed by a 200 kHz filter. Considering the superposition of the two filter transfer functions, the maximum effective demodulation bandwidth at the auxiliary outputs of HF2LI is 147 kHz. Alternatively the 200 kHz bandwidth is achieved with digital sample transfer to the computer by USB cable.

Table 3 summarizes the achievable imaging frame rate with an HF2LI used for signal acquisition for different resolutions. The left-most column shows the number of pixels per frame. The 5% Rate is for instance the frame rate, where the smallest resolvable feature size is increased by 5% due to the demodulation filter. All of these frame rates assume that the smallest resolvable feature is sampled by two pixels. For example, the size of the 512 x 512 pixel frame is 128 x 128 μm . The frame rates of Table 3 assume a zero time for subsequent frames acquisition. The detailed calculations can be looked up in [12].

Table 3. Achievable imaging frame rates with HF2LI

Frame pixels	5% Rate	10% Rate	20% Rate
512 x 512	1.2 fps	1.7 fps	2.3 fps
256 x 256	4.9 fps	6.7 fps	9.4 fps
175 x 175	10 fps	14 fps	20 fps

Therefore high-speed microscopy with 10 or more frames per second is feasible with a minimal trade-off of spatial resolution. Besides coherent Raman and nonlinear absorption microscopy, also nonlinear pump-probe

imaging techniques (such as photothermal microscopy), benefit from the fast detection performance.

The HF2LI also features two signal inputs and 6 demodulators that can be used for combination of different detection methods. One example is a simultaneous measurement of modulation transfer, which is detected on the first channel, and single beam two photon absorption, which is detected on the second channel of the HF2LI at any arbitrary harmonic of the modulation frequency. Accordingly, the optical setup needs to be extended to detect simultaneously the collected pump-probe pulses. Another example is to apply pseudoheterodyne detection method [13] to measure the vibrational phase in the interferometric CARS setup utilizing optical phase modulators.

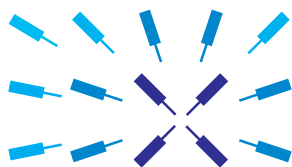
Acknowledgements

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References

- [1] Evans, Potma, Puoris'haag, Côté, Lin, Xie, Chemical imaging of tissue in vivo with video-rate coherent anti-Stokes Raman scattering microscopy, PNAS, 102 (46) 16807-16812, 2005
- [2] Zhang, Slipchenko, Cheng, Highly sensitive vibrational imaging by femtosecond pulse stimulated Raman loss, J. Phys. Chem. Lett., Vol 2, 1248 - 1253, 2011
- [3] Saar, Freudiger, Reichman, Stanley, Holtom, Xie, Video-rate molecular imaging in vivo with stimulated Raman scattering, Science, Vol 330, 2010
- [4] Day, Domke, Rago, Kano, Hamaguchi, Vartiainen, Bonn, Quantitative coherent anti-Stokes Raman scattering (CARS) microscopy, J. Phys. Chem. B, Vol 115, 2011
- [5] Ganikhanov, Evans, Saar, Xie, High-sensitivity vibrational imaging with frequency modulation coherent anti-Stokes Raman scattering (FM CARS) microscopy, Optics Letters, Vol 31 No 12, 2006
- [6] Potma, Evans, Xie, Heterodyne coherent anti-Stokes Raman scattering (CARS) imaging, Optics Letters, Vol 31 No 2, 2006
- [7] Jurna, Garbacik, Korterik, Herek, Otto, Offerhaus, Visualizing resonances in the complex plane with vibrational phase contrast coherent anti-Stokes Raman scattering, Anal. Chem, Vol 82, 2010
- [8] Min, Freudiger, Lu, Xie, Coherent nonlinear optical imaging: Beyond fluorescence microscopy, Annu. Rev. Phys. Chem., Vol 62, 507-530, 2011

- [9] Ye, Fu, Warren, Nonlinear absorption microscopy, Photochemistry and Photobiology, Vol 85, 631 - 645, 2009
- [10] Slipchenko, Le, Chen, Cheng, High-speed vibrational imaging and spectral analysis of lipid bodies by compound raman microscopy, J. Phys. Chem. B, Vol 113, 7681 - 7686, 2009
- [11] Whitmore, Design and construction of linear and nonlinear optical systems for measurements of nanomaterials: Towards chemistry at the space-time limit, Ph.D. Thesis, University of California, Irvine, 2011
- [12] Derivations shown in Zurich Instruments Blogs, www.zhinst.com/blogs/andrin/2011/12/06/scanning-speed-and-demodulation-bandwidth/
- [13] Zurich Instruments application note on pseudoheterodyne detection in optics, www.zhinst.com/applications/pseudoheterodyne



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