
Part II

NONLINEAR OPTICAL SPECTROSCOPY

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This part of the handbook presents a detailed account of the various physical processes that lie at the heart of biological microscopy and are based on nonlinear optics. It consists of three chapters that cover the basic nonlinear optical processes that are used in nonlinear microscopy. These processes are saturated fluorescence, harmonic generation, and stimulated light scattering. It is the intent of the authors of these chapters to present a clear and comprehensive exposition of the physical processes that are crucial to the success of the implementation of these nonlinear optical techniques.

The beginning of the field of nonlinear optics is often, and somewhat arbitrarily, taken to be the discovery of optical second-harmonic generation by Franken and coworkers in 1961 (Franken et al., 1961) but the origins of this field can be traced back much earlier. The earliest report of a nonlinear optical effect known to the present authors is that of G. N. Lewis, who in 1941 reported saturation with increasing excitation strength of the fluorescence intensity of the organic dye fluorescein in a boric acid glass host (Lewis et al., 1941). In broad terms, the interest in using nonlinear optical techniques for microscopy is to extend the capabilities of traditional microscopy. For example, traditional, linear-optics microscopy is limited in the transverse and longitudinal spatial resolution that can be achieved to a value of approximately the wavelength of the light being used. In many circumstances, it is not possible to obtain increased resolution by using a shorter wavelength, because many biological materials do not even transmit light at these wavelengths. Nonlinear techniques hold the promise of achieving high spatial resolution while making use of light of visible wavelengths. A unifying theme in the description of nonlinear optical interactions is the use of the nonlinear susceptibility to quantify the strength of a nonlinear optical interaction. According to this procedure, the nonlinear polarization $\tilde{P}(t)$ is related to the electric field strength $\tilde{E}(t)$ of the applied optical field according to the expansion:

$$\tilde{P}(t) = \epsilon_0 \left[\chi^{(1)} \tilde{E}(t) + \chi^{(2)} \tilde{E}^2(t) + \chi^{(3)} \tilde{E}^3(t) + \dots \right], \quad (\text{II.1})$$

Here $\chi^{(1)}$ is the linear susceptibility, $\chi^{(2)}$ is the second-order susceptibility, $\chi^{(3)}$ is the third-order susceptibility, etc.

The procedure of describing nonlinear interactions in terms of the susceptibility is developed in some detail within this section; see especially the chapter by Boyd.

II.1 TWO-PHOTON EXCITATION FLUORESCENCE SPECTROSCOPY

In microscopic implementations of multiphoton excitation (MPE), in which two or more photons induce the electronic transition from the ground state to the excited state via virtual states, the signal generation is proportional to the intensity of the excitation light squared. Therefore, only in the focal volume of the microscope objective is there significant MPE of fluorescent. Thus, the physics of MPEM defines the optical sectioning capability of the technique. The advantages of two-photon-excitation microscopy as compared to the single photon case typically include minimal volume of signal generation, increased depth penetration, and reduced photodamage of live cells and tissues. These same advantages apply for thick-tissue microscopy using two-photon excitation as compared to confocal microscopy with ultraviolet excitation. The major impediment to using multiphoton spectroscopy is the cost of the femtosecond laser that is the basis of the spectroscopic and imaging instrumentation. Two-photon-excitation fluorescence (TPFM) techniques are currently extremely popular, in part due to the wide variety of fluorescent probes that are available and the rapid development of new probes: organic probes, genetically expressed probes, and quantum dots. All three classes of fluorescent probes are in a state of very active development, and we can expect continuing development of new probes with important utility in neurobiology, cell biology, developmental biology, and clinical diagnostics. In addition, there are a variety of intrinsic probes for TPFM: NAD(P)H, flavoproteins, elastin, and neurotransmitters that are useful for live cell imaging. Genetically expressed fluorescent probes are becoming more common in the laboratory, and ease of use of this technique is one factor that is driving their rapidly expanding usage. We should note that all genetically expressed fluorescent probes are overexpressed, and the appropriate controls must be performed to demonstrate the validity of all experiments. For example, it is critical that the experimenter validate the experiment by demonstrating that the fusion protein is correctly localized and is functional. The large size of green fluorescent protein is a major limitation. In general, it is necessary to demonstrate that the use of extrinsic fluorescent probes is not causing alterations in the cells and tissue.

II.2 SECOND-HARMONIC AND THIRD-HARMONIC GENERATION

Second-harmonic generation (SHG) and third-harmonic generation (THG) both provide the ability to image collagen and elastin in living biological cells and tissues. While SHG is allowed only in acentric systems or noncentrosymmetric media (those without a center of inversion symmetry), THG is allowed in both acentric and centric systems (centrosymmetric media) (i.e., in those systems both with and without a center of inversion symmetry). The depth of focus is due to the physics of the harmonic generation. The generation of the harmonic is proportional to the intensity squared; therefore, the region of harmonic generation occurs in the region of the focus where the intensity is very large. The major advantage of these techniques is that the SHG and the THG signal is generated within live cells and tissues without the need for extrinsic probes, and there is no damage. While intrinsic molecules that provide contrast for SHG and THG are useful for live-tissue imaging, the types of biological molecules that generate signals are very limited. In addition, the efficiency of SHG and THG is

very low. A major thrust in this field is to develop new types of contrast probes for use with SHG and THG in live-cell imaging. SHG microscopy can be used to image collagen, myosin heads in muscle, and microtubules. THG microscopy gives signals when there is a change in the refractive index within the sample and will yield a signal in the presence or the absence of centers of symmetry. These imaging techniques can be used to investigate patterns of collagen in normal biological specimens as well as alterations in collagen structure and organization that can occur with development, aging, and injury and pathology. These noninvasive optical techniques may provide new knowledge on the extremely important process of wound healing and scar formation. We can expect to see the future development of extrinsic probes with specific biological specificity to aid in the further application of these optical imaging techniques. The further development of potential probes that are excited with SHG will be another tool to investigate membrane potentials with applications in neuroscience. For example, these probes may find utility in optical imaging of the brain *in vivo*. With appropriate SHG probes, studies of membrane potentials can be measured with four times the sensitivity compared to two-photon-excitation fluorescence methods. SHG methods do not involve the formation of excited states; however, resonance-enhanced SHG occurs with two-photon excitation fluorescence, and therefore photodamage and photobleaching may still occur. The combination of two or more of these nonlinear optical microscopic techniques will provide new insights into both the basic sciences and clinical and diagnostic medicine.

II.3 COHERENT ANTI-STOKES RAMAN SCATTERING

Coherent anti-Stokes Raman scattering (CARS) spectroscopy and microscopy are emerging techniques for the visualization of live biological specimens with chemical specificity and do not require extrinsic labeling. The technique of CARS requires stable picosecond lasers and/or optical parametric oscillators (OPOs) in the wavelength range appropriate to excite biomolecules in live cells. Two laser beams are required, a pump beam and a Stokes beam. If the beat frequency (difference of these two beam frequencies) is identical to the Raman vibrational frequency of a molecule, then the vibrations are driven coherently and a strong anti-Stokes signal is obtained at two times the pump frequency minus the Stokes frequency. For tissue imaging and live-cell imaging the signal is detected in the epi-direction.

CARS shows promise based on three important factors: it is a noninvasive optical technique, it has high sensitivity, and it is specific to the chemical structure of the molecules that are involved in the CARS processes. The vibronic levels that are excited in CARS are unique chemical markers of individual molecules; therefore, CARS provides a high measure of chemical selectivity. CARS microscopy offers three-dimensional optical sectioning ability due to the fact that the signal generation occurs only at the focal volume of the microscope objective.

With present sensitivity, CARS is an important microscopic technique to image biomolecules such as lipids and water in cells and in cell membranes. A recent paper on chemical imaging of tissue *in vivo* with video-rate coherent anti-Stokes Raman scattering microscopy points out how this technique can be used to image the lipid-rich structures in the skin of a live mouse (Evans, Potma, Puoris'haag, Cote, Lin, Xie, 2005).

We can expect to see future developments in the application of CARS microscopy to the basic studies of lipid movements in cells and tissues, and there will be applications to the study of normal and diseased skin and the study of skin grafts, scar formation, wound healing, and the aging process in skin. CARS may also provide a diagnostic tool for skin pathology. The chemical selectivity with CARS is very high; specific molecules in a cell can be imaged. The nonlinear technique of CARS generates only a signal within the focal volume of the laser. This is the physical origin of the optical sectioning effect and is similar to that of MPEM.

II.4 COMPARISON OF THE THREE NONLINEAR OPTICAL TECHNIQUES

We now present an overview and comparison of the three different techniques in terms of sensitivity, specificity, selectivity, types of probes required, unique information available from each technique, and the experimental complexity of each method.

II.4.1 Sensitivity

It is possible to use MPEM to detect a single molecule that is spatially restrained. SHG, THG, and CARS microscopic techniques have a comparable sensitivity. Recently, CARS imaging and spectroscopy have been demonstrated at video rates in *in vivo* tissue. The sensitivity of SHG and THG is considerably less, as it requires 10^4 to 10^6 molecules for detection.

II.4.2 Selectivity

CARS techniques have high chemical specificity and work with the intrinsic molecules within cells and tissues. For example, CARS can be tuned into the CH_2 vibrational band of lipids. Therefore, the microscopic implementation yields images of the location of specific chemical structures such as lipids. SHG is specific for biological molecules without centers of symmetry. SHG and THG spectroscopic techniques are sensitive to molecular orientation and they show specificity for membranes. CARS spectroscopic techniques are sensitive to chemical structure and therefore can differentiate different cellular and tissue structures based on their chemical composition. Both MPEM and CARS can be used to track the diffusion of chemicals in skin.

II.4.3 Endogenous Versus Exogenous Probes

MPEM has available a wide variety of highly specific molecular probes. Many studies with MPEM used genetically expressed probes that fluoresce. These genetically expressed probes can be used to image the location of specific proteins in live cells and tissues. Some new and promising probes for MPEM are a variety of three-dimensionally confined quantum dots. The use of highly specific fluorescent probes also introduces the possibility of altering the structure and the function of the cells and tissues that are being studied. SHG, THG, and CARS spectroscopic techniques applied to optical microscopes can be used to image many endogenous molecules. There is a great need to synthesize contrast agents that respond to SHG and THG spectroscopic techniques.

SHG and THG microscopy is useful for the imaging of organized and orientated protein assemblies; however, these techniques are not useful to image other biological components due to the low specificity and sensitivity. This limitation points out the need for the development of contrast agents for SHG and THG microscopy. The great advantage of SHG and THG is that they image intrinsic molecules in tissues. CARS microscopic techniques, a nonlinear Raman technique, can provide high-resolution optical imaging together with a high degree of chemical specificity and do not depend on exogenous probes to generate contrast.

II.4.4 Experimental Complexity (Equipment, Data Interpretation)

The main limitation in the microscopic implementation of these nonlinear spectroscopic techniques is the cost and complexity of the femtosecond or picosecond lasers and the technique of their synchronization. While the size and the maintenance of these lasers are constantly being improved, the high cost is still an impediment to many investigators.

II.5 CONCLUSIONS

Photodamage of live cells and tissues is always an important limitation for live-cell imaging. The use of near-infrared excitation minimized both the linear absorption and the photodamage to live cells and tissues. In summary, results obtained over the past several years demonstrate the good promise that nonlinear optical techniques hold for microscopy. For example, Gustafsson (2000, 2005) has shown that the transverse resolution of optical microscopy can be increased through use of spatially structured illumination. The resolution is increased by only a factor of two for the case of linear optics, but is in principle unlimited for a nonlinear response. Also, the use of multiphoton effects for applications in endoscopy has been described by Jung and Schnitzer (2003). Reviews of various nonlinear techniques for biological microscopy have been presented by Masters, 2003; Masters and So, 2004; Zipfel et al., 2003; and Hell et al., 2004. The merging of several of these nonlinear techniques, for example by combining SHG and MPEM, may provide unique information on cellular structure, and function and new insights for diagnostic biomedical imaging.

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