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How to improve your image

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Abstract

The technique of second harmonic generation microscopy is used to obtain pictures of living systems, but the dyes required provide only modest imaging contrast per molecule. The latest dyes give a much better picture.

Optical imaging experiments have provided a wealth of structural, functional and mechanistic insights into biological processes, and at every level of organization: from the subcellular to the cellular, at the tissue level, and even in whole animals. Some of these investigations rely on multi-photon techniques, in which a fluorescent signal is generated from a dye by the near-simultaneous absorption of two or more photons. Writing in the *Journal of the American Chemical Society*, Reeve *et al.*¹ describe a new family of dyes that produce dramatic signal enhancements in a two-photon technique known as second harmonic generation (SHG) microscopy. The dyes could dramatically improve the scope of the technique for biological imaging — especially in studies of membranes.

Two-photon microscopy^{2–4}, of which SHG microscopy is an example, has revolutionized the biological sciences by enabling non-invasive, high-resolution imaging of living samples in real time. Perhaps the most commonly used variant is two-photon-excited fluorescence (TPEF) microscopy, in which a pair of low-energy photons is absorbed by an emissive probe molecule that would normally absorb a single photon at half the wavelength (twice the energy) of the low-energy photons. The resulting electronically excited dye relaxes to a fluorescent state that can be observed. By using low-energy (near-infrared, NIR) photons to generate fluorescence, rather than higher-energy (visible-light) photons, light-induced degradation of biological samples is minimized.

In contrast to TPEF imaging, the dye molecules in SHG microscopy do not absorb photons. Instead, they scatter light at exactly half the wavelength of the incoming radiation. As no real excited states are produced, phototoxic damage to biological samples can in principle be eliminated. Like TPEF imaging, SHG microscopy is based on a nonlinear optical process in which the observed signal scales with the square of the intensity of the incident light; this relationship provides excellent spatial resolution. But whereas the light produced in SHG microscopy is always half the wavelength of the irradiating laser, that produced in TPEF imaging is determined by the spectral breadth of the dye's fluorescence emission band.

Both methods require pulsed-laser excitation, which provides the necessary peak intensity of light for these experiments, typically at NIR wavelengths. This is a particularly useful optical window for studying biological systems, because biological media do not absorb NIR light well, which reduces background noise in the images obtained. Furthermore, because light scattering diminishes with increasing wavelength, NIR light penetrates tissue more effectively than either ultraviolet or visible light.

To obtain high sensitivity and resolution in SHG microscopy, hyperpolarizable probes are required — dye molecules that have electron-density distributions that can be highly distorted

under the influence of an optical field. Reeve and colleagues' probes¹ are based on previously reported 'porphyrin' dyes⁵ (Fig. 1a). Porphyrins provided a good template from which the authors could work, because such compounds have been shown to fluoresce intensely on absorbing low-energy photons⁶, and to be particularly good at absorbing pairs of photons^{7,8} — properties that are crucial for linear and nonlinear optical imaging applications.

When the porphyrin is attached to electron-donating and electron-accepting groups by carbon-carbon triple bonds (C≡C), the electron density of its delocalized electrons can be hyperpolarized in an optical field. The C≡C bonds are particularly important in the design of imaging agents for biological applications, because porphyrin compounds featuring this bonding motif exhibit desirable nonlinear optical properties and exceptional hyperpolarizabilities^{5,9}, as well as strong NIR fluorescence^{6,8}. Yet another reason for the interest in porphyrins is that they are ubiquitous components of the proteins involved in electron migration (cytochromes), oxygen transport (haemoglobin) and oxygen storage (myoglobin). Probes such as those described by Reeve *et al.*¹ might therefore exhibit the biocompatibility and biodegradability required for *in vivo* imaging applications.

Will SHG microscopy supplant TPEF as the standard microscopic method for biological imaging? To answer this question, it should be noted that SHG measurements require hyperpolarizable dyes to be ordered in an asymmetric fashion — a 'noncentrosymmetric' arrangement, for those in the know — over macroscopic dimensions. Reeve *et al.*¹ incorporated structural elements into their dyes (long hydrocarbon chains on the electron-donating portion of the molecules) that ensure asymmetric ordering of the molecules within biological membranes. This enabled the authors to generate SHG micrographs of SK-OV-3 cancer cells¹ (Fig. 1b). But TPEF imaging does not require asymmetric ordering of dyes to generate signals, and will thus always remain a more general two-photon microscopy method.

On the other hand, TPEF imaging is an incoherent process that entails light absorption and, in general, subsequent omnidirectional emission. This combination of processes often causes valuable information to be lost about how dye molecules interact with their local biological environment. In contrast, SHG microscopy is a coherent phenomenon that maintains phase information about the incident radiation. The scattered light observed in SHG imaging therefore consists of highly directed radiation that depends on the distribution and orientation of dipoles in the dye molecules. Accordingly, SHG microscopy can in many instances provide structural information that can't be obtained by TPEF imaging^{2,3}.

SHG microscopy has been used in such diverse applications as probing cell–cell interactions, scrutinizing cellular morphology and even recording waves of electrochemical activity (action potentials) in neurons in real time^{2-4,10}. But thus far, the method has relied on dyes that have only modest hyperpolarizabilities. Reeve and colleagues' compounds¹ are a big improvement, but the authors have another trick up their sleeves that could further enhance imaging contrast per molecule of dye. Porphyrins readily bind dipositive metal ions. When the authors' dyes bind to certain such ions, such as copper ions (Cu²⁺), the resulting complex is non-fluorescent. If these non-fluorescent complexes are used for SHG microscopy, the experimental set-up can be tuned to exploit an effect known as resonance enhancement. This increases the hyperpolarizability of the molecules^{5,9}, thus providing even more sensitive probes for SHG microscopy.

Reeve and colleagues' compounds are the first of a new generation of SHG imaging dyes that will undoubtedly enable multi-photon imaging at higher contrasts and resolution than was previously possible. Because the electron-donating and electron-accepting groups can be readily changed, an enormous range of dyes can be made and optimized for various

applications. This, in turn, will provide new tools for visualizing cellular and subcellular events in living tissue.

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Figure 1. Another way to dye

a, Reeve *et al.*¹ report improved fluorescent dyes for second harmonic generation (SHG) imaging. The molecular structure of the dyes consists of a porphyrin core (blue), to which an electron-donating group (green) and an electron-accepting group (red) are attached. R represents a methyl group, or a hydrocarbon chain to which a polar group is attached. **b**, The authors used their dyes to obtain SHG images of SK-OV-3 cancer cells. (Scale bar, 10 μm . Image reproduced from ref. ¹.)