Biological Second and Third Harmonic Generation Microscopy

This unit describes how higher harmonic generation microscopy (HHGM) is applied to detect native, nonstained cell and tissue structures that were previously only accessible after immunohistochemical or immunofluorescent labeling.

Nonlinear optical microscopy by multiphoton excitation has developed into a popular, and powerful approach that combines different excitation and emission techniques for the three-dimensional (3-D) reconstruction of biological specimens (Denk et al., 1990; König, 2000). The specific approaches include detection of two- and three-photon excited fluorescence, two-photon excited fluorescence lifetime (FLIM), and the second and third harmonic generation. Specimens amenable to multiphoton microscopy include fixed and live samples, thin and thick slices or samples with transparent or relatively opaque properties, as well as intravital microscopy in anesthetized animals (see UNIT 4.11). The most obvious advantages of multiphoton microscopy include: (1) greater tissue penetration up to the millimeter range due to near-infrared excitation and consequently, less scatter; (2) reduced phototoxicity imposed onto cells and tissues due to the inherent confocality exciting only fluorophores in the focal plane and the lack of a detector pinhole resulting in improved optical detection efficiency; (3) the broader excitation range of fluorophores, compared to single photon excitation, allowing multicolor imaging using the same excitation wavelength; and (4) the possibility of combining fluorescence microscopy with other detection modes, including HHGM and FLIM. Therefore, nonlinear imaging is being used for nearly any application in cell biology that requires multicolor imaging, deep tissue penetration, low phototoxicity, and high resolution, similar to confocal microscopy.

Two-photon or multiphoton excited HHGM has become one of the most popular and powerful techniques for the reconstruction of intrinsic structural and molecular properties of cells, extracellular matrix, and bone without the use of dyes or stains (Campagnola et al., 2002; Friedl, 2004). Two modalities of HHGM are presently being used for biomedical applications, the second (SHG) and third (THG) harmonic generation imaging (the principle and theoretical basis for these imaging modalities are detailed in the Commentary). SHG and THG imaging provides structural and molecular information that is complementary to two-photon fluorescence (TPF) imaging and can be simultaneously combined with other two-photon excited modalities, including two-photon photoactivation, two-photon correlated spectroscopy, two-photon single-particle tracking, and two-photon lifetime microscopy. HHGM is most useful in the imaging of connective tissue, collagen fibers and fascia, as well as striated muscle, inflammatory cells, blood vessels, and hair at a resolution near the diffraction limit of visible light.

This unit will provide details on the experimental setup and some biological applications of HHGM, including 3-D connective tissue reconstruction and dynamic imaging of cell motility in 3-D tissues. In the commentary, the physical basics of HHGM are described, additional examples for HHGM are given, and there is a discussion on how to detect and minimize multiphoton-excited phototoxicity.

STRATEGIC PLANNING

Materials detectable by SHG and THG

The first step is to consider whether the molecular structure of interest can be detected by HHGM. Because this is a young technique, progressive use of this technology will lead
Table 4.15.1 Second and Third Harmonic Imaging of Cell and Tissue Structures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation wavelength (nm)(^a)</th>
<th>Molecular property</th>
<th>Reference</th>
<th>Technical comments; substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SHG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrillar collagen</td>
<td>700–1064</td>
<td>Triple coil of coiled (\alpha)-helices</td>
<td>Stoller et al., 2002; Cox et al., 2003; Yasui et al., 2004</td>
<td>Dermis, vessel walls, peritumoral stroma; diminished signal during proteolytic degradation</td>
</tr>
<tr>
<td>Tubulin</td>
<td>760–880</td>
<td>Coil of whole protein units forming tube with polarized ends</td>
<td>Dombeck et al., 2003</td>
<td>Brain slices; dynamic imaging of mitotic spindles at 880 nm</td>
</tr>
<tr>
<td>Myosin</td>
<td></td>
<td>Two (\alpha)-helix polymer proteins coiled in a left hand</td>
<td>Both et al., 2004; Plotnikov et al., 2006</td>
<td>Brain slices; dynamic imaging of mitotic spindles at 880 nm</td>
</tr>
<tr>
<td>Styryl dyes</td>
<td></td>
<td>Forms anisotropic scaffold along plasma membrane the orientation of which is voltage-dependent</td>
<td>Dombeck et al., 2005; Sacconi et al., 2005</td>
<td>Suitable for backward detection; scanning in the msec range; no photobleaching</td>
</tr>
<tr>
<td>FM4-64</td>
<td>1064</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RH 237</td>
<td>880</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>840–880; 1230</td>
<td>Structural symmetry of (\beta) 1:4-linked glycans</td>
<td>Cox et al., 2005</td>
<td>Low S/N(^b)</td>
</tr>
<tr>
<td>Starch (in chloroplasts)</td>
<td>840–880; 1230</td>
<td>Structural symmetry of (\alpha) 1:4-linked glycans</td>
<td>Cox et al., 2005</td>
<td>High S/N(^b)</td>
</tr>
<tr>
<td>DNA</td>
<td>730–800</td>
<td>Unknown</td>
<td>Gauderon et al., 2001</td>
<td></td>
</tr>
<tr>
<td><strong>THG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipids and lipid bodies</td>
<td>1050–1250</td>
<td>High comparative third order susceptibility, (\chi^{(3)})</td>
<td>Debarre et al., 2006</td>
<td>Good backward S/N(^b) THG in weakly absorbing tissue</td>
</tr>
<tr>
<td>Myosin</td>
<td>1230</td>
<td>High comparative third order susceptibility, (\chi^{(3)})</td>
<td>Chu et al., 2004</td>
<td>Good backward S/N(^b) THG in weakly absorbing tissue</td>
</tr>
<tr>
<td>Collagen</td>
<td>1050–1300</td>
<td>Unknown</td>
<td>Kao, 2004</td>
<td></td>
</tr>
<tr>
<td>Hydroxyapatite crystals in tooth enamel</td>
<td>1050–1300</td>
<td>Unknown</td>
<td>Kao, 2004</td>
<td></td>
</tr>
<tr>
<td>Inorganic crystals (CaCO(_3)) in cytolyths, mouse bone, and spinules</td>
<td>1500</td>
<td>High comparative third order susceptibility, (\chi^{(3)})</td>
<td>Oron et al., 2004</td>
<td></td>
</tr>
<tr>
<td>Oxyhemoglobin</td>
<td>1260–1470</td>
<td>High comparative third order susceptibility, (\chi^{(3)})</td>
<td>Schaller et al., 2000</td>
<td>Low S/N(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Detection at \(\lambda_{ex}/2\) for SHG and \(\lambda_{ex}/3\) for THG.

\(^b\)Abbreviation: S/N, signal-to-noise ratio.

to rapid growth of the materials list, therefore a database search on molecular structures and optimum wavelength characteristics should be performed before starting a higher harmonic generation imaging (HHGI) project.

**Structures to be detected:** Second harmonic generation is caused by materials that are structured in crystalline-like lattices that are noncentrosymmetric on length scales on the order of the excitation light wavelength called the coherence length (Chu et al., 2002; Mohler et al., 2003; see Table 4.15.1). Besides metal and ion crystals, several
biological materials contain repetitive or symmetric structural units, including coiled-coil structures and polymeric proteins (Table 4.15.1). Third harmonic generation is a much weaker process, however elicited by different types of materials, particularly phase transitions, such as water-lipid interfaces (Table 4.15.1).

**Combination with fluorescence:** Because HHGI can be combined with fluorescence microscopy and other imaging modalities, it is necessary to consider which excitation and detection wavelengths are required to excite the fluorophores together with SHG or THG (Gauderon et al., 2001; Mempel et al., 2004b). The combination of higher harmonic and fluorescence microscopy has allowed for versatile combinations of cell and tissue imaging in cell biology, histology, and tissue engineering (Table 4.15.1).

**Sample type:** For the imaging of live thick tissue samples (>100 µm thick) of strong light-scattering properties, including connective tissues and lymph nodes, near-infrared excitation offers increased penetration depth in thicker light-scattering samples at a relatively low degree of cellular phototoxicity and thus, is superior to single-photon excitation. The properties of HHGM to reconstruct 3-D ECM structures and cell membranes together with fluorescence show particular promise in the assessment of tissue morphogenesis during development and pathological remodeling, including wound repair and neoplasia.

### DESIGNING A MICROSCOPE SYSTEM FOR HHGM

The hardware setup for HHGM (Fig. 4.15.1) follows, in principle, that of a laser-scanning microscope for visible wavelength emission and detection (see UNIT 4.5) and a two-photon excitation microscope using pulsed near-infrared laser sources (see UNIT 4.11). Any two-photon microscope already includes the capability of HHGI, therefore the setup description here will address specific features only. Besides higher harmonic generation imaging, the setup allows the excitation and detection of fluorophores (see UNIT 4.11), and using additional laser and detector hardware, coherent anti-Stokes Raman scattering (CARS) microscopy, fluorescence lifetime imaging (FLIM; see UNIT 4.14) in parallel and without crosstalk.

The laser beam is generated by a titanium-sapphire laser, either alone or in combination with a synchronously pumped optical parametric oscillator (OPO; Table 4.15.2). Using a cascade of mirrors, the laser beam is directed into the microscope by a computer-controlled xy-scanner consisting of one or two galvanometer-driven mirrors (Table 4.15.3) coupled to an upright or inverted microscope stage. The reconstruction of 3-D specimens requires serial z-sectioning and thus, a computerized z-positioning of sample stage or

**Table 4.15.2 Laser Sources and Wave-lengths**

<table>
<thead>
<tr>
<th>Laser type</th>
<th>Wave length (nm)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titanium:Sapphire</td>
<td>700–1040</td>
<td>Popular and broadly used laser type, but cost-intense; modern systems include computer-automated wavelength tuning and user-friendly maintenance</td>
</tr>
<tr>
<td>Fianium fiber</td>
<td>1064</td>
<td>Useful for measurements of membrane potential</td>
</tr>
<tr>
<td>Chromium fosterite</td>
<td>1230</td>
<td>Greatly reduced tissue autofluorescence at very long excitation wave length; low photodamage</td>
</tr>
<tr>
<td>Optical parametric oscillator (OPO)</td>
<td>1000–1600</td>
<td>Requires a Ti:Sa pump laser at fixed wavelength (e.g., 775 nm); computer-automated tuning available</td>
</tr>
</tbody>
</table>
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4.15.4

Figure 4.15.1  Set-up of a photomultiplier-based multiphoton microscope for higher harmonic generation microscopy. The beam of a mode-locked Ti:Sa laser is passed through an optional prechirp path consisting of two prisms to compensate for shifts in pulse width, followed by an attenuator of the beam intensity, and one or two galvanomirrors for scanning. For tuning of the incident beam above 1 \(\mu\)m, an optical parametric oscillator (OPO) pumped by the Ti:Sa laser is required. After reflection by a dichroic mirror, the beam is focused by the objective lens into the sample. Emission signals elicited in the focal plane of the sample are detected in forward direction by the condenser lens and in backward direction through the objective lens, spectrally separated by a dichroic filter and passed through a band-pass filter for each individual signal-adapted bandwidth before the detector photomultipliers (PMTs). In both directions, the transmitted or reflected laser light is blocked by band pass filters.

Table 4.15.3  Commercially Available Multiphoton Systems

<table>
<thead>
<tr>
<th>System</th>
<th>Website</th>
<th>Technical details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeiss HLSM 510 MP</td>
<td><a href="http://www.zeiss.de">http://www.zeiss.de</a></td>
<td>4 non-descanned PMTs (2 forward, 2 backward), up to 6 descanned PMTs; includes 3-D and 4-D reconstruction software</td>
</tr>
<tr>
<td>Leica TCS MP</td>
<td><a href="http://www.leica-microsystems.com">http://www.leica-microsystems.com</a></td>
<td>4 non-descanned PMTs (2 forward, 2 (4) backward), up to 6 descanned PMTs; includes 3-D and 4-D reconstruction software</td>
</tr>
<tr>
<td>LaVision Biotech</td>
<td><a href="http://www.lavisionbiotec.de/start_product.html">http://www.lavisionbiotec.de/start_product.html</a></td>
<td>8 non-descanned PMTs (4 forward, 5 backward); includes optional multibeam CCD camera-based version</td>
</tr>
<tr>
<td>Prairie Technologies</td>
<td><a href="http://www.prairie-technologies.com/ultima.htm">http://www.prairie-technologies.com/ultima.htm</a></td>
<td>4 non-descanned PMTs in the backward direction</td>
</tr>
</tbody>
</table>
Table 4.15.4  Software Packages for 3-D and 4-D Data Reconstruction and Analysis

<table>
<thead>
<tr>
<th>System</th>
<th>Website</th>
<th>Technical details</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imaris</td>
<td><a href="http://www.bitplane.com">http://www.bitplane.com</a></td>
<td>3-D and 4-D multicolor reconstruction, cell tracking, colocalization,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>microfilament tracing</td>
<td>Fast reconstruction; user-friendly software</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>handling</td>
</tr>
<tr>
<td>V olocity</td>
<td><a href="https://www.improvision.com/">https://www.improvision.com/</a></td>
<td>3-D and 4-D multicolor reconstruction, cell tracking, colocalization,</td>
<td>64 bit version available, user-friendly platform;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>angiogenesis tool</td>
<td>limitations with large datasets</td>
</tr>
<tr>
<td>Amira</td>
<td><a href="http://www.amiravis.com/">http://www.amiravis.com/</a></td>
<td>3-D multicolor reconstruction, volume rendering</td>
<td>Not able to reconstruct 4-D stacks, limited export</td>
</tr>
<tr>
<td>NIH Image</td>
<td><a href="http://rsb.info.nih.gov/nih-image/Default.html">http://rsb.info.nih.gov/nih-image/Default.html</a></td>
<td>Mac and PC version available, fast and powerful tool for data handling and manual tracking; good video manipulation tools</td>
<td>Open-access shareware platform; large number of plug-ins generated by users available; no automated tracking or 3-D surface rendering</td>
</tr>
</tbody>
</table>

objective, as is available for most high-quality microscopes. The detection unit includes beam splitters and band-pass filters designed for the wavelength-resolved detection of emitted light using photomultipliers (PMTs).

The operation of the system including scan-head control and image acquisition is controlled by acquisition routines provided by the manufacturer of the scanning system. Because image acquisition will generate large amounts of data, the hardware and software design should be optimized for maximum throughput and storage capability as well as fast processor speed. These rules are described in detail in UNIT 4.10. However, appropriate image reconstruction and analysis often require the use of additional software designed to handle large data stacks (Table 4.15.4). These data analysis software packages can retrieve primary files generated by most major confocal and multiphoton systems and if required, custom-designed input can be obtained from the manufacturer on demand.

Materials

Upright or inverted microscope and optics suitable for epifluorescence detection including:
- Epi-illuminator (bright field)
- High numeric aperture objective (NA > 0.7)
- Detector unit using photomultipliers, beam splitters and band-pass filters
- Sample stage including computer-driven z-position control (alternatively xyz-position control)
- Heating system to maintain 37°C (optional)

Multiphoton laser including:
- Diode-pump laser coupled to titanium-sapphire laser (Table 4.15.2)
- Incoupling of the laser beam into the scan head via mirrors (beam path)

Laser scanning unit including computer-controlled operation system for scanning conditions, signal acquisition, primary image reconstruction and storage (Table 4.15.3)

Software for 3-D and 4-D image reconstruction (Table 4.15.4)

Vibration isolation table designed for stable mounting of optical instruments
Powermeter to measure laser light intensities along the beam path, including infra-objective level

**Adjust beam path and optics**

1. Place all optical and electronic components including the microscope, except for the computer, on a vibration isolation table.

   *All optical and electronic components including the microscope need to be of the highest quality.*

2. Obtain specific information from the microscope and scan-head manufacturers, which should include the parameters of all mirrors, lenses, and the objective. To reduce absorption or scattering of light, use silver coatings for mirrors.

   *To provide sufficient sensitivity for weak signals, the optical transparency of the total beam path (from laser source to the location of the sample) should be >30%.*

   *For excitation above 1200 nm, the optical elements of most systems (mirrors, tube lens, and scan lens) must be custom-designed for increased infrared transparency.*

   *Upon request, most manufacturers will provide an appropriate alternative system design for microscope, scan head, and detectors.*

3. Set up epifluorescence detection, which is not mandatory, but it is useful for initial sample screening and positioning.

4. Select objectives that allow high resolution (indicated by high NA) and maximum transparency.

   *For intravital microscopy in living animals, long working-distance objectives of NA 0.80 to 0.95 (usually 20× magnification) are popular because of their versatility and deep tissue penetration capabilities (up to 2 mm) and high light sensitivity; however they often do not provide sufficient detailed resolution for cytoskeletal structures or the subcellular distribution of membrane components. Objectives of higher NA are usually limited by their short working distance (≤250 μm); however they provide better subcellular detail, which can be important for imaging of cell and tissue cultures. The choice for oil- versus water- or multi-immersion objective depends upon the type of sample used. For intravital and live-cell microscopy, water-immersion dipping objectives are preferred because they can be directly introduced into the sample medium. For further details on objectives see Unit 4.11.*

5. Detect HHG using photomultipliers in either forward/transmission or backward direction. To avoid loss of signal, use a collection lens in the forward direction with a NA the same or higher, compared to the illumination NA determined by the objective (Fig. 4.15.1).

   *Whereas fluorescence intensities detected in the backward or forward direction are often equal, SHG transmission often shows a 5- to 10-fold stronger intensity, compared to the backward detection, yet using a sensitive objective, the backward detection will still deliver sufficient signal for the detection of collagen and myosin in native state (Fig. 4.15.2).*

   *Because of the inherent confocality achieved by multiphoton excitation, the detectors do not require a pinhole, thus are nondescanned (i.e., do not go again through the scanhead). Besides PMTs, a CCD camera may be placed to detect higher harmonic signals in the forward direction, but not in the backward direction, because the reflected signal mostly comprises scattered photons and consequently, gives low signal-to-noise ratio at the focal plane of the CCD chip. For third harmonic imaging, at least in theory, emission is nonisotropic and might be detected by CCD camera-based systems in the forward and backward direction.*
Figure 4.15.2 Three-dimensional reconstruction of collagen bundles from human dermis. Non-labeled 3-mm thick samples from human dermis were fixed in 4% buffered paraformaldehyde and sliced tangentially. The wavelength of the incident beam was 880 nm, the polarization vertical and horizontal, and the band-pass filters 440/20 (SHG) and 515/30 (autofluorescence). Images were acquired using a 20× N.A. 0.95 water immersion objective, resulting in an optical transverse resolution of 500 nm and a pixel resolution of ~400 nm. The axial resolution was 4 to 5 μm. Images show (A) SHG together with autofluorescence in x-y, (B) the reconstructed x-z projection, and (C) topographic reconstruction of SHG only. EF, elastic fiber; FC, fat cell. Bar = 20 μm. For color version of this figure see http://www.currentprotocols.com.

Select lasers
6. As a laser source, select a tunable titanium-sapphire laser generating femtosecond pulses of laser light to provide maximum flexibility for HHGI in biological applications (Table 4.15.2). Adjust the wavelength within a tuning range from 700 to ~1000 nm. Alternatively, use lasers generating a fixed wavelength (Table 4.15.2).
In general, short-pulse lasers allow smaller spot size and higher resolution. For excitation above 1000 nm, fixed-stage lasers for specific wavelength or a tunable OPO deliver wavelengths up to 1600 nm (Table 4.15.2).

**Select detectors and filters**

7. In contrast to fluorescence emission, the SHG (THG) emission is near exactly half (one-third) the introduced wave-length. Set conditions so that SHG and THG are spectrally separated from other spectra of the emission signal by dichroic mirrors and relatively narrow band-pass filters spanning 10 to 20 nm. The aim is to block the incident laser beam at least twice at the level of the high-pass and band-pass optical filters. All filters and dichroic mirrors should block the wavelengths at which the laser operates (i.e., up to 1000 nm for Ti:Sapphire laser; up to 1500 nm for an OPO-based system). Further set up details are described in Unit 4.11. As alternative to a cascade of dichroic filters, spectral separation can be achieved by a prism and dynamic range mirrors or an optical array detector, as used in the descanned beam path of Leica or Zeiss confocal systems, respectively (Table 4.15.4). As detectors, very high sensitivity PMTs that have a narrow dynamic range or PMTs with lower sensitivity but higher dynamic range may be used, depending on the application.

For most applications in ex vivo samples and intravital microscopy, an overlapping spectrum of emitted HHG and fluorescence spectra at different intensity will be present, which requires a broad dynamic range of the PMT detectors at the expense of sensitivity at the ultra-low signal strength. For applications that include very low SHG and THG and relatively limited intensity, such as the detection of small numbers of multimers, a high sensitivity PMT or avalanche photodiodes to count single photons might be required.

BASIC PROTOCOL 2

DETECTION OF FIBRILLAR COLLAGEN IN CONNECTIVE TISSUE EX VIVO

Fibrillar collagen is a very bright second harmonic generator at excitation range above 800 nm; it is suitable for the quantitative reconstruction of ex vivo and in vivo tissue samples (Fig. 4.15.2). Vertically oriented collagen fibers scatter mostly in the forward direction, whereas horizontally oriented fibers scatter bidirectionally (Zipfel et al., 2003), thus, imaging of horizontally positioned fibers should be attempted by adjusting the orientation of the sample.

For 3-D reconstruction of thick specimens of low optical transparency detection of the backward scatter is required, such as thick slices of human skin ex vivo (Fig. 4.15.2) or murine skin during intravital microscopy (Fig. 4.15.3). Other collagenous tissues that require SHG backward detection due to low transparency are bone and parenchymatous organs.

**Materials**

- Freshly excised piece of tissue or organ
- Medium of choice (e.g., RPMI, DMEM, or PBS; Appendix 2A)
- Additional reagents and equipment for designing a microscope system for HHGM (Basic Protocol 1)

1. Place freshly excised piece of tissue or organ directly into liquid nitrogen (−196°C).
2. Retrieve the sample and position it onto a microscope slide and add 200 to 1000 µl of medium compatible with the cell or tissue type being used (e.g., RPMI, DMEM, or PBS).
3. Position sample at the microscope and dip the objective directly into the medium.

If the interface between sample and objective loses water, the immersion depth can be increased by using a polyethylene ring or placing the sample within a petri dish.
Figure 4.15.3 Intravital SHG microscopy of individual collagen fibers and striated muscle in the mouse dermis. The epidermis of live anesthetized BL6 mice was horizontally removed and z-sectioning up to 400 nm in depth was performed using 800-μm excitation and a 20× N.A. 0.75 water immersion objective, as described (Wolf et al., 2003). SHG was detected using an upright microscope at 400/20 nm in backward direction for both x-y (A, C) and x-z sectioning (B). (A) Depth-encoded reconstruction of collagen fibers. (B) Vertical reconstruction showing the loose ("L") and dense ("D") dermal connective tissue, and (C) false-color encoded horizontal representation of penetration depth. (D) Intravital SHG detection of striated muscle and collagen-rich fascia. Bars = 50 μm (A to C), 30 μm (D) and 10 μm (D, inset). For color version of this figure see http://www.currentprotocols.com.
4. Scan sample at 880 nm. To separate SHG at 440 nm from other signals, use a 440/15 or 440/20 band-pass filter before the PMT.

The autofluorescence emission of native tissues covers the ranges from 480 to 650 nm, thus a dichroic 480-nm beam splitter or below will be useful. Whereas the emission peak of SHG signal shifts with the wavelength of the incident beam, the fluorescence spectrum remains fairly stable.

For the multimodal reconstruction of human skin, SHG is readily combined with green or red autofluorescence, representing mostly elastic fibers, elastolytic (denatured) collagen fibers, and fat cells (Konig et al., 2005). The combined detection of SHG and autofluorescence was used in the reconstruction of human and mouse skin (Campagnola et al., 2002; Wolf et al., 2003), native arterial vessels (Zoumi et al., 2004; Boulesteix et al., 2006), heart valves (Konig et al., 2005), native mouse lymph node (Mempel et al., 2004a), and lung (Debarre et al., 2006).

5. If necessary, increase the intensity of the SHG signal by altering the polarization of the incident laser beam. Alternatively, try rotating the sample. If signal intensity is very low, increase laser power to 50 to 100 mW to lead to sufficient SHG excitation.

The SHG signal elicited by collagen stems from the crystalline-like triple-helical structure whose cross-section has rotation symmetry but not inversion symmetry. The signal stems from surface layers of the collagen fibril but not from inner structures (Williams et al., 2005), and its intensity strongly depends on the tertiary and quaternary fibril structure, as well as ionic strength of the surrounding medium. Dissociation of the fibrillar structure and thus second order susceptibility tensor $\chi$ by a hyperosmotic agent, such as glycerin which is used as transparency-enhancing clearing agent in transcutaneous microscopy, leads to the denaturation and hence reduction of SHG signal of fibrillar collagen which is reversible upon rehydration (Yeh et al., 2003). Structural changes caused by air-drying leads to a 4-fold reduction of SHG (Yasui et al., 2004), denaturation by heating to 30° to 75°C increases the SHG signal by up to 2.5-fold, whereas higher temperature causes complete disintegration and loss of signal (Tan et al., 2005). Conversely, chemical cross-linking reduces effects caused by denaturation (Yeh et al., 2003). For future work, the susceptibility of structural changes in collagen to SHG imaging will be useful to study collagen assembly and remodeling in morphogenesis and disease state.

6. To calibrate the penetration depth of the system, define the top and bottom of the tissue volume and reconstruct the volume by acquiring sequential scans at different $z$-positions.

In human skin using 880-nm excitation, which has a lower absorbance and scattering than lower wavelengths, sensitive reconstruction of autofluorescence is achieved up to a depth of 120 $\mu$m (Fig. 4.15.2B), whereas the decay of the SHG signal is apparent earlier. In contrast to fibrillar collagens, nonfibrillar collagen, basement membranes, epidermal keratin, and fibrin are not known to elicit SHG (Yasui et al., 2004).

**BASIC PROTOCOL 3**

**DETECTION OF SHG IN MOUSE TISSUES BY INTRAVITAL MICROSCOPY**

Whereas in histology and most immunofluorescence applications, samples are processed by fixation and slicing, intravital microscopy in small animal models is based upon minimum change to the specimen observed. Emission thus is preferably endogenous and includes SHG, autofluorescence, and light emitted by endogenously expressed fluorophores in transgenic cells or animals. Here, SHG detection results in an anatomically correct reconstruction of extracellular matrix and certain cell types, such as striated muscle cells.

**Materials**

Mouse sample

Additional reagents and equipment for preparing mouse samples for intravital microscopy (UNIT 4.11), acquiring a $z$-stack (Basic Protocol 2) and, designing a microscope system for HHGM (Basic Protocol 1)
1. Prepare the mouse sample for intravital microscopy, as described in UNIT 4.11. The location of interest needs to be stably positioned and fixed on the microscope stage, e.g., using needles or custom-designed holders.

2. Acquire a z-stack as described in Basic Protocol 2.

3. Reconstruct the 3-D stack from several individual scans by optical superimposition to generate an extended focus in depth representation. Alternatively, use x-z-reconstruction to obtain information about the total depth and signal intensity changes relative to penetration depth.

   In contrast to human skin, mouse dermis is less densely textured and consists of loosely oriented individual rather than densely packed collagen fibers (Fig. 4.15.3A, B). Particularly due to the low scattering of light, high resolution reconstruction of individual fibers by SHG is achieved up to a depth of 400 µm (Fig. 4.15.3).

4. Using the same setup, detect striated muscle cells in vivo, using SHG. If the signal intensity is low, try rotating the sample slightly around the vertical axis to increase signal strength. For maximum SHG, orient the polarization of the incident beam to 45°, not 0° or 180° to the length orientation of the myosin filaments.

   The sarcomeres are 2- to 3-µm long contractile actomyosin units in striated muscle and cardiomyocytes that generate strong SHG (Fig. 4.15.3D; Both et al., 2004) and moderate THG (Chu et al., 2004). The signal is induced by the α-helical structure of the coiled rod region of the myosin filament, not by myosin heads and actin filaments (Plotnikov et al., 2006). In contrast to SHG, THG is much less angle-dependent (Chu et al., 2004).

SIMULTANEOUS DETECTION OF CELLS AND COLLAGEN FIBERS IN VITRO AND IN VIVO

Dynamic imaging of cell-ECM interactions in 3-D in vitro collagen lattices represent a useful, in vivo-like model to reconstruct molecular cell functions in the 3-D tissue-like context (Abbott, 2003). To simultaneously visualize the collagen fibers and the shape and position of live cells, SHG is combined with two-photon excited fluorescence microscopy (Figs. 4.15.4 and 4.15.5).

Materials

- Cells expressing GFP or labeled with fluorescent dye (Support Protocol 1)
- Collagen lattice containing fluorescent cells (Support Protocol 2)

Additional reagents and equipment for designing a microscope system for HHGM (Basic Protocol 1)

1. No specific cell label is required if the cells endogenously express GFP and thereby provide an intrinsic fluorescent signal for detecting their morphology. Alternatively, the cytoplasm or compartment of interest may be labeled with a fluorescent dye, such as Calcein AM or CFSE which label the cytoplasm (see Support Protocol 1).

2. Incorporate the cells within a 3-D collagen lattice (see Support Protocol 2).

3. Select a wavelength between 800 and 880 nm for excitation and 400 and 440 nm (λ/2) for detection of SHG of collagen fibers (e.g., 440/10). For fluorescence, use a dichroic filter below 510 nm and a band-pass filter suitable for the spectrum of FITC to detect the cells (e.g., 515/30) or a long-pass filter (e.g., LP515).

   Because absorption of light by 3-D collagen lattices is much weaker than by dense human tissues, the excitation wavelength of 800 nm has the advantage of strong excitation and emission strength.

4. To detect SHG and TPF in live samples, use simultaneous two-channel scanning. To determine the number of adjacent sections required for complete volume
Multimodal SHG and fluorescence microscopy of invasive cancer lesion. Frozen section (10 µm) of human dermatofibrosarcoma protuberans was stained with anti-CD34 antibody (staining cancer cells) and secondary Alexa 543–conjugated secondary antibody and DAPI and analyzed as described in Figure 4.15.3. The emission of SHG and DAPI was detected at 440/20 and Alexa 543 was detected at 620/40. (A) Two-color representation of SHG and fluorescence in epidermis, dermis, and cancer lesion. (B) SHG of collagen and DAPI signal alone, showing strongly reduced SHG in the cancer lesion. (C) Normalized intensity curves for normal and cancerous tissue show the loss of SHG at the border and within the cancer lesion, as reconstructed along the solid line in (A). For color version of this figure see http://www.currentprotocols.com.

reconstruction, run a preliminary z-stack and perform a frame-to-frame review of the images. If the same horizontal collagen fiber is present in at least one but no more than two adjacent sections and if diagonally sectioned fibers continuously “run” from frame to frame without jumps, sufficient overlap is present to achieve loss-less 3-D image acquisition. If the axial resolution is known, calculate the number of sections required. A stack depth of 20 µm requires at least five consecutive z-sections for an axial resolution of 5 µm (20× objective) or 11 for an axial resolution of 2 µm (63× objective) for loss-less reconstruction.

The aspect and resolution of SHG from collagen matches to great extent the reconstruction using confocal reflection microscopy (Fig. 4.15.5; see UNIT 4.5; Wolf et al., 2003). Using intravital microscopy in anesthetized mice, the combination of fluorescence and SHG allows the dynamic reconstruction of lymphocyte migration and interaction with antigen-presenting cells in the lymph node (Mempel et al., 2004b) and the dissemination of cancer cells in the mammary fat pad towards blood vessels and the immigration therein (Wang et al., 2002).

Quantitative reconstruction of the data set by xyz tracking shows both the fate of migrating cells in cell-cell communication and reverse transmigration in vivo and its relation to existing tissue structures, such as migration in between and along collagen fibers (contact guidance; Wang et al., 2002; Mempel et al., 2004a). Other approaches to image live cells include the tracking of intracellular vesicles in hepatocytes using THG (Debarre et al., 2006) and time-lapse reconstruction of the sarcomere shortening in striated muscle cells in vitro and in vivo (Plotnikov et al., 2006). In all cases, a fast enough acquisition of 3-D data stacks (up to 100 individual slices) requires relatively strong SHG and fluorescence signals in the backward direction and 4-D image reconstruction by a software independent of the scanning routine. Presently available shareware and commercially available software packages for 3-D reconstruction and data analysis are shown in Table 4.15.4.

5. Run a time series by serially acquiring z-stacks at defined time intervals, such as one frame per 15 sec for fast leukocytes or one frame per 5 min for slow tumor cells.

Observation periods may vary from a few minutes to several days, depending on the magnitude of migration dynamics.
Figure 4.15.5  Real-time reconstruction of cancer cell migration in 3-D collagen lattices using simultaneous multiphoton SHG and fluorescence detection (A), compared to confocal reflection and fluorescence microscopy (B). HT-1080 fibrosarcoma cells were labeled with Calcein-AM (green), incorporated into 3-D collagen lattices and monitored by either multiphoton (A) or single-photon excited confocal microscopy (B). Z-stacks of 16-µm scanning depth (4 sections) were obtained at 15 (A) and 5 (B) min time intervals and superimposed. Position changes of selected cells during the observation interval are highlighted by start and end point as well as migration path. Collagen is equally well reconstructed by SHG, compared to confocal reflection microscopy (Friedl et al., 1997). Preserved cell elongation and position change during the entire observation indicate the absence of photodamage in both settings (incident light power in (A) was 15 mW). Bars = 80 µm. Time (min) is indicated in the lower left corner. For color version of this figure see http://www.currentprotocols.com.
SUPPORT PROTOCOL 1

CYTOPLASMIC STAINING OF LIVE CELLS

This procedure is used to stain the cells in 3-D collagen cultures to differentiate the cells from the collagen fibrils.

Materials

- Cultured cells in culture flask or petri dish
- Calcein-AM (Molecular Probes)
- DMSO (Invitrogen)
- Dulbecco’s modified Eagle’s medium (DMEM) or other medium (e.g., PBS; APPENDIX 2A); may contain serum and antibiotics

1. Add a small aliquot of Calcein-AM from stock solution (1 mM; in DMSO) to the medium of the culture in a collagen lattice to obtain a final concentration of 1 µM.
2. Incubate the culture for 60 min at 37°C.
3. Remove the supernatant and wash the culture twice using medium or PBS.

SUPPORT PROTOCOL 2

ESTABLISHMENT OF 3-D COLLAGEN CULTURES

3-D collagen lattices represent a simple yet biologically meaningful alternative to 2-D liquid culture approaches to measure cell functions, such as proliferation, survival, differentiation, and migration (Friedl et al., 1997; Friedl, 2004). This procedure describes how cells are incorporated into the 3-D lattices so they are embedded from the onset of the culture.

Materials

- Adherent cells
- 2 mM EDTA solution in PBS, pH 7.4
- Dulbecco’s modified Eagle’s medium (DMEM) or other medium; may contain serum and antibiotics (APPENDIX 2A)
- 10× concentrated minimal essential Eagle’s medium (MEM; Flow Laboratories) or other 10× concentrated medium
- 7.5% (w/v) sodium bicarbonate solution (Life Technologies)
- 3.0 mg/ml collagen stock solution, acidified (e.g., bovine dermal collagen; Vitrogen; Nutacon BV or rat tail collagen; Becton Dickinson)
- Migration chamber (see recipe)

1. Detach adherent cells (e.g., 2 × 10^5) by incubating in 2 mM EDTA for 2 to 5 min, 37°C. Wash twice with 1 to 10 ml of medium, centrifuging 10 min at 300 × g, 4°C each time. Decant the supernatant and resuspend the cells in 500 µL medium.

   EDTA is preferred over trypsin/EDTA because it does not lead to alterations in surface protein composition caused by the enzymatic removal of adhesion receptors.

2. Add 100 µl of 10× MEM to 50 µl sodium bicarbonate in a small sterile tube. Check that the indicator color changes from yellow (acidic) to purple (basic).
3. Add 750 µl cell-free collagen stock solution and mix until homogenous slightly purple color is obtained.

   Avoid air bubbles while mixing.

4. Add 450 µl cell suspension in medium and mix until homogenous bright pink color is reached. Again, avoid generating air bubbles.

   The pH should now be 7.8 to 8.0. To verify the pH use a pH indicator strip.
5. Add the cell-containing collagen solution into the incubation chamber.

6. Place the incubation chamber into the incubator (37°C, 5% CO₂) for 15 to 30 min for collagen polymerization and equilibration of the gas conditions. The final pH should be 7.4 to 7.5.

    If a commercially available climatized incubator is used with the video microscope, steps 1 to 6 can be modified for ECM culture in conventional petri dishes or microtiter plates.

7. Fill the chamber with warm and previously equilibrated medium.

    This medium can contain chemokines or other factors, such as antibody or pharmacological inhibitors.

8. Close the migration chamber, place it on the microscope stage and proceed with Basic Protocol 4.

**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.*

**Migration chamber**

A migration chamber is a cell incubation chamber, either from commercial sources or self-constructed (Friedl and Bröcker, 2004). Most microscope manufacturers provide small chambers for a small petri dish or specialized incubation chambers for extra small volumes. Alternatively, an incubation chamber can be self-constructed. An example for a self-constructed migration chamber is detailed in Friedl and Bröcker (2004).

**COMMENTARY**

**Background Information**

*Physical principle of two-photon and multiphoton effects*

Two-photon excitation occurs by the near-simultaneous absorption of two or more photons by a molecule normally excitable by one photon of twice the energy and occurs the moment two lower energy photons are simultaneously absorbed by the same molecule (Fig. 4.15.6A). The first photon generates a virtual state of the electron and the second must be absorbed before the virtual state is de-excited (i.e., within the Heisenberg approximation of 10⁻¹⁵ sec). Together, both photons result in an excitation of nearly the same impact as a single higher energy photon excitation of the same molecule. In order to achieve a high enough photon density for two-photon excitation, the irradiant laser light needs to be phase-(mode)-locked and pulsed.

The pulse width of the irradiant beam is determined from an autocorrelation (in time) experiment in which the full-width at half-maximum of the pulse is in the range of 2 to 10 psec (for picosecond lasers) or 100 to 300 fsec (for femtosecond lasers) interrupted by an illumination-free interval of several nanoseconds, which results in a repetition rate of 76 to 96 MHz (Fig. 4.15.6A). Because of the high photon density during the pulse, high probabilities for two-photons or multiple photons to become near-simultaneously absorbed by a molecule is high, provided minimum of tens of milliwatts (for fsec pulses) are present in the focal plane under the objective (corresponding to an overall intensity ranging from MW/cm² up to GW/cm²).

Two-photon excitation is quadratically related to the illumination intensity at the focal point, and only at the focal point is a significant number of photons achieved. Consequently, signal arises only from the scanned focal volume, and thin optical sections are being generated in 3-D samples, whereas little excitation and photodamage occurs outside the focal plane. Therefore, multiphoton excitation lacks photobleaching and phototoxicity in regions above or below the focal plane.

*Higher harmonic generation (HHG)*

HHG includes SHG and THG (Fig. 4.15.6B). SHG results from the sum frequency doubling to produce a narrow band of half the excitation wavelength and twice the energy (Fig. 4.15.6B). THG results from the sum frequency tripling to produce emission at a third of the excitation wavelength and triple
the energy (Fig. 4.15.6B). HHG occurs only in the focal plane providing sufficiently high photon density and, similar to multiphoton fluorescence microscopy, represents an inherently confocal approach allowing 3-D reconstruction from z-slices (Fig. 4.15.6C). Emitted HHG signals are commonly detected by photomultipliers (Fig. 4.15.6C). In contrast to fluorescence, most cases of HHG do not involve the absorption of light, thus relatively little heat is produced. Therefore, pure harmonic generation imaging tends to be much less phototoxic than two-photon or multiphoton fluorescence microscopy.

**SHG**

SHG is a nonlinear coherent scattering process that conserves energy. The summation of the light rays into the second harmonic frequency results from phase matching of the incident light with an environment that encourages summation of frequencies. The resulting signal

\[ \text{SHG} \propto \frac{(p \cdot \chi^{(2)})^2}{\tau} \]

is dependent on the square of the input laser power, \( p \), a susceptibility coefficient, \( \chi \), and the width of the laser pulse, \( \tau \). As the light rays pass through this environment the electric field of the light exerts forces on the electron charge distribution and consecutive changes in a dipole moment, referred to as the electric polarization. The electric polarization (\( P \)) is linear, but for high-energy electric fields, such as an incident laser pulse (\( E \)) of high energy, \( P \) becomes a summation of linear and nonlinear terms. SHG is sensitive to structural molecular alterations caused by external factors such as temperature, folding, osmolarity, and ionic strength; biological structures may vary in their proportion of linear and nonlinear properties. Therefore, SHG should be tried out for each sample and contains an empirical component. The electric polarization is further determined by a susceptibility factor (\( \chi \)) which depends on the environmental and molecular structure and polarization angle with respect to the light rays, as defined by

\[ P_{\text{SHG}}^{(2)} = \chi^{(2)} \cdot E \]

The intensity of emitted light is nonlinearly proportional to the input light frequency but has a quadratic dependence, which means that below a certain threshold at low excitation energy no signal will be obtained whereas a near linear emission is achieved above the threshold. In the linear range, intensity changes can thus be used for quantitative image analysis.

The strength of SHG is further dependent on the size scale to which the noncentrosymmetric (NCS) medium is effective, called the coherence length, \( L_c \), \( \Delta k \cdot L_c \approx \pi \), where \( \Delta k \) is the difference in wavevectors,

\[ k = \frac{2\pi}{\lambda} \]

between the fundamental and second harmonic waves of wavelength, \( \lambda \). The coherence length is dependent on the incident wavelength and must be in the order of the NCS period size.

SHG is an anisotropic process and mainly propagates in the same direction of the incident light, whereas only a minor fraction is backscattered (Fig. 4.15.6C). The ratio of SHG signal for forward to backward direction often ranges above 5:1 (tubulin, fibrillar collagen; Dombeck et al., 2003), thus forward detection is required for low S/N ratio.

**THG**

THG is based on the “third order susceptibility” which means that its signal has a cubic dependence on input intensity:

\[ P_{\text{THG}}^{(3)} = \chi^{(3)} \cdot E \cdot E \cdot E \]

Unlike the physical and geometrical rules of SHG, all materials potentially possess properties to elicit THG to a varying degree, but the THG signal intensity is more sensitive to and requires high average powers and small pulsewidths,

\[ \text{THG} \propto \frac{(p \cdot \chi^{(3)})^3}{\tau^3} \]

Similar to SHG, the susceptibility factor, \( \chi^{(3)} \) in THG is variable and also changes vastly from material to material, thus extensive spectral examination is required for each individual sample type. Near-infrared input elicits third harmonic generation of ultraviolet light of relatively lower penetration depths in scattering samples, whereas the use of excitation above 1200 nm reaches THG signals in the visible range. THG is an isotropic process, therefore emission signal intensities are equally strong in forward and backward directions.

Although the above theoretical considerations predict ubiquitous THG for most biological samples, empirical evidence suggests that THG signals, which are intrinsically weak,
Figure 4.15.6  Principles of single-, two-, and three-photon excited fluorescence (A), compared to second and third harmonic generation (B, C). (A) Excitation of fluorescence. Single-photon excitation results from a continuous photon flux, generating continuous emission. Two- and three-photon excitation are caused by high-energy pulses of light of longer wavelengths. In both cases, the emission spectrum is near identical. (B) Excitation of second and third harmonic generation. Two (top) or three photons (bottom) of pulsed high-energy light are converted to one photon of two- or three-fold energy, respectively, corresponding to half or a third of the wavelength. (C) Beam path of forward and backward SHG induced by triple-helical collagen. Abbreviations: FL, fluorescence emission; S₀, nonexcited state of an electron; S₁, excited state of an electron; Sₙ, short-lived excited state of higher energy level; ω, frequency of the light; λₑₓ, wavelength of excitation beam; λₜ(SHG), wavelength resulting from SHG. For color version of this figure see http://www.currentprotocols.com.
are further dampened by homogeneous out-of-focus regions just above and below the focal point that interfere destructively and delete the net signal.

Critical Parameters and Troubleshooting

Photodamage in live samples

The maximum photon flux and thus signal-to-noise ratio is limited by photodamage which varies for different sample types. Even though the use of (near-)infrared light causes less photodamage to live cells and tissues than visible or UV light excitation, the use of too high peak power required for enhancing weak two-photon and multiphoton effects can severely compromise cell function and integrity. The mechanisms of laser-induced cell damage are not resolved, however a combination of local heat, absorption of incident illumination, and toxic photoproducts of chromophores are plausible explanations. A reliable net indicator of photodamage is the generation of gas bubbles in the sample causing the loss of specific signal of both SHG and fluorescence (Fig. 4.15.7).

As a rule of thumb for the tuning range from 700 to 1000 nm, intensities of up to 7 mW, as measured by a powermeter below the objective, will not cause photodamage and intensities up to 15 mW can be tolerated by live cells over several hours if the lag phase between scanning is sufficiently long (Fig. 4.15.6). Much higher excitation intensities up to 100 mW or more in the focal plane can be used for detecting weak signals of the tissue microarchitecture without signs of photodamage or denaturation, such as collagen and elastin in fixed samples (Dombeck et al., 2005; Boulesteix et al., 2006; Debarre et al., 2006). At illumination >1000 nm, higher intensities are well tolerated. Long-term continuous-time scanning in living Xenopus embryos using excitation at 1280 nm and a power of 75 mW is required to generate strong SHG and THG signals yet lacks apparent toxicity (Sun, 2005). Likewise, excitation of membrane potential dyes at 1064 nm up to 80 mW is tolerated by neuronal cells, suggesting that long-term real-time microscopy by multiphoton excitation will develop towards better tolerability using excitation wavelengths above 1 µm.

Anticipated Results

Other structures detected by HHGI

Microtubules In cells, polymerized tubulin forms the filamentous microtubule network important in cell polarity, vesicle trafficking, and mitosis. Microtubules in the polymerized state, but not after depolymerization by nocodazole, generate relatively weak SHG signals in brain slices (Dombeck et al., 2003). Mitotic spindles can be detected in living cultured cells (Dombeck et al., 2003) or intact embryos (Sun, 2005).

DNA. Condensed chromosomes during mitosis, but not interphase DNA, after extraction and fixation and mounting on a glass slide can be detected by SHGI, using 800-nm excitation wavelength (Gauderon et al., 2001). By contrast, no published example has monitored SHG or THG of nonlabeled DNA in live cells and tissues, suggesting that the sample preparation contributes to the noncentrosymmetry and spatial arrangement of the chromosome structure.

Bone and teeth. Mineralized bone matrix and developing central bone marrow cavity in the mouse embryo is detected by THG up to penetration depths of several hundred microns (Oron et al., 2004). Despite progressive mineralization, both loose and compact bone regions are resolved in detail. In sections from teeth, linear SHG and even stronger THG signal is generated by dentin, most likely originating from the underlying collagen fiber scaffold (Kao, 2004). Particularly inorganic crystals and inorganic complexes in tissue display an abrupt change in refractive index at their surfaces, relative to the surrounding tissue, which is strongly susceptible to THG.

Membrane potential. Styryl dyes are elongated molecules with large hyper-Rayleigh scattering efficiency that get aligned in roughly perpendicular order along the plasma membrane to form anisotropic scaffolds, the orientation of which is sensitive to the transmembrane electric field. Alterations of the membrane potential, as during depolarization, cause electro-optic realignment and thus, anisotropy that is exploited as increased SHG for membrane potential measurements (Pons et al., 2003; Dombeck et al., 2004; Millard et al., 2004; Sacconi et al., 2005). Live-cell studies indicate that the dyes RH 237, FM4-64, and JPW1114 show sufficient signal-to-noise ratio and little toxicity (Dombeck et al., 2005) and can be combined with conventional fluorescent indicators, including Ca2+- and Na+-sensitive dyes. The absolute change in signal intensity amounts to 5% to 20% per 100 mV which is linear to the amount of depolarization and can be calibrated by voltage-gated patch clamp technique (Dombeck et al., 2005; Sacconi et al., 2005). Differences of absolute
SHG sensitivity are specific for each dye, cell type, and subcellular location of the region of interest, and transformed cells appear to develop stronger signals than nontransformed cells (Dombeck et al., 2005; Sacconi et al., 2005). Forward detection in transparent cell culture as well as backward detection (at 80% lower efficiency) from thick tissue slices at high temporal (<1 msec) and spatial resolution can be achieved (Dombeck et al., 2005). Thus, SHG imaging will be an important approach to intravital reconstruction of fast-voltage transients in cell membranes during cell-cell signaling at minimum interference with the electrophysiological properties of the membrane.

Plants. Cellulose and starch are both polymers of glucose. Cellulose is composed of a polymer of β-1,4 linkages and starch of α-1,4 linkages. Individual polymer chains are cross-linked and arrange in a crystal-like order to form fibrils of high tensile strength that are susceptible to SHG. Starch, because of its open helical structure, causes higher SHG intensity than cellulose, which is more linear in structure (Cox et al., 2005).

Lipid bilayers. Although all materials have the susceptibility for THG, lipid membranes contained in aqueous medium have a comparatively high third-order susceptibility due to the formation of water-lipid interfaces. Strong THG signals are generated by lipid membrane and lipid bodies in tissues and in vitro systems, including lipid bodies and vesicles in live hepatocytes (Debarre et al., 2006).

SHG and THG probes. Several extrinsically applied labels exhibit strong SHG signals, either based on their own structure, or after their interaction with cell and tissue components. These include gold nanoparticles for immunological staining (Jumah et al., 2004; Lippitz et al., 2005) and polyhydroxybutyrate (PHB) polymers that generate biodegradable fibrillar structures used for tissue engineering (Deng et al., 2003).

Time Considerations

The acquisition of a single image frame takes 0.2 to 5 sec, depending on the scanning frequency and resolution. Image stack acquisition from the reconstruction of a 3-D volume requires a few up to several hundred images, thus may take several seconds up to minutes for a single data point. Whereas a single microscopy experiment is complete within a few minutes to hours, sample preparation, as well as image reconstruction and data analysis, may take up to days and weeks.
Acknowledgements
The authors would like to thank Volker Andersen for expert system design and helpful discussions and Matthias Gunzer for providing information on software use.

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Contributed by Peter Friedl, Katarina Wolf, and Gregory Harms

University of Würzburg

Würzburg, Germany

Ulrich H. von Andrian

Harvard Medical School

Boston, Massachusetts