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HAL Id: hal-00836429
https://hal-polytechnique.archives-ouvertes.fr/hal-00836429
Submitted on 12 Oct 2013

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Second-harmonic microscopy of unstained living cardiac myocytes: measurements of sarcomere length with 20-nm accuracy

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Received March 22, 2004

We extend second-harmonic generation (SHG) microscopy to the measurement of sarcomere length in unstained living cardiac myocytes with 20-nm accuracy. We quantify individual sarcomere shortening in the presence of saxitoxin and find that it is in agreement with mechanical measurements of atrial tissue contracture. This functional application of SHG microscopy is generally applicable to quantify the physiological effects of drugs on contractile tissue. Our data also suggest that packed myosin heads in sarcomere thick filaments are responsible for the large second-harmonic endogenous signal in muscle tissue. © 2004 Optical Society of America

OCIS codes: 190.0190, 190.4160, 180.0180, 180.5810, 170.0170, 170.3880.

Second-harmonic generation (SHG) microscopy was demonstrated recently as a novel biological imaging technique that is well adapted to the specific observation of labeled membranes or unstained macromolecular structures composed of densely packed, well-organized proteins such as collagen or tubulin. A SHG endogenous signal in skeletal muscle was also reported and was attributed to the actomyosin complexes in sarcomeres, the muscular contractile units.

In this Letter we extend SHG imaging to live unstained cardiac myocytes, and we demonstrate that this technique allows accurate determination of subresolution variations of sarcomere length. In comparison, electron microscopy provides a resolution in the nanometer range but requires fixation and staining. Confocal fluorescence microscopy is less invasive and can be applied to living cells, but it requires the introduction of fluorophores, typically by exogenous staining or fusion protein (e.g., green fluorescent protein constructs) transfection. Moreover, the accuracy of length measurements with fluorescence images is very dependent on chromophore efficiency and photostability. In the following, we show that SHG microscopy allows direct observation of sarcomere contracture in unlabeled muscle cells and provides quantitative measurements with typical 20-nm accuracy. We show that this technique allows one to measure the physiological effects of a toxin on cardiac myocytes. Finally, images of sarcomere shortening provide evidence that the main source of intrinsic SHG signal lies in the myosin heads.

Sarcomeres are the elementary contractile units in cardiac myocytes and in skeletal muscle. They comprise thin actin filaments, with associated proteins, attached to the Z disks at both ends of the sarcomere (see Fig. 1). In the middle of the sarcomere, actin filaments overlap thick filaments of myosin, a protein with a long tail and globular heads. The myosin heads face in opposite directions on either side of a bare central region of the thick filaments, around the M line. During contraction, the myosin heads slide along the actin filaments, which results in sarcomere shortening, with no change in the length of the thin and thick filaments.

Atrial myocytes were obtained by enzymatic digestion of the sinoatrial region of an adult frog heart. In the resting state these myocytes are approximately 300–500 μm in length and 3–5 μm in diameter. They were kept at room temperature in a polystyrene tissue culture dish containing a Ringer solution, composed of (in millimoles per liter) NaCl, 110.5; KCl, 2.5; CaCl₂, 2; MgCl₂, 1; Na pyruvate, 5; HEPES (NaOH), 10; glucose 10, albumine (1 mg/ml); and with pH 7.35. For imaging, myocytes were directly observed in the Ringer solution without staining.

SHG imaging was performed with a home-built laser scanning microscope comprising a femtosecond titanium sapphire laser (Mira, Coherent), galvanometer mirrors (GSI Lumonics), photon-counting photomultiplier modules (Electron Tubes), and laboratory-designed 100-MHz counting electronics. Detection channels were implemented in both the reflected and the transmitted directions to allow for simultaneous detection of two-photon excited fluorescence and SHG signals by use of appropriate filters. For the experiments reported here we used a 0.9-N.A., 60× water-immersion objective lens.
STX at 8-nM concentration inhibits logical and mechanical techniques. We found that diac cells. STX blocks Na
shellfish poisoning toxin saxitoxin (STX) on carto
quantify the physiological effect of the paralytic
signal arises principally from the myosin heads. Images suggest that the bare central area in the thick
the density of harmonophores. Consequently, these
the SHG signal dip is significantly larger than the
continuous illumination.

Typical SHG images are displayed in Fig. 2. Myocytes appear as striped structures that produce a
readily detectable signal, typically peaking at 2 × 10⁷
detected photons/s with 20-mW excitation power.
Similarly to what is found in SHG images of skeletal
muscle, bright areas are attributed to the myosin
filaments and dark areas to the regions around the Z
lines. Looking more precisely at individual sarcomeres, one can distinguish a darker region within the
area that produces a SHG signal. This may partly
be due to destructive interference, since opposite
thick filaments on both sides of the M line exhibit a
centrosymmetric orientation. However, the width of
the SHG signal dip is significantly larger than the
focal volume, which also indicates a local decrease of
the density of harmonophores. Consequently, these
images suggest that the bare central area in the thick
filaments produces no SHG signal and that the SHG
signal arises principally from the myosin heads.

The following, we use the above-described method
to quantify the physiological effect of the paralytic
shellfish poisoning toxin saxitoxin (STX) on car

do not affect cellular and sarcomeric integrity, and the SHG signal has
the same order of magnitude. However, sarcomeres appear to be smaller in STX than in the control, as
revealed by further analysis of the SHG images.

Figure 3 describes the procedure for analyzing SHG
data. The signal profile along a single myocyte is
extracted, and the series of double peaks is fitted
with Gaussians of adjustable heights, center positions,
and widths. This procedure allows one to determine
four relevant lengths, as indicated in Fig. 3B and
summarized in Table 1. The bare length is the
distance between the two SHG peaks in a sarcomere.
The dark length is the distance between two consecutive SHG peaks of neighboring sarcomeres, on
both sides of the Z line. The full width at 1/e²
of the SHG signal in a sarcomere is obtained as
the sum of the bare length and the half-widths at
1/e² of both adjacent SHG peaks. Finally, the total
length of a sarcomere is obtained as the distance
between two SHG peak pairs. All these lengths are
determined as differences in the center positions of
the Gaussian fits. The uncertainty is approximately
10 nm in peak localization and 15 nm in length
determination. In a manner similar to what is com
monly reported in single-molecule experiments, the
achieved accuracy is well below the microscope reso
lution, owing to the remarkable contrast of the SHG
signal. However, we observed small variations
among sarcomeres within a single myocyte and from
cell to cell. Finally, taking this sample variability
The measurement of sarcomere length in unstained heads in the focal volume. As a coherent process, SHG scales as the square of the also consistent with the large magnitude of the signal. This hypothesis on the origin of the SHG signal is significant contribution from myosin heavy chain B. auricle. which is consistent with data already reported for frog 2.14 of length of filament, and there are approximately 300 myosin heads per arrangement and separated by approximately 50 nm, myosin filaments are packed in a quasi-crystalline endogenous SHG arises from densely packed, orga- noncentrosymmetric organizations. Consequently, the central area without myosin heads. Conversely, the overlap of the actin and myosin filaments increases, so the area with only thin filaments decreases. This area corresponds to the dark length measured in our experiments, which consistently decreases on contraction. Therefore these measurements provide evidence that the myosin filaments, and presumably the myosin heads, are responsible for the SHG signal, independent of their overlap with actin. This result is in agreement with reported measurements of genetically modified nematode muscle that demonstrated a significant contribution from myosin heavy chain B. This hypothesis on the origin of the SHG signal is also consistent with the large magnitude of the signal. As a coherent process, SHG scales as the square of the harmonophores’ density and can be obtained only from noncentrosymmetric organizations. Consequently, endogenous SHG arises from densely packed, organized harmonophore distributions. Indeed, myofibril myosin filaments are packed in a quasi-crystalline arrangement and separated by approximately 50 nm, and there are approximately 300 myosin heads per filament, which results in a high density of myosin heads in the focal volume.

In summary, we have extended SHG microscopy to the measurement of sarcomere length in unstained living cardiac myocytes with unprecedented 20-nm accuracy. We demonstrated that this technique provides a quantitative measurement of the subcellular physiological response to a natural toxin, consistent with observed global tissue contracture. This technique is generally applicable to measurement of small sarcomere length variations on application of a toxin, a drug, or an ionic solution. Given the remarkable efficiency and contrast of SHG in sarcomeres, it is straightforward to extend this technique to measurements in intact tissue, including dynamic observation of contraction. In conclusion, SHG microscopy appears to be a promising tool in nanopharmacology of living unlabeled cardiac and muscular tissue.

The authors thank X. Solinas, M. Bierry, and J.-M. Sintes for technical assistance with the microscope design and assembly. M.-C. Schanne-Klein’s e-mail address is marie-claire.schanne-klein@polytechnique.fr.

### References


### Table 1. Sarcomere Characteristic Dimensions in the Absence and in the Presence of STX

<table>
<thead>
<tr>
<th>Samples</th>
<th>Bare Length</th>
<th>Dark Length</th>
<th>Full Width SHG</th>
<th>Sarcomere Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6/60</td>
<td>0.80 ± 0.02</td>
<td>1.51 ± 0.02</td>
<td>1.79 ± 0.02</td>
</tr>
<tr>
<td>STX</td>
<td>10/52</td>
<td>0.75 ± 0.02</td>
<td>1.39 ± 0.02</td>
<td>1.82 ± 0.02</td>
</tr>
</tbody>
</table>

*All dimensions (in micrometers) are determined by Gaussian fitting of SHG images (see text and Fig. 3).*

*aNumber of myocyte images analyzed and total number of sarcomeres fitted.
*bSTX (8 nM) in Ringer solution.