Single-beam multimodal nonlinear-optical imaging of structurally complex events in cell-cycle dynamics

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Abstract

We demonstrate a multimodal nonlinear-optical imaging that combines second- and third-harmonic generation (SHG and THG) with three-photon-excited fluorescence (3PEF) as a means to resolve fine details of the cell structure and trace its transformations throughout structurally complex episodes of cell-cycle dynamics, including the key stages and signatures in cell division. When zoomed in on cell mitosis, this technique enables a high-contrast multimodal imaging of intra- and extracellular signatures of cell division, detecting, via a multiplex, 3PEF/SHG/THG readout, a remarkable diversity of shapes, sizes, and symmetries in a truly single-beam setting, with no need for beam refocusing or field-waveform re-adjustment.

1. Introduction

Nonlinear optics provides a vast arsenal of concepts and tools for \textit{in vivo} bioimaging [1, 2]. In biological tissues, where the capabilities of linear optical methods are inevitably limited because of strong absorption and scattering, nonlinear-optical approaches offer a unique combination of a high resolution, large imaging depth, as well as enhanced brightness and chemical specificity [3–8]. As one of its most powerful resources, nonlinear-optical microscopy enables multiple imaging modalities [9–14] by combining optical readout signals generated via different nonlinear-optical processes—second- and third-harmonic generation (SHG and THG), two- and three-photon-excited fluorescence (2PEF and 3PEF), as well as coherent and stimulated Raman scattering [6–8, 15–21].

Whenever a meaningful combination of such signals can be simultaneously read out with a suitably tailored beam geometry, a wealth of unique microscopic information on a targeted object becomes available, allowing fine structural details of biological cells and intracellular compartments, to be detected at large imaging depths with a high resolution, high brightness, high contrast, and a high chemical specificity [3, 6–8]. However, identifying a suitable beam geometry for multimodal nonlinear-optical bioimaging is anything but trivial. Nonlinear optical processes are highly sensitive to both the intensities of the driving fields and their beam-focusing geometry. Moreover, coherent nonlinear processes are drastically different from their incoherent counterparts in beam divergence, directness, and phase matching. These factors often impose conflicting requirements on beam focusing, laser intensities, laser pulse widths and bandwidths, carrier frequencies, and pulse repetition rates.
Here, we show that these challenges can be addressed with a compact and versatile single-beam nonlinear imaging setting that combines SHG and THG with 3PEF, providing means to resolve fine details of the cell structure, as well as structurally complex episodes within the cell cycle. When zoomed in on cell mitosis, this technique will be shown to enable a high-contrast, high-resolution multimodal imaging of intra- and extracellular signatures of cell division, detecting, via a multiplex, 3PEF/SHG/THG readout, a remarkable diversity of shapes, sizes, and symmetries in a truly single-beam setting, with no need for beam refocusing or field-waveform re-adjustment.

2. Concept

Central to our approach is an integration of multiple imaging modalities based on coherent and incoherent nonlinear-optical processes and resorting to both stained and stain-free imaging techniques all integrated on a compact, single-laser-beam imaging platform. Providing a platform for the implementation of such vastly multimodal imaging in a single-beam format is a homebuilt femtosecond long-cavity Cr:forsterite (CrF) laser (figure 1(a)) operated near the soliton blowup threshold [22–26]. The central wavelength of the short-pulse output of this laser, $\lambda_0 \approx 1.25 \, \mu m$, falls within one of the high-transmission windows of biological tissues, sometimes referred to as the second near-infrared spectral window [27]—the spectral band within which radiation attenuation by soft biotissues is relatively low (figure 1(b)). Moreover, both the second and third harmonics of the 1.25 $\mu m$ output of this laser (sketched in figure 1(b) as signals centered at 625 and 417 nm) fall within the spectral range where the absorption of biological tissues is still not prohibitively high, as it would be the case for THG driven by a Ti:sapphire laser. Multimodal, SHG- and THG-based bioimaging with CrF laser sources is discussed in an extensive earlier literature (see, e.g. [5, 28–30]).

These advantages, however, come at a price. The minimum number of 1.25 $\mu m$ photons needed to drive a fluorescence response from most of the available high-brightness genetically encodable fluorescent markers, including green fluorescent proteins (FPs), is $n = 3$. This brings up an interesting question as to whether three nonlinear-optical processes—SHG, THG, and 3PEF—governed by nonlinear-optical susceptibilities $\chi^{(m)}$ of different orders—$m = 2$ for SHG, $m = 3$ for THG, and $m = 5$ for 3PEF—can all be integrated on a compact single-beam platform. Experiments presented below show that such integration is possible. The short-pulse output of our long-cavity mode-locked CrF laser not only proves highly beneficial for bright SHG and THG,
but also provides a driver for efficient three-photon excitation of a meaningful variety of FPs, which enable, via their shifted fluorescence spectra (as sketched in figure 1(b)), a high-contrast multimodal 3PEF imaging of selectively stained cell structures and intracellular compartments. Moreover, with a careful optimization of the CrF laser output toward a shorter pulse width, a higher average power, and a higher repetition rate [26], the intensity of the multiplex, SHG/THG/3PEF readout can be increased without boosting the laser energy to a level where unwanted photoinduced processes in a biological system become unavoidable.

3. Laser platform and methods

3.1. Laser

Experiments presented below in this paper were carried out with an extended-cavity, ytterbium-fiber-laser-pumped CrF laser, delivering laser pulses with a central wavelength \( \lambda_0 \approx 1.25 \mu \text{m} \) as its output with a pulse width \( \tau_0 \) in the range of 50–80 fs, a bandwidth variable from 22 to 44 nm, and a pulse energy up to \( E_0 \approx 30 \text{ nJ} \) at a pulse repetition rate \( f_0 \approx 30 \text{ MHz} \) [25, 26]. The spectrum of CrF-laser pulses is measured with an infrared spectrometer (IR Spec in figure 1(a)) based on a cooled InGaAs array and designed for the 920–1670 nm wavelength range. The beam profile of the CrF-laser output is characterized with a homebuilt apparatus for z-scanned \( M^2 \) measurements (\( M^2 \) in figure 1(a)).

While the 30 nJ level of laser pulse energy is, of course, way too high to be directly focused on living cells, it provides a comfortable margin of available laser energy to sustain inevitable radiation losses in all the down-the-line optical components (figure 1(a)) needed to deliver the laser beam to the microscope-objective (MO) plane, focus this beam onto a sample, and provide chirp pre-compensation for the minimum pulse width on the sample. Reliably detectable third-harmonic and 3PEF signals were generated in our experiments at a typical level of the on-sample laser driver power of 10 and 25 mW, respectively.

3.2. Laser-pulse characterization

Because the pulse width of the laser driver is central for high-brightness, high-resolution, minimally disruptive nonlinear imaging, special measures are taken in our system for an accurate characterization of laser pulses and finely adjustable pre-compensation for pulse chirping and stretching by downstream optical components. Quantitative pulse characterization is performed in our system by means of frequency-resolved optical gating (FROG) based on SHG in a 50 \( \mu \text{m} \)-thick beta barium borate crystal (FROG in figure 1(a)). A flip mirror (FM in figure 1(a), the full arrangement of mirrors behind the FM is not shown for the compactness of the drawing) allows SHG FROG traces to be measured without any realignment of the optical scheme, providing a full characterization of the spatial, temporal, and spectral mode of the driver field used in nonlinear bioimaging. A typical SHG FROG trace of the CrF-laser output with a pulse energy of about 30 nJ is presented in figure 2(a). The temporal envelope and phase retrieved from this trace (figure 2(b)) reveal a field waveform with a pulse width of about 80 fs and small residual high-order phase distortions.

3.3. Big-picture perspective

In a bigger-picture perspective, a laser system with such a combination of parameters—radiation wavelength, pulse energy, pulse width, repetition rate, as well as peak and average power—is ideally suited for a vast range of laser bioimaging modalities, including high-\( m \) nonlinear microscopy, such as 3PEF imaging. While laser systems for 2PEF imaging [2, 31–33] have become a routine tool of life-science studies [1, 7], laser sources for 3PEF microscopy [20, 21] are still the work in progress that goes through the phase of conceptual and engineering development. The CrF laser system demonstrated in this work helps advance this development via a merger of two ideas. First, this laser combines 3PEF with other, better established and, perhaps, better understood imaging modalities, such as 2PEF, SHG, and THG, en route to a single-beam high-resolution microscopy of morphologically complex cellular systems and structurally complex events in cell-cycle dynamics. Second, the approach that we develop in this work aims to address the problem of low 3PEF yield—an inevitable cost of high-\( m \), \( \chi^{(3)} \) nonlinearity behind the 3PA process—by enhancing the 3PEF power without increasing, wherever possible, unwanted photoinduced effects in a biological system. To this end, we aim to increase the 3PEF power, \( p_3 \propto f_0 \varphi_3 E_0^3 \tau_0^2 \), without boosting too much the laser pulse energy \( E_0 \), by increasing the pulse repetition rate \( f_0 \), reducing the laser pulse width \( \tau_0 \), and choosing the laser wavelength \( \lambda_0 \) in such a way as to minimize radiation loss due to scattering and absorption and to maximize \( \varphi_3 \) of the fluorescence marker as much as possible.

Experiments presented below in this paper suggest that, when integrated into a suitably designed imaging setting, which combines a carefully optimized beam focusing and thoughtfully chosen, high-brightness optogenetic fluorescent markers, our CrF laser is well-placed to confront the challenges of single-beam nonlinear imaging, offering a powerful tool for multimodal microscopy based on multiphoton processes relating to optical susceptibilities \( \chi^{(m)} \) of vastly different orders—from \( m = 2 \) for SHG to \( m = 5 \) for TPEF.
The available alternative laser sources for 3PEF microscopy include an Er-fiber chirped-pulse amplifier (CPA), delivering a frequency-shifted ≈60–65 fs, 1 MHz soliton output at a central wavelength of ≈1.62–1.68 µm [20, 34], a noncollinear optical parametric amplifier (OPA) with a ≈55 fs, 0.8 MHz output at λ₀ ≈ 1.3 µm [35], a monolithically integrated 50 MHz, Er-fiber-based laser with a frequency-shifted 3 nJ, 75 fs soliton output at λ₀ ≈ 1.65 µm [36], and an Yb-fiber–CPA–OPA, yielding ≈90 fs, 1.25 MHz pulses at λ₀ ≈ 1.7 µm and ≈140 fs, 1.25 MHz pulses at λ₀ ≈ 1.3 µm [37]. In our setting, pulse widths as short as τ₀ ≈ 55 fs are achieved right in the microscope sample plane at λ₀ ≈ 1.25 µm and f ≈ 30 MHz. Given that 3PEF relates to a higher-order, χ(5) optical nonlinearity, laser pulses with such parameters are particularly advantageous for 3PEF imaging, opening routes toward practical biomicscopy with enhanced optical sectioning based on the use of higher-m nonlinear-optical processes [38].

3.4. Cells and staining
Imaging experiments were performed on populations of HeLa cells cultured in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum as a supplement at 37 °C in a 5% CO₂ incubator. These cultured cells were then co-stained with two fluorescent markers—SypHer3s and PhiYFP. To this end, the cells were transfected with a mixture of 1 µg of SypHer3s DNA and phiYFP DNA using 3 µl of X-tremeGene 9 transfection reagent per dish. In our experiments, we found no indication that such a fluorescent staining can have any detectable effect on SHG or THG. One of the markers used in these studies, SypHer3s [39], is encoded to express within the entire volume of HeLa cells, with no specificity to any cell compartment, but making HeLa cells clearly detectable against the intercellular space in nonlinear-optical images. As will be shown below in this paper, such staining is instrumental for studying structurally complex episodes of cell-cycle dynamics, such as cell mitosis. The second marker, PhiYFP, is intended for compartment-specific fluorescent labeling. Serving this purpose is a PhiYFP–fibrillarin expression vector, which is targeted...
specifically at nucleolar fibrillarin, thus providing an imaging contrast localized within cell nucleoli. Imaging of nucleoli is an important task of subcellular microscopy, as nucleoli serve as dynamic multifunctional subnuclear structures that play a central role in ribosome and ribonucleoprotein biogenesis, mitosis regulation, cellular response to stress, as well as cell-growth and cell-cycle regulation [40, 41]. Much of the ongoing research is focused on the processes that link nucleoli to a broad variety of human diseases, including multiple genetic disorders, predisposition to cancer, and neurodegenerative diseases [42–44].

3.5. Beam focusing and detection
The CrF-laser beam is focused into a cell culture in an upright-microscopy scheme with a water-immersion 20×, NA = 1.00 Olympus XLUMPlanFLN MO (figure 1(a)). An adjustable Thorlabs BE02-05-C telescope (T) is used to finely tune the diameter of the laser beam in the sample plane. The 3PEF readout from the sample is collected in the epi-direction through the same MO. The sample is scanned laterally (in the xy-plane) and axially (along the z-axis, chosen along the laser beam) with a fully automated high-precision 3D translation stage (figure 1(a)). The spectra of the 3PEF signals from PhiYFP and SypHer3s are centered, respectively, at ≈555 and 515 nm (figure 2(c)). Because the peaks in the spectra of these signals are separated by only 20 nm, special measures need to be taken to discriminate these two signals. In our experimental setting, such a discrimination is achieved by using a dichroic mirror (DM) (Thorlabs MD515 or equivalent, DM2 in figure 1(a)) with a sharp passband edge, as shown by the black dashed line in figure 2(c). Such a mirror transmits the fluorescence signal from PhiYFP, but reflects the fluorescence from SypHer3s, separating these signals into two detection channels. In their respective detection channels, the signals from PhiYFP and SypHer3s are further filtered by suitable bandpass filters, such as Chroma ET510/20 and Thorlabs MF559-34 or equivalent, with transmission spectra as shown by the dotted lines in figure 2(c), to be detected by two photomultipliers (Hamamatsu H7422PA-40 or equivalent, PMT1 and PMT2 in figure 1(a)). The second and third harmonics are collected in the forward direction, using a microscope condenser MC (figure 1(a)), as well as scan and tube lenses (SL and TL in figure 1(a)). An adjustable Thorlabs BE02-05-C telescope (PC in figure 1(a)), consisting of SF-14 prisms. The chirp introduced by this compressor is finely adjusted by varying the distance between the prisms. A set of mirrors (PM in figure 2(b), and stretching in our imaging system is a homebuilt prism compressor (PC in figure 1(a)), consisting of SF-14 prisms. The chirp introduced by this compressor is finely adjusted by varying the distance between the prisms. A set of mirrors (PM in figure 1(a)) is used to couple the laser output into the PC and to couple the optimally pre-chirped pulse out of the PC. As a special feature of our system, the net on-site laser-driver pulse width at the microscope sample plane is monitored by the brightness of the third harmonic from a well-calibrated glass–water interface.

An SHG FROG characterization of a typical added chirp introduced by the key components of our imaging system in its standard configuration yields ≈500 fs² for the high-NA MO, ≈400 fs² for the SLs and TLs along with the DM, and ≈200 fs² for the T and the PBS cube. As an important advantage of our laser system, its central wavelength, λ₀ ≈ 1.25 μm, lies close to the wavelength of zero group-velocity dispersion (GVD) of fused silica and many of glasses used in standard optical components. Because λ₀ is close to the zero-GVD wavelength of the downstream optical components in our setting, the added phase that the laser pulse picks up as it passes through these optical components is small compared to typical added chirp values in imaging systems based on TiS lasers.

3.6. Point spread functions and spatial resolution
In a separate experiment, point-spread functions (PSFs) of SHG, THG, 2PEF, and 3PEF imaging modalities were measured using agarose-immobilized polystyrene beads with well-calibrated diameters varying from 100 to 800 nm. Typical THG and 3PEF images of 100 nm polystyrene beads (475/540, L9904, Sigma-Aldrich) taken in the xz- and yz-planes are presented in figure 1(c). Analysis of the PSFs retrieved from such measurements (figures 1(d) and (e)) leads to the following estimates on the lateral and axial resolution: Δx ≈ 2.9 μm and Δr ≈ 0.61 μm for SHG, Δz ≈ 2.3 μm and Δr ≈ 0.47 μm for THG, Δx ≈ 2.9 μm and Δz ≈ 0.57 μm for 2PEF, and Δx ≈ 2.3 μm and Δr ≈ 0.44 μm for 3PEF. The volumetric resolution provided by 3PEF, ΔV_{3PEF} = (π/6)(Δr_{3PEF})²Δz_{3PEF} ≈ 0.23 μm³, is thus a factor of ≈2.1 higher than the volumetric resolution of 2PEF, ΔV_{2PEF} = (π/6)(Δr_{2PEF})²Δz_{2PEF} ≈ 0.49 μm³.
4. The physics behind a high resolution: coherent versus incoherent processes

As one of its key advantages, our imaging setting combines modalities of nonlinear microscopy based on both coherent (SHG and THG) and incoherent (3PEF) processes. Because of their coherent nature, SHG and THG give rise to a harmonic signal whose buildup is controlled not only by the pertinent nonlinear susceptibility, but also the phase shift of the harmonic field relative to the wave of, respectively, the second- and third-order nonlinear polarization [9, 15, 16, 45]. As imaging modalities, SHG and THG advantageously complement incoherent nonlinear processes, such as 2PEF and 3PEF [46], providing a powerful tool whereby fine structural details and spatial symmetries of biological systems can be detected with a high contrast and a high resolution.

To appreciate how the coherence of harmonic buildup can help resolve the structural features and detect boundaries and symmetries, thus providing a useful modality of laser imaging, we resort to a standard perturbative treatment of harmonic generation, which yields the following result for the power of the Nth harmonic from the volume of a nonlinear medium [47]:

$$P_N(z) = n \omega_0 \varepsilon_0 w_0^2 |A_N(z)|^2$$

where $n = n(\lambda)$ is the wavelength-dependent refractive index, $\varepsilon_0$ is the vacuum permittivity, $c$ is the speed of light in vacuum, $w_0$ is the beam-waist radius, $b = 2\pi nw_0^2/\lambda_0$ is the confocal parameter,

$$A_N(z) = \frac{iN\omega}{2nc} \chi^{(N)} A_N^* J_N(\Delta k, z_0, z)$$

and

$$J_N(\Delta k, z_0, z) = \frac{1}{zn} \exp \left( \frac{i\Delta k z'}{N!} \right)$$

where $z_0$ is the coordinate of the input boundary and $\Delta k = 2\pi N[n(\lambda_0/N) - n(\lambda_0)]/\lambda_0$ is the wave-vector mismatch for $N$th-harmonic generation.

It is straightforward to see from equations (1)–(3) that the harmonic output is highly sensitive to the shift $\Delta \varphi$ of the phase of the harmonic field, $\varphi_h$, relative to the phase $\varphi_g$, of the Nth-order polarization induced in the medium. Specifically, in the case of THG, this phase shift consists of two parts [47, 48], $\Delta \varphi = \varphi_h - \varphi_p = \Delta \varphi_m + \Delta \varphi_g$, related to the material dispersion, $\Delta \varphi_m$, and the geometric phase shift, $\Delta \varphi_g$. The material-dispersion part of $\Delta \varphi$ is given by $\Delta \varphi_m = \Delta k z$, where $\Delta k = 6\pi(n_3 - n_0)z/\lambda_0$ and $n_0$ and $n_3$ are the refractive indices of the medium at the frequencies of the laser driver and its third harmonic. The geometric phase $\Delta \varphi_g$ accounts for a Gouy phase shift of a focused beam relative to a plane wave, $\phi_0$, which, in the case of a Gaussian beam, is written as $\phi_0 = -\arctan(2z/b)$.

In figure 2(f), we compare the behavior of the third-harmonic and 3PEF signals as functions of the diameter of a targeted object $D$ irradiated by a focused laser beam with a beam-waist radius $w_0$ and a confocal parameter $b$. To mimic a typical geometry of beam focusing in our experiments, calculations in figure 2(f) are performed for $w_0 \approx 0.65 \mu m$ and $b \approx 3.3 \mu m$. As long as $D < w_0$, $b$, the 3PEF signal is seen to follow an intuitively clear $D^2$ scaling with the diameter $D$, reflecting the $\sim D^3$ growth of the fluorescent volume and an incoherent, i.e. phase-insensitive addition of 3PEF emitted by space elements within this volume. As the diameter $D$ increases first above $w_0$ and then above the confocal parameter $b$, the 3PEF signal saturates as much of object’s volume is now irradiated by a low-intensity pump or is not irradiated at all.

The $D$ scaling of the third harmonic is drastically different (cf red and blue lines in figure 2(f)). As a signature of coherent addition of third-harmonic fields emitted by all the space elements within an object of volume $\sim D^3$, the third harmonic closely follows a $D^6$ scaling as a function of the diameter $D$. When $D$ starts to exceed $b$, on the other hand, the third harmonic begins to display a signature oscillatory behavior as a function of $D$ as a manifestation of phase-matching effects (the solid blue line in figure 2(f)). Results of experiments performed on well-calibrated fluorescent beads of varying diameters (figures 2(d) and (e)), fully verify these predictions. Specifically, because of the much steeper, $\sim D^6$ scaling of the third harmonic at small $D$, the third harmonic from 100 nm beads is much weaker than the 3PEF. As a consequence, very small, $D < \lambda_0$ structural features that can still be detected in 3PEF tend to get lost in THG images (figure 2(e)).

This analysis of coherent effects in THG offers important insights into the properties of THG images. Specifically, when a laser beam with a confocal parameter $b$ is tightly focused near the center of an object with a diameter $D \gg b$, the Gouy phase shift of the focused beam leads to a coherent cancellation of the third-harmonic output as described by equations (1)–(3). If, however, a tightly focused laser beam comes across an optical inhomogeneity, this coherent cancellation of THG is no longer perfect, giving rise to a nonvanishing third-harmonic signal. Thus, when a typical size of an object—a whole cell or an intracellular
Figure 3. Panoramic (a) and close-up (b) images of a population of cultured HeLa cells co-expressing SypHer3s and PhiYFP recorded using 3PEF (encoded by red and yellow), SHG (green), and THG (blue) all driven with a single CrF-laser beam. Also shown are multiplex, 3PEF/SHG/THG image (merged) obtained as an overlay of 3PEF (red and yellow), SHG (green), and THG (blue) images. The scale bar is 50 µm (a) and 20 µm (b).

Figure 4. Panoramic (a) and close-up (b) single-beam multimodal imaging of early stages of cell mitosis in a culture of HeLa cells using 3PEF (red and yellow), SHG (green), and THG (blue) all driven by a single CrF-laser beam. The nuclear envelope is contoured by a white dashed line. Yellow dots mark the centrosomes. The scale bar is 20 µm.

5. Discussion

5.1. Understanding the multicolor readout and integrating imaging modalities

In figures 3(a) and (b), we present typical images of a population of SypHer3s- and PhiYFP-co-stained HeLa cells recorded using 3PEF, SHG, and THG all driven by a single CrF-laser beam. As one important observation to be made from these images, the 1.25 µm short-pulse output of the CrF laser can drive a high-brightness 3PEF return from both SypHer3s and PhiYFP (red and yellow in figures 3(a) and (b)). Moreover, with SypHer3s-versus-PhiYFP fluorescence signal discrimination as provided by our 3PEF detection scheme, the 3PEF readout from PhiYFP (encoded yellow in figures 3(a) and (b)) is reliably detected against the nucleolus-nonspecific 3PEF signal from SypHer3s (red in figures 3(a) and (b)), enabling a...
Figure 5. Single-beam multimodal images taken with a time interval of 4 min, capturing formation of a microtubule spindle apparatus as a part of cell mitosis (a), (b) and late stages of cell mitosis (c) in a culture of HeLa cells using 3PEF (red and yellow), SHG (green), and THG (blue) all driven with a single CrF-laser beam. The nuclear envelope is contoured by a white dashed line. Yellow dots mark the centrosomes. (d) The $x'z$- and $y'z$-projections of multimodal images of HeLa cells undergoing cell mitosis, with $x'$-, $y'$-, and $z$-axes as shown in figure (a).

high-brightness, high-contrast imaging of PhiYFP-stained intranuclear structures and nucleolar protein machinery.

While the 3PEF readout in our experimental scheme enables a high-contrast imaging of HeLa cells and their nucleoli, but relies on targeted fluorescence staining, SHG and THG can image cells, as well as fine intra- and intercellular structures without any staining (green and blue in figures 3(a) and (b)). THG is especially helpful, as comparison of 3PEF and THG images shows (figures 3(a) and (b)), in resolving the edges and visualizing the boundaries of HeLa cells and intracellular compartments. This ability of THG to detect boundaries and interfaces correlates well with one of the key properties of phase matching [9, 15]. Indeed, in the regime of tight focusing, where a laser beam with a confocal parameter $b_0$ is focused within a cell of size $D_c \gg b_0$, the phase shift between the third harmonic and the third-order polarization that drives THG leads to a coherent cancellation of the net third-harmonic output [45]. Specifically, taking $D_c \approx 25 \, \mu m$ for a typical size of HeLa cells in figures 3(a) and (b) and estimating the confocal parameter of the laser beam in our experimental as $b_0 \approx 2.5 \, \mu m$, we find $D_c \gg b_0$, leading to a strong suppression of THG from the bulk of HeLa cells due to a destructive interference of the third-harmonic fields generated up- and downstream the beam focus. However, when a laser beam is scanned through a boundary of a cell or an intracellular compartment, THG is no longer prohibited, as its geometry does no longer entail coherent signal cancellation, giving rise to a bright third-harmonic output. This third-harmonic signal provides a high-contrast probe for cell boundaries and the edges of intracellular compartments.

With a suitable laser-beam focusing geometry, where the $D_c \gg b_0$ condition is met jointly with $d_0 \ll D_c$, $d_0$ being the laser beam-waist diameter, coherence properties of THG are combined with an enhanced spatial confinement of this process, provided by a strongly nonlinear, $\sim I^3$ behavior of the third harmonic as a function of the laser intensity $I$, to enable a truly 3D imaging of cells, subcellular structure, and intracellular compartments. This ability of THG to resolve the 3D structure of cells and their compartments is illustrated by supplementary video 1 (available online at stacks.iop.org/JPHOTON/3/044001/mmedia), composed of stacks of 2D THG images taken by translating a sample of a HeLa-cell culture along the $z$-direction, i.e. along the laser beam axis (figure 1(a)), with a minimum scanning step of $\delta z \approx 0.2 \, \mu m$. This video shows that, when the laser beam is focused on the upper boundary of a cell, that is, within the area adjacent to the upper cell membrane, the entire cross section of the laser-irradiated cell gives rise to a bright third harmonic, showing up in THG images as a bright-signal area. As the laser beam focus, however, is shifted toward the inside of a cell, cell boundaries and much of the intracellular structure become clearly resolved in THG images against a dark signal coming from most of the inner part of the cell. As can also be
are multiplex images of mitotic division of HeLa cells recorded using SHG, THG, and 53–51 in SHG images as they start to organize a microtubule spindle apparatus (supplementary videos 6 and 7). Accompanied by a formation of a pair of centrosomes (marked with yellow dots), which become resolved in prometaphase. Coupled to this process is a disassembly of nucleoli, readily visible in 3PEF images, which disintegrates at early mitotic stages, such as prophase and metaphase, and eventually leads to a separation of two daughter chromosomes (figure 5(c)). In our imaging setting, SHG from polar microtubules visualizes the formation of the mitotic spindle at early stages of cell mitosis and helps trace its evolution through all the phases of cell division all the way down to late stages of mitosis, where a gradual separation of the daughter cells extends the mitotic spindle (figure 5(c) and supplementary video 9), eventually leading to a rapid spindle disassembly during telophase. As an important complementary insight, THG images of this stage of mitosis visualize a reassembly of nuclear envelopes around the separated daughter chromosomes (figure 5(c) and supplementary video 9), eventually leading to a separation of two new nuclei (shown by white dashed contours in figure 5(c)).

Standing out as one of the most striking features of the sequence of images in figures 3–5 is the high degree of complementarity with which SHG, THG, and 3PEF readouts combine into multiplex images, thus capturing a remarkable diversity of shapes, sizes, and symmetries of subcellular and subnuclear structures involved in cell mitosis. Specifically, the second-harmonic contrast, as can be seen from figures 3–5, is reserved almost exclusively for polar microtubules and structural proteins. As one of its key advantages, the second-harmonic readout from these constituents of mitotic fabric is almost background-free. On the flip side, however, SHG cannot—nor is it intended to—detect other important transients of cell mitosis. Filling

5.2. Multimodal imaging of cell mitosis
We will now demonstrate the ability of single-beam 3PEF/SHG/THG microscopy to resolve structurally complex events in cell-cycle dynamics by applying this technique to mitosis imaging. Mitosis is a precisely regulated dynamic process whereby a eukaryotic cell replicates its chromosomes, produces two identical nuclei, and eventually divides into two identical daughter cells [50, 51]. While the normal outcome of mitosis is controlled with a remarkable accuracy by complex signaling cascades, referred to as mitotic checkpoints, or spindle-assembly checkpoints, uncorrected errors, leading to chromosome misdistribution between the daughter cells, is believed to cause birth defects, tumor development, and the growth of cancer [52, 53]. Developing a suitable imaging technique that would be capable of capturing the key phases of mitosis in all their structural complexity is challenging, as mitosis involves an intricate interplay of coupled processes that occur on drastically different spatial scales, leading to a reorganization of subcellular functional units and giving rise to transient structures widely varying in size, shape, and symmetry.

Shown in figures 3–5 are multiplex images of mitotic division of HeLa cells recorded using SHG, THG, and 3PEF processes all driven with a single laser beam delivered by the CrF laser (figure 1(a)). As one of the signatures of mitotic cell division, THG images in figures 4(a) and (b) show how the nuclear envelope (contoured with a white dashed line) disintegrates at early mitotic stages, such as prophase and of prometaphase. Coupled to this process is a disassembly of nucleoli, readily visible in 3PEF images, accompanied by a formation of a pair of centrosomes (marked with yellow dots), which become resolved in SHG images as they start to organize a microtubule spindle apparatus (supplementary videos 6 and 7).

Through the entire progression of cell mitosis (figures 3–5), assemblies of polar microtubules are seen to give rise to a bright second harmonic as they stretch out (yellow dashed line) from the opposite centrosomes (yellow dots), forming a mitotic spindle as an aid for chromosome separation. Mitotic spindle formation is especially well resolved in the images capturing mid-stage and late phases of mitosis, including metaphase, anaphase, and telophase (figures 5(a)–(c) and supplementary videos 8 and 9). The ability of SHG to image polarized microtubules in a vast variety of cellular milieu, including, most notably, neuronal axons, has been convincingly demonstrated in extensive earlier studies [54–58]. In our imaging setting, SHG from polar microtubules visualizes the formation of the mitotic spindle at early stages of cell mitosis and helps trace its evolution through all the phases of cell division all the way down to late stages of mitosis, where a gradual separation of the daughter cells extends the mitotic spindle (figure 5(c) and supplementary video 9), eventually leading to a rapid spindle disassembly during telophase. As an important complementary insight, THG images of this stage of mitosis visualize a reassembly of nuclear envelopes around the separated daughter chromosomes (figure 5(c) and supplementary video 9), eventually leading to a separation of two new nuclei (shown by white dashed contours in figure 5(c)).

Since SHG in noncentrosymmetric systems is strongly forbidden, the second-harmonic readout can add an important imaging contrast to nonlinear microscopy, providing a probe for structures with a broken center of symmetry [5, 7, 49]. When focused into a culture of HeLa cells, the 1.25 µm short-pulse output of our CrF laser is seen to give rise to a robustly detectable second harmonic (green in figures 3(a) and (b)), which can help map the sites where noncentrosymmetric structures show up as a part of structural transformations inherent in cell-cycle dynamics (supplementary videos 4 and 5).
in this blank in our multimodal imaging setting are THG and 3PEF signals, each providing important information on cellular dynamics, helping resolve, as figures 3–5 show, the key stages and signatures in cell division.

6. Conclusion

To summarize, we have demonstrated a multimodal nonlinear-optical imaging that combines SHG and THG with 3PEF as a means to resolve structurally complex episodes of cell-cycle dynamics. When zoomed in on cell mitosis, this technique has been shown to enable a high-contrast, high-resolution multimodal imaging of intra- and extracellular signatures of cell division, detecting, via a multiplex, 3PEF/SHG/THG readout, a remarkable diversity of shapes, sizes, and symmetries in a truly single-beam setting, with no need for beam refocusing or field-waveform re-adjustment. Such multiplex images can be assembled into meaningful time-lapse video records, such as those presented in supplementary videos 1–9, which can help assess variances in mitotic progression, detect uncorrected errors at various stages of mitosis, reveal mitotic stressors, and resolve mitotic aberrations as markers for potential pathologies, tumors, and the early signs of cancer [52, 53, 59–61].

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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