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High-resolution multi-photon imaging of morphological structures of *caenorhabditis elegans*

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Abstract

In this protocol, we combine two-photon excitation fluorescence to visualize in *caenorhabditis elegans* (*C. elegans*) dopaminergic (DAergic) neurons and their processes with non-linear optical measurements to reconstruct the three-dimensional architecture of the pharyngeal region and the muscular system of the anterior and mid-body region. Femto second laser pulses excite second-harmonic generation (SHG) and third harmonic generation (THG) signals, which show detailed structural information regarding the organization of myofibrils that are arranged around the central pharynx region. The combination of two-photon excitation with SHG and THG imaging is a very powerful tool to study cell morphology, the microarchitecture and tissue arrangement in *C. elegans*.

Keywords

C. elegans; multi-photon microscopy; label-free imaging; SHG; THG

INTRODUCTION

Multi-photon microscopy (MPM) is a powerful technique to study the 3-dimensional (3D) architecture of tissues and organs in the living animal. The major breakthrough in the field was achieved in the last decade with the development of nonlinear imaging modalities, which opened the door to deep tissue imaging, and significantly reduced phototoxicity and photobleaching (Andresen et al., 2009; Denk, Strickler, & Webb, 1990; Helmchen & Denk, 2005).

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The basic principle underlying multi-photon absorption is a process in which two or more photons are absorbed simultaneously, exciting electrons into a higher lying state. This non-linear process is a very rare event and can only be observed at significant rates when the light intensity is high in space and time (Denk et al., 1990). In MPM an infrared (IR) laser is used which emits near-infrared light (NIR) or IR light in ultra-short pulses (in the order of 100 fs) at high repetition rates (80–100 MHz) to excite fluorescence as well as non-linear excitations effects, such as second and third harmonic generation (SHG and THG) (Helmchen & Denk, 2005; Zipfel, Williams, & Webb, 2003). NIR/IR light has the ability to penetrate deep into tissues, resulting in imaging depths up to 1 mm. Due to the longer excitation wavelength less tissue scattering is observed compared to confocal microscopy. Furthermore, MPM fluorescence is only generated when the laser beam is tightly focused restricting the excitation to a tiny focal volume, which significantly reduces phototoxicity and photobleaching above and below the focal plane (Andresen et al., 2009; Oheim, Beaurepaire, Chaigneau, Mertz, & Charpak, 2001). In addition, SHG and THG imaging modalities allow optical sectioning and visualization of cellular and tissue structures without the use of dyes or stains resolution (Campagnola et al., 2002; Friedl, 2004). The structural information obtained by SHG and THG imaging is complementary to two-photon fluorescence signal and is therefore most useful for the visualization of collagen fibers and fascia, striated muscle, blood and fat cells at a submicron spatial resolution (Rehberg, Krombach, Pohl, & Dietzel, 2011; Weigelin, Bakker, & Friedl, 2012).

Here, we will describe the detailed 3D imaging of morphological structures of *C. elegans* using multi-photon excitation fluorescence microscopy combined with SHG and THG imaging. These three complementary imaging modes were used to reconstruct the anatomy of the nematode at subcellular resolution. MPM was used before to image cellular structures and processes in *C. elegans* in vivo (Filippidis et al., 2009; Gualda et al., 2008), however, we considerably improved the spatial resolution as well as SHG signal intensities. As a result we could visualize myofibrils of individual muscles of *C. elegans* in high resolution using SHG imaging.

BASIC PROTOCOL 1

C. ELEGANS COLLECTION, PREPARATION AND ANTIBODY LABELING—This protocol describes immunolabeling of *C. elegans* using a modified picric acid fixation method (Duerr, 2006; Nonet et al., 1997). To visualize the morphologic structure of *C. elegans* including dopamine neurons a *C. elegans* strain expressing green fluorescent protein (GFP) under control of the *dat-1* gene promoter (a tyrosine hydroxylase) was fixed and immunolabeled using a mouse anti-GFP primary antibody and a secondary CyTM3-anti mouse IgG. Successful fixation and immunolabeling requires permeabilization of the tough cuticle, achieved here through liquid nitrogen freeze/thaw and the use of several permeabilizing detergents.

Materials:

- C. elegans* worms on nematode growth media agar plates
- Bowin's fixative (see recipe)

Methanol
B-mercaptoethanol
Liquid Nitrogen
Borate Buffer pH 8, 40X (see recipe)
BTB (see recipe)
BT (see recipe)
AbA (see recipe)
AbA Blocking (see recipe)
AbB (see recipe)
Mouse Anti-GFP Antibody (Roche)
CyTM3-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories)
1.7 mL SliptechTM Microcentrifuge Tubes (Denville Scientific)

Collect *C. elegans*:

1. Generate adult worms on nematode growth media agar plates using standard methods (Brenner, 1974). Remove worms from agar plates using a disposable transfer pipette to gently rinse plate with water. Collect the water and worms in a 15mL conical tube.
2. Centrifuge at 3,000rpm for 5 minutes to pellet worms. Discard supernatant and wash pellet with water, spin and remove supernatant. Lavage 1–2mL of liquid and worms in conical tube.

To avoid shearing worms the opening to the transfer pipette should be >1mm, the tip can be cut at a 45° angle if necessary
3. Resuspend pelleted worms in remaining liquid and aliquot into 2–3, 1.7mL SliptechTM microcentrifuge tubes using a disposable transfer pipette.
4. Wash worms 2X by filling tubes with water, centrifuging at 1,000rpm for 2.5 minutes, removing supernatant, and leaving 250µL of liquid in microcentrifuge tubes

The volume of pelleted worms should be at least 50–100µL

Fix and Permeablize *C. elegans*:

5. Place tubes on ice for approximately 5 minutes
6. To each tube add 400µL Bowin's fixative, 500µL methanol, and 10µL β-mercaptoethanol

The fixative should be mixed fresh and chilled prior to use

7. Freeze tubes in liquid nitrogen and thaw under running water until just melted but not warmed

If staining is weak, this step may be repeated up to 3x to increase cuticle permeability
8. Place tubes in ice and gently rock for 30 minutes

This step can be lengthened up to 1 hr
9. Pellet fixed worms by centrifuging at 1,000 rpm for 2.5 minutes, and gently remove fixative with pipette

All spins from here on are done at 1,000 rpm for 2.5 minutes. Alternatively, if worms become too fragmented, the worms will settle to the bottom of the tubes without spinning, although this adds additional time to the protocol. Fixed worms are fragile; when removing liquid slowly draw it off with a pipette and when adding liquid slowly drop it along the side of the tube.
10. Wash worms 5X by adding 1.4mL BT, gently inverting, spinning, and removing supernatant
11. Wash 3X by gently adding 1.4mL BTB, mixing by gentle inversion, spinning and removing supernatant
12. Wash 4X by adding 1mL BTB and rocking for 1 hr at room temperature, spinning and removing supernatant
13. Wash once more in BTB, spin and remove supernatant
14. Wash 2X with 500µL ABA, mixing by gentle inversion, spinning and removing supernatant

At this point, worms may be stored in ABA at 4°C for several months

Immunolabeling:

15. Add 500µL ABA Blocking to microcentrifuge tube, rock at room temperature for 1hr
16. Using a pipette tip that has been cut at an angle to increase the size of the opening (>1mm), remove 25µL of worms into a fresh microcentrifuge tube
17. Add 100–175µL of ABA containing mouse anti-GFP antibody at 1:000 dilution
18. Incubate overnight at 4°C with rocking

Depending on the antibody, room temperature incubation may also be used
19. Wash 2X with 400µL ABB, mixing by gentle inversion, spinning and removing supernatant
20. Add 400µL ABB and rock 10min, spin and remove supernatant
21. Add 400µL ABB, rock 2hr or as long as overnight at room temperature

22. Spin and remove supernatant. Add 400µL ABA, gently invert, spin, and remove supernatant
23. Add 100–200µL ABA containing CyTM3-conjugated Anti-Mouse IgG at 1:500 dilution. This is a standard dilution commonly used in these types of studies.

From this point forward, the samples should be protected from light as much as possible by using foil
24. Rock at room temperature for 1hr
25. Repeat steps 18–20
26. Wash 2X with 400µL ABB, mixing by gentle inversion, spinning and removing supernatant, leaving 50–100µL of worms and liquid

At this point worms may be stored for several months at 4°C and can be shipped using refrigeration packs

Prepare Slides:

27. Tap tube gently to suspend worms, remove 5µL of worms with pipette tip that has been cut at an angle
28. Place worms on a glass slide; add 20 µl Fluoromount-G (SouthernBiotech) and a cover slip. Seal the slide with fingernail polish.

ALTERNATE PROTOCOL 1

IMAGING OF *C. ELEGANS* WITH MULTI-PHOTON MICROSCOPY—We used a TriM Scope II multi photon system from LaVision BioTec to visualise immune-labeling of neuron as well as SHG and THG imaging to visualise anisotropic structures, such as collagen and muscle fibres.

Materials:

1. Multi-photon microscope setup (TriM Scope II) with single beam scanning and non-descanned detectors from LaVision BioTec (Bielefeld, Germany).
2. Olympus BX51 WI microscope stand that is equipped with high sensitive NDD detectors (PMTs).
3. Coherent Scientific Chameleon Ultra II Ti:Sapphire laser (tuning range 680–1080 nm, 120 fs pulse width, 80 MHz repetition rate).
4. Coherent Chameleon Compact OPO (automated wavelength extension from 1000 nm to 1600 nm, 200 fs pulse width, 80 MHz repetition rate).
5. 20x IR objective lens (Olympus XLUMPlanFI 20x/1.0W, working distance of 2.0 mm).
6. LaVision BioTec ImSpector Software for 3D image acquisition.
7. Pair of x-y galvanometric mirrors for scanning the sample at a scanning speed of up to 1200 lines/s.

8. Dichromatic mirrors and band pass filters: blue (384–406 nm, forward detection), green (475–575 nm, forward detection), red (560–680 nm, backward detection).
9. Collect emission signals in forward direction by a specialised condenser (Olympus U-AAC condenser) and in backward direction through the objective lens.
10. Photomultiplier tubes (PMT; Hamamatsu H67080-01 (blue channel), H67080-20 (green and red channels)).

Protocol steps

Combined fluorescence, SHG and THG imaging:

1. Detect emitted forward SHG signals using optical parametric oscillator (OPO) infrared multiphoton light source at 1100 nm.
2. Use a green-sensitive PMT to detect light passing a band pass filter (525/50).
3. Detect emitted THG signals using an OPO as an infrared multiphoton light source at 1180 nm with 100 mW under the microscope.
4. Use a blue-sensitive PMT to detect light passing a narrow band pass filter (395/11).

Backward excitation of Cy3-labeled neurons:

5. Use OPO as an infrared multiphoton light source at 1100 nm to visualise Cy3-labeled neurons.
6. Use a red-sensitive PMT to detect light passing a band pass filter (620/60).
7. Record SHG and fluorescence signals simultaneously.
8. Record THG sequentially as it does not have an excitation wave-length in common with the others.

REAGENTS AND SOLUTIONS

Use Mili-Q-purified water or equivalent for the preparation of all buffers.

Bowin's Fixative

Prepare a solution containing 7.5mL saturated picric acid, 2.5mL 20% buffered paraformaldehyde, and 0.5mL glacial acetic acid. Prepare fresh.

Paraformaldehyde, 20%

Combine 0.22g paraformaldehyde, 10 μ L 1N NaOH, and 990 μ L water in a microfuge tube, mix and incubate at 60°C for 40min. Prepare fresh.

Borate Buffer pH 8, 40X

Prepare solution of 1M H₃BO₃, adjust pH to 8 and heat to dissolve. Can be stored for several months at RT.

BT

Using 40X Borate Buffer, prepare a solution of 1X Borate Buffer pH 8 with 0.05% Triton X-100. Prepare fresh.

BTB

Using 40X Borate Buffer stock solution, prepare a solution of 1X Borate Buffer pH 8, with 0.5% Triton X-100 and 2% β -mercaptoethanol. Prepare fresh.

ABA

Prepare a solution of 1X PBS pH 7.4, 0.1% Tween-20, 1mM EDTA pH 8, 0.05% NaN_3 , and 0.1% SDS. Store at 4°C for several months.

ABA Blocking

Prepare a solution of ABA with the addition of 2% bovine serum albumin, and 1% dehydrated skim milk. Store at 4°C for one month.

ABB

Prepare a solution of 1X PBS pH 7.4, 0.1% Tween-20, 1mM EDTA pH 8, 0.05% NaN_3 , 0.1% SDS, and 0.2% BSA. Store at 4°C for several months.

COMMENTARY

Background Information

Most classic microscopy techniques are not suitable for imaging thick tissues, while MPM allows the visualization of cellular and tissue structures several hundred micrometers into a biological sample. Light microscopy allows the visualization of cells or tissues without prior staining using techniques, such as Köhler illumination and phase contrast; however a detailed 3D reconstruction of the sample is not possible. Confocal laser scanning microscopy requires fluorescent structures and therefore specific staining procedures, but allows the detection of emitted photons from a single plane. Consecutive images can therefore be assembled to a stack, which allows 3D reconstruction of the tissue morphology. With SHG and THG imaging both, label-free visualization and optical sectioning, can be combined allowing a remarkable 3D high-resolution reconstructions of anatomic tissue structures and interfaces.

SHG and THG are both predominately forward scattering signals, which require excitation by strong infrared lasers with pulses in the fs-range. (Andresen et al., 2009; Friedl, Wolf, von Andrian, & Harms, 2007; Rehberg et al., 2011). SHG emission is observed when two, near-simultaneously arriving photons are combined into one single photon at dense, non-centrosymmetric structure, such as collagen fibers and striated muscle myosin. THG signals detect local differences in third-order nonlinear susceptibility, refractive index and dispersion at water-lipid or water-protein scaffold interfaces. THG imaging allows the visualization of structures which combine three, near-simultaneously arriving photons to one photon, a non-linear optical effect which allows the visualization of fat cells, nerve fibres, lipid bodies, cell membranes and intracellular vesicles. In summary, SHG and THG imaging complements two-photon fluorescence microscopy by obtaining detailed structural

information of a biological sample in 3D and at submicron spatial resolution (Friedl, 2004; Rehberg et al., 2011; Weigelin et al., 2012).

Critical Parameters and Troubleshooting

Laser safety—Light sources used for MPM (Ti:Sapphire laser, OPO) are very powerful pulse lasers (laser class IV). Only qualified and specifically trained personnel should perform laser beam alignments or other operations that carry the risk of laser beam exposure. Proper shielding and interlock during these operations are required as well as protective eyewear.

Laser alignment—Problems with mode-locked laser in MPM frequently arise from:

1. Amplitude noise, beam-pointing instability
2. Dust or dirt on optics (dichromatic mirrors, band pass filters)
3. Reflections from surfaces which reflect light back into the laser cavity
4. Temperature-instability causing beam-pointing drift
5. Poor adjustment or loss of alignment of laser beam to microscope scan head

On a routine basis proper laser alignment and performance of the MPM setup should be checked using sub-resolution fluorescent beads to determine the point-spread function as an indicator for correct system performance.

Simultaneous imaging of multiple labels

Most fluorophores show relatively broad two-photon excitation spectra and allow the simultaneous imaging of multiple labels using a single excitation wavelength and multiple detectors. The choice of appropriate optics, namely suitable dichroic mirror and band pass filters is required to separate the different emitted wavelength without bleed-through from one channel into another.

No image or poor quality image

There might be a number of reasons why you are unable to acquire an image or the obtained image is only of poor quality. Below you will find a brief list of possible reasons why you don't get an image.

1. Laser not switched on or shutters closed
2. Incorrect dichroic mirrors or bandpass filters
3. PMT switched off due to too high laser exposure
4. Inappropriate excitation wavelength
5. Too much laser attenuation

The quality of an image highly depends on the quality of your sample, the fixation procedure and the staining protocol including the selection of appropriate primary and secondary antibodies. In addition, ensure that the objective is clean and that all optical

elements are properly aligned since off-centered elements result in loss of fluorescence intensity, sharpness and spatial resolution. Some chromophores might bleach after repetitive imaging of the same site resulting in dimmer and blurry images. Always start with a low laser power first and stepwise increase the power until you obtain an image of good contrast and signal intensity.

Anticipated Results

The anticipated results depend primarily on the following factors: 1) quality of worm preparation staining procedure and antigen expression levels 2) system configuration and performance of MPM light sources (Ti:Sapphire laser and OPO), 3) possibility to detect SHG and THG signal in forward direction. Although the SHG and THG signals can either be detected in forward or backward direction, emission is significantly stronger in forward compared to backward direction (SHG: ~5 times; THG: ~25 times) (Weigelin et al., 2012).

Time Considerations

Microscope setup and imaging—System setup including warming up of the lasers, adjusting dichroic mirrors and bandpass filters as well as checking the system performance might take 10–15 min. The time required to identify a suitable region for imaging including image acquisition and change of wavelength and settings for Ti:Sapphire or OPO imaging might vary considerably. In general, 1.5h to 3h imaging sessions need to be planned to achieve adequate results.

Image analysis—The time required for analyzing the acquired images, for selecting the optimal regions and for assembling those using the Inspector (LaVision Biotec, Bielefeld) or Imaris software (Bitplane) might take ~2–3h. The Imaris software further allows to process the assembled images and to perform surface rendering plots for optimized 3D reconstruction.

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Figure 1.

NIR/IR multiphoton microscope setup. The beam of a mode-locked titanium-sapphire (TiSa) laser (80 MHz, 120 fs) is divided using a beam splitter (BS). One part is used to pump an optical parametric oscillator (OPO; 80 MHz, 200 fs), the other part is used for imaging the specimen directly. The OPO shifts the TiSa wavelength range of 680–1080 nm to longer wavelengths covering a range of 1000–1600 nm. Both beams pass a beam-shaping device and are combined via a dichromatic mirror (DM) before entering a common scan-head, which allows the simultaneous use of the TiSa and OPO beam. SHG and THG emission signals are collected in forward direction by a condenser (C). Fluorescence light is collected in backward direction through the objective lens. For spectral separation dichromatic mirrors and bandpass filters (F) were used in front of the photomultiplier (PMT). Abbreviations: LBF: laser blocking filter; SL: scan lens; TL: tube lense.

Figure 2. Cy3 labeled dopaminergic neurons (A), SHG signal showing myofibrils (B) and THG signals showing the pharynx (C) of wild type *C. elegans*. D shows the merged image. Insets show regions enclosed by a dotted line box at higher magnification. E-G show individual z-planes of the pharyngeal region (4 μm between individual planes). Bar = 30 μm .

Figure 3.

Imaris surface-rendered plot of Fig.1D. A shows Cy3- fluorescence (red), the SHG (green) and THG signal (blue) in the pharynx and mid-body region of wild type *C. elegans*. B and C show the image depicted in A after removing the SHG (B) or THG (C) signal, respectively. D: side view of D after rotating the x-axis of the object. Bar = 30 μ m.