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Second Harmonic Generation Microscopy in Zebrafish

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Abstract

Modern optical imaging has progressed rapidly with the ability to non-invasively image cellular and subcellular phenomena with high spatial and temporal resolution. In particular, emerging techniques such as Second Harmonic Generation Microscopy (SHG) can allow for the monitoring of intrinsic contrast, such as that from collagen, in live and fixed samples. When coupled with multiphoton fluorescence microscopy, SHG can be used to image interactions between cells and the surrounding extracellular environment. There is recent interest in using these approaches to study inflammation and wound healing in zebrafish, an important model for studying these processes. In this chapter we present the practical aspects of using second harmonic generation to image interactions between leukocytes and collagen during wound healing in zebrafish.

Keywords

Multiphoton; SHG; zebrafish; macrophage; wound healing

1. Introduction

Second harmonic generation (SHG) is a non-linear optical event resulting from the interaction of light with non-centrosymmetric materials, including biological molecules such as collagen, which provides an endogenous source of contrast (Mohler, Millard and Campagnola 2003). This intrinsic property of molecular structures emits photons at exactly half the incident wavelength. The method allows a variety of incident wavelengths to be utilized. Therefore, SHG can be spectrally separated from fluorescent events, permitting the combination of SHG with a diversity of endogenous and exogenous fluorophores (Campagnola, Clark, Mohler, Lewis and Loew 2001; Zipfel, Williams, Christie, Nikitin,

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Hyman and Webb 2003). Additionally, the fact that SHG does not require fluorophore fusions ensures that observations are physiologically relevant and not a byproduct of the fusion construct. A primary use of SHG imaging of tissue has been the examination of collagen during cancer progression, where it is being studied as a potential diagnostic assessment tool since changes in collagen, and corresponding SHG patterns, have been shown to be involved in disease progression (Conklin, Eickhoff, Riching, Pehlke, Eliceiri, Provenzano et al. 2011; Keikhosravi, Bredfeldt, Sagar and Eliceiri 2014; Brisson, Mauldin, Lei, Vogel, Power, Lo et al. 2015; Drifka, Tod, Loeffler, Liu, Thomas, Eliceiri et al. 2015; Tilbury and Campagnola 2015). We recently used SHG to show that collagen fibers in the zebrafish larval tail fin exhibit changes in organization in both chronic and acute inflammatory environments (LeBert, Squirrell, Rindy, Broadbridge, Lui, Zakrzewska et al. 2015). The SHG imaging modality we have used is backwards SHG (Chen, Nadiarynh, Plotnikov and Campagnola 2012) whereby the SHG signal is detected on the same side of the sample as the excitation source. A caveat of this method is that it does not distinguish between different types of fibrillar collagen (Chen et al. 2012) and therefore we refer to the fibers as simply “collagen fibers” rather than specifying collagen type.

The larval zebrafish is an increasingly popular vertebrate model for the study of both immunity and wound healing. The zebrafish innate immune system is highly conserved and represents a powerful model system (Meeker and Trede 2008; Deng and Huttenlocher 2012). Zebrafish are also capable of rapidly regenerating many tissues, including the caudal fin, following tail transection. Additionally, due to their translucency during the first few days of development, they are highly amenable to non-invasive in vivo imaging of cellular behavior under physiologically relevant conditions. Although the innate immune system has been extensively studied during this stage of larval development, much less is known about collagen fiber organization in the developing zebrafish fin and its reorganization during wound healing (Duran, Mari-Beffa, Santamaria, Becerra and Santos-Ruiz 2011)

Our recent study (LeBert et al. 2015) identified a thickening of collagen fibers during fin re-growth following tail transection. Depletion of the matrix metalloprotease, MMP9, impairs wound healing and alters the collagen signature detected by SHG, suggesting that collagen reorganization likely plays an important role during fin re-growth. Studies in other systems also indicate that collagen changes during wound healing (Madden and Peacock 1971; Clore, Cohen and Diegelmann 1979). However, little is known about potential interactions between the immune system and collagen structures during normal and aberrant wound healing. There is, however, evidence that macrophages may influence collagen synthesis and remodeling in damaged tissues (Hunt, Knighton, Thakral, Goodson and Andrews 1984; Madsen, Leonard, Masedunskas, Moyer, Jurgensen, Peters et al. 2013). The combination of SHG with the transparency, genetic malleability and availability of fluorescently-tagged immune cells in zebrafish models promises to provide significant new insight into the relationship between the immune system and extracellular matrix during wound healing and tissue regeneration in live animals.

Although our interests lie mainly within the realm of the immune response during wounding, inflammation and infection, the SHG/zebrafish combination provides an accessible and relevant system for examining collagen changes in different tissues and

organs under a variety of normal and disease states (Sun, Chu, Chen, Tsai, Liu, Lin et al. 2004; Olivier, Luengo-Oroz, Duloquin, Faure, Savy, Veilleux et al. 2010; Kieu, Mehravar, Gowda, Norwood and Peyghambarian 2013). For example, SHG has been used in the zebrafish for studying neutrophil transmigration through collagen during tumor cell invasion (He, Lamers, Beenakker, Cui, Ghotra, Danen et al. 2012). SHG can also be utilized to assess other structured components in the zebrafish, such as sarcomeres in the muscle during statin-induced myopathy (Huang, Hsiao, Lin, Chow, Chang and Liao 2011) and subcellular components, such as mitotic spindles, during early embryonic development (Sun et al. 2004; Olivier et al. 2010; Luengo-Oroz, Rubio-Guivernau, Faure, Savy, Duloquin, Olivier et al. 2012).

The endogenous nature of SHG imaging lends itself readily to using live cells, tissues and organisms. This is particularly powerful in zebrafish where it can be combined with the use of genetically encoded fluorescent tags. There is also the potential advantage of using multiphoton imaging (Denk, Strickler and Webb 1990) for long term imaging of processes that involve changes in collagen organization. One obvious extension of the techniques presented here is the ability to perform in vivo imaging of macrophage/collagen interactions in real time, during wound healing. A challenge of long term imaging during wound healing is the creation of application specific devices that maintain larval viability and permit growth while accommodating the higher resolution lenses preferred to clearly delineate fine collagen fibers. Advances in SHG imaging technologies are permitting the simultaneous collection of both forwards and backwards SHG signal, which will allow more precise delineation of different types of fibrillar collagens (Tilbury, Lien, Chen and Campagnola 2014), and as these imaging systems become widely available, could be applied to understanding collagen dynamics in vivo in zebrafish over time.

Herein we describe a newly established protocol for imaging (Figure 1) and analyzing (Figure 2) collagen fibers using multi photon, SHG imaging, alone and in combination with transgenic, fluorescently labeled, macrophages (Figure 3). By pairing the transparent larval zebrafish with this imaging technique, we are able to examine changes in collagen organization during normal development and after wounding. This technique allows for non-invasive assessment of both collagen fibers during development, wound healing and macrophage response and, potentially, interactions between macrophages and collagen fibers. In the following sections, we provide protocols for performing SHG imaging on fixed transgenic zebrafish that express fluorescently-tagged proteins in macrophages. This includes protocols for preparation of transgenic fish, microscope setup, image acquisitions and post acquisition analysis.

2. Materials

2.1 Zebrafish embryos

1. Zebrafish husbandry according to The Zebrafish Book. Available online http://zfin.org/zf_info/zfbook/zfbk.html. Accessed 17 August 2015.

2.2 Microscope supplies and components

The Laboratory for Optical and Computational Instrumentation (LOCI <http://loci.wisc.edu/>) is an instrumentation laboratory advancing optical and computation techniques, thus much of the instrumentation, acquisition and analysis software presented here was developed at LOCI. Therefore, we describe the set up used to acquire the images presented in the figures. Commercial vendors also provide similar systems and all the components utilized are commercially available.

1. Multiphoton microscope (<http://loci.wisc.edu/equipment/spectral-lifetime-multiphoton-microscope-slim>)
 - a. Microscope: Nikon Eclipse TE2000U inverted microscope
 - b. SHG and MP Emission Detector: Hamamatsu H7422P-40 GaAsP photomultiplier (PMT). Other PMTs can be used including the common multialkali PMTs available on all commercial systems.
 - c. Transmitted Light detector: An infrared-transmitting Si photodiode (Hamamatsu K34213) was used in a BioRad MRC-1024 Transmitted light detector box for detecting transmitted light. Any IR-transmitting photodiode or PMT could be used.
 - d. Objectives: Nikon 10X S Fluor 0.5NA and Nikon CFI Apo Lambda S 40X long working distance water immersion 1.15NA
 - e. Laser: Coherent Chameleon Ultra II Ti:Sapphire laser – tuneable range 700 to 980 nm
 - f. Scanning system: Cambridge Technologies 6210H galvanometer with Prairie Technologies (Bruker) galvanometer control box.
 - g. Laser power control via Pockel Cell: Conoptics Model 350-80LA KD*P Series Electro-Optic modulator with Model 302RM amplifier. Other power control schemes may be used as well.
 - h. Emission filters:
 - i. For SHG: Semrock 445/20 nm BrightLine single-band bandpass filter
 - ii. For Dendra: Semrock 520/35 nm Brightline single-band bandpass filter
 - i. Filter wheel: Sutter Instrument Lambda 10B Optical Filter Changer
 - j. Shutter: Uniblitz Model D122 shutter and driver system
 - k. Z and stage control: Applied Scientific Imaging MS-2000 xyz stage control
 - l. Vibration table: TMC CleanTopII vibration isolation system

2. Multiphoton laser scanning acquisition system: WiscScan 7.2.2 <http://loci.wisc.edu/software/wiscscan>. Commercial scanning packages that can control multiphoton microscopes are also sufficient.
3. Glass bottom petri dish: In Vitro Scientific 35mm dish with 20mm bottom well #1.5 (0.17 mm) cover glass thickness

2.3. Buffers, other reagents and tools for sample preparation

1. 60x E3: To make 1L, add 17.2 g NaCl, 2.9 g CaCl₂ and 4.9 g MgSO₄. Fill to 1 L with reverse osmosis (RO) water. Adjust pH to 7.2 with NaOH.
2. 1x E3: Dilute 60x E3 with RO water to 1x. Add 0.1% methylene blue.
3. E3/PTU: 0.003% 1-phenyl-2-thiourea in 1x E3.
4. 1X PBS
5. #10 Feather surgical blade for transection, #15 scalpel blade for sample preparation prior to imaging
6. 20 gauge syringe needle
7. FisherBrand 200 µl Specialty Pipette Tips, large orifice

2.4 Image Processing Components

Image acquisition software usually contains some image viewing and/or imaging analysis functions. Additional commonly used software includes but is not limited to the following:

1. FIJI (free software; <http://fiji.sc>. Accessed 17 August 2015 (Schindelin, Arganda-Carreras, Frise, Kaynig, Longair, Pietzsch et al. 2012)
2. CTFIRE software: <http://loci.wisc.edu/software/ctfire> (Bredfeldt, Liu, Pehlke, Conklin, Szulczewski, Inman et al. 2014)
3. Bitplane Imaris (<http://www.bitplane.com/go/products/imaris>). Accessed August 17 2015

3. Methods

3.1 Macrophage specific protein expression

For imaging purposes, transient mosaic expression is typically sufficient. However, in certain cases, high levels of expression of the transgene can be cytotoxic or have undesirable phenotypes. In this situation generation of a transgenic line can be beneficial as lines with lower level expression can be identified. Preparation of transient or transgenic zebrafish that express the gene of interest in a macrophage specific manner requires the use of a cell specific promoter such as macrophage-expressed gene 1 (mpeg).

1. Plasmid construction: Use DNA expression vectors containing minimal Tol2 elements for efficient integration flanking the mpeg promoter, the gene of interest and an SV40 polyadenylation sequence (Clontech Laboratories Inc.)

2. Preparation of plasmid DNA: Transformation of the plasmid into regular cloning competent cells, e.g., *E. coli* DH5 α is sufficient. DNA extraction and purification is achieved using a commercially available miniprep kit (Promega Wizard Plus SV Minipreps DNA purification system).
3. Preparation of Tol2 transposase mRNA: transposase is prepared by in vitro transcription (Ambion mMESSAGE mMACHINE) followed by column purification (miRvana RNA isolation kit).
4. Microinjection of zebrafish embryos is a standard routine procedure. Different laboratories will have slightly different setups. For basic setup information and injection procedures, please refer to *The Zebrafish Book*.
5. Expression of the construct is obtained by injection of a 3 nl volume of 12.5 ng/ μ l DNA plasmid with 17.5 ng/ μ l Tol2 transposase mRNA into the cytoplasm of the 1-cell stage embryo.

3.2 Preparation of 35-mm polystyrene bottom petri dishes

Protein coating of dishes is necessary because we find that larvae stick to polystyrene and glass surfaces without coating which can lead to wounds on the zebrafish larvae. The 35-mm dishes are submerged in 1% skim milk for 10 minutes and rinsed several times with RO water and air dried before use.

3.3 Caudal-fin amputation

The caudal-fin region of the larval zebrafish is the preferred region for SHG imaging due to its relatively thin nature and lack of high numbers of pigment cells. Amputation of the caudal-fin is also performed to assess wound healing as well as macrophage recruitment and resolution. In our hands, we find wounding of PTU treated larvae at 2 to 3 dpf to be the most reproducible as larvae anesthetized with tricaine tend to lie flat at this stage of development. Zebrafish at 4 dpf lay at more of an angle, due to the orientation of the yolk sac, which makes amputating and imaging the caudal-fin more difficult.

1. Place larvae in 3 mL of 0.2 mg/mL tricaine in E3/PTU in a protein coated 35-mm polystyrene plate. Let sit for at least 1 minute.
2. Gently insert a feather no. 10 scalpel into the media being careful not to nick the fish. When the blade hits the surface of the media it creates currents that push the larvae along the bottom of the plate making wounding more difficult. It is recommended to keep the blade in the media once it has been inserted.
3. Rock the tip of the blade forward over the caudal-fin, just distal to the notochord, before dragging the blade back over the fin. This dual-cutting motion ensures complete amputation and, in our hands, creates a smooth wound edge.
4. Once wounding is complete, rinse the larvae 3x in E3/PTU for 1 minute.
5. Transfer the wounded larvae to a fresh milk-coated 35-mm polystyrene plate. The wounding plate will contain many small abrasions in its surface, which will cause unintended wounds to the larvae as they pass over them.

3.4 Preparation of fixed samples

Although fixation is not necessary for SHG imaging, fixation permits the imaging of larger numbers of samples from different conditions at various time points over multiple imaging days. This is of particular importance when first defining a phenomenon of interest.

1. For most applications, larvae can be fixed at set time points with the following 4% fixative solution: for 1 mL of fixative add 100 μ L of 10x PBS, 250 μ L of fresh 16% paraformaldehyde (PFA) and 650 μ L of ddH₂O. In situations in which the fluorophore dissipates rapidly upon fixation, we recommend using the following 1.5% fixative solution: for 1 mL of fixative add 801.3 μ L of ddH₂O, 100 μ L PIPES (1M), 93.7 μ L PFA (16%), 4 μ L EGTA (0.5M), 1 μ L MgSO₄ (1M).
2. Samples are fixed directly in 35-mm dishes. Samples are transferred to 4°C O/N.
3. Fixed larvae are washed 3X in room temperature 1X PBS. The larvae can be stored in PBS at 4°C.
4. Using large orifice pipette tip on P200 pipette, 2 to 4 larvae are transferred in a drop (approximately 100 μ L) of PBS to a 35-mm glass bottom dish. To ensure that the caudal-fin lies evenly on the bottom of the glass bottom plate, the caudal-fin is removed from the rest of the body using a scalpel blade, posterior to the yolk sac extension, such that a 1 to 2 mm piece of the caudal fin is retained.
5. The rest of the body is carefully moved out of the drop to the side of the dish using either a syringe needle or a fine tip pipette tip. It is important not to damage the yolk sack as yolk granules will contaminate the drop and interfere with imaging.
6. Because the fins are not attached in any way to the glass bottom, the dish must be transported carefully to the microscope to minimize jostling. Also for this reason more than 4 tail fragments can cause crowding of the tail fins within the drop, resulting in overlap and potential difficulties in keeping track of which tails have or have not been imaged.

3.5 Image acquisition

Image acquisition requires the designation of the x, y and z locations of the sample to be imaged as well as the establishment of imaging parameters (power levels, scan speed, detector gain, etc) appropriate for the sample of interest.

1. In preparation for imaging, a drop of distilled water is placed on the 40X water immersion lens and then the lens is gently rotated out to permit the use of the 10X lens. The glass bottom dish is placed into the appropriate stage holder on the microscope and the 10X lens is used to initially locate the tails. To maintain consistency in fin orientation, the dish is rotated so that the notochord is parallel to the x axis of the image. The 40X water lens is rotated into place using proper microscopy technique and refocused on the fin.

2. Images are collected to include the tip of the notochord and the edge of the tail fin. For younger larvae or early post-wound tails, this may be a single region of interest (ROI). In the case of older larvae or later post-wound times this might include 2 or even 3 ROIs, though a greater number of ROIs could be defined if larger regions of the tail are of interest. These multiple regions of interest locations are marked for subsequent automated image collection. Then the z stack top and bottom are set to accommodate the ROI with the greatest z depth. Although this is usually the ROI about the tip of the notochord, fin tips that are curved, cupped or distorted may require increased z-depth, so z-depth should be checked for all ROIs for a given fin. The z-interval was set at 1 or 2 microns.
3. Once regional information has been set, signal-specific parameters must be set. For a given experiment, imaging parameters should be identical or as similar as possible, with records kept of all settings to ensure consistency and comparability of data across treatments and imaging days. For SHG imaging we utilized 890 nm two-photon laser excitation and a 445 nm emission filter. Note that because of the nature of optical sectioning of angled surfaces such as the tail fin, any given individual z-image will only include a small region of collagen fibers. For Dendra image collection, an 890 nm excitation with 520 nm emission filter were used. Detector gain was kept consistent for both SHG and fluorophore image collection. Laser power was attenuated using a pockels cell and set to provide good contrast without signal saturation. Typically, SHG is a much weaker signal than exogenous fluorescence thus our image acquisition software (WiscScan) permits differential Pockel cell settings for different emission filters. Scan speed was set, and maintained for all samples, to improve signal to noise while minimizing sample exposure. Automated image acquisition was then conducted for the zstacks at multiple regions for both signals, with sequential collection of the two signals. Note that our system includes simultaneous transmitted light collection so brightfield features can be captured the same time as fluorescence and SHG.

3.6 Image processing and analysis (Figure 2)

1. A number of software packages and plugins are available for a variety of image analyses. Our preference is to engage open sources software as much as possible, utilizing commercial software as needed when open source software is not readily available for the task required. Initial image processing for visualization was conducted using FIJI. If multiple ROIs were collected for a given tail, stitching of these ROIs was necessary for viewing the entire tail region. Additionally, because of the nature of the optical section and the angle of tail, SHG images of individual z-sections are often not very informative and benefit from z-projection as an initial processing for visual data assessment. ROI stitching was performed using the “Grid/Collection Stitching” plugin in FIJI ((Preibisch et al., 2009). This provides a stitched z-stack containing both the brightfield and emission channel. For data assessment, the brightfield and emission channel were separated and the emission channel was z-projected. This

can be done manually or semi-automated with macros in FIJI (Schindelin et al. 2012). For data sets consisting of a single ROI, emission z-stacks were z-projected in FIJI. Similarly, simple macros can be written to batch process large number of images. If 2 labels are collected, either the z-stacks or the z-projections can be merged in FIJI to provide information about the relative location of the two signals.

2. The z-projections provide a simple, accessible method for visualizing these data sets, which can then be assessed for interesting changes in collagen fiber organization with different treatments in order to determine the appropriate quantitative measurements to be acquired. Although a variety of quantitative assessments could be accomplished using FIJI or other image analysis software packages, in our experiments we noticed a change in the fiber thickness as wound healing progressed (Figure 2B). Therefore, we utilized the fiber analysis tool, CT-FIRE (Bredfeldt et al. 2014) (Figure 2A) to assess fiber width in the z-projection of the SHG images. CT-FIRE extracts and analyzes a variety of metrics on images of fibers in tissues that can be statistically evaluated. The purpose of CT-FIRE is to allow users to automatically extract collagen fibers in an image and those quantify fibers with descriptive statistics, such as fiber angle, fiber length, fiber straightness, and fiber width.
3. Generating z-projections in FIJI and quantitative analysis in CT-FIRE are important tools for assessing changes in the SHG fiber organization in the larval tail during wound healing and re-growth under varying conditions. However, the tail and the process of wound healing are 3 dimensional entities and benefit from 3D visualization. To accomplish this, we opened z-stacks in Bitplane Imaris (Figures 1, 2 and 3). Z-stacks from all three channels (brightfield, SHG and fluorescence) could be incorporated into a single 3D reconstruction. The different channels can be surface rendered to enhance the visualization. These 3D reconstructions can be rotated and re-sliced at different angles and locations in order to better understand the 3D nature and relationships within the healing tail.

4. Notes

1. Glass bottom plates are commercially available, but an inexpensive and relatively easy alternative is to make them in house. Drill a 16 to 18 mm hole in the center of a 35-mm tissue culture dish bottom. Apply a thin layer of Norland Optical Adhesive 68 on the outside surface around the hole. Place a 22 mm round #1 cover slip on top of the adhesive and press down slightly. The adhesive is cured by UV light. Overnight exposure of UV light in a tissue culture hood or approximately 1 hour on a UV transilluminator is sufficient to cure the adhesive.
2. To make a transgenic line, injected larvae displaying mosaic transgene expression are raised to sexual maturity (as early as 2 months). F0 founders are screened by outcrossing with wild-type zebrafish and checked for transgene

expression in the F1 larvae at 2 to 3 dpf. The positive F1 progeny can then be used for imaging or propagation of the transgenic line.

3. Not all DNA extraction and purification procedures/kits yield DNA of high enough quality to be used for microinjection. Injecting poor quality DNA will result in embryo death. For small amounts of DNA preparation, the Promega Wizard Plus SV Minipreps DNA purification system yields good DNA quality in our experience.
4. The use of PTU treatment is crucial in ensuring high quality image resolution. In our experience, the presence of even small amounts of pigmentation can result in tripping the detector and damage to the sample. The pigment cells absorb the infrared light generating heat. This heat is enough to damage the pigment cell and the surrounding cells. It is therefore important to fully pigment-inhibit as early as 15 hpf, though we find that beginning PTU treatment at 22 to 24 hours is sufficient. The use of non-pigmented zebrafish lines maybe a viable alternative to PTU treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Second Harmonic Generation microscopy of the larval zebrafish tail

(A) Diagram of zebrafish larvae showing region imaged. (B) 3 views of 3D reconstruction of collagen fibers in an unwounded zebrafish larval tail (3 dpf) to illustrate organization of fibers. Image stitched in FIJI from z-stacks of 20 ROIs. Top row show 3D reconstruction of SHG data while bottom row show surface rendering. (Supplemental video 1). Scale bar = 50 microns.

Fig. 2. SHG imaging of fibers in the zebrafish tail reveal quantifiable changes in organization of collagen fibers during wound healing

(A) Schematic illustrating steps involved in quantifying fiber metrics, specifically width, in zebrafish tails after wounding. (B) FIJI-generated projections of SHG z-stacks of the distal ROI showing changes in fiber organization in wounded and unwounded tails from 4 dpf (2 dpw) larvae. Scale bar = 20 microns. (C) Graph showing quantitation of fiber width as determined using CT-FIRE fiber analysis software. (Supplemental videos 2 and 3). Significance of $p < 0.01$ indicated by **.

Fig. 3. Multiphoton excitation of Dendra-expressing macrophages combined with SHG imaging following wounding

3D reconstructions, enface view, of tail transection of multiphoton data of showing the merge of collagen fibers and fluorescently-tagged macrophages as well as the two signals separated. Three views of 3D surface rendering more clearly demonstrate the spatial relationship between the macrophages and the fibers, with macrophages either accumulating at the wound edge or found within the wedge-shaped space between the layers of fibers. Scale bar = 20 microns. (Supplemental video 4).