Nonlinear microscopy of common histological stains reveals third harmonic generation harmonophores

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Since its invention over a hundred years ago, histological analysis using coloured dye staining remains the gold standard for histopathology. While these stains provide critical information for a variety of diagnostic purposes, they offer limited two-dimensional histological information. Extending classical histological analysis to three dimensions requires novel imaging approaches such as multiphoton microscopy. Multiphoton microscopy enables multimodal, three-dimensional imaging of histologically stained samples. Specifically, third harmonic generation (THG), a nonlinear optical process in which three incident photons are combined into one by the sample, allows high contrast imaging of tissues stained with absorbing dyes, which in turn act as harmonophores. While this technique has previously been applied to hematoxylin and eosin (H&E) tissue sections, we extend this approach to other commonly used histological stains to demonstrate further potential applications of the technique. We demonstrate THG imaging of both human skin and liver tissue stained with H&E, Verhoeff-Van Gieson (VVG) and Picrosirius Red stains. We find that these stains provide excellent contrast as THG harmonophores, enabling high resolution imaging of histological samples. THG imaging of the Verhoeff stain enables easy detection of elastic fibers while Picrosirius Red acts as an effective harmonophore for imaging collagen fibers of all sizes.

Introduction

Medical diagnostics have relied on histological staining for the last century thanks to its ability to easily contrast different tissue elements using brightly coloured stains.1 The most popular tool in histopathology is the hematoxylin (H) and eosin (E) stain, most commonly referred to as H&E.2 Hematoxylin is a dye extracted from the logwood tree. Its oxidized form, hematin, is combined with an aluminium salt to form haemalum, a complex that has a high affinity for chromatin in nuclei.3 Its application in histology results in cell nuclei being stained a deep purple colour. Eosin Y, a fluorescent dye, is used to provide contrast by counterstaining basic amino acids, resulting in extracellular matrix elements, connective tissue and cytoplasm being stained a light pink colour.4 In combination, H&E’s ability to differentiate between cells and extracellular tissue elements has made it into the gold standard of histopathological analysis.

Other histological stains provide contrast for different tissue elements. For example, the Verhoeff stain effectively highlights elastic fibers in addition to nuclei by colouring them black.5 Its counterstain, the Van Gieson stain, is used to differentiate collagen fibers in red and other tissue elements in yellow.6 Together, these techniques form the Verhoeff-Van Gieson (VVG) stain, an effective technique for identifying elastic tissue disorders, particularly in dermatology.7

Another useful method for revealing extracellular collagen is the Picrosirius Red stain, a combination of Sirius Red, a poly-azobenzene dye, with an aqueous saturated picric acid solution. This technique highlights collagen in bright red, with other tissue elements being stained in yellow.8 While similar to the Van Gieson stain, Picrosirius Red serves as an important alternative due to its improved stability and its ability to enhance the birefringence of collagen, improving its specificity when combined with polarization microscopy.9 In this fashion, Picrosirius Red has been used for quantitative histological determination of collagen in various tissues.10

Histological investigations are usually performed with basic white light microscopy. However, the tissues under examination need to be thinly sectioned to avoid out-of-focus blur,
providing only a limited two-dimensional snapshot of the tissue. As tissues exist in a three-dimensional (3D) structure, histologists are required to subjectively determine which projections to take during sectioning to ensure representative sampling. 3D reconstruction must either be done mentally during visualization of the slices or computationally after laborious imaging of consecutive sections. Pathological examinations could benefit from 3D histological information, with improved spatial resolution and better image contrast than that provided by light microscopy.

Nonlinear microscopy\textsuperscript{12–16} is a technique that is fast gaining notoriety in the world of biological imaging thanks to its multimodal nature and ability to conduct deep 3D imaging. It utilizes ultrafast lasers to induce multiple photon-matter interactions, that results in the visualization of multiphoton fluorescence and other nonlinear optical contrast mechanisms such as second harmonic generation (SHG) and third harmonic generation (THG).\textsuperscript{17} SHG is the generation of emitted light at exactly twice the frequency of excitation light in a nonlinear optical material.\textsuperscript{18} In tissue, this intrinsic nonlinear property arises in dense non-centrosymmetric structures such as collagen fibers and muscle.\textsuperscript{19} THG, a phenomenon in which three excitation photons are combined into a single photon presenting three times the incident frequency, occurs from interactions, that results in the visualization of multiphoton fluorescence and other nonlinear optical contrast mechanisms such as second harmonic generation (SHG) and third harmonic generation (THG).\textsuperscript{17} SHG is the generation of emitted light at exactly twice the frequency of excitation light in a nonlinear optical material.\textsuperscript{18} In tissue, this intrinsic nonlinear property arises in dense non-centrosymmetric structures such as collagen fibers and muscle.\textsuperscript{19} THG, a phenomenon in which three excitation photons are combined into a single photon presenting three times the incident frequency, occurs from optical heterogeneities within the sample.\textsuperscript{20} THG has been used to visualize many intrinsic interfaces in tissue, including lipid/water interfaces such as myelin,\textsuperscript{21} cell membranes\textsuperscript{22} and other lipid bodies.\textsuperscript{23} Nonlinear microscopy has become an essential technique to conduct label-free histology via SHG and THG.\textsuperscript{24–26}

However, using labels for nonlinear contrast mechanisms such as THG is becoming a topic of interest as it allows for increased specificity while retaining the inherent three-dimensional imaging capability. Materials that exhibit high second and/or third order nonlinear susceptibilities have been called harmonophores.\textsuperscript{27} These harmonophores exploit resonant effects during the multiphoton interaction process to significantly enhance emission while maintaining low incident power. Several intrinsic THG harmonophores have been discovered, including hemoglobin\textsuperscript{28} and hemozoin.\textsuperscript{29–31} In addition, hematoxylin has been found to present significant THG susceptibility, likely due to resonance effects thanks to its nature as an absorptive dye.\textsuperscript{32–34}

In this study, we performed trans nonlinear imaging of unlabelled and histologically stained human skin and liver tissue sections, and demonstrated that in addition to H&E, other histological dyes used in elastic and collagen staining also have the ability to induce strong THG signals in stained tissues. For the first time, we have observed that tissue components stained by VVG and Sirius Red act as THG harmonophores. Elastic fibres and cell nuclei exhibit a strong THG signal upon VVG staining, while Van Gieson labelled collagen exhibits two photon fluorescence (TPF). Collagen stained with Picrosirius Red demonstrates strong third order nonlinear susceptibility through its intense THG signal at low excitation power.

**Methods**

**Nonlinear microscopy**

Nonlinear microscopy of the histological samples was achieved using a customized upright multiphoton microscope (FV1200 MPE, Olympus Canada Inc., ON, Canada) as shown in Fig. 1. The system is equipped with two ultrafast laser excitation sources that provide ∼200 fs pulses: a Ti:sapphire laser (Mira 900F, Coherent, CA, USA) tuned for excitation at 780 nm and an optical parametric oscillator (Mira OPO, Coherent, CA, USA) configured for excitation at 1150 nm. Switching between the two sources is accomplished through two motorized flip mirror mounts (MFF101, Thorlabs, NJ, USA). Laser excitation power is controlled by a custom computerized variable attenuator composed of a half wave rhomb retarder (FR600HM, Thorlabs, NJ, USA) mounted in a motorized rotational stage (PRM1Z8, Thorlabs, NJ, USA) combined with a Glan-Laser polarizer (GL10, Thorlabs, NJ USA). Excitation laser power is measured immediately prior to beam entrance to the microscope using a thermal power sensor and console (S175C/PM100D, Thorlabs, NJ, USA). The polarization of the excitation beam is selected after power control using an achromatic quarter waveplate (AQWP10M-980, Thorlabs, NJ, USA) mounted on a rotational cage mount (CRM1, Thorlabs, NJ, USA). A 25× 1.05 NA water immersion multiphoton objective (XLPL25XWMP, Olympus Canada Inc., Ontario, Canada) focuses the excitation light on the histological tissue samples. The light emitted in the forward direction is collected with a 0.9 NA dry top lens condenser and directed towards two photomultiplier (PMT) detectors after splitting by a dichroic mirror. A 770 nm short pass infrared-blocking filter (FF01-770/SP32, Picrosirius Red demonstrates strong third order nonlinear susceptibility through its intense THG signal at low excitation power.

![Fig. 1 Experimental nonlinear microscopy imaging setup. Abbreviations: PMT, photomultiplier tube; SP, short pass; LP, long pass. Briefly, the Verdi laser pumps the Ti:sapphire laser, which can either be used directly for imaging or directed to an optical parametric oscillator to achieve higher excitation wavelengths. Both laser outputs are directed into a power and polarization control module prior to entry to the microscope base.](image-url)
Semrock, New York, USA) is used to block scattered excitation light.

THG imaging was conducted using the OPO (1150 nm, 15-55 mW) for excitation and a 400 nm filter (380-420 nm filter, ET400/40X, Chroma Technology, Vermont, USA) for detection. SHG and TPF imaging was achieved by excitation with the Ti:sapphire laser (780 nm, 8-35 mW). SHG was collected using the same filter as THG, while TPF was collected using three channels: “blue” (460-500 nm filter, BA460-500, Olympus Canada Inc., Ontario, Canada), “green” (515-555 nm filter, BA515-555, Olympus Canada Inc., Ontario, Canada) and “red” (570-630 nm filter, ET600-60, Chroma Technology, Vermont, USA). SHG was collected at the same time as the “red” TPF channel, while “blue” and “green” were collected together. Two dichroic mirrors (TP550LPXR and TP505LPXR, Chroma Technology, Vermont, USA) were used to separate the channels within each set. Brightfield images were acquired on the same imaging setup using a color CMOS camera (DCC1645C, Thorlabs, New Jersey, USA).

Tissues and histological staining

Human skin (TTR003-25EA) and liver (TTR012-25EA) control tissue sections were obtained from MilliporeSigma, ON, Canada along with all histological reagents unless otherwise specified. Each set of tissue sections was first deparaffinized using passages in xylene. H&E staining was performed using Harris hematoxylin solution (HHS16) and alcoholic eosin Y solution (HT110116) in accordance with the “HHS” Sigma-Aldrich procedure. VVG staining was achieved using the HT25A elastic stain kit in accordance with the “HT25” Sigma-Aldrich procedure. Picrosirius Red staining solution was performed using a Sirius Red (3655548) solution in saturated picric acid (P6744). Each stain was performed on separate, sequential sections of each tissue type.

Results and discussion

Nonlinear microscopy of unlabelled tissue highlights collagen via SHG

We first performed nonlinear microscopy of unlabelled tissue sections to evaluate the features observable through native two photon autofluorescence, SHG and THG. We first excited the unlabelled skin and liver samples with 780 nm illumination to visualize TPF and SHG (Fig. 2).

Fig. 2A and B displays blue TPF exhibited by skin tissue sections where Fig. 2A shows part of the epidermis and the underlying dermis and Fig. 2B displays the reticular dermis, mostly composed of type I collagen and elastic fibers. The autofluorescence exhibited in this channel seems to be largely nonspecific with the exception of elastic fibers, which are composed of intrinsically auto-fluorescent elastin.17 Fig. 2C and D displays blue TPF of liver tissue sections, specifically connective tissue associated with portal areas within the liver. Fig. 2E-H highlights the green TPF while Fig. 2I-L represents the red TPF emitted by the skin and liver tissue sections. Elastin is emphasized more prominently in the green TPF of the skin sections (Fig. 2E and F) while red blood cells are readily observable in the green and red TPF of liver sections (Fig. 2K and L).

SHG imaging of both tissue types (Fig. 2M-P) efficiently highlights fibrillar collagen within the skin dermis and connective tissue of the liver portal areas. It has been shown previously that SHG is a vital tool of label-free nonlinear microscopy, due to its high specificity for non-centrosymmetric materials with high second order nonlinear susceptibility such as collagen and to a lesser extent muscle.35-38 In our study, we use the SHG channel as the control channel, to track readily observable fibrillar collagen structures within the skin dermis samples and the liver connective tissue samples.

The samples were then excited with 1150 nm illumination to observe THG, highlighting the presence of optical heterogeneities within the tissues (Fig. 2Q-T). Most prominently, THG reveals the stratified squamous keratinised epithelium (keratinocytes) in skin samples (Fig. 2Q), in addition to red blood cells in the liver tissue sections, visible thanks to their highly resonant hemoglobin content28,39 (Fig. 2T). Elastic fibers (Fig. 2R) also provide a slightly above background THG signal (Fig. 2R).40 While THG is useful for unlabelled imaging of lipids, interfaces, and highly resonant harmonophores, its capabilities in label-free histological investigations are restricted due to low specificity outside of resonant harmonophores.

Overall, while nonlinear microscopy of unlabelled tissue sections readily visualizes collagen and native tissue autofluorescence though SHG and TPF respectively, it does not provide sufficient specificity and contrast for full histological investigations. It also appears that native tissue autofluorescence can be seen across all three of the TPF channels that were imaged, providing no additional information. As such, we will not be including the blue TPF channel in subsequent figures.

H&E stained tissue sections visualized by nonlinear microscopy reveals high contrast nuclear imaging via THG

The first histological stain that was investigated is H&E, the most widely used stain in histopathology. Two skin samples and two liver samples were stained and imaged first with white light microscopy (Fig. 3A-D). The H component resulted in cell nuclei in both tissue types being stained a deep purple while other tissue elements including collagen and cellular cytoplasm were dyed pink by the E component. Fig. 3A shows part of a hair follicle, while Fig. 3B displays sweat glands, both structures located in the reticular dermis in skin samples. Fig. 3C demonstrates part of a liver lobule with red blood cells (stained red-pink, no nuclei) and hepatocytes (nuclei in purple). Fig. 3D showcases a portal area including hepatocytes and Kupfer cells (nuclei in dark purple/black).

TPF imaging of these H&E stained samples can be found in Fig. 3E-H (green TPF) and Fig. 3I-L (red TPF). The features displayed in these channels are those most stained with eosin Y, a highly fluorescent dye that binds to basic amine acid residues.41 Cell cytoplasm (top) and collagen fibers (bottom) are visible in Fig. 3E. Fig. 3F displays staining extracellular tissue
Fig. 2 Nonlinear microscopy of unlabeled skin and liver tissue sections. First and second columns: skin; third and fourth columns: liver. A–D: Blue TPF; E–H: green TPF; I–L: red TPF; M–P: SHG; Q–T: THG. A–P are excited by 780 nm while Q–T were excited by 1150 nm. Global scale bar in A is 10 µm.
elements, most notably collagen (later verified by SHG) and elastin. In the liver, hepatocyte cytoplasm is readily visualized in addition to red blood cells within the sinusoids (Fig. 3G). Fig. 3H again demonstrates cytoplasm and extracellular matrix staining. The aforementioned features are similarly displayed in the red TPF channel (Fig. 3I–L).

Fig. 3M–P illustrates SHG imaging of the H&E stained tissue sections where the SHG signal is readily attributed to the presence of fibrillar collagen and not enhanced by the H&E staining. On the other hand, THG imaging (Fig. 3Q–T) exhibits clear contrast of cellular nuclei stained by hematoxylin. It has been previously shown that hematoxylin presents strong resonance enhancement of THG as the virtual transition states of the THG process when excited at 1230 nm correspond to real electronic energy states of the molecule.33 Specifically, hematoxylin possesses absorbance bands at two and three times the fundamental excitation frequency (second and third virtual states). As we excited our H&E samples at...
1150 nm, these correspond to 575 nm and 383 nm, respectively, which are included in the absorbance spectrum of hematoxylin. It has previously been shown that aggregation of hemalum complexes within cell nuclei significantly increases THG emission.

To verify the power dependency of the SHG and THG channels, we collected SHG/THG emission at different incident laser powers. As can be seen in Fig. 4, the SHG signal grows with a second order dependence \((x^{1.95})\) while THG follows a third order power dependence \((x^{3.02})\) verifying that the THG channel is indeed capturing THG emission.

**Nonlinear microscopy of VVG stained tissue sections highlights elastic fibers and cell nuclei by THG**

Next, we investigated the nonlinear properties of VVG stained skin and liver tissue sections. We first visualized the stained tissue sections with white light microscopy (Fig. 5A–D). The Verhoeff component of the stain colours both elastic fibers and cell nuclei in black. The Van Gieson component, composed of picric acid and acid fuchsin, provides a pink counterstain to collagen and other extracellular components. Fig. 5A presents the epidermal layer of a skin section, whereas Fig. 5B demonstrates a sweat gland in the reticular dermis. Fig. 5C and D shows interlobular connective tissue within the liver sections containing elastic fibers stained in black.

During initial experiments, significant photobleaching and damage was observed when tissue sections were illuminated at 780 nm at low power (15 mW). Therefore, we limited our excitation to 1150 nm illumination for our examinations of VVG. Fig. 5E–H shows emission in the range of 570–630 nm, which covers both SHG and red TPF emission within the same channel. The features exhibited within skin tissue sections show a combination of SHG signal from fibrillar collagen

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**Fig. 4** Power curve of H&E stained tissue samples showing the second order dependency of SHG emission and third order dependency of THG emission.

**Fig. 5** Nonlinear microscopy of VVG stained skin and liver tissue sections. First and second columns: skin; third and fourth columns: liver. A–D: White light microscopy; E–H: SHG + red TPF (570–630 nm); I–L: THG; E–L are excited by 1150 nm. Global scale bar in E is 10 µm.
fibers previously shown in H&E and unlabelled tissue samples along with fluorescence from the acid fuchsin (pink) stained tissue elements. This includes all forms of collagen and other extracellular matrix elements. In addition, we expect some contribution from native tissue autofluorescence in red TPF. Acid fuchsin is known to act as a fluorophore when excited at 543 nm, which is within the two photon excitation range at 1150 nm.42

Fig. 5I–L demonstrates THG imaging of VVG stained tissue sections where we observe significant emission from Verhoeff stained tissue components in both skin and liver tissue. Specifically, we readily observe cell nuclei (Fig. 5I and J) and stained elastic fibers (Fig. 5J–L). As the Verhoeff stain primarily consists of a iron hematoxylin complex,5 which binds readily to elastic fibers, it is apparent that the binding of these complexes to elastic tissue displays significant third order nonlinear susceptibility, enabling us to visualize them by THG. The resonance enhancement process likely proceeds similarly to the hematoxylin component of H&E staining, where real energy levels within the molecule coincide with the second and third virtual transition states of the THG process.

To verify if the emission in the 380–420 nm channel is due to THG, we conducted a power dependency study (Fig. 6), where we observed a third order dependency \((x^{2.01})\) associated with the VVG-THG signal. To the best of our knowledge, this is the first application of VVG as a THG harmonophore for high contrast elastic fiber and nuclei imaging.

**Picrosirius Red acts as a highly selective collagen specific THG harmonophore**

Picrosirius Red is a stain regularly used for histological analysis of collagen content in tissue.43 The stain consists of Sirius Red, an azo dye also known as direct red 80. The dye is mixed with a saturated solution of picric acid prior to application, which enhances the intensity of the stain. Collagen within tissue stained with Picrosirius Red appears as bright red against a yellow background. During staining, the elongated, anionic structure of the Sirius Red dye molecule binds to cationic collagen fibers, also enhancing the natural birefringence of collagen.8

In our next experiment, we stained skin and liver tissue sections with Picrosirius Red and first examined them with white light microscopy. Fig. 7A and B demonstrates the staining of skin tissue samples where Fig. 7A shows a section of the epidermis and underlying dermis while Fig. 7B highlights a blood vessel within the dermis. Fig. 7C and D shows staining of connective tissue within liver sections where Fig. 7C presents part of a portal triad.

We then performed nonlinear microscopy using 780 nm excitation to evaluate TPF and SHG of the tissue sections. Fig. 7E–H displays green TPF of skin and liver tissue sections, respectively. The green TPF signal imaged seems to be largely due to nonspecific autofluorescence within the tissues as previously seen in the unlabelled tissue sections (Fig. 2E–H). However, the red TPF of the tissue sections most prominently show the Picrosirius Red staining, highlighting areas of collagen that were stained bright red in the dermis (Fig. 7I and J), around a blood vessel in the skin (Fig. 7J), and in the connective tissue of the liver (Fig. 7K and L). It has been shown that Picrosirius Red staining observed in fluorescent imaging displays a highly specific and sensitive, red fluorescent signal for the evaluation of collagen in tissue sections.44 In addition to the highly specific collagen signal, we observe weak nonspecific autofluorescence similar to that seen in the green TPF channel.

Fig. 7M–P displays the SHG signal from the 780 nm excitation where we observe fibrillar collagen within the skin and liver sections. The areas highlighted by SHG seem to correspond well to the features observed in the red TPF (Fig. 7I–L). Most notably, the contrast obtained by SHG appears to be more selective of larger collagen fibers (Fig. 7N) whereas the Sirius Red TPF highlights a larger number of fibers, including smaller bundles of collagen (Fig. 7J). This is likely due to the size dependent nature of SHG signal from collagen fibers. As such, when detection sensitivity is adjusted below signal saturation, only the larger fibers can be visualized due to their strong signal and weaker signals from smaller fibers may not be visualized. This effect does not occur for Sirius Red stained collagen since the fluorescence is equally attributed to all collagen fiber sizes.

Finally, we excited the Sirius Red stained tissue sections with 1150 nm to collect THG images (Fig. 7Q–T). Unlike the unlabeled tissue sections in Fig. 2Q–T, where we only observed weak THG signal at 45 mW excitation, Sirius Red staining provided significant THG emission up to PMT saturation at a third of the power (15 mW). The features exhibiting THG emission overlap with the collagen detected by SHG while demonstrating a larger number of fibers similar to the red TPF. As such, it appears that the collagen stained with Picrosirius Red exhibits a high third order nonlinear susceptibility to be visualized by THG. In comparison to the features seen by red TPF, THG provides increased contrast of the collagen against a significantly lower background due to the specificity needed for
THG emission. We did not observe any autofluorescence in the THG channel in comparison to the red TPF. To validate the origin of the contrast mechanisms detected within the SHG and THG channels, we performed a power dependency study (Fig. 8). The SHG signal held a quadratic power dependency ($x^{1.93}$) while the THG signal showed a cubic power dependency ($x^{2.95}$). To the best of our knowledge, this is the first application of Picrosirius Red as a THG harmonophore for collagen detection.

In comparison to the traditional SHG-based detection of collagen, THG of Picrosirius Red provides greater detail across all fiber sizes, higher signal and is less sensitive to fiber orientation within the tissue. SHG emission is highly dependent on the fiber orientation in regards to the laser polarization. In addition, SHG is not sensitive to fibers that are oriented parallel to the laser propagation direction. In contrast, THG emitted by a harmonophore such as Sirius Red is not subject to these limitations.

The origin of the significant third order nonlinear susceptibility displayed by Sirius Red is not yet known. Azobenzene dyes, such as Disperse Red 1, have been shown to exhibit third
order nonlinear susceptibility, which has been attributed to the trans–cis transition within the dye molecule. Sirus Red is a poly-azo dye, suggesting that it could benefit from the same mechanisms as other azobenzene dyes for nonlinear susceptibility. Additionally, resonance through electronic nonlinearity could enhance this effect. It can be suggested that there is overlap between the virtual states invoked by THG and the real electronic energy levels of Sirius Red. Specifically, the molecule presents an absorbance peak at 528–529 nm with significant absorbance until 560 nm (ref. 49) as well as a smaller shoulder around 372 nm. This could result in overlap with the second (575 nm) and third (383 nm) virtual states involved with 1150 nm excitation, leading to a resonance enhancement of THG emission. An additional factor for the nonlinear susceptibility of Sirius Red could be due to the enhanced birefringence of collagen by the dye molecule. It has been shown that THG can occur in the bulk of birefringent materials thanks to enhanced phase matching conditions. By using circular polarization excitation along with a polarization analyzer at the detector, THG could be used to evaluate the birefringence of materials.

As THG requires ultrafast laser excitation, that can be used to simultaneously investigate multiple modalities, it is important to consider the thermal impact of multiphoton excitation on the sample. Commonly, this would require the use of nanothermal indicators, such as quantum dots, with spectral detection capabilities, to properly evaluate the thermal impact at the 3D focal volume. In mouse brain tissue, it has been estimated that two photon microscopy produces around 1.8 °C increase per 100 mW excitation. As our method utilizes excitation powers ranging from 8 mW to 55 mW, we expect the thermal impact to be minor provided there is no linear absorbance of the excitation wavelength in the tissue.

THG imaging presents improved resolution over white light microscopy thanks to its use of a tightly focused laser beam and the cubic dependence of its point spread function on excitation light intensity. The resolution of THG imaging could be significantly improved through the use of image scanning microscopy (ISM) schemes where the point detector is replaced with an array of photodetectors. This enables computational pixel reassignment that can enhance spatial resolution. ISM has previously been demonstrated for two photon microscopy and second harmonic generation imaging.

To increase the specificity of THG harmonophore based histology beyond the selectivity offered by traditional histological dyes, targeted attachment of harmonophores through different labelling modalities could be explored. Cellular organelles have been specifically labelled with gold nanoparticles that act as plasmon-resonant harmonophores for THG. In addition, lipid-enclosed quantum dots functionalized with antibodies have been used to detect cellular receptors in breast cancer cells via THG imaging. Most recently, carotenoid-based labels that act as THG harmonophores have been used to label specific cell structures.

The use of THG to visualize harmonophores in common histological dyes brings several potential advantages to medical diagnostics. THG imaging enables better contrast than white light microscopy, along with the inherent possibility for 3D imaging. In addition, visualizing histological features such as cell nuclei and collagen reorganization in separate detection channels enables easier quantification by cell counting and area segmentation. A THG based approach could be automated for direct objective evaluation of histological features.

**Conclusion**

In this work, we identified two new applications of common histological dyes as third harmonic generation harmonophores for specific and high contrast imaging: Verhoeff Van Gieson and Sirius Red. The techniques were demonstrated using human skin and liver tissue sections and characterized by multimodal nonlinear microscopy, specifically two photon fluorescence, second harmonic generation and third harmonic generation imaging. We verified hematoxylin as a THG harmonophore for high contrast nuclear staining in accordance to previous studies. We distinguished that the iron hematoxylin of the Verhoeff stain acts as a strong THG harmonophore in tissue, where it binds to elastic fibers and cell nuclei. We also demonstrated that Sirius Red presents significant third order nonlinear susceptibility upon binding to collagen in tissue samples, allowing for enhanced resonance and highly specific high contrast collagen detection by THG.

The utilisation of THG imaging to visualize common histological dyes presents great potential for three-dimensional histological imaging at low cost due to the extremely low power requirements for resonant THG imaging. Other histological dyes could be examined as potential THG harmonophores through electronic resonance enhancement, rendering them potentially powerful tools for the future of three dimensional histology.
Conflicts of interest

There are no conflicts of interest to declare.

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References