THG imaging of lipid body profiles in diagnosis of biological samples

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Abstract

Nonlinear optical imaging techniques are at the forefront of biomedical research over the last years. The ability of label free imaging, diffraction limited resolution, high penetration depth, intrinsic three dimensional (3D) sectioning and low phototoxicity make this technology a perfect diagnostic tool in many biomedical applications. This review briefly refers to the non-linear microscopy modalities, depicts their advantages and focuses on recent applications of third harmonic generation microscopy in recognizing lipid body structures in biological specimens. Specifically, this study concentrates on the efficiency of THG signal quantification to be used as an innovative diagnostic tool in the follow-up of mouse embryo development, in distinguishing mouse BV2 microglia cell activation and allowing classification of human breast cancer cells.

Keywords: Third harmonic Generation, Label free imaging, Lipid Bodies, Nonlinear Microscopy Quantification analysis, Mouse embryos , BV2 glial cells , Breast cancer cells

1. Introduction

Nonlinear optical imaging is a rapidly developing technology in biomedical diagnosis. During the past decade, nonlinear imaging techniques were proven to be extremely useful in life sciences revealing valuable and complementary information about the specimens under investigation. The efficiency of nonlinear optical processes in diagnosis, allowed widespread applications in diverse biological samples such as cells, model organisms and tissues (C. K. Chen & Liu, 2012; S.-Y. Chen et al., 2010; S.-Y. Chen, Wu, & Sun, 2009; Chu et al., 2003; Gavgiotaki, Filippidis, Kalognomou, et al., 2015; Rehberg, Krombach, Pohl, & Dietzel, 2011; Tsai, Chen, Shieh, Lou, & Sun, 2011; Tuer et al., 2010; Weigelin, Bakker, & Friedl, 2012).

The term "nonlinear" means that the response of a material to an applied optical field depends in a nonlinear manner on the strength of the applied field (Boyd, 2008).

A brief description of the applications of multimodal nonlinear microscopy techniques in biomedical imaging and life sciences is presented in the first part of this review. The next section is dedicated to the application of THG imaging quantification analysis, which is mainly based on the lipid profiles in different biological samples and processes. Specifically, this section focuses on the evaluation of mouse microglia BV2 cell activation, the discrimination of different human breast cancer cell lines and the *in vitro* examination of pre-implantation mouse embryo development.

2. Nonlinear microscopy

Nonlinear microscopy became a well-established technique with the development and optimization of mode locked stable, reliable, femtosecond laser sources. Tightly focused femtosecond laser beams are necessary for the effective creation of the nonlinear phenomena from the biological specimens. The nonlinear signals are produced only from the limited focal volume of the excited laser beam, decreasing thus dramatically the out of focus plan phototoxicity and photobleaching phenomena. The use of infrared wavelengths enables low

absorption and reduced scattering from the sample, providing deeper penetration depths. Moreover, employment of these high resolution modalities allows acquisition of intrinsic 3D delineation images of the specimens under investigation. Another great advantage of such technology is the ability to evaluate several different nonlinear effects simultaneously, by performing one single scan. Consequently, different contrast mechanisms can be used to record parallel images of the same structure. Multicontrast microscopy appears to be very useful when different nonlinear responses reveal different functional structures of the same biological object (Sparrow, 2008).

The most widely used contrast mechanisms in nonlinear optical microscopy are third harmonic generation (THG), second harmonic generation (SHG) and two photon excitation fluorescence (TPEF).

In TPEF, two photons are absorbed almost simultaneously (each photon provides half of energy) to excite the fluorophore (a molecule that fluoresces) from the ground to the first excited state. After some non-radiative decay processes, a fluorescent photon is emitted and can be collected to generate an image. The probability of two photon absorption by a fluorescent molecule follows a quadratic dependence on the excitation radiance. However, TPEF suffers from photobleaching, as all other fluorescence techniques (So, Dong, Masters, & Berland, 2000; Thomas, van Voskuilen, Gerritsen, & Sterenborg, 2014).

SHG and THG are both nonlinear, coherent, scattered processes, related to the interaction of intense light with matter. Compared to TPEF, no exogenous fluorophores are needed. SHG describes the generation of light waves that are twice the frequency (half of the original wavelength) of the incident waves, while THG describes the generation of light waves with triple frequency (one-third of the original wavelength) of the incident waves. SHG and THG microscopy provide superior axial resolution due to quadratic and cubic dependencies of the signals on the illumination intensity, respectively. Moreover, long term observations

become possible, since harmonic generation does not involve energy deposition to the sample and therefore biological specimens do not suffer from side effects (e.g. photobleaching, thermal effects).

In SHG microscopy, second harmonic signal is obtained when the light passes through materials with a specific molecular orientation and especially, when the material consists of non-centrosymmetric molecular structures. In the case of biological materials, intense SHG signals are recorded from collagen, microtubules (tubulin) and myosin thick filaments, because these are highly polarizable due to their large non-centrosymmetric structures (Carriles et al., 2009).

In THG microscopy, third harmonic light is generated at the focal point of a tightly focused ultra-short pulsed laser beam. No net THG signal is obtained from a homogeneous, normally dispersive medium. However, when the nonlinear medium is not uniform, THG signal is elicited by interfaces and optical inhomogeneities. This nature of the THG process renders it highly sensitive to in-homogeneities, while the efficiency of signal generation strongly depends on the relative size of the in-homogeneity and the focal volume. Thus, THG has been used to investigate a variety of biological samples because of its intrinsic property to provide valuable information about morphology, structure and anatomy of the specimens at microscopic level.

In biological samples, THG signal is enhanced by the presence of multilayered structures, like membranes and lipid bodies. It has been shown that in THG imaging, the primary sub-cellular source of contrast, are lipid bodies (LBs) (Debarre et al., 2006). Therefore, such approach can be used to study lipid bodies in complex environments.

Lipid bodies are vital structures for the cell and play a crucial role in multiple cellular processes. Lipids in human health and disease have gained a central role in biomedical research. Although during the past decades, LBs were considered as lipid storage and trafficking organelles in the cell, it is now well established that LBs are highly regulated structures involved in inflammatory and infectious diseases (Digel, Ehehalt, &

Füllekrug, 2010; Martin & Parton, 2006; Walther & Farese, 2012). Increased cytoplasmic lipid accumulation in cells, has been observed in a number of clinical and experimental metabolic diseases (Abramczyk et al., 2015; Bozza & Viola, 2010; Swinnen, Brusselmans, & Verhoeven, 2006; Walther & Farese, 2012). The shape and the localization of LBs appear to correlate with the development of metabolic disorders (Daemen, van Zandvoort, Parekh, & Hesselink, 2016).

Many modern technologies, including mass spectrometry (MS), nuclear magnetic resonance (NMR) and microfluidic devices, are utilized to identify, quantify, and understand the structure and function of lipids in biological systems (Daemen et al., 2016). However, these are complex techniques, where visualization of LBs needs the use of fluorophores and fixation. In contrast, the non-destructive THG modality can identify LBs without any labeling, providing thus information about their structure and position in a variety of samples.

3. Applications

The need for improved, new diagnostic tools in biomedical studies has driven the demand for the development of novel, label free, non-destructive techniques, leading thus to the applications of nonlinear microscopy. THG has been successfully used to solve diverse biological problems (Coda, Siersema, Stamp, & Thillainayagam, 2015; Gavgiotaki, Filippidis, Kalognomou, et al., 2015; Kuzmin et al., 2016) and allow comprehensive description of complicated biological processes.

THG modality is increasingly promising for clinical diagnostics and monitoring serious diseases (J. Adur et al., 2012; Gavgiotaki, Filippidis, Kalognomou, et al., 2015; Nadiarnykh, Thomas, Van Voskuilen, Sterenborg, & Gerritsena, 2012; Sasanpour, Rashidian, Rashidian, & Vossoughi, 2010). Additionally, many studies utilized THG microscopy for LB visualization and quantification in different biological issues.

Debarre et al. characterized the morphogenetic movements in developing

Drosophila embryos by employing THG quantification techniques (Debarre et al., 2004). Later on, the same group of authors focused on the characterization of biological liquids, by using the THG imaging modality. Analysis of THG images of cells, showed large discontinuities around dense inclusions of lipid structures. Moreover, the authors speculated that lipid bodies in tissues are expected to provide 1–2 orders of magnitude higher THG signals as compared to the signals generated at an interface between two aqueous media (Débarre & Beaurepaire, 2007).

In additional studies, Belisle et al. utilized THG imaging as a diagnostic tool for the detection of malaria-infected blood cells. The diagnosis was based on the quantification of S/N ratios of THG signal in cells (Bélisle et al., 2008). Later on, Watanabe et al. demonstrated the dynamic behavior of LBs in mouse embryology at different developmental stages (Watanabe et al., 2010). Bautista et al. discriminated quantitatively the composition of LBs by employing polarized third harmonic generation (Bautista et al., 2014). Wu et al. used a combination of THG and SHG modalities to quantify the structural changes of collagen in mouse. They found that SHG and THG could be used as a proper diagnostic tool for collagen remodeling *in vivo* (Wu, Hsieh, Tsai, & Liu, 2015). Recently, Kuzmin et al. used third harmonic generation as an imaging tool for diagnosis of human brain tumors (Kuzmin et al., 2016).

3.1 Evaluation of BV-2 microglia cell activation

In this section THG imaging microscopy was used to investigate the activation state of BV2 microglia mouse cells. BV2 cells are resident microglia cells that play a significant role in the Central Nervous System (CNS) and are connected to many pathological conditions. The activation of these cells is connected to many serious CNS inflammations such as Alzheimer's, Parkinson's, Huntington's and schizophrenia.

Many studies were focused on the activation of glial cells during the past years. Ling, investigated the lumbosacral spinal cord into adult rats by using electron microscopy (Ling, Dahlström,

Polinsky, Nee, & McRae, 1992). Xie, investigated the activation of glia cells when inducing neuron death, by using fluorescence microscopy techniques (Xie, Smith, & Van Eldik, 2004). Later on, Stence et al. employed confocal microscopy to identify changes in the dynamic behavior of activating microglia (Stence, Waite, & Dailey, 2001).

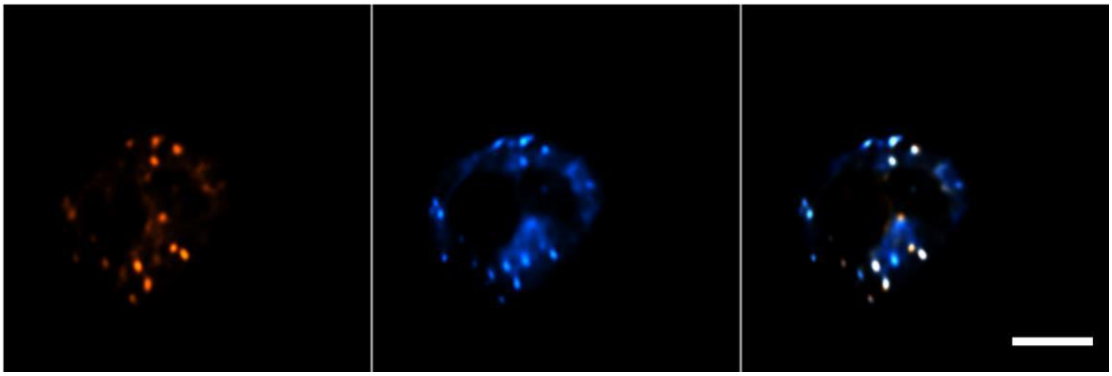

Figure 1: Identification of sub-cellular organelles of BV2 microglia cells that produce high THG signals. THG and TPEF images were collected simultaneously. 2D images of BV2 cell stained with nile red (THG in orange, TPEF in blue). The white color on the right merged image depicts the co-localization of TPEF and THG signals. Scale bar denotes 10 μm.

Lipid bodies are functionally intracellular organelles and have been shown to be important in microglia activation (Khatchadourian, Bourque, Richard, Titorenko, & Maysinger, 2012). THG has several unique technical and application advantages in comparison to other imaging techniques. As already mentioned, the main contributor of cellular structures in THG imaging are the lipid bodies (Debarre et al., 2006). The efficiency of this technique to visualize lipid structures without the need of staining makes it a perfect tool to evaluate microglial activation.

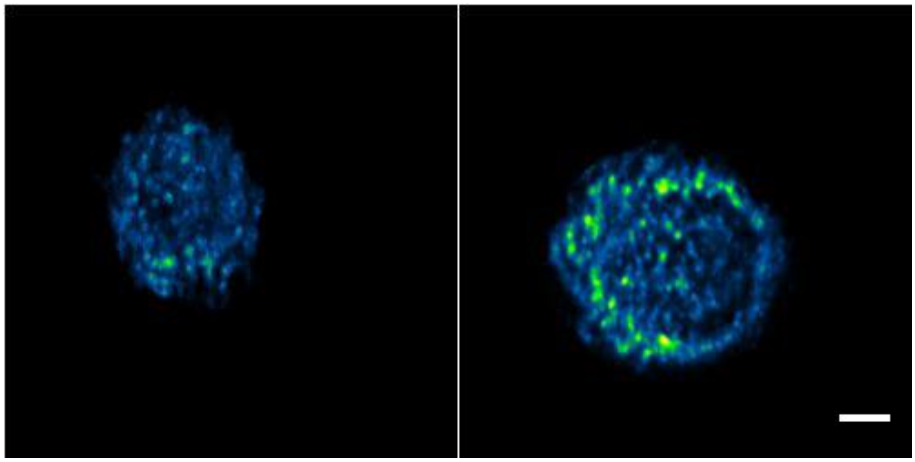

Figure 2: THG images of BV-2 cells (a) control (b) activated. The scale bar depicts 5 μm.

Thus, THG techniques were used to delineate and quantify the intracellular structures in control and activated microglia cells (Gavgiotaki, Filippidis, Kalognomou, et al., 2015).

Upon staining of BV2 cells with a nile red for LB detection, the cells were processed to THG imaging (Figure 1). The results showed that the major subcellular source of high THG signal is emitted by LBs, confirmed thus previous studies (Debarre et al., 2006).

Using THG image analysis, it could be shown that activation of BV2 cells with lipopolysaccharide (Gavgiotaki, Filippidis, Kalognomou, et al., 2015) revealed higher amounts of LBs as compared to control non-activated cells (Figure 2). In order to quantify the intracellular THG signal, a proper algorithm (MatLab) that was setting a constant threshold to calculate only the area of the organelles in the cytoplasm that produce high THG signal (mostly LBs), was used. The results showed that the mean total area of THG values of the activated cells was almost ten times higher than those of non-activated cells, implicating that the THG microscopy technique is a proper tool for the illustration and discrimination of BV2 microglia cells during activation.

3.2 Discrimination of Breast cancer cell lines

Cancer, considered to be a disease with truly global reach and research focusing into its causes and treatment, is on the spotlight. Various studies have

utilized label free THG imaging to observe morphological changes that arise in cancer cells and cancerous tissues (Adur et al., 2011; Javier Adur et al., 2012; J. Adur et al., 2012; Chang et al., 2008; Coda et al., 2015; Harpel et al., 2016; Kuzmin et al., 2016; Nadiarnykh et al., 2012; Sasanpour et al., 2010; Tai et al., 2005; Tuer et al., 2010; Wu et al., 2015).

Breast cancer cell lines have been widely used to investigate breast cancer pathobiology and new therapies. By applying the THG imaging technology to breast cancer cells (Gavgiotaki, Filippidis, Psilodimitrakopoulos, et al., 2015), it was shown that high THG signals could be detected from the cellular and nuclear membranes as well as from the intracellular LB structures (Figure 3). In THG imaging, nucleus is generally presented as a black area in the cell, due to the dense chromatin structure, which does not allow nonlinear light scattering.

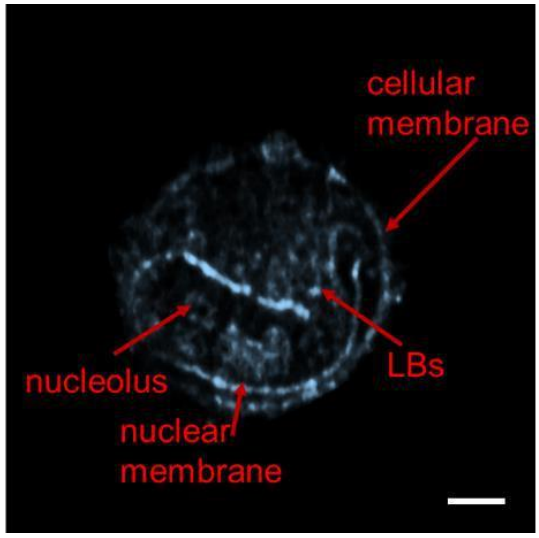

Figure 3: 2D THG image of a cancer cell. Scale bar depicts 5 μm.

All cancer cells were characterized by irregular size and shape, enlarged nucleus and nucleolus, as well as an increased amount of intracellular signal. There were profound differences in the nuclear architecture of cancer compared to normal cells, which show nuclei with regular and ellipsoid shape. Such morphologies are in perfect agreement with other studies (Chiotaki, Polioudaki, & Theodoropoulos, 2014; Lin, Zheng, Wang, & Huang, 2014; Webster, Witkin, & Cohen-Fix, 2009; Wolberg, Street, & Mangasarian, 1999; Zink, Fischer, & Nickerson, 2004).

The breast cancer cell lines used in that study were classified as luminal, Triple negative or Her2 positive. Peripheral blood leukocytes (PBMCs)

were used as controls for comparison reasons. Each cell line presented a specific THG signaling pattern. Specifically, it was observed that in Her2+ cells, THG signal originated from the intracellular structures (mainly LBs), while THG signal in the triple negative cells mostly originated from the nuclear and cellular membranes. In luminal and control cells, THG signals arose from both the membranes and the sub-cellular components (Figure 4).

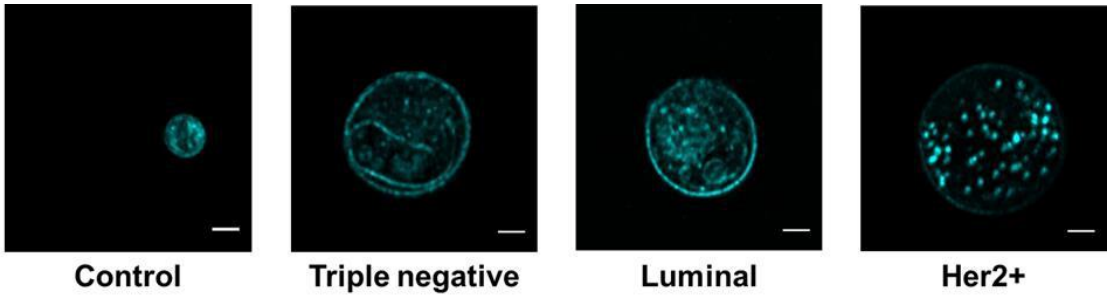

Figure 4: THG signal distribution (maximum z projections) of different cell types. Scale bar depicts 5 μm.

Additional information related to the activation state of different cell types was extracted by quantifying of the collected THG signals. Such analysis was based on the calculation of the mean total area by setting a constant intensity threshold of THG signals that corresponded mainly to LBs and cellular membranes. It was observed that breast cancer cells presented an increased lipid profile in comparison with control cells (PBMCs), while it was shown that each cell line followed a specific compartmentalization of THG signal. Therefore such methodology may contribute and offer new insights for better understanding the pathogenesis of breast cancer.

3.3 Qualification and Quantification of Pre-implantation Mouse Embryo Development

Several studies demonstrate the ability of THG imaging to provide information related to morphological changes and complex developmental processes of embryos during the past years (S.-Y. Chen et al., 2006; Chu et al., 2003; Debarre et al., 2004; Hsieh et al., 2008; Jesacher et al., 2009; Sun et al., 2004; Supatto et al., 2005) (Aviles-

Espinosa et al., 2010; Débarre, Olivier, Supatto, & Beaurepaire, 2014; Kyvelidou et al., 2016; Luengo-Oroz et al., 2012; Mahou et al., 2011; Thayil et al., 2011; Tserevelakis, Filippidis, Megalou, Fotakis, & Tavernarakis, 2011).

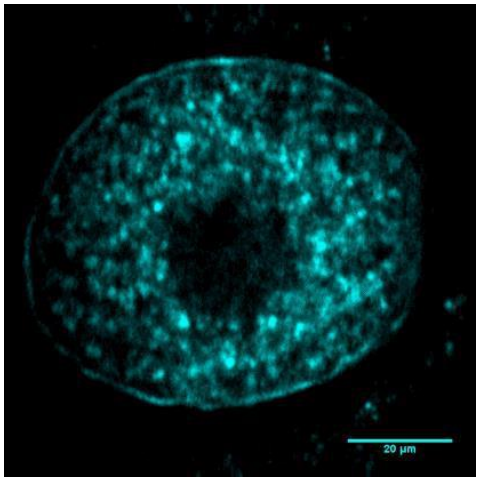

Figure 5: 2D THG image of a mouse embryo (zygote). The membrane of the embryo, the LBs structures and the black area which depicts the nucleus can be observed. Scale bar depicts 20 μm.

THG microscopy has been used to evaluate pre-implantation mouse embryo health (Kyvelidou et al., 2011). Until now many diagnostic tools have been applied in embryology, including confocal scanning microscopy and differential interference contrast technique (DIC). However, these techniques consist of complex systems and offer a view of the intracellular structures by using fluorophores of other markers. The major advantage of THG imaging in embryological studies is that minimum preparation is needed, while there is no need of staining of the samples.

THG was used as an alternative method for detection of lipid bodies, in order to provide valuable information as to the energetic status of pre-implantation embryos and time evolution of the different developmental stages.

The study of pre-implantation embryo development stage and its energetic profile was indirectly correlated to embryo health. In THG imaging, the nucleus appeared as a black spot inside the embryo because of its homogeneity, disallowing thus any THG signal generation (Figure 5).

Following embryo development, the position of the nucleus and lipid bodies could be defined, while the disappearance of nucleus could be observed during mitosis. Embryo patterning was studied through THG imaging in different developmental stages (Figure 6). It was found that THG imaging could easily detect the pronuclei and the intracellular structures (mostly lipid bodies) in embryos at the different developmental

stages. Moreover, the use of a proper algorithm for THG signal quantification revealed important information about the energetic profile of each developmental stage of pre-implantation mouse embryo. Specifically, it was shown that the highest LB content was observed at the 2-cell stage embryos and decreased thereafter (Kyvelidou et al., 2011).

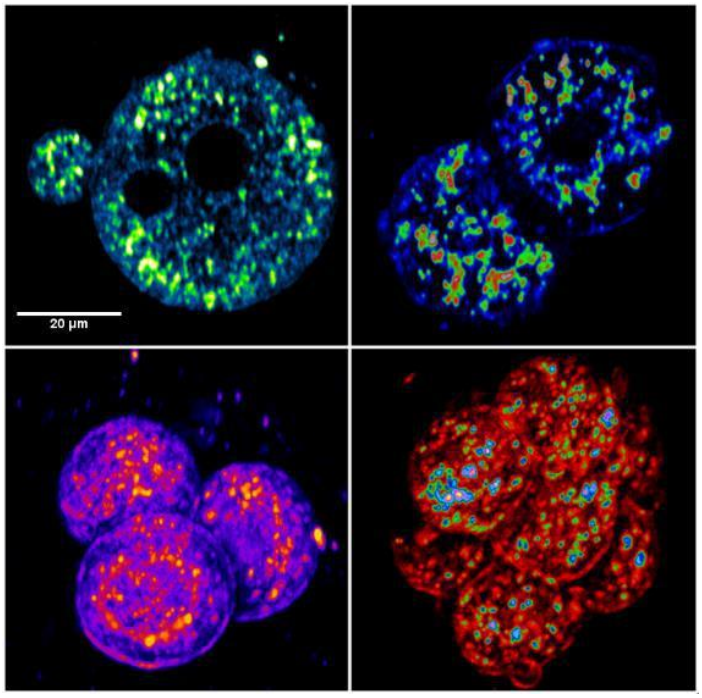

Figure 6: Different developmental stages of mouse embryos (1, 2, 4, and 8 cell stage embryos) imaged via the detection of THG signals.

Therefore, it was proved that the THG tool could easily provide reliable criteria for the pre-implantation embryo patterning during development. THG imaging is superior to other techniques, because it does not require embryo manipulation, fixation or staining and could be implemented in the future to human embryo selection as well.

4. Conclusions

Nonlinear optical imaging modalities are increasingly promising for clinical diagnostics and the monitoring of serious diseases, as they can probe the biological sample with high resolution without damaging it.

THG imaging, through its ability to map lipid bi-layers and LBs in the intracellular compartments, becomes an

ideal tool for examination of many biological settings. Application of this technology has been successfully extrapolated to BV2 cell activation, breast cancer cell line classification and distinction of mouse pre-implantation embryo health. LBs are emerging as important organelles in cellular processes, since they are linked to various biological functions including lipid metabolism, cells signaling and inflammation. Therefore, THG imaging is being offered as a suitable non-invasive tool for study.

Concluding, THG is beneficial for performing deep microscopy without the need for external labeling. THG modality can be used as a new, label free, non-destructive, high resolution technique, alternative to fluorescence and dye-based approaches, for lipid biology research. The quantification of the recorded THG signals provides reliable biological criteria, through a novel approach for studying the activation state of cells. The developed methodology will potentially be proven extremely useful as an innovative diagnostic tool to understand inflammation, pathogenesis of cancer disease as well as evaluation of the pre-implantation embryo health.

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