Higher-harmonic generation microscopy beyond the diffraction barrier based on re-scan strategies for optical data acquisition (HARMOPLUS)

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ABSTRACT

Microscopy techniques that exploit nonlinear optical process by which a sample emits lights in the visible upon excitation with infrared photons have attracted important attention in the past decades. Among these, Second Harmonic Generation Microscopy (SHG) and Third Harmonic Generation Microscopy (THG) have been demonstrated as powerful tools for the 3D visualization of tissues and advanced materials. The main objective of HARMOPLUS is to demonstrate that the contrast mechanisms of these techniques can be harnessed to provide resolutions beyond the diffraction barrier, when combined with the underlying concepts of the award-winning Re-scan Confocal Microscope (RCM). Our efforts showed that an RCM system can be easily modified to enable Re-scan Second/Third Harmonic Generation Microscopy, and additional imaging techniques based on nonlinear effects such as Re-scan Two-Photon Excitation Fluorescence Microscopy.

Keywords: Re-scan Confocal Microscope, Second Harmonic Generation Microscopy, Two-Photon Excitation Fluorescence

1. INTRODUCTION

Given their valuable contrast mechanisms and optical sectioning capabilities, nonlinear optical microscopies (NLO)[1,2] have emerged over the past three decades as very powerful and important optical characterization tools. Among these Second Harmonic Generation Microscopy (SHG), a technique in which two incident (infrared) photons are combined into a single emitted photon with double the energy via a nonlinear process involving virtual states, has gained important interest as a result of its superb potential for the label-free characterization of fixed, *ex-vivo* and *in-vivo* tissues[3-5]. Third Harmonic Generation Microscopy (THG), which involves similar principles but achieved with three-photons instead of two, is also emerging as an important bioimaging tool[6]. Both have also brought significant added value to the field of materials sciences, enabling a variety of applications[7-9]

The importance of light microscopy at sub-diffraction resolutions is well highlighted by the 2014 Nobel Prize in Chemistry that was awarded *"for the development of super-resolved fluorescence microscopy"*[10]. Although their advent immediately generated huge impact in life sciences, the most “notorious” fluorescence based Super-resolution Microscopy (SRM) techniques such as STED[11], or STORM[12] face a series of important limitations due to the lack of chemical sensitivity and dependence on fluorescent probes, which restricts their use to a limited range of samples, mainly of biological origin. Even in the case the latter, the advantages of fluorescence SRM techniques come accompanied by a series of drawbacks related to the fact that exogenous and genetically engineered contrast agents can influence the behavior of live specimens which are imaged, while also leading to cyto- and phototoxicity. Furthermore, recent studies suggest unpredictable anomalous processes related to the distribution of contrast agents in biological samples[13]. Such limitations and concerns keep scientists motivated to innovate towards alternative avenues of overcoming the diffraction barrier in the form of optical imaging techniques not requiring contrast agents (“label-free”). SHG/THG imaging based on the intrinsic contrast of samples holds important potential for overcoming many of the issues above mentioned but achieving sub-diffraction resolutions is still a challenge.

To address this we have developed a multimodal prototype system which exploits the ingenious concepts of the Re-scan Confocal Microscope (RCM)[14-16] to enable super-resolved Re-scan SHG/THG imaging, and additionally, Re-scan Two-Photon Excited Fluorescence (TPEF), also a NLO technique. Our work shows that the advantages of RCM over conventional CLSM, such as increased resolution and sensitivity are also available with the new Re-scan workmodes, demonstrating the versatility of this technology, and its potential to enable a new wide range of applications focused on super-resolved label-free NLO imaging. Based on the Re-scan concept, such applications will become available with easy-to-use systems fit for every-day use.

2. STATE OF THE ART

Fluorescence based SRM succeed in overcoming the resolution limits imposed by diffraction, offering typical resolutions in the range of 20-100nm[11,12,17]. However, the current state-of-the art in label-free SRM is yet to reach such performances. Among the first notable attempts to achieve super-resolved images based on harmonic generations have been reported by Masihzadeh O.[18] and Liu J. et al.[19], who succeeded in reducing the size of the Point-Spread Function (PSF) by manipulating the polarization state of the incident light. While these strategies are very ingenious, they also present an important disadvantage, consisting in their high-sensitivity to effects such as circular dichroism or birefringence that interfere with the polarization state of the excitation light, and cannot be neglected in thick specimens. A more recent approach that overcomes these problems, has been proposed by Field et al.[20] who introduced multi-photon spatial frequency-modulated imaging (MP-SPIFI), a technique demonstrated to provide super-resolved images in both SHG and TPEF. The authors experimentally showed a resolution improvement of 2x and demonstrated a theoretical limit of 4x. Although the great value of this work cannot be disputed, the intricate experimental setup raises some concerns on whether this method can be made available in easy-to-use systems that can produce reliable datasets for a non-expert user. In this regard, we find important to mention the work of I. Gregor et al.[21], who reported a way to achieve super-resolved SHG and TPEF images using an image scanning system that can be easily obtained by lightly modifying a conventional setup for multiphoton microscopy. Under illumination with a 900 nm laser beam, the authors demonstrated in the case of rat tail collagen I hydrogels the possibility to resolve collagen fiber distances of 550 nm with a contrast of 100%, which was not possible with conventional SHG imaging. Also important to mention is the very recent work of A.M. Barlow et al.[22], who demonstrated that super-resolved SHG and TPEF images can also be collected with the commercial Airyscan scan detector from Zeiss. With their approach (also based on Image Scanning Microscopy (IMS) as described by Enderlein[23]), they demonstrated a 1.7x improvement over the diffraction limit in the case of collagen fibers. The great advantage of this last work with respect to the future widespread of super-resolution imaging based on harmonic generations stands in the market share of Zeiss, one of the leading microscopy companies, and the number of Airyscan units available in laboratories worldwide.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

The main objective of HARMPLUS was to demonstrate super-resolved harmonic generation imaging based on the Re-scan concept, and that such imaging modalities can provide similar advantages in terms of resolution and sensitivity as RCM. This objective has deep implications for facilitating the widespread of super-resolved SHG/THG imaging (and NLO imaging, in general) due to the versatility and flexibility of Re-scan systems. Commercialized at present by Confocal.nl, these systems are modular in nature. An RCM unit can be coupled to an optical microscope featuring a lateral port, to collect images with a low-cost or high-end scientific camera, under illumination with independent laser sources coupled into the RCM unit by an optical fibre. Given this modular structure, RCM systems are gaining high interest, as they can be configured in a myriad of ways in terms of illumination sources, cameras, microscopes, etc. Furthermore, image acquisition is performed by means of the μManager[24] open-source software, which allows the user to customize open-source plugins contributed by the imaging community or write novel ones to enable new image acquisition strategies or visualization methods.

En route to achieving the planned objective we have built a proof-of-concept multimodal system featuring RCM, Re-scan SHG/THG and Re-scan TPEF, that can be used to visualize biological species and advanced materials. With respect to the former, this system enables biochemical fingerprinting and structural mapping of cellular and sub-cellular structures or tissues in a non-invasive and non-destructive manner with or without fixing and/or labelling, given its complementary contrast mechanisms. This multimodal characterization approach at sub-diffraction resolution can be harnessed to investigate with high accuracy a large variety of fundamental biological processes, including cell cycle dynamics, cell death, cell differentiation, etc. Furthermore, this system allows exploring the architecture of non-stained tissues at an incredible level of detail, as shown next. With respect to this, given that an RCM unit adapted for Re-scan SHG can be easily coupled to optical microscopes available in clinical settings, histopathology labs can immensely benefit from this development, as it can enable modern histopathology workflows complementing their traditional assays. For example, by resolving the collagen architecture at supe-resolution histopathologists can identify subtle modifications that precede or develop during cancers.

4. PROJECT RESULTS

To achieve the set objectives, we have modified a standard RCM unit to enable multimodal imaging with RCM, Re-scan SHG/THG and Re-scan TPEF. While coupling the fs-laser (required for achieving non-linear optical effects) via an optical fibre could also be possible, we preferred to couple it by free-space to avoid a spreading of the pulses, and to have the possibility to more easily modify different properties of the beam (e.g. size, shape, polarization, etc.) in order to benchmark their effect on the imaging outputs. For this, a free-space port has been added to the case of the RCM unit, and used to

introduce the fs-laser traveling by air in the optical path, that was initially used for RCM imaging[14]. A dichroic mirror ZT775sp-2p (Chroma) was used to combine the Continuous Wave (CW) beam(s) used for RCM imaging (405nm, 488nm, 633nm) with the fs-laser beam used for Re-scan SHG/THG (860nm/1290nm). The standard dichroic mirror and emission filter of a typical RCM unit have been kept in place, ZT405-488-561-640-NIR-rpc & ZET405-488-561-640m (Chroma), as they don't interfere with collecting the SHG/THG signals generated under the above-mentioned illumination settings. Two additional short pass filters, ET750sp-2p8 (Chroma), have been introduced before the camera to block the NIR excitation light. To extract the SHG/THG signals (or TPEF signals) with a bandpass filter we designed a retractable 3D-printed filter holder, which allows switching between RCM and NLO workmodes. For SHG imaging of collagenous tissues a bandpass filter was inserted, ET430/24x (Chroma); and for TPEF imaging (of samples not emitting SHG light) no filter was used.

To perform multimodal imaging with the considered modalities, the custom-modified RCM unit has been coupled to a Nikon inverted microscope equipped with a 100x/1.42 APO objective. For RCM imaging an Omicron laser combiner featuring three lines (405nm, 488nm, and 638nm) has been coupled by optical fiber via the default RCM illumination port. For Re-scan SHG and TPEF a fs-beam originating from a Chameleon Vision II (Coherent) Ti-Sapphire laser with ~140 fs pulses, a repetition rate of 80 MHz, tuned at 860 nm and ~100mW power has been introduced in the RCM unit via the custom-made free-space port. For Re-scan THG imaging the fs-beam has been fed to an Oria IR XT (Radiantis) Optical Parameter Oscillator (OPO), to result into a 1290nm beam; work is still in progress for demonstrating this workmode. Imaging has been performed with a low-cost, non-cooled, Chameleon3 USB3, Model: 5.0 MP, 2448 X 2048, MONO, 35 FPS, SONY IMX264 camera, with a unit cell size of 3.45 μm (H) × 3.45 μm (V).

One of the important issues that had to be addressed in terms of illumination with a NIR fs-laser beam was the lack of triggering possibilities for line blanking. In a typical RCM system, the CW laser is switched off during the return movement of the scanner in order to avoid ghosting effects (blur) that may arise due to the fact that the angular speed of the scanner and the re-scanner are not exactly the same for the forward and backward movement of the scanners. This leads to a phase difference of the scanner relative to the re-scanner. This problem is normally solved by hardware triggering, which synchronizes the on/off status of the laser with the forward/backward movement of the scanner. Implementing a similar strategy with a fs-laser beam is not feasible as such high-frequency on/off modulation interferes with the pulse locking. To avoid this, a plugin has been designed to identify an optimal offset in the phase of the re-scanner, which alleviates ghosting effects. This plugin enables a calibration procedure consisting in acquiring multiple images of an a priori known scene (e.g. micrometer ruler) at different, incremental, scanner phase offset values, and identify the phase offset that results in the sharpest image. Following this calibration procedure an optimal phase offset is identified and used to collect images on the sample of interest.

In Fig. 1 we showcase the resolution advantage of Re-scan SHG and Re-scan TPEF over conventional, diffraction limited SHG and TPEF imaging. As thoroughly described in the work of De Luca et al.[14] improved resolution with the Re-scan concept is achieved when the angular amplitude of the camera scanner (re-scanner) is double the angular amplitude of the sample scanner (Sweep factor = 2, in Confocal.nl RCM terminology); the resolution available when the amplitudes of the two scanners match (Sweep factor = 1) is similar to the resolution of a conventional confocal laser scanning system (diffraction limited). The images depicted in Fig. 1 are collected at Sweep factor 1 (bottom row) and Sweep factor 2 (top row). We can observe that Re-scan SHG images collected on a rat-tail tendon section exhibit significantly higher resolution compared to SHG images collected at conventional resolution. Re-scan TPEF is also shown to resolve in more detail subtle features of a herbaceous plant stem (Omegon) compared to conventional TPEF. We feel it is important to mention that in high-end RCM systems cooled s-CMOS cameras are typically used, while the images presented in this article have been collected with a non-cooled camera, with quantum efficiency < 70%.

The reason for using collagenous tissues for demonstrating the resolution advantage of Re-scan SHG relates to the importance of SHG imaging for tissue state assessment. Given that collagen is the main component in the extracellular matrix of mammalian tissues, SHG imaging of this protein with non-centrosymmetric structure provides important information on how the structure of tissues is modified along the progression of various pathologies including cancers. Recent method for artificial intelligence hold significant potential to augment this usefulness of SHG imaging, and to this end, in an experiment partially supported by HARMOPLUS, we showed that fine-tuning pre-trained Deep Neural Networks with limited sets of SHG/TPEF images (healthy vs. dysplastic epithelial tissues) leads to achieving classification accuracies exceeding 95%[25]. This preliminary experiment has been implemented on SHG/TPEF images collected with a conventional system, and work is in progress for demonstrating how the improved resolution available in Re-scan SHG/TPEF can boost the classification performance.

While an experiment on thoroughly benchmarking the resolution capabilities of Re-scan SHG using $BaTiO_3$ nanoparticles is still in progress, the preliminary results suggest a very consistent resolution improvement over the diffraction limit, exceeding the resolution improvement of RCM over conventional CLSM.

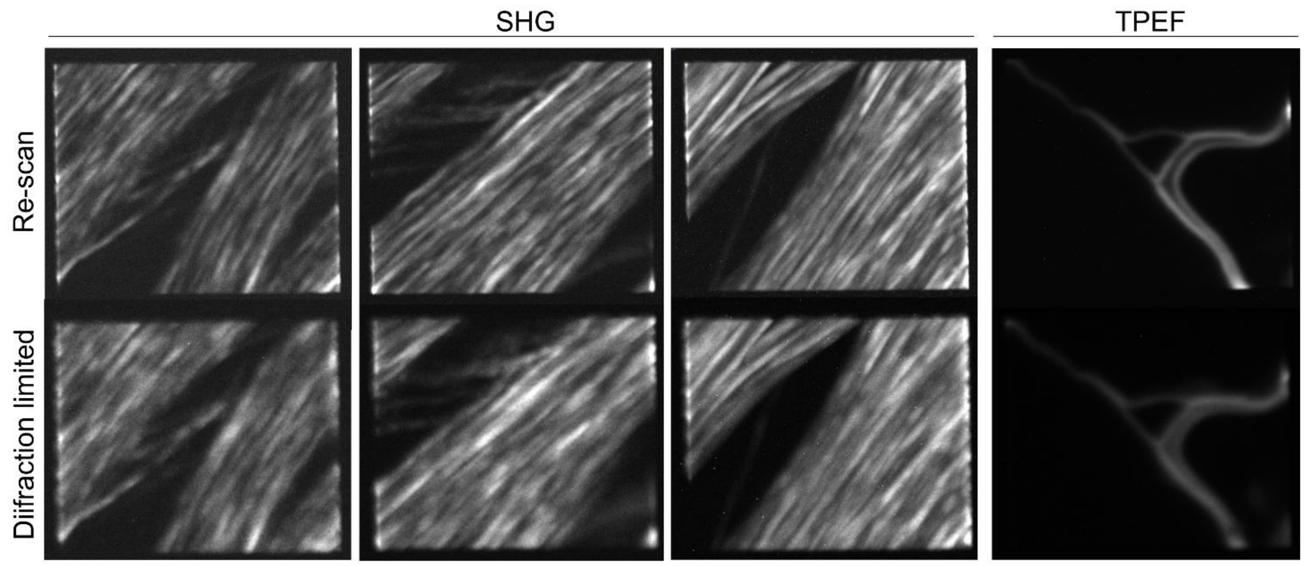

Fig. 1 Re-scan Non-Linear Optical Microscopy. (left) Re-scan SHG vs. diffraction limited SHG images collected on rat-tail tendon. (right) Re-scan TPEF vs. diffraction limited TPEF images collected on an herbaceous plant stem.

5. FUTURE PROJECT VISION

5.1. Technology Scaling

The HARMOPLUS project achieved its set objectives, at TRL 3: Experimental proof-of-concept. We envision that Phase 2 of Attract would allow taking the demonstrated technology further, at the borderline of TRL7: TRL 7 – “system prototype demonstration in operational environment” and TRL 8: TRL 8 –“system complete and qualified”. The main steps required to scale up the proposed technology, namely super-resolution label-free Re-scan Harmonic Generation Imaging, are the following:

- Tests for Safety Certification of the modified RCM unit to be used with Class IV lasers, required for non-linear optical microscopies.
- Developing illumination strategies to enable the coupling of fs-lasers by optical fiber instead of free-space, to enhance user safety.
- Improvement of acquisition speed to ensure compatibility with video-frame rate imaging by developing a novel illumination method that uses either synchronized resonant scanners, or a single scanner to perform both tasks (sample and camera scanning).
- Developing artificial intelligence methods custom designed to match the specifics of Re-scan Harmonic Generation Microscopy. Such methods will address four important topics: a) improvement of resolution and speed[26], b) virtual cross-modality[27] c) digital staining[28], and d) automated quantitative analysis (e.g. object segmentation[29], tracking, classification[25], etc).
- Demonstration of the functionality and usefulness of the envisioned Smart High-Speed Re-scan Harmonic Generation Microscope in three-types of end-user labs: cell biology, histopathology and chemical engineering labs.

5.2. Project Synergies and Outreach

To efficiently address the activities that are required to scale up the proposed technology, briefly discussed under the previous sub-section, we consider that an optimal consortium configuration would require (besides the two initial partners, CO: UPB and P1: Confocal.nl):
P2: an SME dealing with development of laser sources, with interest in developing affordable fs-illumination sources tuned to the requirements of Re-scan Harmonic Generation Microscopy.
P3: academic/SME partner with expertise in electronics, and interest in developing novel data acquisition schemes for high-speed Re-scan imaging
P4: academic/SME partner with expertise in artificial intelligence and cloud-based computing, and interest in developing methods tuned to the specifics of Re-scan imaging.
P5: SME partner with expertise in image deconvolution.
P6: End-user lab with expertise in cell biology
P7: End-user lab with expertise in histopathology
P8: End-user lab with expertise in chemical engineering.

To augment the impact of the developed technology and accelerate the scaling up, outreach activities will represent an important focus. In this regard we will submit in the first year of implementation of the potential Phase 2 project a COST proposal aimed at constituting a

hub for relevant stakeholders in super-resolved label-free imaging. This hub will gather instrumentation specialists, computer scientists with interests in imaging oriented artificial intelligence, and end-users of super-resolution imaging systems. In case this COST Proposal will be funded, specific activities such as Short-Term Scientific Missions, ITC Conference Grants, Workshops, etc., will be harnessed for the advent of an eco-system revolving around super-resolved label-free imaging. In the alternative case, in which COST support will not be gained, we envision the creation of a European Cluster for Label-Free Imaging. Various routes to achieve funding for creating this hub (including EC funding, or private funding) will be carefully assessed.

5.3. Technology application and demonstration cases

In a potential Phase 2 project we envision the participation of three end-user labs, that will facilitate the system prototype demonstration in operational environment (TRL 7). An end-user lab with expertise in cell biology will demonstrate the usefulness of the proposed technology with respect to enabling novel fundamental research routes that can sched light on still unresolved aspects of cell life and death. An end-user lab with expertise in histopathology will demonstrate the usefulness of the proposed imaging system for resolving subtle tissue architecture features not available to conventional imaging systems. The main focus in this research direction will be placed on identifying cues that can signal the onset of cancers. An end-user lab with expertise in chemical engineering will demonstrate the usefulness of the proposed imaging system for characterizing optical properties of (nano)biomaterials, which enable/facilitate their functionalization for nanomedicine. This end-user will be in close dialogue with the cell biology and histopathology end-users.

5.4. Technology commercialization

During the life-span of the Phase 2 extension of HARMOPLUS, a detailed business plan will be put in place, which is required for a swift translation of the TRL7-8 system to the market. Intellectual Properties (IP) developed in the project will be harnessed to keep-on top of competitors, and to efficiently monetize the gained know-how via IP licensing routes. One of the key features of the RCM systems are versatility and affordability, and to this end we plan to liaise with relevant companies showing interest in bundling complementary equipment (e.g. cameras, lasers, etc) with the Re-scan Harmonic Generation Microscope in all-in-one packages at different levels of price and performance, addressing various market segments. A consulting company will be hired to design optimal dissemination and advertisement routes, which will favour a wide exposure of the developed technology to potential end-users.

5.5. Envisioned risks

The ambitious goal of scaling up the developed technology requires the expertise of both academic and private partners. SME's are mainly preoccupied to invest time in developments that can swiftly bring added value to their portfolio of IPs or marketable products, while academic groups are more tempted to invest time in work that leads to publications in top-journals, even if an immediate effect of their efforts on the market value for their SME partner is difficult to predict. Satisfying both philosophies can be at times challenging, and the impossibility to timely identify solutions pleasing to all involved parties represents an implementation risk

5.6 Liaison with Student Teams and Socio-Economic Study

HARMOPLUS Phase 2 will nominate an experienced person to liaise with relevant MSc. level student teams, to familiarize them with the developed technology and its potential for advancing various fields of research or for enabling novel industrial applications. In this regard, a YouTube channel with lectures presented by the consortium partners, in terms easily accessible to MSc. level students will be organized, and an online forum will be published. Internship positions will be created and advertised on various online and offline channels. To contribute to the expert-driven socio-economic study that will be organized by ATTRACT, HARMOPLUS will make available to the coordinators of this study all required information. Furthermore, all team members will commit time to participating in interviews, polls, etc.

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