frontiers in Physics

EDITORIAL
published: 14 February 2020
doi: 10.3389/fphy.2020.00017

Editorial: Advances in Label Free Tissue Imaging With Laser Scanning Microscopy Techniques

Stefan G. Stanciu[1]*, *Christophe Silien*[2]* and *Paolo Bianchini*[3]*

[1] Center for Microscopy-Microanalysis and Information Processing, Politehnica University of Bucharest, Bucharest, Romania, [2] Modelling Simulation and Innovative Characterisation, Department of Physics, Bernal Institute, University of Limerick, Limerick, Ireland, [3] Nanoscopy and NIC@IIT, Istituto Italiano di Tecnologia, Genoa, Italy

Keywords: laser scanning microscopy, tissue imaging, second harmonic generation microscopy, two photon excitation fluorescence microscopy, Brillouin microspectroscopy

Editorial on the Research Topic

Advances in Label Free Tissue Imaging with Laser Scanning Microscopy Techniques

Significant efforts are being spent at the time being for transferring various laser scanning microscopy (LSM) techniques to the realm of tissue characterization, because of their potential to circumvent some of the most important disadvantages of traditional histopathology approaches based on excisional biopsy and tissue staining. Although conventional histopathology is currently regarded as a golden standard for the diagnosing pathologies that reflect in tissular modification (e.g., cancers), limitations such as long diagnosis time, invasiveness, artifacts, sampling error, time consumption, high costs, and interpretive variability make such approaches to be impractical in many scenarios, while also placing considerable pressure on the sustainability of healthcare systems around the world. The potential of LSM techniques to contribute to overcoming these aspects derives from their "non-invasive" character. They can exploit various endogenous optical signals generated by tissues upon interaction with a laser beam and are able to provide optical sections (virtual biopsies) that reflect the tissular architecture at controlled depths. Many studies reported to date showed that LSM techniques can provide label-free information of similar pathologic relevance to the information collected for characterization/confirmation purposes with traditional histopathology approaches. These techniques are thus capable of probing optical properties of tissues with deep implications for resolving important anatomical and physiological aspects which represent hallmarks for disease predisposition and progression. To date techniques such as Confocal Laser Scanning Microscopy (CLSM) [1], Fluorescence Lifetime Imaging (FLIM) [2], Two-Photon Excited Fluorescence Microscopy (TPEF) [2–6], Second Harmonic Generation Microscopy (SHG) [5, 6], Third Harmonic Generation Microscopy (THG) [4], Coherent Anti-Stokes Raman Scattering Microscopy (CARS) [3, 7], as well as other LSM variants such as the Brillouin Microscopy [8] have already been demonstrated to be powerful tools for investigating tissue morphology, functionality, and biochemical composition with high spatial and temporal resolution. In the opinion of many, these techniques, together with investigations approaches based on their combined use, will soon become the central element of the default tissue characterization frameworks for both *ex vivo* and *in vivo* assays. Furthermore, emerging LSM techniques exploiting various ingenious strategies to achieve superresolved images in a label-free manner [9–12] are also likely to be transferred soon toward applications addressing tissue imaging.

The Frontiers in Physics Research Topic entitled "*Advances in Label Free Tissue Imaging with Laser Scanning Microscopy Techniques*" presents a series of articles focusing on different LSM techniques, in terms of technical aspects and applications. The comprehensive review of Mazumder et al. discusses the principles of the most notorious techniques based on non-linear

Edited and reviewed by:
Qiyin Fang,
McMaster University, Canada

***Correspondence:**
Stefan G. Stanciu
stefan.stanciu@cmmip-upb.org
Christophe Silien
christophe.silien@ul.ie
Paolo Bianchini
paolo.bianchini@iit.it

Specialty section:
This article was submitted to Medical Physics and Imaging, a section of the journal Frontiers in Physics

Received: *16 January 2020*
Accepted: *21 January 2020*
Published: *14 February 2020*

Citation:
Stanciu SG, Silien C and Bianchini P (2020) Editorial: Advances in Label Free Tissue Imaging With Laser Scanning Microscopy Techniques. Front. Phys. 8:17. doi: 10.3389/fphy.2020.00017

optical (NLO) effects, namely TPEF (together with TPEF based FLIM), SHG (including particular variants such as those focused on polarization dependence or circular dichroism), and CARS. The authors also discuss important applications addressing cell and tissue imaging, with a special focus being placed on collagen imaging, the most important protein in the extracellular matrix of mammalian tissues. The subject of multimodality is discussed as well in this review, and provided examples of combining NLO techniques in different ways, e.g., TPEF+FLIM+SHG, SHG+CARS, or FLIM+CARS, are very useful for understanding how complementarity can be exploited to resolve important aspects not accessible by a single technique. Kumar and Kumar, focus in their mini-review on a specific application of SHG imaging, namely the assessment of collagen in articular cartilages, which is important for the efficient diagnosis, treatment-planning, and monitoring procedures in orthopedic diseases. The authors discuss in detail the reasons why SHG is important for examining and understanding articular cartilages, while also demonstrating based on existing literature that this imaging technique is complementary to the standard clinical methods, being capable to elucidate aspects not available with other imaging modalities. For example, SHG surpasses the resolution limitations of other non-invasive techniques such as radiography or magnetic resonance imaging (MRI), which also lack molecular level specificity, while its optical sectioning capabilities can substitute in specific cases direct assessment methods such as arthroscopy and histology. The potential of SHG for taking tissue imaging beyond what is possible with routinely used investigation techniques, is also nicely presented in the original works of Goto et al., Tokarz et al., and Scodellaro et al., all these experiments addressing polarization-resolved SHG, in combination with other complementary techniques. Goto et al. introduce a new methodology for collecting polarization-resolved SHG and TPEF signals based on a LSM system equipped with a spinning-disk confocal scanner, capable to achieve high-speed intravital imaging of living tissues. The superior-temporal resolution that this system exhibits over typical systems used for TPEF and SHG is very important for analyzing *in vivo*, fast dynamics of non-centrosymmetric biological molecules. Tokarz et al. employ SHG, THG, and TPEF for imaging normal and tumorous pancreatic tissues stained with hematoxylin and eosin (H&E), showing that these techniques can bring significant added value not only with respect to imaging unstained tissues, but also in the case of studying tissues prepared with traditional histopathology protocols. We find this to be very important given the fact that these modalities can provide complementary information to conventional histopathology, augmenting thus the potential of H&E stained slides which probably represent at the time being the most common vehicle for tissue characterization and diagnostics. In this regard, the authors show that based on several polarization SHG parameters (e.g., the full width at half maximum of $\chi^{(2)}{}_{xyz}$'/$\chi^{(2)}{}_{zxx}$' occurrence histograms or degree of linear polarization of the SHG signal), normal pancreatic tissues can be distinguished from pathological tissues affected by tumors, while THG is capable of revealing differences in the nuclear morphology for the considered tissue types. Based on the fluorescence of Eosin, TPEF, which is intrinsically available in SHG/THG systems, is shown to be useful for placing SHG/THG data into a well-understood histopathological context. This is because medical experts are mainly trained to distinguish between healthy and pathological tissue states based on images collected with brightfield microscopy on H&E stained tissues and TPEF images replicate part of their content. Scodellaro et al. demonstrate in their work that the diagnostic potential of SHG can be considerably augmented by means of digital image analysis algorithms. They apply their previous method named μMAPPS [13], to analyze entire fixed tumor sections in the specific purpose of extracting the microstructural collagen fibrils angle (θ_F) along with anisotropy (γ) parameters. This experiment, implemented on mouse tissues inoculated with tumor cell lines for colon or breast cancer, shows that converting the above-mentioned measures into dispersion plots in the Fourier space using a 2D phasor algorithm makes possible the subsequent use of clustering algorithms for automatically grouping tissue regions exhibiting similar properties. This is important for enabling novel tumor diagnosis protocols that are fast and easy to implement. The immense usefulness of image analysis algorithms for boosting the utility of data sets collected with NLO microscopies is also demonstrated in the ingenious work of Saitou et al., who employ a conventional machine learning technique named Bag-of-Features (BoF), previously used in couple of other experiments dealing with both TPEF [14] and SHG [15] imaging, to automatically distinguish between TPEF spectra/images collected on normal and pathological liver tissues. They show that combining spectrally resolved TPEF datasets and BoF enables the discrimination of binary tissue states (healthy vs. pathological), and also distinguishing between various stages of a specific disease (in this experiment, namely, liver fibrosis). As discussed by the authors, the proposed methodology can be easily adapted and extended to address other chronic liver diseases (and not only), thus potentially using it in tandem with systems for TPEF endomicroscopy based on miniaturized probes [6] could play an important role for a precise assessment of the liver in real-time. A series of perspectives for using BoF methods in association with LSM techniques to further help in this purpose were previously discussed in Stanciu et al. [16]. Last but not least, in a very useful and interesting study, Elsayad focuses on the emerging technique named Brillouin Microspectroscopy, which represent an important tool for probing the mechanical and viscoelastic properties of biological samples (and materials) in 3D [8, 17]. In this work, a major challenge of Brillouin techniques (both spectroscopic and imaging oriented) is addressed, namely that relatively weak Brillouin light scattering signals together with their subtle spectral variations are observed in many biological samples, yielding significant consequences for subsequent data analysis. Elsayad shows that by using phasor analysis (also discussed in the work of Scodellaro et al., in the context of polarization-resolved SHG imaging) one can more easily distinguish variations in the noisy Brillouin spectra, facilitating thus applications such as cell-sorting or medical diagnostics in terms of speed and ease of implementation. Very importantly, the proposed method is shown to enable the identification of subtle functional variations

in the spectra that are not available with routinely used Brillouin spectroscopy approaches.

To conclude, we believe that this collection of articles stands as convincing proof that LSM techniques are indeed important for imaging tissues. However, a series of significant challenges still lie en route their widespread and reliable use in clinical settings. Difficulties in collecting LSM datasets inside the human body due to miniaturization or stability constraints still exist (the latter due to intrinsic tissue motion), and insufficient penetration depth of the laser beam or high-scattering of the optical signals of interest are still a problem when it comes to probing particular relevant regions in the tissue volume. Furthermore, data interpretation difficulties also cannot be neglected, as histopathologists are trained to recognize diagnostic features in images exhibiting very specific properties in terms of color schemes, scales, visible structures, etc., which complicates their analyses of LSM images. Nonetheless, technological progress in the field of LSM imaging is emerging at fast pace, and recent techniques for virtual-staining [18, 19], that can transform LSM images into representations more accessible to histopathologists are sure to play an essential role in facilitating the interpretation of LSM data by histopathologists. Both are required for the deeper penetration of LSM techniques into the clinical realm.

AUTHOR CONTRIBUTIONS

SS, CS, and PB wrote and reviewed this manuscript.

ACKNOWLEDGMENTS

The Editors express their appreciation to the authors for submitting their fine work, and for their valuable efforts for improving it based on the comments received from the appointed referees. The Editors are also thankful to the reviewers who participated in this effort, identifying issues that had to be addressed and providing thoughtful suggestions for the authors to improve their work. SS acknowledges the support of Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI) in Romania via grant PN-III-P1-1.1-TE-2016-2147 (CORIMAG).

REFERENCES

1. Kiesslich R, Goetz M, Vieth M, Galle PR, Neurath MF. Technology insight: confocal laser endoscopy for *in vivo* diagnosis of colorectal cancer. *Nat Rev Clin Oncol*. (2007) **4**:480. doi: 10.1038/ncponc0881
2. Skala MC, Riching KM, Gendron-Fitzpatrick A, Eickhoff J, Eliceiri KW, White JG, et al. *In vivo* multiphoton microscopy of NADH and FAD redox states, fluorescence lifetimes, and cellular morphology in precancerous epithelia. *Proc Natl Acad Sci USA*. (2007) **104**:19494–9. doi: 10.1073/pnas.0708425104
3. Weinigel M, Breunig H, Kellner-Höfer M, Bückle R, Darvin M, Klemp M, et al. *In vivo* histology: optical biopsies with chemical contrast using clinical multiphoton/coherent anti-Stokes Raman scattering tomography. *Laser Phys Lett*. (2014) **11**:055601. doi: 10.1088/1612-2011/11/5/055601
4. Witte S, Negrean A, Lodder JC, De Kock CP, Silva GT, Mansvelder HD, et al. Label-free live brain imaging and targeted patching with third-harmonic generation microscopy. *Proc Natl Acad Sci USA*. (2011) **108**:5970–5. doi: 10.1073/pnas.1018743108
5. Liang W, Hall G, Messerschmidt B, Li M-J, Li X. Nonlinear optical endomicroscopy for label-free functional histology *in vivo*. *Light*. (2017) **6**:e17082. doi: 10.1038/lsa.2017.82
6. Dilipkumar A, Al-Shemmary A, Kreiß L, Cvecek K, Carlé B, Knieling F, et al. Label-free multiphoton endomicroscopy for minimally invasive *in vivo* imaging. *Adv Sci*. (2019) **6**:1801735. doi: 10.1002/advs.201801735
7. Pezacki JP, Blake JA, Danielson DC, Kennedy DC, Lyn RK, Singaravelu R. Chemical contrast for imaging living systems: molecular vibrations drive CARS microscopy. *Nat Chem Biol*. (2011) **7**:137. doi: 10.1038/nchembio.525
8. Palombo F, Fioretto D. Brillouin light scattering: applications in biomedical sciences. *Chem Rev*. (2019) **119**:7833–47. doi: 10.1021/acs.chemrev.9b00019
9. Zanini G, Korobchevskaya K, Deguchi T, Diaspro A, Bianchini P. Label-free optical nanoscopy of single layer graphene. *ACS Nano*. (2019) **13**:9673–81. doi: 10.1021/acsnano.9b05054
10. Liu N, Kumbham M, Pita I, Guo Y, Bianchini P, Diaspro A, et al. Far-field subdiffraction imaging of semiconductors using nonlinear transient absorption differential microscopy. *ACS Photon*. (2016) **3**:478–85. doi: 10.1021/acsphotonics.5b00716
11. Gong L, Zheng W, Ma Y, Huang Z. Saturated stimulated-Raman-scattering microscopy for far-field superresolution vibrational imaging. *Phys Rev Appl*. (2019) **11**:034041. doi: 10.1103/PhysRevApplied.11.034041
12. Field JJ, Wernsing KA, Domingue SR, Motz AMA, DeLuca KF, Levi DH, et al. Superresolved multiphoton microscopy with spatial frequency-modulated imaging. *Proc Natl Acad Sci USA*. (2016) **113**:6605–10. doi: 10.1073/pnas.1602811113
13. Radaelli F, D'Alfonso L, Collini M, Mingozzi F, Marongiu L, Granucci F, et al. μMAPPS: a novel phasor approach to second harmonic analysis for *in vitro-in vivo* investigation of collagen microstructure. *Sci Rep*. (2017) **7**:17468. doi: 10.1038/s41598-017-17726-y
14. Stanciu SG, Xu S, Peng Q, Yan J, Stanciu GA, Welsch RE, et al. Experimenting liver fibrosis diagnostic by two photon excitation microscopy and bag-of-features image classification. *Sci Rep*. (2014) **4**:4636. doi: 10.1038/srep04636
15. Saitou T, Kiyomatsu H, Imamura T. Quantitative morphometry for osteochondral tissues using second harmonic generation microscopy and image texture information. *Sci Rep*. (2018) **8**:2826. doi: 10.1038/s41598-018-21005-9
16. Stanciu SG, Tranca DE, Stanciu GA, Hristu R, Bueno JM. Perspectives on combining nonlinear laser scanning microscopy and bag-of-features data classification strategies for automated disease diagnostics. *Optic Quan Electron*. (2016) **48**:320. doi: 10.1007/s11082-016-0589-8
17. Prevedel R, Diz-Muñoz A, Ruocco G, Antonacci G. Brillouin microscopy: an emerging tool for mechanobiology. *Nat Methods*. (2019) **16**:969–77. doi: 10.1038/s41592-019-0543-3
18. Rivenson Y, Wang H, Wei Z, de Haan K, Zhang Y, Wu Y, et al. Virtual histological staining of unlabelled tissue-autofluorescence images via deep learning. *Nat Biomed Eng*. (2019) **3**:466. doi: 10.1038/s41551-019-0362-y
19. Cahill LC, Giacomelli MG, Yoshitake T, Vardeh H, Faulkner-Jones BE, Connolly JL, et al. Rapid virtual hematoxylin and eosin histology of breast tissue specimens using a compact fluorescence nonlinear microscope. *Lab Invest*. (2018) **98**:150. doi: 10.1038/labinvest.2017.116

Conflict of Interest: PB is involved in Genoa Instruments as advisor and co-founder.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.