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EDITORIAL

The New Digital Pathology: Just Say NLO

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Modern optics and spectroscopy offer promising noninvasive solutions that have the potential to improve tissue-based diagnosis, as demonstrated by the extensive use of nonlinear laser scanning microscopy for tissue imaging in the past decade. Nonlinear optical (NLO) microscopy offers powerful approaches for tissue imaging at the subcellular level as it can provide morphological and functional information without the use of exogenous labels. Second-harmonic generation (SHG) microscopy is a coherent microscopic modality able to provide background-free images of anisotropic hyperpolarizable repetitively patterned biological molecules, such as collagen, muscle, or microtubules. During the last years, SHG microscopy has been successfully applied to the imaging of non-centrosymmetric molecules inside cells [1], cellular membranes [2], brain [3], and biological tissues [4]. The capabilities of SHG microscopy in providing background-free high-resolution imaging of collagen has already been demonstrated in several tissues. Even though a single collagen molecule has high hyperpolarizability, when arranged in bundles in fibrous structures, an extremely strong SHG signal emerges, enabling selective high-resolution imaging in connective tissues. SHG microscopy can be used for the investigation of structural organization and fibrillar orientation of collagen in human dermis, keloid, fibrosis, thermally treated samples, and in tumor microenvironments. Due to its sensitivity to the organization of molecular emitters, SHG microscopy can be used to detect altered tissue physiology in muscle, bone, and cartilage as an indicator of disease. Further, the investigation of collagen organization in the tumor microenvironment can be related to invasiveness as a useful prognostic indicator.

This imaging approach offers several advantages with respect to standard histopathological examination for tissue diagnostics. First, since SHG microscopy is not dependent on dyes, stains, or labels, it can be performed on unprocessed tissue samples and also on fresh biopsies. Second, SHG microscopy provides background-free images of collagen, unlike immunohistochemistry, which requires specific collagen labeling for (i.e., Verhoeff-Van Gieson [5]) and does not provide background-free images. Third, the optical process at the basis of SHG is coherent; hence, the generated signal strongly depends on the organization of the emitters within the focal volume, making SHG extremely sensitive to subtle changes in collagen organization. Finally, the acquired SHG images are digital, which can be automatically processed by automated image analyzers. The growing need for more accurate, objective, and faster diagnosis has driven researchers to combine digital imaging with routine diagnostics. The term "Digital Pathology" refers to clinical practice focused on the use of computer technology for the development of high-resolution images from slides containing tissue samples. Although digital pathology is transforming diagnostic procedures, the largely used hematoxylin and eosin staining requires digitization using slide scanners, whereas the SHG approach can be easily adapted to digital pathology.

As demonstrated in the paper published in this issue of *Digestive Diseases and Sciences* [6], the authors use SHG microscopy and scoring methods to detect alterations of the

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collagen in the extracellular matrix of colonic tissue slices, including specimens of normal mucosa, low-grade dysplasia, high-grade dysplasia, and tumor, with readouts including averaged intensity per pixel and anisotropy. In particular, the scoring methods based on these two factors are able to discriminate malignant from benign tissue, while providing information regarding subtle changes in the collagen extracellular matrix as an early precursor of malignancy. The advantage of such approach consists in providing an objective quantitative evaluation of the collagen organization in biological tissue samples. The limitations mainly reside in the fact that the evaluation method is based on SHG intensity, whereas intensity-independent method could be preferable for a quantitative evaluation of the collagen architecture and a proper scoring of the pathology. Up to now, several intensity-independent methods for connective tissue classification with SHG have already been successfully employed [7]. Nevertheless, SHG microscopy alone is not able to provide an exhaustive imaging modality for biological tissue imaging and pathological assessment. A fully exhaustive morpho-functional characterization of the tissue requires implementing SHG microscopy in combination with other NLO techniques, able to provide label-free high-resolution images of cells, lipids, and other molecules, in a multimodal approach. The combination of SHG with two-photon excited fluorescence (TPF) [4] microscopy and nonlinear vibrational techniques, such as Coherent Antistokes Raman Scattering (CARS) [8] or Stimulated Raman Scattering (SRS) [9] microscopy, has the capability of specifically highlighting different tissue components. In particular, while TPF reveals the distribution of endogenous fluorophores such as NADH, flavins, elastin, and others, SHG microscopy offers direct high-resolution imaging of collagen structures, although vibrational techniques enable more detailed characterization of the chemical-molecular composition of the tissue under investigation. Hence, the combination of the above-mentioned microscopic techniques has the potential to provide morphological and chemical information in tandem, realizing a morpho-chemical characterization of a biological specimen [10]. The advantages of having such technologically advanced diagnostic tools can reduce healthcare costs by reducing interpretation time while providing more accurate diagnosis. Currently, the "gold standard" diagnostic technique is microscopic examination of conventionally processed and stained histologic section, a time-consuming process with high economic (working time for the pathologist) and social costs (stress and anxiety for the patient). The proposed method represents a powerful diagnostic tool assisting the pathologist having the potential to help an experienced pathologist to render a diagnosis in a shorter period of time, while improving the diagnostic accuracy of the novice pathologist. Further, considering that the method is label-free, in the near future it could be extended to in vivo clinical diagnostics, thus reducing the number of unnecessary biopsies. Nonetheless, much work needs to be done before this methodology is recognized as a diagnostic standard, including training pathologists to the new kind of images, reducing the cost for laser sources required for these techniques, standardizing the acquisition and analysis processes. Given these multiple advantages, these techniques could soon find a stable place in a clinical setting.

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