Multimodal Nonlinear Microscopy: Histopathology of Brain Tumors

Biophotonics provides - amongst others - "all-optical" technology for clinical diagnostics. Here we focus on the description of a novel biophotonic tool to assist in the diagnosis and localization of brain tumors. Currently, staining microscopy is the gold standard used in histopathology to determine tumor properties, but it is virtually impossible to apply this technique in vivo.

All For One

During neurosurgery precise tumor border detection presents a central challenge. By combining three nonlinear microscopic imaging techniques to a multimodal microscopy approach, similar information on the morphology and chemical composition of unprocessed tissue can be extracted. Much greater depth penetration can be achieved in comparison to conventional light microscopy. This all-optical approach fuses coherent anti-Stokes Raman scattering (CARS), second-harmonic generation (SHG) and two-photon excited fluorescence (TPEF) imaging into a single microscopic setup. These three optical spectroscopic methods and their implementation into a single instrument are presented here. The setup has been used to study the morphology and chemical composition of (ex vivo) brain tissue of a domestic pig as a model for human brain tissue and human brain tumor samples from biopsies. The experimental techniques presented are contact-free and label-free all-optical techniques. Thus, they are potentially applicable in vivo, opening the door to label-free diagnostic and surgical guidance for significant improvements in online tumor border detection.

Deeper

Due to the availability of stable laser sources for ultra short pulses the development of nonlinear imaging techniques was intensified, resulting in the introduction of TPEF, SHG and CARS microscopy in life sciences in the last decade of the 20th century. Using near infrared light, the depth penetration in tissue is greatly enhanced to several hundred µm, enabling the investigation of thick tissue specimens as opposed to just "scratching the surface" with visible light.
Furthermore, the nonlinear nature of signal generation with low energy NIR photons greatly reduces phototoxic effects and improves the 3D resolution. This is due to the signal being solely generated within the laser focal region, where the photon density is high enough to cause nonlinear signals.

In contrast to normal fluorescence, TPEF is based on nonlinear absorption, i.e. two photons are simultaneously absorbed to electronically excite a molecule, which emits the fluorescence photon to be detected (fig. 1A). Relying on the fluorescence properties of the analyte molecules, TPEF is restricted to imaging autofluorescent species, which include, for instance, NADH, flavins, keratin, retinol and elastin in biological samples. However, SHG and CARS are coherent nonlinear scattering processes. In SHG two NIR photons are fused to a single scattered photon of twice the photon energy (fig. 1B). With SHG, molecular structures lacking inversion geometry can be visualized. This is especially true for the structural protein collagen in connective tissue, but also of acto-myosin and tubulin, or in general (hidden) interfaces. CARS is the most informative and generally applicable imaging process. In principle, all types of molecules can be visualized by CARS, in contrast to SHG and TPEF, which are limited to certain molecular species. CARS requires two pulsed lasers of different wavelengths to simultaneously illuminate the sample. If the frequency difference of both lasers matches a vibrational resonance, this vibrational level becomes selectively populated. Further photons are then coherently scattered off this vibrational state, generating scattered light at the anti-Stokes frequency (fig. 1C). In our study, CARS was used to display the spatial distribution of lipids in tissue, which is an important marker used to differentiate between cancerous and normal brain tissue, by focusing on the CH-stretching vibration.
Figure 1D displays the basic layout of the multimodal nonlinear imaging setup, allowing for joint TPEF, SHG and CARS imaging. The output of a pulsed Ti:Sa laser is split to pump an optical parametric oscillator to generate the tunable pump wavelength for CARS, while the other fraction serves as the Stokes beam. For SHG and TPEF either the pump or the Stokes laser alone can be used, and a suitable detection window can be selected by the appropriate choice of filters. After recombinining both pulse trains spatially and temporally, the lasers are fed into a commercial laser scanning microscope. Dichroic beamsplitters and filters are used to separate the three signals, which are detected by photomultiplier tubes.

**Looking Inside**

The method has been tested on brain tissue sections of a domestic pig (Sus scrofa domestica) and the obtained morphology has been compared with H&E stained sections, i.e. the current gold standard in histopathology (fig. 2). The cerebellum is composed of the enveloping arachnoid membrane, followed by layers of gray and white matter. The gray matter itself consists of three sublayers: the molecular layer, the Purkinje cell layer and the granule cell layer. Through nonlinear imaging the membrane is visualized by SHG (blue), since it is primarily composed of collagen generating a strong SHG signal. The white matter is rich in lipids, resulting in a strong CARS signal (yellow), while the distribution of autofluorescent species within the gray matter allows the differentiation of its three sublayers (cyan). Thus, our work showed that the morphology and structure of the brain tissue can be visualized with accuracy and precision similar to conventional H&E staining histopathology.

Multimodal imaging has been applied to investigate human brain tumor samples (fig. 3). The H&E stained image is shown on the left for comparison, while the CARS image at the CH-stretching vibration of 2850 cm\(^{-1}\) is displayed on the right. The large dark area in the center left part of the CARS image is a solid lung tumor metastasis. The green area around it corresponds to brain matter. Within the brain matter a few tumor islets are visible, appearing darker. The clear CARS image contrast arises as brain matter is very rich in lipids, while cancerous tissue usually lacks nutrients like lipids due to its fast metabolism. Therefore, even CARS microscopy alone is sufficient to differentiate between cancerous and normal brain tissue.

**Application**

It was shown that multimodal nonlinear imaging is similarly accurate in detecting tissue morphology as H&E staining, but the technical setup is considerably more
complicated, bulky and expensive. Therefore, this research does not aim to replace cheap and precise staining histopathology. The main advantage of this innovative biophotonic technique is its applicability in vivo. Implementation into surgical endoscopes could provide optical guidance during brain surgeries or needle biopsies and significantly improve the surgeon's precision in removing cancerous tissue, while better preserving functional brain tissue. As a first step towards this goal a miniaturized microscopic setup will be constructed and directly tested in hospitals.

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