ABSTRACT

Over the past decades, photonic (optical) microscopy has emerged as an indispensable tool for investigating the structural, molecular, and functional composition of biological and biomedical specimens on the microscale. Microscopic examinations have 'enlightened' our understanding about how life manifests itself below the resolution limit of the human eye, and have revolutionized a wide range of scientific fields, from basic biological research over translational medicine to everyday clinical practice. Recent advancements of photonic technology further broadened the overall capabilities of photonic microscopy by exploiting specific types of light-matter-interactions as the underlying contrast mechanism. However, each microscopy modality is still limited in the wealth of extracted information due to a predetermined set of detectable biological moieties, in the applicability to *in vivo* specimen due to potentially toxic labeling strategies, and in the imaging depth due to a low penetration of light into tissue. These limitations thereby impede both a comprehensive examination of a living biological specimen as well as the extraction of information of value regarding the organism's condition.

An auspicious approach to overcome the current limitations of photonic microscopy is the integration of several compatible imaging modalities into a single hybrid microscope. The trend of hybridizing various microscopy technologies is increasingly popular, as it allows researchers to precisely select modalities with imaging performances well suited for the scientific task. In examinations of specimens that range from single-cells and tissue slices to small model organisms, multi-photon microscopy and optoacoustic (photoacoustic) microscopy have exhibited highly promising capabilities. The former utilizes reading of non-linear optical phenomena induced by distinct biological compounds when encountered with light of high intensity. The latter relies on detecting acoustic waves generated by thermoelastic expansion when exciting molecule-specific optical absorption. Due to their functioning principles, both of these advanced microscopy technologies feature deep imaging, high spatial resolution, *in vivo* applicability, and potentially label-free employment. Thus, they are well suited for the study of specimens that are well established in biological and biomedical research, such as plated cells, organoids, zebrafishs, mice, and human histopathology slices.

This work is dedicated to the further advancement of hybrid multi-photon and optical-resolution optoacoustic microscopy for biological and biomedical investigations at the junction between development of the optical setup in terms of hard- and software,

utilization of the multi-modal imaging to study endogenous or newly designed contrast agents, and exploration of novel approaches to perform optoacoustics on the microscale. The first part introduces the hybrid multi-photon and spectral optical-resolution optoacoustic microscope (HyMPSOM), an optical system that merges seven modalities into a single device and facilitates simultaneous multi-modal investigations with subcellular resolution, deep imaging, fast acquisition, and high sensitivity. Its functioning principle is elaborated in detail, including a devised synchronization method based on a global trigger signal for concurrent operation of the modalities, as well as an imaging procedure based on a combined laser- and sample-scanning schemes for fast image capturing. Moreover, a novel approach is presented to characterize the optoacoustic total impulse response and to correct for it on the signal level using a spatial matched filter algorithm, which improves the signal-to-noise ratio and the axial resolution of optoacoustic microscopy significantly and, thus, further strengthens the optoacoustic imaging capabilities to complement the multi-photon modalities of HyMPSOM.
The second part concerns the utilization of the multi-contrast imaging capability of HyMPSOM to investigate biological and biomedical specimen as well as to explore novel designed multi-modal contrast agents. HyMPSOM is first characterized towards its ability to image *in vitro*, *in vivo*, and *ex situ* samples ranging from single cells and organoids over zebrafish larvae and mice to histopathological tissue slices. Next, HyMPSOM is employed to study the microstructural composition of human carotid atheroma specimen giving new insights into the interplay of key tissue moieties, such as the microvasculature and the connective tissue, involved in the disease progression of atherosclerosis. Transferring these imaging capabilities to living specimens, HyMPSOM is used to investigate the process of wound healing in mouse ears on the microscale and precisely uncovers spatio-temporal tissue responses denoting specific healing phases. Moreover, specifically developed contrast agents for multi-modal microscopy are studied with HyMPSOM on the single-cell level. First, the iron-carrier protein ferritin is mediated to accumulate in lysosomes for generating multi-modal contrast and for facilitating the interaction of HyMPSOM with single-cells via photothermal ablation. Second, bioconjugated C_{70}-fullerenes are testified to be bio-compatible and to give rise to various imaging modalities. They therefore constitute a highly promising platform for photodynamic therapy. Third, CaSPA, a calcium sensor for optoacoustics, has been shown to undergo signal changes in the presence of calcium ions, which allowed HyMPSOM to depict the intrinsic beating of cardiac organoids.
The final part presents technical extensions of HyMPSOM for performing microscopic optoacoustic sensing and imaging beyond the standard mode of operation. Two approaches to conducting optoacoustic microscopy in the frequency domain are elaborated; in one case, the microscope was equipped with sinusoidal-modulated continuous wave lasers, and in the other case with laserdiodes overdriven to generate bursts of pulses. Both techniques allow for simultaneous multi-wavelength optoacoustic mi-

croscopy by loading the excitation sources onto specific frequencies, whose associated optoacoustic signals are subsequently separated in the frequency space. Furthermore, an all-optical optoacoustic sensing setup is integrated into HyMPSOM based on detecting acoustic waves via a π-FBG sensor, which enabled the imaging of thick samples, such as the mouse abdomen in epi-configuration. Lastly, an optoacoustic flow cytometer is conceptualized, which, based on referencing the received optoacoustic signals to simultaneously recorded light-scattering, achieves in-flow detection and distinguishing of chromoproteins expressed in *E. coli* cells.
Conclusively, the herein devised and further developed hybrid microscope HyMPSOM evolved to a versatile platform evincing the operational capability of advanced multimodal microscopy for biological and biomedical research. By fusing several powerful microscopy modalities into a single device, HyMPSOM is characterized by a multifaceted and unique potential to comprehensively study specimens from single-cells to small animals, to exploit multi-modal contrast to gain new insights into the microscopic properties of living species, to assist the development of novel contrast agents, and to advance the field of optoacoustic technologies on the microscale.