Translational Clinical Molecular Spectroscopy

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ABSTRACT

Effective and early cancer and infection diagnosis, as well as personalized therapy, necessitate new methods of differential diagnosis and represent an outstanding medical task. Within this contribution we will highlight our recent efforts in translating spectroscopic approaches with focus on Raman spectroscopy towards routine clinical applications. In the first part of this contribution, a series of innovative multi-contrast marker free spectroscopy approaches (both microscopy and endoscopy based) for a precise intraoperative tumor margin control and reliable tumor classification to initiate an individualized therapy plan as quickly as possible. The second part highlights our most recent efforts to use Raman spectroscopy for diagnosing infectious diseases. These efforts include (I) predicting the immune response based on the patient's health, (II) quickly identifying the infection-causing pathogen and, in the case of bacterial infections, its resistance pattern; and (III) assessing the patient's response to treatment. An essential, often neglected point in the research of novel diagnostic methods is the sample preparation. Therefore, promising techniques based on particles and chips focusing on the enrichment of bacteria will be presented. The introduced approaches comprise the entire process chain i.e. from sampling to the final diagnostic result, and have a high potential to significantly reduce the critical parameter ‘time’ to initiate a personalized lifesaving therapy as compared to the gold standard microbiology. Novel, multi-user infrastructures are needed to bring these recent advances to patients faster. The Leibniz Centre for Photonics in Infection Research developing market-ready light-based diagnostic devices and novel infectious disease treatments is introduced.
Keywords: spectral histopathology, nonlinear imaging, infections, antibiotic sensitivity test, sample preparation

1. INTRODUCTION

Over the last decade, the use of molecular spectroscopy in medical diagnostics and therapy (biophotonics) has steadily increased. The combination of optical technologies and the capabilities of artificial intelligence (AI) will play an important role in the clinical diagnosis of cancer and infectious diseases in the future, as they have the potential to improve diagnostics and thus advance patient-specific targeted therapy [1-4]. From the start, the emphasis is on constructive collaboration between clinicians and technologists, which is critical for the efficient implementation of innovative ideas and their translation into market-ready products. The detection of tumor margins during intervention is critical to effective tumor treatment. Interventional surgery sparingly resects healthy tissue is the main treatment for solid tumors. Typically, the removed tissue is histopathologically processed in two dimensions for a thorough marginal check to ensure that it is tumor-free [3]. However, the surgery must be halted, and intraoperative frozen section diagnosis lacks the specificity and sensitivity of embedded tissue histology. Coherent anti-Stokes Raman scattering (CARS) in combination with two-photon excited autofluorescence (TPEF) and second harmonic generation (SHG) has been shown to be a powerful approach for stain-free clinical imaging in recent years [5]. In almost all clinical areas, infections can have a significant impact on the success of modern surgery, oncology, or stem cell therapy [6]. Rapid, efficient and reliable diagnosis of the pathogens and the corresponding host response are essential for the success of treatment and the patient's chances of survival. Classification of immune status by immunophenotyping enables a holistic description of infection states [7, 8] Using easily available peripheral leukocytes, host response profiling via Raman spectroscopy is achievable. A rapid identification of the infection causing pathogen and in case of bacterial infections its resistance pattern and response to treatment are of essential importance for the therapy decision [9]. Raman microspectroscopic characterization and identification of bacteria from bulk to single bacterial cells serve as the foundation for new rapid and effective microbiological diagnostics [10-13].

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In addition, numerous sample preparation techniques compatible with Raman spectroscopy have been developed [14-16]. Antibiotic susceptibility testing is a special focus in the field of optical and spectroscopic pathogen detection. After a few hours, a pathogen's response to an antibiotic can be identified. With this time-sensitive information, therapy can be switched early on to a suitable antibiotic [17, 18]. To expedite the delivery of these recent advances to patients, novel infrastructures that are accessible to a variety of users are required. The Leibniz Centre for Photonics in Infection Research[1] is an exemplar of infrastructure that is currently being developed; it aims to facilitate the development of market-ready light-based diagnostic products and novel treatment approaches for treating infectious diseases. As a translational center, it is supported financially by the German Federal Ministry of Education and Research and will be based on a systemic approach that enables ideas for the light-based diagnosis and therapy of infectious diseases to be developed, tested, and brought to market maturity rapidly. As a worldwide unique infrastructure, the centre bridges the gap between the outcomes of research and the introduction of solutions for physicians and patients to the market. Leibniz-IPHT, Leibniz Institute for Natural Product Research and Infection Biology Hans Knoll Institute, Friedrich Schiller University Jena, and University Hospital Jena are constructing the centre.

2. MULTI-CONTRAST MARKER FREE SPECTROSCOPY APPROACHES

Imaging techniques such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), endoscopy, and others, which are used as standards in the preparation of tumor resection procedures, can only show the pathological correlate of the tumor disease i.e., where and to what extent the tumor has already spread. In contrast, intraoperative diagnostics during surgery primarily consist of the use of magnifying glasses, endoscopy, and microscopy to visualize the tumor area with higher magnification, in addition to direct visual inspection by the surgeon. The gold standard for confirming a final diagnosis, as well as staging and grading, is histopathological examination histopathological examination of the removed tumor tissue [3]. A differential diagnosis is time-consuming and difficult. On the one hand, it entails microscopic examination of the sample after staining with a standard dye (e.g., hematoxylin-eosin, HE) and supporting methods such as the use of antibody-based characterization methods (immunohistochemistry), fluorescence-in situ hybridization (FISH), DNA-based methods, or biochemical assays to obtain additional molecular information that can be used to confirm and supplement the primary histological. Furthermore, because the pathologist works with fixed and embedded tissue samples in most cases, it is not always possible to perform histopathology in the operating room to obtain histopathological findings while the patient is still in the operating room. In the future, the combination of optical technologies with the powerful capabilities of artificial intelligence (AI) for automated analysis of multimodal images generated will play a significant role in the real-time clinical diagnosis of tumor diseases [3, 4]. In recent years, it has been demonstrated that coherent anti-Stokes Raman scattering ("coherent anti-Stokes Raman scattering," CARS) in combination with two-photon excited autofluorescence ("two-photon excited autofluorescence," TPEF) and the generation of the second harmonic generation (SHG) is a powerful approach for stain-free clinical tissue diagnostics, capable of precisely localizing the tumor intraoperatively (i.e. ex-corpore in vivo as a frozen section procedure or potentially also in vivo)[19-22]. Molecular vibrations unique to each molecule can be detected using conventional Raman spectroscopy. Raman spectroscopy can thus detect the molecular composition as well as the morphology of complex samples such as biological cells or tissues with little or no sample preparation. Because pathological changes in the biochemical composition and structure of biomolecules are associated with pathological abnormalities, the Raman spectrum of tissues provides a sensitive and specific fingerprint of its nature and condition. This makes Raman spectroscopy an ideal tool for label-free histopathological tissue examination [23]. However, because of the small Raman scattering cross-sections, using Raman spectroscopy for intraoperative imaging (point-by-point scanning of larger tissue areas to generate hyperspectral Raman images) is far too time-consuming. Nonlinear coherent Raman methods, such as CARS microscopy, can be used in this case. When compared to conventional Raman spectroscopy, CARS microscopy has the significant advantage of producing not only point measurements, but also image data with only one characteristic Raman active vibration. Other nonlinear multiphoton effects, such as TPEF and SHG, are also co-generated in the CARS process. CARS implementations can thus be considered multimodal. CARS typically employs a frequency difference of 2850cm-1 for imaging lipid distribution and visualizing membranes. SHG detects non-centrosymmetric protein assemblies, particularly collagen fibers in connective tissue, whereas TPEF locates the distribution of autofluorescence

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of molecular species such as the fluorescent proteins keratin and elastin or cofactors such as NAD(P)H or FAD. The image data are combined to produce a false-color image, which allows the morphological and chemical composition (morphochemistry) of unfixed tissue sections to be determined without the use of markers [24]. Artificial intelligence, i.e., machine and deep learning approaches, are required to translate the morphochemical information encoded in multimodal spectroscopic images into medically relevant information. Because the tissue has not been altered, the same slice can be analyzed with different imaging techniques, for example, if a suspicious region needs to be examined in greater detail [25]. The CARS/SHG/TPEF approach has been translated into a compact microscope that is suitable for clinical use in the context of intraoperative reliable frozen section diagnostics [26]. Overall, the outlined multimodal approach in the form of a compact clinically applicable microscope (slide scanner) combined with innovative automated image analysis routines has great potential as a powerful tool for computer-aided spectral histopathology for automatic tissue type/disease prediction during surgery. As a result, it provides the opportunity to address unmet medical needs by reducing time in the operating room through immediate feedback and reducing workload through automation. Endospectroscopic probe concepts are required for the implementation of a nonlinear multimodal imaging approach for in vivo tissue screening. Three endoscopic probe concepts for multimodal imaging will be briefly summarized below. The needle endoscope is employed in neurosurgical procedures. It provides excellent imaging in a compact device. The probe head contains no moving or electrical components [27]. A novel concept for applications requiring flexible endoscopes has been developed. The imaging fiber is used for illumination in this case. This allows for 3µm spatial resolution, 500µm field of view, and 10,000 pixel resolution without the use of moving parts [28]. Furthermore, an innovative piezo-controlled distal scanning fibre probe for nonlinear multimodal spectroscopic imaging could be realized [29]. The developed compact probe concept can produce high-quality trimodal nonlinear images of unstained tissue that are comparable to images produced by bulky laboratory setups using commercial laser scanning microscopes. The developed compact probe concept can produce high-quality trimodal nonlinear images of unstained tissue that are comparable to images produced by bulky laboratory setups using commercial laser scanning microscopes. In the evaluation and interpretation of data, artificial intelligence and deep learning techniques will play an increasingly crucial role. Significant progress will be made in the diagnosis of tumour diseases in the future, which will lead to better patient care and patient care in the long run, as well as significant cost savings.

3. RAMAN SPECTROSCOPY FOR DIAGNOSING INFECTIOUS DISEASES

Over the last two decades, climate, demographic, and technological changes have altered the landscape of infectious disease risk. Novel infections mainly spread at the local level before becoming global pandemics. Diseases that were once under control are reemerging, and the increasing development of antimicrobial resistance necessitates new health-care strategies [6]. To develop effective diagnostics and therapeutics for severe infections, a thorough understanding of the host response to infection with one or more pathogens is required. Leukocytes are an intriguing research subject because they orchestrate the immune response against infection and hence can shed light on the host response [30]. During infection and sepsis, the activation of innate and adaptive immunity causes molecular and functional alterations in leukocytes [31]. However, there are currently only a few, mostly time-consuming methods that allow for precise characterization of the host response. In this regard, new photonic approaches have already yielded promising results. Raman spectroscopic methods, in particular, enable direct and non-destructive characterization of patient samples, such as blood plasma but also immune cells [8, 32, 33]. Raman spectroscopic methods enable direct and non-destructive evaluation of biological specimens e.g., cells or tissues. The principle of this examination method is to irradiate a biological specimen with laser light for a few seconds (0.5 to 30 s) and record the inelastically scattered light as a spectrum. This spectrum reflects the molecular signature of the sample. The typical pattern of a cell's Raman spectrum is characterized by overlapping signals of peptide bond vibrations (-CO-NH-), various CH vibrations from lipid and protein structures, characteristic contributions from functional amino acids like phenylalanine and tryptophan, as well as typical signals from nucleic acids or other macromolecules such as polysaccharides [34-36]. Automated microscopy in combination with Raman spectroscopy represents an innovative strategy for cellomic analysis [37, 38]. When embedded in a meaningful experimental design, Raman spectroscopy can be used to rapidly characterize cellular phenotypes with minimal effort. Spectral modifications are induced by changes in the cells' biochemistry, for example triggered by physical or chemical stimulations, the invasion by pathogens or other stress factors [8, 32, 39-42]. However, the resulting changes cannot be read directly from the spectrum. For this purpose, chemometric data analysis is necessary. Various chemometric tasks can be performed using machine learning approaches, such as clustering and classification methods, or calculating a substance's concentration using a regression model. This requires the development of a test model that

indicates whether the spectral information can be used to answer a defined research question [43]. For investigation at the single-cell level, a high throughput screening Raman spectroscopic (HTS-RS) device was developed that enables rapid and label-free cell screening to achieve statistically relevant results [38]. Using this set-up, one Raman spectrum from each cell is generated separately through an automated cell recognition software, allowing thousands of cells to be captured in a short amount of time. The potential of HTS-RS has already been demonstrated in the study of infection biology issues. A Raman-based white blood count demonstrated the ability to classify leukocytes into their respective lineages and produce numbers comparable to machine counting [38]. Raman spectral fingerprints of blood-derived leukocytes were utilized to differentiate between inflammation, infection, and sepsis in hospitalized patients [8]. Neutrophils are the first immune cell type recruited to infection sites, including viral infections, and they perform both protective and pathogenic activities [44]. Raman spectroscopy can nondestructively and label-free extract information on the activation state of leukocytes to differentiate between different host response scenarios. It could be shown that leucocytes activated either by bacterial or fungal pathogens differ in their spectral phenotypes, which offers new Raman-based diagnostic approaches [32, 45]. Rapid and reliable diagnostics with pathogen identification and the generation of a resistogram are especially important in septic and critically ill patients. The MALDI-TOF system and various PCR-based systems are currently available for rapid pathogen diagnostics. These systems can typically identify the pathogen in a very short period of time, but they do very little to reliably and rapidly test for resistance. Resistance cannot be tested using the MALDI-TOF instrument, whereas PCR-based methods can only detect individual resistance genes. In the positive case, a susceptibility pattern for individual pathogens can be inferred, but these conclusions are very limited, particularly for Gram-negative pathogens. There is also purely phenotypic resistance, which has mechanisms that are not always fully understood. Overall, the mechanisms of resistance are so diverse and varied that therapy must be adjusted based on phenotypic testing. Antimicrobial sensitivity can be read out by Raman micro spectroscopy at cellular level before colony growth is detectable which simplify testing, reduces time and costs. Milestones have reached to make the unique advantages of Raman spectroscopy accessible for the next generation diagnostic platforms [14, 17, 46]. The combination of Raman spectroscopy and microscopy yields a novel microbiological tool for characterizing bacterial pathogens. Working at the microscopic level allows bacteria to be studied, for example, their response to anti-infectives, after much shorter interaction times than is possible with macroscopic systems like turbidimetry or the agar diffusion assay. Isolation strategies for clinical diagnostics are being developed in order to reliably and non-destructively isolate the small number of bacteria and make them available for Raman spectroscopic analysis. On-site sample preparation strategies are on the way usable on chip- or cartridge based miniaturized setups, streamlined AST strategies and data pipelines for diagnostic information are interconnected and coordinated with each other. All these innovations were only fully exploited once the right infrastructure is in place. Medical device regulations, modern data science and clinical practice will closely be integrated to translate photonic technologies into clinical diagnostics. The Raman spectroscopic pathogen diagnostics principle of directly sampling pathogens as infectious units has the potential to significantly accelerate and simplify microbial diagnostics.

4. CONCLUSION

Unmet medical needs include the fast increase in cancer due to an aging population, the rapid spread of life-threatening infectious diseases, and the emergence of bacteria resistant to antibiotics. An effective and early diagnosis and individualized treatment of cancer and infections is a formidable medical challenge requiring the development of novel diagnostic techniques. In recent years, spectroscopic techniques have demonstrated their ability to address the aforementioned medical issues. The presented approaches encompass the full process chain, i.e., from sample preparation to the final diagnostic result, and have the potential to drastically reduce the essential parameter 'time' to commence a tailored life-saving therapy compared to the gold standard procedures. The infrastructure provided by the Leibniz Centre for Photonics in Infection Research will encompass all stages of product development, from validation via analysis of real patient samples to professional support in product design and small-scale manufacture. There will be resources available to aid with the implementation of standard operating procedures and comprehension of pertinent legal restrictions. Industry and government must be involved from the outset to ensure a seamless introduction of novel diagnostic and treatment processes to the market.

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