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Advanced multimodal laser imaging tool for urothelial carcinoma diagnosis (AMPLITUDE)

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1. Overview

Bladder cancer (BC) is the eleventh most diagnosed cancer worldwide. The age-standardized incidence rate (per 100 000 person/years) is 9.0 for men and 2.2 for women [1]. Urothelial carcinoma (UC) represents about 90% of all bladder tumors, thus carrying an enormous social and economic burden [2]. UCs are classified in different stages and grades, depending on their invasiveness and on their degree of cytological abnormalities. The key aspect for a positive prognosis is the early and accurate diagnosis of the lesion stage, in order to identify the most aggressive disease forms and treat them promptly. It is well known that tissue metabolism constitutes a basic mechanism, which is at the base of many pathologies, especially BC. Being able to detect and characterize tissue metabolism and molecular fingerprints at the cellular level could be a key aspect in characterizing the pathology and enabling both early detection and therapy monitoring.

The new European Union Horizon 2020 project called AMPLITUDE, the 'Advanced Multimodal Photonics Laser Imaging Tool for Urothelial Diagnosis in Endoscopy', starting in January 2020, proposes the development of an advanced multi-modal imaging tool exploiting new laser technologies in an approach combining confocal and non-linear imaging to fulfil unmet clinical needs in terms of the specificity and accuracy of urothelial cancer diagnosis and therapy monitoring. The project is coordinated by Tampere University (Finland) and carried out in cooperation with leading European research organizations including Aston Insitute of Photonic Technologies—AIPT (UK), Consiglio Nazionale delle Ricerche—CNR (Italy), Institute of Photonic Sciences—ICFO (Spain), University of Milan-Bicocca, Modus Research and Innovation Ltd. (UK) and University of Florence (Italy), as well as industrial partners: Ampliconyx Oy (Finland), Femtonics Ltd. (Hungary), HC Photonics (Taiwan), and LEONI Fiber Optics GmbH (Germany).

2. Clinical needs

The best way to manage UC disease is to provide a quick and accurate diagnosis of the tumor, in particular for its stage and grade, as the most aggressive forms should be treated promptly. In fact, it is reported that a three month delay in the treatment of an aggressive and advanced stage disease has a severe impact on prognosis [3]. There are low- and high-grade USs, according to their aggressiveness, plus a variant histology that is usually more aggressive, like squamous cell cancer or a nested variant. The stage varies according to the depth that the bladder tumour reaches, as shown in figure 1 [4].

There are Carcinoma *in situ* (TIS), plain areas aggressive and high-grade, Ta tumours, mostly papillary, limited to the mucosa (both high- or low- grade), T1 tumour, which invades lamina propria and usually

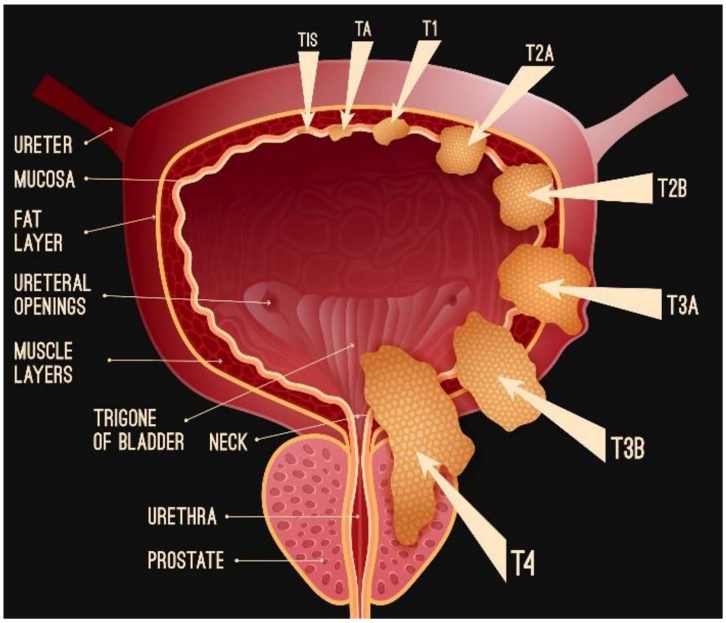

Figure 1. Illustration of different pathological stages of bladder cancer. Stage TIS: flat cancerous cells within the cells lining the bladder; Stage TA: just in the lining cells; Stage T1: into the first deep bladder layer; Stage T2A: just into the bladder muscle; Stage T2B: deeply into the bladder muscle; Stage T3A: just into the bladder fat; Stage T3B: deep into the bladder fat; Stage T4: invading other organs around the bladder (prostate, cervix, vagina).

high-grade, T2 tumours that invade bladder muscle layer and high-grade, T3 tumours that reach the peri vescical fat and T4 tumors that invade the areas around the organs.

Transurethral resection bladder tumors (TURBTs) are the current standard of care to treat BC at its first presentation and to obtain the tumor histology to provide a first staging and grading, but they have some limitations. Specimens obtained through TURBT may be without a detrusor muscle, and thus inadequate for proper staging. This situation brings drawbacks on clinical outcomes, for example a second TURBT may show both tumor persistence and/or recurrence and/or disease upstaging [5]. Detrusor muscle absence at the first TURBT raises the recurrence rate, and provides an up to 45% risk of further muscle invasive disease [6, 7]. In addition, the resection technique may also alter tumor cito-architecture due to the resection of itself and to thermal damage of sensitive areas within the specimens. In fact, a dedicated pathologist is necessary to increase diagnostic accuracy and to reduce misinterpretations [8]. Currently, a new TURBT technique, en-bloc resection, is debated as an alternative to traditional TURBT to improve specimens quality, but it is not yet the standard of care [9]. Another important issue in UC treatment is the lack of sensitivity of white light cystoscopy, meaning some tumors may be missed, especially Carcinoma *in situ*, which is also associated with a higher rate of disease progression [10]. Carcinoma *in situ* is a flat lesion that can be missed when using white light. To improve diagnostic accuracy, techniques such as narrow band imaging and blue light cystoscopy with hexylaminolevulinate may improve the detection rate. Nevertheless, at present, their use is limited and has not improved in specificity when compared to traditional white light cystoscopy [11].

Thus, better tools to obtain an improved detection rate of flat tumors and a swift, accurate and reliable histological analysis are necessary. In clinical practice a better tumor visualization accompanied by a more rapid and accurate diagnosis of both tumor grade and stage could have a significantly reduction on UC recurrence and progression, and could thus improve overall cancer survival.

3. Ultrafast laser sources at 1700 nm

The ability of light to penetrate tissue is highly dependent on tissue composition, with optical scattering profoundly affecting the penetration depth. With an unmet and increasing demand for deeper tissue imaging, the use of near-infrared instead of visible- or UV-light, is increasingly appealing, in particular when the wavelengths fall within 'biological windows' where light has much deeper penetration [12, 13]. Within these windows, four distinctive wavelength regions have been identified: the first biological window spans the wavelength range from 700–950 nm, the second biological window spans the region from 1000–1350 nm, the third biological window covers 1550–1870, and the fouth biological window is within 2100–2300 nm, with each window providing increased transparency with respect to biological matter. Biological windows 1 and 2

have been extensively investigated for many years using widely available light sources. However, the third biological window wavelength range offers several potential highly appealing advantages for deep tissue imaging that are not currently fully exploited. Firstly, the longer wavelength decreases Rayleigh scattering (which varies as the inverse fourth power of the wavelength) and due to Mie scattering (which varies as $1/\lambda^n$ with $n \geq 1$) (we can expect much less scattering (five times in comparison with a 1 μm wavelength for soft tissue) [13, 14] and higher contrast images (SNR = ~ 5 dB at 1700 nm compared to SNR = 0 dB at 1300 nm for 1.1 mm penetration depth [15]) than the first or second biological windows due to the nonlinear effect based on discrepancy of scattered and signal light absorption by the tissue [16]. Taking into account the possible penetration depth and reduced effect of optical scattering, the use of these longer wavelengths for both excitation and emission are therefore, in principle, highly favourable for photonics imaging systems. A high interest in short pulse laser sources emitting in the third biological window has been further escalated by the success of recent brain related studies [17, 18] demonstrating high-quality (spatial resolution ~ 1 μm) imaging down to ~ 1.3 mm deep into the tissue.

The only possible commercial solution for this spectral region so far is a supercontinuum source. However, this source is prohibitively expensive for use in cost-effective biomedical imaging and to use it only for ~ 10% of the whole wavelength range is not an effective solution. Such broadband sources also introduce higher noise levels for the imaging system since the white noise components are distributed in the entire temporal frequency region [15].

Scientifically demonstrated lasers have a much broader coverage compared to the available market options. A number of ultrafast fiber lasers emitting in the 1600–1800 nm range were developed using Bi-doped fibers [19–22]. The main disadvantages of these lasers were a small fiber core diameter (~2 μm) leading to rather low energy pulses at the output of bismuth lasers and a large cavity length required due to the low gain per meter coefficient for Bi-doped fibers limiting the standard fundamental repetition rate of such sources to ~ 10 MHz.

Other possible approaches to design fiber pulsed sources operating in the spectral region around 1.7 μm include Raman shifting the pulsed radiation emitted by an Er-fiber laser [17, 23] and special Tm-doped active fibers in combination with optical elements suppressing amplification at 1.85 μm [24, 25]. The all-fiber femtosecond Raman-based laser approach was notable for the shortest pulses (65 fs [17]) in this wavelength region so far.

Tm-doped fiber lasers operating at 1.7 μm are intrinsically more efficient than Bi-doped fiber lasers and higher level doping allows shorter cavity lengths resulting in higher pulse repetition rates [24]. Progress in the fabrication of highly effective Tm-doped fibers enabled our team in 2016 to demonstrate for the first time a widely tuneable, mode-locked fiber laser capable of producing sub-picosecond pulses between 1705 and 1805 nm [24]. This initial success was further expanded by using a specially designed photonic crystal fiber, which enabled two tuning ranges (1788–1831 nm and 1702–1764 nm) of a thulium doped mode-locked all-fiber laser [25].

This recent progress in widely tunable ultra-short pulsed lasers gives an opportunity to gather consistent information about the optical response of tissues within the third biological window, which will be one of the primary goals of AMPLITUDE. The laser sources covering the 1650–1800 nm wavelength range will be developed and studied to obtain systematic and broad knowledge about optimum laser parameters for deep and high-resolution imaging within the third biological window.

4. Project concept

The AMPLITUDE project aims to harness the novel features of the new laser sources to develop an innovative multi-modal imaging system to be used in a clinical setting and to combine the imaging modalities in an endoscopic probe format providing unique diagnostic capabilities. The multimodal approach is based on three techniques, which together will provide precise and detailed information to determine the tumor stage and grade. The first technique is three-photon imaging (figure 2) using a novel ultrashort-pulsed laser operating in the third biological window to enable deeper tissue penetration in the mm rangeand improved image contrast. Part of this laser beam will be nonlinear frequency-doubled to obtain a short-pulse light source at around 850 nm for autofluorescence-based metabolic imaging. Both wavelengths will be delivered to the tissue in one channel. Frequency doubling of the 1700 nm pulsed laser radiation gives access to the first bio-window, where imaging and fluorescence imaging of key metabolic activity indicators NADH and FAD is possible [26]. Other intrinsic fluorophores such as porphyrin, collagen, elastin, keratin, lipofuscin and melanins also have roles in several relevant physiological processes and diseases such as cancer. Multiphoton microscopy can provide functional imaging in a non-invasive way [27]. A periodically poled lithium niobate (PPLN) crystal will be used for frequency-doubling to generate 850 nm in the femtosecond regime by highly efficient nonlinear wavelength conversion processes [28]. A specific

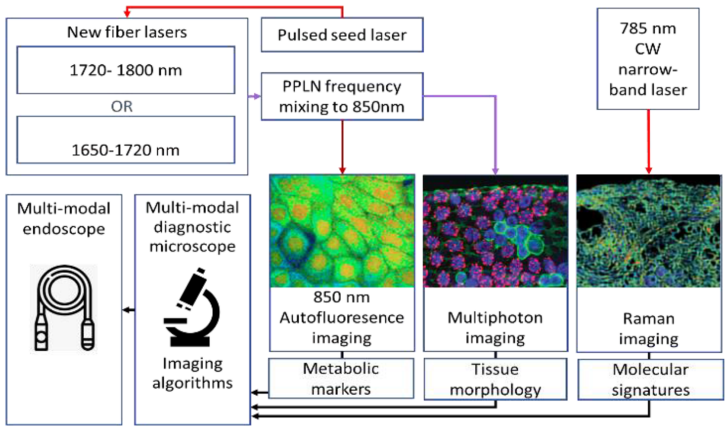

Figure 2. Illustration of the new bio-window targeting sources and multi-modal imaging approach of AMPLITUDE.

PPLN for the 1700/850 nm broadband SHG will be developed in AMPLITUDE and exploited for two-photon excited autofluorescence (TPEF) from endogenous fluorophores. These measurements, performed on cell models, will provide useful morpho-functional information to be related to the spectroscopic and metabolomic data obtained from the same samples.

Finally, Raman spectroscopy will be realised by a second narrow linewidth laser diode illuminated at 785 nm. This will provide an exhaustive characterisation of the examined tissue at the molecular level. The data obtained will be correlated to the metabolic fingerprints and panel of metabolites identified by metabolic profiling with the aim of combining the profiling capability offered by state-of-the-art metabolomic methods with the non-invasiveness, speed and ease of use peculiar to fiber-based optical spectroscopic technology. This will aim to provide a high-accuracy diagnosis of BC, grading and staging in-vivo in the course of clinical investigation; a key-to-success for a positive prognosis on UC.

Combining the morphological and structural information provided by these modalities, the proposed system will provide higher resolution, faster and more accurate imaging, helping clinicians to efficiently diagnose and monitor the effects of an applied treatment.

The low phototoxicity of 1700 nm laser radiation due to the lower single-photon absorption effect and the fact that it is beyond the visible wavelength absorption of organic molecules (flavins and porphyrins) is expected to be extremely beneficial. The label-free procedure proposed by AMPLITUDE will allow us to avoid the use of administered fluorophores and their phototoxic effects. Reduced phototoxicity will positively affect the reproducibility and quality of the imaging results by minimising cell damage.

5. Conclusion

A multi-modal imaging tool for urothelial cancer diagnosis and therapy monitoring will be developed in the AMPLITUDE project. The approach is mainly based on an ultrashort pulse fiber laser operating in the third biological window combined with frequency doubling for multi-photon imaging and TPEF, as well as a narrow linewidth continuous-wave laser emitting at 785 nm for Raman spectroscopy. Both microscope and endoscope formats will be developed and assessed in clinical environments.

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