Review Article

Value of multiphoton microscopy in uro-oncology: a narrative review

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Background and Objective: Multi-photon microscopy (MPM) is a 3-dimension fluorescence imaging technique that combines the excitation of two low-energy photons, enabling less photo-bleaching and deeper penetration of the imaged tissue. Two signals are detected, autofluorescence (AF), from natural intracellular fluorophores [such as nicotinamide adenine dinucleotide phosphate (NADP) and flavine adenine dinucleotide (FAD) transformation], and second harmonic generation (SHG), a physical property of the laser enhancing non-centrosymmetric structures such as collagen fibers. MPM can give both visual and quantitative information of a fresh tissue (without the need of processing, cutting or staining the tissue), aiding in the progress towards optimizing a real-time imaging device. The objective of this review is to show the value and benefits of the use of MPM in uro-oncology.

Methods: A structured literature review was performed using PubMed and Web of Sciences, including all articles with the following keywords: "multiphoton microscopy", "two-photon microscopy", "non-linear microscopy", "second harmonic generation", "urology", "prostate", "bladder", "kidney", "upper tract", "oncology", "surgical margins", "frozen section". Articles were reviewed to summarize the use of this tool in performing biopsies, assessing surgical margins, staging and grading complementary tool, and real-time imaging.

Key Content and Findings: A total of 476 articles were identified with these keywords, and later screened for inclusion. We finally included 47 publications that were relevant to our topic. The advantages of this technique have led to its application in the management of several cancers, allowing cellular description as well as quantitative measurements of AF or SHG and their correlation with clinical outcomes.

Conclusions: MPM has shown great improvement in providing a real time assessment of fresh tissue, giving oncologic diagnosis, performing *in vivo* imaging and quantitative analysis of the tissue as well as increasing precision of the diagnosis. This nonlinear optical technique has the potential of guiding both biopsy and surgery, as well as helping the surgeon with interesting additional tissue information intra-operatively.

Keywords: Multi-photon microscopy (MPM); connective tissue; *in vivo* imaging; non-linear microscopy; biopsy

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Introduction

Multi-photon microscopy (MPM) is a non-linear laser microscopy technology that has become the Gold Standard for fluorescence microscopy in fresh tissue and thick sections (1,2). MPM is a combination of two techniques: a two-photon excitation fluorescence, enabling autofluorescence (AF) in unstained living tissue, through intrinsic emissions of nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide (FAD) within cells (3); second harmonic generation (SHG) (4), that allows the identification of non-centrosymmetric structures like collagen fibers trough scattering phenomena. The tissue penetration of this technology can go up to 1 cm, when combined to chemical clearing techniques (5).

During the past decade, novel imaging techniques have been used to increase the accuracy and precision of cancer detection at different levels and resolutions. These include elastography (6), optical coherence tomography (7), confocal microscopy (8), and multiphoton microscopy (MPM) (9). Some of these techniques have been compared to Gold Standard imaging or pathology techniques, and can be complementary to them (8,10,11). MPM has started to be used in clinical studies setting, aiming at a new era of both diagnostic and prognostic in the oncology field, and there is a need to know, as clinicians, what could be the benefits of using this technology in the everyday practice.

The objective of our literature review was to highlight the latest updates regarding the use of MPM in uro-oncology, and to bring our future perspectives of adding this technology into the clinical field. We present the following article in accordance with the Narrative Review reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-21-973/rc).

Methods

Using Web of Science and PubMed databases, we performed a structured review of the literature, selecting only English articles with no limits in timeline. The keywords featured in the search were: "MPM", "two-photon microscopy", "non-linear microscopy", "second harmonic generation", "urology", "prostate", "bladder", "kidney", "upper tract", "oncology", "surgical margins", "frozen section". We first selected all the publications that included both "two photon microscopy", "MPM", "non-linear microscopy" AND one of the urology words such as "kidney", "bladder", "prostate", or "urology". A total of 476 articles were identified with these keywords, and later screened for inclusion; original articles and reviews were included, while abstracts and opinion articles were excluded. We then excluded all the articles that weren't relevant to our uro-oncology topic, with a lot of articles that were focused on the nephrology aspect of the kidney. We also excluded articles that were purely described of microscopy techniques and that had no translational approach to the clinic (*Table 1*). We finally included 47 publications that were relevant to our topic.

Mutliphoton in various uro-oncology uses

Prostate cancer (PCa)

Visual biopsy tool for cancer tissue detection

Gold standard histopathology (GSH) for PCa diagnosis is based on a fixed, paraffin-embedded tissue sample biopsy, stained with hematoxylin and eosin (H&E). A large discrepancy between the Gleason score of biopsy samples and radical prostatectomy specimens has been reported (43% to 70% concordance) (12). El Hajj *et al.* (13) showed an upstaging (> T2) in 20.6% of cases, and upgrading (Gleason score >6) in 44.9% of cases after prostatectomy.

Visual biopsy tools have been gaining medical interest due to their simplicity and practicality (14). By allowing visualization of fresh, unprocessed and *in vivo* tissue, MPM acts as a complementary technology to the GSH. Recently, Yang *et al.* have proven the utility of MPM in imaging of unstained tissue, and have demonstrated its integration with a deep learning process (15).

MPM has previously shown its capability in recognizing both prostate and periprostatic structures with a resolution similar to histopathology. While studying surgical margins of prostate sections, Yadav *et al.* (16) were able to differentiate periprostatic structures such as arteries or nerves, and distinguish characteristic features of the intraprostatic structures with the help of MPM, discriminating intraprostatic and periprostatic alterations in benign and malignant lesions. For this publication, a combination of both AF and SHG was used. Later on, Durand *et al.* showed the capacity of this technology in an *in vivo* fashion on an animal model, being able to show the prostate neurovascular bundles of a rat under general anesthesia (17), without any phototoxicity on the living tissue after laser imaging, using the same imaging technique (AF + SHG).

Future perspectives

The GRIN lens system probe is a 1-mm diameter and 8-cm length. portable and compact multiphoton endoscope. The small diameter of this device allows it to be used inside a

Table 1 The search strategy summary

Items	Specification
Date of search	01/02/2020–30/05/2020
Databases and other sources searched	PubMed, Web of Science
Search terms used	"non-linear microscopy", "two photon microscopy", "multiphoton microscopy", "urology", "uro-oncology", "prostate cancer", "kidney cancer", "bladder cancer", "artificial intelligence", "liver cancer", "melanoma", "breast cancer", "colon cancer", "lung cancer", "second harmonic generation", autofluorescence"
Timeframe	From 1990 to 2020
Inclusion and exclusion criteria	Inclusion criteria: all articles (original articles, meta-analysis, clinical trials) between 1990 associating two photon microscopy and onco-urology, in English language
	Exclusion criteria: all articles written in another language than English, all articles including nephrologic aspect of the kidney, and abstracts and opinion articles
Selection process	First selection of all articles including multiphoton microscopy and all uro-oncology use (prostate cancer, bladder cancer, kidney cancer, and then in dermatology, lung cancer, pancreatic cancer, breast cancer, liver cancer, for last table in the MPM application in other cancers. A total of 476 articles were screened to exclude all non-English articles, opinion articles, and abstracts. Once the abstracts were read, we excluded articles that were not relevant to the topic, either being too technical on the microscopy technique or focusing on other aspects of the organ (i.e., nephrology articles on the use of MPM in kidneys). A total of 47 articles were selected for final inclusion

needle to inspect and to identify suspicious tissue sites (18). By incorporating MPM to the GRIN lens system probe, targeted biopsies could be carried out or the device could be used for intraoperative assessment of tissue margins.

MPM has also proven to be capable of *in vivo* visualization of spermatic cord nerves in rats that were then ablated with the laser being used at a higher energy (19). By combining these different approaches, MPM could have the potential to not only visualize a tumor microscopically but also treat it directly in a real-time fashion.

Complementary tool in grading and staging by tumor micro-environment (TME) assessment

TME is an important component in PCa development. The prostate glandular architecture is surrounded by stroma, a collection of loose connective tissue consisting of collagen fibers, fibroblasts, auto fluorescent myoblasts, elastin fibers, vasculature, nerves and immune components. The collagen fibers which are the main structural and mechanical component of the stroma, produce strong SHG signal. By allowing detection of collagen fibers in a tissue through the use of SHG, quantitative analysis can potentially refine grading and therefore staging of the disease (18,20).

Several studies have shown correlation between orientation of collagen fibers and cancer's aggressiveness. In a given plane, the direction along which majority of the fibers tend to align, is defined as the preferred orientation, and it has been showed that malignant samples tend to have a higher degree of preferred alignment along a single direction compared to the non-malignant ones (21), and has been confirmed in other studies. Penet *et al.* (22) demonstrated how SHG microscopy can be used to identify collagen 1 (Col1) fiber patterns in the xenografts as well as in human samples. Cancer associated fibroblasts (CAFs), the principal source of Col1 fibers in tumors, showed an increase in aggressive tumors, correlating with higher Col1 fiber volumes in these tumors. This demonstrated the role of CAFs in supporting aggressive cancer. Higher levels of TGF-β (a growth factor that increases Col1 production) were also found. They also showed how the tumor extracellular matrix (ECM) plays an important role in facilitating metastasis.

Nerve sparing and surgical margins tool

Frozen sections and imprint cytology are currently used to confirm real-time margin status of oncology specimens but are often unreliable (23). MPM has the potential to be used as an intraoperative tool for margin assessment and nerve sparing management. Post-prostatectomy patients have positive surgical margins (PSMs) in 13.8% to 22.8% of

cases (24), and a sexual dysfunction of 6% to 27% (25) even after using intraoperative frozen section technique.

One of the major concerns of a surgeon performing a prostatectomy is the neurovascular bundle injury. MPM can provide improved visualization of the periprostatic nerves leading to improved outcomes of nerve-sparing radical prostatectomy and therefore preservation of individual nerves (16).

Cavernous nerves (CNs) are birefringent because of both the microtubule's arrangement in nerve axons alignment and the myelin sheath covering the nerve axons. The distance between CNs and prostate surface varies during their course from hundreds of micrometers to a few centimeters, making it difficult to predict their path and location (26,27).

Yoon *et al.* (7) compared MPM to polarization-sensitive optical coherence tomography (PS-OCT), in differentiating prostatic nerves from their surrounding periprostatic tissues. They concluded that the PS-OCT image may show birefringent fibrous structures at a greater depth, but MPM visualized individual nerves separately (4–10 µm nerve diameter *vs.* 154 µm). The limitations of the MPM were the smaller field of view (700 µm × 700 µm *vs.* 5 mm × 5 mm for OCT) and shorter depth (50 µm *vs.* 140–280 µm, SHG technology).

Bladder cancer

Imaging is playing a huge part in both diagnosis and prognosis of bladder cancer, with recently a new MRI validation score (28). *In vivo* techniques have been also used, bringing efficient staging devices in the operating room for a real time diagnosis of a bladder tumor. These instruments include high-resolution micro ultra-sound for bladder tumors (29), confocal laser endomicroscopy (30) and MPM imaging, which was successfully tested on animal models (31).

Diagnostic tool

MPM has already been tested in the evaluation of human bladder biopsies (32). A study by Jain *et al.* was able to demonstrate a sensitivity of 90.4, specificity of 76.9%, and a high predictive value (94%) in cancer detection. In their study, architecture (papillary versus flat) was correctly determined in 56 of 65 cases (86%), and cytologic grade (benign/low grade) was assigned correctly in 38 of 56 cases (68%) (33). A more recent study performed by Jain *et al.* gave even better results (Sensitivity and specificity of 97% and 100% respectively, and positive and negative predictive value of 100% and 98%. Interobserver agreement, k, was 0.93) (34).

Complementary tool in staging and grading

MPM can determine the depth of the tissue imaged, compare collagen and elastin fibers in the lamina propria and in the smooth bladder muscle. It has also assisted in illustrating on a rat model, the occurrence of collagen recruitment during the loading of the bladder wall (SHG technology) (34). This technology has demonstrated the deformation of the bladder wall during loading, confirming that unfolding of rugae and rearrangement of the lamina propria fibrils occurs when the bladder is extending (35). The phenomenon of collagen recruitment also has a correlation with age. The differences in the bladder wall architecture and in the connective tissue between young and old mice were also described by MPM (34,36).

Future perspectives

By acquiring MPM images of the different layers of the bladder wall, and supplementing them to *in vivo* techniques, a real-time optical diagnosis of bladder tumors could be described. This technique could therefore avoid an unnecessary second look trans-urethral resection of the bladder tumor (37). As shown in other cancers (21,38) collagen plays a key role in cancer tumorigenesis and development. Collagen stiffness is a promoter for bladder cancer progression as well (39). Incorporating collagen quantitative measurements to the MPM images could therefore provide information on both the diagnosis and prognosis of a bladder tumor in a real-time fashion, as it has previously in PCa (21). However, there are some drawbacks to this interpretation, since only clearing techniques have been able to image tissues up to 1 mm (2), limiting the translation to the everyday clinic for now.

Kidney cancer

Diagnostic tool

The use of MPM in the evaluation of kidney tumors was first studied in 2015 by Jain *et al.* (10), in which a blinded analysis of fresh unprocessed tissue (neoplastic and benign) for subcategorizing malignant kidney tumors was performed with the help of two uro-pathologists. Since MPM is not able to give intra-nuclear details, the subtyping was based on the nuclear-cytoplasmic ratio (N/C ratio) and nuclear pleomorphism characteristics, rather than the intra-nuclear characteristics used in Furhman's tumor grading. The diagnostic accuracy of tumor subtyping by the two uro-pathologists with MPM was 95%. Tumors were then further divided into non-papillary and papillary, based on their architecture. Furthermore, non-papillary tumors

Table 2 Multiphoton imaging anatomopathological characteristics of various renal tumors

Histology	Oncocytoma	Atypical oncocytoma	rCC	ChrCC	Eosinophilic variant of chrCC	Papillary rCC	Papillary urothelial carcinoma
Pattern	Acinar	Acinar	Sheets of cells separated by delicate branching vascular tissue	Trabecular growth pattern	Acinar	Papillae lined by single cell layer	Papillae lined by multilayered cells
Cell characteristics	Small, abundant	Small, abundant	Cells with fat droplets in cytoplasm (low-grade) and lacking fat droplets (high-grade)	Large cells, distinct cell borders	Small, abundant, uniform	Large histiocytes	Multiple cell layered papillae
Cytoplasm	Homogenous	Homogenous	Homogenous	Wispy and scant	Homogenous	Abundant	Homogenous
Nuclei	Central signal-void	Central signal-void	Signal void	Pleiomorphic, perinuclear halo	Perinuclear halo	–	–
IG	Larger and brighter than chrCC	Discrete	–	Small and sand-like (not seen on H&E)	Coarser	–	–
Distribution of IG	Apical and perinuclear (indicating mitochondrial origin)	Apical	–	Diffuse (indicating microvesicle origin)	Diffuse	–	–
SHG	Collagenous tissue surrounding (stroma)	Collagenous tissue surrounding	Fine network of vascular channels	Collagenous tissue surrounding (stroma)	Signal void	Fibro-vascular core	Fibro-vascular core

rCC, renal clear cell carcinoma; ChrCC, chromophobic renal cell carcinoma; H&E, hematoxylin and eosin; IG, intracytoplasmic granules; SHG, second harmonic generation.

were classified on the basis of cytoplasmic features. Clear-cell renal cell carcinoma (RCCs) and chromophobe RCCs (chRCCs) had distinct identifiable features.

In another study by Jain *et al.* (40), using AF and SHG, MPM was used to differentiate two entities: oncocytomas (typical and atypical) and chRCC. These two tumors are often considered to be part of the same morphological spectrum of diseases but require contrasting paths of therapeutic management.

In this study, MPM imaging of these tumors showed auto fluorescent intracytoplasmic granules, with distinct sizes, shapes, distribution and different wavelength of auto fluorescence. The eosinophilic variant of chRCC also had a distinct auto fluorescent nucleus and perinuclear halo. Via a blinded MPM and morphometric analysis, diagnostic accuracy for oncocytomas and chRCC was found to be 100% and 83.3%, respectively (*Table 2*).

Pediatric tumors (nephroblastomas amongst other types of tumors) have also been assessed by MPM (41). All the typical features of pediatric tumors can be identified with distinct architectural changes on MPM. MPM could illustrate the three typical components of nephroblastoma: (I) epithelial component, (II) solid tumor cells (which are small with a hyperchromatic nuclei and scant cytoplasm) and (III) mesenchymal differentiation. All of these studies used a combination of AF and SHG.

Surgical planning tool

A kidney biopsy may not provide enough information for cancer detection, leading to a 30% rate of unnecessary nephrectomies for benign lesions (42). Therefore, the diagnostic yield of kidney biopsy, when it comes to small renal masses, needs to be improved.

An *in vivo* biopsy of mouse kidney has already been carried out by Li *et al.* (31), using a rigid MPM probe able to fit inside a biopsy needle. The probe used in their study was capable of performing three-dimensional (3D) two-photon (AF) optical biopsy, allowing a real-time optical diagnosis of the renal mass without the need of fine needle aspiration biopsy. This method was therefore capable of

lowering the potential complications of a renal biopsy, such as hematomas (30), by enabling the actual need of a biopsy.

Future perspectives

Although MPM provides a good three- dimensional view of the tissue, the maximum depth of the tissue imaging is approximately 100 µm (z stacks) in a classical use of the MPM. To be comparable to slide based microscopy, optical sectioning techniques are needed in order to perceive tissue images deeper than this limit (5). As a novel technique, a study demonstrated the use of chemical clearing techniques in addition to MPM to visualize tissues up to a depth of 1 mm (2). In contrast to histology that uses Bright Field microscopy on 5 µm sections to avoid blurring of the nuclei, optical sectioning techniques like MPM create 2 µm distance sections, allowing for a more defined visualization of the tissue. In addition, microtome cutting artefacts and folding of the tissue that occur in traditionally processed H&E sections were avoided using this technique.

In vivo assessment; MPM-assisted surgery

One of the possibilities of *in vivo* assessment with MPM could be the evaluation of ischemia-reperfusion injury (IRI) following a partial nephrectomy. Using time-lapse imaging, or imaging *in vivo* the same kidney for several days (43), MPM is able to demonstrate the development of acute tubular necrosis by picking up the flattening of the epithelium, swelling of lumen and shedding of the epithelial cells (44). MPM can also be used to detect certain tagged proteins and molecules by using antibodies against them (CD44+ cells) (44). MPM imaging of the same kidneys 1 to 2 days after IRI (Ischemia reperfusion injury) was found to have a significantly increased (10-fold) number of CD44+ cells in peritubular capillaries surrounding proximal tubule segments. Serial MPM imaging of the same glomeruli in the same kidney and animal for several days were able to show the migration of renal cells in the intact living kidney, explaining the remodeling and repair after injury (44).

Future perspectives

Another potential use of MPM would therefore be the imaging of oncology markers, which correlate to aggressiveness of these tumors. These could potentially be imaged *in vivo*, in order to determine both their diagnosis and prognosis concurrently.

Innovation and other cancer use

Other cancers

MPM has been widely studied in other solid tumors, either for scientific or for clinical purposes, and has even become part of the routine clinical evaluation for some oncologic pathologies, such as dermatology tumors. We organized a brief summary of the major studies involving MPM's application in diagnosis of solid tumors (*Table 3*).

Deep learning

Deep learning has been, for more than ten years, used into various specialties, such as medical imaging and histopathology (15).

Two major studies have been incorporating multiphoton microscopy with deep learning.

Huttunen *et al.* (45) used 4 pretrained convolutional neural networks (CNNs), to analyze slides with SHG and fluorescence contrast. The CNNs were able to differentiate between healthy tissue and the tissues associated high grade serous carcinoma. Results showed high sensitivity (95.2%±2.5%), specificity (97.1%±2.1%), and accuracy (96.1%±1.1%) with fine training VGG-16.

Liang *et al.* (46) also used deep learning to process SHG images of collagen networks in glutaraldehyde-treated bovine pericardium (GLBP), a common tissue for the fabrication of bioprosthetic heart valves and vascular patches. The deep learning was used to predict tissue elastic mechanical properties. The trained model was capable of identifying the overall tissue stiffness with a classification accuracy of 84% and was able to predict the nonlinear anisotropic stress-strain curves with average regression errors of 0.021 and 0.031. We can therefore imagine a future integration of deep learning with MPM images in the clinical scenario, allowing a better diagnosis and prognosis of the disease.

Conclusions

This narrative review describing the value of MPM in uro-oncology is, to our knowledge, the first review to properly detail the benefits of the microscope in all the oncologic sub-specialties of urology.

MPM is a new emerging technology with potential to be used in multiple scenarios, especially in the uro-oncology field, such as kidney cancer, PCa or bladder cancer. Our review covers a wide variety of literature, showing the relevance of MPM in the current scenario of our specialty.

There are multiple advantages of MPM technology. It can first be used as an *in vivo* tool especially to assist surgeries and grade cancers, providing a complementary assistance in decision making. As outlined previously,

Table 3 List of major articles involving MPM use in various medical specialties

Organ	References	Microscopy technique	Clinical use	Year
Brain	Identification of distinctive features in human intracranial tumors by label-free nonlinear multimodal microscopy. Galli *et al.*	SHG + TPEF	Diagnostic use in identifying and subtyping of brain tumors and brain metastasis of various tumors	2019
Lung	Multiphoton microscopy: a potential "optical biopsy" tool for real-time evaluation of lung tumours without the need for exogenous contrast agents. Jain *et al.*	AF + SHG	Diagnostic tool for lung tumors and their subtypes	2014
Breast	Label-free imaging of blood vessels in human normal breast and breast tumor tissue using multiphoton microscopy. Xi *et al.*	SHG + TPEF	Diagnostic tool to differentiate between properties of blood vessels in normal breast tissue and breast tumor tissue	2019
	Collagen analysis by second-harmonic generation microscopy predicts outcome of luminal breast cancer. Natal *et al.*	SHG	As a prognostic tool by quantitatively assessing collagen fibers within tumors	2018
	Direct comparison between confocal and multiphoton microscopy for rapid histopathological evaluation of unfixed human breast tissue. Yoshitake *et al.*	AF + SHG and confocal microscopy	Diagnostic use	2016
Liver	Label-free classification of hepatocellular-carcinoma grading using second harmonic generation microscopy. Lin *et al.*	SHG	Diagnostic and prognostic use. algorithm for assessing the grade of the HCC tumor	2018
	Automated classification of hepatocellular carcinoma differentiation using multiphoton microscopy and deep learning. Lin *et al.*	SHG + TPEF in conjunction with AI	Diagnosis and grading of HCC on basis of differentiation	2019
Pancreatic	Characterization of pancreatic cancer tissue using multiphoton excitation fluorescence and polarization-sensitive harmonic generation microscopy. Tokarz *et al.*	MPF, SHG & THG	Diagnostic use in pancreatic cancer	2019
Ovarian	Automated classification of multiphoton microscopy images of ovarian tissue using deep learning. Huttunen *et al.*	AI in conjunction with SHG + TPEF	Diagnosis of high-grade serous carcinoma of ovary	2018
Colorectal	Non-labeling multiphoton excitation microscopy as a novel diagnostic tool for discriminating normal tissue and colorectal cancer lesions. Matsui *et al.*	AF + SHG	Diagnostic to quantify differences between cancerous tissue and normal colorectal tissue in fresh colorectal specimens	2017
Cardiovascular	Multiphoton imaging of collagen, elastin, and calcification in intact soft - tissue samples. Gade *et al.*	MPF + SHG	Diagnostic/prognostic. Protocols for simultaneous and quantitative analysis of collagen, elastic content and calcification in fresh tissue samples of arteries and bladder	2018

MPM, multi-photon microscopy; SHG, second harmonic generation; TPEF, two-photon excitation fluorescence; AF, auto fluorescence; AI, artificial intelligence; MPF, multiphoton excitation fluorescence; THG, third harmonic generation; HCC, hepatocellular carcinoma.

when MPM is employed, nerve-sparing surgeries (radical prostatectomies) could be potentially performed in a more accurate way. This technology can also study the TME, and aid in uncovering newer findings, such as the orientation of collagen fibers in more aggressive tumors, as a prognostic tool. In addition, MPM imaging could also differentiate tumors that look similar on histopathology [oncocytomas (typical and atypical) and chRCC], and develop more accurate ways of subcategorizing malignant kidney tumors, based on both nuclear-cytoplasmic ratio (N/C ratio) and

nuclear pleomorphism characteristics. MPM can also be used as a quantitative analyzing tool, incorporating collagen quantitative measurements to the MPM images that could therefore provide information on both diagnosis and prognosis in a set tumor.

There are certain drawbacks for this technology. First, it is costly, and needs specific rooms as most of the MP microscopes are stored in a specific room for laser and cooling system to be fully effective. Trained personals are required to make use of it, as it is an unfamiliar imaging analysis for the eye trained on the conventional H&E-stained slides. It can therefore act as a complementary tool but not completely replace histopathology, since no randomized controlled trials against GSH have been organized up to date. Finally, deep learning models have been investing this imaging modality, but need greater number of test values in order to accurately train an AI model.

To bring MPM from bench to bedside, there are a few limitations that we need to address; first, the use of MPM requires time: the image acquisition can be slow (around 1 to 5 frame/second, a frame being a 512 nm × 512 nm size image). This can be a drawback to an *in vivo* requirement for surgery or biopsy tool. The explanation is both by the scanning of an entire field of view with only one laser, and the acquisition of 3D images by moving the tissue and objective with the help of a stepper motor. A faster acquisition can be obtained with a technology called multiphoton multifocal microscopy, but will definitely lower the quality of the image, making it hard to use in an easy "user-friendly" approach for the clinician in the images' interpretation. Durand *et al.* (17) demonstrated the average speed of an *in vivo* assessment on animal model, which can go up to 75 min, therefore being a major limitation in a surgical procedure. He was also able to show the limitation of the *in vivo* use with a living tissue: being uneven to the microscope scanning, the tissue was hard to image with the same quality on the whole surface, leading to motion artefacts on some specific images. To have a fully "user-friendly" MPM in a clinical setting, we would therefore need a machine that will: have faster image acquisition/capability of imaging uneven surfaces with the same quality and same depth/automatically adapt the laser intensity depending on the type of surface that is being scanned/overcome the use of a cover slip in order to flatten surfaces. Another limitation is the size of the multiphoton microscope, often needing a dedicated room for its purpose. MP microscopes need to be in a dark environment, to limit the light penetration on an imaged tissue and therefore modify the intensity of the laser imaging. Lasers are often heavy and need a cooling system, making it for now difficult in a small operating room or a consultation space. Portable devices such as MPM endomicroscopes (47) but also GRIN endoscopes (18) or rigid probes (31), have been recently developed for in an-vivo use. These devices can perform AF imaging near histopathological resolution. The rigid probe (31) is compatible with a 14-gauge biopsy needle, allowing the microscope to image inside a tumor that would be guided by either macroscopically by ultrasound or CT scan. 3D images are realized, by the field of view is still very small (120 µm) and limited in terms of focus scanning range (200 µm), but the image acquisition was faster than traditional MPM (10 frames per second), and *in vivo* images of disorganized cellular cells were shown, able to detect a kidney tumor *in vivo*, and can be described as an imaging biopsy. As for the GRIN endoscope, this device contains a 1-mm large and 8 cm long probe, and weighs less than 2 lbs. where are added the optical components, such as scan mirrors, scan lenses, and objective. The total system length of the portable device is approximately around 27 cm, and was able to image unstained mouse lung tissue *ex vivo*. The limitation of this device is the power of the laser combined with a fast imaging acquisition rate, that could give motion artefacts *in vivo* tissues; however, in their study, Huland *et al.* were able to maximize their frame rate, making it adequate to overcome these motion artefacts.

Finally, *in vivo* studies have been limited to animal settings, and great care should be taken regarding the imaging depth analysis, since rat tissues are always thinner than human tissues. For now, imaging techniques have been limited to a maximum depth of 100 to 200 µm for most of the tissues, making it extremely difficult in an everyday clinical use of this technology. We can imagine that the different layers of a tissue could be identified in a 3-D setting on a human model (most of the urothelium layers are above 200 µm), but the field of view would limit the interpretation compared to histopathology: a microtome section of an entire tissue given to the pathology department has the benefit to look at a 1 cm wide field of view in a matter of seconds or minutes depending on the pathologist's experience. If we were to compare this histopathology interpretation period with the acquisition and interpretation time of the MPM imaging, there is with no doubt a notice difference in favor of histopathology, as we showed an acquisition time of more or less one hour in our review for MPM technology. Clearing techniques (5) would help in

defining a deeper resolution of an MPM imaged tissue, but needs a real preparation of a tissue, losing all the benefits of a fresh and unfixed image that could be scanned in a everyday clinical setting. With all these limitations, we can now understand the difficulties that are needed to be challenged for the translation of this developing technology to the clinic.

There are certain limitations to our study. First, we did not perform a systematic review, because of the heterogenicity of the publications. We chose to do a narrative review, as the studies we included differed widely in terms of methodology and conclusions. Therefore, conclusions could be subjective due to the choice of publication inclusion, hence why we decided to include all publications that were relevant to our topic. Finally, we decided to mostly describe the clinical benefits of this technology, causing a selection bias in the publications that we included (translational studies). This selection bias was purposely performed as we chose to focus in the "on-field" use of this microscopy imaging technology.

MPM has therefore been showing great improvement in having a real time assessment of fresh tissue oncology diagnosis, *in vivo* imaging as well as quantitative analysis of the tissue, thereby increasing precision of the diagnosis. This nonlinear optical has the potential of guiding both biopsy and surgery, helping the surgeon with interesting additional tissue information intra-operatively. Therefore, we could one day imagine the use of this technology in both clinic and operative rooms, if it becomes more costly accessible and size-compatible in the future. More studies will need to be done to correctly evaluated this technology in the translational field, such as randomized controlled studies against GSH.

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Footnote

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