Peer Review File

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This paper introduces the interesting and important topic of the current and potential applications of multiphoton microscopy in uro-oncology. It is valuable to have information about this technology and its potential for clinical applications. There are few papers of this kind and the overall concept is quite interesting. However, there are some serious concerns that limit enthusiasm for publication of this manuscript in its current form.

Writing style: Unfortunately, the manuscript looks like it was hastily put together without proper editing (many typos, in many places sentence structure is simplistic or grammatically incorrect). Greater care needs to be taken in editing the paper in terms of punctuation and sentence structure. Phrases are at times a little too casual and detract from the credibility of the paper. For example, Deep learning has been a hot topic for the past few years.

Thank you for your interesting comment, we have tried modifying the writing style of the manuscript to give a more scientific aspect to it.

Limitations on imaging depths.
MPM is still limited to a few hundred microns, without fixation and optical clearing. MPM imaging in vitro of rats, for example, is already insufficient to image through the entire wall without optical clearing. The wall thickness of the human bladder is substantially greater than that of mice and rats. Therefore, the MPM that work in rats and mice will not necessarily translate to humans. For example, on page 6 and 7, the authors discuss the possibility of using MPM in the clinic to diagnose bladder cancer. It is stated that: 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 prostate cancer (39). However, the techniques referred to in the prior paragraph are for in vitro imaging in rat bladder. The suggestions for future perspectives are intriguing, however there is a need for a detailed discussion of the technical barriers for translation to the clinic.

A further technical challenge for imaging depth is the strength of the autofluorescence signal (for example from elastin) which can reduce the quality of the SHG imaging data for collagen.

Care should be taken throughout the paper to be precise about which technologies are used in which studies. Statements, such as that in the introduction, where it is stated that MPM has the ability to penetrate up to 1cm into the tissue should be qualified to explain that this is only for optically cleared samples.

We have added the details of each study regarding the use of AF or SHG, and also discussed the depth of images in a specific paragraph of discussion

These substantial technological challenges are not discussed sufficiently. It would be extremely useful to the readers to discuss the issue of imaging depth up front in a single section of the paper and explain how the various approaches might address this or what new technical developments are needed for clinical translation.

Clinical Devices

The manuscript summarizes some current technology that enables the use of MPM in living systems using probes. This is exciting but needs more elaboration to understand the technical limitations of these systems and how they compare to commonly used in vitro systems. What is the GRINS lens system- never defined just mentioned in the paragraph starting on page 182.

We have tried to describe in a more precise way the different instruments used with MP lasers and microscopes.

In summary, the coupled presentation of a summary of current work along with views of the authors on future perspectives is valuable and intriguing. However, there is insufficient information about the technical requirements for this translation. Without such information it is not possible to understand how readily these possibilities could be realized.

It would be extremely important to elaborate on specific technical capabilities that would be needed for clinical translation and of these, which are currently available and which would require further development.

This topic has now been discussed in the discussion section

A few other points:

Methods:

The approach to selecting the articles needs more detail. It was not clear whether they articles needed to have all the keywords. Presumably they did not since 476 articles were identified. How were these papers later “screened for inclusion”? It is not at all clear how this set was reduced to 48 publications “that were relevant to our topic”.

Methodology has been modified according to your comments

Other:

The images in some of the figures look like they have been taken from other papers. The quality is not good, for example, in Figure 1. Also, was permission obtained to use these figures?

We have now removed two of the images, and got permission from Matthieu Durand to use his picture in the publication