Peer Review File

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Reviewer A

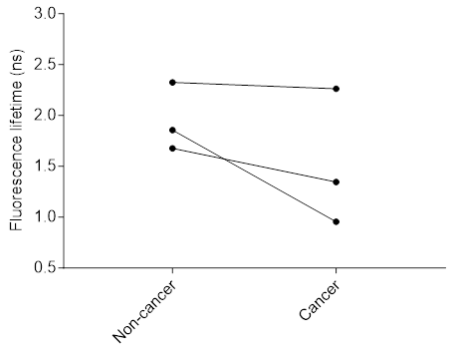
The authors' study is a fundamental research comparing lung cancer and normal lung ex-vivo using fibre-based fluorescence-lifetime imaging microscopy. The authors demonstrated that fluorescence lifetime is reduced in lung cancer compared to normal lung.

Comment 1:

This study includes cases of lung adenocarcinoma. Pathologically, lung adenocarcinoma is divided into several subtypes. In particular, lepidic adenocarcinoma grows by replacing the existing alveolar epithelium, so it resembles normal lung tissue. Was lepidic adenocarcinoma included in this study? If it was included, please tell us about the fluorescence intensity and lifetime image.

Reply 1:

Thank you for the comment and yes, we had three cases of predominantly lepidic adenocarcinoma. As shown in table 1 this study included 11 adenocarcinoma, 6 squamous cell carcinoma, 1 pleomorphic carcinoma and 3 mixed. Of all the adenocarcinoma, 5 had a predominant solid pattern, 3 were predominantly acinar and 3 were predominantly lepidic. The result of the observation of a lifetime shift is not changed by the presence of lepidic adenocarcinoma compared to other subtypes with the 3 datasets isolated in the plot below.



We agree that images of lepidic resemble normal lung and indeed on CT scanning this may present as a pure ground glass opacification. Clinically these are managed differently compared to solid lesions (which if adenocarcinoma are more likely to be another subtype) The main purpose of this manuscript was a proof of concept study for ex vivo samples.

Change in text:

To the manuscript we have included a breakdown of the subsequent pathological diagnoses and stages of cancers in the table.

Comment 2:

The authors state that this study suggests that fluorescence-lifetime imaging microscopy may have potential as a guiding tool for bronchoscopic biopsy. Currently, EBUS-GS is used as a guiding tool in clinical practice. If there are any advantages of this method compared to EBUS-GS, please mention them.

Reply 2:

We thank the reviewer for this comment. We believe this technology will sit in a separate space than EBUS. In our, and UK practice, we predominantly use EBUS for the safe aspiration and staging of lymph nodes/masses within the mediastinum. We do not typically use EBUS for more peripheral lung nodules or masses. In those circumstances we believe this technology will have a significant advantage as using a fibre based system we can have real time feedback as to whether we are at a lung lesion, and then are able to use this to guide biopsy procedures.

In the manuscript we have clarified that we envisage the use of this for peripheral lung nodules and masses.

Change in text:

Lines 236-238 now state: “We envisage the application of this technology particularly for peripheral lung nodules or masses to provide real time feedback that the lesion has been reached and where procedures such as endobronchial ultrasound (EBUS) are typically not used.”

Comment 3:

Please change the "1" in Stage 1B on line 204 to Roman numerals.

Reply 3:

This change has been made in the text.

Change in text: Stage 1B changed to Stage IB at line 205.

Comment 4:

I believe that RROC on line 220 is a typo for ROC. Please correct it.

Reply 4:

The typo has been corrected.

Change in text: RROC has been changed to ROC at line 221.

Reviewer B

Comment 1:

If commercialized, it is expected to be useful as an auxiliary device for biopsy using a bronchoscope for lung cancer. The paper used ex vivo lungs, but I am curious whether similar results are seen in inflated lungs.

Reply 1:

The reviewer is correct that the intention, if commercialized, is to provide a method of real time assessment for guiding biopsy at the bedside. The work presented is an early proof of concept and was carried out on excised tissue samples following resection with unprocessed tissue samples taken from the patient and measured within the next hours. It is the scope of future work to confirm that the signatures presented are confirmed *in-vivo* through clinical trial and is out with the scope of this report. We currently have an ongoing clinical study, trial number ISRCTN10996089 (https://doi.org/10.1186/ISRCTN10996089) utilizing the technology for this application. There is a body of work in the literature showing the applicability of FLIM *in-vivo,* albeit in different organs, including showing the preservation of FLIM signal moving from *ex-vivo* to *in-vivo*, for example:

Marsden M, Weyers B, Bec J, Sun T, Gandour-Edwards R, Birkeland A, et al. Intraoperative margin assessment in oral and oropharyngeal cancer using label-free fluorescence lifetime imaging microscopy. IEEE Transactions on Biomedical Engineering. 2021;68(3):857–68.

Hassan, M.A. *et al.* (2023). FLIm-Based in Vivo Classification of Residual Cancer in the Surgical Cavity During Transoral Robotic Surgery. In: Greenspan, H., *et al.* Medical Image Computing and Computer Assisted Intervention – MICCAI 2023. MICCAI 2023. Lecture Notes in Computer Science, vol 14228. Springer, Cham. https://doi.org/10.1007/978-3-031-43996-4_56

The point is covered in the discussion line 257-266, which we have expanded and include the second recent reference above.

Changes in text:

Lines 260-264

"FLIM has been demonstrated as an in-vivo tool for distinguishing cancer from non-cancerous tissue by Hassan et all for the oropharynx with Marsden *et al* demonstrating fluorescence lifetime trends of healthy and cancerous oral tissue *in vivo* are unchanged,"

Hassan 2023 reference added.

Reviewer C

Review on the manuscript entitled "Fibre-based fluorescence-lifetime imaging microscopy: A real-time biopsy guidance tool for suspected lung cancer"

In this manuscript, Susan et al. reported using auto-fluorescence imaging to distinguish cancer with non-cancer tissue in NSCLC. The tests were conducted on ex vivo tissues after surgical removal. The major concerns are:

Comment 1:

> How will this method be utilized in a clinic? The approach demonstrates that the use in ex vivo tissue. However, it is unsure how will this approach being used during surgical removal, since the aim is to distinguish cancer vs non-cancer tissues during surgical resection.

Reply 1:

> The presented work is proof of concept and indeed uses *ex-vivo* tissue samples measured following resection. As mentioned in a previous response there is evidence in the literature of preservation of FLIM signals *in-vivo*. The reported system is presented as a tool to aid in the guidance of biopsy for assessment of nodules of masses where cancer is suspected, I real time, not during the resection of a confirmed tumor. There is in the future, the potential to ascertain whether this can be used for assessing margins after resection has taken place. The fibre-based tool fits down a standard bronchoscope to potentially enable rapid access and real time assessment of tumors and guide the most appropriate location for biopsy sampling.

Change in text;

> Lines 236-241:
> "We envisage the application of this technology particularly for peripheral lung nodules or masses to provide real time feedback that the lesion has been reached and where tools such as endobronchial ultrasound (EBUS) are typically not used. Furthermore, there is potential for the use of the tool to assess the margins of a lesion following resection when coupled with an appropriate bronchoscopy navigation tool."

Comment 2:

> How does this approach compare to current well-established procedures, such as CT scanning?

Reply 2:

> This procedure will always be done as an interventional procedure following

the finding of an abnormality on CT scan. Indeed, CT screening is likely to be implemented across Europe over the next few years, where we will find many suspected cancers (nodules or masses on the CT scan). These subsequently require pathological confirmation and this technology will be used in the process of pathological confirmation.

Comment 3:

Will different tumor subtypes and histological structures impact their results? Seems from the HE slides, there is already distinct differences between cancer and non-cancer tissues, the need for using this auto-fluorescence approach seems needless.

Reply 3:

The goal of the presented system is to guide the initial biopsy locations of tumors and increase the "hit rate" on the acquisition of tissue biopsy for assessment which is a common problem in the clinical workflow and will reduce the need for multiple time-consuming biopsies.

Change in text:

In addition to the above changes: Line 236: "and increasing the biopsy "hit-rate".