Reviewer's report

**Title:** Infrared Micro-Spectral Imaging: Automatic Distinction of Cell Types in Axillary Lymph Node Histology

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**Reviewer:** MICHEL MANFAIT

Reviewer's report:

The authors present here two methods based on HCA and ANN for analysing FTIR spectral images of axillary lymph nodes. The importance of rapid histological detection of such tissues is relevant but the methods used are not new. The concept of "automatic distinction" is misleading because while this is true for the HCA method, the ANN still needs input concerning the groups and training.

Although the results presented are convincing, the number of cases studied seem too small.

This research work can be published in BMC Clinical Pathology after considering the revision remarks.

**Major Compulsory Revisions**

1) The title is misleading because it mentions the automatic distinction of cell types. However, based on the resolution used here (25 µm:pixel), it seems difficult to identify cell types. It would be more appropriate to replace "cell types" by "tissue types".

2) The abstract is too general and seems to lack concrete results emanating from this study.

3) They have also used two approaches for image analysis, HCA and ANN, with the latter being a faster method. However, they never concluded on which method is better for spectral diagnosis.

4) On the one hand the authors state that the method is rapid but on the other hand conclude that data acquisition is still quite time consuming. This ambiguity must be removed.

**Minor Essential Revisions**

5) Materials and Methods

Page 7, the authors should make it clear why for deparaffinised tissues they use a different substrate and work in reflection mode.
Page 8: Rephrase 1st sentence: Spectroscopic imaging data was acquired using an infrared spectrometer (make and model) coupled to....

- If the measurement area is 5 mm x 5 mm, and having all other conditions the same, why do the measurement times vary from 100 to 220 minutes?

- What is the basis of the spectral quality test used by the authors? This must be made clearer.

- The authors state that the spectra were vector normalised in the 1800-900 cm\(^{-1}\) region, but finally the spectral hypercube dimensionality also includes the 3100-2800 cm\(^{-1}\) region, which has not been normalised. This looks ambiguous and needs to be commented.

- The authors use two approaches, absorption and reflection modes (for deparaffinised samples). As noted above, the rationale for this choice is not clear. Also, it does not seem that they have carried out other pre-processing procedures to correct for optical effects that contaminate the chemical information, especially in the reflectance mode. Can the authors comment on this.

- On page 11, the authors state that the image analysis by HCA for reconstructing an image can take several hours and is not compatible with rapid diagnosis. The IR image acquisition itself takes a few hours. Is this not also incompatible with rapid diagnosis?

- Deparaffinisation is known to affect tissue composition but preserves morphology. Can spectral diagnosis, which is based on tissue composition, depicts such changes?

- It seems that a complementary study by Raman spectroscopy on similar samples has been undertaken (see ref. 18). In this work, the authors do not compare their findings with this previous work. Can they comment on that?

Discretionary Revisions

- Page 6, line 6: The authors state that « A chemical image of the tissue section can then be constructed that is similar to a stained image....". This statement must be corrected because they do not mention in what way the chemical image is similar to the stained one. To my point of view, the basis of a spectral image constructed on biochemical information is different from a stained section, which mainly reflects the morphological information.

- Typographical error. I guess the authors mean "...within a matter of minutes".

- Last sentence: I guess the authors mean they want to diagnose disease based on the spectral datasets and not to
diagnose spectral datasets.

Page 7, line 9: "depth of 6 µm" should be replaced by "thickness of 6 µm".

In the legends to figures, the dark dots are not assigned.

The authors compare images obtained by HCA and ANN methods. It is clear that the ANN method is faster although it seems to give less number of color-coded groups. Other classification methods have proved to be also quite fast. What are the real advantages of ANN compared to such methods?

In an automation process of spectral diagnosis, can the loss of information on going from HCA to ANN affect the outcome, for example in precancerous situations where minor changes are to be depicted?