Author's response to reviews

Title: Automated Recognition of Cell Phenotypes in Histology Images based on Membrane- and Nuclei-Targeting Biomarkers

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Author's response to reviews: see over
Dear Editor,

Please find attached a revised version of the article entitled “Automated Recognition of Cell Phenotypes in Histology Images based Title: on Membrane- and Nuclei-Targeting Biomarkers” by B. Karacali, A Vamvakidou, and A. Tozeren (1708430466132582).

Also included with this cover letter is our detailed response to reviewer comments. We are grateful to the reviewers and the editors of BMC Medical Imaging for providing us with insightful evaluation of our manuscript. We hope that you will find the revised manuscript appropriate for publication in the journal and look forward to hearing from you soon.

Sincerely yours,

Aydin Tozeren
Detailed Response to Reviewer Comments

Reviewer: Klaus Werner Kayser

Major Requested Revision:

The authors assume that the applied markers only react with antigens etc. located in the cell membrane or nuclei (which is true for the majority, but not for all cases). As they only consider pixel in their clustering matrices, some remarks should be given how to detect stainings not associated with membranes etc.

In the revised manuscript, we have added a paragraph on the computational assessment of sub-cellular stains in the Discussion section (see page 13 paragraph 2).

There are some "open accessible" measuring systems in the internet available that provide automated measurements of immuno-stained histological images (EAMUS.de), and which have been described in detail recently (Kayser et al. Diagnostic Pathology, 2006). The authors should discuss the pros and contras of their pixel-addressed system to those that are "object-addressed".

We have now included a comparison of our approach with the object-addressed approaches in the literature in the Discussion section of the manuscript (see page 13 paragraph 2).

The authors should mention that their system addresses segmentation of pixels "related to a specific color". The question arises whether it is able to quantify the color intensity, as this has not been mentioned and described in the article.

Our approach is based on clustering tumoroid cross-section image regions that exhibit statistically significant differences in the amount at which they express the stain. Beyond this basic separation of varying staining intensities, it is not designed to measure the exact amount of staining at a given pixel. This point is clarified in the Methods section of the revised manuscript (see page 7 paragraph 1).

I assume that the system measures images of PAP-stained slides (brown color). The authors should mention this in their chapter material and methods.

The reviewer is correct. We have now explicitly stated that the slides were PAP-stained in the Methods section (see page 5 paragraph 2).

The authors should give the corresponding image/microscope magnifications in all their included figures.

All images used in the manuscript were acquired at 40× objective of the Coolscope Digital Microscope as stated in the Methods section. In the revised manuscript, we have included this information in all figures containing tumoroid cross-section images.
Minor Considerations and Revisions: None

Reviewer: George Nikiforidis

Major Requested Revision:

The core contribution of this paper is unclear. Automated recognition of cells stained with different biomarkers in cross section images has been extensively studied. The method of constructing the cross section images has been presented elsewhere, by the same authors in reference [29] found in this manuscript, and the image analysis algorithms are well known in quantitative analysis of cross section images. These cannot be considered as advancement. The authors should clarify the contribution of this paper.

This manuscript develops a novel image analysis technique that integrates automated detection and quantification of sets of biomarkers that are (1) cell membrane associated and (2) nuclear bound in determining cell cluster phenotypes in vitro cell aggregates. Our previous work, like many others in the literature, focused on the cell nuclei and their spatial distribution in the tumor tissue. In this article, we use both the cell nuclei and cell membrane boundaries (decorated with biomarkers) in the determination of cell phenotypes using computer vision. We have focused our research in this article on the image analysis of invitro tumoroids as they are being increasingly used in cancer research. Very little is available in the literature on the structural and morphological composition of breast tumoroids. Significant differences exist between in vitro tumoroids and breast tumors of the same size in the composition of cells, extracellular matrix and the metabolic parameters. These differences in the microenvironment reflect on the spatial texture of tumoroids, as observed in the planar views (images) of tumoroid cross sections. Our preliminary experiments with tumoroid image processing indicated that the computational algorithms used in studying histology slides obtained from surgically resected or biopsied specimens do not necessarily perform well on tumoroid cross section images. This is mainly due to the much higher number density of overlapping nuclei in tumoroid cross sections, making standard segmentation and filtering operations less effective for tumoroid cross section images.

The review of the literature is incomplete. Although the authors do mention various commercial image analysis platforms for general processing of histology images, they do not reference methods presented in literature for automated recognition of cells stained with different biomarkers, which is the subject of this effort. The authors should sufficiently review the literature in this field. In this way the core contribution of their paper might become clearer.

We have expanded the review of the existing literature on processing of histology slides stained with different biomarkers in the revised manuscript (see page 3 paragraph 2, and page 4 paragraph 1).
The authors determine the number of optimal clusters for the k-means clustering by ‘visual evaluation’. It is not clear who performed this ‘visual evaluation’. This is a task for experienced experts (i.e. histopathologist, cytologist etc). This should be clarified since it affects the validity of the results. Additionally, if cells stained with different biomarkers are visually distinguishable, then what exactly is the gain by using image analysis algorithms? It is clear that for whole cross section images with hundreds or thousands of cells the automatic recognition of regions with different biological meaning is an important and handful process. However, I am not sure if only this may support a publication. The authors should discuss these issues.

Since a ground truth image dataset is missing to evaluate the accuracy of various clustering algorithms and the efforts in the computer vision literature for determining the optimal clustering adaptively remain inconclusive, visual evaluation stands as the only viable alternative to pick an agreeable setting for unsupervised clustering. As the reviewer points out, automated characterization of staining percentages across tumoroid cross sections is crucially important for fast and repeatable quantification of tumoroid composition in evaluating prospective cancer drugs. As we have responded to the previous comment of the reviewer, the efficacy at which this can be accomplished on tumoroid cross section images stained for membrane- or nuclear-bound markers has not been studied.

To generalize the k-means clustering algorithms, the authors use a reference image to find cluster centroids based on which they determine similar clusters in new images using a k-nearest neighbor approach. It is important to discuss how this reference image was produced, based on what criteria and why this reference image can be considered as representative?

The reference image shown in Figures 3 and 4 of the manuscript was selected for the sole reason that it exhibited all the regions of interest for studying the tumoroid cross-section images in our dataset in abundance, and therefore, presented the most complete and statistically accurate description of the data for training the subsequent classifiers. This clarification is included in the Methods section of the revised manuscript (see page 6 paragraph 4).

Another important issue that needs to be addressed is the production of simulation images. Firstly, only circular nuclei are used, however, in figure 3 of the current manuscript it is obvious that real tumor cells have arbitrary shapes and sizes. From this aspect the simulation is not representative of the morphology of cells. Secondly, the spatial distribution of cells in synthetic images was chosen uniformly, which is again not representative of the real spatial distribution. From this aspect the simulation is not representative of the topology of cells. Thirdly, after placing the synthetic spots, a smoothing operation is performed without any explanation of why. The authors have not taken into account the Point Spread Function of the imaging system, which is important when one attempts to create synthetic images representative of real images. Under these circumstances, these synthetic images cannot be considered as representative of real
images, thus, the results of segmentation algorithms on these images cannot be used to assess the performance in real images.

We agree with the reviewer. The task of generating absolutely real-like cross-section images is very complicated and multi-faceted and in itself is a huge project. Indeed, we are currently engaged in our group in a project that aims to generate statistically viable simulations of differently stained cross-section images. The simplistic simulations shown in Figure 2 are intended to provide a point of reference to the readers elucidating the operations of the image segmentation algorithms on cross-section images stained for membrane- and nuclei-bound biomarkers, and not as a realistic assessment of the respective algorithms’ performances. We have included clarifying remarks in the revised manuscript to establish the merits of the simulated images and the corresponding segmentation results (see page 8 paragraph 3, and page 13 paragraph 1).

The authors state that they ‘took stain diffusion into account’, but it is not explained how. Staining diffusion may have a significant impact. This should be clarified.

In cross-section images, diffuse staining is observed due to the targeting of biomarkers in cell cytoplasm before they are transported and anchored in their respective compartments, in our case, either the membrane or the cell nucleus. In our classification approach, this type of staining is represented by the E4 and P3 classes respectively in Ecad- and PR-stained cross-section images.

The authors use morphological filtering. The effect of morphological filtering in nuclei is sometimes aggressive (i.e. making irregular-shaped nuclei more circular, smaller etc). Thus, the values of morphological parameters that the authors determine (i.e. eccentricity) are affected. Since the authors extract interesting biological conclusions based on these morphological parameters, they should discuss the impact of morphological filtering in nuclei (does it alter the size and shape of nuclei?).

The sizes of morphological filters used in our method are 0.85µm and 0.52µm, and are much smaller than a typical cell nucleus with a diameter of 10-15µm. In our method, the smaller DNA spots with cross-sectional areas no larger than ~40µm² that can potentially be altered geometrically by these structuring elements are presumed to be of cytoplasmic origin and eliminated from the analysis. For viable nuclei, the morphological operations are therefore not expected to alter the geometry to a discernable extent (see page 10 paragraph 2).

Minor Considerations and Revisions: None