Reviewer Report

Title: A methodological approach to correlate tumor heterogeneity with drug distribution profile in mass spectrometry imaging (MSI) data

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Reviewer Comments to Author:

This is an interesting paper addressing how MSI can be effectively used to better understand the link between drug and the characteristics of malignant tissue. While it is not surprising that the physicochemical properties of both the tissue and the drug are important to passive tumor penetration and local exposure, MSI provides an important opportunity to understand the spatial and temporal dynamics of this process, and the development of effective computational workflows is vital. A few questions/suggestions for the authors follow:

- To what extent can the most prominent histochemical changes occurring post-bevacizumab treatment be captured by m/z range and other experimental settings studied in this untargeted MSI experiment? Since it used a limited mass range, certain important changes (e.g., in cell-surface protein expression, lipid membrane composition, etc.) may not have been measured. The authors may have addressed this question in their cited previous work, but it would be helpful to provide some additional context.
- Since the focus of this paper is methodology, evaluation of the approach against known ground-truths is critical. In that regard, the efforts of the authors in developing a synthetic dataset and evaluating the methods on it is appreciated. There are a few ways that this assessment could be expanded to provide additional information about the robustness of the workflow. For example, Additional File 3 includes plots showing the synthetic data and some of its characteristics, but to what extent do the statistical properties of the synthetic data compare with those of real MSI datasets? The SL method was recommended, but how sensitive is it to the selection of the weight matrix? If it is sensitive, are there any recommendations for selecting the weight matrix based on data characteristics? When bridging to the experimental data, has the method been tested on MSI datasets (including synthetic ones) with available complementary ground-truth labeling to help evaluate the extent to which identified clusters map to known differences? It is mentioned that peaks that were present in less than 20% of the tissue were removed to focus on more common ions. To place this 20% cutoff in context, what was the coverage area of the clusters identified? It seems possible that this step may omit significant portions of tissue heterogeneity. For future applications of this workflow, how should this cutoff threshold be selected? Overall, how robust are the results/workflow recommendations to the choices of distance metric and clustering index?
- Given the dose of the drug administered, how much exposure within the tumor is expected based on pharmacology, and how might this affect the output? It would also help to provide more explanation of Figure S1 in Additional File 7. In it, the concentration (units undefined) of the drug in each cluster appears to be very similar, across both cell lines and treatment arms; however, the comparison between

the clusters and the LISA maps appears to suggest differently. Also, interpretation of the LISA maps of drug exposure in Figure 3 and the data in Table 1 in terms of histology is briefly in the Discussion, but it would be helpful to expand on this.

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