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Spatial Probabilistic Mapping of Biomolecular Ensembles in Tissue via Mass Spectrometry Imaging

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Tissues are a major focus of clinical research and histopathological diagnosis for a wide range of diseases. Understanding the complex biomolecular manifestations of disease within tissues by characterizing its morphology and biomolecular information content paves the way for exploring the fundamental mechanisms of pathogenesis and for identifying diagnostic and prognostic biomarkers and potential therapeutic targets. Among the many tissue-investigation techniques, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has evolved into a label-free core technology for visualization and spatially-resolved *ex vivo* analysis of biomolecules directly from tissue samples.

Ion images, i.e. false color renderings of mass-to-charge ratio (*m/z*) intervals of interest, are used as the fundamental investigation tool in MALDI-MSI for conveying the spatial distribution of molecules-of-interest (MOIs, e.g., metabolites, drugs, lipids or proteins) within biological tissues that are often compared to external histopathology annotations. They are considered as the gold-standard by MSI researchers against which the biomarker discovery methods are validated. However, the conversion of raw MSI data into ion images for visualization, spatial interpretation and molecular analysis, has not changed since the inception of the technology. Moreover, the generated ion images can be prone to technical artifacts, user input- and user perception-bias.

This work introduces a computational framework, *moleculaR*, which proposes a coherent spatial probabilistic approach for mapping tissue MOIs and allowing for a user-independent spatial visualization and interpretation of MOIs' distribution in tissue samples via MSI. *moleculaR* uses user-independent assignment of *m/z* intervals for capturing MOIs based on the device- and measurement-dependent mass resolving power along with Gaussian-weighting of observed peak intensities for improved reliability of metabolite/lipid/drug signals in MSI. Instead of relying on a subjective qualitative judgment of the end-user concerning the observed spatial distribution of an MOI within a tissue sample, *moleculaR* proposes molecular probabilistic maps (MPMs), which apply pixel-wise spatial significance testing of MOI intensities against a complete spatial randomness (CSR) model inferred from the signal intensities of that same MOI. The framework also allows for spatial statistical comparisons of different tissues (cross-tissue MPMs, or CT-MPMs) and for collective projections of metabolite ensembles onto a single tissue plane, followed by computation of collective projection probabilistic maps (CPPMs). Ultimately, computed "hotspot" and "coldspot" spatial contours provide user-independent and probabilistic localization of tissue areas where an MOI has a statistically significant non-random relative spatial abundance or deficiency, respectively. Furthermore, this work extends the above concept to spatial quantitative mapping in tissues based on drug dilution series proposing a generalized nonlinear calibration model as a replacement for the traditional linear model that could better model drug-intensity response in the presence of noise and technical variability.

The framework has been tested and validated on data acquired from various MALDI-MSI instrument platforms featuring different tissue samples including isocitrate dehydrogenase-wild type (IDH-WT) glioblastoma (GB), IDH-WT and -mutant glioma, gastrointestinal stromal tumor (GIST), wild-type mouse brain, porcine liver and APP NL-G-F Alzheimer's disease mouse model tissue samples in addition to simulation-based experiments mimicking a MALDI-MSI ground-truth which have been developed and utilized to test the proposed workflows. The results highlight *moleculaR*'s capabilities of i) improving MOI signal reliability, ii) providing objective and data-driven designations of areas which exhibit statistically significant non-random spatial patterns of MOI intensities independent of how an end-user may perceive its (i.e. MOI's) spatial relative abundance or deficiency and iii) enabling spatially-resolved investigation of ion milieus, lipid remodeling pathways or complex scores like the adenylate energy charge within the

same image. On the other hand, spatial quantitative mapping based on generalized nonlinear calibration and cross-tissue probabilistic mapping can be used to provide valuable insight into drug-tissue penetration. In particular, spatial quantitative mapping of the drug imatinib in a cohort of GIST tissue samples revealed striking inefficiency in imatinib penetration into GIST liver metastases, despite the abundant imatinib levels beyond the limit of quantification (LOQ) observed within the corresponding healthy liver tissues surrounding the metastatic GIST.

In conclusion, the results suggest that *moleculaR*, with its core concept of spatial probabilistic mapping of biomolecules in tissues, shall replace or complement ion images for the spatial analysis of MOIs because of its valuable benefit of enabling probabilistic localization of non-random patterns of MOI signal intensities and shall further foster the role of MALDI-MSI as a valuable technique for investigating the spatial distribution of biomolecules and drugs in tissue samples. *moleculaR* has been made available for the scientific community as an open-source R package.