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Leiden
The Netherlands

Engineering a metabolomics workflow to capture single-cell diversity in 3D models

Hetzel, L.A.

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Appendix

Summary

Nederlandse samenvatting

Curriculum vitae

List of publications

Acknowledgements

Summary

Cellular heterogeneity plays a decisive role in disease progression, therapeutic response, and the emergence of drug-resistant subpopulations. Yet, its functional biochemical basis often remains unresolved because current analytical strategies either average away single-cell diversity or lack the contextual relevance needed to connect metabolic phenotypes to biological behavior. This thesis addresses these limitations by engineering an end-to-end single-cell metabolomics workflow capable of capturing biochemical diversity in biologically relevant 3D environments. The workflow integrates computational innovations, advances in gas-phase separation, and microfluidic organ-on-chip technologies to expand the depth, robustness, and contextual relevance of single-cell metabolic measurements.

Chapter I provides the conceptual foundation of the thesis. It highlights the consequences of cellular heterogeneity in clinical and biological settings—particularly in cancer, where metabolic plasticity enables distinct subpopulations to survive therapeutic stress. The chapter outlines how conventional bulk assays and 2D culture systems obscure these metabolically divergent cells and argues for methods that measure functional phenotypes at single-cell resolution. Single-cell metabolomics (SCM) is introduced as a uniquely powerful tool because it captures what cells *are doing* biochemically, rather than only what they *could do* based on gene or protein expression. The chapter concludes by motivating the need for workflows that not only perform SCM robustly, but also maintain fidelity when applied to physiologically relevant 3D models.

Chapter II addresses one of the central bottlenecks in single-cell metabolomics: the absence of computational workflows capable of handling the noise, missingness, and complexity inherent in untargeted single-cell MS datasets. To overcome this, the chapter introduces **MeDUSA**, an R-based platform that performs data extraction, quality

assessment, filtering, normalization, imputation, and statistical analysis in a modular and biologically informed manner. The pipeline implements mass-defect filtering, intensity-based selection, and cell-specific quality checks to remove exogenous contaminants while preserving true biological heterogeneity. A suite of statistical tools—from PCA and T-tests to machine learning—enables untargeted biomarker discovery and annotation via HMDB and LipidMaps. MeDUSA serves as the computational backbone for all experimental chapters that follow.

Chapter III focuses on the instrumental limitations of SCM, particularly the challenge of achieving adequate sensitivity and metabolic coverage in samples with extremely low analyte abundance. This chapter establishes a new analytical workflow integrating **High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS)** with live single-cell high-resolution MS. By leveraging orthogonal gas-phase filtering, FAIMS suppresses background noise, increases selectivity, and enhances detection of biologically relevant metabolites. Systematic evaluation across compensation voltages (CVs) revealed strong CV-dependent differences in detectable features and demonstrated that no single CV captures the full metabolic landscape. When applied to single-cell lysates, FAIMS expanded metabolite coverage and improved mass accuracy, revealing metabolic features otherwise obscured in non-FAIMS measurements. Together with the computational tools of Chapter II, this chapter forms the technical foundation required for analyzing metabolic heterogeneity in 3D biological systems.

Chapter IV applies the integrated single-cell workflow to a vascularized 3D organ-on-chip model to explore how cellular heterogeneity influences the uptake of tamoxifen, a clinically relevant anti-cancer drug. The introduction of extracellular matrix (ECM) components required adaptations in sample preparation to reduce viscosity and maintain measurement stability. Chemical background noise in direct-infusion MS further necessitated FAIMS filtering, which substantially improved signal detection. Using live single-cell microsampling, the study reveals how individual cells in a 3D architecture vary in drug uptake, emphasizing the

importance of microenvironmental gradients and structural context. This chapter demonstrates the transformative potential of combining 3D models with single-cell metabolomics to study drug response heterogeneity.

Chapter V broadens the scope by reviewing the expanding interface between microfluidics and 3D biological models, with a particular focus on organoids and spheroids. The chapter analyzes how device architecture, fabrication materials, flow profiles, and trapping mechanisms shape the suitability of microfluidic systems for different biological applications. It discusses the advantages and limitations of PDMS, polymers, glass, and emerging manufacturing strategies such as 3D printing. By comparing design trade-offs—including molecule absorption, imaging compatibility, and cell-recovery strategies—this chapter clarifies how microfluidic platforms can be optimized to support advanced metabolomics workflows, including those developed in this thesis.

Chapter VI synthesizes the overall findings and discusses future challenges. The thesis demonstrates that a functional, scalable, and context-aware SCM workflow is achievable when computational rigor, instrumental enhancement, and micro physiological modelling are combined. At the same time, additional complexities remain: co-culture systems will require new sampling strategies to accommodate cells of different sizes, the integration of imaging-based single-cell modalities will demand further computational harmonization, and higher-throughput sampling techniques—such as segmented flow—may be necessary to increase scalability. Nonetheless, the platform established here provides a robust foundation for future single-cell studies in increasingly complex biological environments.

Overall, this thesis presents a unified framework for studying functional metabolic heterogeneity from the level of computational pre-processing to live single-cell sampling in 3D models. By expanding both the depth and contextual relevance of single-cell metabolomics, it supports a future in which metabolic phenotypes play a central role in understanding disease progression and guiding therapeutic strategies.