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

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Chapter I

General introduction and scope

Clinical Implications of cellular heterogeneity

Cellular heterogeneity and its implications in biological and clinical settings are critical frontiers in modern biomedical research, especially in physiologically relevant contexts[1,2]. Generally, there is a lack of understanding of the full impact of cellular heterogeneity. This is despite current research showing that it affects treatment outcome in diseases such as cancer where metabolically heterogeneous ecosystem cell populations possess different drug susceptibilities and proliferative behaviors[3]. Furthermore, the heterogeneity is further exacerbated by tumor metabolic plasticity that enables cancer cells to adapt their biochemical pathways in response to environmental pressures, nutrient availability, and therapeutic stress[4]. Despite the stated impact of cellular heterogeneity, conventional bulk-level analytical approaches and two-dimensional (2D) cell culture systems still represent the majority of techniques used to study biological systems. Bulk methods average across entire cell populations, which masks the metabolic phenotypic variability of single cells[5]. This in turn shrouds the mechanisms of differential drug response and disease progression, concealing clinically relevant subpopulations, such as drug-resistant cells or cancer stem cells, that drive treatment failure[6]. On the other hand, 2D cell culture systems fundamentally do not represent the physiology of the disease[7]. Therefore, they do not capture the spatial-temporal dynamics and microenvironment gradients that are characteristic to the tumor microenvironment[7–10]. These two factors combined represent a substantial barrier to advancing precision medicine. While recent technological advances have enabled the profiling of cellular heterogeneity at the genomic and transcriptomic levels through single-cell RNA sequencing, the functional metabolic phenotype remains largely inaccessible at the single-cell resolution in 3D biological contexts. This gap between genomic knowledge and functional readouts has profound implications for drug development and patient stratification, as metabolic heterogeneity frequently drives phenotypic diversity and therapeutic resistance independent of genetic variation[11–13].

Single-cell metabolomics

A potential way forward is single-cell metabolomics (SCM) where the metabolic fingerprint of each cell is mapped out. Therefore, SCM presents a

direct readout of how the cell adapts, differentiates and responds to stress via changes in its biochemistry. This becomes especially relevant in cancer, where metabolic reprogramming is a cornerstone of tumor progression and therapy resistance. For example, cancer subpopulations can switch between glycolysis and oxidative metabolism based on nutrient access, drug exposure, or the surrounding microenvironment conditions. These transitions determine cellular survival and response to drugs. Furthermore, at single-cell resolution, SCM can show how cells vary in drug metabolism and uptake, which directly influence drug sensitivity. Finally, SCM empowers discovery of functional biomarkers that classify cells based on what they do, not only what genes they carry as well as identify possible biomarkers for rare subpopulations, which will improve drug efficacy. In a nutshell, SCM will help not only in defining heterogeneity, but translating this heterogeneity into practical, clinically actionable insights that can be used in future, more effective treatments.

Current technology for single cell metabolomics

Currently, mass spectrometry remains the gold standard for SCM due to its high biochemical resolution and sensitivity. It has been used successfully in several SCM studies to reveal hidden subpopulations in cancers, identify the unique metabolic profile of circulating tumor cells, and for single-cell drug monitoring.
However, despite these advancements, current technologies either prioritize spatial resolution or throughput over physiological relevance. For example, mass spectrometry imaging (MSI) based methods such as MALDI, while possessing high throughput capabilities, comes at the expense of perturbations to the cells being measured. This is due to its dependence on extensive sample preparation that also precludes measurements in native envrionments, i.e., cell culture media. While MALDI can generate excellent static chemical snapshots, it comes at the expense of destroying the model in question, as well as altering its metabolome. This precludes any possibility of studying metabo-temporal dynamics in a cellular context, let alone studying it in a relevant 3D model due to the sample prepartion needed[14]. Other MSI techniques empasize resolution instead of throughput such as Secondary Ion Mass Spectrometry (SIMS). SIMS offers high subcellular spatial resolution (down to ~50 nm). However, due to fragmentation occurs during energy deposition into the

sample, which limits the coverage of intact bioactive molecules needed for robust phenotyping. It also shares the same limitations with MALDI when it comes to the extensive sample preparation needed in addition to sampling in vacuum in the case of SIMS. Consequently, both approaches generate terminal endpoints of analysis. Therefore, they fail to capture the rapid turnover rates and metabolic fluxes. Furthermore, both approaches are incapable of measuring cells embedded in 3D matrices without extensive sample preparation such as fixing and sectioning the model[15].

In order to address this, an improved analytical workflow is needed that moves beyond profiling at the cost of altering the metabolome and destroying the model in the process. On that front, methods that work in ambient conditions such as, Live Single-Cell Mass Spectrometry, nano-DESI, and the Single /T-probe have emerged to allow for *in situ* sampling and subseuqent analysis with high sensitivity[16]. Live Single-Cell Mass Spectrometry, pioneered by Masujima et al., directly aspirates intracellular content via a capillary under microscopic observation to link cellular morphology with metabolic snapshots. Using this method, researchers could successfully identify metabolic profiles of circulating tumor cells in patients' blood, investigate cancer drug uptake heterogeneity, as well as integration with other analytical modalities. However, while this technique provides a snapshot into cellular behavior, it might induce stress response in cells due to extensive manipulation prior to the sampling process.[17] Alternatively, the Single and T-probes attempt to provide a dynamic picture of the cellular metabolome. This is achieved via continuous pico-liters sub-cellular sampling using an offline, or in-line probes embedded into the cell in question. Using a co-axial dual bore capillary, this method enables microextraction and in-situ metabolic analysis under ambient conditions with even less disruption to the cell in question. However, the small bore size also makes it more liable to clogging when sampling from complicated 3D matrices, and the dynamic sampling comes at the cost of sacrificing throughput. Furthermore, with all the aforementioned techniques, metabolic coverage remains an issue. This is largely due to the minute sample volumes involved.

Assuming that the issue with metabolic coverage can be addressed, the translation of SCM-MS measurements will still be hindered by the lack of

computational methods to process single-cell datasets. Standard bulk-based data processing pipelines assume normality, low degree of missingness, Gaussian noise distributions, and some degree of quality control. All are missing in untargeted SCM-MS datasets. While some separation based pipelines such as XCMS, Metaboanalyst, and Mzmine or imaging based tools such as Metaspace can be adapted for direct infusion,[18] they still lack the tools needed to handle the high degree of missingness and noise generated from single-cell experiments. The final challenge standing in the way of translating SCM-MS experiments into biological insights lies in adapting the current technologies, wet and dry, to be adapted to complex 3D models like spheroids and organoids that faithfully recapitulate the *in vivo* environment[19]. Live SCM has the potential to study the interactions between individual cells in 3D context, but technology is not ready yet. There are three technological breakthroughs that are needed: **First**, novel data processing pipelines that are specifically tailored for single-cell metabolomics (SCM). **Second**, improved SCM methods with higher sensitivity and coverage for better biological translatability. **Finally**, the physical integration of SCM methods with 3D culture models.

Scope and outline of the thesis

The underlying idea of this thesis is that **the lack of methods capable of studying SCM in 3D context hinders the use and impact of single cell studies in clinical studies**. In order to address this, an improved end-to-end single-cell analytical workflow that can analyze a wide range of bioactive molecules as well as drugs in relevant biological/clinical conditions is needed. Therefore, the aim of this thesis is to develop such an analytical workflow, first by establishing the data processing and analysis pipeline needed to process single-cell data. Then by improving the sensitivity and coverage of current methods to improve the interpretability of the data. Finally, by applying the developed methods to investigate the impact of single-cell heterogeneity on anti-cancer drug uptake in a biologically relevant vascularized 3D organ-on-chip model. The standard live single-cell metabolomic workflow is compared to the improvements proposed in this thesis in Figure 1.

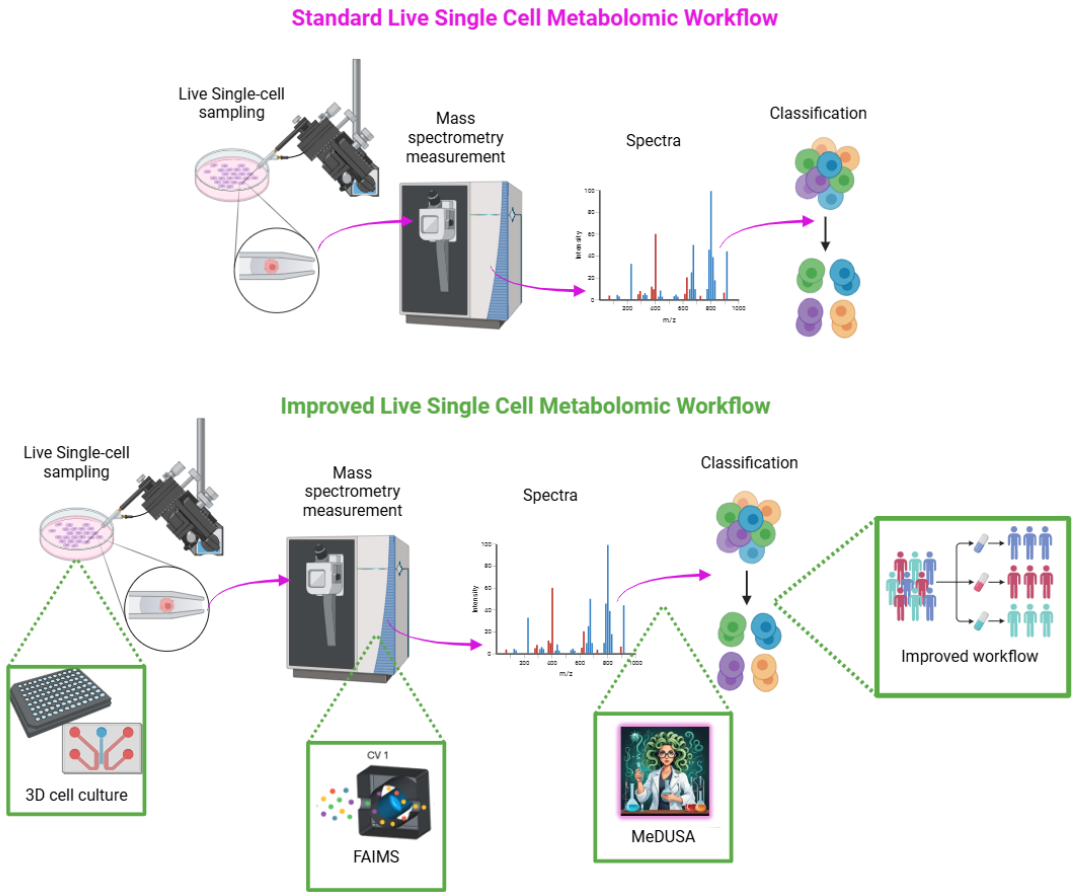

Figure 1 Comparison of the standard live single-cell metabolomic workflow (top) to the improved analytical techniques proposed in this thesis (bottom). Each green box highlights a proposed improvement and where it belongs in the process. The last box highlights the improvement to diagnostics and therapeutic analysis.

Chapter II aims to mitigate the current bottlenecks in single-cell metabolomics data processing and analysis, especially the lack of suitable computational pipelines and methods that can handle the unprecedented degree of noise, missingness, and dimensionality associated with single-cell datasets. The aim of such a computational pipeline should be to preserve the biochemical uniqueness of single cells due to their inherent heterogeneity, while removing as many biologically irrelevant signals as possible in an untargeted manner. Therefore, steps such as quality checks,

and filtering should be emphasized in the pipeline. In addition, such a pipeline should enable untargeted biomarker discovery using univariate, multivariate, or machine-learning methods. The **computational** platform developed in **Chapter II** will be used to analyze the single-cell datasets generated in **Chapters III and IV**, as well as any other studies that generate similar single-cell datasets.

Chapter III aims to improve metabolic coverage to boost biomarker discovery. The aim is to develop a novel untargeted single-cell metabolomics workflow by integrating High-field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) with live single-cell high-resolution mass spectrometry. The primary focus will be on leveraging the orthogonal gas-phase filtering by FAIMS to improve selectivity, suppress chemical background noise, and consequently, increase both the sensitivity and the depth of metabolite coverage. This chapter will first investigate how to adapt the current sampling and sample preparation of live single-cell MS to FAIMS, and how to optimize various FAIMS parameters to improve sensitivity and metabolomic coverage of SCM. The data acquisition platform developed in Chapter III will be used in Chapter IV for SCM in 3D cell systems.

Chapter IV aims to extend the SCM platform to live sampling of individual cells in a 3D in-vitro model for imaging guided precision sampling, and specifically, from a high throughput microfluidic model using a 384 well plate format. The aim is to adapt the microfluidic platform to allow live sampling, and to overcome challenges like extracellular matrix (ECM) contamination. The suitability of the developed method will be validated quantifying anti-cancer drug uptake heterogeneity in hepatocellular carcinoma cells under a drug-induced gradient.

Chapter V aims to provide a comprehensive review of the use of microfluidic devices in the field of organoids. Since the physiological relevance of SCM studies is reliant on the model system, **Chapter V** establishes best practices for organoid cultivation and their maximal utilization in assays. The review aims to provide an overview of challenges in applying conventional assays to organoids and how microfluidic devices may overcome these issues. This chapter will provide a guiding framework on the options and considerations for device selection and

development based on the specific application and developmental stage of the organoid model.

In **chapter VI** the main findings of this thesis are summarized and discussed. The developed analytical and computational workflow are evaluated, and further possible improvements discussed such as integration with other analytical modalities, overall throughput, and data fusion with multiomics and fluxomics, and future perspectives of SCM for disease monitoring and drug discovery are provided.

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