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

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Chapter VI

Conclusions and Perspectives

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General Conclusions

The central aim of this thesis was to develop a comprehensive and context-aware workflow capable of capturing functional metabolic heterogeneity at the single-cell level, particularly within biologically relevant 3D environments. This level of analysis enables the identification of multiple cell types in environments which were assumed to have only one type. The improved diagnostic and therapeutic evaluation capabilities can ultimately lead to more efficient patient diagnosis and treatment.[1,2] The development of the workflow required advancing three interconnected pillars of single-cell metabolomics (computation, instrumentation, and biological application) and integrating them into a unified platform that can resolve individual biochemical cellular behavior in experimental environments that more accurately reflect their native physiology.

Together, the chapters of this thesis form a cohesive framework that advances single-cell metabolomics toward truly functional, context-dependent measurements. By aligning computational algorithms tailored for SCM, improved chemical sensitivity, and physiologically realistic models, this thesis lays the groundwork for future studies aiming to decode how single-cell diversity drives disease progression and therapeutic outcomes in complex biological systems.

To establish a robust computational foundation, **Chapter II** introduced MeDUSA, a framework that enables single-cell datasets to be processed in a way that preserves biological diversity as much as possible while minimizing noise and artefacts. One of the major goals was to create a start-to-finish package that was designed for scientists, not statisticians. To ensure the package was versatile for scientists not necessarily proficient in R, functions had to be interchangeable, modular and include user-defined parameters.

The R package is divided into five main sections: data extraction, filtering, processing, statistics, and annotation. Each section contains “magic” functions that allow the user to perform multiple tasks in one step, which is

beneficial for a starting point of the analysis and for scientists new to metabolomics analysis. The functions maintain a consistent output format which is the expected input of most functions, making the package modular and easy for users to customize. The data extraction includes peak smoothing, centroiding and condensation of the scans over time to a single intensity per mass for the measurement. If the user decides to implement another algorithm besides the default binning for alignment, as long as the output remains the same format, other algorithms will work easily with all other functions in MeDUSA. After the data set has been filtered so only proper cellular peaks remain, the data is prepared for statistics with normalization, imputation, and log functions. Multiple statistical tests are available in the package so the user can choose which best suits their data. The output of the statistical analyses can then be input into the function comparing *m*/*z* values in the measurement to HMDB and LipidMaps for identification.

While MeDUSA addressed a significant gap in the field of single-cell computation, it could benefit from some optimization in future versions. In the pre-processing, the data extraction and aligning peaks functions are quite computationally heavy, therefore potentially causing the system to crash. It is advised to reduce the computation load through optimizing with other packages, changing the order of processing functions, or eliminating the need for the work to be performed in a Docker container. Furthermore, in its current implementation, MeDUSA is not compatible with MS/MS datasets. To achieve this, parts of the pipeline can be adapted and incorporated into existing tools for processing MS/MS datasets. Finally, the default parameters for the various functions in MeDUSA have been chosen based on high resolution orbitrap single cell datasets. Therefore, users of other instrumentation should adapt the parameters if needed.

The field of single-cell metabolomics continues to advance with new analytical techniques. With each analytical advancement, MeDUSA remains a strong base, but does require either additional or amended functions that can be easily fit into the package due to the modularity. For example, Chapter III of this thesis introduces high field asymmetric ion mobility spectrometry (FAIMS) as an analytical tool. MeDUSA currently

does not consider the output of this tool and how to incorporate it into the workflow. While the base functions of MeDUSA remain useful, the advantage of an end-to-end pipeline would be lost. Additionally, as segmented flow becomes a viable option for single-cell metabolomics, it is clear that MeDUSA lacks the functions to separate the signals of individual cells in one continuous measurement. Finally, there have been many advancements in single-cell omics with imaging, such as MALDI developments, and MeDUSA has not been assessed for potential integration with this type of data, however, integration of imaging data could be a future expansion.

Building on the computational base, **Chapter III** tackled the technical limitations that restrict metabolic coverage and sensitivity when analyzing the content of individual cells with high field asymmetric ion mobility spectrometry (FAIMS). We developed a novel workflow to combine FAIMS with direct-injection mass spectrometry (DIMS). The workflow consists of offline cellular lysis and FAIMS mass spectrometry (FAIMS-MS) acquisition method recommendations. The impact of the compensation voltage (CV) on detected peaks was critical to proposing an untargeted acquisition method. To understand the implications of varying CV on the metabolic coverage in an untargeted approach, both standards and single-cell lysis were individually measured covering a mass range from 50 to 1100 *m*/*z* with multiple scan range windows and varying CV.

For many standard compounds, non-FAIMS measurements produced higher absolute intensities. Notably, for half of the compounds, the CV that yielded the highest intensity differed from that producing the highest S/N. For the single-cell measurements, a global comparison of detected features across mass ranges showed that FAIMS and non-FAIMS measurements exhibited similar overall trends. Examining feature counts across 11 CVs demonstrated that no single CV resulted in a highest S/N for all, or even most compounds. Individual *m*/*z* windows further emphasized CV-dependent variation, with certain mass ranges favoring specific CVs in each ionization mode. The extent of metabolic coverage was evaluated by comparing annotations generated with and without FAIMS across multiple filtering stages. Notably, FAIMS initially yielded substantially more unique

annotations, but stringent filtering reduced these totals, ultimately resulting in limited overlap between the two methods.

The study evaluated the effects of FAIMS and CVs on signal intensity, signal-to-noise ratio S/N, and metabolic coverage. The general decrease in signal intensity with FAIMS was expected due to changes in ion transmission. However, improvements in S/N were observed in only a few compounds, and no single CV provided optimal performance for all.

It was expected that an intact cell could be injected and would lyse as a result of the voltage from the ionization source, as this was the general practice for single-cell measurements. It was surprising that the electrical field of FAIMS somehow interfered with this. If the proposed sample preparation induces too much dilution or sample loss in a specific application, future studies could investigate the underlying cause of the intact cell not generating a spray and optimize the protocol to mitigate the effect.

Comparisons of datasets with and without FAIMS showed that FAIMS reduced noise but also increased missingness after filtering. Since FAIMS is largely advertised for its ability to reduce chemical noise, the reduction itself was not surprising, however, we expected to see a more dramatic improvement in the S/N. Despite this, FAIMS and no-FAIMS methods detected largely different subsets of biologically relevant features, with surprisingly limited overlap between them.

The study in thesis serves as a starting point for implementing FAIMS in single-cell metabolomics. Further investigations should explore smaller CV increments, electrode temperature settings, and carrier gases. Additionally, potential confounding factors outside of the instrument should also be considered, such as ionization solvent. Further computational analysis on the measured data could reveal patterns in the effect of FAIMS (specifically the CV) on classes of metabolites, and which adducts were the most common. The annotations were made with the common, assumed adducts.

In proteomics, FAIMS is used with both data independent and data dependent acquisition (DIA and DDA, respectively).[3] Preliminary

metabolomic studies benefit from the broad analysis of untargeted, evaluating a large mass range. However, biological studies require a deeper, quantitative, and confident analysis, which could be provided with DIA and DDA MS^2 analysis. Since FAIMS provided a nearly unique dataset, it stands to reason that the fragmentation of DIA and DDA analyses would also be unique, thereby providing a higher degree of confidence in the annotation, and potentially limiting the false positives.

The study in this thesis tested FAIMS coupled with only one mass spectrometer and ionization source. In order to make global assertions about the use of FAIMS for single-cell metabolomics, other ionization techniques should be considered. For instance, nanospray desorption electrospray ionization (nano-DESI) can be used for single-cell analysis,[4] and it would be interesting to investigate the modifications needed to incorporate FAIMS. MALDI is also a technology that is continuously used for single-cell metabolomics. FAIMS could be evaluated with MALDI, exploring the effect of the matrix on the measurement as well as the CV. Additionally, FAIMS has the potential to isolate isomers, therefore it may be possible to add confidence to the MALDI annotations as opposed to increasing the coverage. In this thesis, we have recommended cycling through CVs with stitching in the acquisition method, however, our recommendation was based on an ambient ion source that provides a constant stream of ions. The acquisition method would have to be modified to accommodate the pulsed supply of ions in MALDI. Just as FAIMS was first developed for and validated with single-cell proteomics, TIMS-ToF has shown promising results in advancing single-cell proteomics.[5,6] As TIMS-ToF also utilizes ion mobility to separate the analytes, it would be interesting to compare the coverage of TIMS-ToF with FAIMS for single-cell metabolomics.

The analytical and computational workflow developed in Chapters II and III were applied in **Chapter IV** as an integrated workflow to a vascularized 3D organ-on-chip model. The goal was to use a high throughput microfluidic 3D organ-on-a-chip platform that is compatible with robotic workflows. To make such a chip with a 384 well plate format compatible for the microsampling of individual cells, several modifications to the

hardware had to be made to allow the use of a micropipette for sampling from the inlets within individual wells. The modifications and developed workflow were evaluated with a relatively simple biological application. The novel workflow including a new sample preparation protocol allowed us to preserve, in principle, the dynamic properties of the 3D microenvironment through live microscopy while performing comprehensive single-cell biochemical measurements, thereby enabling a more accurate investigation of tumor heterogeneity and therapeutic response.

With modifications to the chip in place, a drug gradient was established. Measurements taken during 24 hours of drug perfusion confirmed that the applied drug gradient remained. Following gradient validation, cells were sampled and analyzed from the 3D model. To maximize signal-to-noise and enable detection of tamoxifen and its metabolite in the diluted lysates, FAIMS was incorporated into the workflow. Accurate mass measurements, isotopic patterns, and MS/MS fragmentation confirmed the identities of the compounds. To refine spatial correlation, each cell's precise position within the chip was recorded by imaging at the time of sampling, and tamoxifen abundances were mapped onto these coordinates, revealing a clear gradient in single-cell drug uptake.

Gradients are fundamental drivers of biological behavior and they contribute to substantial heterogeneity in cellular responses. By exposing cells to a controlled drug gradient, our approach enabled direct measurement of concentration-dependent uptake at the single-cell level. Consistent with expectations, cells nearest the drug source exhibited higher tamoxifen abundance than those farther away; however, the measured intensities did not linearly mirror the gradient, reflecting inherent heterogeneity in cellular uptake likely arising from differences in transporter expression and intracellular metabolism. These findings underscore the necessity of single-cell analytical technologies to accurately capture drug response distributions across 3D environments.

The complexity of matrices such as Matrigel introduces analytical challenges, as its rich protein composition obscures mass spectrometric

detection. Mass spectrometry methods that utilize a different ionization source, such as REIMS, did not suffer as much of a detrimental effect of the matrix, as they often measure the signal due to the matrix next to the cells. While other platforms did not target tamoxifen specifically to know definitively if Matrigel interferes, we found it surprising that the tamoxifen metabolites could not be detected through any residual ECM without the use of FAIMS.

While the study discussed in this thesis increased the complexity of the model from traditional 2D single-cell models, it lacked several biological assets that would undoubtedly contribute to analytical complications. Introducing multiple cell types would increase the biological authenticity of the model, however, different cell types have different sizes and shapes. The size difference could warrant the use of different size capillaries, and this could subsequently negatively impact the downstream data analysis. Additionally, while great care is taken to capture only one cell in the capillary, capturing two different cell types would render the sample obsolete as the metabolic profile could not be attributed to an individual phenotype. Theoretically, the platform is suitable for a co-culture, however, optimization would be necessary, and modifications would be expected.

Another consideration for the platform proposed is the throughput. Live single-cell sampling has several options for automation[7], however, each automation option has stringent requirements. The specialization of the techniques would require further modification of either the sampling platform or the chip discussed in this thesis. The modifications may be worth the effort though, as then the sample preparation could be performed online, decreasing time and sample loss. A segmented flow single-cell sampling platform could be the most promising option, requiring the least amount of modification to each platform to enable a successful coupling.

Finally, **Chapter V** positioned this workflow within the broader landscape of organoid and microfluidic technologies, outlining the design considerations that will guide future biological applications.

A portion of the chapter highlighted how the intended application of a microfluidic platform shaped every aspect of its design. Despite sharing foundational design principles, devices required distinct features depending on whether they were used to nurture organoids from single cells, manipulate preformed structures, or incorporate additional cell types. Fluid flow remained a central requirement, both to deliver nutrients and to impose physiologically relevant shear stresses, but the specific flow profiles and channel geometries varied widely. Some applications demanded precise control over organoid size, while others relied on features to sort or isolate organoids according to size. Certain devices emphasized the ability to recover organoids for downstream analysis, whereas others were optimized strictly for on-chip imaging, drug delivery, or exposure to chemical gradients. As organoids increasingly recapitulated patient heterogeneity and complex microenvironments, devices also incorporated co-cultures, controlled gradients, and specialized trapping strategies to enhance biological relevance.

Another section of the chapter emphasized that the fabrication of microfluidic devices was inherently constrained by the material properties and manufacturing capabilities available. The choice of substrate was dictated by the optical, chemical, and permeability requirements of the biological assay. In many cases, no single fabrication method was sufficient, prompting the integration of multiple materials or layered construction to accommodate complex features. Ultimately, the fabrication strategy had to balance optical requirements, chemical compatibility, geometric constraints, scalability, and downstream analytical workflows, leading to an increasingly diverse and sophisticated landscape of microfluidic platforms for organoid and spheroid research.

Many of the devices discussed were designed with formation of organoids as the primary objective. Since many studies require organoids to be relatively the same size, this is a valid objective, however, this often requires isolation which limits the signaling from other cells. This may be acceptable if the study is focused on the direct effect of a drug on the cells, however, the clinical relevancy is decreased as cellular signaling is a major component of the microenvironment influencing the metabolome and cell

behavior. For single-cell metabolomics, it is critical that the microfluidic device has an opening accessible to a microcapillary and imaging. Additionally, the metabolome is susceptible to even slight variations in the microenvironment, so features should support and facilitate a biologically relevant microenvironment and limit disruptions. The field of metabolomics can benefit greatly from automation, and single-cell specifically is enhanced with minimal sample loss and dilution. Microfluidic devices have the potential to incorporate sample preparation step on chip, eliminating the need for transfer.

Final Perspectives

As biological models become more complex to better mimic the *in vivo* conditions with multiple cell types, vasculature and 3D structures, complex matrices, and interstitial stresses, the analytical instrumentation and methodology must be tailored to have the ability to properly assess the models. Consequently, as the experimental landscape and measurement toolkit continue to mature, the data generated and the way we analyze it will also evolve. The work in this thesis was built on established live single-cell sampling and mass spectrometry of 2D cells in relatively simplistic microenvironments. First, the data analysis pipeline for the existing technology was developed. It was clear that biological relevancy would require 3D models, however, the instrumentation and related methodology advancements were established with the simplistic 2D models. Finally, the methodology could be optimized for and implemented in the more relatively simplistic 3D models. The future holds potential for this technology to be further optimized and developed for the more complex, clinically relevant models.

The methods developed in this thesis address some of the main bottlenecks in live SCM. However, they need to be contextualized within the broader computational and analytical landscapes. On the computational front, recent developments in MZmine 3 allowed for improved compatibility with multimodal mass spectrometry methods such as ion mobility, LC-MS, GC-MS, and MSI.[8] Furthermore, tools such as METASPACE enable

researchers to perform machine learning false discovery rate-controlled annotation which reduces the incidence of false positive hits during annotation.[9,10] However, despite these advances, neither tool are currently optimized for the extreme missingness, noise, and variability that are characteristic of single-cell direct infusion datasets as well as the lack of proper quality control samples. MeDUSA addresses these specific issues, albeit, with some limitations of its own as well. Improved future versions of MeDUSA could incorporate METASPACE isotopic-based algorithms for annotation to reduce FDR, as well as MZmine's visualization and statistical modules.

Moving beyond the computational realm, the methodological advances in chapters III & IV allowed for improved SCM coverage as well as the performing SCM in the context of 3D cell cultures. In the wider context of the SCM field, researchers showcased that LC-MS can be combined with capillary microsampling for live single-cell lipidomic analysis, resulting in improved selectivity and coverage[7,11] compared with the direct infusion-based methods employed in this thesis. Consequently, even more improvements in coverage can be gained if the FAIMS method can also be integrated with the aforementioned LC-MS method. Moreover, in contrast to the whole microsampling approach used throughout this method, other groups succeeded in repeated nano sampling of single cells followed by MS measurement which enables longitudinal studies on single cells. These advances can be incorporated in the workflows developed in this thesis to enable repeated SCM studies with high coverage in 3D models.

A critical aspect that deserves attention is the throughput of single-cell analysis technology. In order for large-scale, robust clinical studies to benefit from the hidden biochemical insights illuminated with single-cell metabolomics, the throughput must improve, likely with automation such as segmented flow. This advancement will certainly progress the field of single-cell metabolomics, but it will also have repercussions that trickle into measurement and data analysis methodologies. The data analysis pipeline will need to be capable of accurately deciphering between a cell and a buffer, and handling multiple cells in one measurement. This will also include programmatically processing two or more phenotypes in one

measurement. While code is being developed for targeted metabolomics, untargeted analyses with various phenotypes and different solvents will likely pose a greater challenge. A microfluidic platform is being developed simultaneously that utilizes segmented flow, so once both are validated, they will both potentially require modifications to integrate. FAIMS for single-cell metabolomics was not tested with any form of automation, so it is not clear if it will maintain the same performance. The oils used in segmented flow do not ionize, and therefore do not produce a mass spectrometry signal, however, this may require electrode cleaning steps. Optimization will determine if this can happen online and how often it should take place. If it cannot happen online, the robustness of the study may decline as the instrument requires calibration after cleaning. Aside from the instrumentation, the models themselves may need adaptation. The features contributing to the biological relevancy of the model must be maintained while also allowing for the sampling and imaging of the preferred automation technique.

This thesis discussed the use of FAIMS for single-cell metabolomics, however, multi-omics (proteomics, metabolomics) and fluxomic studies are gaining traction in biological evaluations.[12] After the evaluation of FAIMS for single-cell metabolomics in targeted and untargeted studies, it seems reasonable that the utility of FAIMS could extend to multi-omics and fluxomic studies as well due to its selectivity and coverage improvements. FAIMS development could offer significant payoffs for multi-omics and fluxomic single-cell platforms. While FAIMS for single-cell metabolomics followed similar trends as those seen in proteomics regarding effect of CV and distribution of peaks at various CVs, there was a greater percentage of data lost in metabolomics studies. The data presented in this thesis indicated that FAIMS and non-FAIMS measurements detected different peaks, ultimately generating unique data sets. Consequently, by alternating between FAIMS and non FAIMS the overall molecular coverage is increased for a single-cell measurement. Furthermore, in developing multi-omics and fluxomic methodologies, an evaluation of which combination of modes would be optimal is required. A proper evaluation would likely

have to be conducted in multiple stages to limit the number of variables, concluding with a large-scale validation of a complete pipeline.
For the advancement of drug discovery and disease prognosis, researchers are compelled to continuously adapt the analytical techniques. It is imperative that we all keep an open mind to the developments and advancements in the field. As 3D models, especially microfluidic devices, gain traction, it would be easy to limit ourselves to the technology that is currently capable of analyzing these samples. For example, mass spectrometry imaging techniques are well suited for microfluidic devices.[13] However, there are limitations and challenges. For instance, these techniques work best with a monolayer of cells, and as the models gain complexity, it becomes more difficult to justify a monolayer. Mass spectrometry imaging relies on surface measurements, requiring sectioning and realigning the data for samples with relatively considerable depth.[14] The techniques presented in this thesis are adaptable to accommodate complex 3D culture models. Additionally, techniques such as MALDI require the sample to be fixed, potentially limiting the biological applications or downstream analysis. While the tamoxifen study did not necessarily require the cells to be alive during sampling, the platform could be used in such instances that this is necessary, for example in migration studies. While the device used for the drug gradient study required some modification to be used with the sampling setup and FAIMS required some optimization to the target metabolites, the foundation has been laid to improve the biochemical insights in these complex models.

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