Universiteit Leiden
The Netherlands

Mapping the unseen to uncover the unknown: spatial analysis of neuromuscular disorders

Heezen, L.G.M.

Chapter 6

Discussion

Short summary of Chapters

The studies presented in this thesis focused on deepening our knowledge of the basic pathomechanisms in dystrophinopathies, its accompanying tissue alterations and the identification of molecular markers and cell types involved in these tissue alterations.

Over the past years, scRNA-seq has massively increased our understanding of tissue compositions, cellular interactions, and developmental processes. Especially in heterogeneous tissues such as the brain, this single-cell resolution led to the discovery of many cell subtypes. Mapping out the expression patterns of the *Dmd* gene and its isoforms throughout the central nervous system aids in getting a better understanding of cell types that might be affected by the lack of *Dmd* and the consequent clinical manifestations in patients suffering from a dystrophinopathy (**Chapter 2**). This mapping was done by adopting publicly available scRNA-seq datasets. Even though there are many benefits to using data produced by this technology, several challenges and future directions remain that will be discussed later on.

Besides understanding the pathomechanisms, there is a need for biomarkers for muscular dystrophies in general and dystrophinopathies specifically in this thesis. Biomarkers have, as discussed before, multiple functions amongst which the monitoring of early diagnosis and detection, disease progression and treatment response assessment. We discuss the potential use of DT-MRI metrics as a noninvasive biomarker in NMDs, by focusing on the potential use and validation in BMD patients in **Chapter 3**. Even though this study shows promising results on quantifying fiber size differences without the need of invasive muscle biopsies, some limitations and potential future developments will be discussed.

Finally, we introduced a new way to investigate the histopathological tissue changes in both murine (**Chapter 4**) and human (**Chapter 5**) skeletal muscle by using spatial transcriptomics as one of the first in the world to apply this technique to the skeletal muscle. It has opened up doors to map gene expression patterns in a spatial context of affected and non-affected skeletal muscle. To reveal gene transcripts and cell types involved in these histopathological changes and pinpoint molecular markers, which may be interesting therapeutic targets. However, the presented results also come with various discussion points. Not only on potential limitations within our study, but also a general discussion on the application of this new technique within the neuromuscular field and future directions to take.

6.1 BRAIN COMORBIDITIES IN DMD RESEARCH

While the focus of this thesis is on the muscle degeneration in dystrophinopathies and not on the brain comorbidities that DMD patients suffer from, there is great potential in a [1] better mapping of *DMD* expression and its function as well as the use of [2] spatial transcriptomics to study brain comorbidities in DMD research. These two research opportunities will thus be highlighted in this discussion.

6.1.1 Incomplete mapping of DMD expression and its function

The focus of most research on dystrophinopathies was on the muscle wasting characteristic of the disorders and not the cognitive defects that are present in a big percentage of the patient population. It is an often overlooked part of the disease, as it is not causative for the early death these patients suffer from. However, as stated in **Chapter 1**, the brain comorbidities are often perceived as more impactful on the quality of life by patients and their caretakers, compared to the limitation in mobility caused by the muscle degeneration (Hendriksen et al., 2020).

Luckily, there is more research nowadays on this aspect of the disease (Vaillend et al., 2025; Catapano et al., 2025). Researchers are for example looking into the lack of various dystrophin isoforms in mouse models and the effect this has on cognitive and behavioral processes (Verhaeg et al., 2024). These dystrophic mouse models, exhibiting some of the human cognitive and behavioral deficits, are being well phenotyped and for instance used for gene therapy studies (Van Putten et al., 2020; Vaillend, Billard & Laroche, 2004; Bellissimo et al., 2023; Saoudi et al., 2021). Restoring brain dystrophins, improves the behavioral deficits of the dystrophic mice (Sekiguschi et al., 2009; Goyenvalle et al., 2015).

These are promising trends within the field, yet, full understanding of *DMD* expression and the functions of all its isoforms within the brain remains limited. In **Chapter 2**, we used publicly available scRNA-seq data as well as newly gathered scRNA-seq data from dystrophic mouse brains to aid in a better mapping of the expression patterns of this gene and its isoforms throughout brain regions and over time. However, this data was not complete. The data obtained from the dystrophic mouse models was limited to the hippocampus and cortical area, whereas some of the affected domains (e.g. anxiety, executive functioning, depression) lie (partly) outside of these brain areas or would favor a more specific annotation (e.g. amygdala, basal ganglia, thalamus, prefrontal cortex,

subgenual anterior cingulate cortex) (Vaillend et al., 2025). Moreover, only one time point and a limited number of animals were included. It would thus be interesting to increase group size and age variation as well as the isolation of more specific brain regions to better map Dmd expression. While this mapping may seem as basic knowledge, it is a highly informative tool to increase our understanding of the functioning of different dystrophin isoforms in the brain. Expression is not random and is related to the function of the cell type (McKenzie et al., 2018). Deepening our knowledge on the cell types expressing dystrophin, and its related functioning or dysfunctioning when dystrophin is lost, can shine light on the cognitive impairments seen in dystrophinopathies and eventually aid in the utilization of proper neuropharmacology in this patient group.

6.1.2 The use of spatial transcriptomics on the DMD brain

An increased transcriptomic characterization of dystrophic mouse models and the expression of the dystrophin isoforms throughout the brain should not be limited to scRNA-seq or long-read RNA-seq. The field will greatly benefit from an spatial mapping of the expression patterns to overcome the issues presented in our single cell work and in the single cell field in general.

Affected domains and behavioral deficits DMD patients suffer from, such as anxiety and executive functioning, are not represented in the brain by a single neuronal cell type. Rather, these are complex behavioral functions exhibited by groups of cells, networks, throughout the brain. While scRNA-seq data is well-suited for expression mapping, tissues are deconstructed and networks within the brain would be disturbed. Spatial transcriptomics would allow for a spatial mapping of the *DMD* expression while retaining spatial context, allowing for more in-depth analyses of the dystrophin-expressing cell types such as cell-cell communication.

Recent advances within the spatial field allow for spatial isoform transcriptomics, which is a technology that was already utilized on mouse brain sections and thus limited optimization is needed to apply this to dystrophic mouse models as well (Lebrigand et al., 2023). Moreover, it would of course be of great interest to apply any spatial transcriptomics technology to brain tissue of a DMD patient. However, this tissue is very limited and difficult to obtain. When possible, it would be specifically of interest to select brain areas involved in working memory, executive functioning, intellectual functioning, anxiety, depression and reading such as the amygdala, hippocampus, cortical areas and the thalamus.

Until then, it could already be informative to spatially map *DMD* expression of control brain, adult and developmental, that is publicly available (e.g. Qian et al., 2025; Langseth et al., 2021). Given the speed of developments within this field, future work will most likely shed more light on the yet unanswered questions and knowledge gaps presented here.

6.2 WHAT UNSEEN WAS REVEALED BY SPATIAL MAPPING

In this thesis, we set out to better understand the histopathological tissue changes such as the muscle to fat transition, inflammation, calcification, fiber size differences and fibrotic tissue formation in dystrophinopathies. We sought to find potential biomarkers that may be of use to monitor disease progression, or the identification of molecular markers that underlie/are involved in these histopathological tissue changes.

6.2.1 Molecular mapping led to better understanding of the pathophysiology

In **Chapters 4 and 5**, we analyzed dystrophic (and healthy) skeletal muscle biopsies, both murine and human. Various genes have been identified that are involved in disease progression and specific histopathological tissue changes such as fibrosis in de D2-*mdx* mouse (*Vim*, *Fn1*, *Thbs4*) or fat accumulation in boys suffering from BMD and DMD (*PLIN1*, *LPL*, *PLIN4*, *SCD*, *ADIPOQ*) (Heezen et al., 2023; Heezen et al., 2025).

Further validation of these genes and their potential use as targets for future therapies should be studied further in functional tests as well as the validation of these markers across different (spatial) platforms. Moreover, besides genes, cell types involved in disease progression such as fibroadipogenic progenitor cells have been identified as well. We have revealed how different cells communicate with each other to drive muscle degeneration and fat accumulation, but validation of these findings is limited and future studies should look into this.

In **Chapter 5**, we used a publicly available single-nucleus RNA sequencing dataset to deconvolute the cell types in our Visium data. This allowed for further investigation of the cell populations and their cell-cell interactions. A limitation of this analysis was that the immune cell population was lacking, it would be very interesting to enrich the data with data presented in the work of Massier and colleagues which has a big dataset consisting of multiple studies of human white adipose tissue that capture e.g. immune cells, adipocytes, vascular cells and FAPs

(Massier et al., 2023). An integration of such seems to be of importance as FAPs, thought to be involved in the muscle to fat transition, send out signals to immune cell types such as the M2-like macrophages (Massier et al., 2023).

Recent work investigating intramuscular fat depositions in pigs reveals that genes involved in TGF-B signaling were specifically enriched in fat depositions and FAPs (Wang et al., 2025). Their study combined spatial transcriptomics and single-nucleus RNA sequencing revealing markers such as *ADIPOQ*, *PLIN1*, *PDGFRA*, *COL1A1* and *FABP4* that have also been identified in **Chapter 5**. Suggesting that these intramuscular fat depositions and their regulation may be similar across species. TGF-B signaling could thus be of great interest to dive into further as this may be a therapeutic target for dystrophinopathies as well. This idea is supported by the many colleagues in the field that indeed focus on this pathway (Luna-Angulo et al., 2024; Zhou & Lu, 2010; Yao et al., 2021; Kemaladewi et al., 2014). Another, even more recent study, also looked into intramuscular fat depositions in cattle using single-nucleus RNA sequencing (Ueda et al., 2025). They too find genes such as *FABP4*, *SCD* and *ADIPOQ* highly expressed. As well as confirming a marker such as *LPL*, that we identified in **Chapter 5** in human intramuscular fat, is also found in the intramuscular fat compartments in cattle. Moreover, they report of a specific subtype of FAPs that would transition into intramuscular adipocytes expressing marker which include *ICAM1*, *TGFBR2*, *TGFBR3*, *MME*, *EBF* and *BMPER* (Ueda et al., 2025). Whether these FAPs could also be the drivers in human skeletal muscle fat depositions remains to be investigated in future studies.

6.2.2 Bridging biopsy and MRI: towards integrated biomarker discovery

Histological analysis of skeletal muscle biopsies has given the field many insights into the pathological tissue changes. They capture many of the features such as fibrosis, inflammation, fibre-size variation, necrosis and regeneration and therefore have been used as endpoints in an open-label study as well as a phase II clinical trial (McDonald et al., 2021; Comi et al., 2022). Nonetheless, biopsies are invasive and capture only a small part of an entire muscle, which may not be representative to the rest of the muscle. Moreover, the repeatability of this muscle biopsy collection is difficult, leaving the researcher with less generalizable results across muscles and participants.

In **Chapter 3**, we investigate the use of DTI-MRI and the random permeable barrier model (RPBM) to capture fiber-size variation and permeability of the lower leg in BMD patients. This non-invasive imaging approach was combined with histological analysis of muscle biopsies from the opposite leg of the patient. The study was limited to only combine fiber-size variation and fibrosis measures from histological analysis to the imaging outcomes, but showed promising correlations between the two. It would be interesting to combine more detailed histological as well as transcriptomic analysis (invasive) with imaging (non-invasive) to ultimately build a map that could be used in the clinic for e.g. treatment response, disease progression prediction and drug efficacy assessment. Though this may seem like a far-fetched idea, the spatial field is growing rapidly and collaborative efforts between preclinical and clinical researchers in the neuromuscular field could make this into a reality.

6.3 CHALLENGES IN SPATIAL ANALYSIS

One of the limitations of scRNA-seq, the loss of spatial location, is overcome with spatial transcriptomics which allows for analyzing gene expression in a spatial context. Even though this big limitation is overcome by this new technology, other challenges arise with the generation and handling of spatial transcriptomics data.

Nature Methods crowned the spatially resolved transcriptomics technology as 'Method of the Year 2020'. Fast forward to 2025, the field has expanded tremendously. The number of technologies out there increased rapidly, both commercially available (Visium, Xenium, MERFISH, GeoMx) as well as labs that developed their in-house technology and published valuable research with those (EEL FISH, Borm et al., 2023). With this rise, the number of bioinformatic tools, analysis packages and pipelines also exploded. Tackling various problems and questions on which we will later come back such as spatial clustering, deconvolution strategies, cell-cell communication and the identification of spatially variable genes. Moreover, the generation of all this data is causal for the rise of various databases to store these spatial omic-related data such as SpatialDB, Aquila, SOAR, STellaris and others (Fan, Chen & Chen, 2020; Zheng et al., 2023; Li et al., 2022; Li et al., 2023).

The enormous acceleration of spatial research is also reflected in the number of publications over the past years (Figure 1), especially after being named Method of the Year in 2020. With new techniques and new ways of answering research questions, new problems arise in this (somewhat) undiscovered territory. Where the new technique offers a lot of potential, there is little consensus on how to analyze the data which poses several challenges that will be discussed below.

Overall, the advancement in computational methods has an important role in extracting meaningful biological signals from raw data, and computational biologists and biomedical scientists should invest more time in working together mapping out unresolved issues.

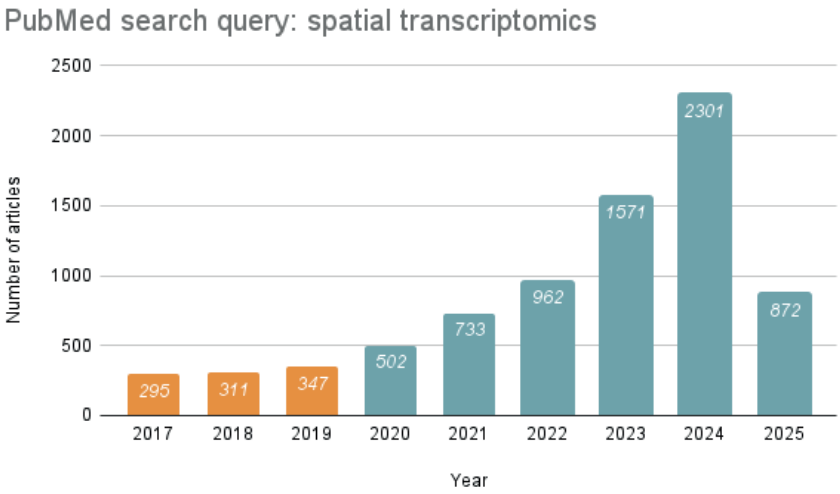

Figure 1. Number of publications with 'spatial transcriptomics' on PubMed over the past 9 years. (Information accessed on PubMed on April 1st 2025).

6.3.1 Structuring space: annotating the cellular landscape

One of the key steps in many standardized scRNA-seq pipelines is the clustering of cells. There are many tools available, essentially all clustering cells based on the similarity in their gene expression profile.

How these cells are organized, or clustered, based on their similarity differs among clustering algorithms (e.g. graph-based, density-based, centroid-based, tree-based) (Combe et al., 2015; Traag, Waltman & Van Eck, 2019; Ester et al., 1996; Lloyd, 1982; MacQueen, 1967; Ward et al., 1963). The most common ones used are the default settings in Seurat and Scanpy. Seurat and Scanpy are the two leading scRNA-seq analysis toolkits, they use Leiden clustering by default based on a kNN graph (Wolf, Angerer & Theis, 2018; Satija et al., 2015; Hao et al., 2024). This will allow the researcher to cluster cells into different 'types' which is of great importance in single cell analysis.

Similar algorithms exist for spatial data and are extensively used (e.g. **Chapter 4**). However, contradictory to single cell analysis, we are not only interested in what type of cell/spot this is, but rather, where is this cell/spot and to what region or niche environment does it belong to? This conceptual shift in clustering is essential for method development and application. because next to the gene expression profiling, there is an added axis, the location in tissue, and methods must (ideally) integrate spatial proximity, tissue structure and maybe even histology. However, there is no consensus yet on how to do this properly, which method to select for your biological research question, if any. Do you need to run: STAGATE, Spagcn, BASS, GraphST, BayesSpace, or SEDR for example (Long et al., 2023; Hu et al., 2021; Li & Zhou, 2022; Dong & Zhang, 2022; Zhao et al., 2021; Xu et al., 2024)? Perhaps, the major consideration should first be whether you need clustering to answer your research question and if yes, which type of clustering would be most suitable.

In **Chapter 5**, we try to overcome these challenges with tissue annotation in spatial transcriptomic data by working with module scores. We annotate the tissue based on Hematoxylin and Eosin staining as well as averaged gene expression scores (module scores). First, we annotate fat in the tissue and gene expression matrix based on manually constructed masks. Afterwards, we calculate the gene expression score for every remaining spot of a combination of several genes that would best annotate a certain cell or tissue type, e.g. connective tissue characterized by the expression of genes such as *FMOD, COL1A1, COL1A2* and *THBS4*. A big disadvantage of this approach is that we have to perform this step-wise. Meaning, that we have to choose which cell or tissue type to annotate first (the less abundant cell types such as neuromuscular junction), giving a slight bias to these types over the other, more prominent cell or tissue types later on (such as 'skeletal muscle'). However, this approach was most suitable for this current research question as we had performed multiple experiments over a longer time period and in different labs. Because of these factors, differences in sequencing depth, slide effects, and technical effects lead to batch effects that in turn impact the outcome of the clustering tools. Therefore, by using these module scores, we treat every sample in the exact same way as we, at the time, did not find a suitable clustering method that would be able to deal with these potential batch effects successfully.

Luckily, it is a rapidly expanding field and recent studies are benchmarking the multitude of tools that have become available over the past years for, for example spatial clustering (Yuan et al., 2024; Sun et al., 2025), as well as deconvolution (Li et al., 2023; Yan et al., 2023) and the

detection of spatially variable genes (Chen et al., 2025; Li et al., 2023, Kang et al., 2025) etc. A big benchmarking study tested and compared N = 13 clustering tools on N = 12 Visium (10x Genomics) spatial datasets and N = 22 imaging-based spatial derived datasets (Yuan et al., 2024). The authors come up with a thorough spatial clustering assessment framework to assist researchers in choosing the right tool for their dataset and biological question.

However, several (big) challenges remain. First of all, unsurprisingly, there is not a one size fits all method. These tools are tested on a decent number of datasets, but detection of small regions within tissues, the lack of multi-slice analysis capability and large-scale scalability (dataset size, memory and computation time) remain problematic. This poses several challenges and opportunities for future work in the spatial neuromuscular field which will be discussed in section **6.4** of this thesis.

6.3.2 Bigger datasets, bigger problems

Where in 2021 it was still possible to publish with N = 1, as a proof-of-principle study, nowadays to publish in highly regarded journals, the size of the dataset comes into play. Especially when researching highly heterogeneous diseases, such as NMDs, it is of importance not to base your conclusions on a dataset which is not representing the patient's heterogeneity.

However, many spatial transcriptomics techniques are expensive and sometimes require specialized equipment, limiting their widespread accessibility. These can be barriers to large-scale studies or to labs with fewer resources. As the technology matures, reducing costs and improving scalability will be essential for its broader adoption in both basic and clinical research. Two examples of this technology development are Open-ST developed by the lab of Nikolaus Rajewsky and Nova-ST developed by the lab of Stein Aerts (Schott et al., 2024; Poovathingal et al., 2024) . These spatial methods are aimed to study the molecular organization of tissue in 2D, is 3D-scalable, easy-to-use, high-resolution and cost-efficient. It is completely open-source, both experimentally and as a computational resource. It will be exciting to see what method developments like this can do in the future investigations and the scalability of studies. Application of such a method, or in general a decrease in price for these experiments, would allow us in both **Chapter 4 (and 5)** to

increase the number of individuals per group. With this, instead of only comparing data points (spots) within samples, we could compare across dystrophic models for example.

Besides increasing the number of included samples, one can also think of multi-omic integration as a solution to enrich your datasets (Li et al., 2022; Abdelaal et al., 2020; Biancalani et al., 2021; Vahid et al., 2023). As advanced as spatial transcriptomics is, it is also still only a snapshot of complex, heterogenous and multidimensional (in space and omics) tissue. To fully understand biological processes within these complex tissues, we cannot ignore the fact that we are only looking at the transcriptome snapshot of a tissue section from a bigger organism. For this reason, it is of great interest to combine (the data of) multiple technologies and dimensions (e.g. proteomics, scRNA-seq, metabolomics, lipidomics, etc.) to sketch a more complete picture. However, combining spatial data with other modalities, such as scRNA-seq, or proteomics, remains challenging. Tools such as SpaGE, Tangram and CytoSPACE allow for integration and alignment of scRNA-seq data to various forms of spatial data, aiming to enrich the sparse spatial datasets (Abdelaal et al., 2020; Biancalani et al., 2021; Vahid et al., 2023). This is a promising development in the spatial field, especially for imaging-based technologies where researchers may be limited by the measured number of RNA transcripts. The tools are not flawless yet, as for example the performance and cell classification of Tangram was negatively influenced by a difference in cell-type ratio between the two modalities (Cui et al., 2023).

Most of these tools need the scRNA-seq and spatial data to come from the same tissue, which in reality, is not always the case with limited funds and sample availability. Further development of the computational tools to overcome the bias of integrating data from different samples, experimental platforms or imaging modalities would allow many researchers to implement such tools and publicly available datasets as we did in **Chapters 4 and 5**.

While imaging-based spatial platforms may be limited by the total number of RNA transcripts they can detect, sequencing-based technologies are limited by their spatial resolution. It thus remains a challenge to spatially map the whole-transcriptome at single-cell resolution. Ongoing efforts are pushing the technologies further towards making this possible (Xenium, MERFISH, etc.), but it comes with a cost (Chen et al., 2015; Janesick et al., 2023). Another way to push these discoveries is by optimizing the computational tools

that allow for data integration, alignment and prediction. Future datasets will grow bigger through many ways, by integration of data modalities, by co-measurement of different modalities or for example by improved technology pushing the number of transcripts measured in a single tissue. This holds great promise for upcoming studies and will bring new challenges to overcome with concerns such as computational capacity and memory. And while it is exciting to work in a young and innovative field that keeps producing new tools, techniques and data to work on. It can be tough to keep up with the latest developments and choose the 'correct' analysis method. The lack of consensus is a big challenge as well as an opportunity for the future of the field. The lack of consensus on which technique to use for which type of research (hypothesis-driven vs. hypothesis-generating), how to normalize your data, which clustering algorithm to apply, how to perform cell deconvolution with your spatial data, how to integrate and align the data and many other steps in the analysis is something many researchers are now trying to overcome. Overcoming this lack of consensus is done by benchmarking and systematically comparing different tools available for these various steps mentioned above (Li et al., 2022; Hu et al., 2024; Salim et al., 2025; You et al., 2024; Lin & Qu, 2022; Du et al., 2025; Li et al., 2023). Establishing such frameworks will help researchers select the appropriate spatial transcriptomics technology and analysis tools for their specific biological questions.

6.4 FUTURE OF SPATIAL TRANSCRIPTOMICS IN NEUROMUSCULAR RESEARCH

Spatial transcriptomics is a coming of age technology that has proven its use and has grown tremendously since the late 2010's (Moses & Pachter, 2022). While most of these technologies are built using brain tissue, the application of the techniques has spread towards practically any tissue type (both fresh frozen and FFPE preserved). For neuromuscular disorders, and general muscle functioning, various studies have now published valuable research applying spatial transcriptomics to skeletal muscle (e.g. Heezen et al., 2023; Coulis et al., 2023; Wang et al., 2025; Hsu et al., 2024; Kaplan et al., 2024; Monceau et al., 2024). However, even though we are closer than ever before to capture and profile the transcriptome in space, it still comes with some caveats. For this final part of the thesis, the focus will be on the challenges and opportunities that are there for spatial neuromuscular research.

6.4.1 Challenges in the spatial neuromuscular field

6.4.1.1 Sample availability and quality

To perform spatial transcriptomics experiments, one needs biopsies of good quality. Meaning, they have been sampled and stored properly to ensure good RNA quality and tissue integrity. Nowadays, standard diagnosis procedure for DMD (and BMD) consists of performing a physical examination, followed by blood tests for creatine kinase (CK) levels and genetic testing to identify the presence of a mutation in the dystrophin gene. Essentially, there is no need for surgical intervention in these patients to obtain a muscle biopsy for their diagnosis, which used to be the case prior to genetic testing. This results in less biopsies in this modern era and only when a doctor wants to obtain more information, a muscle biopsy is requested.

Luckily for the patients, modern diagnostic techniques have almost diminished the need for biopsies. However, this also has an impact on basic research. We have encountered multiple times that there were no biopsies available (of good quality). Since we are working on rare disorders, this increases the challenge further and we even had to collaborate with a lab in the United States of America, to be able to perform the experiments planned. This was a great collaboration and offered us additional knowledge, but it would not have been necessary if the biopsies available at our own site had been sampled and stored properly. Informing the doctors on how to properly freeze and store skeletal muscle

biopsies, improved the quality of hereafter-taken biopsies tremendously. More collaborative efforts in wants and needs from the preclinical and clinical setting will greatly improve not only sample collection, but also hypothesis generation, research course setting and critical thinking.

6.4.1.2 Skeletal muscle is a special type of tissue

Another challenge for neuromuscular researchers aiming to perform spatial transcriptomics experiments is to keep in mind that most of these techniques and analysis methods are built for/upon highly structured tissue types such as the brain, liver and various tumor types. This is noticeable when we have a look at the papers from the most commonly used commercially available spatial technologies (Table 1; Ståhl et al., 2016; Merritt et al., 2020; Chen et al., 2015; Rodriques et al., 2019; Janesick et al., 2023; He et al., 2022). One can imagine that the data of a highly structured sample, such as the brain (and its cortical layers), is for example easier to cluster compared to a homogenous tissue type such as healthy skeletal muscle.

Technology	Company	Primary tissue type	Paper
Visium	10x Genomics	Brain and cancer tissue	Ståhl et al., 2016
GeoMx DSP	NanoString	Lymphoid, colorectal tumor and autoimmune tissues	Merritt et al., 2020
MERFISH	Vizgen	Human fibroblast cell line	Chen et al., 2015
Slide-seq	Curio Biosciences	Brain	Rodriques et al., 2019
Xenium	10x Genomics	Breast cancer	Janesick et al., 2023
CosMx	NanoString	Nonsmall cell lung and breast cancer	He et al., 2022

Table 1. Primary tissue types used in technology development of the most prominent commercially available spatial transcriptomics technologies.

Similarly, single cell (and consequently spatial) analysis pipelines usually include a step in the standard preprocessing workflow where they calculate the percentage of reads that map to the mitochondrial genome in order to filter out cells/spots that have a certain percentage (usually >5%) of these mitochondrial counts (**Chapters 2 and 4**). This percentage of reads mapping to the mitochondrial genome is used as a proxy for cell health. A higher percentage of mitochondrial mapping (mt%) is linked to the phenomena that suggest either cell stress, cell death or low-quality of cells. Namely, damaged or dying cells often have leaky membranes which leads to the loss of cytoplasmic mRNA, while mitochondrial transcripts that are protected by the mitochondrial membrane stay intact and thus enriched in such a cell (Ilicic et al., 2016). This is why in single cell or spatial analysis, these cells or spots are filtered out to ensure the analysis reflects biologically meaningful and viable cells, not artifacts.

However, skeletal muscle is a high energy-demanding tissue type that has a high mitochondrial content because of the natural functioning of the muscle with muscle contraction, high ATP turnover and mitochondrial biogenesis in oxidative fibers (Diaz & Moraes, 2008). By default, these muscle fibers have a higher expression of mitochondrial genes, even in healthy, viable cells. It can thus be considered unfair to filter out these cells/spots as it is a normal feature and not a quality-control artefact that the researcher has to deal with. Yet, within the neuromuscular field, there is no consensus on how to deal with this during the analysis. By removing these cells/spots from the dataset, one might remove healthy muscle cells and potentially create a certain bias towards glycolytic or non-skeletal muscle cells (e.g. immune, endothelial cells). Moreover, these mt% may even differ between healthy and diseased samples, adding weight as another compounding factor in the analysis. It is of importance to create a consensus on how we, as neuromuscular investigators, deal with this issue. This thesis suggests three suggestions to discuss:

[1] Do not filter out the cells with high mitochondrial counts and do not filter out the mitochondrial reads. One could label the cells/spots with a high mt% for careful interpretation. However, one might also even use the mt% for clustering of cell types (oxidative - glycolytic). The danger is that if mitochondrial gene expression is not accounted for, clustering algorithms may group cells primarily by mt%, as these genes have a significantly higher expression compared to other genes and thus dominate the signal. Which could cause a clustering based on these expression patterns rather than by biologically meaningful differences.

[2] Combine a relaxed mt% threshold with other quality control metrics. Filtering is usually already done by accounting for multiple metrics, however the mt% cut-off should probably be higher for skeletal muscle as well (more relaxed filtering). When a high mt% is present together with a low number of genes and counts (overall RNA molecules) per cell/spot, this is more likely due to poor quality of the cell/spot. Whereas a high mt% combined with high number of genes and counts likely reflects real metabolic activity.

[3] Instead of plotting and filtering the mt% across all cell types, visualize this mt% after clustering to see which clusters naturally have a higher mt% (e.g. oxidative fibers) and which ones likely are affected by cell damage/death. By considering the cell type first, the filtering of the mitochondrial reads will use cell-type-specific thresholds.

6.4.1.3 Deconvolution of single fibers

Finally, the skeletal muscle cell is a unique cell in its shape, size and nuclei formation which has implications for downstream spatial analysis that is good to keep in mind. A myofiber, a skeletal muscle cell, is a giant cell with (sometimes) hundreds of nuclei (myonuclei). These nuclei share the same cytoplasm, but can be transcriptionally specialized depending on their location along the myofiber (e.g. near neuromuscular junction or at the distal fiber region) (Folker & Baileys, 2013; Roman & Gomes, 2018). Standard spatial analysis tools assume that one nucleus is one cell, but this assumption does not hold in skeletal muscle. Especially with imaging-based, high spatial resolution technologies, the cell segmentation based on nuclei is incorrect and needs to be adjusted to skeletal muscle fibers. This can be done based on a membrane staining, followed by transcriptional identification of the cell type. The caveat may be that too little transcripts are detected for proper identification and grouping of cells is needed. When working with cross sectional tissue sections in array-based spatial transcriptomics technologies, such as Visium (**Chapters 4 and 5**), the spots may sometimes capture multiple cells, but when dealing with a hypertrophic fiber, it may span over multiple spots. This violates the assumption that each spot corresponds to one cell or a mix of nearby cells.

Deconvolution strategies allow for an estimation of the cellular composition of each spatial location (spot), this can be done with tools such as SPOTlight, RCTD or Tangram

(Elosua-Bayes et al., 2021; Cable et al., 2022; Biancalani et al., 2021). However, these deconvolution algorithms make use of a few typical assumptions that are not valid when applied to skeletal muscle (Table 2).

Assumption	Valid in muscle?	Issue/implication
Each spot contains a mix of distinct cells	⊘	Spots may all contain parts of the same multinucleated fiber
Cells are mononucleated and spatially bounded	⊘	Myofibers are multinucleated cells and myonuclei are shared within one cytoplasm
Gene expression is cell-specific and discrete	⚠	Expression varies within one fiber due to myonuclear heterogeneity
Spots reflect local neighborhood structure	⚠	Not always: expression changes may reflect position along a fiber, not proximity of different cells

Table 2. Assumptions of cell deconvolution strategies and its implications on the use of them when applying to skeletal muscle.

Future work should look into making this deconvolution more suited for skeletal muscle, by e.g. using membrane bound staining as the ground truth of the cell boundary. This will avoid running the risk of over-deconvoluting (tools might infer multiple cell types in a single fiber segment), setting false boundaries (abrupt changes in gene expression along a fiber may be misinterpreted as cell-type boundaries) and the potential loss of resolution (when transcriptional features of individual myonuclei would be blurred in bulk spot signals).

6.4.2 Opportunities and future perspectives

The challenges discussed above, directly offer opportunities for the neuromuscular field. While it can be challenging to work with tissue that is not on the priority-list for development of technologies and computational tools, one can use this to its advantage and step into this apparent gap. A few opportunities that are a bit more out of the previous scope deserve the attention to be addressed below as potential future work for neuromuscular research.

6.4.2.1 A slice is not a muscle, the need for 3D

What we currently capture with the techniques presented in this thesis (**Chapters 4 and 5**) are single sections of an entire muscle. A 10 micron tissue section of an entire muscle (group). While it offers us insight into the molecular landscape of that tissue section, there is of course a lot to gain. As said, a slide is not a muscle and there is a need for a 3D representation.

Multiple studies underscore this need and have come up with various ideas or solutions. The focus can for example be on the alignment and integration of various experiments and adjacent tissue sections (Zeira et al., 2022), while this does not overcome the problem of high costs linked to running these experiments. Open-ST, as discussed before, does reduce the costs drastically and allows for a 3D reconstruction (Schott et al., 2024). Moreover, very recent work shows the applicability of an AI framework that predicts 3D volumetrics from 2D spatial transcriptomics data (Almagro-Peréz et al., 2025).

Current claims on findings using spatial transcriptomics for NMDs are being made with caution, as it is known that there is transcriptomic variation not only between muscle groups but also within muscles already in healthy skeletal muscle, let alone diseased muscle with more variation (Abbassi-Daloii et al., 2023). More work can be done to improve both the computational alignment and reconstruction as well as data-collection and technology development (e.g. tissue clearing and whole mount spatial transcriptomics) for 3D spatial mapping (Fang et al., 2024). These advancements will aid research within the neuromuscular field. It would be interesting to dive deeper into the localization of fat infiltration. From what we have observed in cross sectional skeletal muscle tissue slices with fat depositions, it does not appear completely random. It has been speculated that it has to do with the mechanical force on the muscle bundles. The mechanical force being more eminent on the proximal and distal ends compared to the muscle belly (middle).

Many MRI studies have looked into the presence of fat infiltration in dystrophinopathies, they also look into which muscle type has more fat infiltration and if MRI parameters can be used as a proxy for fat infiltration, but do not consider where within the muscle this is more or less prominent (Hooijmans et al., 2017; Johnston et al., 2015; Kim et al., 2013). Performing spatial experiments in 3D could shed light onto whether these fat infiltrates are localized randomly or whether it is non-random. Exploring this could be done by applying a technique such as Tomo-seq, which allows for processing an entire tissue instead of a single slice and spatially reconstructing the transcriptome for that tissue (Kruse et al., 2016; Seurbert et al., 2024). This technique has been applied on skeletal murine muscle before (Martinez Mir et al., 2024). An interesting experiment would be to combine a non-invasive technique such as MRI which will allow for mapping the fat depositions along the muscle in a murine model that has fat infiltration before processing the muscle for Tomo-seq (Khattri et al., 2023). This way, we could map the transcriptomic changes along the muscle and simultaneously link this to MRI imaging, see whether one could build a predictor of fat replacement and identify whether there is a specific pattern of fat deposition in terms of localization linked to e.g. mechanical stress.

6.4.2.2 From snapshot to temporal trajectory

Besides the fact that we are currently capturing a single section of a 3D tissue, we also capture one moment snap frozen in time instead of looking at a temporal trajectory. Increasing the spatial and temporal dimensions in spatial transcriptomics research will enhance studies and their findings.

Spatial analysis over a time trajectory allows for identification of molecular markers that drive, try to hinder and clean-up tissue lesions such as inflammation, fibrosis, fat infiltration, necrosis, etc. In **Chapter 4** we applied an algorithm (RNA velocity), developed for scRNA-seq analysis, to resolve the potential future state of a Visium spot in our affected D2-mdx mouse model based on the proportion of spliced and unspliced transcripts. The velocities, gained after running the algorithm, indicate the direction and strength of a change in the transcriptional state of each spot. This analysis, though not performed before and with some caveats, revealed specific genes that potentially contribute to future tissue alterations. Being able to map spatiotemporal trajectories will aid in validating and adding knowledge to these findings.

Finally, the transcriptome, mapped by spatial transcriptomics, is only one asset of cell functioning and thus only part of the snapshot. Other aspects include e.g. the proteome, metabolome, epigenome and genome. Where Nature Methods renowned spatial transcriptomics 'Method of the Year 2020', it has recently praised spatial proteomics 'Method of the Year 2024' (Karimi et al., 2024). And rightfully so, while we often use transcriptomics to say something about the protein dynamics, as the function lies with the protein, the correlation between mRNA and protein is estimated to be at a mere 40% (Kishi et al., 2022; Wirth et al., 2023). Luckily, various methods have been developed or extended to profile multiple modalities from the same piece of tissue, providing a more comprehensive understanding of the cell state compared to transcriptomics alone (e.g. Vickovic et al., 2022; Wu et al., 2024; Vandereyken et al., 2023). Future work within the neuromuscular field could focus on capturing more than a single spatial modality, gaining basic knowledge as well as a direct translation between for example transcript and protein level.

6.4.2.3 An unresolved challenge to be resolved: how much dystrophin is needed?

Beyond filling in the unknown puzzle pieces by mapping the underlying histopathological pathways with multiple modalities, would be to target the defect and tackle the lack of dystrophin. But, how much dystrophin needs to be restored in order to overcome the defect caused by this lack? This question remains a big topic within the field and there is no consensus yet on the answer to it. Using spatial transcriptomics could be a way to get to the answer.

In healthy muscle, all nuclei along the muscle fiber are expressing DMD (Figure 2a), whereas in dystrophic muscle, the nuclei carry the mutated transcript and do not express DMD (Figure 2b). This in turn leads to the lack of dystrophin production, leaving the fibers more vulnerable to mechanical stress and contraction-induced muscle damage. The comparison of healthy and diseased tissue is what has been investigated throughout this thesis (**Chapters 4 and 5**) and has given us valuable information on the possible drivers (genes) and cell types involved in the histopathological tissue changes observed in dystrophinopathies (e.g. fat, fibrosis, inflammation and calcification).

However, the longstanding question that is relevant for all studies related to dystrophin restoration in boys suffering from DMD relates to how much dystrophin expression is actually needed to protect the skeletal muscle from further degeneration?

In a study with *mdx* mice, a 15% homogenous dystrophin expression seemed enough to protect against the contraction-induced damage and muscle strength changes correlated to dystrophin expression levels (Godfrey et al., 2015). Whereas in another review, considering data from human and murine studies, 20% of endogenous dystrophin expression, uniformly expressed along the muscle fibers, is suggested to be enough to largely prevent disease progression (Wells, 2019).

There are different therapeutic strategies being developed (and applied) to overcome the progressive muscle wasting in DMD boys (Chakkalakal et al., 2005). The ones that try to restore the protein expression in skeletal muscle are thought of as the 'direct' approaches. This can be done by improving the endogenous synthesis (e.g. antisense oligonucleotide mediated exon-skipping to restore the open reading frame in dystrophin mRNA or read-through stop codon strategies of premature mutations), or by introducing an intact gene or micro-gene using viral vectors or cellular systems or perform genome modifications using CRISPR-Cas9 (Wilton et al., 2007; Odom et al., 2007; Duan, 2018; Sampaolesi et al., 2006; Verhaart & Aartsma-Rus, 2019; Barton-Davis et al., 1999; Dunant et al., 2003). There are currently still both technical challenges (i.e. delivery to skeletal muscle) and concerns regarding safety (i.e. the vector induced immune response) for these therapeutic strategies that complicate the effectiveness and use. Many researchers are working hard to improve therapeutics and overcome these challenges, but with the currently available treatment strategies we do not reach a high dystrophin expression restoration and thus the question of how much is needed is still very relevant.

If we were to apply spatial transcriptomics, especially the currently available approaches with high spatial resolution, to treated dystrophic muscle (murine and human), using for example micro-dystrophin gene therapy and antisense oligonucleotide mediated exon-skipping, we could look into various effects of the treatment (Figure 2c). For once, we can compare corrected nuclei and uncorrected (still mutated) nuclei to one another along the same muscle fiber. This allows us to look further into the nuclear domains and the effect of correcting a mutated nuclei on downstream targets. Besides, one can delve further into the cell types involved in the secondary effects of a dystrophin loss around the mutated nuclei and delve into cell-cell communication or even communication between nuclear domains of uncorrected and corrected nuclei within the same muscle fiber. This allows us to look further into the nuclear domains and the effect of correcting a mutated nuclei on downstream targets.

Besides, one can delve further into the cell types involved in the secondary effects of a dystrophin loss around the mutated nuclei and delve into cell-cell communication or even communication between nuclear domains of uncorrected and corrected nuclei within the same muscle fiber. Moreover, with this spatial approach we can calculate the amount of dystrophin expression and relate this to functional outcome as well as histopathological changes and thereby aid in answering this longstanding question of how much dystrophin restoration is needed.

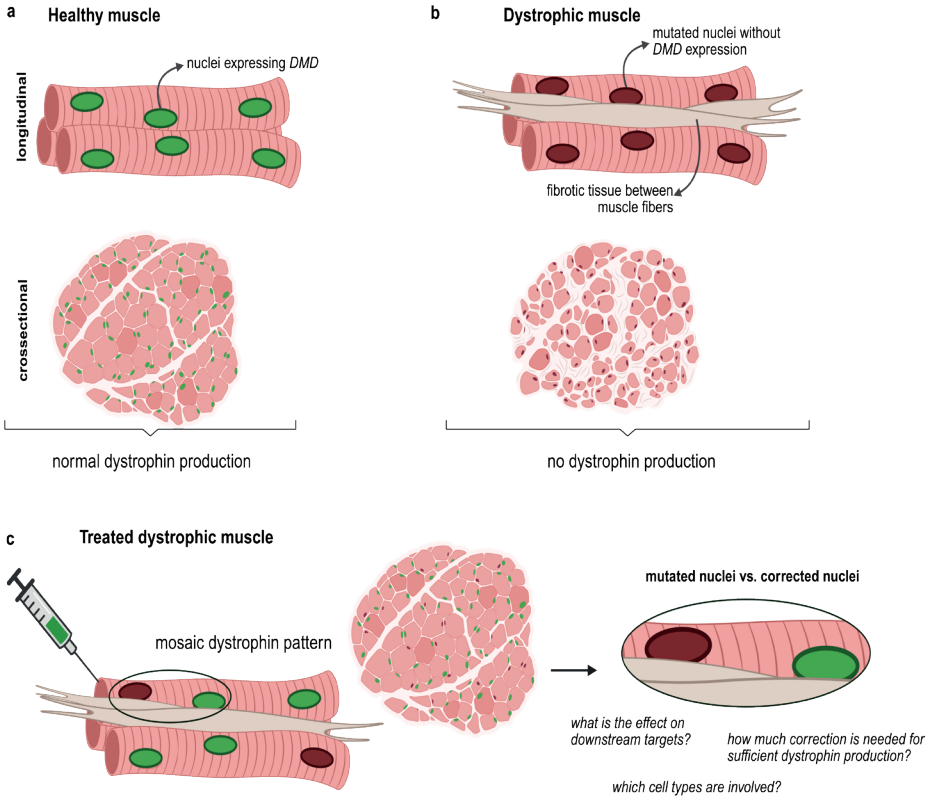

Figure 2. Future spatial studies should look into dystrophin correction therapy to answer the main question on how much dystrophin is needed. (a) DMD expression and dystrophin production in healthy skeletal muscle (b) DMD expression and the lack of dystrophin production in dystrophic skeletal muscle (c) Spatial analysis of treated dystrophic muscle will allow for comparison between mutated and corrected nuclei.

6.5 CONCLUDING REMARKS

This thesis showcases the use and benefit of mapping the transcriptome for an increased understanding of NMDs (**Chapters 2, 4 and 5**). It also underlines the need for biomarker identification in order to better predict and follow-up disease progression of patients suffering from dystrophinopathy (**Chapters 3, 4 and 5**). The main technique used for identifying these molecular markers throughout this thesis and in work beyond is spatial transcriptomics (Visium and Xenium, 10x Genomics). This new technology offers a lot of opportunities, but as discussed above, new technologies and its implementation also comes with the accompanying challenges. This new era of spatial analysis seizes to expand into multi-modal approaches, bringing us closer to drawing a more complete picture of the tissue of interest. Future research may also shed light on the localization and function of *DMD* in the brain, which will help neuropharmacological advancement and basic understanding of brain comorbidities in DMD patients. Moreover, further validation and functional testing of identified markers in skeletal muscle tissue changes may aid in development of additional treatment strategies targeting the secondary pathology in dystrophinopathies.

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