Supplemental Methods

Reagents

Reagent or resource	Source	Product ID	Online links
DMEM, high glucose	ThermoFisher Scientific	11965092	https://www.thermofisher.com/order/catalog/product/11965092
Fetal bovine serum (FBS)	Omega Scientific, Inc.	FB-02	https://omegascientific.com/product/fetal-bovine-serum-usda-certified-heat-inactivated/
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher Scientific	15140122	https://www.thermofisher.com/order/catalog/product/15140122
Ultroser™ G serum substitute	Crescent Chemical Co.	67042	http://www.creschem.com/ultroser-g-list.aspx
TrypLE™ Express Enzyme (1X), phenol red	ThermoFisher Scientific	12605010	https://www.thermofisher.com/order/catalog/product/12605010
ITS	ThermoFisher Scientific	51300044	https://www.thermofisher.com/order/catalog/product/51300044
Alt-R S.p. HiFi Cas9 Nuclease V3	IDT	1081060	https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-enzymes
Alt-R CRISPR-Cas9 tracrRNA	IDT	1073190	https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system
MERSCOPE 140 gene panel, VZG170	Vizgen, Inc.	10400001	https://vizgen.com/reagents-and-consumables/
MERSCOPE Sample Preparation Kit	Vizgen, Inc	10400012	https://vizgen.com/reagents-and-consumables/
MERSCOPE Cell Boundary Stain Kit	Vizgen, Inc	10400118	https://vizgen.com/reagents-and-consumables/
MERSCOPE Non-Beaded Slides	Vizgen, Inc	10500002	https://vizgen.com/reagents-and-consumables/
MERSCOPE 140-gene imaging kit	Vizgen, Inc	10400004	https://vizgen.com/reagents-and-consumables/

Custom Cellpose Training

We used the deep learning-based segmentation software (Cellpose)(Stringer et al. 2021) to segment the mononuclear cells (MNCs) and nuclei. Mononuclear cell segmentation was performed using a combined DAPI (stains nuclei/chromosome) and cell boundary staining (stains cytoplasm) image, with cell boundary staining in the green channel and DAPI in the blue channel. We took the maximum projection over the images of 7 layers across the depth direction (10 μm interval in between) to obtain the largest extension of the MNCs and nuclei footprint. During segmentation, the diameter parameter of Cellpose was set as 24 pixels (~26μm) which is close to the averaged diameter across the MNCs across samples. Using Cellpose's model training utility, we built a custom cell detection model based on the native "CP" model to perform cell segmentation. Nuclei segmentation was performed using only the DAPI images. We again took the maximum projection of the 7 layers to obtain the largest possible nuclei footprint across depth. The diameter parameter was set as 16 pixels (~17μm), and a custom nuclei detection model trained with Cellpose's training utility (also based on "CP") was used for detection.

To train the custom models, we first generated a 1500 * 1500 pixel (1 pixel ~ 1μm) image that contains the representative mononuclear cells (MNCs) and nuclei by visual inspection. This image has cell boundary staining in green channel and DAPI-labeled nuclei in blue channel. To train the custom MNC segmentation model, we Use the cellpose GUI software to automatically segment the sample image with the "CP" option selected in the "model zoo" section. CP is one of the basic fluorescence cell segmentation model included in cellpose. After the automatic segmentation, we use the GUI's manual cell selection utility to select MNCs that was not detected by the algorithm, and remove false detections. We next

save the selection result in a temporary folder and use the “train new models with image+masks in folder” in the “model” drop down menu to train the custom model. The custom model was tested on the sample image using the utilities in the “custom model” section in the GUI. If there were still missing MNCs or falsely selected MNCs, we manually fixed the selection, saved the fixed selection results and train the custom model again.

To train the nucleus segmentation model, we used a similar protocol above and also used the “CP” model as base model. The difference is that we only use the blue channel of the sample image for automatic segmentations and manual selection fix.

For the final MNC and nuclei segmentation model, we examine their performance with the nine example patches shown in supplemental Figure 1. For MNC selection, we exclude all MNC selections that within our manually selected myotube regions. For the remaining MNCs, two types of false positives are noted: part of the multi-nuclei cells are selected as a MNC, or the complete multi-nuclei cell labeled as MNC. For nuclei selection, false positives come from composites that contain multiple nuclei, or over segment one nuclei into two.

Based on these observations, we define model sensitivity as the ratio between the number of automatically detected correct MNC (or nuclei), and the total manually counted number of correct MNC (or nuclei). We also define model accuracy as the ratio between the false positive MNC (or nuclei) detection, and the total automatically detected MNC (or nuclei). For MNCs, we manually added additional MNCs (false negatives) based on visual inspection. For nuclei that are compactly gathered and not detected by the algorithm, we inferred the boundaries of individual nuclei with both visual inspection on the staining and the

transcriptomic density. Overall, across the 10 sample regions the sensitivity for MNC is 0.858 ± 0.039, sensitivity for nuclei is 0.989 ± 0.007, the accuracy for MNC is 0.976±0.007, and the accuracy for nuclei is 0.986 ± 0.003.

After training and validation, the segmentation process on the remaining ROIs was automatic and no manual intervention was included. Final segmentation results were presented as a mask containing individual MNCs or nuclei footprints. Based on the masks, we calculated the boundaries of MNCs and nuclei, then determined the transcripts inside based on their spatial coordinates given by the MERFISH system.

Unsupervised clustering of mononuclear cells and nuclei

MNCs or nuclei across all samples were pooled together for clustering analysis. Unsupervised clustering was performed using the R package Scrattch.hicat (Tasic et al. 2018)(https://github.com/AllenInstitute/scrattch.hicat). This package performs iterative clustering on the dataset based on differential gene expression profiles, and making successive finer splits until no more child clusters fulfilled the predefined differential expression criteria(Tasic et al. 2018) are detected. In this study, we focused on adjusting the following parameters: q.diff.th and de.score.th. For a group of cells that are going to be separated into two clusters, one cluster is named “foreground” which contains most of the cells expressing the up-regulated genes, and the other is named “background”, q.diff.th represent a threshold that helps determine whether foreground and background are established based on the proposition of cells showing high expression levels of the up-regulated genes. De.score.th, on the other hand, helps to determine whether foreground and background are separable by overall differential gene expression. We aimed to maximize both parameters while ensuring

the interpretability of the resulting clusters. The final parameters for all nuclei were q.diff.th=0.55, de.score.th=200; for MNCs, the parameters are q.diff.th=0.18, de.score.th=111. To improve the robustness of our clustering results, we further performed consensus clustering by performing the above clustering process (with the same parameters) using a random subsample of 80% of cells and repeating for 100 times, followed by final clustering based on the co-clustering probability matrix using Louvain clustering algorithm(Tasic et al. 2018).

Identification of myotube clusters

Transcripts counts of single myotube and non-myotube region were normalized using a similar method to “LogNormalize” in Seurat, except the data were divided by the corresponding area, but not the total counts of genes of the region. The normalized data from different batches were converted and merged into a Seurat object. The first the top 20 highly variable genes were identified with “FindVariableFeatures” function using the “vst” method. The expression data corresponding to these features were scaled using the “ScaleData” function and used to carry out principal component (PC) analysis. The batch effects were observed, so we ran Harmony for batch correction with “RunHarmony” function. To cluster the data, we applied Seurat’s “FindNeighbors” using 10 dimensions from the Harmony-corrected reduced dimensions, followed by “FindClusters” at resolution 0.5. For the UMAP representation, we applied the “RunUMAP” from the Seurat to the selected PCs. Seurat-4.1.3, harmony-0.1.1 and standard R packages-4.2.1 were used.

Differential expression analysis

Differential expression analyses of transcripts were conducted with Bioconductor package DESeq2 (v1.36.0) (Love et al. 2014). To control the high statistical power brought by a large number of cells from samples, pseudobulk was calculated for each sample by separately aggregating MNCs, nuclei, and myotubes/non-myotube regions. For MNCs and nuclei, pseudobulk profiles were aggregated by summing up all the intra-cell (or intra-nuclei) raw transcript counts of each sample, with or without separated by the clusters determined in the unsupervised clustering step. For myotubes and non-myotube regions, pseudobulk profiles were aggregated by summing up the raw transcript counts across all myotubes (or non-myotube regions) of each sample. In the final analysis results, p-value was adjusted using BH algorithm.

For differential expression analysis between myotube and non-myotube regions, due to the concern that myotube and non-myotube regions covers large areas and thus are more subject to background noise compared to nuclei and MNC regions, we applied a gene count threshold based on the intra myotube (non-myotube region) blank gene counts for each genotype, and only use the genes with average intra-myotube (non-myotube region) gene counts higher than blank gene counts for differential gene analysis (Supplemental Figure 7C-E).

Pseudotime analysis

To obtain a batch-effect-corrected gene expression matrix for downstream analysis, we utilized Seurat(Hao et al. 2021) to compute the integration anchors (FindIntegrationAnchors) of the area normalized MERFISH data, and then integrated (IntegrateData) the batches using these anchors. This corrected Seurat object was used as inputs to create a Monocle(Trapnell

et al. 2014; Qiu et al. 2017; Cao et al. 2019) newCellDataSet. Differential expression between clusters was calculated using “differentialGeneTest“ function in Monocle. Differential expression between clusters was calculated using “differentialGeneTest” function in Monocle. “DDRTree” method was used for dimensionality reduction, and the pseudotime trajectory plot was generated using the “plot_cell_trajectory” function.

Co-expressed transcript module analysis

For the single myotube MERFISH data, the gene co-expression network analysis was performed using the R package WGCNA (v.1.72.1) (Langfelder and Horvath 2008). Genes are not always informative for co-expression or modules detection as their expression can be linked to technical biases(Lemoine et al. 2021). We removed genes with low variation between pairwise samples ($p > 0.05$) in the differential expression test of Monocle (differentialGeneTest).

We next tested the blanks genes (Decoding barcodes not linked to a gene), which work as false positive misidentification controls, and found that the background level counts may cause technical biases. To avoid the biased background noise effect, we set up a threshold for the area normalized and batch corrected MERFISH data based on the blanks’ values. The genes whose values are higher than 0.015 in at least 30 myotubes were kept for WGCNA analysis. The function "TOMsimilarityFromExpr" was used to calculate the TOM similarity matrix, then "flashClust" function of package flashClust (v.1.2.3) was applied for cluster analysis. The "cutreeDynamic" function was used to identify the modules consisting of groups of genes with higher value of the connection strength and shared functions. Both networkType and TOMType were set as "unsigned". The soft-thresholding power, cut height, and minimal

module size were set as 4, 0.94, and 8, for non-DUX4 target genes respectively. Intramodular connectivity, which is defined as the sum of a gene's connection strengths within the same module, was calculated by the function "intramodularConnectivity".

We performed gene set enrichment analysis of genes in the modules using the online tool gProfiler(Reimand et al. 2007). GO analyses for molecular function cellular component biological process were carried out sequentially, followed by pathway enrichment utilizing the KEGG, Reactome, and WikiPathways databases. The statistical domain scope was used for the analyzed non-DUX4 target genes as custom background. The significance threshold is the g:SCS threshold. The user threshold is 0.05. Cluster analysis of the DUX4 target genes in the FSHD1 and DEL5 myotubes were done similarly, with the soft-thresholding power, cut height, and minimal module size set as 4, 0.8, and 3. Their module tree plots were generated by "plotDendroAndColors" fuction in WGCNA package. The fourth power of correlation, accompanied by its corresponding positive or negative sign, of DUX4 target genes and selected non-DUX4 target genes were visualized using Cytoscape 3.9.1 software.

Linear mixed effect modeling

Linear mixed-effect modeling (LME) is used to address the repeated measurement issues that occur when doing statistical tests with multiple items from the same sample (for example, multiple myotubes come from the same sample in Figure 2 I,J). The LME model utilizes linear regression to estimate the difference between variables by examining the significance of regression coefficients. Importantly, it ("fitlme" in MATLAB) introduces the concept of "fixed effect" and "random effect". "Fixed effect" represents the parameters that do not vary during the linear regression, and practically, represent the actual variables that to be

tested. “Random effect” represents random variables that have impact the “fixed effect”, and practically, often represent the groupings of the “fixed effect” variables(Yu et al. 2022). The model will represent the data in the following format:

$$y = X\beta + Z\mu + \varepsilon$$

In which y is the measured data from the experiment (for example, nuclei counts in each myotube), X represent the “fixed effect” variable that going to be tested (for example, genotypes of each myotube in y), Z is the “random effect” variable (for example, the sample each myotube in y comes from), and ε is the residual error(Yu et al. 2022). The significance of the regression variable β will be used as the statistical test result and the significance level is $p < 0.05$. Compared with a paired *t*-test or repeated measures ANOVA, LME can handle unbalanced designs and missing values, and has greater statistical power in the presence of missing values(Stobart et al. 2018; Indersmitten et al. 2019; Yu et al. 2022).

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