# Documentation for Static muscle cell image analysis code

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## 1. Preprocessing

For static, stained images open:

*Batch\_Binarize\_Percentile\_240110.ijm*

A screenshot of a computer

AI-generated content may be incorrect.An ImageJ/macro that averages slices of images at multiple depths if required, removes general background artifacts, binarizes to a given threshold and saves image files in a format to be imported into the metric extraction software.

**Input:**

1. Match fluorescence channels. ‘Myonuclei channel’ is the myogenic marker (eg. Myogenin or pericentrin).
2. Apply threshold % intensity for segmenting images. It is worth experimenting with Image-> Adjust->Threshold… to try different thresholds and checking the effectiveness before commiting.
3. Z-projection gives an averaged projection of slices at different depths if required.
4. Select whether batch mode is on or off.

## 2. Using the analysis code

## a) Fluorescence-stained images

For automated/Semi-automated Matlab code which counts numbers of myotube and myocyte nuclei in a stained image run Matlab code *StaticImageMetricsBatch240110.m* in the same directory as your image files.

For

**Choosing Metrics**

**A screenshot of a computer

AI-generated content may be incorrect.**

Select which metrics are required. If BTX staining is available include ‘Acetylcholine receptors’. ‘Striation images’ outputs images of the mid-sections of individual myotubes for manual striation analysis.

**Automated/Semi-automated**

It is recommended to begin with semi-automated analysis in order to assess the accuracy of the segmentation and labelling and determine whether manual labelling is required.

**A screen shot of a cell phone

AI-generated content may be incorrect.**

**Inputs:**

**A screenshot of a computer

AI-generated content may be incorrect.**

'Pixel width (microns):', [Important to get this right!!]

'Experiment:', [Default to D1 if unsure]

'Start Position:'[position or experiment number],

'Number of Positions:'[position or experiment number],

'Enhancement factor:', [if image is too dull then increase this for better visual]

'image length:' [Size of the section of the image to be analysed, in microns. Default is 500 microns. Make it smaller than the full image size.]

‘Dilation factor:’ [Leave as default unless too many artifacts (see below)]

‘% Myo-marker threshold:' [Proportion (0-1) of myogenic marker within nucleus above which cells are labelling as a myonucleus. This will need to be tuned for dynamic staining seg pericentrin].

‘Mean nuclei pixel size:’[Used as a baseline to estimate nuclei numbers in clusters and discount small regions of dead cell nuclei/artestifacts. Can be measured by sampling in ImageJ first].

‘Min myotube length:’[Threshold for smallest myotube length to be counted. Below which are considered artefacts]

‘Min myotube image size:’[Size of image sample taken for comparison of striations]

**Manual check**

**A screenshot of a computer screen

AI-generated content may be incorrect.**

The myotube nuclei appear in blue, myoblast nuclei red and myogenic marker in yellow.

Nuclei may be mislabelled. This is especially likely in clusters of nuclei (see image above). To correct this, left-click on miss-labelled myoblasts to change them to myotube nuclei and right-click on myotube nuclei to change them to myoblasts.

Images with a higher density of myotubes contain more of these mis-labelled nuclei. If there are a lot of them, try changing *Dilation factor* in the inputs to reduce the amount. If this does not help, try manually binarizing the images with a different threshold in ImageJ/FIJI.

**Feature outputs**

Dead\_cell\_count -> Number of nuclei smaller than ½ of the mean nucleus area. Assumed apoptotic.

Dist\_mean -> Mean distance between nuclei in multinucleated myotubes. **(Written to .csv)**

Dist\_SD -> Standard deviation of distances between nuclei in multinucleated myotubes. **(Written to .csv)**

Myoblast\_estimate -> Number of single nuclei cells

MyonucleiEstimate -> Number of myotube nuclei (total individual nuclei + estimated number in clusters)

MatureMyonucleiCount -> Number of myotube nuclei with >50% myogenic staining of nucleus.

Myonuclei\_per\_um -> average myonuclei per micron length of myotube.

Findex -> Fusion index, assuming all single nuclei cells are myocytes.

MCmean0 -> mean myocyte nucleus area at Day 1 (in pixels). Used to estimate number of nuclei in clusters.

MCmean -> mean myocyte nucleus area in current image (in pixels).

MTDens -> Proportion of myotubes/mm2.

Reference\_Width -> Average myotube width. Calculated via total area/total length of myotubes.

TotLength -> Total myotube length in image.

Proportional\_Area -> Area of myotubes in image.

## b) Unstained images

For semi-automated Matlab code which counts numbers of myotube and myocyte nuclei in unstained image.

Run the Matlab code *StaticCellBinarizeSeg.m* in the same directory as your image files.

\* If you have pre-processed binary images run *StaticCellSeg.m*

**Input:**

'Pixel width (microns):', [Important to get this right!!]

'Day:', [Default to 1 if unsure]

'Position:'[position or experiment number],

'Enhancement factor:', [if image is too dull then increase this for better visual]

'image length:' [Size of the section of the image to be analysed, in microns. Default is 500 microns. Make it smaller than the full image size.]

‘Dilation factor:’ [Leave as default unless too many artifacts (see below)]

**Manual check**

The code defines myotube nuclei in blue and myocyte nuclei in white.

Clusters of myotube nuclei are often only labelled with one dot… this is fine and is sorted out by the code.

There are often a few nuclei miss-labelled. To correct this, left-click on miss-labelled myocytes to change them to myotube nuclei and right-click on myotube nuclei to change them to myocytes.

Don’t worry about the exact positioning of the click, the co-ordinates are not important. If you accidently click the wrong button just click the other button to cancel it out.

There are two types of artifact you need to account for.

1. Myocytes (or other single nuclei cells) which are very close to a myotube can be recorded as myotubes if they overlap (see figure 1, circle 1). In this case, right-click on the blue dot to remove it from the count.
2. Myotube nuclei can be counted more than once if they are in a sparse region of actin (see figure 1, circle 2). In this case, right-click on any extra blue dots until you have 1 blue dot per nucleus (or overlapping cluster of nuclei)

A close - up of a logo

Description automatically generated with low confidence

Images with a higher density of myotubes contain more of these mis-labelled nuclei. If there are a lot of them, try changing *Dilation factor* in the inputs to reduce the amount. If this does not help, try manually binarizing the images with a different threshold in ImageJ/FIJI.

## c) Manual feature analysis

Code for manual method for obtaining statistics on myotube nuclei spatial distribution and proportion of striated myotubes.

Run the Matlab code *ManualNucQIStats1120.m* in the same directory as your image files.

**Inputs**

'Pixel width (microns):'[see above],

'Day:'[see above],

'Position:'[see above],

'Enhancement Actin:'[Increase if actin is too dull],

'Enhancement Nuclei:'[Increase if nuclei are too dull],

'image length:'[see above],

'Sample number:'[How many myotubes sampled per image],

'Record striations?:'[‘yes’ ‘no’ . Only recommended for higher resolution images where striations are apparent]

**Measurement**

White \*’s are randomly generated markers on the image to randomise sampling.

Select the nearest myotube to a given \* (if none then press *spacebar*).

Click on each of the nuclei in the myotube in order from one end to the other then press *spacebar.*

If you are analysing striations, choose the most appropriate option for the myotube you are observing.

Repeat for all \*’s in the image.

**Outputs**

GlobalMeanNucDist= Global mean of the average distance between nuclei in each myotube.

GlobalSDNucDist= Global mean of the standard deviation of distances between nuclei in each myotube.

GlobalCoeff\_var = Mean coefficient of variance (standard deviation in distance/mean distance) of nuclei in myotube cells. This is a metric of uniformity. The lower the value, the more uniform.

GlobalTot\_length = Mean myotube ‘length’ (distance between extreme nuclei). Does not provide useful information when myotubes are bigger than the field of view.

## d) Myotube orientation statistics

For statistics on alignment and orientation of cells, run Matlab code ‘*Orientation\_extractor.m*

Select the image file required. It works best with fluorescence staining of myotubes but can work with brightfield images. There is no need to pre-process the image.

**Inputs**

‘Min. branch length’: [Minimum length of cell section to use when binarising image, in pixels]

‘Min cell length:’ [Used to remove orphaned segments in post-processing]

‘Bin angle:’[Smaller angle gives a greater chance of discretising cells which overlap with an acute angle but will mean less chance of recombining a cell which is curved]

‘Max image hole:’[Threshold for hole size for preprocessing binary image]

'Binary dilation factor:'[Dilation factor for removing fuzz etc at image border]

'Display images(1/0):'[Are images displayed during run time]

**Feature outputs**

Total\_cells -> count of myotube cells used in image.

Angle\_region\_props -> List of cells regions in each angle bin with associated properties.