## Documentation for myotube analysis code

## Note on binarizing in FIJI

\*Thresholding in FIJI gives you more control over how the binarized image looks (but introduces more user bias). If you do the thresholding in FIJI then follow these additional red instructions.

## Preprocessing

In ImageJ/FIJI check images and remove background artifacts (Process->Remove background)

Save Nuclei image as pos*x*-D*y*-DNA.tif, where *x* is the position number and *y* is the day of cell maturation. If you are not defining positions set *x* = 1.

Save Actin image as pos*x*-D*y*-Actin.tif

\* If binarizing in FIJI, go to Image-> Adjust->Threshold…

\* Save Binary Actin image as pos*x*-D*y*-ActinBW.tif

## Nuclei count

Semi-automated Matlab code which counts numbers of myotube and myocyte nuclei in a stained 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.

**Now for the fiddly bit…!**

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. If it is still too many then contact me and I can look over the image.

**Outputs**

MTEst2 -> Number of myotube nuclei (total individual nuclei + estimated number in clusters)

MCTot -> Number of single nuclei cells

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).

**Save your data as running the next code may clear it!!**

## Myotube nuclei statistics

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… it’s quite crude so feel free to use another method.

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.