Directionality software version v_2017, for 64-bit PC.

README FIRST

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1. Installation

Run the "direction_v2017_pkg.exe" file in a PC computer. It will first install a MCRInstaller.exe (if not installed before), and then generate a direction_v2017.exe file. This will slow down the first run of the program but allow you to run it even if you have no access to Matlab.

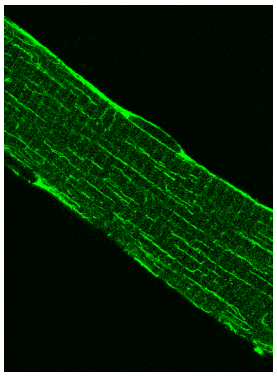
2. Selection and preparation of the images

Select **single .tif images** of good quality (minimal saturation). The software does not handle image series, JPG or PNG images). Grayscale images are fine. RGB images also as long as microtubules are in the green channel, which is the only one analyzed by TeDT.

If necessary, use Photoshop to align the fiber axis with the horizontal (a to b). This step is not required by the software but is necessary to make measurements of vertical vs. horizontal directionality consistent. If your fiber is not straight, you may need to divide it into 2-3 parts.

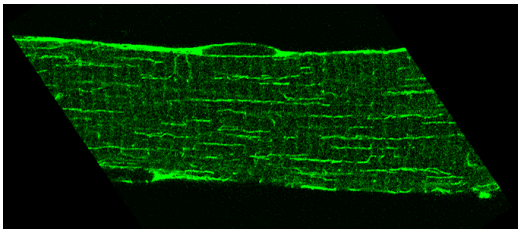
You can crop your images to have a constant length/height ratio (rectangle, c, or square, d). Keep plasmalemma and nuclei out of the cropped areas (c-d).

Place all images to be analyzed in a folder. The results will appear in the same folder.

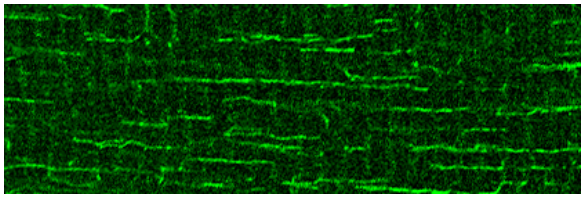


a

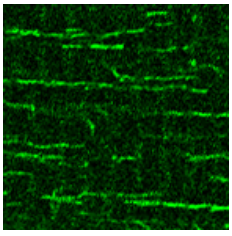
b



c



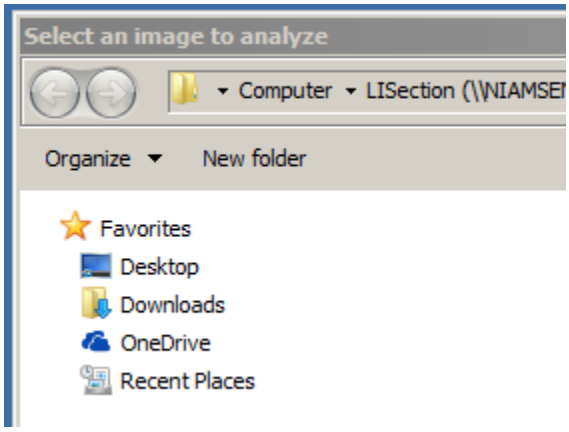
d



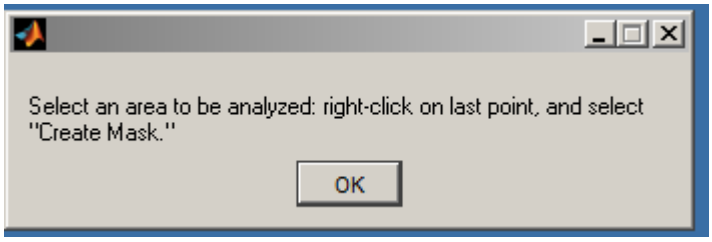
3. Running TeDT

Run the “direction_v2017” program. It opens a navigation window entitled "select an image file to analyze" (e). Navigate to the image folder and select your first image.

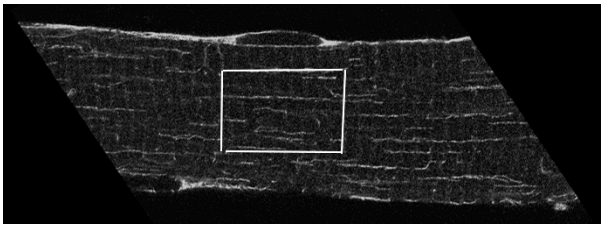
e



The following instruction window opens.

f 

Click OK. The instruction window disappears and a window with your image in black and white opens. To magnify it, drag the corners out. Then draw an ROI; left-click on 4 points and right-click to close the shape (g).

g 

A small text window appears. Select “create mask”. The image disappears.

Selection of peaks and valleys.

The next dialog window (h) shows a graph and asks you to select its peaks. Depending on the size of your image and amount of microtubules, the graph can be smooth or craggy, as is the case in this example. Great precision is not important. In this example you could decide there are 2 peaks. After you have selected the peaks, a similar window opens, that asks you to select the same number of valleys (i).

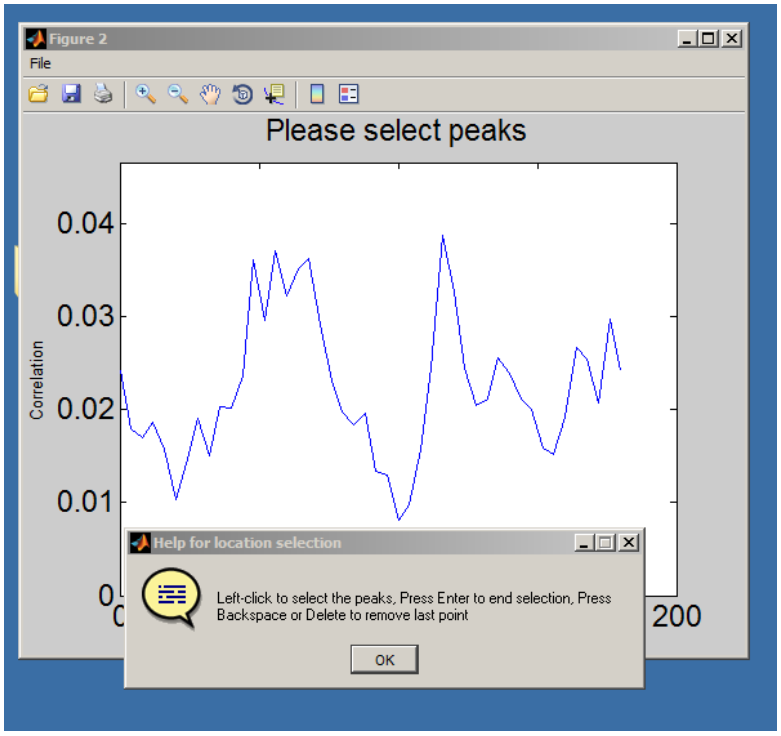


Figure 2
File
Please select peaks
0.04
0.03
0.02
0.01
0
Correlation
200
Help for location selection
Left-click to select the peaks, Press Enter to end selection, Press Backspace or Delete to remove last point
OK

h

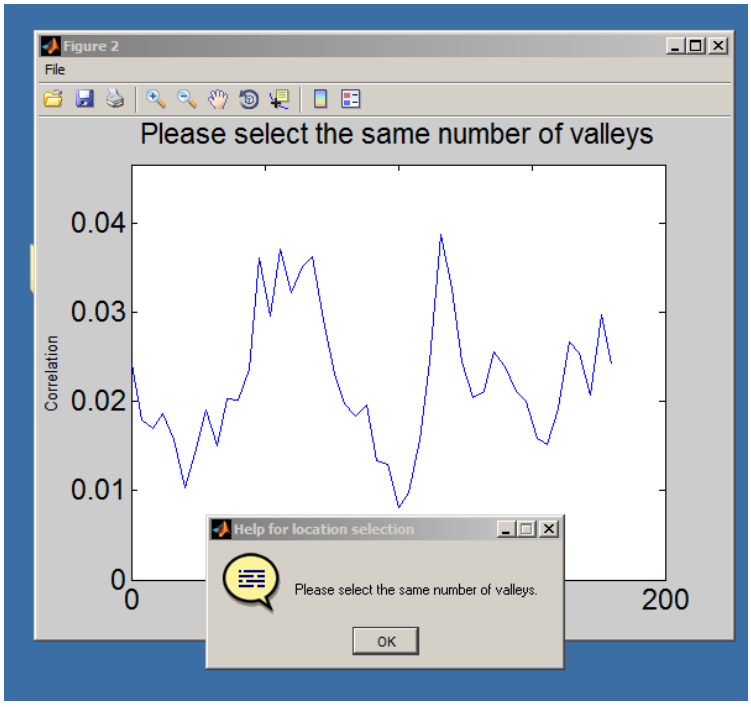
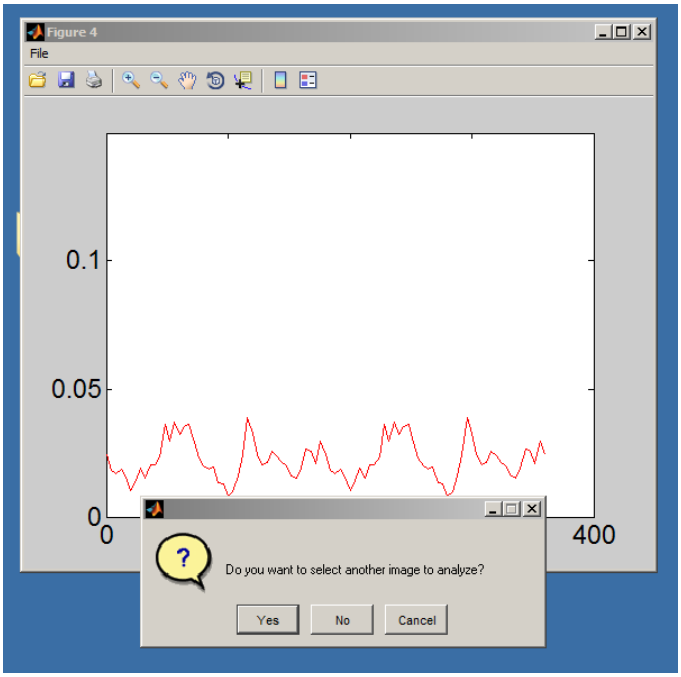


Figure 2
File
Please select the same number of valleys
0.04
0.03
0.02
0.01
0
Correlation
0
200
Help for location selection
Please select the same number of valleys.
OK

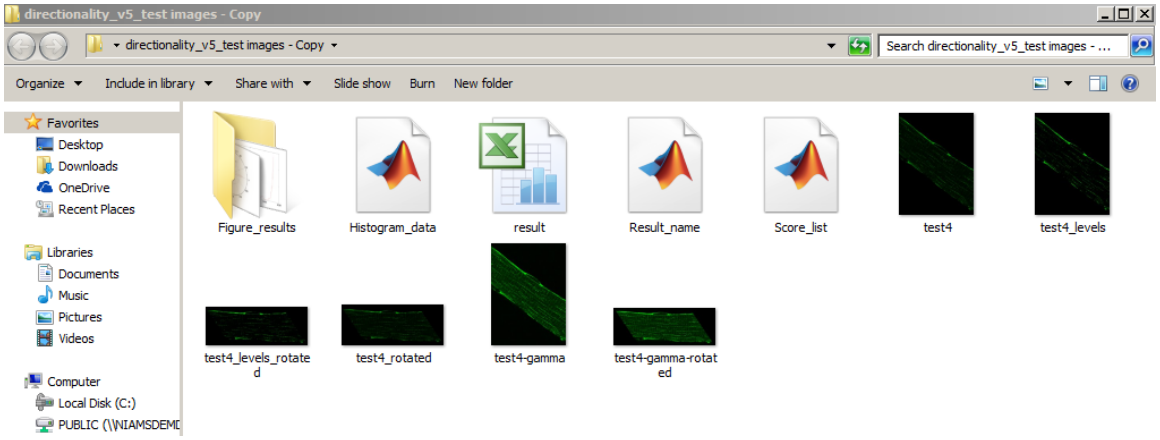
i

The software then performs the calculations and gives you the option to continue with another image or to exit (j).



j

Result files can be found in the folder that contains the analyzed images (k).



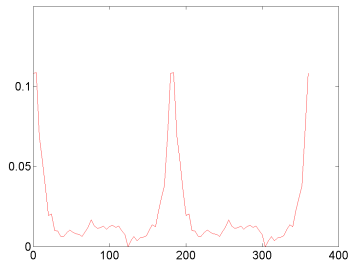
k

4. Understanding the results

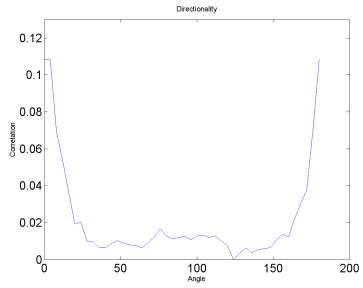
The folder "Figure_results" (inside the image folder), provides images, plots, Matlab files and one Excel file.

The images show the ROIs drawn in the beginning of the analysis (g).

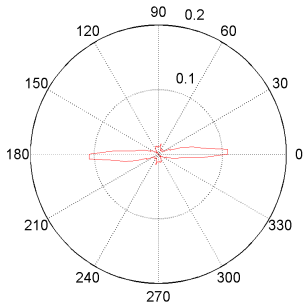
There are three directionality plots: two in cartesian coordinates (360 and 180 degree ranges respectively, l-m) and one in polar coordinates (n). Again, the larger the surface analyzed, the smoother the profiles. The results are given in Matlab files, which you can only open if you run Matlab. The excel data cover the same information.



l



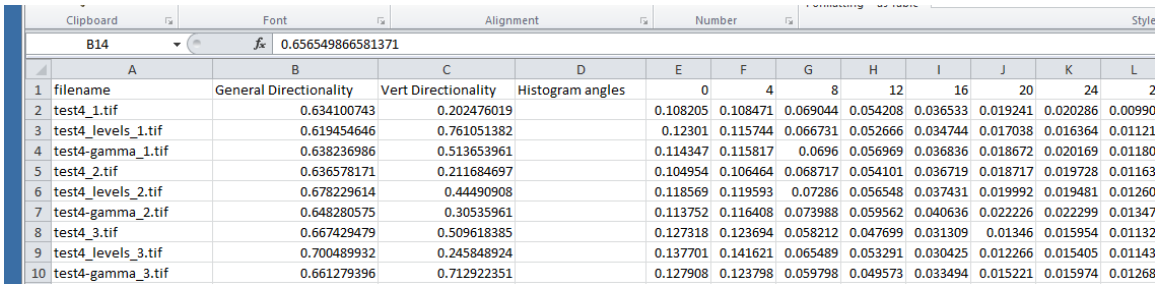
m



n

Directionality scores

Excel provides for each image a general directionality score (column B), and a vertical directionality score (column C). The histogram data are in columns E and following. Each column gives the directional score for one angle (from 0 to 180 degrees at intervals of 4 degrees). Use these data to draw the histogram charts in other programs if needed or to average data from several images.



Clipboard | Font | Alignment | Number | Style

B14 | 0.656549866581371

	A	B	C	D	E	F	G	H	I	J	K	L
1	filename	General Directionality	Vert Directionality	Histogram angles	0	4	8	12	16	20	24	2
2	test4_1.tif	0.634100743	0.202476019		0.108205	0.108471	0.069044	0.054208	0.036533	0.019241	0.020286	0.00990
3	test4_levels_1.tif	0.619454646	0.761051382		0.12301	0.115744	0.066731	0.052666	0.034744	0.017038	0.016364	0.01121
4	test4-gamma_1.tif	0.638236986	0.513653961		0.114347	0.115817	0.0696	0.056969	0.036836	0.018672	0.020169	0.01180
5	test4_2.tif	0.636578171	0.211684697		0.104954	0.106464	0.068717	0.054101	0.036719	0.018717	0.019728	0.01163
6	test4_levels_2.tif	0.678229614	0.44490908		0.118569	0.119593	0.07286	0.056548	0.037431	0.019992	0.019481	0.01260
7	test4-gamma_2.tif	0.648280575	0.30535961		0.113752	0.116408	0.073988	0.059562	0.040636	0.022226	0.022299	0.01347
8	test4_3.tif	0.667429479	0.509618385		0.127318	0.123694	0.058212	0.047699	0.031309	0.01346	0.015954	0.01132
9	test4_levels_3.tif	0.700489932	0.245848924		0.137701	0.141621	0.065489	0.053291	0.030425	0.012266	0.015405	0.01143
10	test4-gamma_3.tif	0.661279396	0.712922351		0.127908	0.123798	0.059798	0.049573	0.033494	0.015221	0.015974	0.01268

The **general directionality score** was introduced in Liu & Ralston (2014). It is normalized (0 to 1) and reflects the presence of preferred orientations among microtubules. For example in a grid-like MT organization, microtubules have mostly 0 and 90 degrees orientations while in a random distribution there are no preferred orientations. The grid-like organization (wild-type fibers e.g.) has a higher score than the random organization (*mdx* fibers e.g.). However, things are more complicated in real life. For example, some muscle fibers in other mouse models have mostly all longitudinal microtubules. They have, consequently, a higher score than WT fibers, because, mathematically, one orientation gives the highest score.

However, we were searching for a way to differentiate normal from mdx-like fibers. The presence of transverse (vertical) microtubules is one of the easiest detectable differences between normal and *mdx*-like MTs (Randazzo et al., 2019). Wenhua Liu therefore developed a new score, the **vertical directionality score**. This score is not normalized. The value for an array of vertical lines is ~ 35.0. The score for a nice grid organization is ~7.0 or higher. A score below one is typical of an image with very few to no transverse microtubules. For this score it is essential that the long axis of the muscle fiber in the image be horizontal.

The vertical directionality score (equation below) is derived from the normalized directionality scores H_D obtained as described in Liu & Ralston (2014). Instead of calculating the general directionally, by summing up the moments around the peaks, the vertical directionality is using a similar approach to calculate the moments near the vertical angles, by summing the moments from 80 to 100 degrees.

$$Dv = 100 * Range(H\theta) * (1 - \gamma * \sum_{\theta=80^{\circ}}^{100^{\circ}}[\left(\theta - \frac{\pi}{2}\right)^2 * H\theta])$$

The normalization factor γ is the same as defined in equation 8 in Liu & Ralston (2014). *Range* $(H\theta)$ is the direction score range from 80° to 100°.

Final Remarks

The more we look at muscle microtubules, the more we're impressed by their complexity. Genotype and muscle fiber type both affect microtubule patterns. The fiber type factor is complicated in the mouse, which lacks entirely slow or entirely fast muscles. The slowest muscle (Soleus) contains only about 60% of slow fibers in the adult mouse vs. 90% in the rat. The mouse fast muscles (EDL for example) have a non-negligible proportion of intermediate type fibers (type IIA). When comparing microtubule directionality in different mouse models, it is necessary to compare the same muscles. Finally this directionality software complements but does not replace presentation of microtubule images.

If you use the results of TeDT in a publication, please acknowledge Drs. Wenhua Liu and Evelyn Ralston (Light Image Section, NIAMS, NIH) for sharing the software, quote the 2014 Liu & Ralston paper and write us a mail about the publication. Thanks!

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References

Liu, W., and Ralston, E. (2014) A new directionality tool for assessing microtubule pattern alterations. Cytoskeleton (Hoboken) doi: 10.1002/cm.21166 Epub 2014 Febr 4.

Randazzo D, Khalique U, Belanto JJ, Kenea A, Talsness DM, Olthoff JT, Tran MD, Zaal KJ, Pak K, Pinal-Fernandez I, Mammen AL, Sackett D, Ervasti JM, Ralston E (2018) Persistent Upregulation of the β-tubulin tubb6, linked to muscle regeneration, is a source of microtubule disorganization in dystrophic muscle. Hum Mol Genet. 2019 April 1, 28 (7), 1117-35. doi.org/10.1093/hmg/ddz035