

The stack to be segmented should be a 3-color RGB (24-bit) confocal stack containing the signals of the nuclear stain (TOPRO in our case, could be DAPI as well), plus two additional nuclear markers (such as gene alleles). The stack should have a sufficient thickness, so that a decent fraction of nuclei is embedded entirely within the stack. As a final output, the program creates small stacks containing individual nuclei. The procedure consists of two steps: a) segmentation of the stack, performed on 8-bit file containing only the TOPRO signal, b) extracting the individual nuclei out of the original RGB stack. Thus, before segmentation, one should prepare a separate 8-bit stack containing only the TOPRO channel from the corresponding original RGB file. The procedure below describes the segmentation of the example stack provided within this online folder.

1. Download “**TRKB\_BDNF\_TOPRO.tif**” which is your original RGB (24-bit) stack; you can also download the corresponding 8-bit “**TOPRO.tif**”, or you can make it yourself using ImageJ (Image→Color→Split channels).
2. Download, and unpack the Segmentation magick.zip file. Open the unpacked “Segmentation magick” folder.
3. Double click on the “**segmentation\_magick.exe**” icon. As a result, two windows appear: a “*console*” window, showing the current status of the segmentation process, and an “*Open Image File*” window.
3. In the “*Open Image File*” window, check “**TOPRO.tif**” 8-bit stack to be processed. While segmentation is in progress you should not close the “console” window. After segmentation is completed, two files are created and saved: “**TOPRO\_contours.tif**”, and “**TOPRO.msk**” that contain respectively the nuclei contours to be reviewed, and “masks” with geometrical information.
4. In the “Segmentation magic folder” open “**cutting\_tool.exe**” file. As a result, two windows appear: a “console” window showing the current status of the operation, and a “*tk*” window.

5. In the “*tk*” window check “Cut entire nuclei” option and click “Start”; as a result, an “*Open Image File*” window appears.

6. Using the “Open Image File” window, open the original “**TRKB\_BDNF\_TOPRO.tif**” file; then “*Open Mask File*” window appears.

7. In the “Open Mask File”, check the “**TOPRO.msk**” file created before. The individual nuclei are being cut out of the stack based on the information contained in “masks”, and are saved in the same folder in which “**TRKB\_BDNF\_TOPRO.tif**” image file has been saved.

8. Review every individual nucleus, delete if it is not properly segmented, or if it is not neuronal. The folder “nuclei TRKB\_BDNF\_TOPRO”, provided within the online folder, contains only the nuclei that passed the “quality control”.