Instructions for reproducibility of Ignácz et al. 2023 MBoC Brief Report entitled „*Dendritic effects of genetically encoded actin labelling probes in cultured hippocampal neurons*”

To evaluate **FRAP recordings**, as seen in **Figure 1** and **Supplementary Video 1**,

1. first create smaller images of the spines that you want to analyse, and
2. create a ROI for the spine, a dendritic reference, and background.
3. Name the ROI files identically to the cut-out images.
4. Segment your time-lapse recordings using „*Running\_Ilastik\_model-macro\_multi-ch*” macro.   
   Name the image postfix „\_segmented” in the code, and make sure that you select the correct timelapse-image model to run (“*Timelapse spine models”* / either *actin* or *homogen* SD-timelapse, corresponding to the expressed construct).
5. After you have the segmented images and the ROI files ready, run “*Actin-spines-FRAP*” macro.
6. The macro will generate excel tables with 3 columns: a spine intensity, a reference branch intensity, and a background intensity column.
7. Using Excel, do the following:
   1. subtract background from the spine and reference ROIs in every time-point (rows).
   2. divide spine intensity by reference intensity in every time-point (rows).
   3. calculate the mean of the 10 pre-bleach time-points.
   4. divide the 10 pre-bleach and the post-bleach time-points by the mean of the 10 pre-bleach time-points.

To evaluate **filopodial motility**, as seen in **Figure 2** and **Supplementary Video 2**,

1. Create a substack of the GFP or mCherry channel, containing the post-bleach time-points.
2. Run “*Running Ilastik model macro multi-ch*” macro, with the necessary adjustments for the channels. Select the Ilastik model from “*Timelapse spine models”* / either *actin* or *homogen* SD-timelapse, corresponding to the expressed construct.
3. After the segmentation is ready, open the small images and threshold them from 0 to 2 values. This will include the spines (coded with 1 a.u. pixel value) and shaft (2 a.u. pixel value) but exclude edge and background.
4. Use a gaussian blur of 2px on the resulting image.
5. Select a polygon ROI around the spine, and use *Dendritic Filopodial Motility Analyzer* plugin downloaded from <https://cnblab.elte.hu/dfma>
6. Save the coordinates for later use.

To evaluate **spine morphological parameters** as seen in **Figure 3E-H**,

1. Create “*stage0”* images by max-projecting and cutting out regions of interests, to result in single-channel 16-bit greyscale images.
2. Use “*Running Ilastik model macro multi-ch*” macro, with the post-fix “stage1” and to create the first stage images. Choose the right model for the image in question from the *strong/weak actin* models for spine-enriched signal, and *strong/weak EGFP* for more evenly distributed signal.
3. Use “*universal\_Stage1-correction*” macro to manually cut out the non-interesting image parts and supervise the segmentation, also correct possible errors on the images, using the drawing tools. The color can be set as a shade of grey corresponding to the different segmentation categories.

**TIPS:** *In this step you can create a polygon selection around the dendritic branch that you want to analyse, then press ‘clear outside’. It is important to set black/white as colors at this step, and set them back afterwards.*

1. Use “*Running Ilastik model macro multi-ch*” macro 2 more times: once with the *Skeleton* model, and once with the *Stage2* model, and as before, change the post-fix accordingly. Use the corrected stage\_1 images for further analysis in both cases.
2. Use “*Spines\_Stage2-Skeleton\_correction*” to correct both the Skeleton and the Stage2 segmentations.

**TIPS:** *Make sure that you only have a single spine head and neck in the stage2 images per spine, when you correct the images. Also, for the skeletons, have a standard thickness, as fluctuations in thickness can be perceived as branching in skeleton analysis.*

1. In the end you need to have a \_stage0, \_stage1, \_stage2 and \_skeleton version of the images. These are what you need to analyse using “*Spines Analysis*” macro. Using the macro, you will end up with a csv table for each analysed image, with the spine morphometric details that you need to summarize to a new excel table. Also, the Analysis macro creates a .zip file with the ROIs that you can use for intensity measurements.

To evaluate **spine-to-shaft signal distribution** as seen in **Figure 3B**,

1. Have all the the .czi images and the spine ROIs in a folder
2. Open “*Intensity\_measurements\_spines*” macro, and check for the post-fix in raws 9 and 13. Set them to your own system of nomenclature.
3. Run the macro on your images. It will result in an excel sheet for each image and an updated ROI file that contains the dendrite and background ROIs as well. In the last rows of the macro you will find which column corresponds to which data in the excel sheet.

To evaluate **dendritic tree elaboration** as seen in **Figure 4,**

1. First do the batch-processing of the images with Ilastik. For this, use “*Running Ilastik model macro multi-ch*” macro on your images. At line 11, modify the postfix for the saved file to “Sholl.tif”, for the further processing steps. Also, include or exclude the initial lines according to your image type (multi- or single-plane, multi- or single-channel). Include the binarizing steps at the end, as the next steps are going to use binary images.
2. After you have the images, use “*Sholl-correction*” macro to correct the images. Typically, remove signal from other cells in the image or background that has been mistakenly segmented as foreground. Also remove axons and large dendritic spines if possible and correct dendritic tips if they are detected as fusion with a branch.
3. Analyse Sholl profiles with Analyze/Sholl/Sholl analysis.
4. For endpoints and total length, use “*Dendrite-length*” macro to first segment the binary image further, then to correct the segmentations. Endpoint number will appear in the log window, and skeleton data will appear as a Results table. The Results table contains the different branch lengths which you can copy into an excel sheet and sum up at the end of the column.