**Analysis of Ca+ signal image experiments**

All the needed scripts are saved into the **Scripts** folder.

Image Processing

1. Setup.

* **Naming** of each experiment consistently:
  + Date + line + Abeta treatment + Drug or Vehicle (i.e. 2021.11.18\_EDi030\_Ab+\_Drug)
  + IMPORTANT: by now the name may affect the script Split\_Conditions.rmd (it searches for Ab+/- and Drug/Vehicle)
* **Output** of the experiment:
  + Each experiment is recorded as a .avi movie.
  + .lif file with the four experiments.
* As example, in the pilot study, three replicates were made, where 4 wells were tested in each replicate (experiments):
  + 2 wells treated with AD human brain homogenate Abeta immunodepleated (concentration 15pmol/L, 24h).
    - 1 well treated with CT1812-6 (concentration x).
    - 1 well treated with Vehicle (DMSO concentration x).
  + 2 wells treated with AD human brain homogenate Abeta mock immunodepleated (0 pmol/L, 24h).
    - 1 well treated with CT1812-6 (concentration x).
    - 1 well treated with Vehicle (DMSO concentration x).

2. From raw to image stacks.

* Save the .lif file into a new folder called **1\_Raw**.
* Run the script “**Lif\_to\_Stacks.ijm**”. Just drag and drop into ImageJ and press run.
* It creates a new folder (**2\_Stacks**) with the stacks of images of each condition.

3. Correct the time drift (registration).

Optional. In some experiments the cells or the camera moves. If so, apply the following correction.

* Run the script “**Drift\_correction.ijm**”. Just drag and drop into ImageJ and press run.
* Select the folder where the image stacks are saved (2\_Stacks).
* Takes long time (may be 20 minutes per stack).
* It creates a new folder (**3\_Aligned**) with the aligned stack of images.
* Compare the stacks saved at 3\_Aligned with the 2\_stacks.

4. Identify neurons (segmentation).

* Run the script **Segmentation\_mip** in Matlab.
* **read\_stackTiff.m** should be present in the same folder of the script.
* Similar to array tomography, there are two main parameters to choose:
  + Filter size (window size): Should be similar to the size of the object.
  + Background (c. factor): the sensitivity, higher the value, more tolerance.
* Also can be chose the size of the objects.
* The output is a max intensity projection (2D) that are save into a new folder (**4\_Masks**).
* Parameters are saved at the same folder as .xls file.

5. Select axons (watershed).

* Run the script **Watershed.ijm** in ImageJ.
* It breaks the mask into smaller pieces (hopefully axons and cell bodies).
* Tolerance of watershed can be changed into the script.
* A new folder is created (**5\_Watershed**).

6. Find intensities over time.

* Create a new folder called **6\_Intensities**.
* Move to the folder the watershed images (5\_Watershed) and the original stacks (2\_Stacks or 3\_Aligned).
* Run the script **Intensity\_all\_cells.m** in Matlab.
* **read\_stackTiff.m** should be present in the same folder of the script.
* Mean intensities are recorded for every object in every frame as well as the size of the object. Can be changed to i.e. Max Intensity.
* A new folder (**7\_Analysis**) and a file (**Intensities.csv**) will be created.
* Move to the top folder the 7\_Analysis folder.

Signal Processing

7. Intensity processing: Smooth

Optional. Recommended to remove low variations of intensity.

* Run **Smooth.rmd** in R.
* Need to be changed the path to the Intensities.csv.
* Can be decided the size of the smoothing (bandwidth).
* Can be checked individual cells as example.
* A new file is created “inputname”\_Smooth.csv(i.e. **Intensities\_Smooth.csv**).
* IMPORTANT: The Intensity column are now smoothed values.

8. Intensity processing: Bleaching correction

Optional. Recommended to correct the loss of intensity over time due to bleaching.

* Run **Bleaching\_correction.rmd** in R.
* Need to be changed the path and filename (i.e. Intensities\_Smooth.csv).
* Can be decided the type of correction (polynomial or top-bottom linear). Set to top-bottom linear).
* Can be checked individual cells as example.
* A new file is created “inputname”\_BleachCorr.csv(i.e. **Intensities\_Smooth\_BleachCorr.csv**).
* IMPORTANT: The Intensity column are now corrected values.

Signal Analysis

9. Split conditions (treatments)

Optional. Provably needed in most experiments.

* Run **Split\_Contitions.rmd** in R.
* Need to be changed the path and filename (i.e. Intensities\_Smooth\_BleachCorr.csv).
* Can be decided at which frames split the data and the names given (i.e. Baseline, Treatment,…), the drug (i.e. vehicle,…) and the pretratment (i.e. Abeta, positive or negative).
* Can be checked plotted conditions cells as example.
* A new file is created “inputname”\_Conditions.csv(i.e. **Intensities\_Smooth\_BleachCorr\_Conditions.csv**).
* IMPORTANT: New columns are created for the new variables (i.e. Treatment, Drug, Abeta).

10. Stats

Here depends on what to analyze: Single experiments, merge all experiments, intensities,…

By now there are the following scripts to be explored:

* **Single\_Experiment\_Stats.rmd**.
  + The input is the .csv file of an experiment with the intensities.
  + Is performed the analysis of absolute values of Baseline and Treatment frames and the ratio Treatment/Baseline in every object.
  + IMPORTANT: Two .csv files are created: inputname**\_BaseTreat.csv** and inputname**\_ratio**.**csv**.
* **Merge\_Experiments\_tool.rmd**.
  + The input are the .csv from different experiments (i.e. inputname\_ratio.csv created in the Single\_Experiment\_Stats.csv from three individual experiments.
  + Single cases can be removed.
  + A column “Experiment” is created.
  + All the experiments are concatenated and saved as .csv (i.e. **All\_Experiments\_ratio.csv**)
* **All\_Experiments\_Stats.rmd**.
  + The input are all the experiments in two files. One with absolute intensities of the Baseline and Treatment frames. And the other with a ratio of intensities Tratment/Baseline in every object
  + IMPORTANT: The input files are created by the Single\_Experiment\_Stats.csv and Merge\_Experiment\_tool. But can be created by you if preferred.