***Macro for processing HEK 293T titrations***

The data is processed in two steps. First, the images are processed to remove noise pixels, which have a strong impact on the ratio values. We also use a local-thresholding algorithm on the red-channel instead of a fixed value due to the different levels of expression obtained with transient transfection of the cells. The excellent translocation of the indicator into the membrane allows removing most of the cytosolic signal during this step as well.

**Script 1: Process data**

name=getTitle()

bname=File.nameWithoutExtension

green="C1-"

red\_488="C2-"

run("Split Channels");

selectWindow(green+name);

rename("green");

selectWindow(red\_488+name);

rename("red\_488");

// Noise reduction

selectWindow("green");

run("Gaussian Blur...", "sigma=1 stack");

selectWindow("red\_488");

run("Median...", "radius=2 stack");

// Creating Thresholding Stack using 1 value of red 488 channel

selectWindow("red\_488");

run("Duplicate...", "duplicate");

run("Auto Local Threshold", "method=Phansalkar radius=60 parameter\_1=0 parameter\_2=0 white stack");

run("Divide...", "value=255.000 stack");

rename("threshold\_red");

// Thresholding channels

imageCalculator("Divide create 32-bit stack", "green","threshold\_red");

rename("thresholded\_green");

imageCalculator("Divide create 32-bit stack", "red\_488","threshold\_red");

rename("thresholded\_red\_488");

// Getting ratio

imageCalculator("Divide create 32-bit stack", "thresholded\_green","thresholded\_red\_488");

//run("Brightness/Contrast...");

setMinAndMax(0, 4);

run("Fire");

rename("ratio\_488");

**Script 2: Execute after drawing the ROI’s.**

file="File\_name"

open\_path="\\ROI’s directory"

saving\_path="\\saving directory"

roiManager("Open", open\_path+file+".zip");

selectWindow("ratio\_488");

roiManager("Show All");

roiManager("Multi Measure");

saveAs("Results", saving\_path+file+"\_ratio488.csv");

selectWindow("thresholded\_green");

roiManager("Show All");

roiManager("Multi Measure");

saveAs("Results", saving\_path+file+"\_green.csv");

selectWindow("thresholded\_red\_488");

roiManager("Show All");

roiManager("Multi Measure");

saveAs("Results", saving\_path+file+"\_red488.csv");