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Open software, select ①“protocols” and ②“create new”

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Select ① “procedure” and ② click “set temperature”. In the pop-up window, set temperature to 37˚C and gradient to 1˚C, click OK. ③Select your plate type of choice from the dropdown menu. ④ Add imaging step by clicking “image, select inverted imager and click “OK”.

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① Add 3 imaging channels and set them to DAPI, RFP and brightfield. ② Tick “Montage” box and set desired number and location of images. 2x2 for a 48 well plate is a common choice. If desired, change the overlap to represent different parts in each well. ③ Select which wells are to be imaged. A new window will open where wells of interest can be selected.

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① Click “focus options” to set focus mode. A new window will open. Each channel needs to be set individually. Commonly wells are autofocused on the “DAPI” channel. ② All other channels are set to “fixed focal height from first channel” and set by clicking “OK”. Close window.

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Start pre-setting data reduction steps by clicking ① “Data Reduction” and select ② “Image Preprocessing”. Setting up this step reduces fluorescence background. Accept default settings by selecting “OK”. A new set of images will be created with the prefix “TSF”. Original images will be preserved.

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Set up a step to count cells in wells by selecting ① “Cellular Analysis”. Select “TSF DAPI” images from the “channel” dropdown menu. Further details must be set after imaging has been performed. This step can also be considered data reduction. Always make sure to keep original images.

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Prepare analysis of RFP (calcification) signal by selecting ① “Statistics”. In the pop-up window ② label step and select “TSF RFP” as input channel. ③ Tick “upper value” and “lower value” boxes. ④ Tick box for “Total Area” in the lower list. ⑤ Select “none” in the “Colour effect” column. In the pop-up window ⑥ click “Custom” and tick “Background” box. This will colour code the results from low – high for easy assessment. Press OK.

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In order to create a meaningful read out, the RFP signal “total area” needs to be divided by cell count. To set-up this step, ① Press “Ratio” on the left side. ② In the pop-up window select for Data in 1 “RFP quantification: Total area” from drop down menu. Further select “Cell count” as data input 2.

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①Finally select a “New Data Set Name” and press OK and OK again. ② Select “Color Effect” as described previously ③ In the upper left corner select “File” and save file as protocol (.prt file type).