SPAchip® cell-based Assays- Image Analysis Pipeline

Reference Software: ImageJ/FIJI 2.14.0

Image acquisition:

Follow the instructions in the user protocol of the corresponding CytoCHECK SPAchip® detection kit to select the parameters and conditions for obtaining the images of your experiment.

1. Select brightfield channel so that you can easily focus your sample and help you when segmenting the image if it is needed. For SPAchips shining in green and cells marked in red, commercial Alexa488 and calcein red channels are suggested.
2. Take at least four planes in Z axis within a separation of approximately 1µm (3µm as total slice size). Maximum projection will be useful for image segmentation.
3. To ensure representative sampling, define at least four fields to capture the images inside each culture well.
4. Try to work under the minimum possible exposure times and laser/LED power to preserve the viability of living cells and optimize the procedure. Each channel could need different exposure parameters.
5. Take one test with the defined conditions before going through the next working block to assure cells and chips are correctly focused in your whole plate.

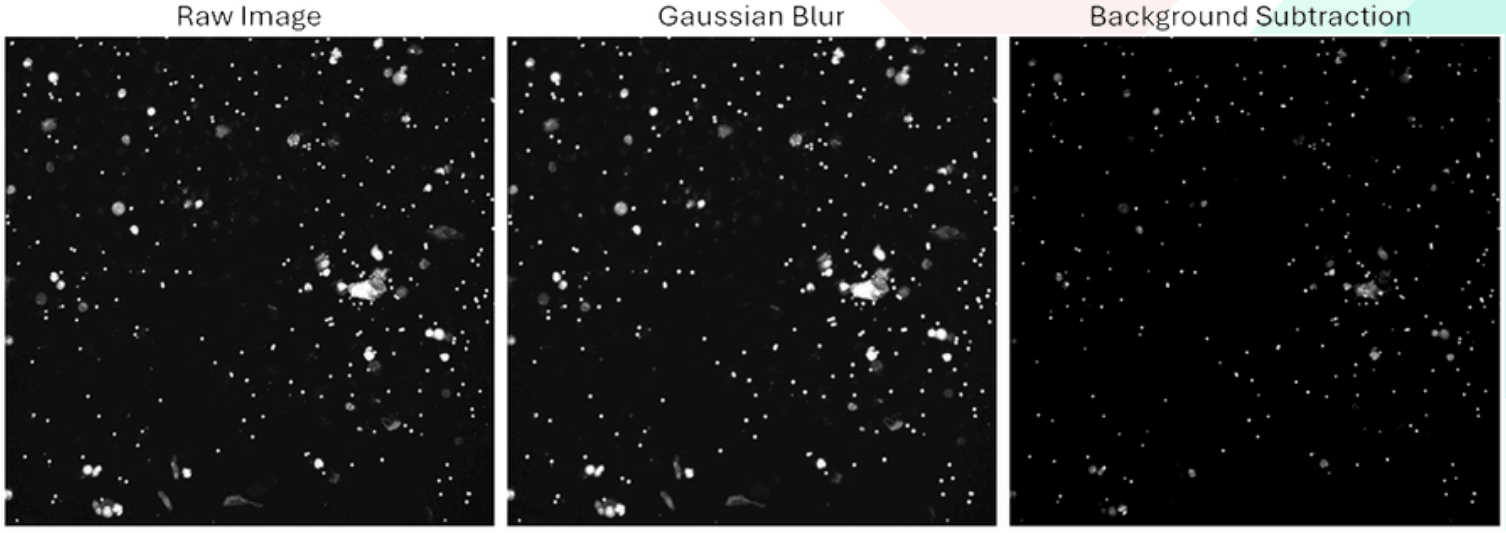
Image Analysis:

- **Define the input image(s):**

The images at the different planes in Z axis can be projected using a maximum projection (Image>Stacks>Z Project).

- **Preprocess the image (if needed):**

Over the maximum projection of the channel in which SPAchip® were acquired, run Gaussian blur (Process>Filters>Gaussian Blur) (i.e. Sigma (Radius):2). Follow through substraction of the background (Process>Substract Background). Duplicate the image with the background substracted (Image>Duplicate).



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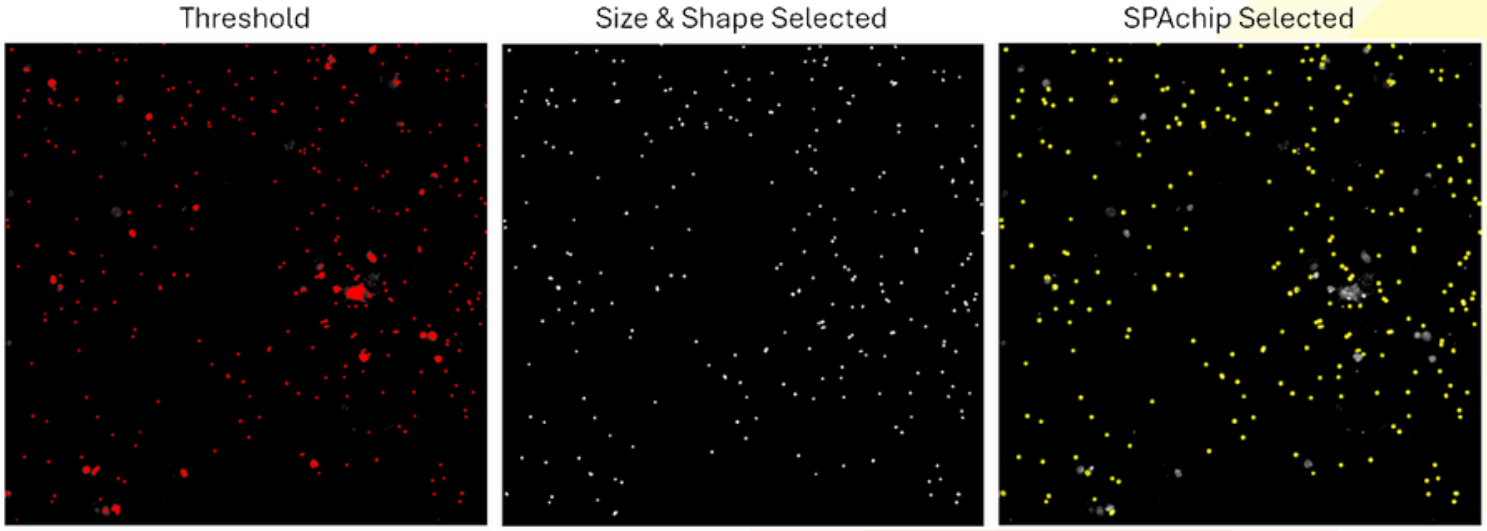
- **Find total SPAchip®:**

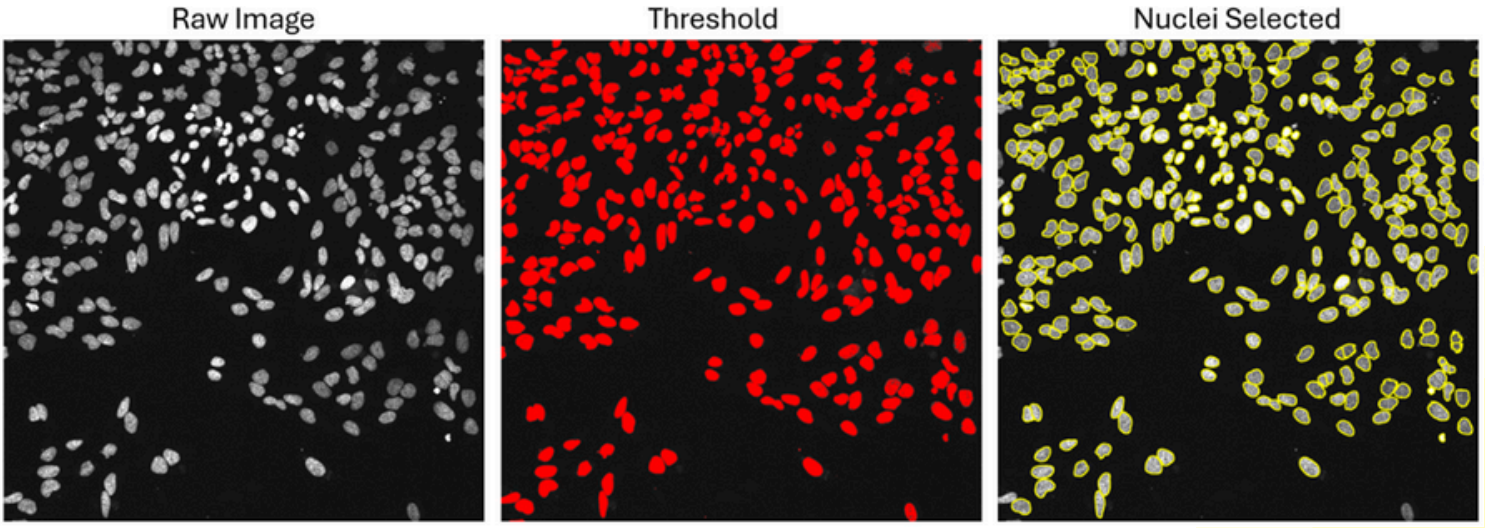
Adjust the Threshold to identify all the SPAchip® present in the image (Image>Adjust>Threshold). Generate a mask over the thresholded image (Edit>Selection>Create Mask). Separate the SPAchip® that are too close to each other using the watershed option (Process>Binary>Watershed). Continue with the segmentation of the SPAchip® based on size and circularity (Analyze>Analyze Particles) (IMPORTANT: in the option Show it should display Masks). Invert the image generated (Edit>Invert). Generate a selection over the selected SPAchip® (Edit>Selection>Create a Selection) and added it to the ROI manager (Analyze>Tools>ROI Manager>Add (t)). This region can be renamed to the "Total SPAchip" (at the ROI Manager window, select the ROI and Rename). The mean intensity of each individual SPAchip® in the selected population of "Total SPAchip" can be measured, see below.



- **SPAchip® intracellular detection via cell segmentation:**

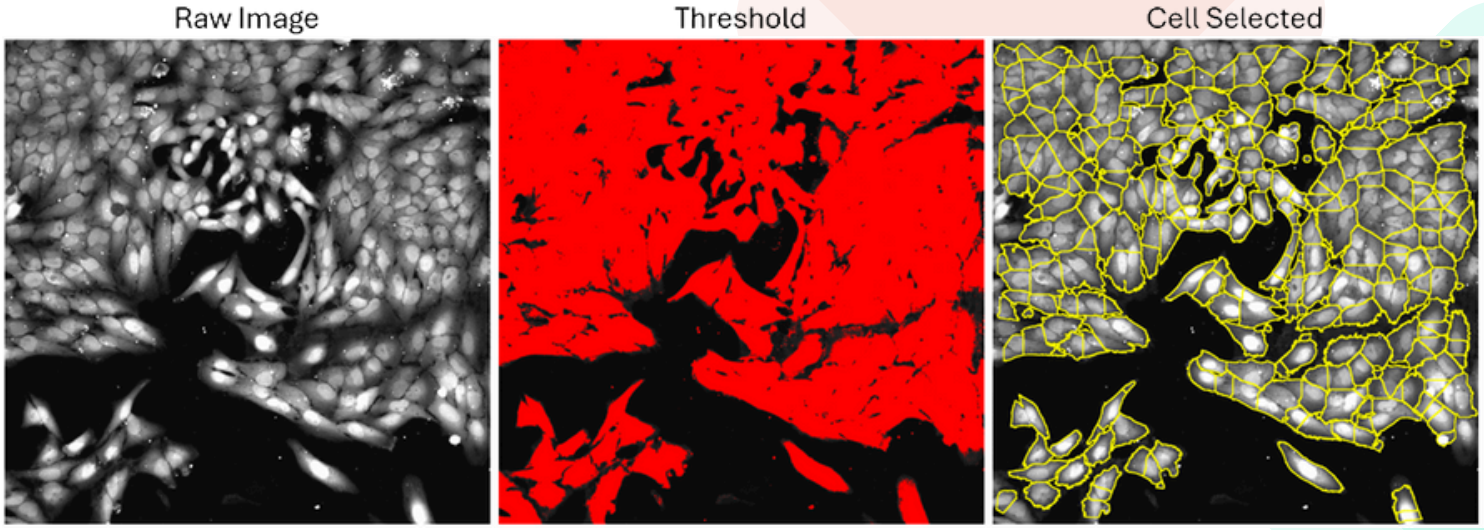
i. Nuclei Segmentation:

Duplicate the channel where the nuclei were imaged (Image>Duplicate), run a threshold selection (Image>Adjust>Threshold). Generate a mask from the thresholded image (Edit>Selection>Create Mask). Fill the empty regions within the nuclei (Process>Binay>Fill Holes) and follow separating the nuclei that are too close to each other using the watershed option (Process>Binary>Watershed). To get rid of micronuclei, you can further segment the nuclei through particle analysis, to try to eliminate the smallest objects (Analyze>Analyze Particles) (IMPORTANT: in the option Show it should display Masks). Invert the image generated (Edit>Invert). Generate a selection over the selected nuclei (Edit>Selection>Create a Selection) and added it to the ROI manager (Analyze>Tools>ROI Manager>Add (t)). This region can be renamed to the "Nuclei" (at the ROI Manager window, select the ROI and Rename).



ii. Cell Segmentation:

Using a cell marker or digital phase contrast you can identify the cells to be able to distinguish, afterwards, whether the SPAchip® are inside or outside the regions defined as cells. As in the previous steps, you will duplicate the channel were the cytoplasm or the digital phase contrast was acquired (Image>Duplicate), run a threshold selection (Image>Adjust>Threshold). Generate a mask over the thresholded image (Edit>Selection>Create Mask). Separate the cells that are too close to each other using the watershed option (Process>Binary>Watershed). Fill the empty regions within the cell (Process>Binay> Fill Holes). To get rid of the smaller particles, you can further segment the cells through particle analysis, to try to eliminate the smallest objects (Analyze>Analyze Particles) (IMPORTANT: in the option Show it should display Masks). Invert the image generated (Edit>Invert). Generate a selection over the selected cells (Edit>Selection>Create a Selection) and added it to the ROI manager (Analyze>Tools>ROI Manager>Add (t)). This region can be renamed to “Cell” (at the ROI Manager window, select the ROI and Rename).



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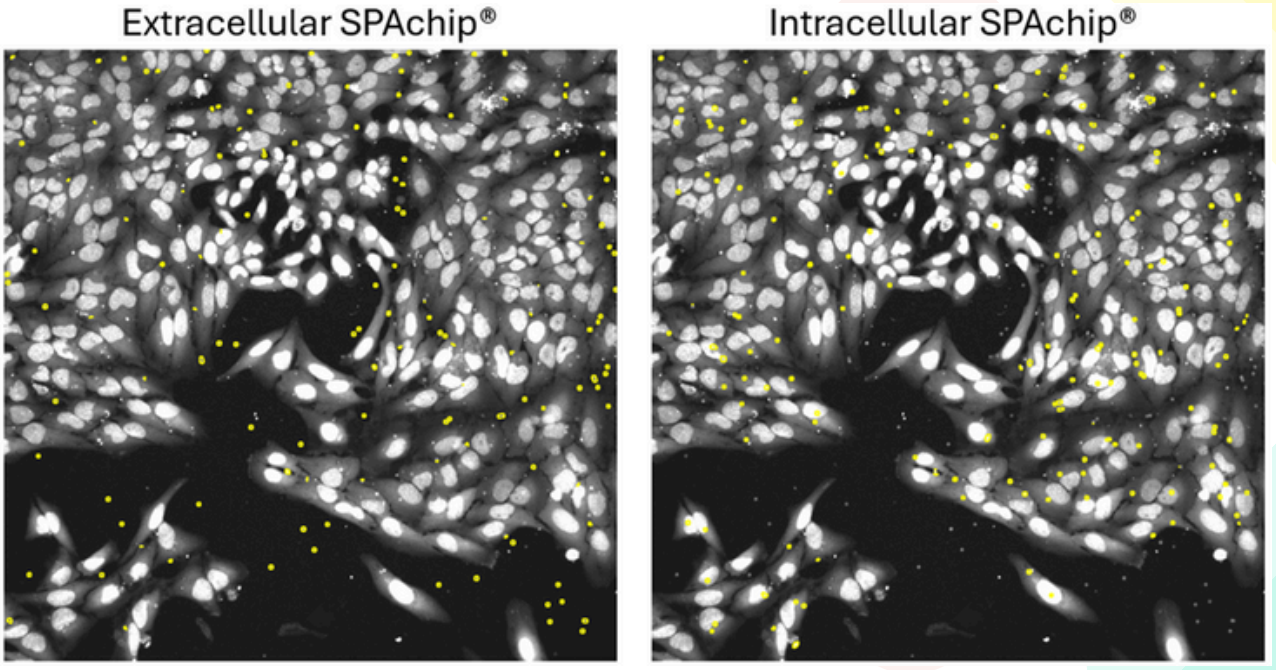
iii. Define extracellular SPAchip®:

Once the cells have been identified you can define only the SPAchip® that are outside the cells. At the ROI manager combine the regions of “Total SPAchip” with “Cell” (ROI Manager>Select “Total SPAchip” and “Cell”>More>OR (Combine)). Through this process you will generate a new region that will consist of the Cell and the subgroup of SPAchip® not present inside the cell (extracellular). Add this new region to the ROI manager (ROI manager>Add(t)). You can rename this region at the ROI manager to “Cell+Extra” (ROI Manager>Rename). In order to identify the extracellular SPAchip®, at the ROI manager exclude the region of the “Cell” from the “Cell+Extra” (ROI Manager>Select “Cell” and “Cell+Extra”>More>XOR). Add this new region to the ROI manager (ROI Manager>Add(t)). You can rename this region at the ROI manager to “Extracellular SPAchip” (ROI Manager>Rename).

iv. Define intracellular SPAchip®:

Once the extracellular SPAchip® have been identified you can identify the intracellular SPAchip®. At the ROI manager select the region “Total SPAchip” and exclude the “Extracellular SPAchip” (ROI Manager>Select “Total SPAchip” and “Extracellular SPAchip”>More>XOR). Add this new region to the ROI manager (ROI Manager>Add (t)). You can rename this region at the ROI manager to “Intracellular SPAchip” (ROI Manager>Rename).



- **Calculate and measure SPAchip® Spot Intensity Properties:**

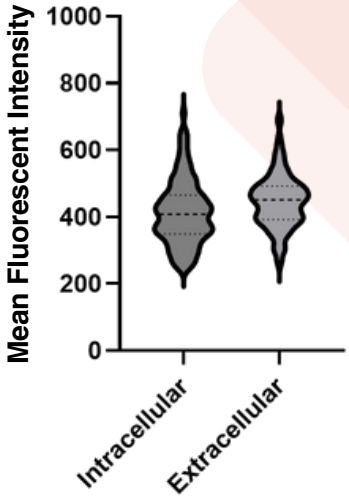
Once all the regions have been identified, we can measure the mean intensity of each individual SPAchip®. Select the channel where the SPAchip® were imaged, with the background subtracted. At the ROI manager, select the region “Intracellular SPAchip” and individualize each SPAchip® (ROI Manager>More>Split). Afterwards, select all the new regions, which correspond to each individual intracellular SPAchip®. At the ROI manager, measure the mean intensity of each individual intracellular SPAchip® (ROI Manager>Measure).

Export the values provided into a .csv format for further statistical analysis. Close the results window. If you want to analyze the extracellular SPAchip, delete the regions of each individual intracellular SPAchip®. At the ROI manager, select all the individual intracellular SPAchip® and delete (ROI Manager>Select the regions> Delete). Select the region "Extracellular SPAchip" and individualize each SPAchip® (ROI Manager>More>Split). Afterwards, select all the new regions, which correspond to each individual extracellular SPAchip®. At the ROI manager, measure the mean intensity of each individual extracellular SPAchip® (ROI Manager>Measure). Export the values provided into a .csv format for further statistical analysis.

	Area	Mean	StdDev	Min	Max
1	0.003	258.484	11.682	240	286
2	0.012	489.117	125.552	252	671
3	1.09E-04	304	0	304	304
4	4.34E-04	459.75	108.325	335	572
5	1.09E-04	300	0	300	300
6	0.012	397.287	105.372	241	587
7	0.02	299.078	44.29	241	386
8	0.002	338.682	63.73	244	442
9	0.012	429.818	112.922	247	609
10	0.012	409.345	103.793	241	573
11	0.016	345.106	73.559	241	485
12	0.011	397.324	92.255	244	547
13	0.006	454.691	115.734	241	620
14	0.01	566.374	248.569	241	1091
15	1.09E-04	286	0	286	286
16	2.17E-04	319	24.042	302	336
17	0.009	354.356	60.8	245	445
18	1.09E-04	329	0	329	329
19	8.68E-04	459.125	234.037	242	891
20	0.002	345.933	72.845	244	483

Quantitative and Statistical Data Analysis:

Perform the desired quantitative and statistical analysis using a statistical program package such as GraphPad Prism 10.



A4Cell can help you

We understand this process can be time-consuming and tedious. To better assist you, our image analysis experts have developed different pipelines (ImageJ/FIJI Macros) designed to streamline your image analysis workflow and help you achieve your desired results more efficiently. Please feel free to contact us if you need further suppport at: info@a4cell.com.