SPAchip® cell-based Assays- Image Analysis Pipeline

Reference Software: Harmony 5.1 (Revvity)

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. Define 4 fields to capture the images inside each culture well.
4. Try to work under the minimum possible exposure time and power of laser so as not to compromise 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):**

Upload the original input image data from your selected experiment as the first step of your image analysis. These images will be analyzed by the following image analysis steps. If different Z-stacks were acquired, decide if individual planes will be analyzed separately, or if an analysis of the maximum projection is preferred.

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

The quality of the images can be improved using a filter mask to adjust saturation, smooth the image to reduce the noise, etc.

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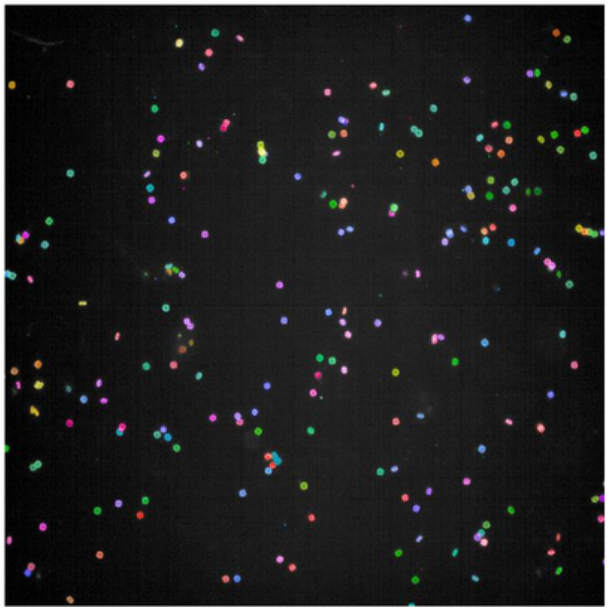
- **Find total SPAchip®:**

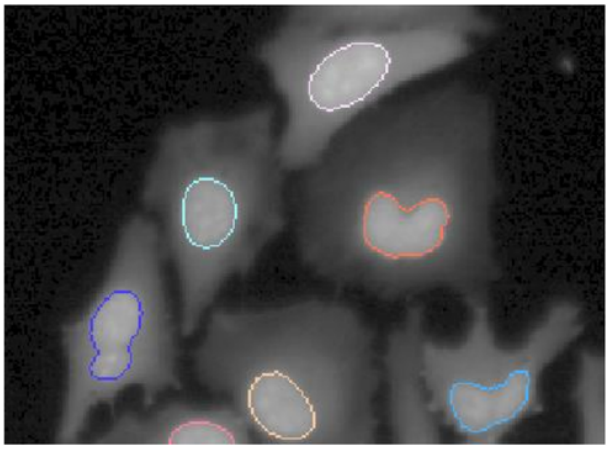
Select “Find Spots” using the appropriate fluorescence channel (depending on the SPAchip® detection kit). Adjust parameters such as relative spot intensity, splitting sensitivity, detection sensitivity, and background correction to optimize the total of spots detected.



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

i. Nuclei Segmentation:

Select “find nuclei” building block to detect nuclei in a nuclei-stained image using the appropriate fluorescence channel (for example, stained with Hoechst 33342 for live cells). Test the different methods and values such as common threshold, area size, splitting coefficient, individual threshold, and contrast, to find the right nuclei segmentation for your specific cell type. If needed, to improve the detection of “find nuclei” step (and if for example small or big areas are detected as nuclei), use “select population” and adjust area size and circularity parameters of the nuclei.



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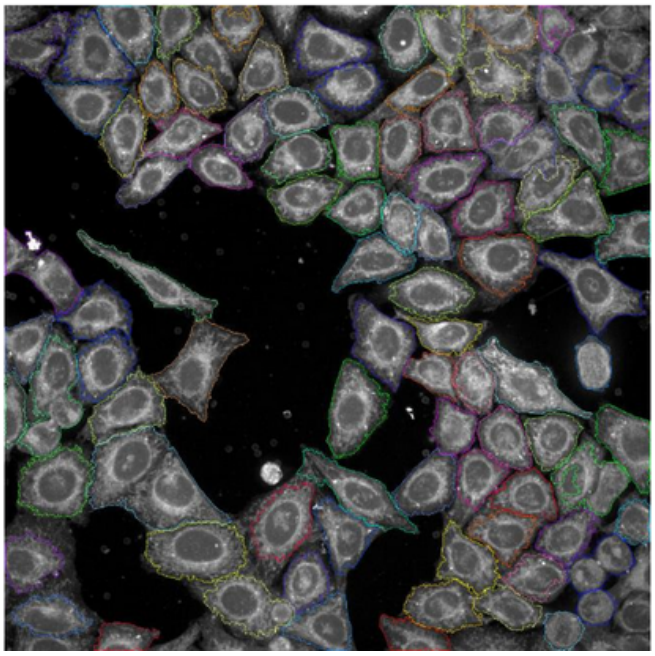
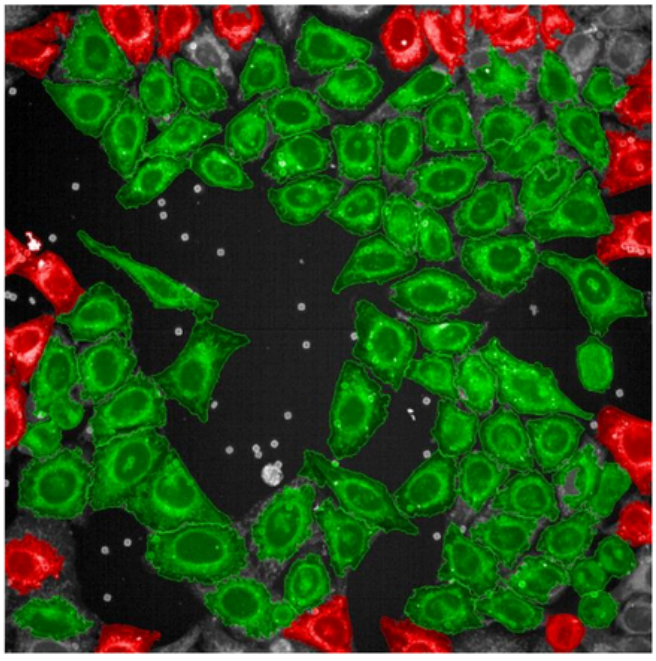
ii. Cell Segmentation:

Select “find cytoplasm” building block to detect the cytoplasm around the previously detected nuclei. Use the appropriate fluorescence channel (for example, stained with Cellmask). Test the different methods and adjust parameters as needed to comprise the whole cell size (it will vary depending on the cell line). Restrict the region size and optimize the common and individual threshold to optimize the segmentation results.



iii. Select Cell Population (if needed):

Use “select population” to remove cells within the borders of the well.



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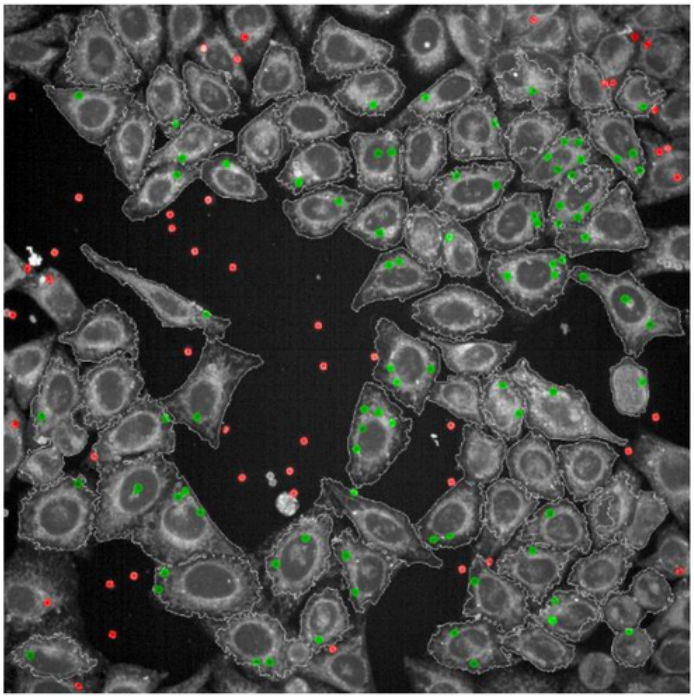
iv. Define intracellular SPAchip®:

Define the cell as the region of interest (ROI) to include only the spots inside the cells. To define the cells, select nuclei and cytoplasm as restrictive areas. Calculate morphology properties of spots: size area (µm²), roundness, and ratio width to length. Calculate intensity signal of the spots. We recommend restrict the intracellular SPAchip® to detected spots within the following parameters: size area >5 and <20 µm² ; roundness > 0.5-0.6 and ratio width to length > 0.5-0.6 but the criteria will be defined depending how restrictive the analysis need to be.



iv. Define extracellular SPAchip®:

First, define the cell as the region of interest (ROI) to include only the spots inside the cells. To define the cells, select nuclei and cytoplasm as restrictive areas to outline the mask region. Then, use select population and use inverted mask in order to invert the mask region; the selected spots will not overlap the mask region, comprising the extracellular SPAchip®. Calculate morphology properties of spots: size area (µm²), roundness, and ratio width to length. Calculate intensity signal of the spots. We recommend restrict the extracellular SPAchip® to detected spots within the following parameters: size area >5 and <20 µm² ; roundness > 0.5-0.6 and ratio width to length > 0.5-0.6 but the criteria will be defined depending how restrictive the analysis need to be.

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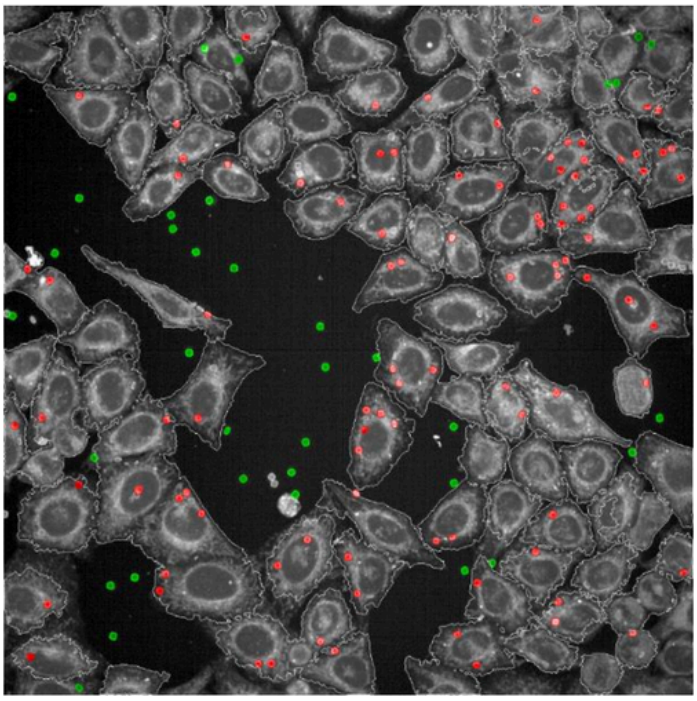

- **Calculate and measure SPAchip® Spot Intensity Properties**

Export corrected and uncorrected intensity values and their standard deviations of both intracellular and extracellular SPAchip®. The fluorescence channel was previously selected during spot detection step.

	F	G	H	I	J	K	L	M
ber o	SPAchips	SPAchips	SPAchips	SPAchips	Nuclei - N	Nuclei - N	Nuclei - C	Nuclei - C
5	14	101,53	7,64842	0,88218	68	0,08824	0,07353	0,92647
5	6	73,0633	6,94539	0,84552	111	0,04505	0,03604	0,96396
5	12	98,684	7,4746	0,87839	97	0,10309	0,10309	0,89691
5	7	75,3747	7,43652	0,82829	84	0,07143	0,07143	0,92857
5	12	80,3579	7,75272	0,85392	118	0,08475	0,08475	0,91525
5	18	96,9135	6,46382	0,86664	90	0,17778	0,15556	0,84444
5	11	88,5245	6,03867	0,85804	125	0,096	0,088	0,912
5	14	84,6169	7,87688	0,89324	118	0,1017	0,09322	0,90678
5	5	76,9502	7,84311	0,88193	135	0,01481	0,01481	0,98519
5	15	97,4082	6,34124	0,8403	129	0,09302	0,08527	0,91473
5	13	75,1767	7,09574	0,84619	98	0,09184	0,09184	0,90816
5	13	84,3235	5,50901	0,86089	107	0,07477	0,07477	0,92523
5	14	95,9137	6,14854	0,85659	117	0,10256	0,09402	0,90598
5	7	87,5711	10,9263	0,76923	86	0,05814	0,04651	0,95349
5	11	74,9234	7,86755	0,89277	80	0,0875	0,0875	0,9125
5	5	94,3316	7,64842	0,89795	94	0,03191	0,03191	0,96809
	11,0625	86,604	7,31356	0,8595	103,563	0,08266	0,07702	0,92298
5	38	98,1697	7,75699	0,8617	98	0,37755	0,2551	0,7449
5	10	113,095	7,04119	0,89237	99	0,10101	0,10101	0,89899
5	23	88,4452	8,97859	0,83181	111	0,1982	0,17117	0,82883
5	23	112,998	7,63029	0,87806	109	0,14679	0,12844	0,87156
5	33	92,6237	7,65966	0,86655	112	0,23214	0,21429	0,78571
5	25	90,7043	6,88636	0,86001	123	0,21951	0,17886	0,82114
5	32	117,194	8,31476	0,86957	107	0,25234	0,20561	0,79439
5	31	80,9629	6,83947	0,84313	121	0,19008	0,18182	0,81818
5	25	91,9862	7,20343	0,83385	96	0,16667	0,125	0,875
5	21	95,8604	6,31078	0,89377	77	0,22078	0,18182	0,81818
5	11	94,8681	7,16803	0,91553	74	0,05405	0,04054	0,95946
5	13	132,15	8,53628	0,83561	81	0,12346	0,12346	0,87654
5	35	92,7428	6,80743	0,83463	75	0,29333	0,17333	0,82667
5	17	108,02	6,62045	0,85504	70	0,18571	0,14286	0,85714
5	26	103,956	8,60582	0,86146	92	0,23913	0,19565	0,80435
5	15	81,7765	7,60516	0,84817	91	0,14286	0,13187	0,86813
	23,625	99,7221	7,49779	0,86133	96	0,19648	0,15943	0,84057

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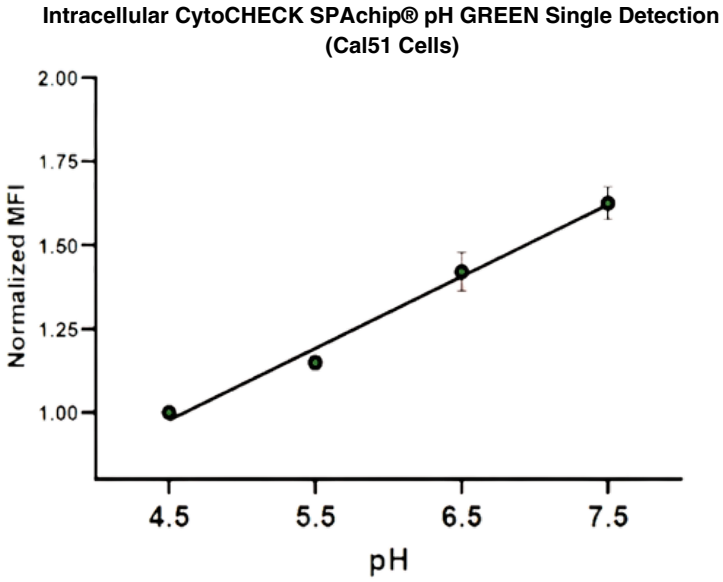
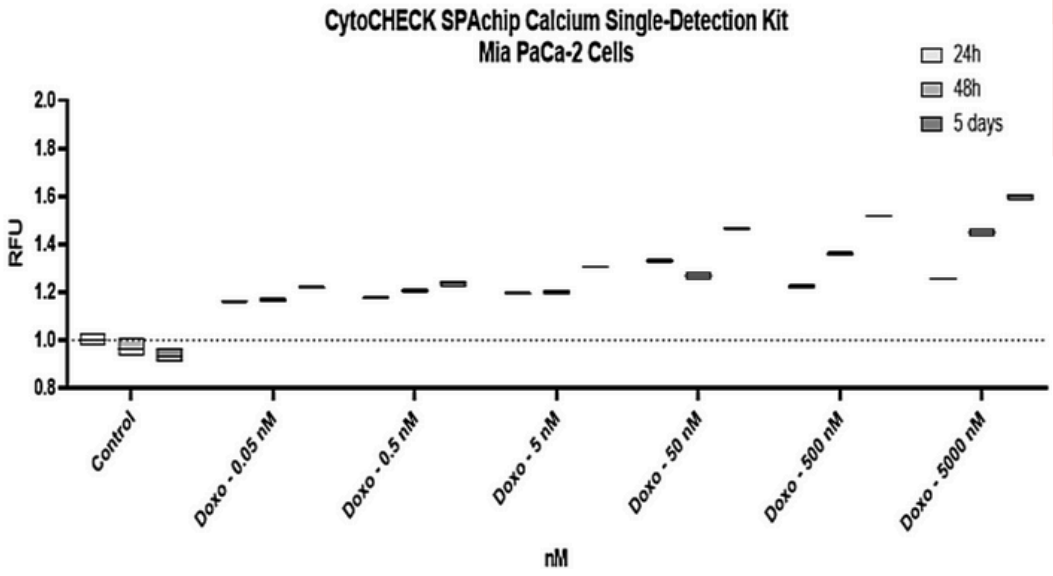
Quantitative and Statistical Data Analysis:

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

1. Image Analysis (described above): select SPAchip® fluorescent channel signal and measure the intensity of segmented Regions of Interest (ROIs) in every SPAchip® with the image analysis software.
2. Quantify the mean fluorescence of extra and intracellular SPAchip® populations.
3. Export your data to a spreadsheet software.
4. To quantify intracellular pH, use the fluorescence of the calibration wells to plot fluorescence units vs calibration pH. Interpolate the values of the sample wells to obtain the intracellular pH value of the sample wells.



Calibration curve interpolation / normalization to control conditions.



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An example of statistical data analysis is described below:

Use fluorescence intensity values corresponding mean values of all objects per condition and error bars representing standard deviations. For statistical analysis, perform multiple comparisons analysis using Two-way ANOVA test. For compound interpolation, linear regression can be performed in order to obtain the pH or Calcium values for the different drug treatment at different concentrations and times. Graphs can be plotted using normalized values of relative fluorescence units (RFU) calculated by image analysis (SPAchip® Spot Intensity values) following the methodology stated in the previous sections.

A4Cell can help you:

We understand this process can be very long and tedious. To help you our experts in image analysis have generated different pipelines (Harmony 5.1 (Revvity)) that can speed up the process. So, you can get your desired results. Please feel free to contact us at: info@a4cell.com.

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