

HCSan2 Users Guide

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High-Content Scan2 (*HCSan2*) is an *ImageJ* macro that performs an automated high-content analysis of two-channel fluorescence images. In the interactive mode the user can use the ROI Manager to view the cell/ROI corresponding to each measurement, a feature that facilitates the optimization of assay parameters. An Excel-compatible report and zipped folder containing images of each cell/ROI are generated, allowing users to archive and publish results at the cellular level.

High-content screening is based on a cell-by-cell analysis of multi-channel images similar to the one shown in Fig. 1:

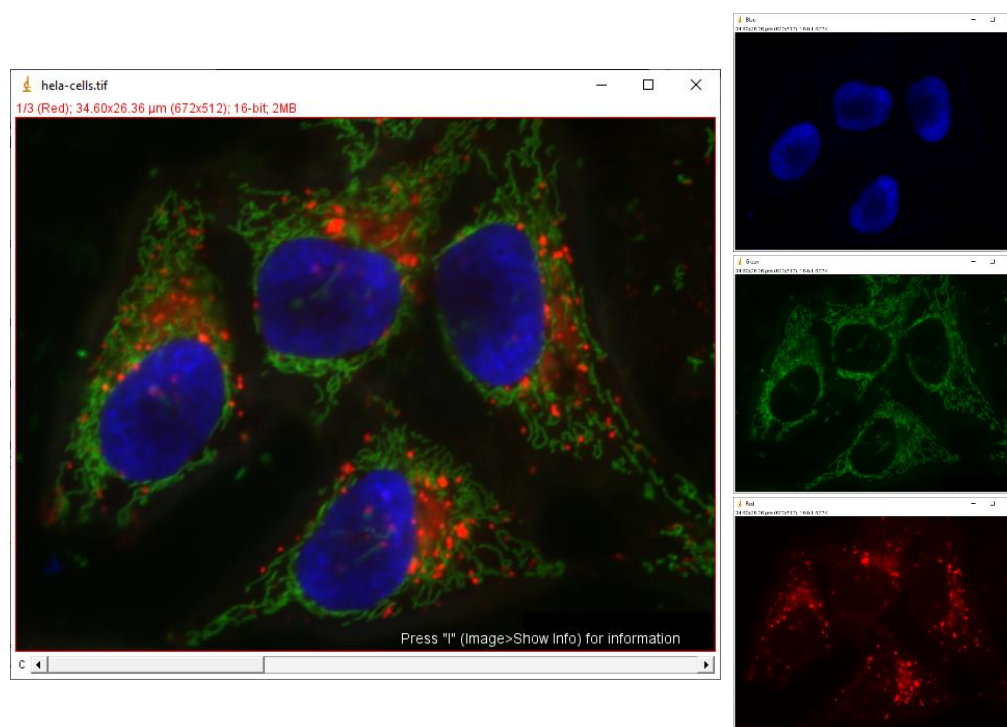


Figure 1 Three-channel fluorescence image of HeLa cells with blue nucleus, green mitochondria, and red lysosomes.

Left: 16-bits/channel composite color image, Right: Individual channel images.

Image from *ImageJ*: *Files>>Open Samples* courtesy of Tony Collins, creator of the *ImageJ* for Microscopy collection of plugins at <<http://www.macbiophotonics.ca/imagej/>>.

The key components of high-content analysis are:

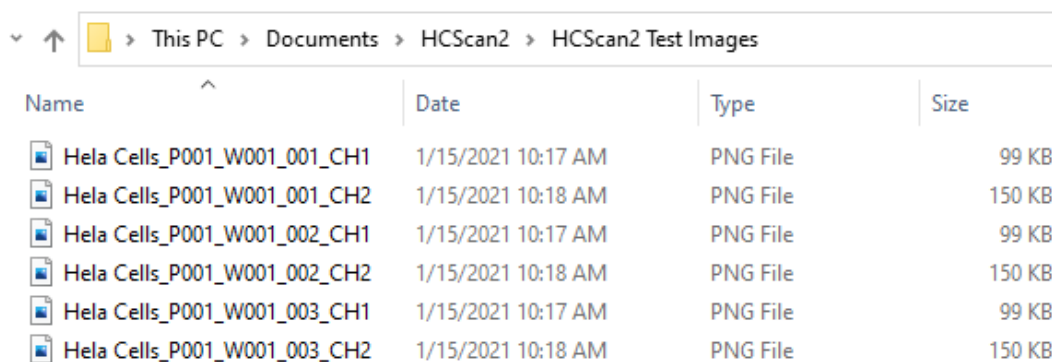
1. *CH1* images contain fluorescence-labeled nuclei. These are used to locate the cells. As Fig. 1 illustrates, in addition to being very bright, the labeled nuclei are relatively large and fairly circular in shape. These morphological parameters are used to distinguish them from other bright objects, e.g., fibers from wipes.
2. *CH2* images contain fluorescence-labeled target compounds. These appear as small spots or diffuse areas of luminance confined to the nucleus or cytoplasm of the cell.
3. Circle and Ring masks are created from the nuclei boundaries. The intensities measured under these masks in the *CH2* images indicate the concentrations of the target compounds in those regions of the cell.

HCSan2 Images

Prepare your multi-channel images in a file folder with the channels stored as separate, 8-bit, gray-scale images and titled as

```
name1_CH1.type
name1_CH2.type
name2_CH1.type
name2_CH2.type
.
.
.
```

The *name* field can be any valid file name characters, including sequence number, and the *type* field can be any file type supported by *ImageJ*. The name fields must match for each image pair. For example, your image folder might appear as in Fig. 2.









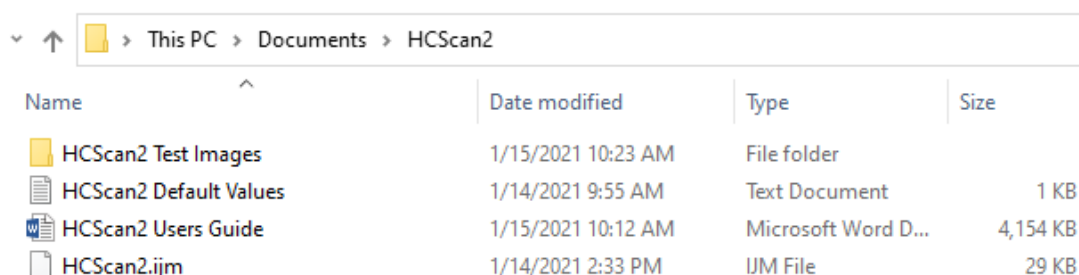
Name	Date	Type	Size
 Hela Cells_P001_W001_001_CH1	1/15/2021 10:17 AM	PNG File	99 KB
 Hela Cells_P001_W001_001_CH2	1/15/2021 10:18 AM	PNG File	150 KB
 Hela Cells_P001_W001_002_CH1	1/15/2021 10:17 AM	PNG File	99 KB
 Hela Cells_P001_W001_002_CH2	1/15/2021 10:18 AM	PNG File	150 KB
 Hela Cells_P001_W001_003_CH1	1/15/2021 10:17 AM	PNG File	99 KB
 Hela Cells_P001_W001_003_CH2	1/15/2021 10:18 AM	PNG File	150 KB

Figure 2 Image folder.
Image name includes Plate, Well and Sequence numbers

How to Run HCSan2

Copy the *HCSan2* folder to your *Documents* folder. This folder contains the *HCSan2* macro (.ijm), the Users Guide (.docx), a file of default assay parameters (.txt), and a set of test images, as shown in Fig. 3.



Name	Date modified	Type	Size
HCSan2 Test Images	1/15/2021 10:23 AM	File folder	
HCSan2 Default Values	1/14/2021 9:55 AM	Text Document	1 KB
HCSan2 Users Guide	1/15/2021 10:12 AM	Microsoft Word D...	4,154 KB
HCSan2.ijm	1/14/2021 2:33 PM	IJM File	29 KB

Figure 3 Copy *HCSan2* folder to *Documents*.

If you have multiple users on your computer there may be problems with other users accessing your *Documents* folder.

To install *HCSan2*, launch *ImageJ* and click on the *Plugins>> Macros>>Install* menu item, see Fig. 4.

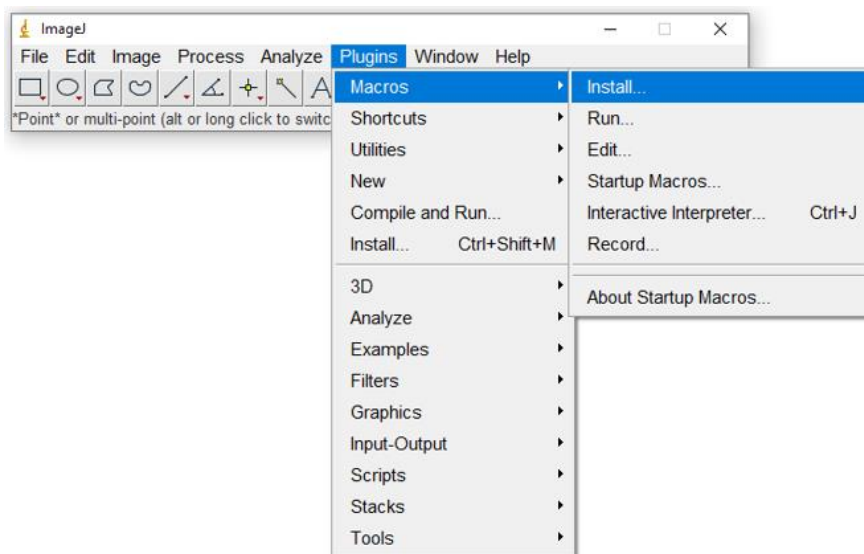


Figure 4 In *ImageJ*, select the *Plugins>> Macros>>Install* menu item.

The *Install Macros* dialog will appear and allow you to browse to the *HCSan2* folder and select *HCSan2.ijm*, see Fig. 5.

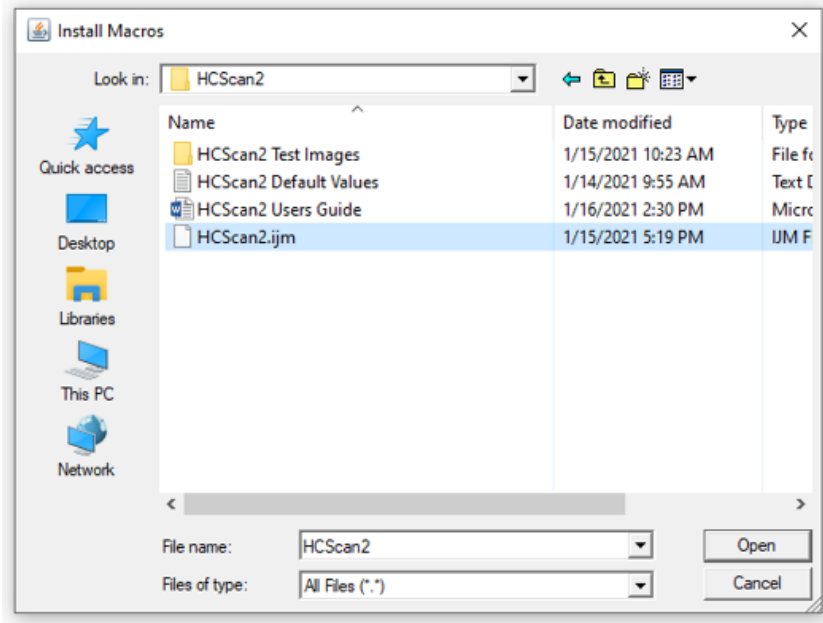


Figure 5 In the *Install Macros* dialog browse to the *HCSan2* folder and click on *HCSan2.ijm*.

Installing *HCSan2* will put it in the *Plugins>>Macros* list, see Fig. 5, and you can launch it by clicking this entry.

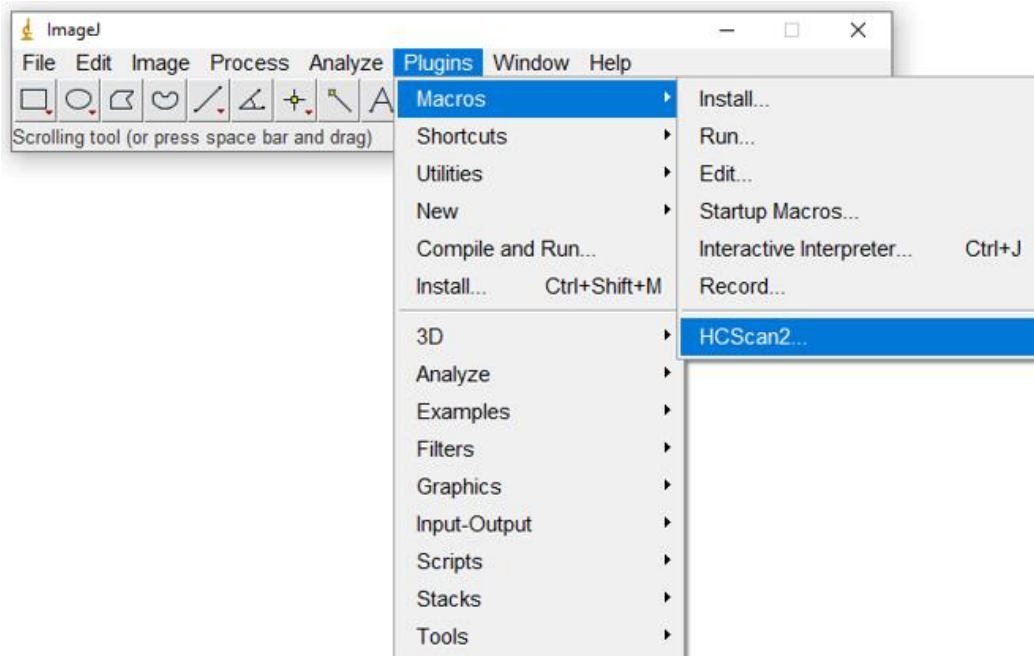


Figure 5 After *HCSan2* is installed, you can run it by clicking on *HCSan2* in the *Plugins>>Macros* list.

After *HCSan2* is launched you will see the *Assay Parameters* dialog shown in Fig. 6. These parameters are described in Appendix A, at the end of this document.

The values displayed are the ones currently stored in the file *HCSan2 Default Values.txt*. Check the *Load Default Values* box to load values from a different file, then click the *OK* button to execute that command. The *Open Defaults File* dialog will allow you to browse to the file you want to load. The *Assay Parameters* dialog will reappear after you select the file and click the *Open* button, and will contain the assay parameters you just loaded.

You can edit the values in the *Assay Parameters* dialog. To save them for future analyses, check the *Save as Default Values* box, then click the *OK* button. The *Save Defaults File* dialog will allow you to browse to the folder you want and save the default values in the file of your choice. The *Assay Parameters* dialog will reappear after you select the file and click the *Open* button.

Figure 6 *HCSan2* assay parameters.

To begin the analysis, click *OK* with both *Load Default Values* and *Save Default Values* unchecked. Clicking *Cancel* will stop the macro without beginning the analysis.

At the beginning of the analysis *HCSan2* will ask you to select an image file. Use the *Open File* select window to browse to the folder containing the image you want to analyze, select it and click *Open*, see Fig. 7. It does not matter which channel of the two-channel image pair you select, *HCSan2* will open both of them.

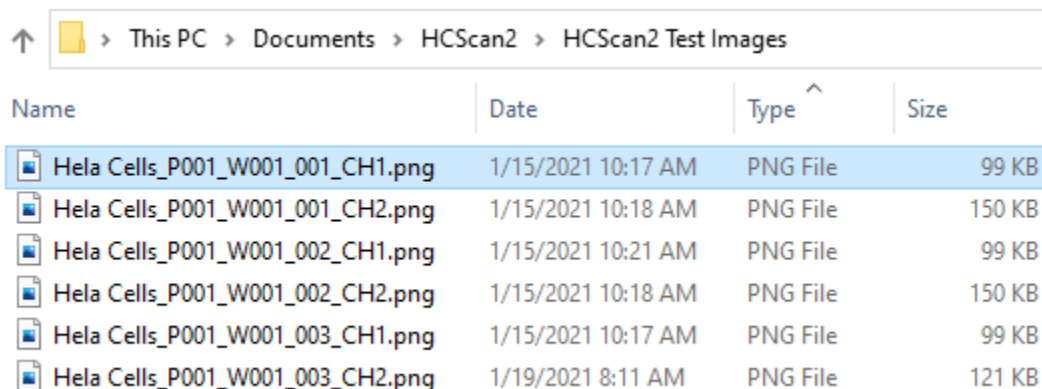


Figure 7 HCSan2 image selection window. Select one file of the two-channel image pair and click *Open*.

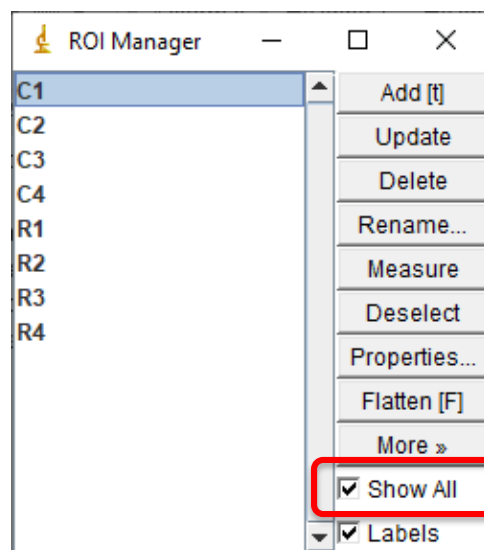
HCSan2 Results

When the analysis is finished your desktop will display the following

1. *ImageJ* menu and tool bar
2. *CH1* image
3. *CH2* image
4. *Results* table
5. *ROI Manager*

The *ROI Manager* contains a combined list of circle and ring ROI's. The circle ROI's are listed first, and are labeled C1, C2,... The ring ROI's have the same labels, but begin with "R". You can select an ROI by clicking, as shown in Fig. 8.

Figure 8 *ROI Manager* used to control the display of ROI's on the analyzed images.



The selected ROI is selected is highlighted on the active image, as shown in Fig. 9. If the *Show All* box of the *ROI Manager* is, unchecked, only the selected ROI is displayed. If *Show All* is checked, all ROI's are displayed and the selected ROI is

highlighted in a contrasting color. Note that checking *Show All* displays the ring ROI's because they are at the end of the list and are drawn on top of the circle ROI's. The *ROI Manager* has a number of tools, listed on the right side, that can be used to further control ROI display.

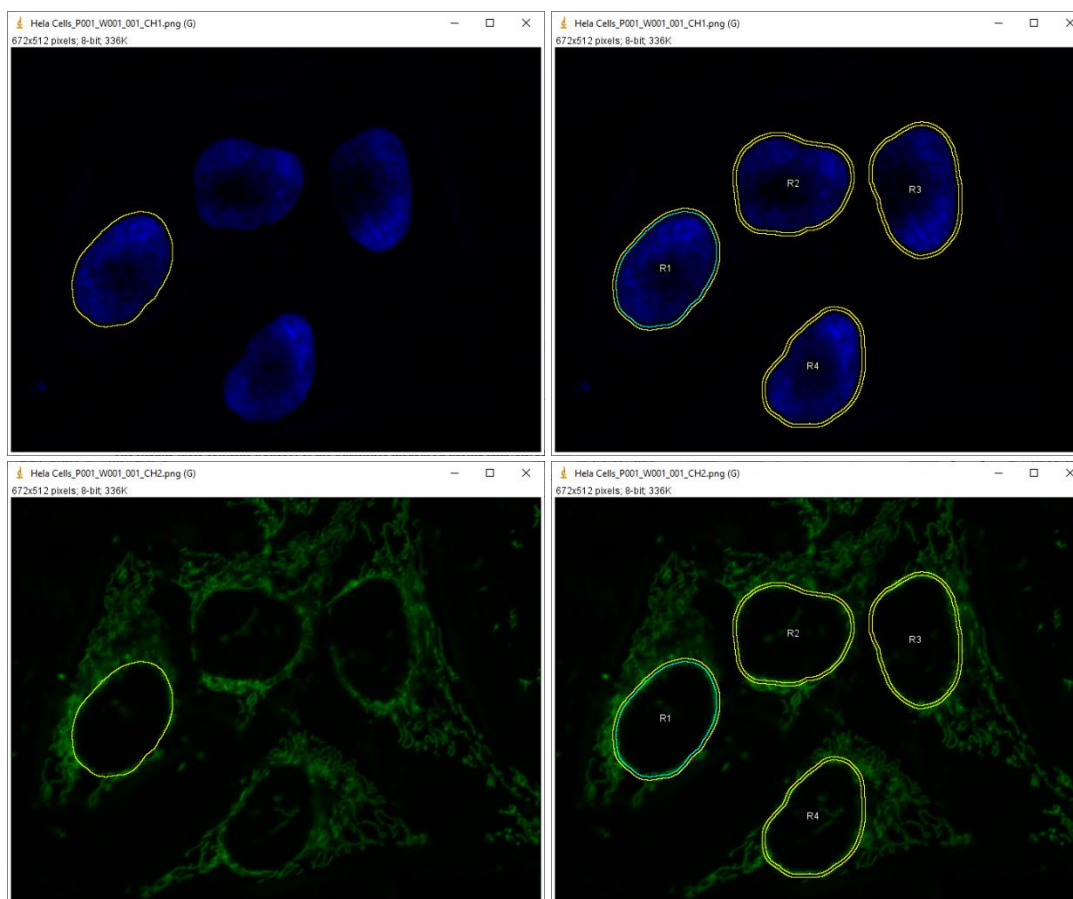
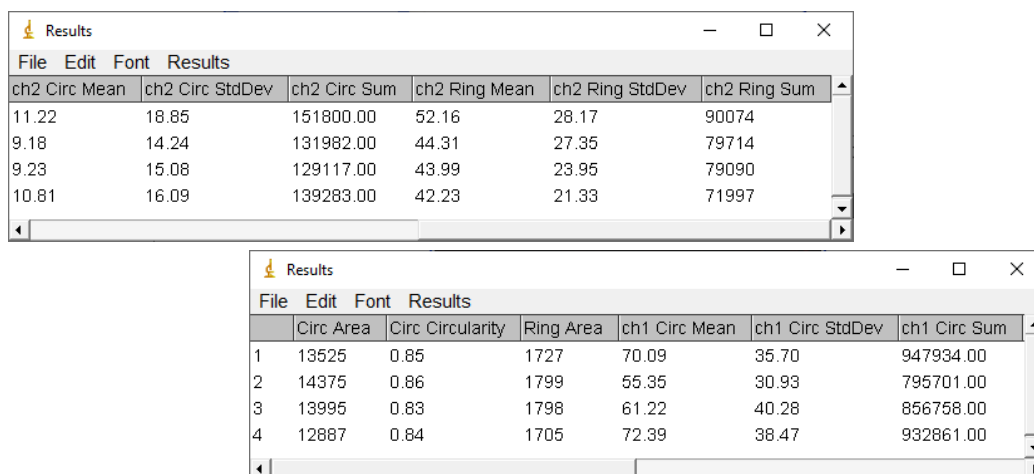


Figure 9 Overlays displayed for ROI C1 selected (as shown in Fig. 8).
Left: *Show All* box unchecked, Right: *Show All* box checked

The *Results* table contains a subset of the quantities measured during analysis, see Fig. 10. The first column is label of the object as it appears in the *ROI Manager*. The next columns give the area and circularity of its circle mask followed by the area of its ring mask. The area and circularity are the morphological parameters used to select valid objects. The intensity data are next, first the circle mask intensities in *CH1*, which are also used to select valid objects, followed by the circle and ring mask intensities in *CH2*, which are the target measurements.

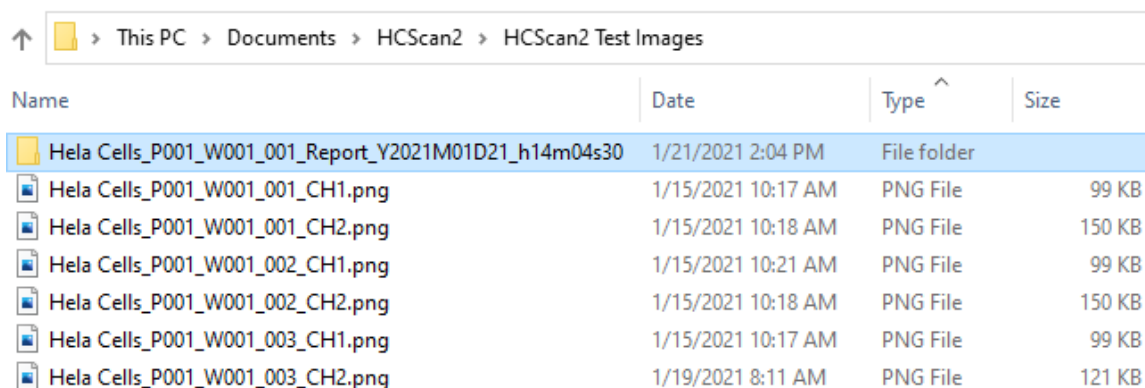


ch2 Circ Mean	ch2 Circ StdDev	ch2 Circ Sum	ch2 Ring Mean	ch2 Ring StdDev	ch2 Ring Sum
11.22	18.85	151800.00	52.16	28.17	90074
9.18	14.24	131982.00	44.31	27.35	79714
9.23	15.08	129117.00	43.99	23.95	79090
10.81	16.09	139283.00	42.23	21.33	71997

	Circ Area	Circ Circularity	Ring Area	ch1 Circ Mean	ch1 Circ StdDev	ch1 Circ Sum
1	13525	0.85	1727	70.09	35.70	947934.00
2	14375	0.86	1799	55.35	30.93	795701.00
3	13995	0.83	1798	61.22	40.28	856758.00
4	12887	0.84	1705	72.39	38.47	932861.00

Figure 10 Results table contains a subset of the measured quantities.

A new report folder will appear in the folder that contained the image you just analyzed, see Fig. 11.

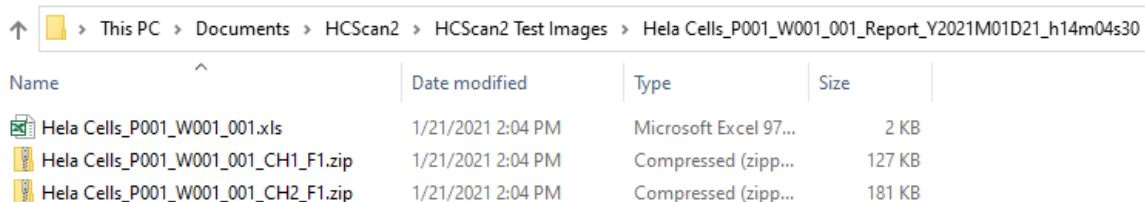


Name	Date	Type	Size
Hela Cells_P001_W001_001_Report_Y2021M01D21_h14m04s30	1/21/2021 2:04 PM	File folder	
Hela Cells_P001_W001_001_CH1.png	1/15/2021 10:17 AM	PNG File	99 KB
Hela Cells_P001_W001_001_CH2.png	1/15/2021 10:18 AM	PNG File	150 KB
Hela Cells_P001_W001_002_CH1.png	1/15/2021 10:21 AM	PNG File	99 KB
Hela Cells_P001_W001_002_CH2.png	1/15/2021 10:18 AM	PNG File	150 KB
Hela Cells_P001_W001_003_CH1.png	1/15/2021 10:17 AM	PNG File	99 KB
Hela Cells_P001_W001_003_CH2.png	1/19/2021 8:11 AM	PNG File	121 KB

Figure 11 Analysis report folder in folder containing image.

The report folder will contain the following files, as shown in Fig. 12

1. An *Excel* compatible file of analysis results
2. A *.zip* file containing the *CH1* image fields with ROI's
3. A *.zip* file containing the *CH2* image fields with ROI's



Name	Date modified	Type	Size
Hela Cells_P001_W001_001.xls	1/21/2021 2:04 PM	Microsoft Excel 97...	2 KB
Hela Cells_P001_W001_001_CH1_F1.zip	1/21/2021 2:04 PM	Compressed (zipp...	127 KB
Hela Cells_P001_W001_001_CH2_F1.zip	1/21/2021 2:04 PM	Compressed (zipp...	181 KB

Figure 12 Contents of analysis report folder.

All of the results, including assay parameters are saved to the *Excel*-readable file (.xls). The report file may be opened by double clicking; however, you will have to click *YES* in response to the *Excel* question.

The two .zip files that contain fields of the analyzed image with ROI's. These images can be opened using *ImageJ* and look just like those shown in Fig. 9, except that the *ROI Manager* cannot be used to select individual ROI's. You can copy and paste these images into Excel or Word documents.

Note About Memory

Unlike other Windows applications ImageJ will only use the memory allocated to it. You can change the allocated memory to equal 75% of total RAM via the menu command: "*Edit/Options/Memory*". Specifying more than 75% of real RAM results in virtual RAM being used causing ImageJ to become very slow and unstable. See <http://rsb.info.nih.gov/ij/docs/install/>.

Appendix A Assay Parameters

The parameters *HCSan2* uses to configure the analysis are described below:

1. *Field Rows* and *Field Columns*: Used to specify the number of rows and columns of non-overlapping fields into which the image is divided for analysis. This feature allows the macro to be run on computers that do not have enough memory to analyze an entire image. Fields are numbered 1 through $Rows \cdot Columns$ from left-to-right and top-to-bottom, as shown in Fig. 11. Setting both parameters to “1” will analyze the entire image as one field.

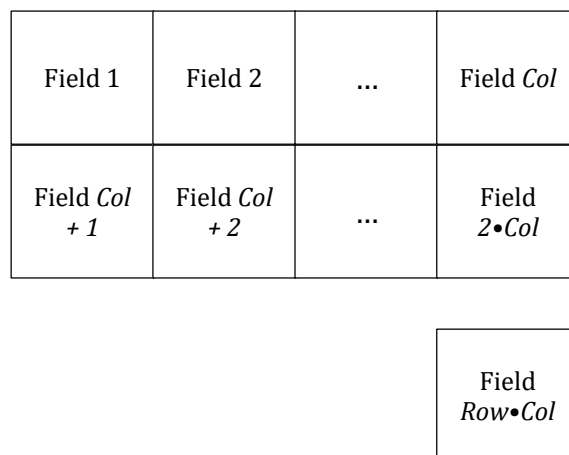


Figure 11 Field numbering scheme.

2. *Analyze Field*: Used to analyze a single, specified field of the row-column array described above. Setting this parameter to 0 will analyze all fields of the array. A parameter value other than 0 will allow you to review the analysis of the specified field.
3. *Stop Analysis after _____*: Stops the macro at points in the analysis so you can *interactively set* various assay parameters. For example, if you stop after the images are loaded you can manually execute the background removal tool with different rolling ball radii, then manually try different threshold methods and values.
4. *Stop for Analysis Review*: Checking this box will stop the analysis after the specified image field. The macro will leave the *CH1* and *CH2* images on the desktop so you can examine the results using the ROI Manager.
5. *Rolling Ball Radius*: Used to remove the background on both *CH1* and *CH2* images. The *Before* image in Fig. 12 has an uneven background that is higher on the left than on the right. The plot below the image represents the pixel values in the horizontal strip slightly below the center of the image. Imagine rolling a ball along the underneath surface of the image and setting every pixel touched by the ball to zero. The result is shown on the right: All that remains in the intensity profile of the image are the peaks that were too small for the ball to enter. The

radius of the ball must be larger than the radius of the smallest object of interest in *CH1*.

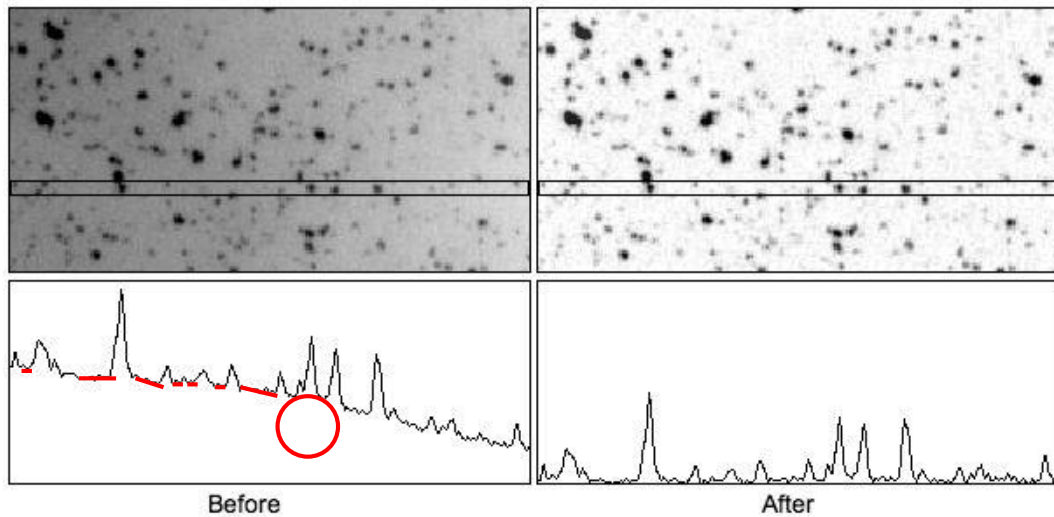


Figure 12 Rolling ball background removal.

(Figure from http://imagejdocu.tudor.lu/doku.php?id=gui:process:subtract_background)

6. *Smooth Radius*: Used to agglomerate *CH1* objects inside smooth boundaries. Object boundaries must be drawn in regions where object pixel intensities are very close to background pixel intensities, see Figure 13. Thus, boundary shape is strongly affected by noise. Smoothing an image before thresholding results in smoother object boundaries.

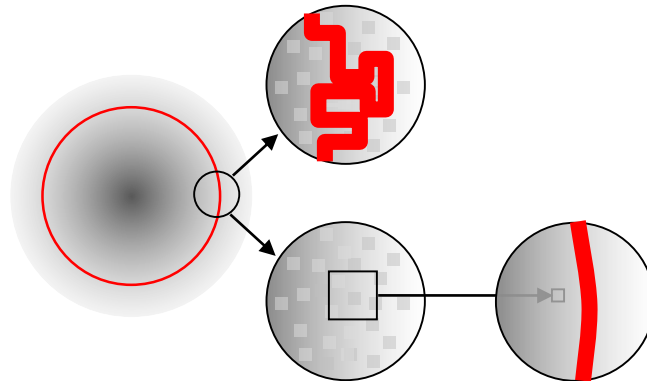


Figure 12 Particle boundary smoothing.

Left: Particle boundary lies in region of image strongly affected by noise.

Top: Thresholding results in jagged boundary.

Bottom: Smoothing the image before thresholding

Right: results in smoother boundary

7. *Min Area*: Used to reject small objects in *CH1*. This is the minimum area of valid circle masks.

One of the results measured in this analysis is the standard deviation of pixel values within a mask. Since the standard deviation is not defined for less than two pixels the minimum area of circle and ring masks is internally constrained to be greater than two pixels.

8. *Max Area*: Used to reject large objects in *CH1*. This is the maximum area of valid circle masks.

The maximum area of circle and ring masks is internally constrained to be less than $image\ width/2 \cdot image\ height/2$.

9. *Min Circularity*: Used to reject *CH1* objects based on shape. Circularity is defined as

$$Circularity = 4\pi \frac{Area}{Perimeter^2},$$

and varies between 0 (fiber) and 1 (circle), see Fig. 14. Since *CH1* objects are assumed to be circular, only the *Min Circularity* parameter is configurable.

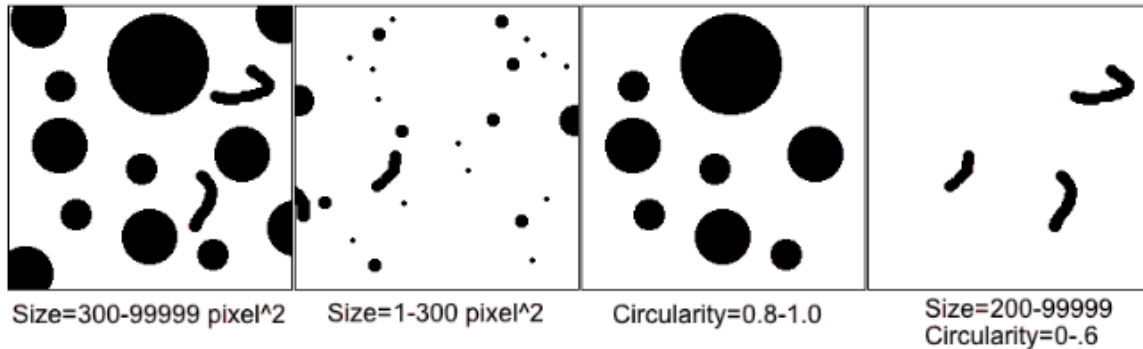


Figure 14 Effect of size and shape parameters on rejection of *CH1* objects.

(Figure from http://imagejdocu.tudor.lu/doku.php?id=gui:analyze:analyze_particles)

10. *Ring Width*: Specifies the width of the ring mask used to measure target intensity in *CH2* that is outside the *CH1* object.

Image Segmentation

Automatic thresholding is used to segment images into objects and background. Both isodata and triangle methods are commonly used in bioassays, see Fig. 15. Isodata is a valley-finding method and is recommended when the number of background and object pixels is comparable (*mBack* and *mObj* are the means of the background and objects, respectively). Triangle is a cliff-finding method that is useful when object pixels are sparse.

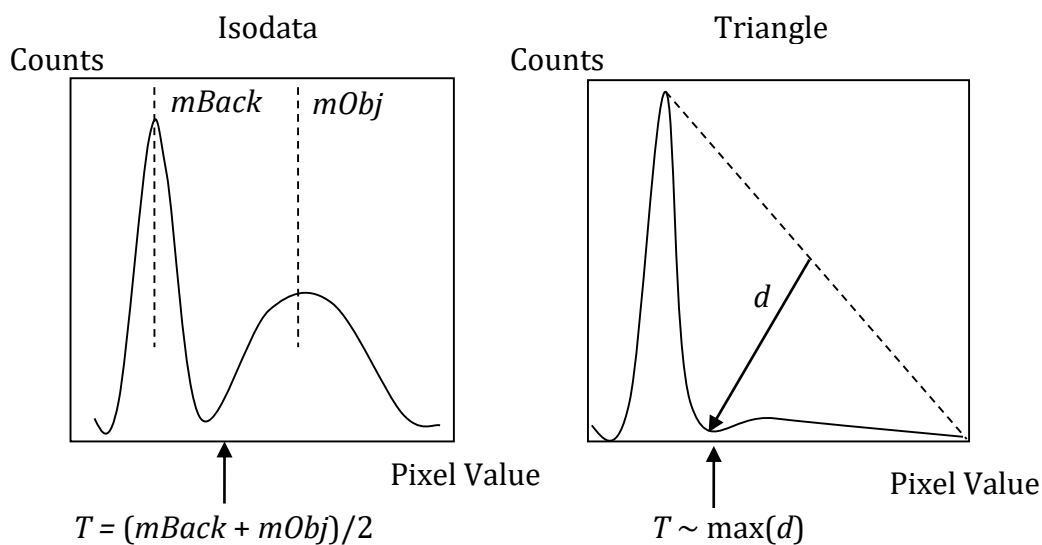


Figure 15 Automatic image segmentation methods.