

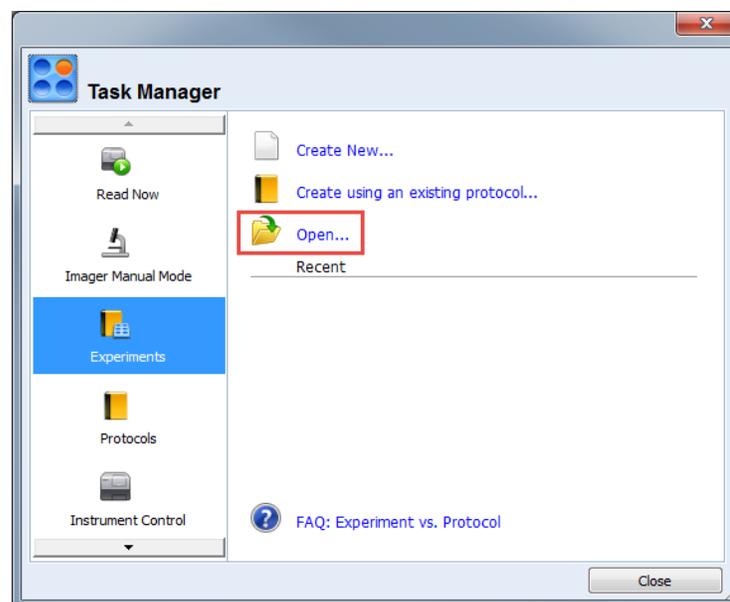
# Agilent Seahorse XF Imaging & Cell Counting Software

## Adjusting Analysis Parameters in BioTek Gen5™ for Difficult to Count Cell Lines

This document describes how to use BioTek's Gen5™ software to re-analyze images initially captured and analyzed with Agilent's Seahorse XF Imaging & Cell Counting Software.

### Opening the Gen5 Experiment File

- 1) Launch Gen5. After the splash screen disappears, the Task Manager window will be displayed.
- 2) From the Task Manager, click **Experiments > Open...**



**Figure 1.** Gen5 Task Manager

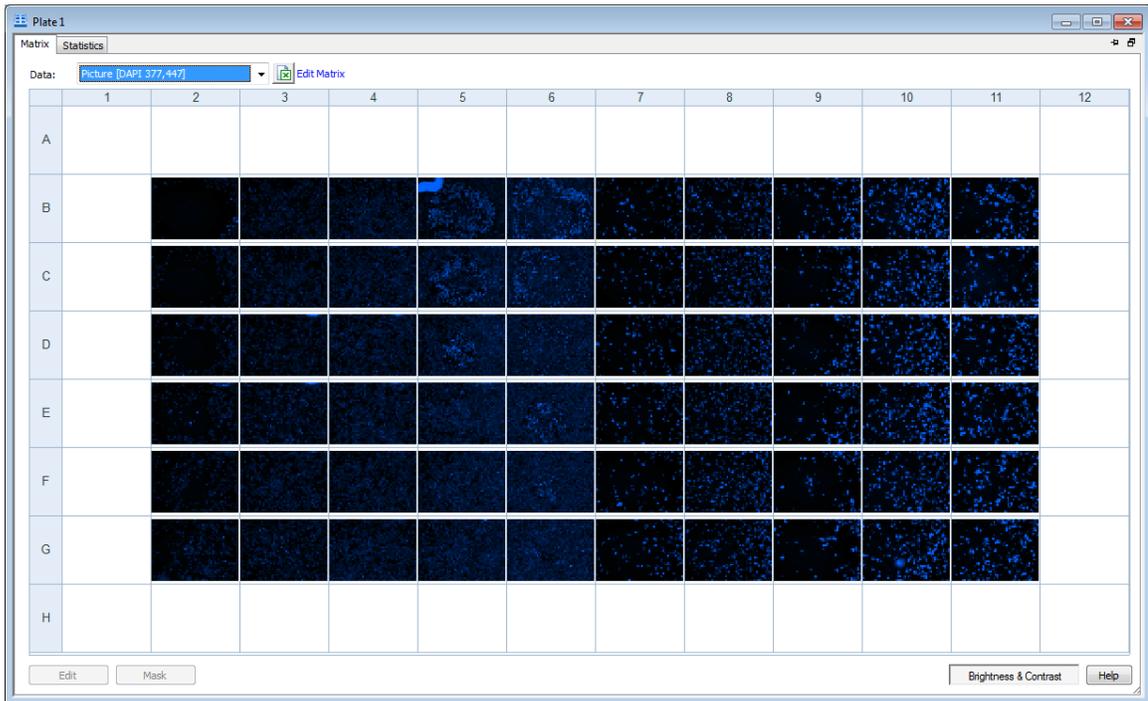
- 3) Navigate to the Gen5 Experiment file you wish to re-analyze. By default, Agilent's Seahorse XF Imaging & Cell Counting Software saves Gen5 experiment files to **C:\BioTek**. Experiment files containing fluorescent cell count analyses are named with the following naming convention:

PostAssay\_FL\_XFeXX\_YYYY-DD-MM--HH-MM-SS.xpt

Use the date and time stamps to identify the appropriate Gen5 experiment file to open.

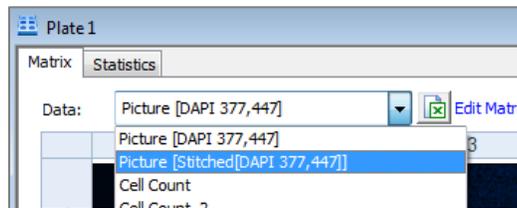
## Adjusting Cell Count Analysis Parameters

- 1) From the menu bar, click **Plate > View** to open the Plate View window.



**Figure 2.** Plate View window

**IMPORTANT:** When reviewing images collected from an **XFe24** plate, switch to the **Stitched** image set prior to viewing images. Use the data dropdown menu to switch image sets.



**Figure 3.** Changing image sets

- 2) In the Plate View window, click on a thumbnail to view the full resolution image in the Image Review window. Choose a representative image to use as a guide for setting analysis parameters.

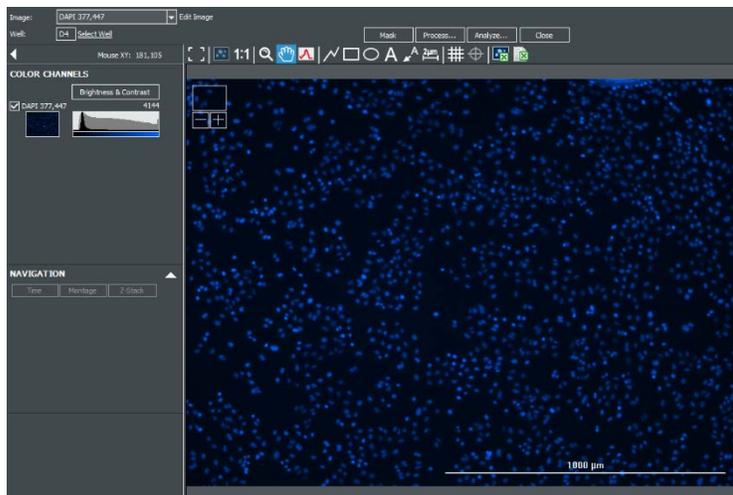


Figure 4. Image Review window

- 3) Click the Analyze... button at the top of the Image Review window.

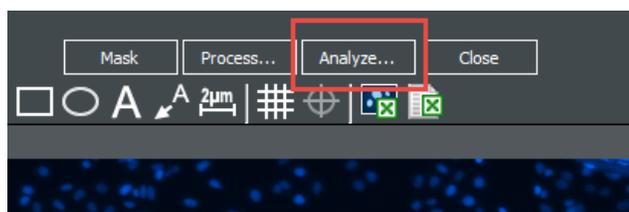


Figure 5. Analyze button

- 4) In the next window, select "I want to edit an existing Image Analysis step, and choose the **first** analysis step in the list ("Cellular Analysis Cell Count").

NOTE: The first Analysis step listed uses a standard method to identify individual nuclei. If your nuclei are extremely clumped and cannot be easily segmented, select the **second** analysis step ("Cellular Analysis Cell Count\_2...") and skip to the **Analyzing Densely Clustered Nuclei** section below.

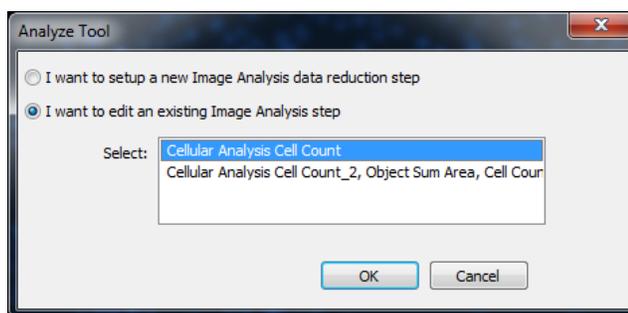


Figure 6. Edit existing Image Analysis step

- 5) In the Analysis Tool window, you can visualize how objects are masked (segmented) in the representative image. For an accurate count, each nucleus should be associated with a single yellow mask. You can inspect masks by clicking on them directly.

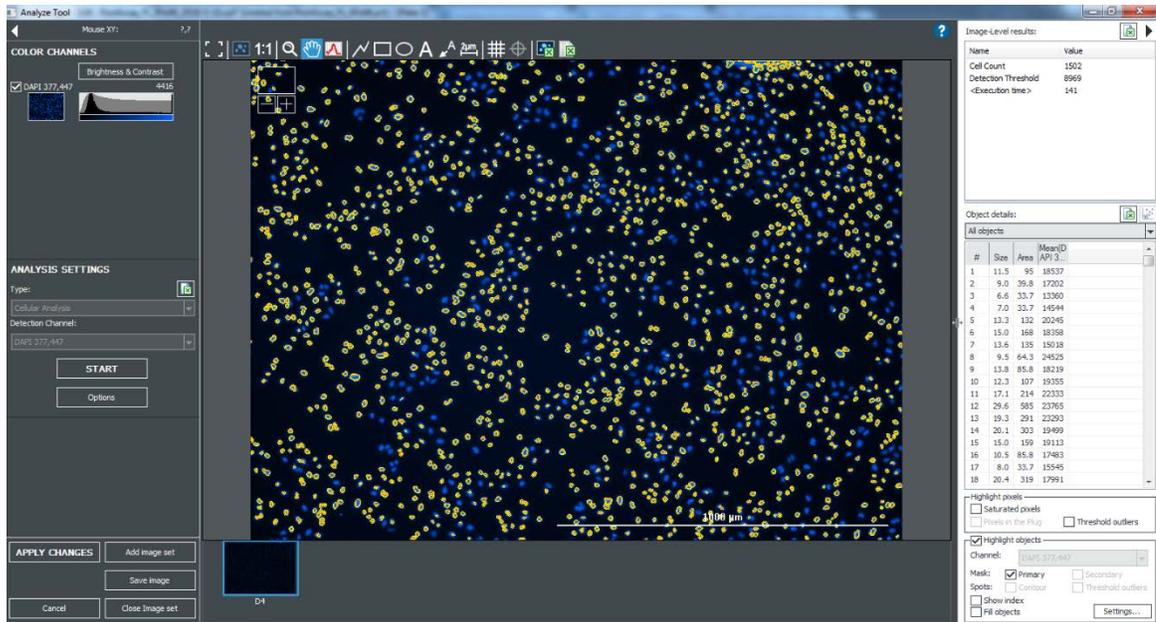


Figure 7. Analyze Tool Window

OPTIONAL: Add additional representative images to the **Analyze Tool**. Click the **Add image set** button and choose the image you'd like to add. Note, the final analysis will run on all images in your plate after exiting the **Analyze Tool** window.

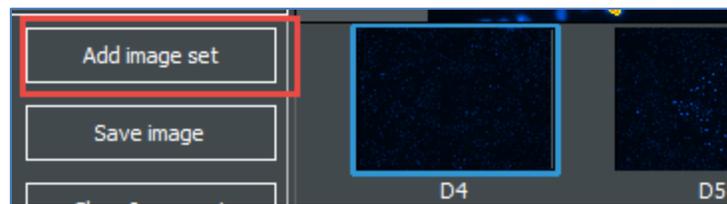
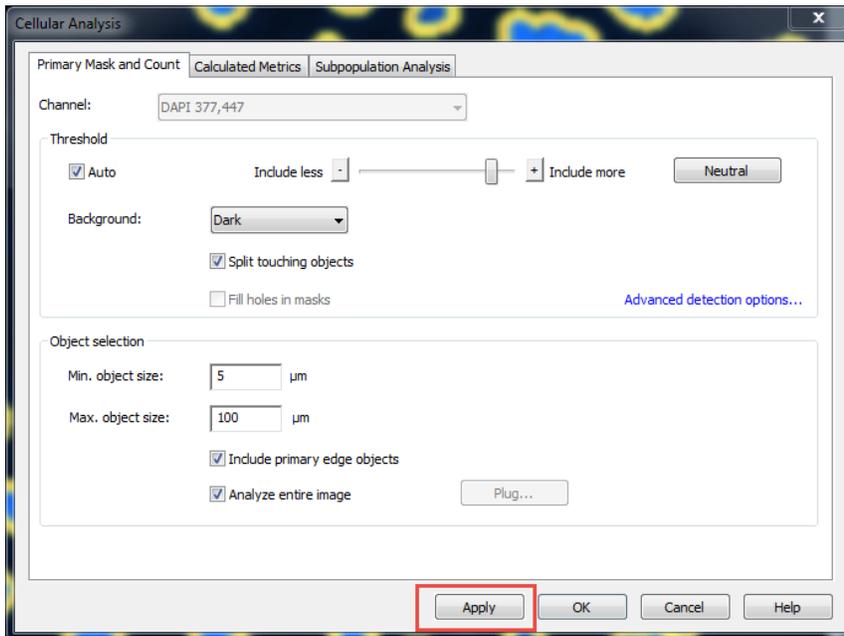
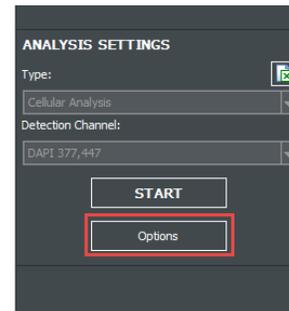


Figure 8. Add image set button

- 6) Click the Options... button under **Analysis Settings**, and try adjusting the settings described below. Anytime you modify a setting, click "Apply" to view the changes to the object masks.



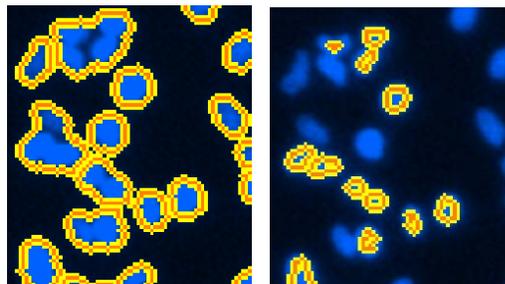
**Figure 10.** Cellular Analysis Options window. Click apply to visualize changes to object masking.



**Figure 9.** Analysis Options button

- Threshold    Include less  —————  Include more

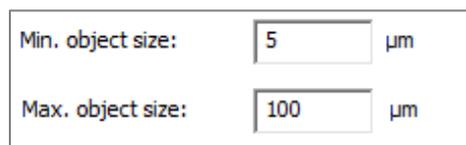
Threshold is the most important setting for accurate object identification. If nuclei are excluded from the count (unmasked), move the slider toward “Include more”. If multiple nuclei are masked as single objects, move the slider toward “Include less”. Click apply each time you adjust the slider to visualize the effect on nuclei identification.



**Figure 11.** Inaccurate object masking

- Object Size

You can limit the objects (or nuclei) to be counted by defining minimum and maximum object sizes. Consider modifying the min. and max. object sizes to exclude small/large debris from the count.

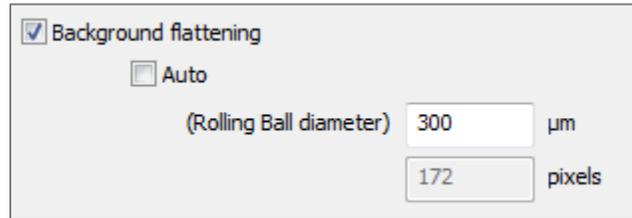


**Figure 12.** Object size range

- Advanced Detection Options: Background Flattening

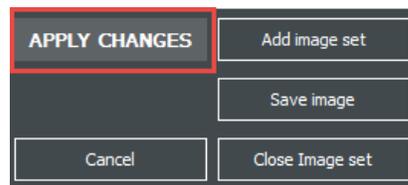
IMPORTANT: Only change this setting if adjustment of the settings above fail to accurately identify objects.

This option corrects for local background variations in an image. Achieving an even background is necessary for threshold based object detection. To adjust, uncheck the box next to Auto and enter a new rolling ball diameter (typically smaller than the default value). Always use a rolling ball diameter larger than your typical object size.



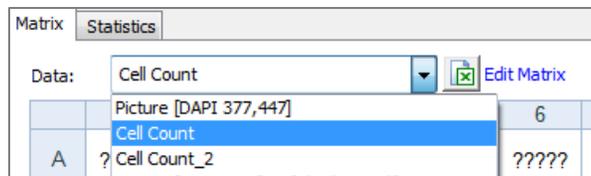
**Figure 13.** Advanced Options: Background flattening

- 7) When you are satisfied with your changes, click OK in the Cellular Analysis Options window, and then click “Apply Changes” in the Analyze Tool window. Gen5 will now re-analyze all the images in the experiment file.



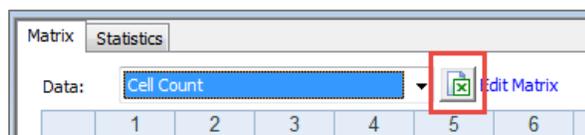
**Figure 14.** Apply Changes button

- 8) Return to the Plate View (Figure 2) window and select “Cell Count” in Data dropdown menu. The Plate View window will now display the total number of nuclei counted in each well.



**Figure 15.** Display Cell Count

- 9) Click the Excel button at the top of the Plate View window to export the cell count data to Excel. Once exported to Excel, the data can be imported into Agilent’s Wave software.



**Figure 16.** Export to Excel

## Counting Densely Clustered Nuclei

NOTE: This section describes an alternative analysis method for counting nuclei. Please follow steps 1-4 of the previous section (**Adjusting Cell Count Analysis Parameters**) before proceeding.

- 1) In the analysis tool window (Figure 7), click the “Options” button (Figure 9) on the left side of the screen.
- 2) In the Cellular Analysis Options window (Figure 10), use the slider to adjust the threshold. After each adjustment, click the “Apply” button to visualize the effect on object masking. For this analysis, Gen5 estimates cell count by measuring the total area of nuclei in an image. Therefore, it’s acceptable to segment clustered groups of nuclei into single masks. See Figure 17 for an example.

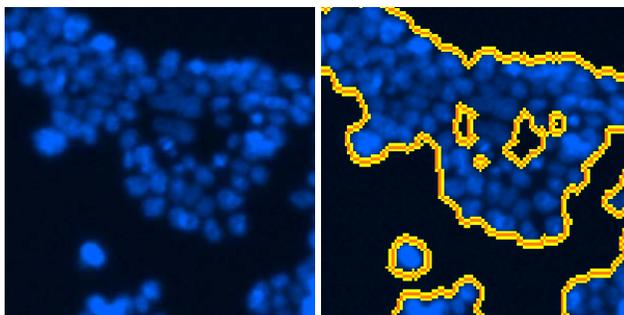


Figure 17. Appropriately masked nuclei

- 3) When you are satisfied with your changes, click OK in the Cellular Analysis Options window, and then click “Apply Changes” (Figure 14) in the Analyze Tool window. Gen5 will now re-analyze all the images in the experiment file.
- 4) Return to the Plate View (Figure 2) window and select “Counts by Area\_2” in the Data dropdown menu. The Plate View window will now display the total number of nuclei counted in each well.
- 5) Click the Excel button (Figure 16) at the top of the Plate View window to export the cell count data to Excel. Once exported to Excel, the data can be imported into Agilent’s Wave software. For additional information about data import into Wave, please contact Agilent Cell Analysis Support ([seahorse.support@agilent.com](mailto:seahorse.support@agilent.com)).

Well	Data	Value
	Object Area[Single Cells]	
	Picture [DAPI 377,447]	6
A	Cell Count	?????
	Cell Count_2	
B	Picture [Histogram[Size] (Cell Count)]	
	Object Sum Area	115
	Cell Count[Single Cells]	
C	Object Area[Single Cells]	
	Counts by Area_2	95.9
	Picture [Histogram[Size] (Cell Count_2)]	
D	Create new Matrix...	198

Figure 18. Display alternative cell count