Takara Bio USA

Shasta™ CellSelect® Software User Manual

Cat. Nos. 640282 & 650015
software v1.0.3
(040125)

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I. Introduction

The **Shasta Single Cell System** (Cat. No. 640282) has been engineered to dramatically increase the pace of biological discovery.

With unparalleled cell isolation, cell selection, and sample throughput, you now have control over your single-cell analyses and the ability to obtain the data you need to move forward with your research.

- **Power**—isolate thousands of cells of any size and process multiple chips per day
- **Control**—choose which cells to process using image analysis software
- **Insight**—process up to eight different samples per chip and leverage experimental flexibility for greater biological insight

Shasta CellSelect Software (referred to as CellSelect software hereafter) analyzes images generated with Shasta CELLSTUDIO™ Software (referred to as CELLSTUDIO software) and provides researchers with the following capabilities:

- Automated or manual image analysis and selection of isolated cells for downstream processing—including complicated cell types, such as cardiomyocytes with multiple nuclei
- Assessment of cell staining to determine viability (live/dead) or cells of choice
- Modify parameters and rerun analyses with the new settings

This software guides you through each step of the image analysis process:

- Prompts you to load images and settings into the software
- Provides functions in sequential order:

Figure 1. CellSelect software function menu icons. This image will be repeated in the procedure (Section III) to guide you through the workflow.

After the samples are dispensed into the chip, CELLSTUDIO software:

- scans the chip
- uses CellSelect software in the background to analyze the wells
- CellSelect saves the WCD (data) file and the filter file.

When the analysis is complete, the user can optionally open the saved WCD file using CellSelect software, examine the data, make any desired changes to analysis parameters, reprocess, and generate a new filter file for use with CELLSTUDIO software performing downstream dispenses.

A. Release Notes

1. Version 1.0.3

- Minor bug fixes

2. Version 1.0.2

- Compatibility with a new feature in CELLSTUDIO software (v1.0.1) that enables users to select CellSelect analysis settings from the *Start new experiment* view (Shasta Single Cell System User Manual, Section VII.D)
- Minor bug fixes and performance improvements

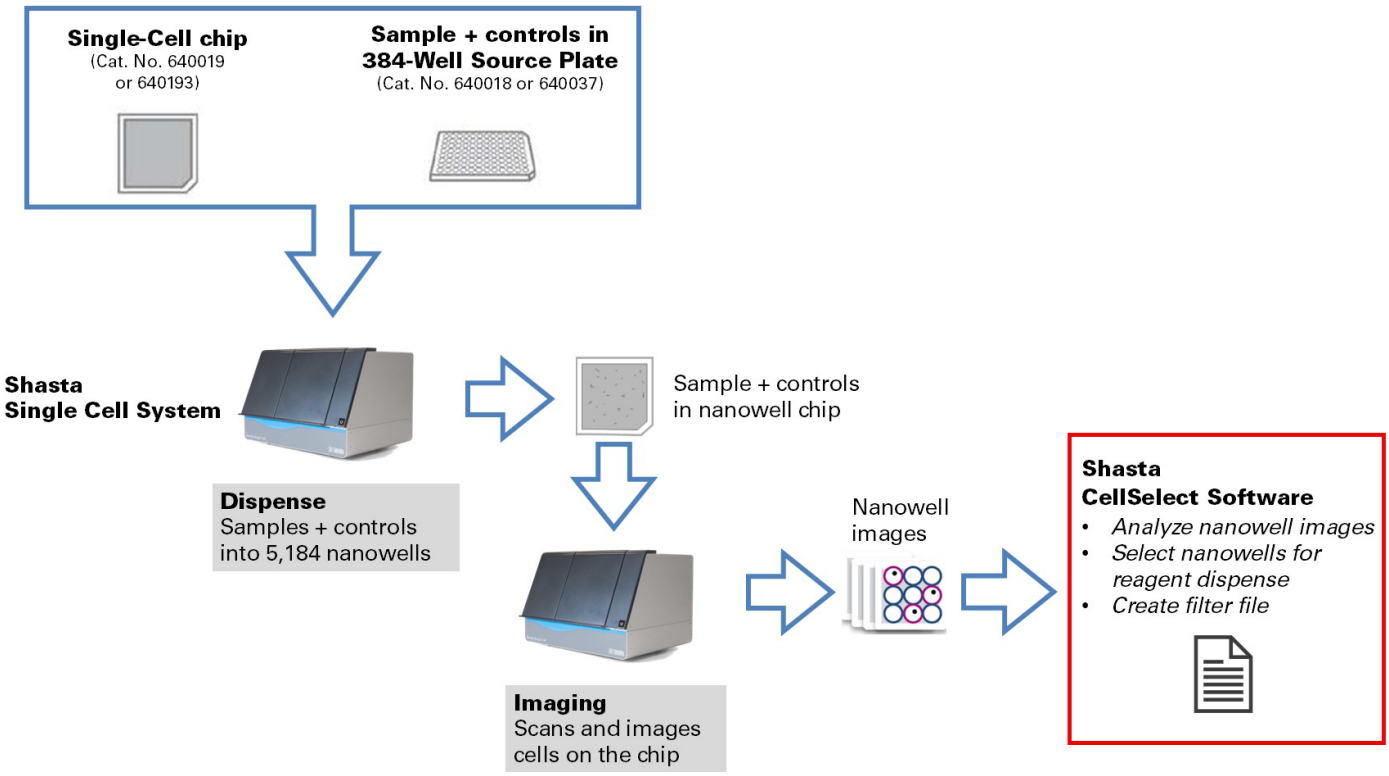


Figure 2. Workflow diagram depicting single-cell isolation, imaging, and image processing steps.

II. Overview: User Interface

Icon for Shasta CELLSTUDIO Software
(for dispensing and imaging)

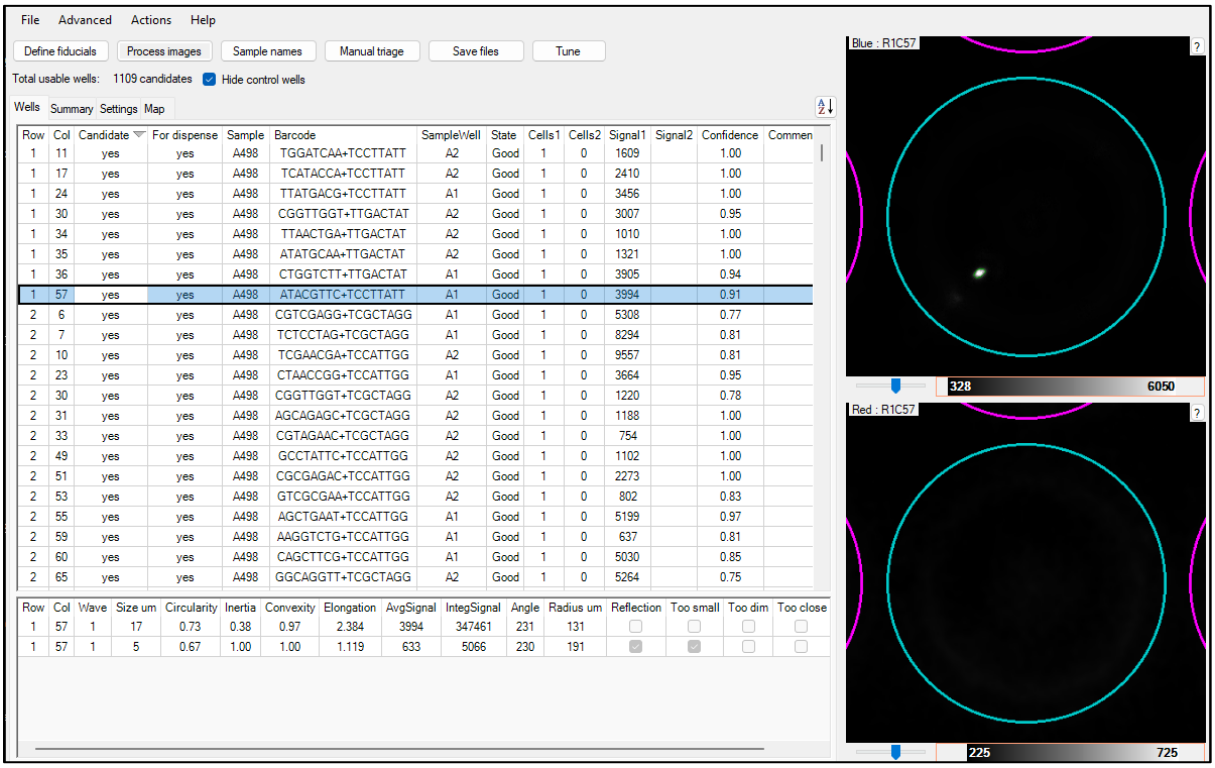
Icon for Shasta CellSelect Software
(for image analysis)

Figure 3. Icons for the Shasta CELLSTUDIO Software and Shasta CellSelect Software.

After running CellSelect, the *Main* screen will display. It includes the following tabs: *Wells*, *Summary*, *Settings*, and *Map*.

A. Wells

The data table on the *Wells* tab lists all the nanowells in the chip and the status of any cells found. A description of each column header for the Wells table (upper table in Figure 4, below) can be found in Appendix C.A, "Wells Data Table". The description of the columns of the cell details view (bottom table in Figure 4) can be found in Appendix C.B, "Cell Details Table".

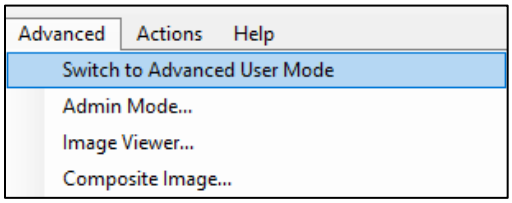


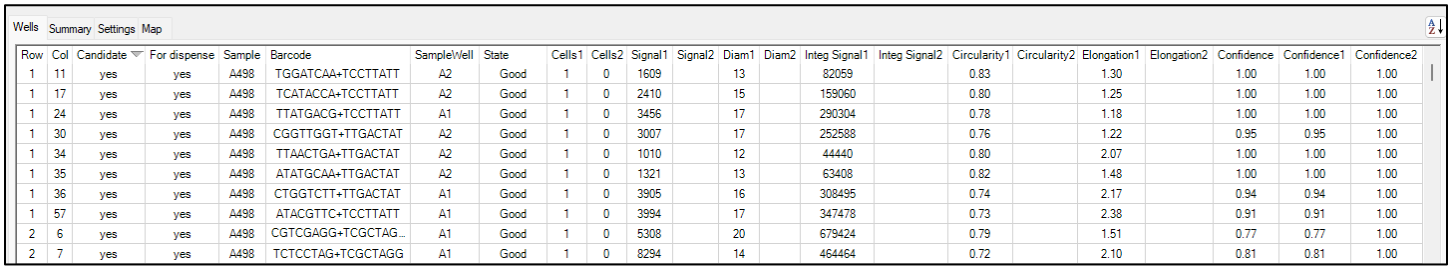
Row	Col	Candidate	For dispense	Sample	Barcode	SampleWell	State	Cells1	Cells2	Signal1	Signal2	Confidence	Commen
1	11	yes	yes	A498	TGGATCAA+TCCTTATT	A2	Good	1	0	1609		1.00	
1	17	yes	yes	A498	TCATACCA+TCCTTATT	A2	Good	1	0	2410		1.00	
1	24	yes	yes	A498	TTATGACG+TCCTTATT	A1	Good	1	0	3456		1.00	
1	30	yes	yes	A498	CGGTTGGT+TTGACTAT	A2	Good	1	0	3007		0.95	
1	34	yes	yes	A498	TTAACTGA+TTGACTAT	A2	Good	1	0	1010		1.00	
1	35	yes	yes	A498	ATATGCAA+TTGACTAT	A2	Good	1	0	1321		1.00	
1	36	yes	yes	A498	CTGGTCTT+TTGACTAT	A1	Good	1	0	3905		0.94	
1	57	yes	yes	A498	ATACGTTC+TCCTTATT	A1	Good	1	0	3994		0.91	
2	6	yes	yes	A498	CGTCGAGG+TCGCTAGG	A1	Good	1	0	5308		0.77	
2	7	yes	yes	A498	TCTCCTAG+TCGCTAGG	A1	Good	1	0	8294		0.81	
2	10	yes	yes	A498	TCGAACGA+TCCATTGG	A2	Good	1	0	9557		0.81	
2	23	yes	yes	A498	CTAACCGG+TCCATTGG	A1	Good	1	0	3664		0.95	
2	30	yes	yes	A498	CGGTTGGT+TCGCTAGG	A2	Good	1	0	1220		0.78	
2	31	yes	yes	A498	AGCAGAGC+TCGCTAGG	A2	Good	1	0	1188		1.00	
2	33	yes	yes	A498	CGTAGAAC+TCGCTAGG	A2	Good	1	0	754		1.00	
2	49	yes	yes	A498	GCCTATTC+TCCATTGG	A2	Good	1	0	1102		1.00	
2	51	yes	yes	A498	CGCGAGAC+TCCATTGG	A2	Good	1	0	2273		1.00	
2	53	yes	yes	A498	GTCGCGAA+TCCATTGG	A2	Good	1	0	802		0.83	
2	55	yes	yes	A498	AGCTGAAT+TCCATTGG	A1	Good	1	0	5199		0.97	
2	59	yes	yes	A498	AAGGTCTG+TCCATTGG	A1	Good	1	0	637		0.81	
2	60	yes	yes	A498	CAGCTTCG+TCCATTGG	A1	Good	1	0	5030		0.85	
2	65	yes	yes	A498	GGCAGGTT+TCGCTAGG	A2	Good	1	0	5264		0.75	

Row	Col	Wave	Size um	Circularity	Inertia	Convexity	Elongation	AvgSignal	IntegSignal	Angle	Radius um	Reflection	Too small	Too dim	Too close
1	57	1	17	0.73	0.38	0.97	2.384	3994	347461	231	131	☐	☐	☐	☐
1	57	1	5	0.67	1.00	1.00	1.119	633	5066	230	191	☑	☑	☐	☐

Figure 4. *Wells* data table.

You can view additional data by selecting **Advanced > Switch to Advanced User Mode**.





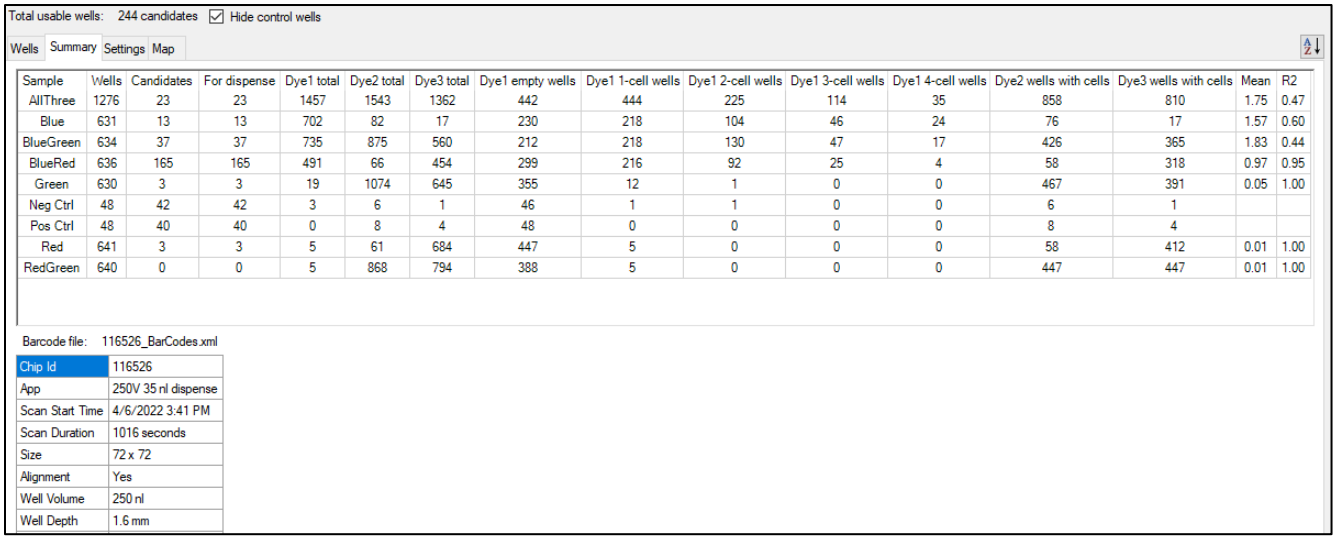
Row	Col	Candidate	For dispense	Sample	Barcode	SampleWell	State	Cells1	Cells2	Signal1	Signal2	Diam1	Diam2	Integ Signal1	Integ Signal2	Circularity1	Circularity2	Elongation1	Elongation2	Confidence	Confidence1	Confidence2
1	11	yes	yes	A498	TGGATCAA+TCCTTATT	A2	Good	1	0	1609		13		82059		0.83		1.30		1.00	1.00	1.00
1	17	yes	yes	A498	TCATACCA+TCCTTATT	A2	Good	1	0	2410		15		159060		0.80		1.25		1.00	1.00	1.00
1	24	yes	yes	A498	TTATGACG+TCCTTATT	A1	Good	1	0	3456		17		290304		0.78		1.18		1.00	1.00	1.00
1	30	yes	yes	A498	CGGTTGGT+TTGACTAT	A2	Good	1	0	3007		17		252588		0.76		1.22		0.95	0.95	1.00
1	34	yes	yes	A498	TTAACTGA+TTGACTAT	A2	Good	1	0	1010		12		44440		0.80		2.07		1.00	1.00	1.00
1	35	yes	yes	A498	ATATGCAA+TTGACTAT	A2	Good	1	0	1321		13		63408		0.82		1.48		1.00	1.00	1.00
1	36	yes	yes	A498	CTGGTCTT+TTGACTAT	A1	Good	1	0	3905		16		308495		0.74		2.17		0.94	0.94	1.00
1	57	yes	yes	A498	ATACGTTC+TCCTTATT	A1	Good	1	0	3994		17		347478		0.73		2.38		0.91	0.91	1.00
2	6	yes	yes	A498	CGTCGAGG+TCGCTAG...	A1	Good	1	0	5308		20		679424		0.79		1.51		0.77	0.77	1.00
2	7	yes	yes	A498	TCTCCTAG+TCGCTAGG	A1	Good	1	0	8294		14		464464		0.72		2.10		0.81	0.81	1.00

Figure 5. Additional well information displayed in Advanced User Mode.

B. Summary

The data table on the *Summary* tab displays tallies for controls, samples, and all categories in the results table. A description of each column header can be found in Appendix C.C, "Summary Table".

The *Summary* data table includes the barcode filenames and downselect information, if any.



Sample	Wells	Candidates	For dispense	Dye1 total	Dye2 total	Dye3 total	Dye1 empty wells	Dye1 1-cell wells	Dye1 2-cell wells	Dye1 3-cell wells	Dye1 4-cell wells	Dye2 wells with cells	Dye3 wells with cells	Mean	R2
AllThree	1276	23	23	1457	1543	1362	442	444	225	114	35	858	810	1.75	0.47
Blue	631	13	13	702	82	17	230	218	104	46	24	76	17	1.57	0.60
BlueGreen	634	37	37	735	875	560	212	218	130	47	17	426	365	1.83	0.44
BlueRed	636	165	165	491	66	454	299	216	92	25	4	58	318	0.97	0.95
Green	630	3	3	19	1074	645	355	12	1	0	0	467	391	0.05	1.00
Neg Ctrl	48	42	42	3	6	1	46	1	1	0	0	6	1		
Pos Ctrl	48	40	40	0	8	4	48	0	0	0	0	8	4		
Red	641	3	3	5	61	684	447	5	0	0	0	58	412	0.01	1.00
RedGreen	640	0	0	5	868	794	388	5	0	0	0	447	447	0.01	1.00

Figure 6. *Summary* data table with barcode filenames and downselect information.

NOTE: The Downselect table is only visible if downselect is in effect. See Section III.H, "Downselect (Optional)" for more information on the downselect function.

C. Settings

In the *Settings* tab, you can adjust options related to the display, image processing, post-processing, and other configuration parameters. There are several algorithms available in CellSelect software: V1, V2, V3, and Cardiomyocytes.

The 'V3' algorithm is the default processing algorithm. If your cell types are cardiomyocytes, you will want to select the 'Cardiomyocytes' algorithm (Section 3, "Cardiomyocytes Algorithm", below).

NOTE: The 'V1' algorithm was used in the original ICELL8 CellSelect Software; 'V2' was the original ICELL8 cx CellSelect Software algorithm. These two options are included in Shasta CellSelect Software only for legacy purposes.

We **strongly** recommend that you become familiar with a parameter before editing it.

- The settings that are most commonly adjusted, "Candidate Logic Selection" and "Thresholds", are shown in Sections 1 and 2, below.
- The unique features of the Cardiomyocytes algorithm are described in Section 3.
- The option **Actions > Compare with Existing Result File** can be used to compare the results after changing a Setting value to the results just prior to the change. For more information about this functionality, please see Appendix F, "Comparing Result Files".
- If you need to reset the parameters in the *Settings* window back to the default, see Appendix D.A, "Restore to Defaults".

Appendix D lists information about other, less commonly modified parameters.

The settings can be edited either by clicking the [Edit…] button under the *Settings* tab or through the **Advanced > Settings…** menu (Figure 7). Figure 8 shows the *Analysis Settings* window.

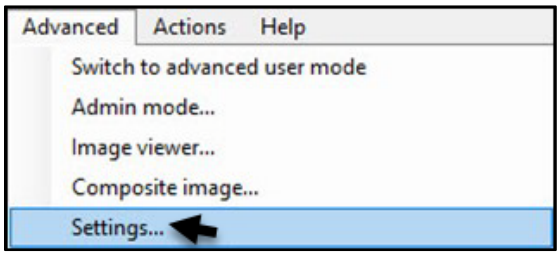


Figure 7. How to access the Settings editor. (**Left**) Through the [Edit…] button on the *Settings* tab. (**Right**) Through **Advanced > Settings…**

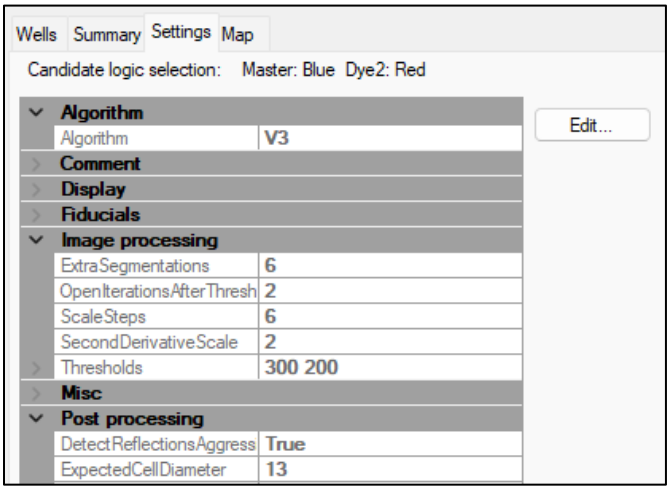


Figure 8. *Analysis settings* editor window. Setting values can be customized here.

1. Candidate Logic Selection

The logic for defining what qualifies as a "candidate" well (candidate selection) is determined through two steps:

1. The main dye needs to be identified and defined as Dye1. Objects that are visible with this dye are considered cells and therefore pass to the next test.
2. Objects in a secondary channel (Dye2) or third channel (Dye3) that are colocalized with cells identified in the Dye1 channel are used to test whether a well will be designated as a single-cell candidate.

A well will be flagged as 'Inconclusive' if there are objects identified in the secondary or tertiary channel(s) that are not colocalized with a cell in the primary (Dye1) channel.

To define the algorithm rules:

1. Click on the […] button to configure the channels that determine the logic rules.

Figure 9. Accessing the *Candidate Logic Selection* window.

This will bring up the *Candidate Logic Selection* window.

2. Select the 'Dye1' primary channel used to identify cells.
   - If two dyes were used during staining and imaging, the second dye option will automatically be selected as 'Dye2' when the other dye is designated
   - If three dyes were used, select the color to serve as the secondary (Dye2) and tertiary (Dye3) channels

   When dye selection is complete, click [OK].

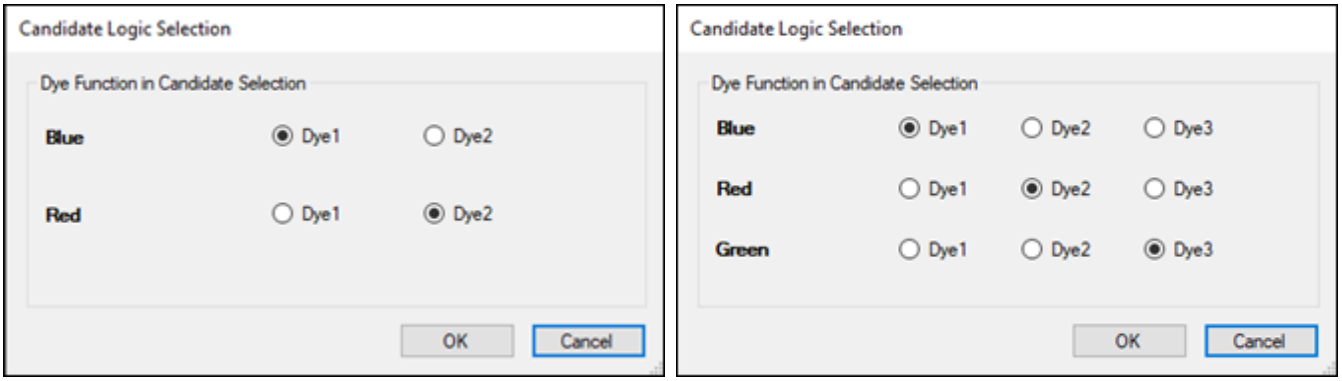


Figure 10. The *Candidate Logic Selection* window. **(Left)** Two color channels. **(Right)** Options when three color channels were used during imaging.

3. Click on the [TT…] button in the *Analysis settings* window.

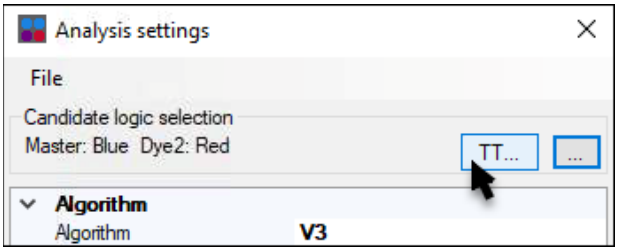


Figure 11. Accessing the *Truth Table* window.

This brings up the *Truth Table* (TT) window (Figure 12).

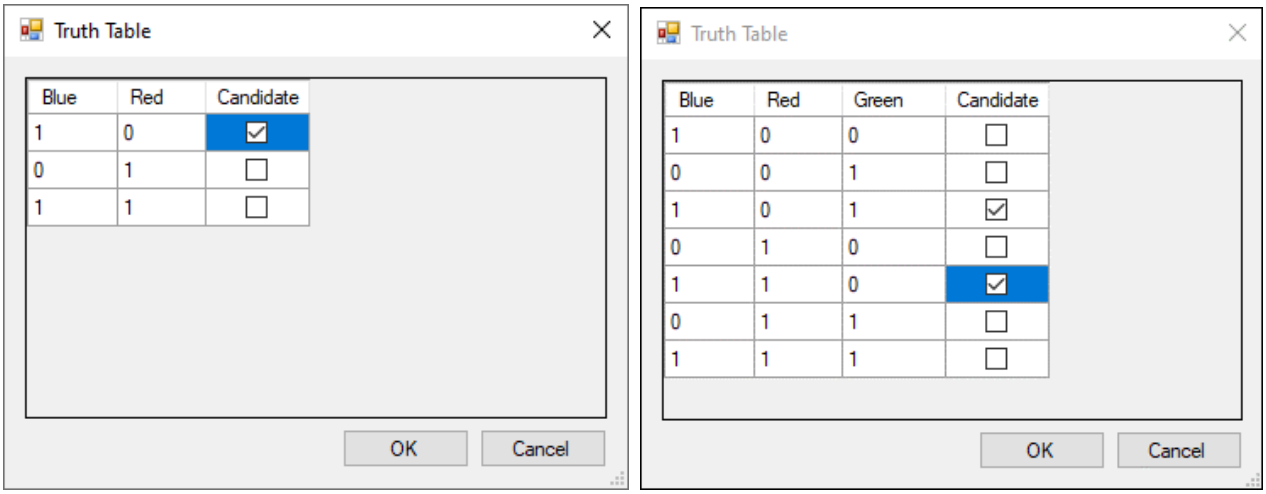


Figure 12. The *Truth Table* window. Dye logic rules are configured here. **(Left)** Refinement options for candidate definition for two-color channel imaging. **(Right)** Options available for three-color channel imaging.

4. Check the box or boxes by which you want to define a candidate well. For each color, a value of '1' means that an object should be detected in the well when scanned with that channel, while a '0' indicates that an object should not be detected for that color range.

   Any combination of boxes may be checked at once to indicate which wells are candidates.

   Example:

   – **Two color channels**
   In the image on the left in Figure 12, the checkmark in the first row indicates a candidate well will have an object detected (1) in the Blue channel where there is no co-located object (0) in the Red channel.

   If the reverse situation was desired (no object in Blue, object in Red), you would uncheck the first box (1, 0) and check the second box (0, 1).

   – **Three color channels**
   In the image on the right in Figure 12, two candidate scenarios are defined.

   - An object is detected in the Blue and Green channels, but NOT in the Red (1, 0, 1)
   - An object is detected in the Blue and Red channels, but NOT in the Green (1, 1, 0)

2. Thresholds

Thresholds are the other most common parameter that might need to be modified. The Thresholds setting defines a differential of how much brighter an object needs to be, compared to its local neighborhood, to be included in the analysis. The default values work well for many applications, but you might want to adjust this value in some cases: for example, when the cells didn't stain well or stained too well or if there is high background brightness.

A lower "Threshold" value detects a larger number of cells; however, if the thresholds are too low, the software will identify many wells as "TooManyCells" and report a very low number of cells.

1. Expand the item to edit the individual values for "Thresholds".

Figure 13. Expanded "Thresholds" option in the *Settings* window.

2. If you want all channels to have the same value, type that value into the "All" field.

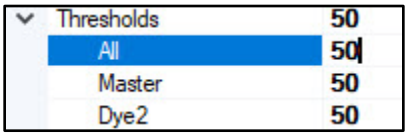


Figure 14. Selecting the "Thresholds" > "All" option in *Settings* window to customize it.

3. When the setting is collapsed, you will see two values separated by a space. If all items have the same value, you will only see one value listed.

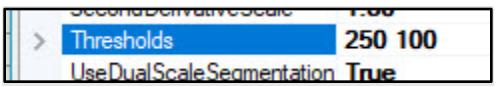


Figure 15. The *Settings* "Threshold" section, displaying multiple sublevel values in the collapsed view.

3. Cardiomyocytes Algorithm

The algorithms include a setting for cells which may be elongated and/or have more than one nucleus. To detect these cells, select 'Cardiomyocytes' in the *Analysis settings* window on the "Algorithm" selection entry.

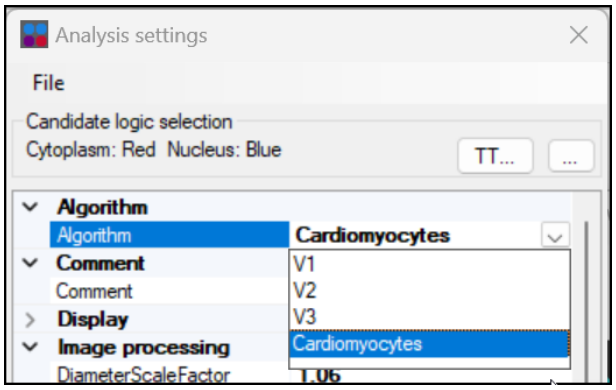


Figure 16. Selecting 'Cardiomyocytes' from the "Algorithm" analysis setting.

The most frequently used settings for this algorithm type are the "Candidate Logic Selection", "Truth Table", and "Thresholds" for each color channel, which works the same way as in the algorithm described above.

For a three-channel experiment, the Candidate Logic Selection in 'Cardiomyocytes' mode provides for cytoplasm and nuclei to be assigned to any of the three available channels, but only those two assignments become part of the candidate well logic. The third channel is informational only.

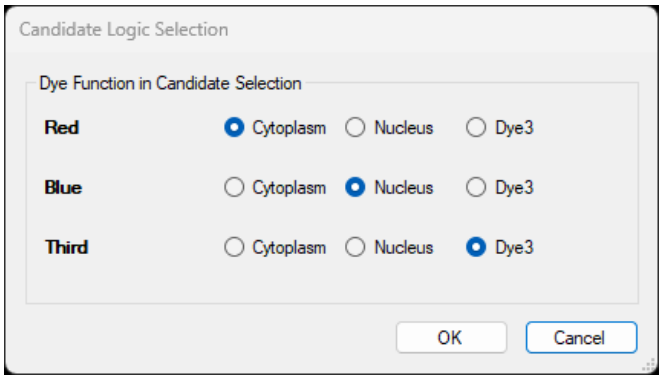


Figure 17. Cardiomyocyte-mode *Candidate Logic Selection* window for a 3-color channel dataset.

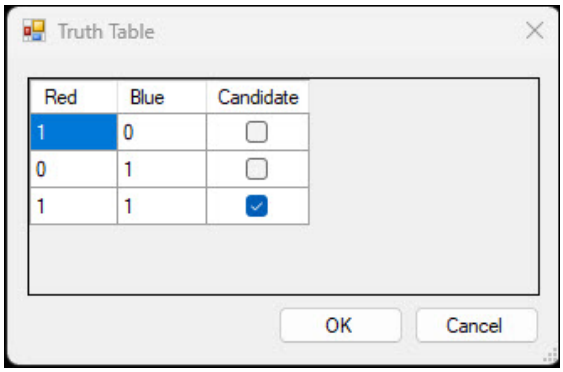


Figure 18. The *Truth Table* in 'Cardiomyocytes' mode. Only the assigned cytoplasm and nucleus channels are used, even if the dataset has three channels.

There are cardiomyocyte-specific settings that become active in the *Post processing* section for the 'Cardiomyocytes' algorithm mode:

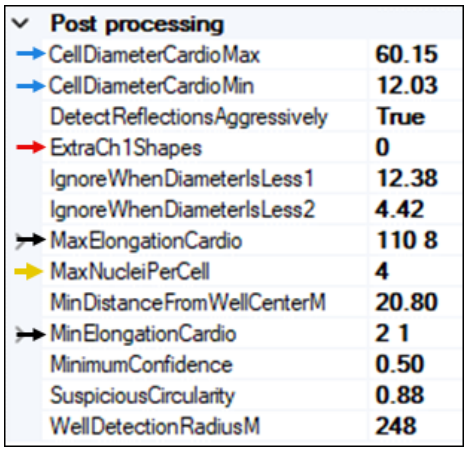


Figure 19. The *Post processing* options in the *Settings* menu unique to the cardiomyocyte algorithm.

- "MaxNucleiPerCell" (highlighted by the yellow arrow) specifies the maximum number of nuclei expected within the body of a detected cell. The value can be defined as 1, 2, 3, or 4.

  In the *Wells* tab, if the "AvgNucleiSep" column is enabled (Figure 21), the average nuclei separation will be displayed. This is the distance between nuclei in cells with two nuclei, or in cells with three or more nuclei, the average of each nearest-neighbor separation.

- The settings highlighted by blue arrows,"CellDiameterCardioMax" and "CellDiameterCardioMin", are equivalent to the "CellDiameterMax" and "CellDiameterMin" settings in the V3 algorithm

- The settings highlighted by black arrows, "MinElongationCardio" and "MaxElongationCardio", are specific for cells identified as cardiomyocytes, which are typically more highly elongated.

- "ExtraCh1Shapes" (highlighted by the red arrow) has a default value of '0' but may optionally be set to 1 to allow one extra detectable object in a well in the 'Cytoplasm' dye channel before the well will be disqualified as a candidate.

- The "SuspiciousCircularity" parameter is not active in the Cardiomyocytes algorithm.

Figure 20 (below) shows an elongated cardiomyocyte cell, with three nuclei visible in the second (blue) channel, while Figure 21 shows the *Wells* tab for this chip, with the "AvgNuclei Sep" cell for this well highlighted by a red box. The value is calculated to be 23.66 pixels; with a 1.6 µm/pixel calibration, this works out to about 38 microns.

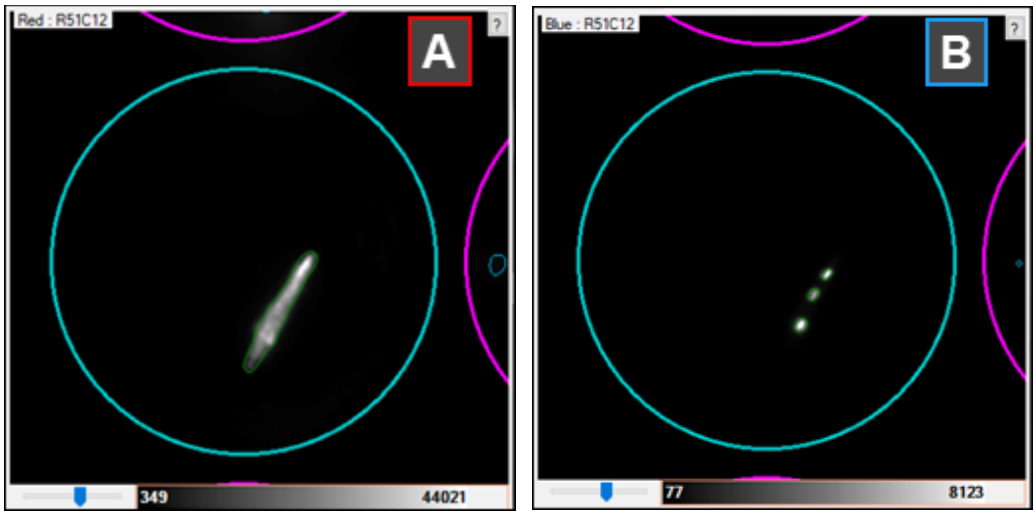


Figure 20. *Main* screen images of a cardiomyocyte cell with three nuclei. These images (A and B) would normally be stacked as Top and Bottom within the software, shown here side by side for greater clarity. **(Panel A)** The image of the cardiomyocyte cell detected in the red channel. **(Panel B)** The outline and identification of the three nuclei of the cell in the blue channel.

Wells | Summary | Settings | Map

Row	Col	Candidate	For dispense	Sample	Barcode	SampleWell	State	Cells1	Cells2	Signal1	Signal2	Confidence	AvgNucleiSep
37	27	yes	yes	sample	AACGTTACTCG	A2	Good	1	3	31479		0.94	14.50
41	7	yes	yes	sample	GTTGCAGACGG	A1	Good	1	3	24798		0.87	10.29
51	12	yes	yes	sample	GAACCGCGAAC	B2	Good	1	3	21714		0.79	23.66
51	48	yes	yes	sample	GTCTACTGAAG	B2	Good	1	3	32375		0.87	10.00
62	33	yes	yes	sample	CGGTTGCGTTG	C2	Good	1	3	25184		0.88	10.51
14	41	yes	yes	sample	AACGGATAATA	B1	Good	1	4	22447		0.89	15.79

Figure 21. The *Wells* table for the cardiomyocyte cell depicted in Figure 20. The well shown in Figure 20 is highlighted in blue in the table here. The red box highlights the "AvgNucleiSep" (Average Nuclei Separation) value determined by scanning the nuclei in Figure 20, Panel B.

4. Saving Settings to an XML File

If you have adjusted the settings to optimize candidate wells, either manually or using the [Tune] function (Section III.J and Appendix G), and are planning to repeat the experiment with similar parameters such as the same sample type or workflow application, we recommend saving a copy of the settings into a file (XML) to expedite analysis in the future. This saved file can then be used as a custom file when setting up the "Analysis" settings during new experiment setup in CELLSTUDIO software (Shasta Single Cell System User Manual, Section VII.D, "Start New Experiment").

1. With a results file loaded, either choose the *Settings* tab and click the [Edit] button or go into the menu **Advanced > Settings…** to bring up the *Analysis settings* window.
2. From within the window, go into **File > Save…**

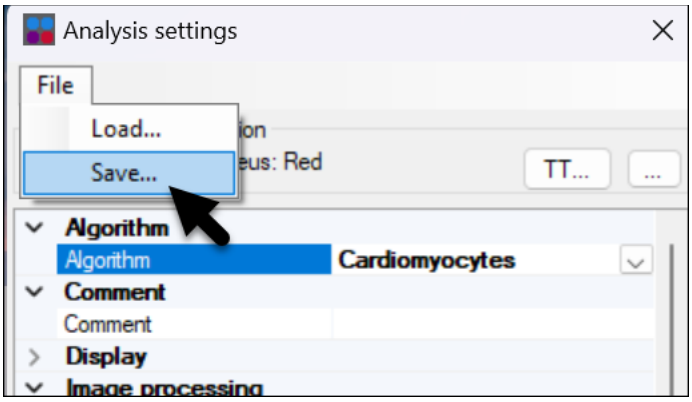


Figure 22. The Save… menu option in the *Analysis settings* window.

3. Save the file with a meaningful name and in a location where you can easily find it later. You can quit out of the settings, if desired.

5. Loading a Saved Settings File

If you have saved customized analysis settings to an XML file, either following the instructions above ("Saving Settings to an XML File") or after performing automated threshold detection (Appendix G), the settings can be imported back into CellSelect software during a well candidate analysis.

1. Load an imaging results file (`.wcd`) into CellSelect (see Section III.B, "Load Images").
2. From the main CellSelect interface, either choose the *Settings* tab and click the [Edit] button or go into **Advanced > Settings…** to bring up the *Analysis settings* window.
3. In the window, go to **File > Load…** (see Figure 22).
4. Select your saved file and rerun your analysis.

D. Map

The table on the *Map* tab displays different data categories in bar graphs and graphic maps. Click the drop-down menu and graph icons to select the data category and graph type (Figure 23). The "Measure" drop-down box options are identical to the *Wells* column names; definitions of the options can be found in Table 6 in Appendix C.A, "Wells Data Table".

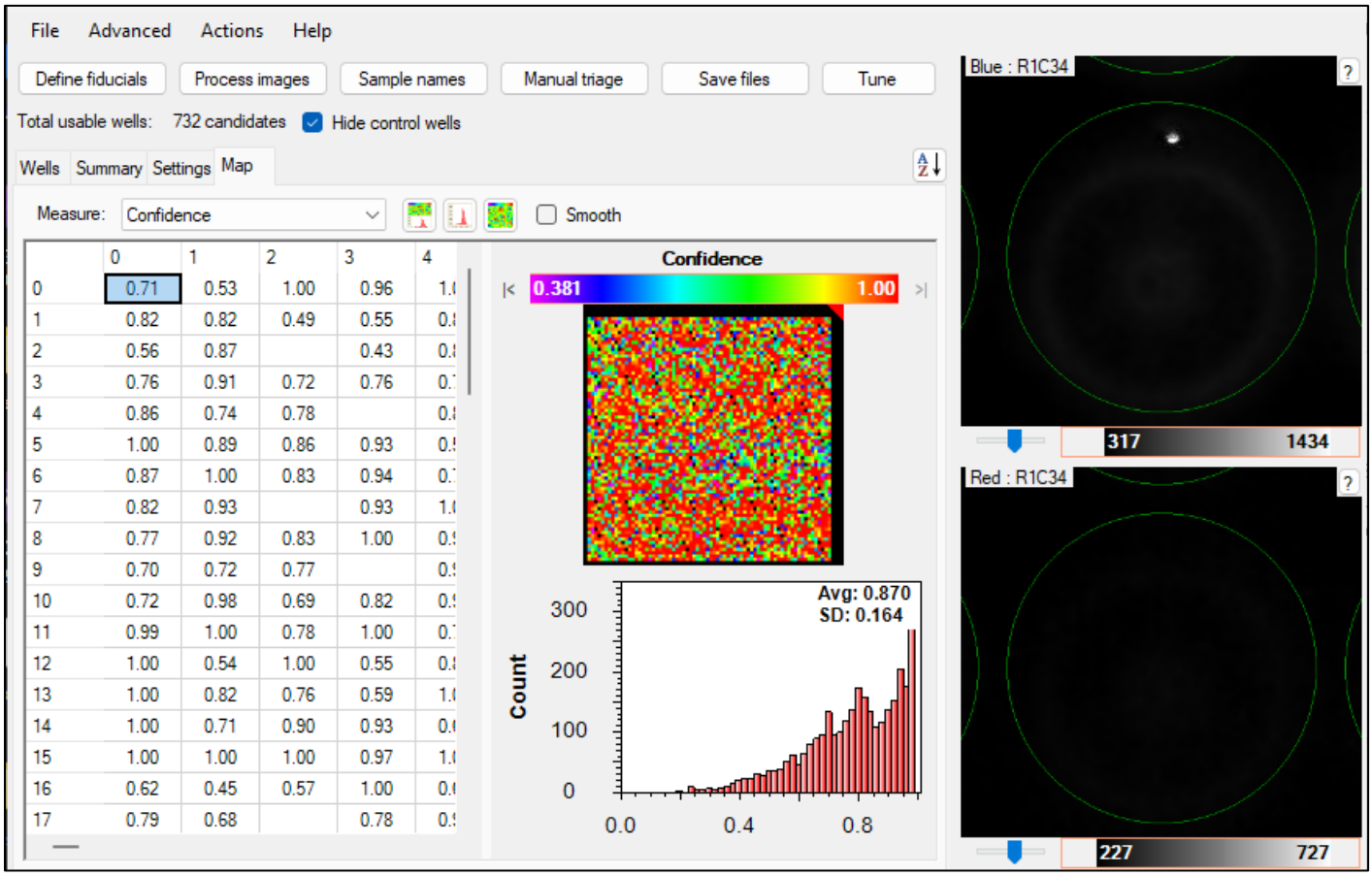


Figure 23. *Map* tab.

III. Procedure

A. Overview

When doing a chip scan using Shasta CELLSTUDIO software, the data automatically transfers to CellSelect software. The image files are then processed in the background to generate a filter file for later dispensing reagents.

If needed, it is possible to run CellSelect on its own, either on the Shasta system computer or as a stand-alone application, to view the image data, adjust existing parameters, or load a different barcode file, as described below.

1. **Load Images:** Open a saved `.wcd` file from the CellSelect Software **File** menu.
2. **Load a Different Barcode File (optional):** Barcodes are already selected when you dispense cells in the Shasta Single Cell system. However, this menu item allows you to associate a different barcode file to the run for analysis.
3. **Process Images:** Start image processing. The software re-analyzes every well image and determines which wells are considered candidates for additional testing. (Candidate selection logic is described in Section II.C.1, above).
4. **Save Files:** Save details of all well analyses as well as the filter file for dispensing reagents to selected candidate wells.

Each step, including manual nanowell analysis, is described in this section.

B. Load Images

You can open CellSelect software directly after scanning from CELLSTUDIO software, using the [Open in CellSelect] button on the Summary page.

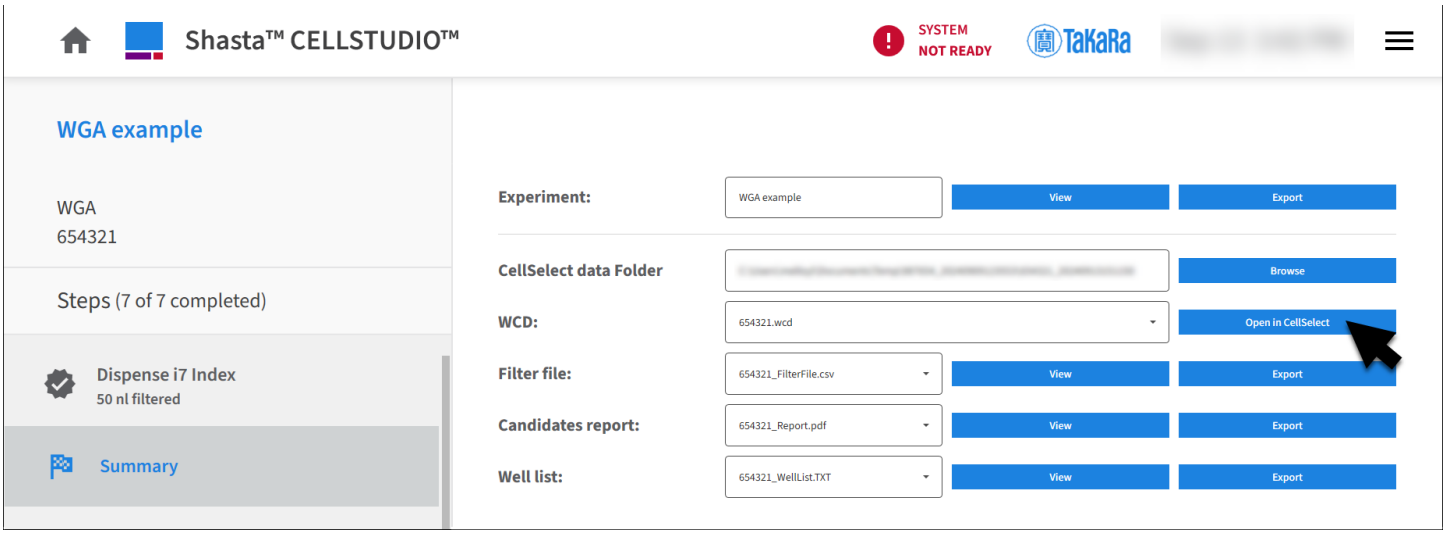


Figure 24. How to open CellSelect software from the Shasta CELLSTUDIO interface. The [Open in CellSelect] button is indicated by the black arrow.

You can also open the software on a separate system, if installed. Contact field_support@takarabio.com for the instructions for how to install and use CellSelect software on a standalone system.

1. From the Shasta CellSelect Software

1. Launch CellSelect Software by clicking the `CellSelect` icon: 

2. In the *Main* window, navigate the menu **File** > **Open Chip Folder**… item.

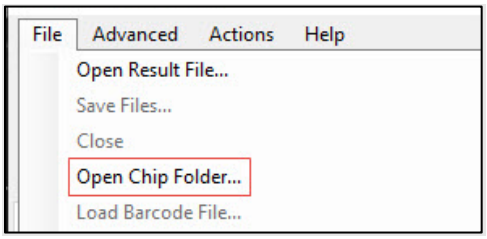


Figure 25. Where to find the File > Open Chip Folder… menu item.

3. Navigate to the folder where the analysis files (files with the extension `.wcd`) should be located.
4. Select the folder in the navigation window, then click the [Select folder] button.

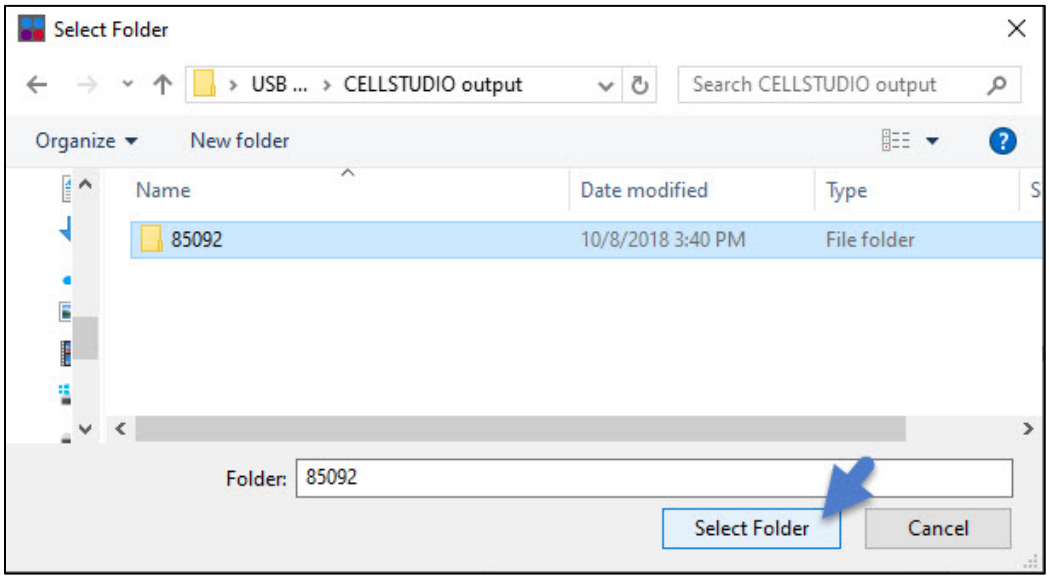


Figure 26. Selecting a chip folder. Do **NOT** double-click and open the folder.

- If there is one `.wcd` file in the folder, that file will be loaded
- If there is more than one `.wcd` file in the folder specified**,** the software will prompt you to select one (Figure 27)

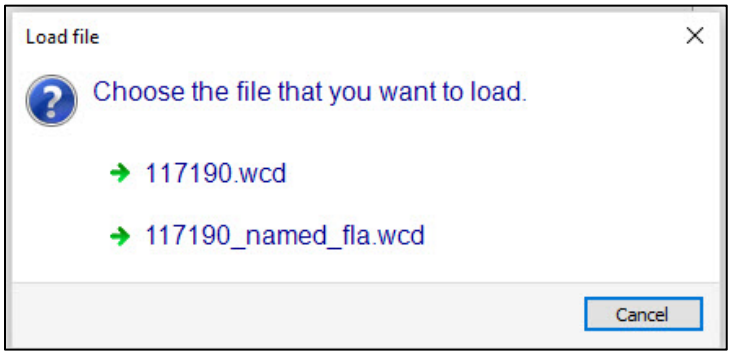


Figure 27. The *Load file* window prompt if multiple `.wcd` files are present in the selected folder.

2. From the Open Result File… Menu Option

You can also open a specific analysis file (`*.wcd`) by using the **File > Open Result File...** menu option.

3. Drag and Drop onto the CellSelect Software Icon

Alternatively, you can drag and drop a folder from Windows Explorer into the application icon.

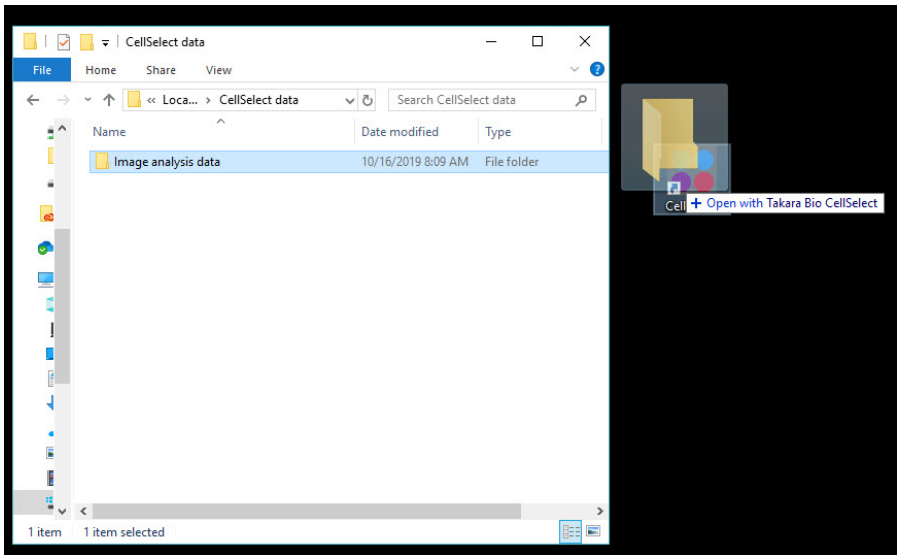


Figure 28. Drag and drop a folder of analysis data onto the CellSelect Software icon to open a results file. If multiple `.wcd` files are contained in the folder, it will behave similarly to the cases of Figure 27.

C. Load a Different Barcode File (Optional)

Barcodes are selected either during the cell dispense step or when the images are scanned by the Single Cell system. However, this menu option allows you to associate a different set of barcodes with the results file for the CellSelect software analysis process.

NOTE: If an incorrect index set was selected during the experimental setup (Shasta Single Cell System User Manual, Section VII.D, "Start New Experiment"), the fix is to load the correct barcode file following the steps below.

1. With the results file (`*.wcd`) open, in the *Main* window, click **File > Load barcode file…**.

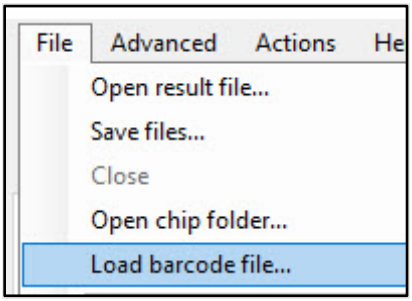


Figure 29. File menu.

2. Navigate to the folder location and choose the barcode XML file you want to apply instead.

   Select the XML file based on whether the indices provided by Takara Bio are associated with a prevalidated application or a custom-defined barcode file.

1. Takara Bio prevalidated application barcode files

The following files are included by default for the Takara Bio prevalidated applications:

Table 1. Prevalidated application barcode XML files

Filename	Application
Shasta_Short_SetA.xml Shasta_Short_SetB.xml	mRNA-seq
Shasta_Long_SetA.xml	Total RNA-seq
	Whole genome amplification (WGA)

These XML files contain the barcode sequences for each nanowell location. The file location folder is located at:

```
C:\ProgramData\Takara\CellSelect\AssayMaps\
```

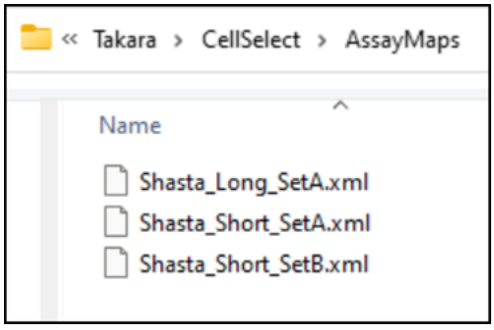


Figure 30. Location of the barcode files preloaded in CellSelect software.

2. Custom Indexes

If custom barcode index files were defined and incorporated into the CELLSTUDIO dispense application (Shasta Single Cell System User Manual, Section VII.D, "Start New Experiment"), then the barcodes will be imported automatically into CellSelect software.

If, however, you need to apply a different barcode file in CellSelect software, an XML file will need to be created. Refer to the Shasta Single Cell Advanced Features User Manual, Section IV.F, "Export Barcode Set" for the procedure to do this.

When selecting the barcode XML file to upload, navigate to the folder location where the exported XML file was saved.

D. Process Images

The [Process images] function analyzes all 5,184 nanowells through the image files generated by CELLSTUDIO software during chip imaging.

1. Click [Process images]. The software will analyze the sets of multiwell images and automatically identify and select all nanowells that contain viable, single cells (i.e., candidates) and controls based on the parameters defined under Settings. The number of images depends on the dispense pattern for the application in CELLSTUDIO software.

NOTE: For each image, the following criteria must be satisfied for the software to identify a cell as being a candidate for downstream analysis:

- The cell must pass candidate logic rules (described in Section II.C.1)
- It must be the only object visible in the nanowell
- It must satisfy defined size/shape parameters

These conditions can be modified in the *Settings* window. See Section II.C and Appendix D for more information on the settings parameters.

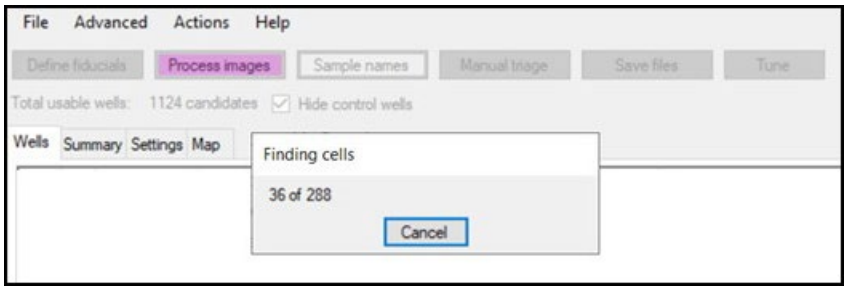


Figure 31. Image processing, in progress.

2. After processing is complete, the software will provide a prompt for saving results. Click [Yes] and input a file name prefixed with the chip number, `<chipID>_<Date>.wcd`, where `<chipID>` is the chip number identifying the chip (found on the edge, Figure 32) and `<date>` is the date the image was processed.

   Example: `116454_053024.wcd`

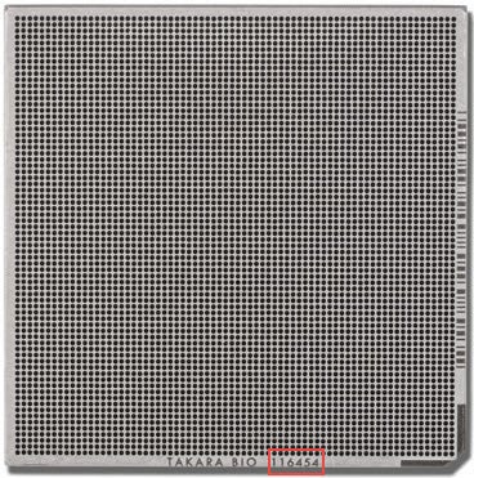


Figure 32. Single-Cell 350v chip. The chip ID is located next to the Takara Bio branding, on the bottom when the chamfered edge is on the lower right.

NOTE: The file name must start with the chip ID; do not modify that part of the name since it is used to keep track of various related files.

The software will then do the following:

- Save the result file under the specified name (e.g., `116454_053024.wcd`). The results file will contain the selected nanowells and setting parameters.

- Generate the filter file for the dispense of reagents (e.g., `116454_053024_FilterFile.csv`). The selected wells in the filter file will be displayed in the *For Dispense* column.
- Generate a PDF file called `116454_053024_Report.pdf`, which contains a short summary of the results.
- Generate a text file called `116454_053024_WellList.txt`, which contains the content of the *Wells* table in a form that can be read easily by downstream analysis software.

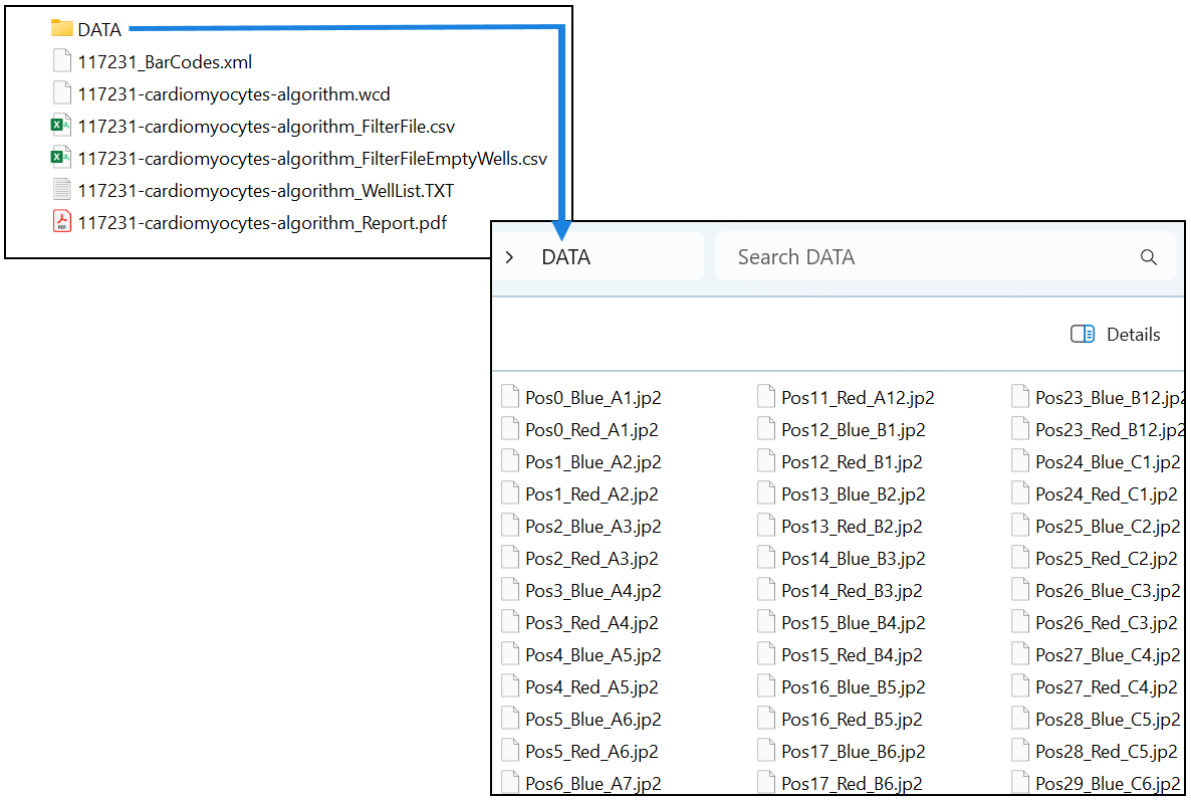


Figure 33. Files generated by CellSelect Software. The chip ID in this example is '117231'. You can load the results along with the settings from the saved `*.wcd` file.

3. After the images are processed, the well classification and summary of the results will be available for review (Figure 34).

NOTE: Images must be reprocessed after changing the analysis settings to transform the displayed results to reflect the new settings.

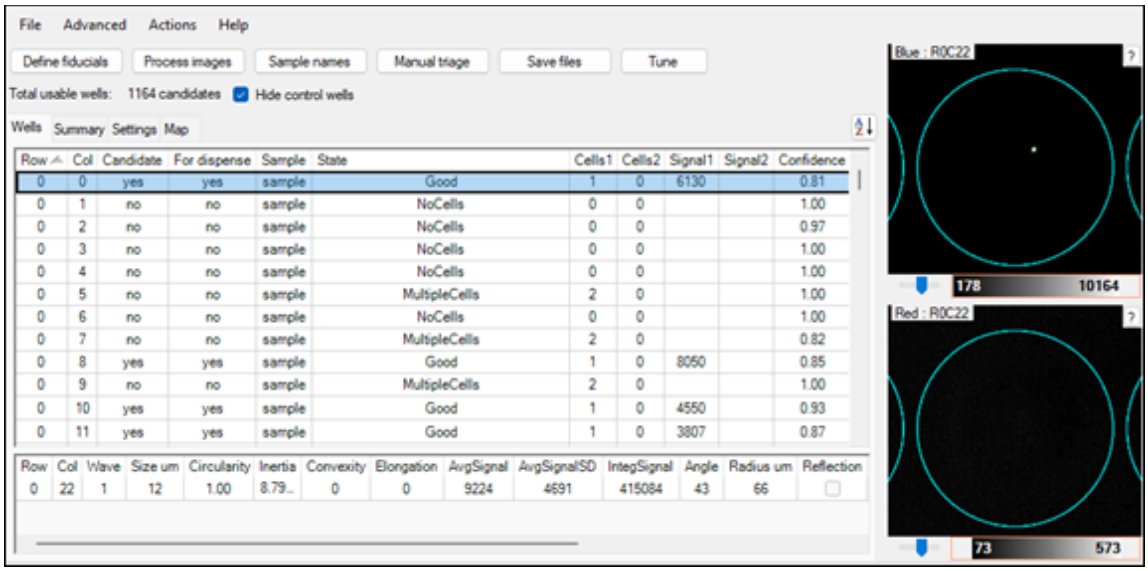


Figure 34. Example of the *Wells* tab with results data post-image process.

E. Change Sample Names

Click [Sample names] and enter a single sample name or use the source plate configuration layout from CELLSTUDIO software.

- "One sample" changes ALL names to the name you enter (the default name is 'Sample')
- "Multiple samples" changes the default name to whatever you edit it to, e.g., to grid names from the source plate (Figure 35)

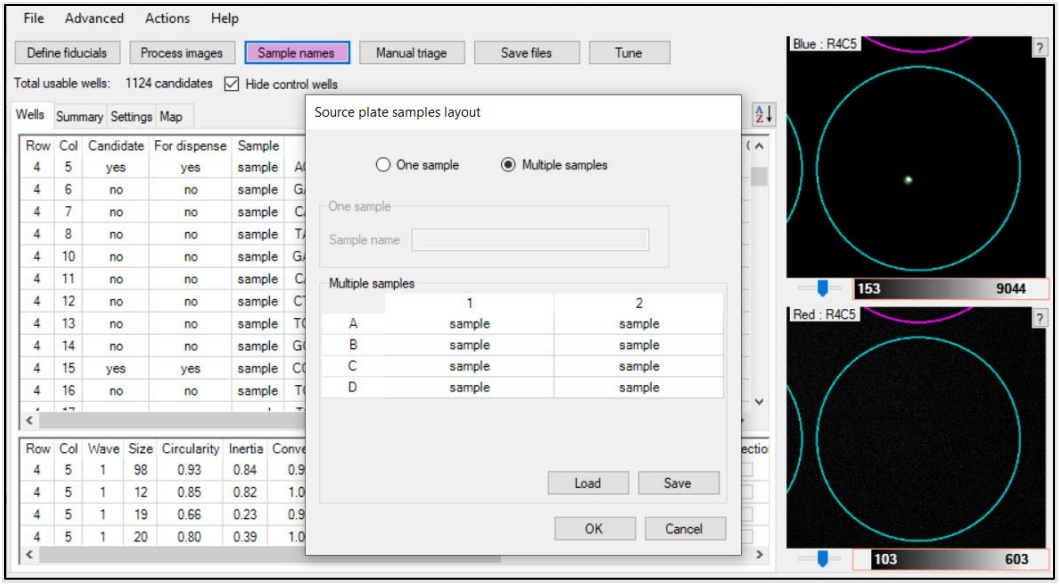


Figure 35. Inputting multiple sample names.

F. Review Images

1. Consolidate All Candidate Wells to the Top

1. Click the *Wells* tab and sort the nanowell data in the "Candidate" column by clicking the "Candidate" header (Figure 36). The software will sort all selected nanowells based on the "Candidate" value.

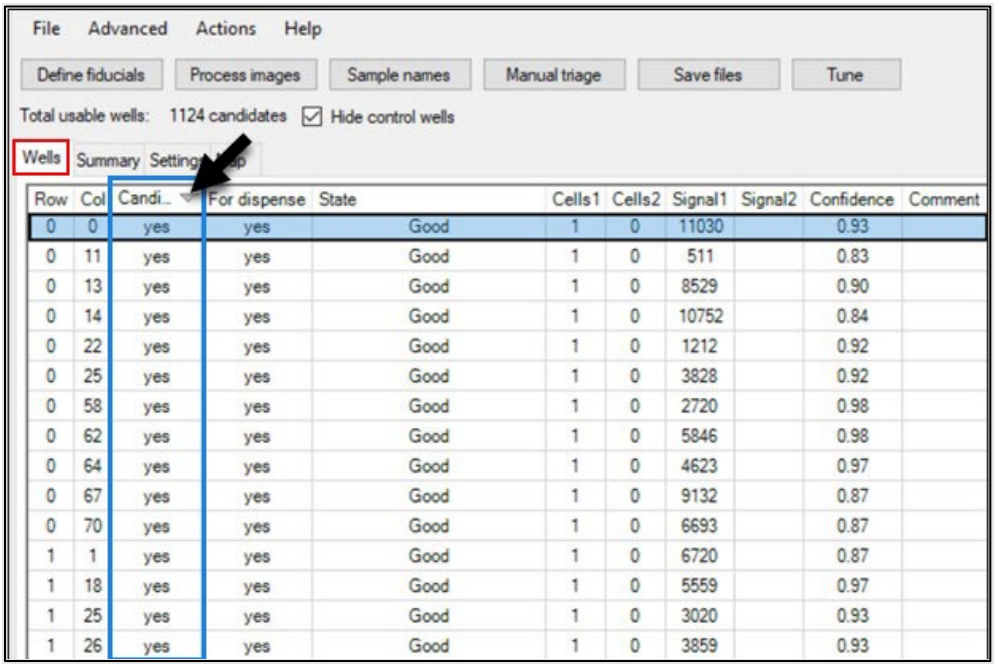


Figure 36. Sorting the "Candidate" column to consolidate all selected nanowells to the top of the table.

2. Click any row for closeup views of the selected nanowell using the imaging filters.

2. View/Edit Results List

If desired, you can examine any selected nanowell and view it as a single-well or multiwell image (Appendix A.E, "Well Images").

1. In the *Main* window, click **Advanced > Image Viewer…** (Figure 37). A multiwell image highlighting the nanowell selected in the results table (on the *Wells* tab) will appear.
2. Arrange the windows as demonstrated in Figure 37. When you click on any of the rows in the results table (on the *Wells* tab, left), the software will present corresponding single-well images (middle), and display the corresponding multiwell image in the *Image Viewer* window (right). Similarly, the corresponding row in the Wells table will be highlighted by double-clicking on a well in the *Image Viewer*.

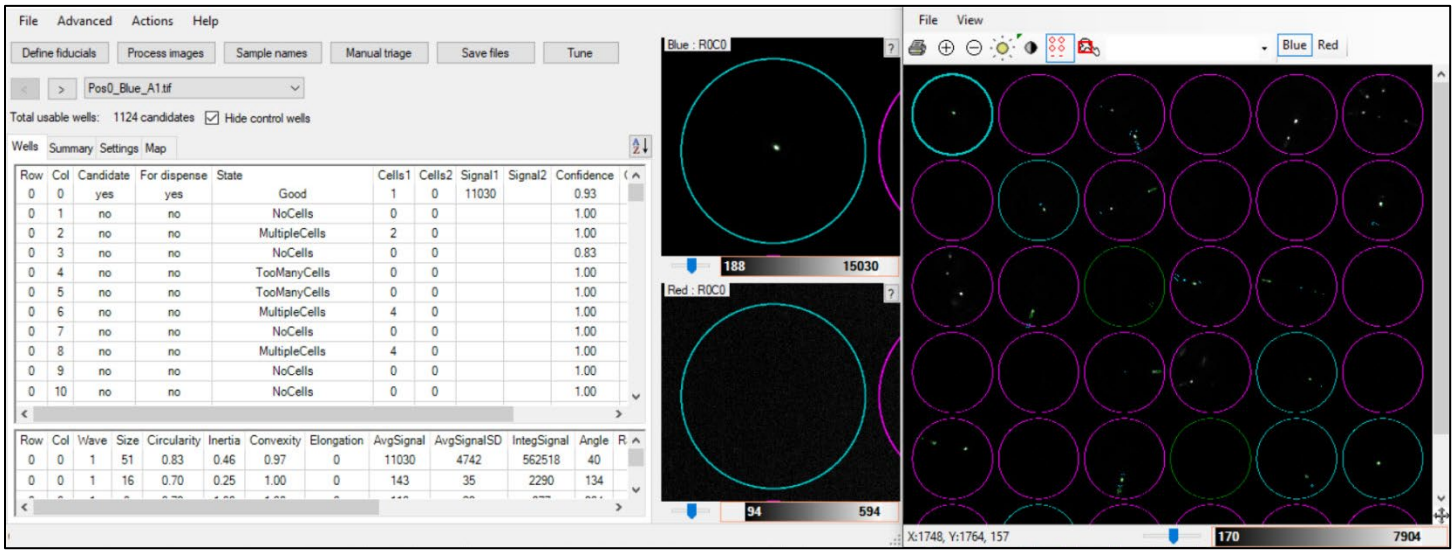


Figure 37. Opening and arranging windows for optimal image viewing. The image on the right is the multiwell image in Image Viewer. The images in the center show a single cell in a nanowell stained by Blue dye **(top)** and the absence of Red stain in the corresponding position **(bottom)**.

3. Use the tools in the *Image Viewer* toolbar to critically examine the cells (see Appendix A.F, "Image Viewer", for details on using the Image Viewer Tools).
4. To manually exclude one or more candidate wells, right-click the highlighted row(s) and select **Exclude selected wells** (Figure 38). To exclude several candidate nanowells or include nanowells the software initially excluded, consider using the manual triage function described in the section below (Section III.G).

You can also force non-candidate wells to be included in subsequent dispenses by selecting them and choosing **Include selected wells** in the right-click menu.

- Nanowells that were manually excluded contain a flag 'GoodButExcluded' in the "State" column
- Nanowells that were manually included contain a flag 'ManualUse' in the "State" column

The Wells and Summary tables are updated immediately after any manual overrides. The result files need to be re-saved after performing manual overrides, to save changes.

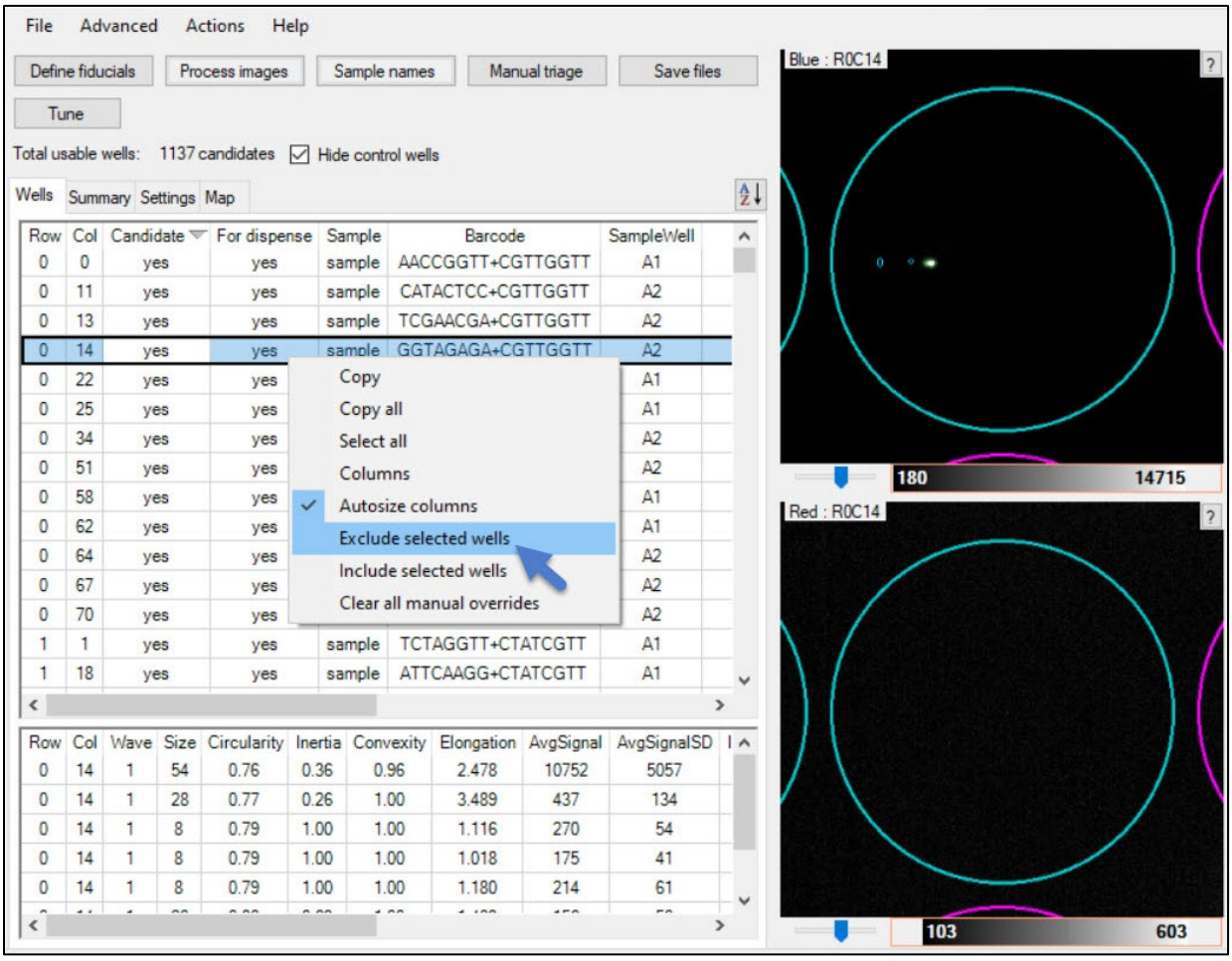


Figure 38. Excluding candidate wells.

G. Use Manual Triage (Optional)

The manual triage function opens a dialog box to quickly review and reject (or bypass) consecutive nanowells down the *Wells* table.

1. Click [Manual triage] to open the corresponding window.
2. Examine each consecutive nanowell image and click [Reject - Next Well] to exclude a candidate nanowell and move on to the next candidate nanowell or click [Next] to retain a candidate nanowell and move on to the next candidate nanowell.
3. You may also click [Use - Next Well] to use a nanowell that was not determined to be a candidate by the software.
4. Add a comment to the selected nanowell by typing it into the "Comment" field and pressing the **[Enter]** key. You can also double-click on one of the already-defined comments in the list to edit the content.

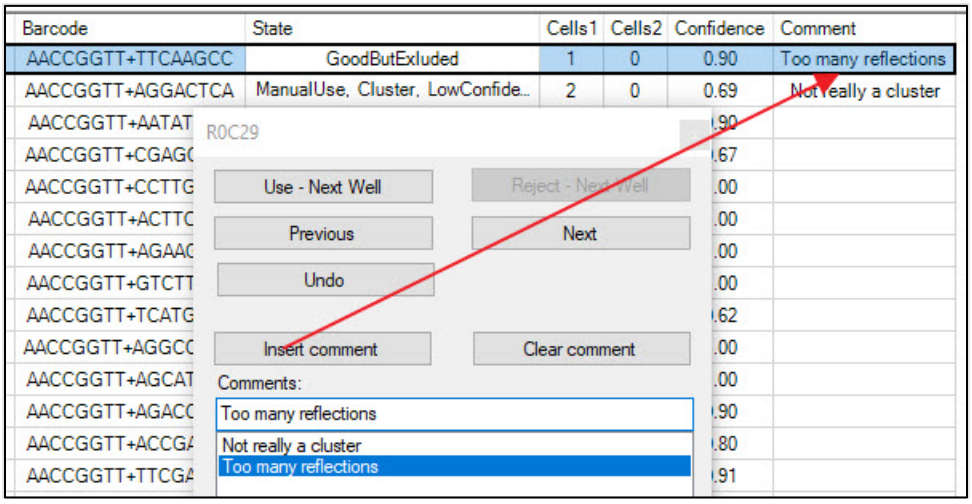


Figure 39. Adding comments to selected nanowells.

Best practice recommendation

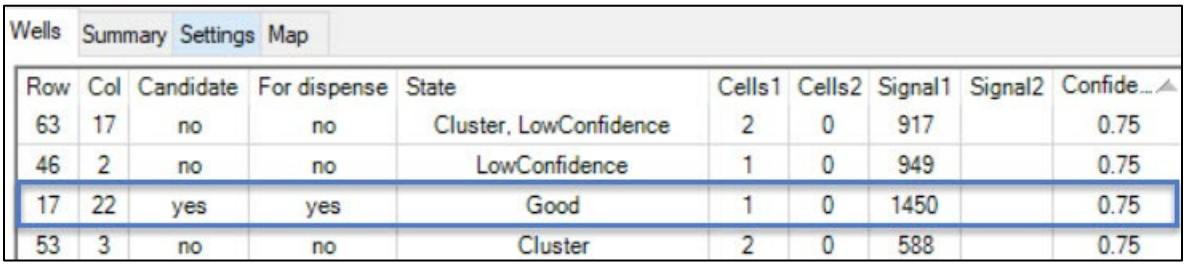
Although for some users and in some instances, triaging every nanowell on the chip may be desired, we recommend doing a subset using the following procedure:

1. Sort wells by increasing confidence level by clicking the column header.
2. Scroll down in the table to where the confidence level value is close to the MinimumConfidence setting (Appendix D.K).
3. Wells that are near the MinimumConfidence level and identified as candidates or non-candidates are those which may need manual triage.

Depending on the outcome, the MinimumConfidence value setting could then be increased or decreased to better fit with those observations, providing you with even greater confidence in the automated candidacy detection.

Example

In the following two figures, the MinimumConfidence is set in the software to a value of 0.75. The *Wells* information was sorted in increasing confidence. Below 0.75, all nanowells were marked as "Candidate" = 'No' but scrolling down (in increasing confidence values) a 'Yes' appeared with a "Confidence" = '0.75' (Figure 40).

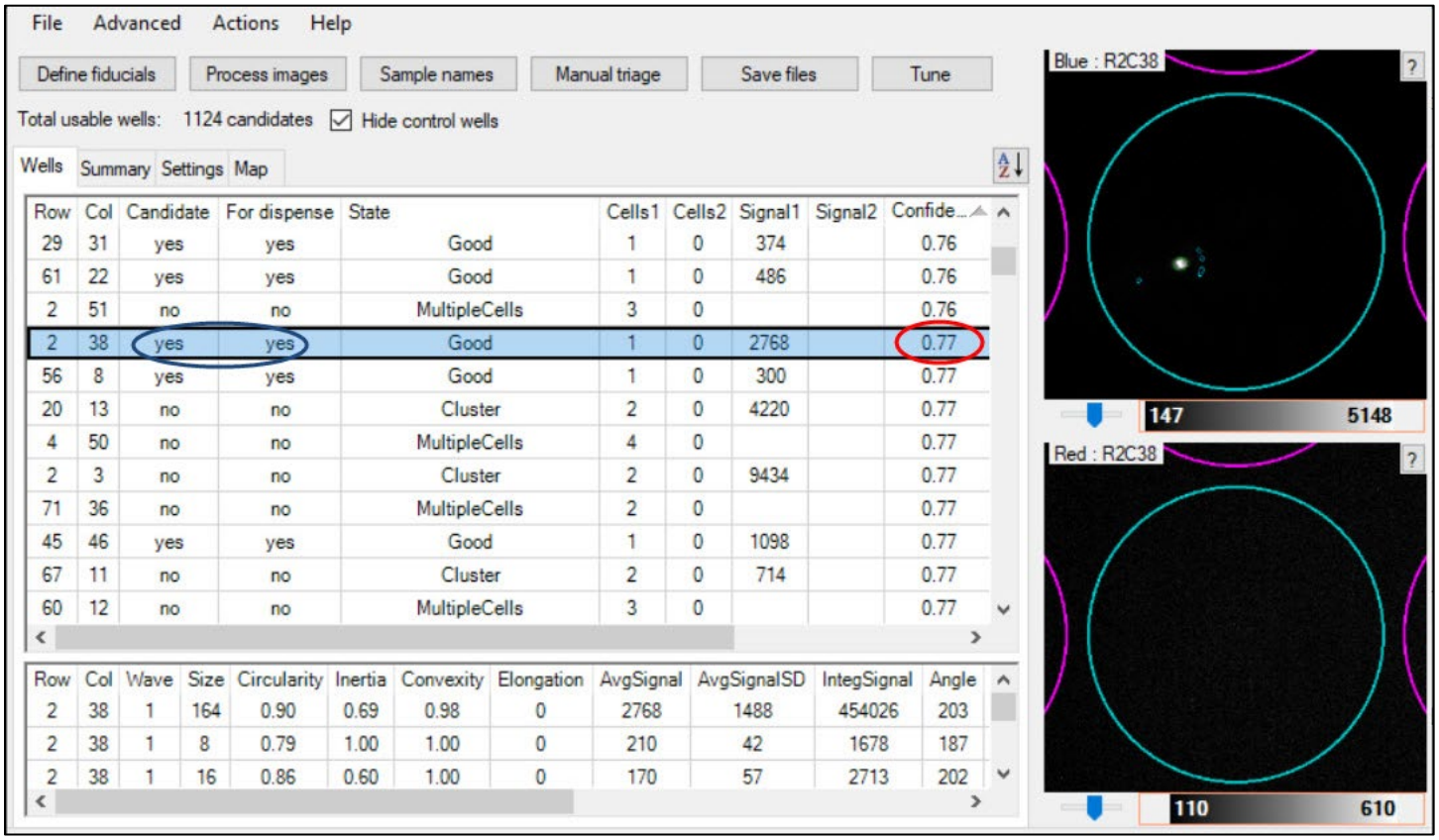


Row	Col	Candidate	For dispense	State	Cells1	Cells2	Signal1	Signal2	Confide...
63	17	no	no	Cluster, LowConfidence	2	0	917		0.75
46	2	no	no	LowConfidence	1	0	949		0.75
17	22	yes	yes	Good	1	0	1450		0.75
53	3	no	no	Cluster	2	0	588		0.75

Figure 40. Example: a candidate well identified with confidence at the minimum threshold setting.

The wells before and after the one identified in Figure 40 make for a good starting place for manual triage.

In reviewing the "Candidate" = 'Yes' rows in the example, many were correct or identified misshapen cells which might be okay. Then the nanowell in Figure 41 was encountered. Marked 'yes', but with a calculated Confidence of 0.77, the image displayed for the blue channel (top) shows indications of being a multiplicate. (For more information about Confidence, see Appendix D.K.)



Row	Col	Candidate	For dispense	State	Cells1	Cells2	Signal1	Signal2	Confide...
29	31	yes	yes	Good	1	0	374		0.76
61	22	yes	yes	Good	1	0	486		0.76
2	51	no	no	MultipleCells	3	0			0.76
2	38	yes	yes	Good	1	0	2768		0.77
56	8	yes	yes	Good	1	0	300		0.77
20	13	no	no	Cluster	2	0	4220		0.77
4	50	no	no	MultipleCells	4	0			0.77
2	3	no	no	Cluster	2	0	9434		0.77
71	36	no	no	MultipleCells	2	0			0.77
45	46	yes	yes	Good	1	0	1098		0.77
67	11	no	no	Cluster	2	0	714		0.77
60	12	no	no	MultipleCells	3	0			0.77

Row	Col	Wave	Size	Circularity	Inertia	Convexity	Elongation	AvgSignal	AvgSignalSD	IntegSignal	Angle
2	38	1	164	0.90	0.69	0.98	0	2768	1488	454026	203
2	38	1	8	0.79	1.00	1.00	0	210	42	1678	187
2	38	1	16	0.86	0.60	1.00	0	170	57	2713	202

Figure 41. Example: low-confidence well marked as a candidate by the software that was accepted by the user. The image displayed for the blue channel (top) shows multiple objects, an indication that it might be a multiplicate.

In this case, the user decided to not dispense to this nanowell by clicking [Manual triage] > [Reject - Next well] and moved on. Figure 42 shows how the *Wells* information is displayed after this step was taken.

Wells | Summary | Settings | Map

Row	Col	Candidate	For dispense	State	Cells1	Cells2	Signal1	Signal2	Confidence
2	38	yes	no	GoodButExcluded	1	0	2768		0.77

Figure 42. Well information after the user rejected the well. The "For dispense" field is updated to 'no', meaning it will be excluded from reagent dispense at the next phase of the application workflow, and the "State" has changed to 'GoodButExcluded'.

H. Downselect (Optional)

Downselect is used when a user wants to decrease the number of candidate wells, to a value less than the number of candidates identified by the specified algorithm and manual triage. Downselection may be done for several reasons:

- To save on the amount of reagents dispensed downstream,
- To increase the sequencing depth for each individual sample,

- If you have multiple samples, to help you normalize the number of single cells for each sample, or
- If you have duplicate barcodes. In this case, downselection is required.

  If the indexes being used (and therefore the barcode XML) is set up for indexes that, if the full number of requested nanowells are indexed, would result in wells with identical barcodes, the software will prompt the user to downselect. This ensures that, after the index dispense, the barcodes will be unique for each well across the chip.

  The software will automatically pick one candidate per barcode and will try to pick the best candidate based on the *Settings* parameters. As a result, the number of wells listed in "For dispense" may be fewer than what was requested.

1. If you would like to downselect nanowells, in the *Main* window, click **Actions > Downselect**.

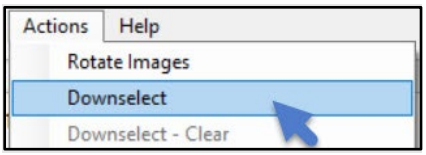


Figure 43. Accessing the Downselect menu item.

2. Enter the desired number of nanowells for controls and sample wells in the *Summary* tab.

NOTE: If you enter a number greater than the number of wells listed, the software will automatically select up to the maximum possible number (a quick and easy way to enter all the candidate wells, especially when working with multiple samples).

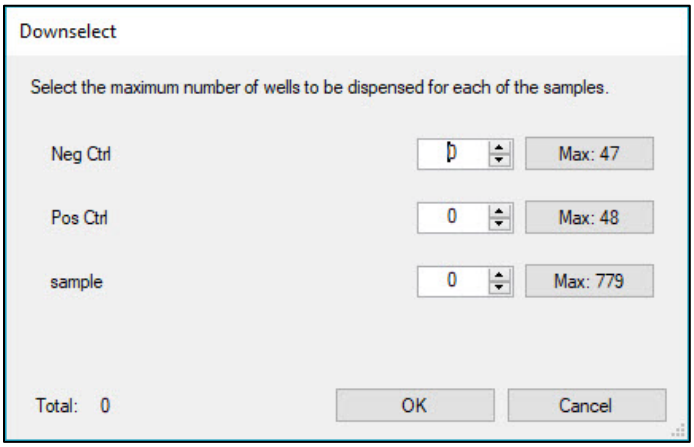


Figure 44. Enter the number of nanowells for controls and samples.

3. Save files after downselecting the number of wells to update all relevant files. Use the resulting filter file (`.csv`) for dispensing reagents in CELLSTUDIO software.

Clearing all downselect selections

When downselect options have been enabled, the menu item **Downselect - clear** becomes available under the **Actions** menu.

Click **Actions > Downselect - clear** to erase all configured downselect choices.

I. Save Files

1. Click [Save Files] and enter a new file name to save any changes you have made to the results file. We strongly recommend using a new file name to avoid overwriting the original results file. The file is saved with the `.wcd` file extension. The related files (WellList, Report, and FilterFile) will also be updated with the new file name.

NOTE: You should always save the `*.wcd` file in the same folder where the associated images are located. When you copy or move files to another hard drive or a network drive, you should always move or copy the entire folder with all the files it contains.

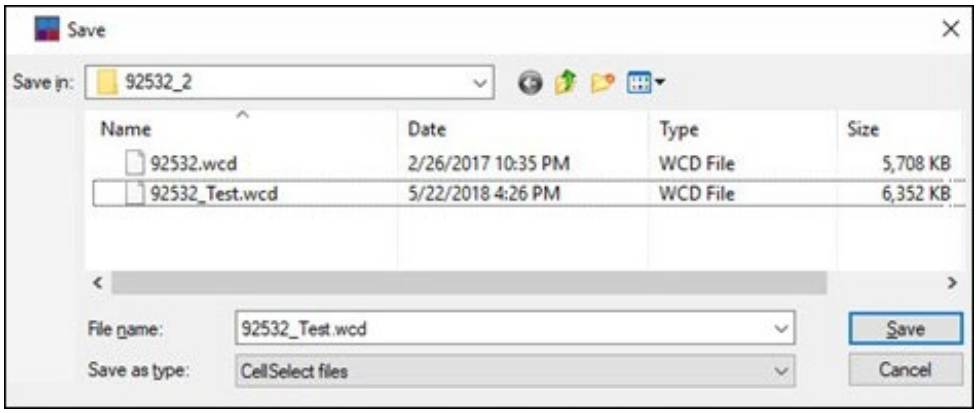


Figure 45. Saving the results file.

2. After the file is saved, the *Required Source Plate Volume* window will pop up, similar to Figure 46.

   When preparing the 384-Well Source Plate(s) for reagent dispense through CELLSTUDIO software, ensure the wells contain the minimum volume displayed in the message, based on the dispense volume for the reagent.

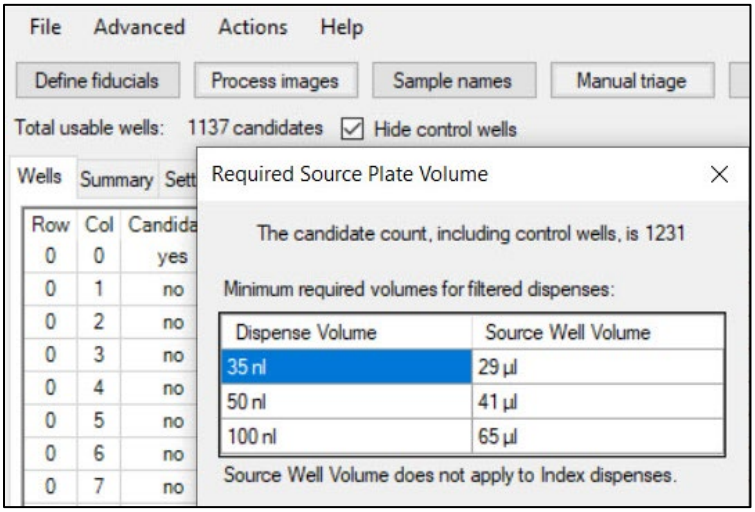


Figure 46. The *Required Source Plate Volume* window. This window automatically pops up after saving the output files by clicking the [Save files] button or under **File > Save Files…**.

J. Tune

Tune can be useful for unique staining protocols or experiments with certain cell types. By default, CellSelect software is programmed with settings baselined from a combination of K562 or NIH3T3 cell lines stained with Hoechst 33342 and propidium iodide (the procedure described in the Single-Cell Minimal Cell Handling and Staining Protocol for Suspension and Adherent Cells Protocol-At-A-Glance). Using [Tune] allows users to customize the analysis settings to accommodate potential differences seen in their experiments, such as image brightness or cell size.

Tuning is a way for the user to input decisions, such as deciding the number of cells in individual wells, based on a manually selected subset of images in the chip folder image files. Once tuned, these settings can be saved and used for subsequent chips imaging the same cell types under the same experimental conditions, thereby simplifying and speeding up the triage process.

For details and the procedure to use this function, please see Appendix G.

Appendix A. Software Reference

A. *Main* Window

The *Main* window contains all the primary functions in one place. These functions are described in detail in this section.

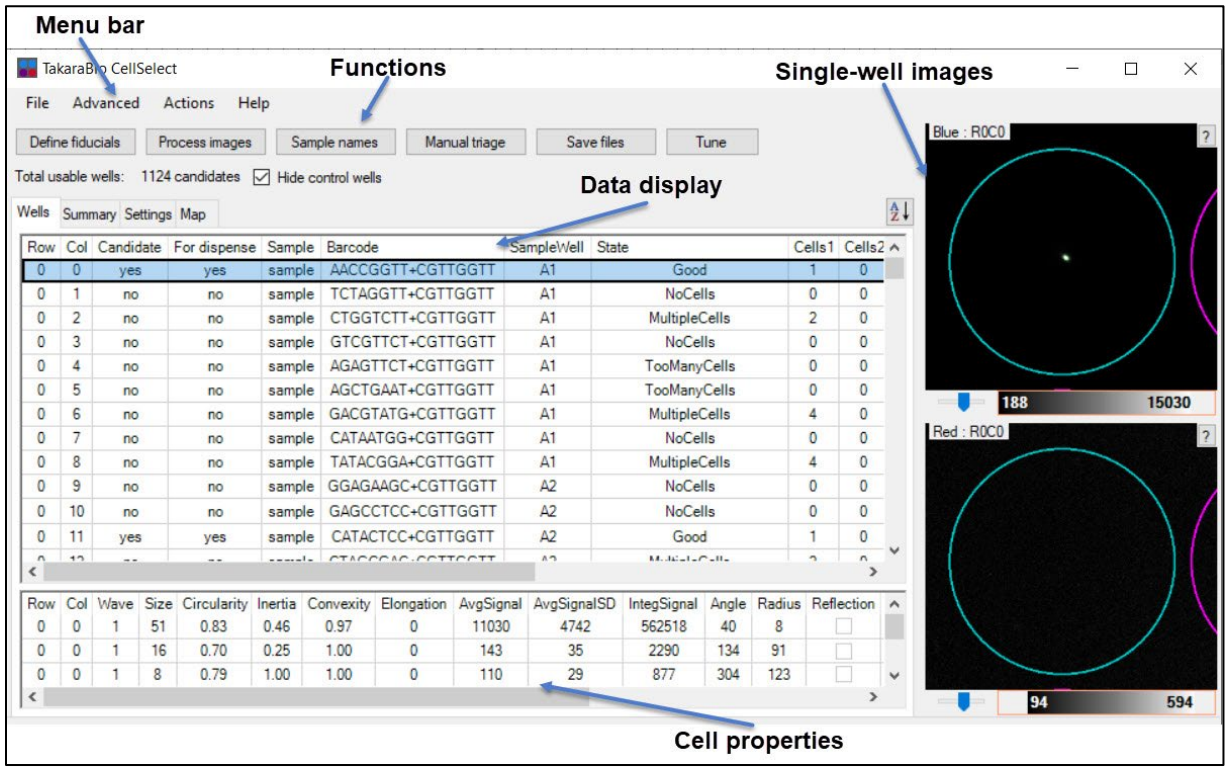


Figure 47. *Main* window.

B. Navigation tips

CellSelect software uses tools and actions in its graphical user interface that are familiar to most users, enabling you to quickly learn and make the best use of the software.

Right-click: CellSelect software makes liberal use of the right-button mouse click to offer context-specific menu options. In Figure 48, the example menu shown will pop up when right-clicking while hovering over any of the cells with data values.

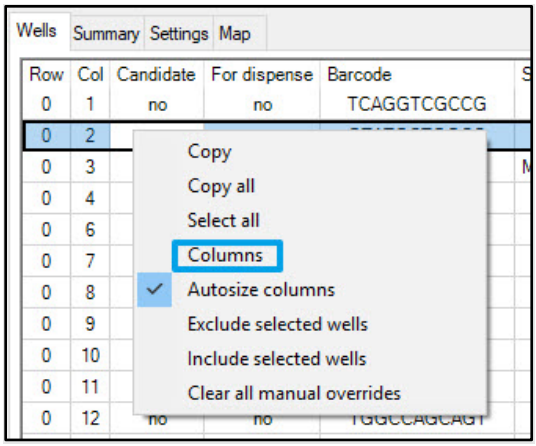


Figure 48. Example right-click menu.

Column display: On several data and analysis screens, you can choose the information to be displayed by right-clicking anywhere within the table (not in the column headers) and choosing **Columns** (Figure 48). This action will open a *Selected fields* window with checkboxes next to the information categories (Figure 49). Click the checkboxes to select or deselect the columns that you want to display or hide. These selections will be remembered the next time the program starts.

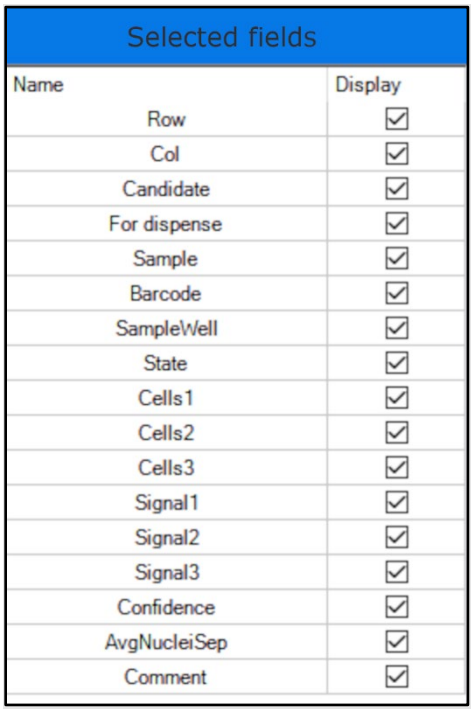


Figure 49. Column heading options.

Column/window width: Column widths can be adjusted by clicking the line between column headings and dragging left or right. In these instances, the appearance of the cursor changes to the ↔ symbol. Various window/section widths can be adjusted as well. Click the left border of the *Image Viewer* window until the mouse pointer changes to the ↔ symbol, then drag the border left or right (Figure 50).

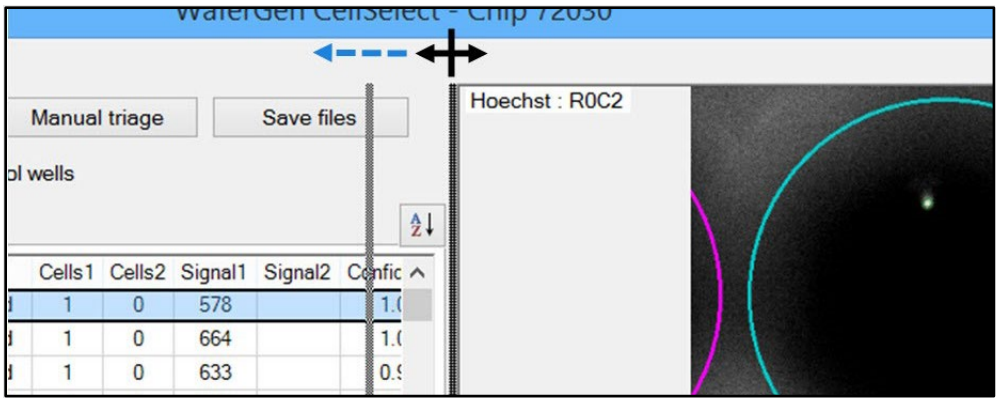


Figure 50. Adjusting the width of the *Image Viewer* window.

Select and copy: Right-clicking in many of the various data tables also triggers the option to select or copy the data as described in the table below:

Table 2. Right-click commands for data tables in the *Main* window.

Command	Function
Copy	Copies all selected rows to the clipboard.
Copy all	Copies the entire table, including the header row, to the clipboard.
Select all	Selects all rows. Click anywhere in the table to deselect all rows except the clicked row.
Columns	Launches the *Selected fields* window to customize the columns to be displayed.
Autosize columns	When turned on, the software automatically adjusts the column widths to accommodate the length of the data being displayed.
Include/Exclude selected wells*	Includes rows that were not selected as candidates and/or excludes rows that were previously selected as candidates.
Clear all manual overrides	Removes all Include and/or Exclude actions.

*Visible only when rows are selected (left click or click and drag the mouse).

Scroll: You can scroll in the software using the arrow keys on your computer keyboard, moving the vertical or horizontal scroll bars, or using the scroll wheel of your mouse.

In the *Image Viewer* window (Appendix A.F), you can press the **[Ctrl]** key on the keyboard while moving the scroll wheel to zoom.

C. Menu Bar

Table 3. Main window menu items.

Menu item	Option	Function
File	Open result file…	Opens a previously saved result file (`.wcd`) (Section III.B).
	Save files…	Processes and saves the results for the current chip* (Section III.I).
	Close	Closes a result file open in the app.
	Open chip folder…	Opens the chip folder containing images created by CELLSTUDIO software (Section III.B).
	Load barcode file…	Loads the configuration file that identifies each well in the chip with a barcode (Section III.C).
Advanced	Switch to Advanced User Mode / Switch to Standard GUI	(default: Standard GUI) Advanced mode displays additional technical attributes (columns) of each well. This option name will change depending on which mode is currently active. Select the mode presented in the menu to toggle between the two view options.
	Admin Mode…	Password protected. This section is for use in troubleshooting by Takara Bio FAS and/or Technical Support Scientist.
	Image Viewer…	Adjusts image size, brightness, contrast, and well overlay. See Appendix A.F (below), for more details.
	Composite Image…	Displays the multi-channel image.
	Settings…	Displays the preconfigured settings for single-cell analysis (Section II.C and Appendix D).
Actions	Rotate images	Rotates all images 90° in the image folder and saves the rotated images to a separate folder. (This action is only needed if a mistake was made during dispensing. Contact field support before taking this action.)
	Load Sequencing Results	Load in per-well sequence data from an external XML file.
	Downselect	Selects the number of wells to be dispensed for each of the samples (Section III.H).
	Downselect – clear	Removes existing Downselect settings (Section III.H).
	Compare with Existing Result File	Compares the current imaging results loaded into CellSelect software with the results of a different saved `.wcd` file. For example: to view the differences between two different cell detection parameter settings on the same set of data.
	Load Custom Dispense Info	Load a customized per-tip `CellDispenseOrder.xml` file. This is not a standard part of the Shasta workflow.
	Load Standard (72x72) Cell Dispense Info	Load the standard per-tip dispense order file from the default `CellDispenseOrderOf.xml.zip` file in the `C:\ProgramData\Takara\CellSelect` folder.
Help	About	Displays the software version and End User License Agreement (EULA).
	Open user manual…	Opens the version of this manual stored within the software.

*When applying changes to a results file, we strongly recommend saving the modified version under a new file name so that the original version is maintained as a backup.

D. Understanding the Software Color Code

To make wells containing candidate cells easier to spot, the software overlays these candidate wells with a teal-colored circle in both the single- and multiwell images. Wells containing non-candidate cells (such as dead cells) are outlined with pink-colored circles.

A well currently displayed in the single-well image is marked with a brighter overlay than surrounding wells in the multiwell image (see example in Figure 51, below).

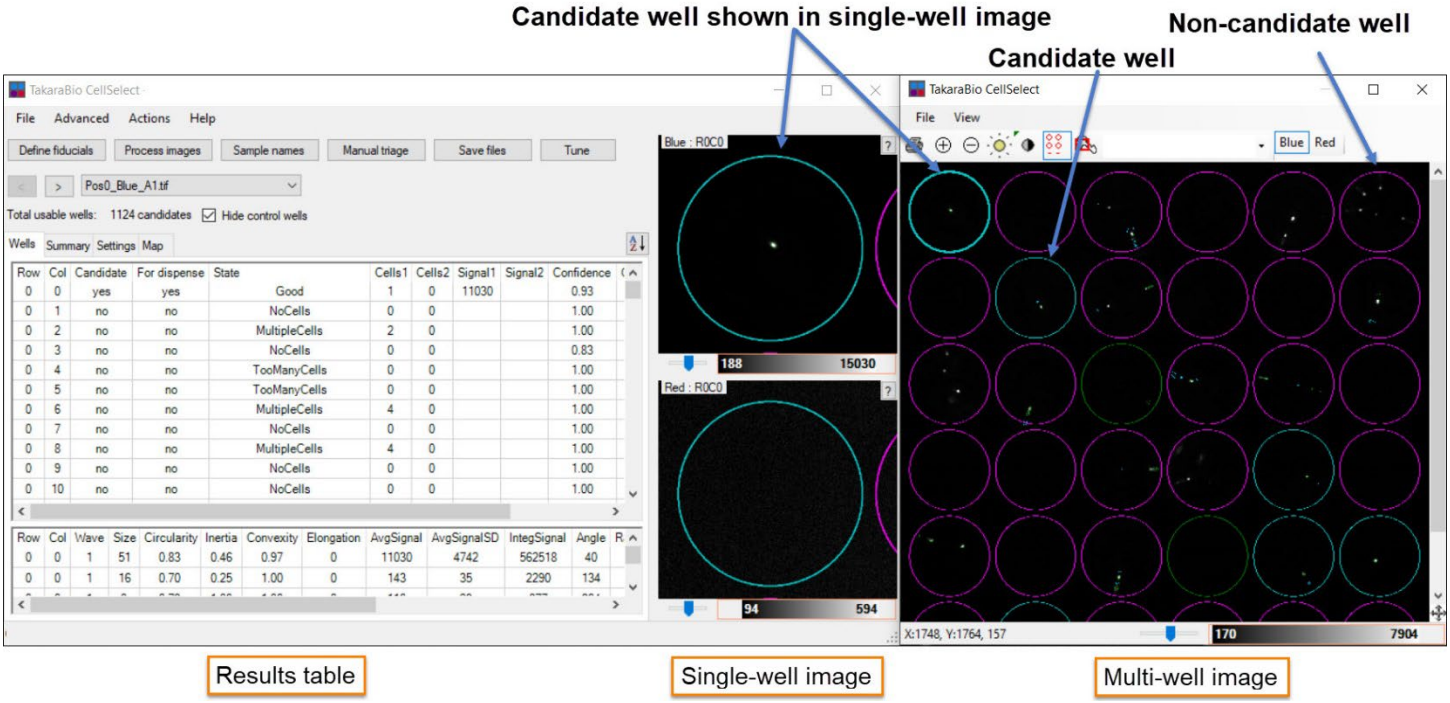


Figure 51. Understanding nanowell color codes. The differing brightness of the selected well (first column, first well down) can be noticed when compared to the nonselected candidate well down one row and one column to the right of it.

Other color indicators, used on objects within the well, include:

- **Green outline**—indicates that the software algorithm identifies a cell based on morphology and intensity
- **Yellow outline**—indicates that the software algorithm identifies an artifact that is too small to be a cell and is ignored
- **Blue outline**—indicates a reflection artifact (a rare occurrence)

To understand how the software determines the status of each nanowell, refer to Table 5 in Appendix B.

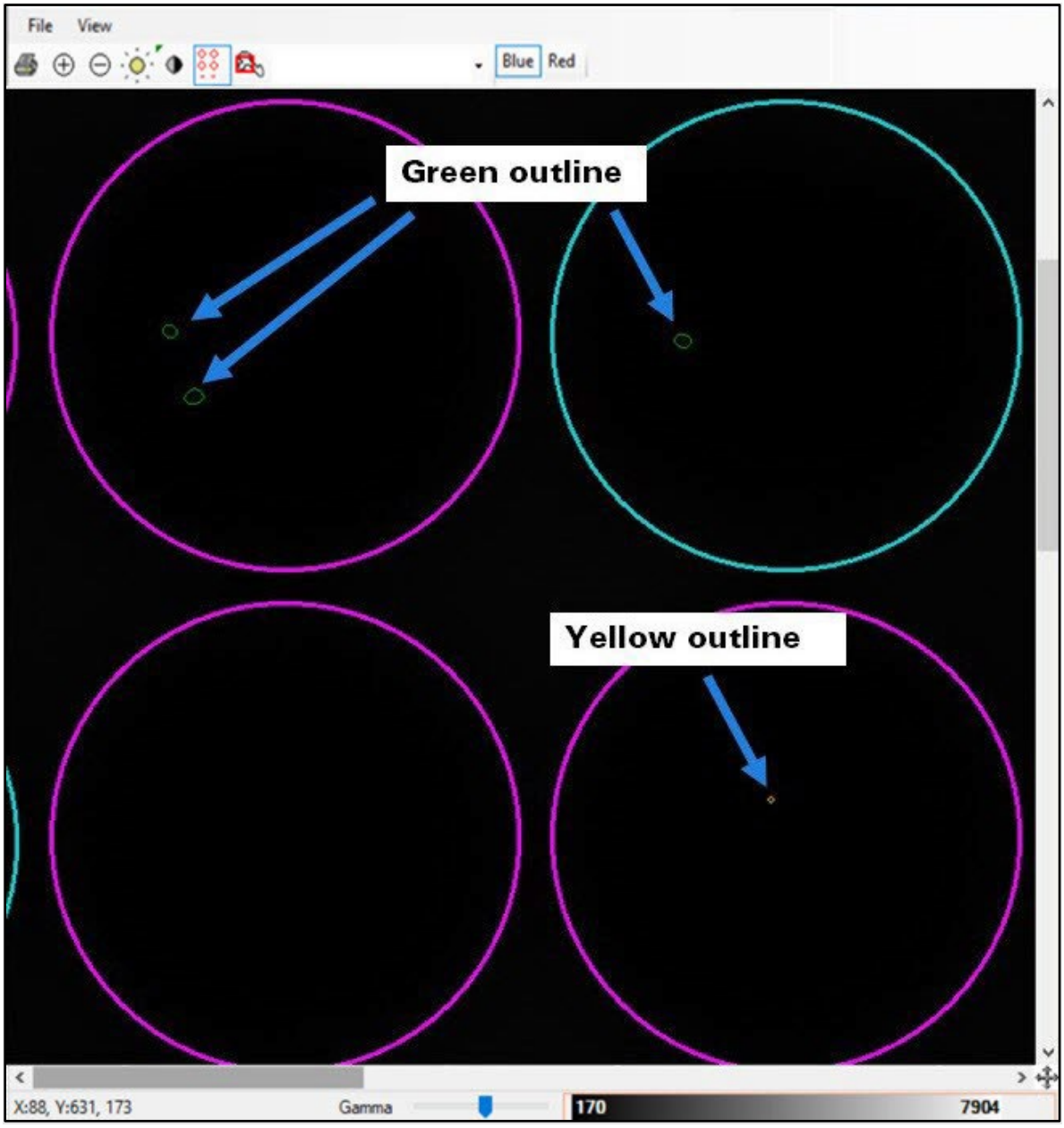


Figure 52. Closeup of wells, showing cell outlines. Green outlines are highlighted in the top wells, while a yellow outline is exampled in the well on the bottom right.

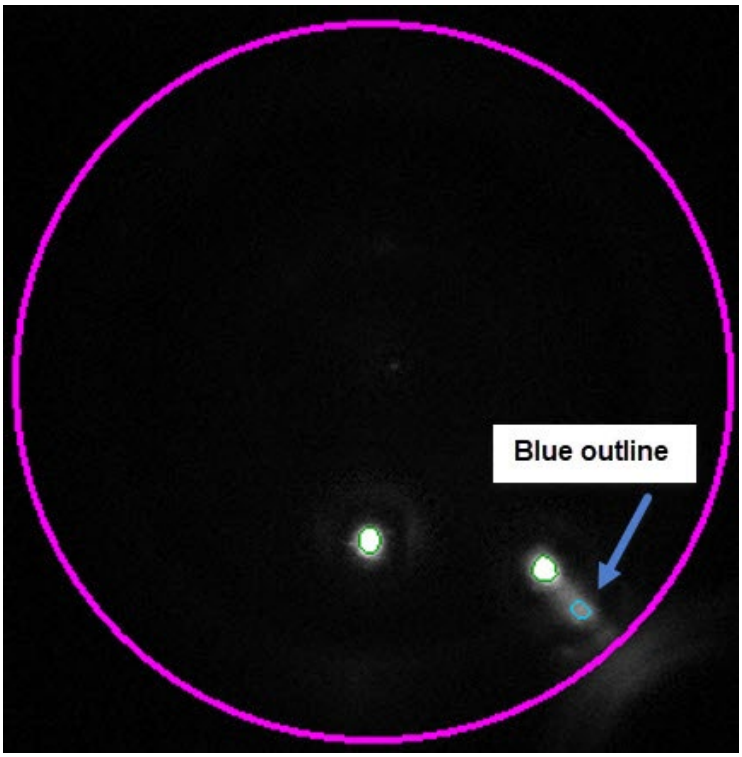


Figure 53. Closeup of single well, showing cell outlines. A blue outline is exampled in this image.

E. Well Images

The well images allow you to look at the objects in the well. One image of the well will be shown for each filter used for scanning the chip.

For example, if the chip was scanned with the Blue and Red channels, two images will be shown, with Dye1 as the top image and Dye2 as the bottom image. A good candidate is a single cell that fits the shape and size parameters of a live cell and follows the candidate logic (see "Candidate logic selection" in Section II.C). If the chip were scanned in only one color, only one image will be shown; if scanned with three colors, three images will be shown.

Inspection of the well images can give the user information about the shape and quality of their cells.

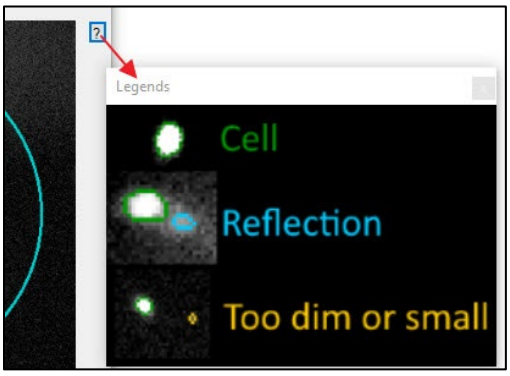


Figure 54. How to access the Legends window. Click on the [?] icon in the single-well image window to pop it up and help interpret the object outlines in image viewer. The green outline indicates the detection of a cell, light blue indicates a reflection that is not considered a cell but reduces the confidence of the well, and a yellow outline indicates that the object is too small or dim to be a cell, which also reduces the confidence of the well. By default, the display of the dim or small objects (yellow) is off. It can be turned on in **Advanced > Settings…** with "PaintOutlines" (Appendix D.N).

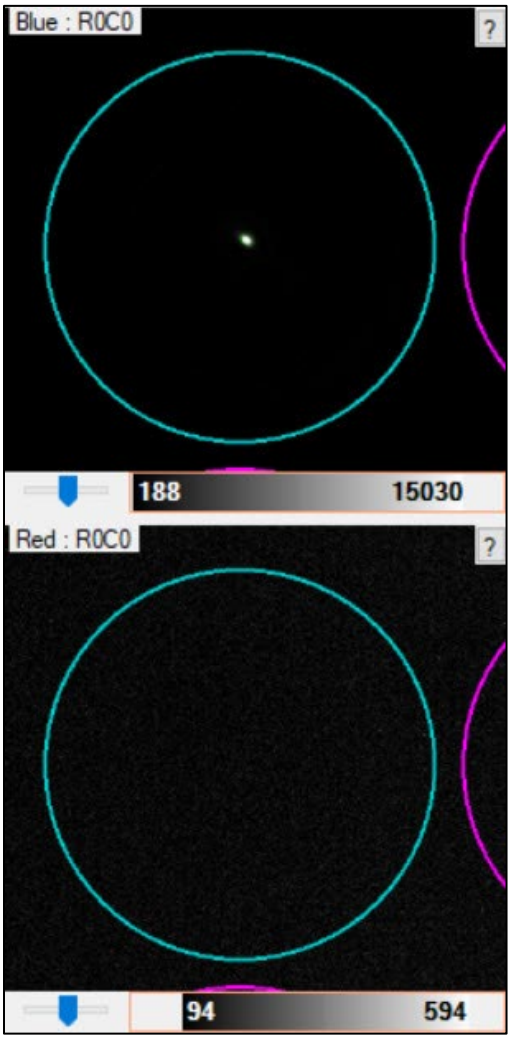


Figure 55. Single-well image from a two-color chip scan of cells stained with Hoechst (Blue channel) and propidium iodide (Red channel). The top image shows a signal (cells that have absorbed Hoechst dye), which indicates the presence of a live cell; the bottom image would show a signal from propidium iodine staining (if present). In this example, the well matches the designated candidate logic selection parameters (see Section II.C.1) and is, therefore, a candidate. If objects appear in the bottom image only or both the top and bottom images, they are either dead cells, other debris, or an imaging artifact.

F. Image Viewer

The *Image Viewer* window is accessible via the **Advanced** drop-down menu in analysis mode and displays multiwell images (6 x 6 nanowells).

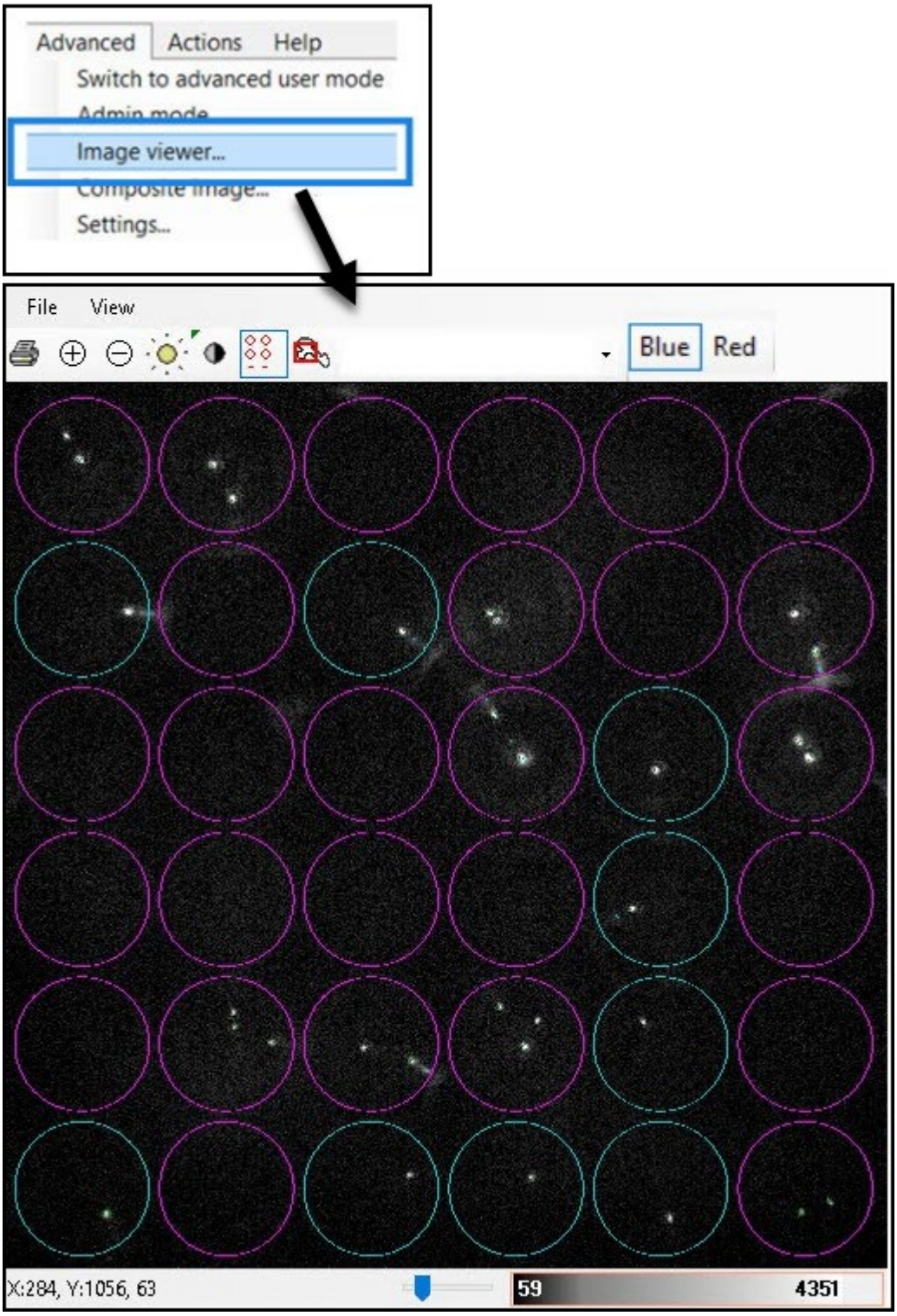


Figure 56. ***Image Viewer*** **window.**

Image Viewer Toolbar Icons

Figure 57. *Image Viewer* toolbar icons.

Zoom icons

The zoom icons are for increasing or decreasing the size of the multiwell image. Clicking the [Zoom in] icon or resizing the viewer window while holding down the **[Shift]** key triggers the *Image Viewer* window to resize itself to occupy the same area as the displayed image. Another way to zoom in/out is to press the **[Ctrl]** key while turning the scroll wheel on the mouse.

If you hold down the [Shift] key while re-sizing the *Image Viewer* window, the system will attempt to adjust the final zoom factor and window size so that the windows frame fits the displayed image exactly.

Image contrast icon

Fluorescence images generated by the Shasta system typically have a very large dynamic range. Clicking the [Image contrast] icon opens the *Image contrast settings* window, which allows you to adjust the mapping of the 65,535 image intensity levels to 256 shades of gray (Figure 58). Drag the control points with the mouse to adjust the mapping.

The gamma value represents the shape of the mapping curve. Small gamma values result in better visibility of dim objects; large values result in better discrimination of very bright objects.

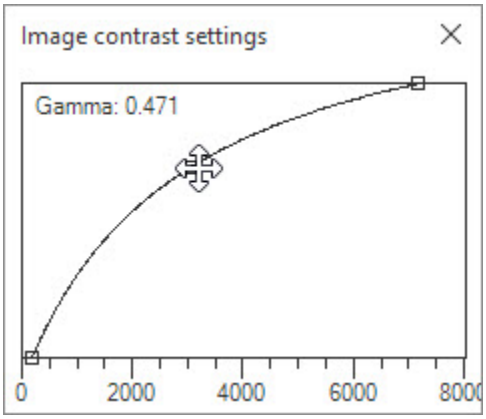


Figure 58. *Image contrast settings* window.

The gamma value can also be adjusted directly from the *Image Viewer* or single-image view with the Gamma trackbar.

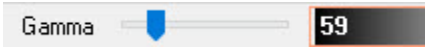


Figure 59. Gamma trackbar slider on the *Image Viewer*.

Image contrast slider

In addition to the [Image contrast] icon on the *Image Viewer* toolbar, the slider at the bottom of each single- and multiwell image can be used to adjust image contrast (Figure 60). Simply click and drag the left and right edges of the grayscale bar or the whole bar itself. Scrolling the mouse wheel while the mouse is over the center of the grayscale bar changes the shape of the curve and is equivalent to dragging the center control point in the image contrast tool (Figure 59). Clicking the buttons to the left and right of the grayscale bar quickly resets the mapping limits to 0 and 65,535, respectively. Alternatively, you can also double-click on the left or right edges of the bar. Right-clicking on the toolbar changes the underlying color map.

Figure 60. Adjusting image contrast using the slider.

Auto contrast icon

Use the [Auto contrast] icon to adjust the upper and lower image contrast and to specify when and how the Auto contrast operation is applied. Clicking the [Auto contrast] icon while holding down the **[Ctrl]** key opens the *Auto contrast settings* window, which allows you to adjust the settings. This feature is also available in the *Image Viewer* (**Advanced > Image Viewer…**).

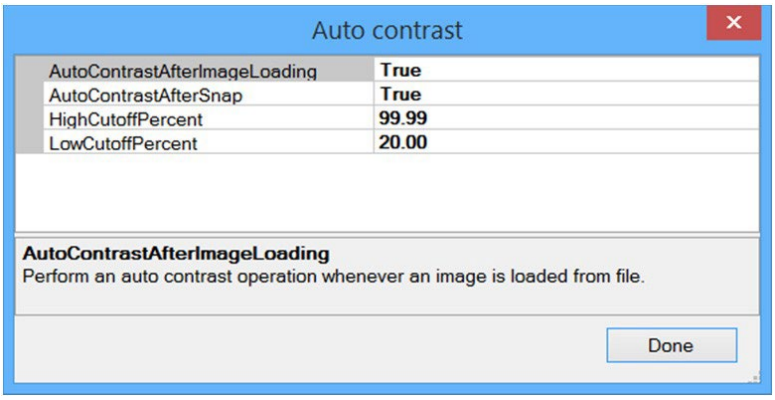


Figure 61. *Auto contrast settings* window. The descriptions of the parameters are summarized in Table 4 (next page).

Table 4. Auto Contrast Settings.

Option	Function
AutoContrastAfterImageLoading	Performs an auto contrast operation whenever an image is loaded from a file
AutoContrastAfterSnap	Performs an auto contrast operation whenever an image is taken with the camera
HighCutoffPercent	The high threshold is set such that all pixels above the HighCutoff percentage are colored white
LowCutoffPercent	The low threshold is set such that all pixels below the LowCutoff percentage are colored black

Map window icon

Use the [Map window] icon to display the entire image. After selecting this icon, drag or resize the red rectangle to change the viewed portion of the image.

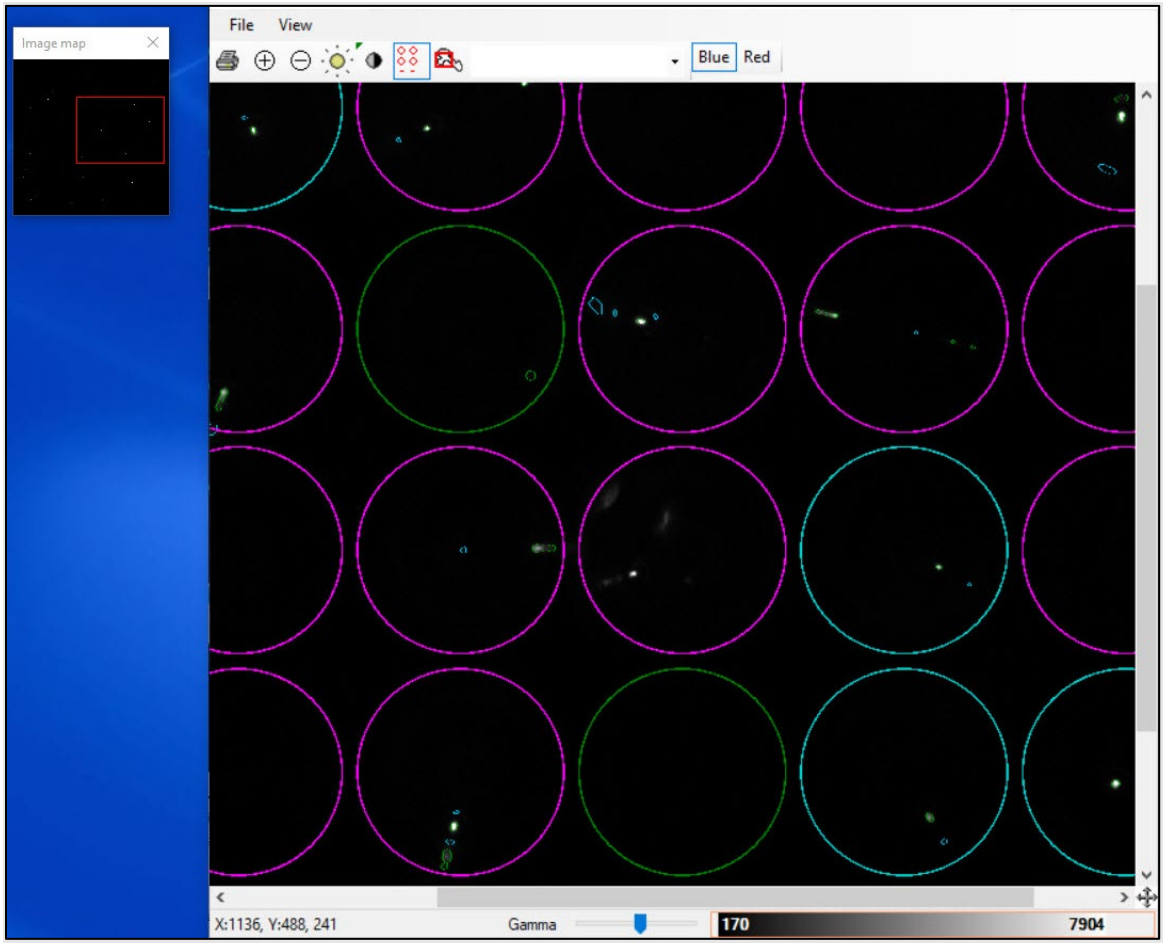


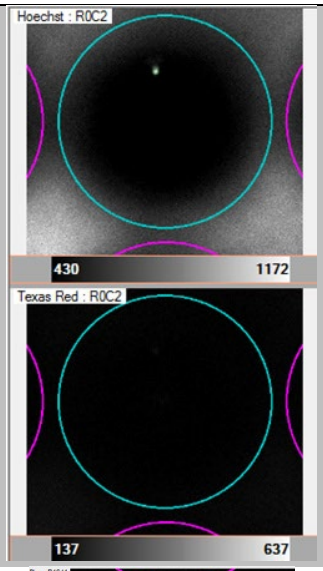
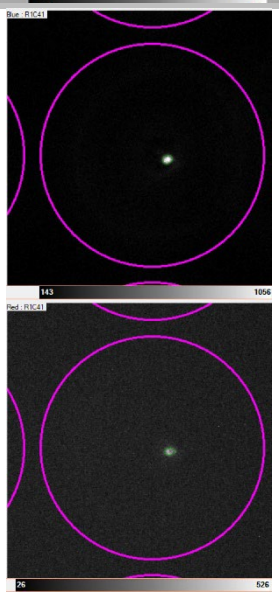
Figure 62. Viewing a subsection of an image using the [Map window] icon and *Image map* window. The red rectangle mentioned in the description can be seen in the small box in the upper left.

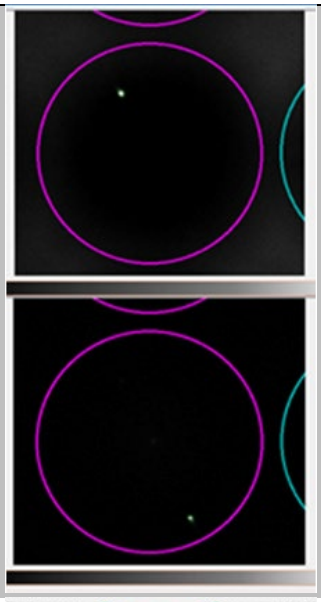
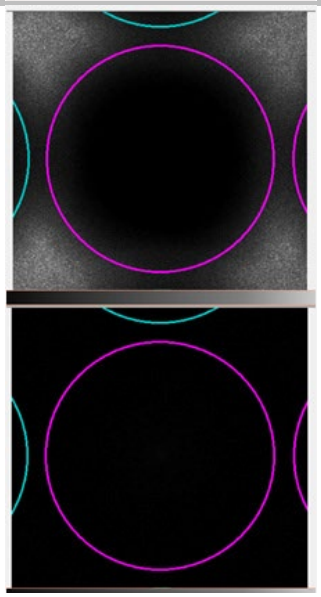
Appendix B. Interpreting the "State" Value in the *Wells* Tab

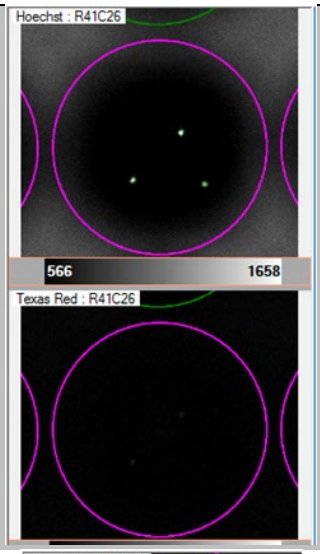
The following parameters apply to the images in Table 5, below. For more information about the "State" parameter, see Table 6 in Appendix C.A.

- **Top image:** Blue staining (Hoechst) indicates the presence of a cell or intact nuclei, provided that the object meets established size and shape parameters and does not appear in the bottom image
- **Bottom image:** Red staining (propidium iodide) indicates the presence of a dead cell, a well bottom, or an artifact

Table 5. Nanowell state descriptions and sample images.

State	Conditions (all conditions must be met)	Image example
Good	Single cell that meets the designated candidate selection logic (i.e., signal displays in the top image (Dye1 channel), but not in the bottom image (Dye2 channel))	
FailsLogic	Cell appears visible in top image (Dye1 channel) but also has a signal in the bottom image (Dye2 channel)	

State	Conditions (all conditions must be met)	Image example
Inconclusive	One cell appears in the top image; one or more cells appear in the bottom image No cells occupy the same location in both views	
NoCells	No cells present in either view	

State	Conditions (all conditions must be met)	Image example
MultipleCells	Multiple cells appear in the top image; no cells appear in the bottom image	
LowConfidence	Cell is not clear The Confidence for a well can be the reduced by various factors: • Unusual cell size • Cell shape that is not round • Cell is dim • There are other objects in the well that are not cells If the confidence falls below the MinimumConfidence threshold, the well is not a dispense candidate.	

Appendix C. User Interface Table Column Descriptions

A. Wells Data Table

Table 6. Column names and descriptions of the data table on the *Wells* tab (Section II.A)

Column name	Field description
Row	Row coordinate of nanowell; starts at 0
Col	Column coordinate of nanowell; starts at 0
Candidate	Well may be used for reagent or index dispense
For dispense	Well will be used for reagent or index dispense

Column name	Field description
Sample	The sample name
Barcode	Index2+Index1 for dual index. Otherwise, the pre-printed barcode sequence
SampleWell	Position in source plate
State	Summary of well results
Cells1	Number of cells in channel 1
Cells2	Number of cells in channel 2
Signal1	Average brightness of cell in channel 1, only available if there is 1 cell in the well
Signal2	Average brightness of cell in channel 2, only available if there is 1 cell in the well
Confidence	Confidence that the well has been called correctly
Comment	A user comment, assigned to the well during manual triage

Table 7. Additional column names in the *Wells* tab when in Advanced User Mode (Section II.A)

Column name	Field description
Size1	Diameter of cell in channel 1, in microns
Size2	Diameter of cell in channel 2, in microns
Integ Signal1	Average brightness times size of cell in channel 1.
Integ Signal2	Average brightness times size of cell in channel 2.
Circularity1	A perfectly circular cell in channel 1 has a circularity value of '1'.
Circularity2	A perfectly circular cell in channel 2 has a circularity value of '1'.
Confidence1	Confidence that the well has been called correctly in channel 1.
Confidence2	Confidence that the well has been called correctly in channel 2.
Source well	Well location of sample in the source plate.
Dispense tip	The tip that has been used during cell dispensing.
Drop index	Value representing the dispense order per aspiration. Multiple wells may get the same Drop index when they are filled simultaneously. Starts from '1' after each aspiration.
Global drop index	Value representing the dispense order. Multiple wells may get the same Global drop index when they are filled simultaneously.
Image1	Channel 1 image filename.
Image2	Channel 2 image filename.

B. Cell Details Table

The cell details are displayed in a table below the well-details table, also on the *Wells* tab. The content of this table applies to the well that is highlighted in the main data table above it.

Table 8. Column names and descriptions of the object details table under the *Wells* tab (Section II.A)

Column name	Field description
Row	Row coordinate of nanowell; starts at 0
Col	Column coordinate of nanowell; starts at 0
Wave	Channel index
Size	Cell size (microns)
Circularity	Based on ratio of area vs length of perimeter
AvgSignal	Average pixel value
AvgSignalSD	Standard deviation (SD) of average pixel value
IntegSignal	Integrated signal: sum of all pixel values
Angle	Angle of object in a coordinate system that is centered in well
Radius	Estimated distance from the well center
Reflection	If the box in the cell is checked, the signal is treated as a reflection
DetectionLevel	Consists of two digits <AB> A = 0 is not displayed A > 0 indicates that the object was detected at a larger filter setting B = 0 indicates a cell B > 0 indicates an object that falls below the detection threshold. Larger values of B indicate dimmer objects.
Too small	Cell is not counted because it is too small. Triggered by "IgnoreWhenDiameterIsLess1" or "IgnoreWhenDiameterIsLess2" (Appendix D.I)
Too dim	Cell is not counted because it is too dim
Too close	Cell is not counted because it is too close to the well center. Triggered by "MinDistanceFromWellCenterM" (Appendix D.J)

C. Summary Table

Table 9. Column names and descriptions of the data table under the *Summary* tab (Section II.B).

Column name	Field description
Sample	Sample name
Wells	Number of wells with this sample
Candidates	Number of wells that are candidates
For dispense	Number of wells that will receive reagents or indexes
Dye1 total	Total numbers of cells that were detected in channel 1 (Dye1)
Dye2 total	Total number of cells that were detected in channel 2 (Dye2)

Column name	Field description
Dye3 total	Total number of cells that were detected in channel 3 (Dye3)
Dye1 empty wells	Number of wells have no cells in channel 1
Dye1 1-cell wells	Number of wells that have 1 cell in channel 1
Dye1 2-cell wells	Number of wells that have 2 cells in channel 1
Dye1 3-cell wells	Number of wells that have 3 cells in channel 1
Dye1 4-cell wells	Number of wells that have 4 cells in channel 1
Dye2 wells with 1 cell	Number of wells that have 1 cell in channel 2
Dye2 wells with cells	Number of wells that have 1 or more cells in channel 2
Dye3 wells with cells	Number of wells that have 1 or more cells in channel 3
Mean	Estimated Poisson lambda parameter
R2	Comparison value of the observed sample distribution with the expected probability distribution (Poisson goodness of fit)

Appendix D. Advanced Settings

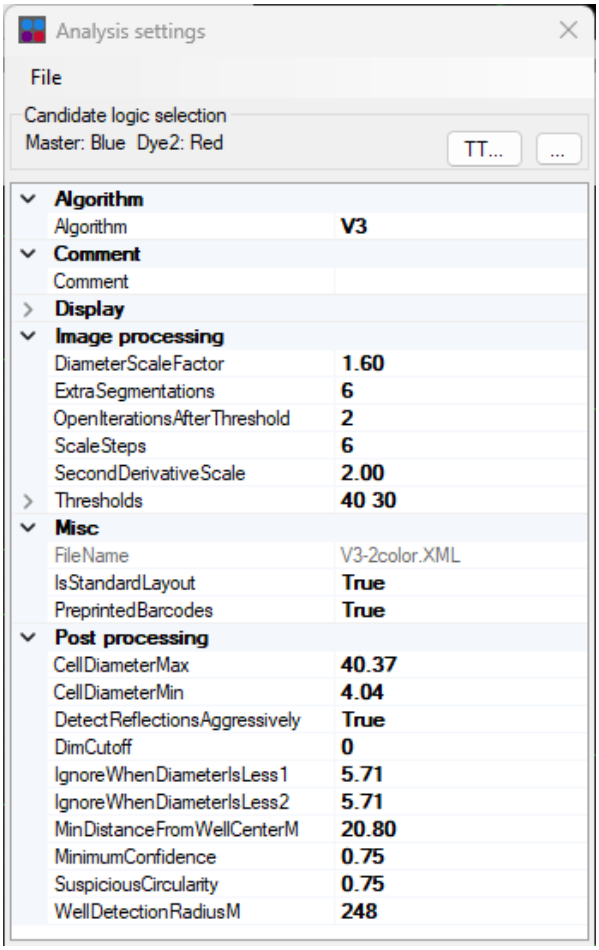


Figure 63. ***Analysis settings*** **dialog.**

The sections below discuss several of the options listed in the *Settings* control. For each section, the section name corresponds to the setting option name. Section A below describes how to restore all the settings to the default.

A. Restore to Defaults

If you wish to return to the default settings for single-cell analysis:

1. Select **File** > **Load…** (Figure 64).
2. Select the appropriate XML file for the type of chip being used.

Table 10. Chip XML file selections.

Chip type	Cat. No.	XML file name
Single-Cell 250f Chip	640193	`AnalysisSetting_250nL_chip.xml`
Single-Cell 350v Chip	640019	`AnalysisSetting_350nL_chip.xml`

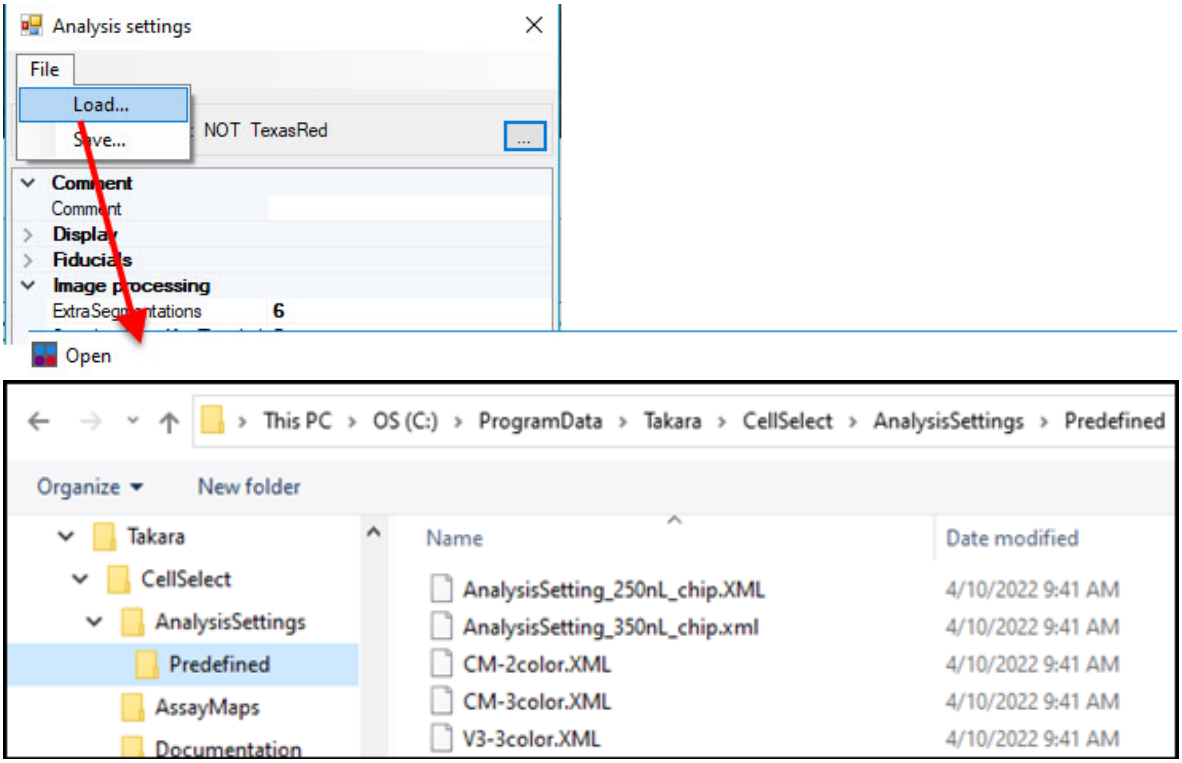


Figure 64. Loading/reloading the XML file for single-cell analysis.

B. Algorithm

There are four options for this setting, V1, V2, V3, and Cardiomyocytes.

- V3 is the default algorithm for general-purpose use, with Truth Table logic and tuning
- Cardiomyocytes is like V3 but optimized for cells containing multiple nuclei and/or with unique shape/morphology

NOTE: The 'V1' algorithm was used in the original ICELL8® CellSelect Software; 'V2' was the original ICELL8 cx CellSelect Software algorithm. These two options are included in Shasta CellSelect Software only for legacy purposes.

C. CellDiameterMax, CellDiameterCardioMax

"CellDiameterMax" is the default parameter name for V1, V2, and V3; "CellDiameterCardioMax" is the name in the cardiomyocyte algorithm. This number is the maximum valid object size, measured as the diameter in microns of the circle with the same enclosed area as the object.

D. CellDiameterMin, CellDiameterCardioMin

"CellDiameterMin" is the default parameter name for V1, V2, and V3; "CellDiameterCardioMin" is the name in the cardiomyocyte algorithm. This number is the minimum valid object size, measured as the diameter in microns of the circle with the same enclosed area as the object.

E. DetectReflectionsAggressively

CellSelect will categorize dimmer shapes that are nearby a brighter shape and on the same radial line from the center of the well as a reflection from the well sidewall and not a separate object. Due to chip variations, a reflection may not appear precisely on the radial line to the computed well center, so an angle tolerance must be used.

With this parameter set 'True', the angle tolerance is increased, resulting in more shapes considered as reflections instead of separate cells, thereby improving the probability of being identified as a single cell.

Set this value 'False' to be more conservative about accepting wells as a single cell, or 'True' to increase the number of well candidates.

F. DiameterScaleFactor

The Shasta instrument uses an optical image of cells to calculate their effective diameter in microns, reported in the "Size" column of the *Wells* tab, and for parameters that select cells according to their size in microns. This calculation requires a "DiameterScaleFactor" calibration in units of microns per pixel.

For large objects, the Shasta optical imaging scale is 1.6 microns per pixel; this value is appropriate for K562 and larger cells. However, the effective scale factor changes due to optical blur effects and make sthe outline of smaller objects in the image appear larger than the actual object size. Experiments with PBMC cells from 7–12 microns in size found an effective scale factor of 1.06.

If the sample uses cells smaller than K562 cells, the "DiameterScaleFactor" can be adjusted for better accuracy. If this number is customized, it will also update the other parameters dependent on cell size in microns (CellDiameterMin/Max, IgnoreWhenDiameterIsLess) to maintain the same selected wells as identified by the original scale factor.

If the DiameterScaleFactor needs adjustment to improve accuracy for your sample cell size, see Appendix E, "Cell-Size Calibration Procedure".

G. DimCutoff

Cells with a master color channel that are too dim, specifically that they have an average pixel value ("Signal1" in the wells table) less than the specified value for "DimCutoff" will not be considered as good cells for the purposes of selecting candidate wells. This control uses the master color channel only.

H. ExtraSegmentations

The decision of whether a given pattern is one larger object or a collection of objects is determined by evaluating it at different characteristic length scales. The "ExtraSegmentations" variable sets the number of different such length scales.

NOTE: User changes to this value are not recommended.

I. IgnoreWhenDiameterIsLess1, IgnoreWhenDiameterIsLess2

Any artifact in Channel 1 or Channel 2, respectively, with a measured diameter below this threshold (in microns) will be ignored. It will not be counted as a single cell, nor will it disqualify a well that has a regular cell.

If an ignored object is close to this threshold, the well confidence will be reduced.

J. MinDistanceFromWellCenterM

With V-bottom Single-Cell chips, debris sometimes accumulates at the bottom (center) of the wells. If the "Thresholds" parameter (Appendix D.O) is lowered, the signal of this debris might be interpreted as a dim cell, although it does not impact confidence. If the value of "MinDistanceFromWellCenterM" is greater than zero, that value is used as the radius of a circle centered on the bottom of the well; cells imaged inside the circle are ignored.

K. MinimumConfidence

To make the manual review of candidate wells more efficient, the system assigns a confidence value to each call that it makes. The confidence value will be in the range 0–1, with 0 being the least confident and 1 being the highest. During a manual review of the wells, it often is safe to skip wells that have confidence close to 1. The confidence may be 1 if there is clearly a candidate or clearly **not** a candidate.

Confidence decreases due to various factors:

- The cell is far larger or smaller than expected
- There are small artifacts in the well
- The cell is not round
- The cell is located close to the edge of the well, as defined by the "WellDetectionRadiusM" (Appendix D.P)

Confidence penalties are usually multiplicative.

Example

A well may be assigned a 0.9 confidence penalty for the cell not being round enough and another 0.95 penalty for the presence of small objects in the well. The total confidence for the well is determined to be 0.855 (0.9 * 0.95).

If the confidence of a well falls below the threshold defined by the "MinimumConfidence" setting value, the well is marked as not being a candidate. If you want to have a higher level of confidence in the candidate calls, you should increase this value; to increase the number of candidates, you should decrease the value.

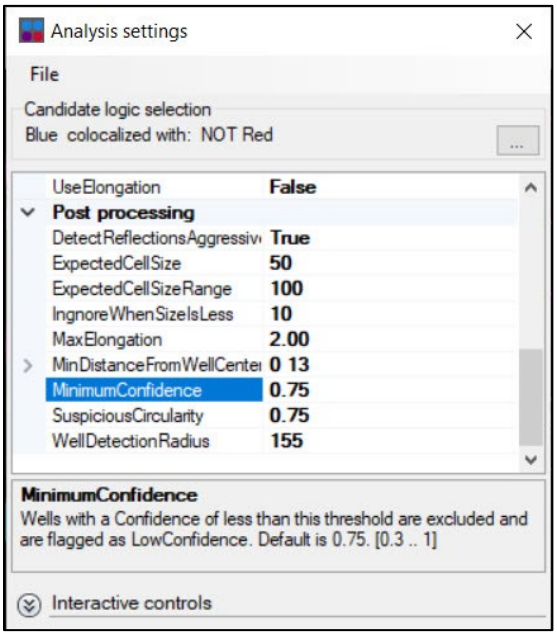


Figure 65. "MinimumConfidence" option in *Settings*.

L. OpenIterationsAfterThreshold

As part of the process of detecting an object from a set of pixels, a morphological opening operation, which tends to reduce pixel noise around the edges of a shape, is performed in sequence the number of times defined by the "OpenIterationsAfterThreshold" value after the thresholding step, which can range from 1 to 3.

NOTE: User adjustment of this value is not recommended.

M. PaintOutline

If "PaintOutline" is set 'True', a colored pixel outline is drawn around each detected shape, as shown below at left. Note that shapes considered to be reflection artifacts are colored in blue instead of green, as mentioned below. If it is set 'False', the interior area of each object is filled in with a solid color, as shown below at right.

To permit the brightness of each cell to be visible, the recommended setting is 'True', so the central area of the cell remains visible.

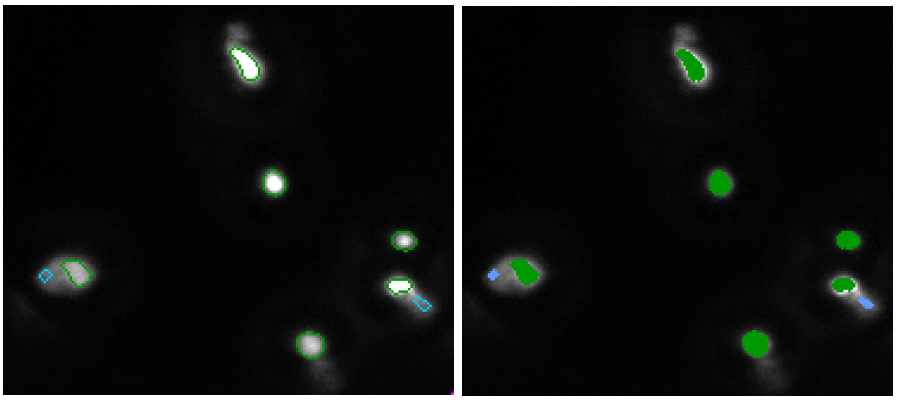


Figure 66. Example images comparing the "PaintOutline" setting values. (Left) PaintOutline value is 'True'. **(Right)** PaintOutline value is 'False'. The 'False' value fills in the outline, obscuring the detected object image.

N. PaintOutlines

To better see objects in single-well images (Appendix A.E, "Well Images"), "PaintOutlines" can be enabled to highlight objects which are determined to be too dim or too small. The outline of this object will be yellow.

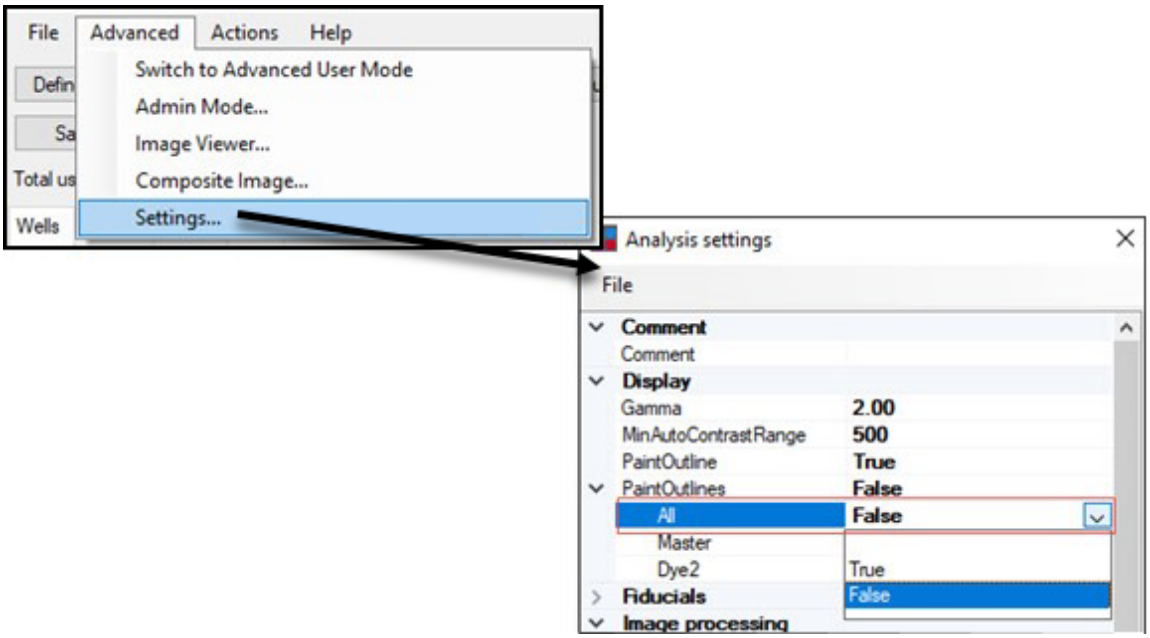


Figure 67. Accessing the "PaintOutlines" option in the *Analysis settings* window.

By default, the value is set to 'False'; it can be enabled by selecting 'True' in the drop-down boxes in either of the individual dye options or 'All' for all dyes being used.

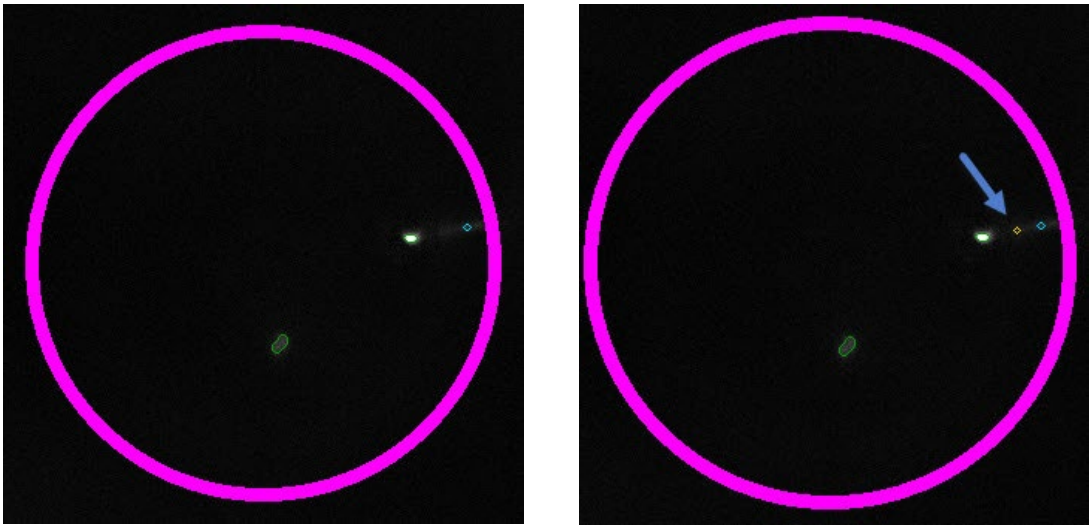


Figure 68. The same noncandidate well with "PaintOutlines" disabled and enabled. (Left) disabled is the default mode. **(Right)** enabled, a yellow-outlined object, highlighted by the arrow, displays.

O. Thresholds

Refer to Section II.C.2, "Thresholds" for details.

P. WellDetectionRadiusM

This setting is the maximum distance, in microns, of a cell from the expected well center. Objects inside the circle defined by this radius setting are considered cells; outside objects are ignored.

Q. ScaleSteps and SecondDerivativeScale

The default algorithm which defines segmentation favors structures that have a radius of approximately:

2 * SecondDerivativeScale

Structures that have a size that is significantly different tend to be suppressed by the software.

The scale-space approach evaluates the images at several scales and, if one or more structures at the same location are visible at multiple scales, it tries to make a determination which scale is the most appropriate. This approach allows CellSelect software to more reliably detect objects of different sizes. Each scale choice is specific to the object, i.e., an image can have objects that were detected at different scales.

The size of the value has the following impact on the images:

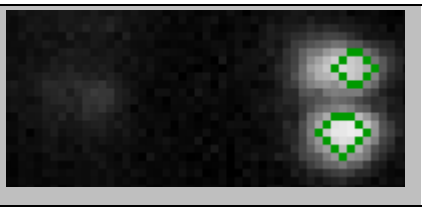
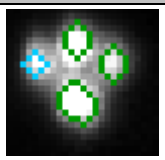
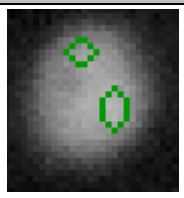
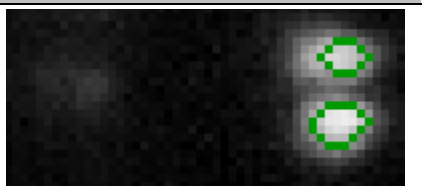
- Small values of "Scale" detect small objects, even when they are close together, but larger objects may be oversegmented
- Large values of "Scale" are better at detecting large objects, but they also tend to blur smaller objects together

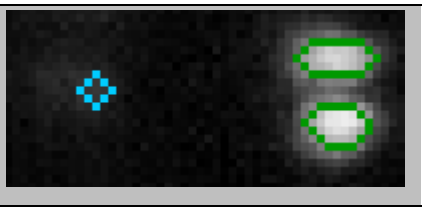
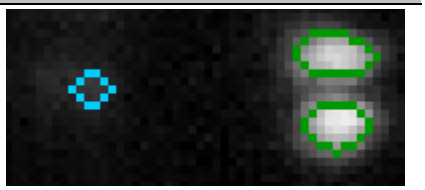
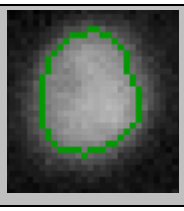
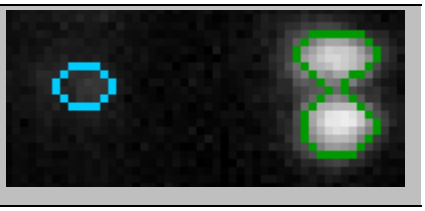
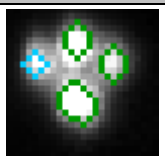
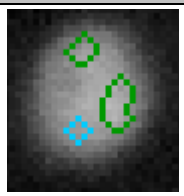
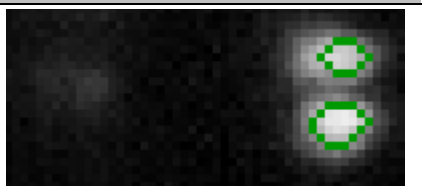
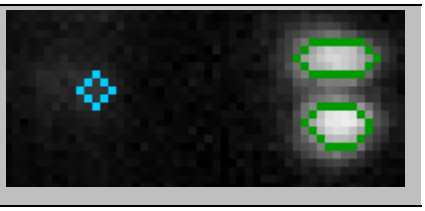
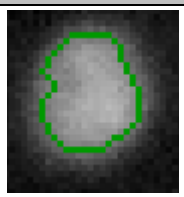
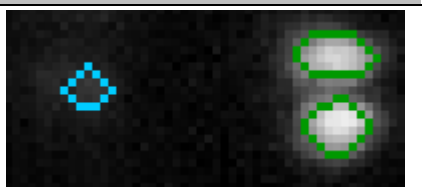
Table 11 shows the effect of changing the setting values for "ScaleSteps" and "SecondDerivativeScale".

Example

In the last row of Table 11, although the parameters (ScaleSteps = 4, SecondDerivativeScale = 0.75) result in a merged blob, it is not very round, i.e., has low circularity, and therefore will be classified as a cluster. Objects outlined in blue are interpreted as a reflection.

Table 11. Effects of changing "ScaleSteps" and "SecondDerivativeScale".

Scale steps	SecondDerivativeScale	Example images		
1	0.75			
1	1.0			

Scale steps	SecondDerivativeScale	Example images		
1	1.5			
1	2			
1	2.5			
2	0.75			
3	0.75			
4	0.75			

R. Algorithm-specific Settings for V1, V2, and V3

1. Gamma

Gamma affects the brightness of the well image initially displayed on the screen and does not affect the numerical analysis results or well candidate decisions. Small values result in better visibility of dim objects, while large values result in better discrimination of very bright objects. The as-displayed gamma value can also be adjusted at any time in the *Wells* tab, using the blue slider at the lower left-hand corner below the well image (see Figure 59 in Appendix A.F, "Image Viewer").

2. MinAutoContrastRange

This sets the minimum range for the display auto contrast to make brighter or darker objects more easily visible. Valid entry values range from 255–2,000.

This affects image display appearance only and does not affect the numerical analysis results or the well-candidate decisions.

3. SuspiciousCircularity

Circularity for a cell is defined as:

$$\text{circularity} = 4\pi * \text{area} / \text{perimeter}^2$$

A perfectly round cell would be assigned a circularity of '1', elongated or other oddly shaped cells are given a circularity value of less than 1.

If the circularity of a cell falls below the threshold defined by this setting value, the confidence of the well will decrease. The penalty grows larger as the assigned circularity value gets smaller.

S. Algorithm-specific Settings for Cardiomyocytes

1. ExtraCh1Shapes

Cardiomyocyte cell preparations may contain extra debris. This setting allows users more flexibility to include or exclude candidate wells if one additional extra shape is identified in the cytoplasm (first) color channel.

- A value of '1' indicates that one extra detected shape (artifact) is allowed in a well in addition to the cardiomyocyte and still be eligible as a candidate well.
- A value of '0' will exclude a well from being labeled a candidate if any extra shapes are detected.

2. MaxNucleiPerCell

The "MaxNucleiPerCell" parameter may be set to a value ranging from 1–4, indicating the maximum number of individually detected nuclei which may be present in a single cell.

3. MinElongationCardio, MaxElongationCardio

The minimum and maximum elongation, as a ratio of length to width, required for an object to be considered a valid cardiomyocyte.

T. Interactive controls: Fast Image Analysis

To quickly evaluate the effect of changes to the image processing settings, you can use the interactive controls from the **Advanced > Settings…** menu.

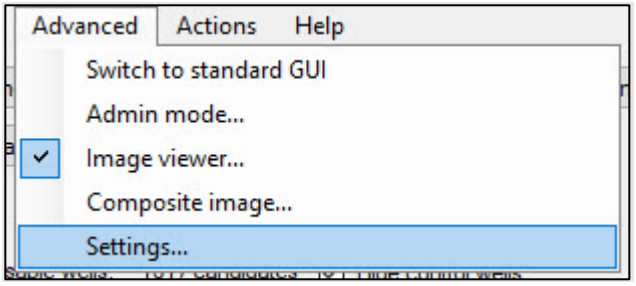


Figure 69. Selecting Settings… under the Advanced menu view.

The interactive controls are located at the bottom of the *Analysis settings* dialogue window.

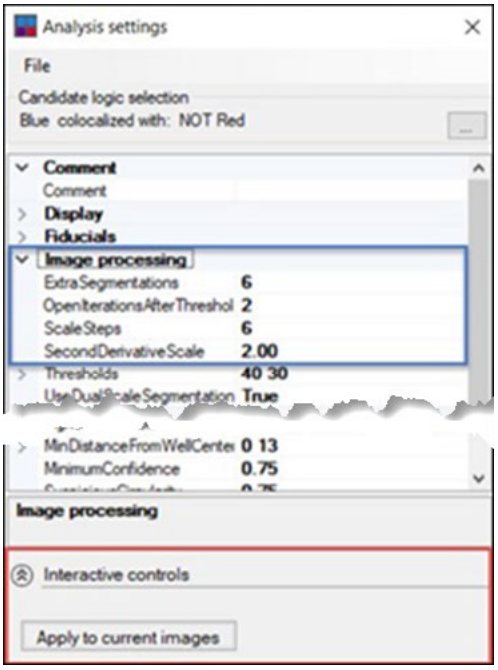


Figure 70. Example *Analysis settings* window. The interactive controls section is highlighted by the red rectangle and the Image processing section is outlined in blue.

If you change the image processing parameters, click [Apply to current images] to run the algorithm on the selected images, which contain 36 wells. The results will appear in the *Wells* table, all the wells not in the reprocessed images are removed, and the cell outlines appear in the image viewer.

Appendix E. Cell-Size Calibration Procedure

NOTE: Refer to Appendix D.F, "DiameterScaleFactor", for more information about the setting described in this appendix.

The CellSelect software reports of effective cell diameter are based on the number of pixels contained within the imaged cell outline. The geometric scaling factor is set by the camera objective at 1.6 microns per pixel, which will be within 20% of the expected value for cells large enough to cover many pixels, e.g., K562 cells. For smaller cells, such as PBMCs, the resolution limit of the optics spreads out the cell image across more pixels than a system with unlimited resolution would do, causing the effective scale factor in microns per pixel to be reduced.

If the average size or your cell sample is smaller than the diameter of common mammalian cell lines (16 microns), you may experience better reported cell sizes by changing the "DiameterScaleFactor" value factor in the CellSelect Analysis Settings window. Use the following steps to derive a new recommended value:

1. Obtain an average cell size reading of your sample cell, using a standard cell-size measurement tool.
2. After dispensing and scanning your chip, examine a representative sample of reported cell sizes from the "Size um" column at the lower panel in the CellSelect main tab.

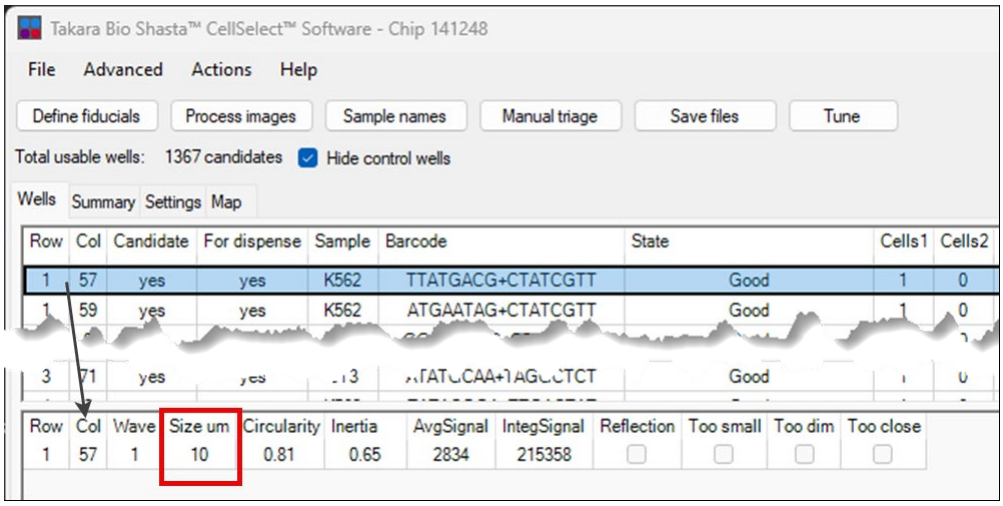


Figure 71. Where to find the "Size um" column on the CellSelect *Wells* tab tables. The column is highlighted by the red box.

3. Find the ratio of CellSelect reported cell size to the standard instrument reported cell size.

$$\frac{\text{<Average CellSelect cell-size value> (from Step 2)}}{\text{<Average measured cell-size> (from Step 1)}}$$

Example:

If the average "Size um" value in the CellSelect Wells table is 14.3 µm but the standard value measured by your tool is 12.2 µm, the ratio is:

$$\frac{14.3\ \mu m}{12.2\ \mu m} = 1.17$$

i.e., too large by a factor of 1.17.

4. To calculate a new "DiameterScaleFactor" specific to your sample cell, use the formula:

$$\frac{\text{<Original DiameterScaleFactor> (from CellSelect settings)}}{\text{<Calculated ratio> (from Step 3)}}$$

Example:
If the original "DiameterScaleFactor" is '1.60' and the calculated ratio is 1.17 (Step 3), then the new "DiameterScaleFactor" value would be:

$$\frac{1.60}{1.17} = 1.368$$

5. In CellSelect *Settings*, modify the "DiameterScaleFactor" value to match the value calculated in Step 4.

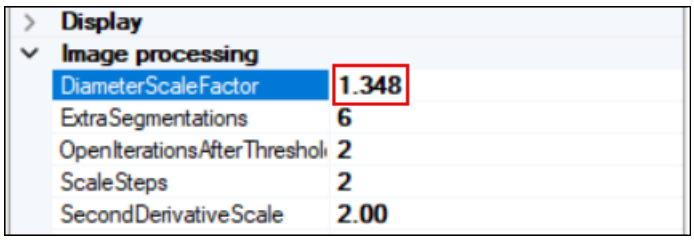


Figure 72. Updating the "DiameterScaleFactor" to a new calculated value. The red box highlights the calculated results from Step 4 being input into the value field.

Appendix F. Comparing Result Files

With new assays, adjusting the analysis parameters in *Settings* will probably be necessary so that the algorithm correctly produces candidates; this concept is covered extensively during training by a Field Application Scientist.

To help researchers determine the optimal parameters, CellSelect Software includes a result file comparison feature. To use it,

1. Process the images with one set of parameters.
2. Save the result file (`*.wcd`).
3. Re-enter the Settings editor in **Advanced > Settings** or via the [Edit…] button from the *Settings* window.
4. Modify one or more parameters.
5. Analyze the images a second time.
6. Select **Actions > Compare with Existing Result File**.
7. In the *Open chip results* dialog window, select the previously saved `*.wcd` file (from Step 2).

NOTE: It only makes sense to compare results that belong to the same chip.

Once the reference file is loaded, the system displays a *Summary of differences* window and a *Comparison results* window.

In the two tables, many items are labeled A and B. 'A' refers to results from the run that is currently loaded (from Step 5), and 'B' refers to the results in the `*.wcd` file on disk (from Step 2).

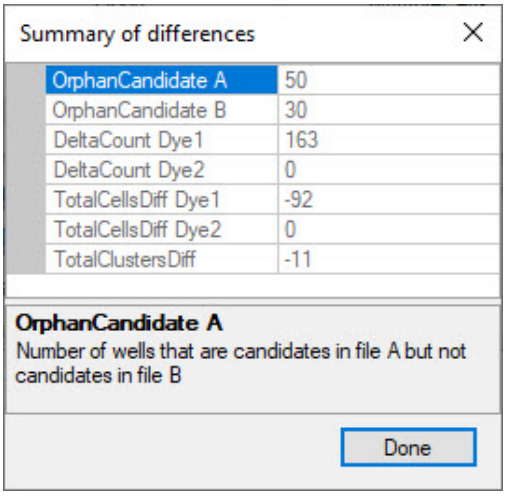


Figure 73. Example of the *Summary of differences* window. The descriptions of the parameters are described in Table 12.

Table 12. Description of the fields of the Summary of differences window.

Field name	Description
OrphanCandidate A	Number of wells that are candidates in the currently loaded data table but not candidates in file B
OrphanCandidate B	Number of wells that are not candidates in the currently loaded table data but are candidates in file B
DeltaCount Dye1	Number of wells where cell counts for Dye1 (Dye1) are different
DeltaCount Dye2	Number of wells where cell counts for Dye2 are different
TotalCellsDiff Dye1	Difference in total number of cells for Dye1 (Dye1)

Field name	Description
TotalCellsDiff Dye2	Difference in total number of cells for Dye2
TotalClustersDiff	Difference in total number of clusters

The example in Figure 73 is the result of only changing the Dye1 Threshold from '40' to '45'. The immediate effect is that the total number of cells for Dye1 is reduced by 92 (–92). Values related to Dye2 stayed the same (0).

The number of cells can sometimes be misleading. If the threshold is too low or there are artifacts in an image, the system may detect more than 20 objects in a well. In that case, it assumes that the well is unusable, ignores all cells, and the well is flagged with the status 'TooManyCells'. Also, there can be cases where a higher threshold may cause a cell to be flagged as a cluster, which is counted as two cells instead of one.

In the example in Figure 73, there are 80 wells (OrphanCandidate A + OrphanCandidate B) where the candidate call has changed.

The "Compare options" drop-down in the *Comparison results* window provides three options to choose what differences are displayed:

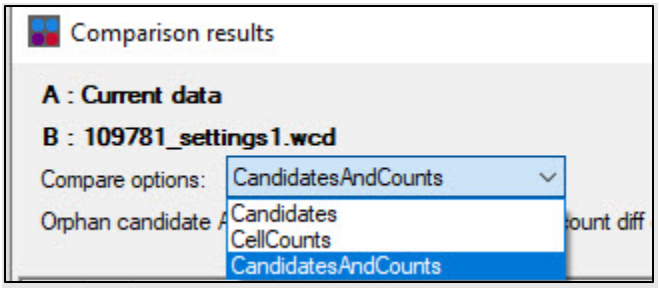
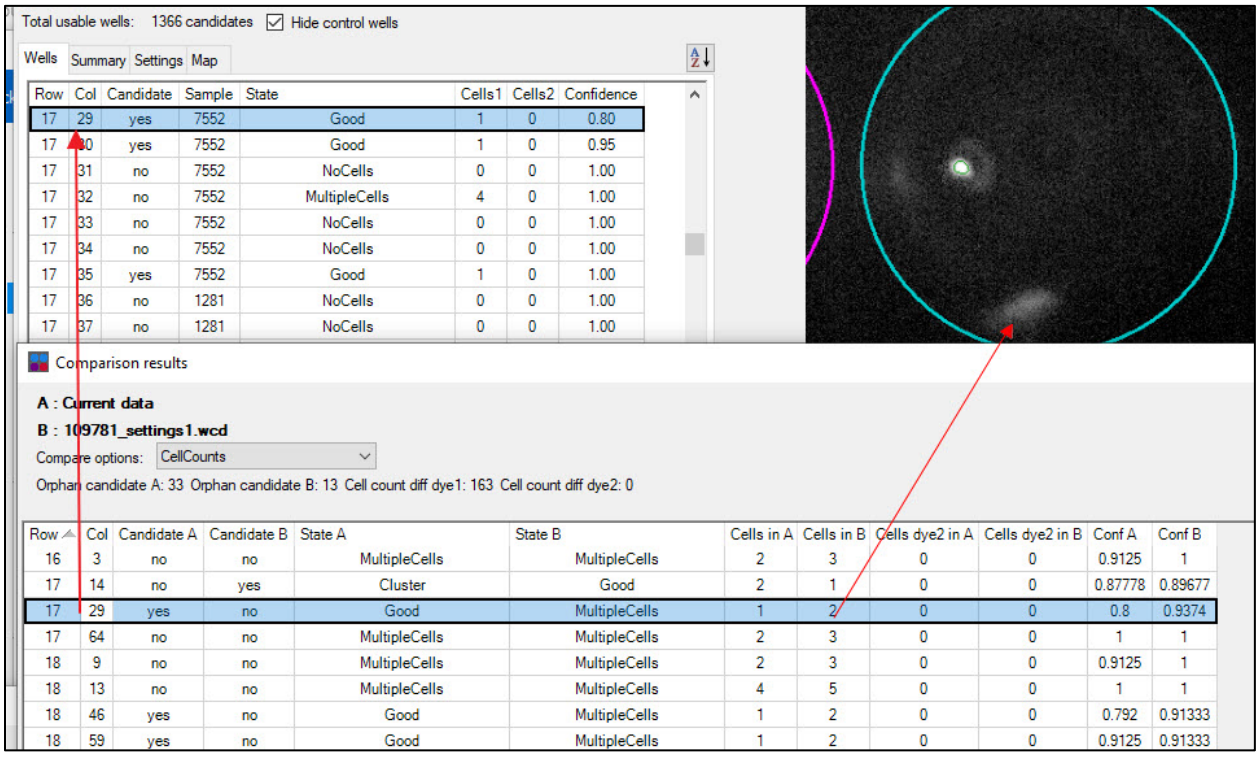


Figure 74. "Compare options" drop-down menu in the *Comparison results* window. The descriptions of the drop-down options are described in Table 13.

Table 13. Description of the potential values of "Compare options".

Option name	Option effect
Candidates	Show only wells where there is a difference in the Candidate call
CellCounts	Show only wells where the cell count for Dye1 or for Dye2 is different
CandidatesAndCounts	Show all wells that are different

When you click on a row in the table, that row will also be selected in the *Wells* table and the corresponding well image will be displayed. Cells in the 'B' dataset are not outlined in the *Image Viewer* if they would not be outlined by 'A', but often you can infer by comparing the image with the information from the B columns in the table as in Figure 75.



Row	Col	Candidate	Sample	State	Cells1	Cells2	Confidence
17	29	yes	7552	Good	1	0	0.80
17	30	yes	7552	Good	1	0	0.95
17	31	no	7552	NoCells	0	0	1.00
17	32	no	7552	MultipleCells	4	0	1.00
17	33	no	7552	NoCells	0	0	1.00
17	34	no	7552	NoCells	0	0	1.00
17	35	yes	7552	Good	1	0	1.00
17	36	no	1281	NoCells	0	0	1.00
17	37	no	1281	NoCells	0	0	1.00

Row	Col	Candidate A	Candidate B	State A	State B	Cells in A	Cells in B	Cells dye2 in A	Cells dye2 in B	Conf A	Conf B
16	3	no	no	MultipleCells	MultipleCells	2	3	0	0	0.9125	1
17	14	no	yes	Cluster	Good	2	1	0	0	0.87778	0.89677
17	29	yes	no	Good	MultipleCells	1	2	0	0	0.8	0.9374
17	64	no	no	MultipleCells	MultipleCells	2	3	0	0	1	1
18	9	no	no	MultipleCells	MultipleCells	2	3	0	0	0.9125	1
18	13	no	no	MultipleCells	MultipleCells	4	5	0	0	1	1
18	46	yes	no	Good	MultipleCells	1	2	0	0	0.792	0.91333
18	59	yes	no	Good	MultipleCells	1	2	0	0	0.9125	0.91333

Figure 75. An example of the *Comparison results* window and how to read the information on it. The highlighted row in *Comparison results* corresponds to the highlighted row under the *Wells* tab. The arrow from the "Cells in B" column of the report relates to the dim image the arrow points to in the well image.

After reviewing several wells with differences, you can decide which set of parameters produces 'better' results (see caveat below). If it is set 'A', the data in memory, you can save the result files and repeat the optimization comparison by changing additional parameters.

A few things to keep in mind:

- Changing parameters will frequently produce better outcomes for some wells but worse for others. There is usually not one set of settings that works best in all situations.
- Sometimes, it is also difficult for the user to decide which call is accurate. Sometimes, different users have different opinions. Which option is 'best' can often only be determined by correlating the imaging with the sequencing results obtained for those cells.

Appendix G. Automated Threshold Detection

A. Quick Start Guide

1. Open the Auto Tune Window

Open the desired dataset with the CellSelect application. Press the [Tune] button to access the *Auto Tune* window:

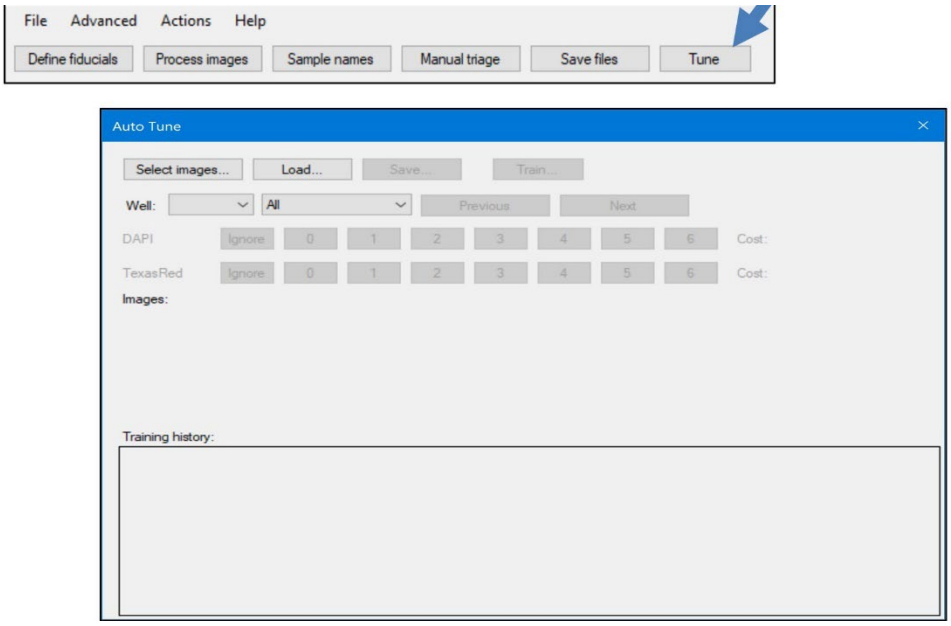


Figure 76. The *Auto Tune* window.

2. Select Images (6 x 6 wells) for Training

In the *Auto Tune* dialog, press the [Select images…] button to bring up both the *Image Viewer* and *AutoTune Image Selector* dialogs:

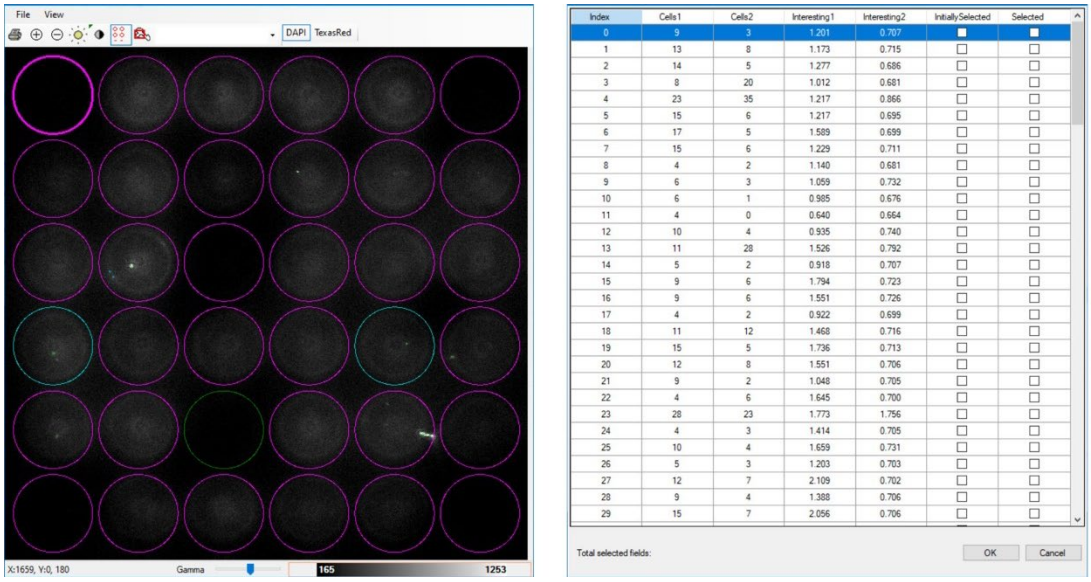
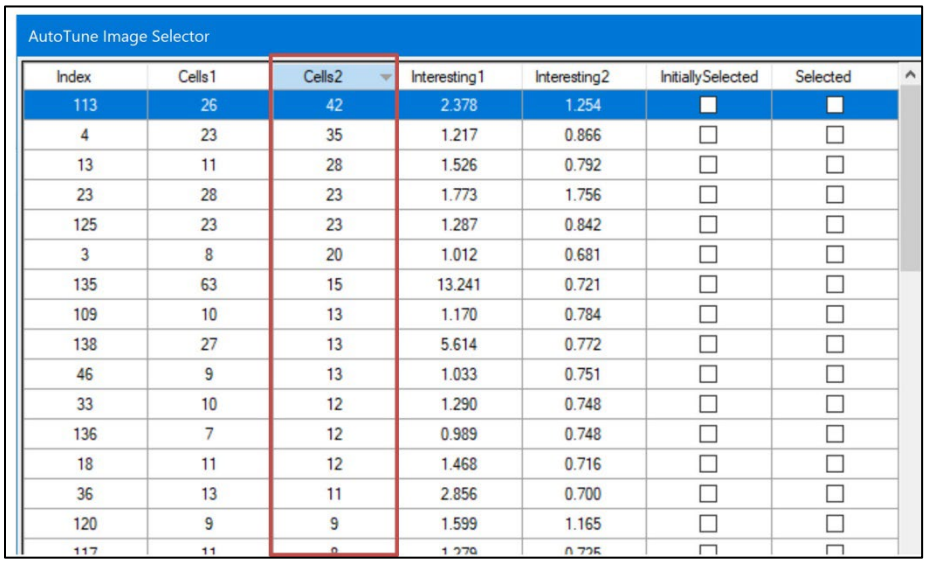


Figure 77. *Image Viewer* and *AutoTune Image Selector* windows.

3. Choose Five Optimal Images

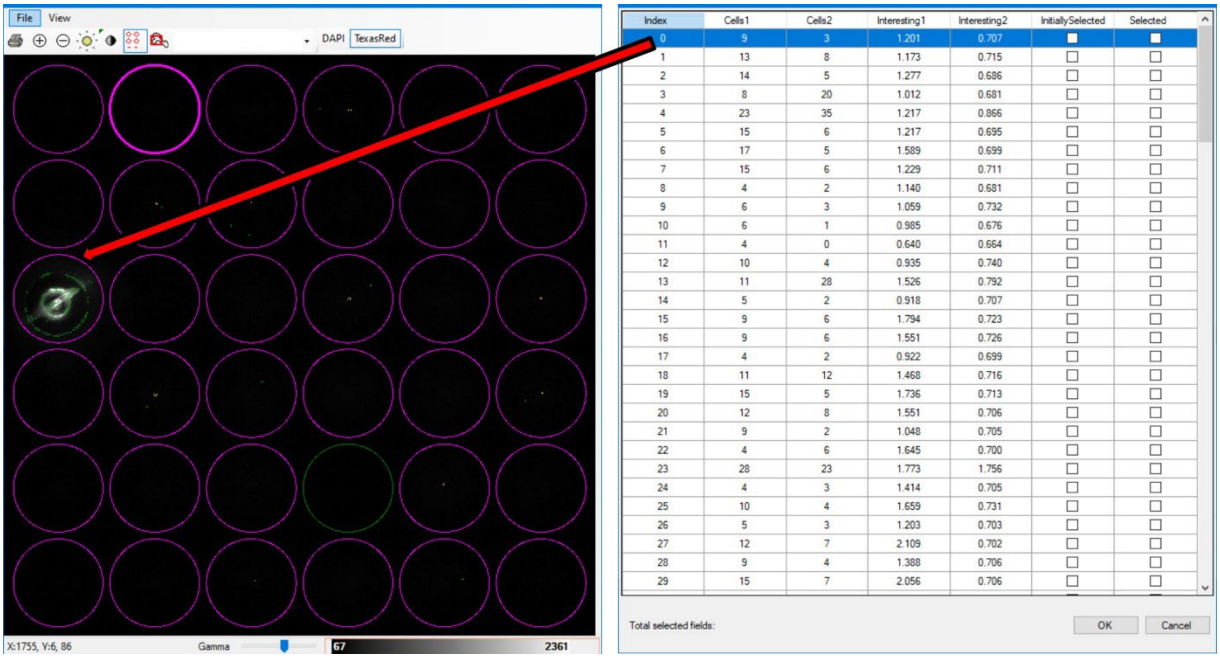
1. In the *AutoTune Image Selector* dialog, sort (descending order) on the "Cells2" (or whichever channel has the least number of cells) column:



Index	Cells1	Cells2	Interesting1	Interesting2	InitiallySelected	Selected
113	26	42	2.378	1.254	☐	☐
4	23	35	1.217	0.866	☐	☐
13	11	28	1.526	0.792	☐	☐
23	28	23	1.773	1.756	☐	☐
125	23	23	1.287	0.842	☐	☐
3	8	20	1.012	0.681	☐	☐
135	63	15	13.241	0.721	☐	☐
109	10	13	1.170	0.784	☐	☐
138	27	13	5.614	0.772	☐	☐
46	9	13	1.033	0.751	☐	☐
33	10	12	1.290	0.748	☐	☐
136	7	12	0.989	0.748	☐	☐
18	11	12	1.468	0.716	☐	☐
36	13	11	2.856	0.700	☐	☐
120	9	9	1.599	1.165	☐	☐
117	11	8	1.279	0.725	☐	☐

Figure 78. *Image Selector* dialog sorted on "Cells2" column.

Avoid choosing images with high cell counts, but showing a lot of imaging artifacts such as debris and reflections:



Index	Cells1	Cells2	Interesting1	Interesting2	InitiallySelected	Selected
0	9	3	1.201	0.707	☐	☐
1	13	8	1.173	0.715	☐	☐
2	14	5	1.277	0.686	☐	☐
3	8	20	1.012	0.681	☐	☐
4	23	35	1.217	0.866	☐	☐
5	15	6	1.217	0.695	☐	☐
6	17	5	1.589	0.699	☐	☐
7	15	6	1.229	0.711	☐	☐
8	4	2	1.140	0.681	☐	☐
9	6	3	1.059	0.732	☐	☐
10	6	1	0.985	0.676	☐	☐
11	4	0	0.640	0.664	☐	☐
12	10	4	0.935	0.740	☐	☐
13	11	28	1.526	0.792	☐	☐
14	5	2	0.918	0.707	☐	☐
15	9	6	1.794	0.723	☐	☐
16	9	6	1.551	0.726	☐	☐
17	4	2	0.922	0.699	☐	☐
18	11	12	1.468	0.716	☐	☐
19	15	5	1.736	0.713	☐	☐
20	12	8	1.551	0.706	☐	☐
21	9	2	1.048	0.705	☐	☐
22	4	6	1.645	0.700	☐	☐
23	28	23	1.773	1.756	☐	☐
24	4	3	1.414	0.705	☐	☐
25	10	4	1.659	0.731	☐	☐
26	5	3	1.203	0.703	☐	☐
27	12	7	2.109	0.702	☐	☐
28	9	4	1.388	0.706	☐	☐
29	15	7	2.056	0.706	☐	☐

Figure 79. *Image Viewer* showing a well with debris and reflections.

2. Choose images with high cell counts by checking the checkbox in the "Selected" column.

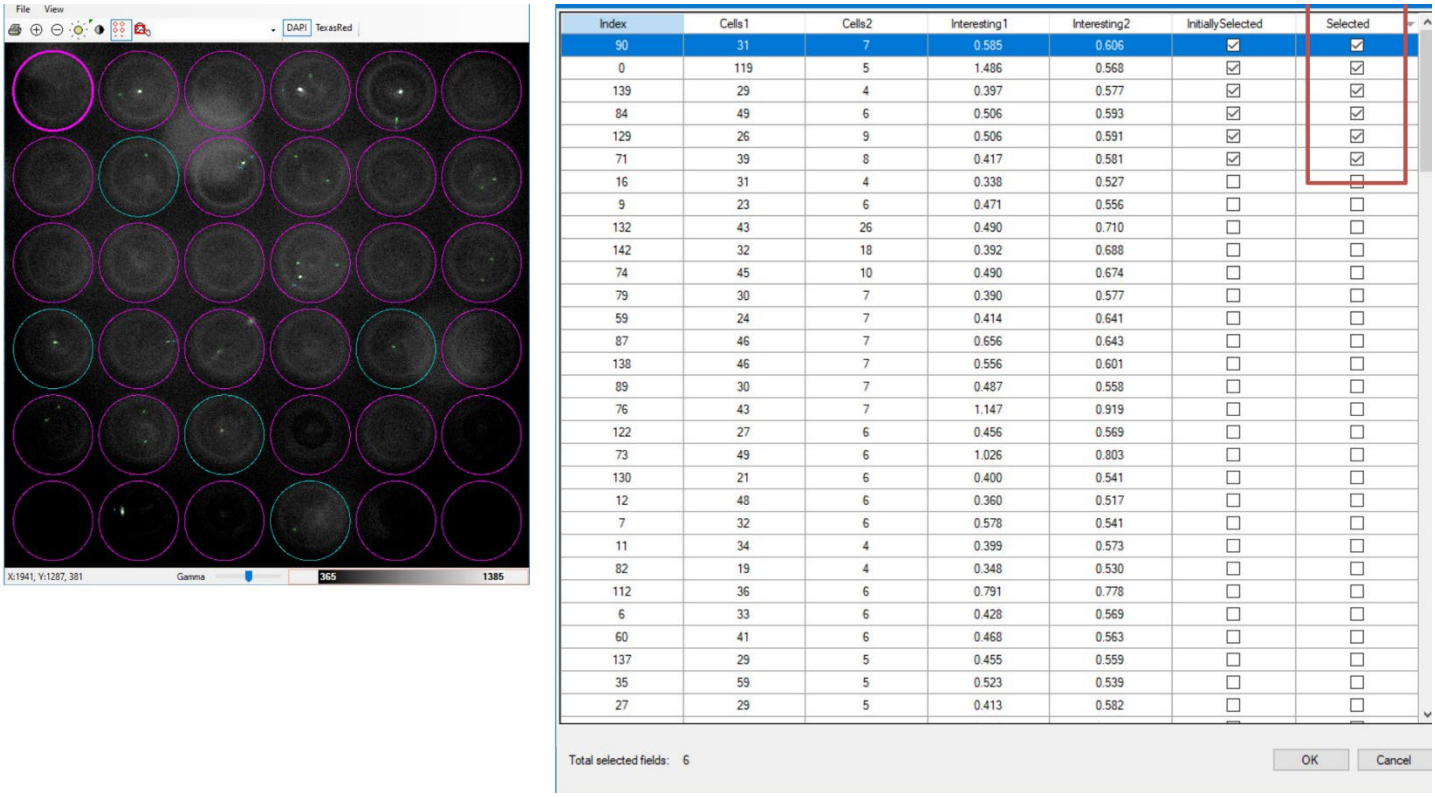


Figure 80. Selected images shown in the *AutoTune Image Selector* window.

3. After selecting the desired images, close both the *Image Viewer* and *Image Selector* dialogs.

4. Specify the Number of Cells in Each Well

After closing the *Image Viewer* and *AutoTune Image Selector* dialogs, you will be left with the *Auto Tune* dialog:

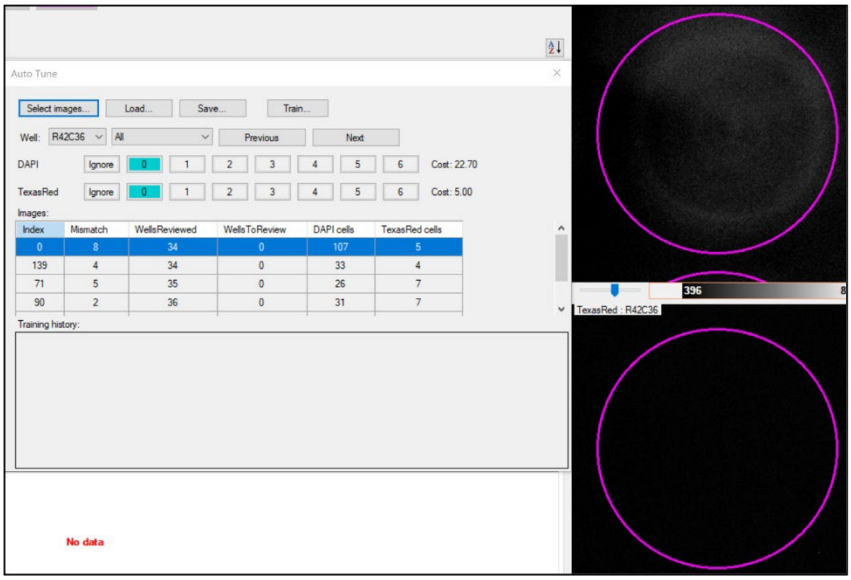


Figure 81. *Auto Tune* window with well selection shown in *Main* window

1. Use the [Previous] and [Next] buttons to examine each well and channel in the selected images. If there were 5 images selected, there will be 180 (5 images x 36 wells) wells to be examined.
2. As the [Previous] and [Next] buttons are used, the well and channel images in the *Main* window are updated to show the selected well.
3. For each well, record the number of cells contained in each channel. This value may differ from that originally reported.
4. For wells with a lot of debris or reflections, select the [Ignore] button.
5. After all wells have been examined, save training history. This action will allow user decisions to be saved for future use.
6. Press the [Train…] button.
7. The training algorithm will execute for 5 to 15 minutes. During this time no user input is required.

5. Process the Images with the New Cell Detection Thresholds

1. After the training is complete, close the *Auto Tune* window.
2. A dialog asking if image processing is desired is displayed. Answer 'yes'.
3. Image processing requires 2-5 minutes for execution. When this process is complete, the auto-tune procedure is finished.
4. Save files in order to save the analysis with the optimized CellSelect parameters.

B. Advanced Auto-tune Features

Once you have the initial results or have loaded a `.wcd` file, press the [Tune] button.

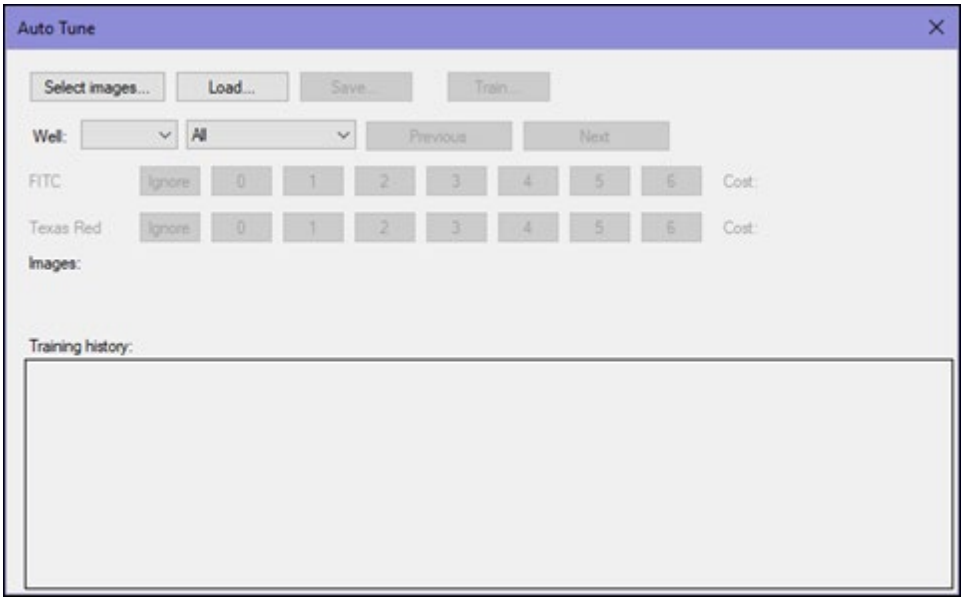


Figure 82. The *Auto Tune* window.

When the *Auto Tune* window is first opened, the only actions available are [Select images…] and [Load]. [Load] is used to implement previously saved training data.

1. Select Images

If this is the first time doing an auto tune for this `.wcd` file, press [Select images…]. This will bring up the *AutoTune Image Selector* window.

Like the "Choose Five Optimal Images" step above (Appendix G.A.3), the system analyzes all images to locate interesting ones and displays the results in a table:

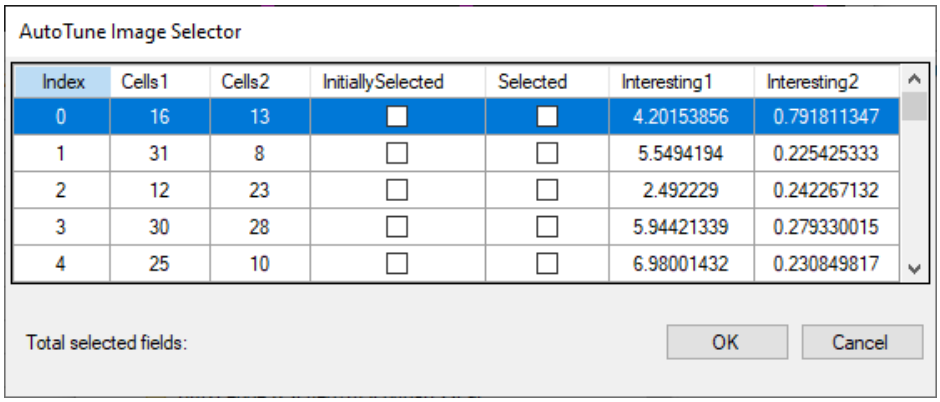


Figure 83. The *Auto Tune Image Selector* dialog window.

The static values in the table are interpreted to help select wells to review. You can click on a column header to sort by that column:

- "Index" column identifies the image location
- "Cells1" and "Cells2" show the number of cells identified from the loaded analysis file and refer to the number of cells for the Dye1 and Dye2 channels of the imaging, respectively
- "Interesting1" and "Interesting2" represent the likelihood of finding cells in the image based on the image histograms

1. Review an image by clicking on a row in the table. Select which color to display by clicking on the channel color buttons in the *Image Viewer* toolbar. Figure 84 uses a red square to highlight where to find the channel color buttons, while 'Blue' is the selected value.

Figure 84. The *Image Viewer* window during auto-tune.

2. Select an image to be included in the training set by toggling the "Selected" checkbox.

   You want to select at least three image locations that have a good number (1–4) of cells in each of the colors. Five or more images is recommended. Press [OK] when done.

   NOTE: The system needs a variety of cell characteristics (small, big, bright, dim, round elongated, etc.) to be trained optimally. If the learning set does not include a sufficient number of training cells of a particular characteristic, the final result will be less than optimal.

After selecting images, the "Well" dropdown box will be filled with the nanowell coordinates of the selected images. Keep in mind there are two kinds of coordinates:

- The usual nanowell coordinates R0C0 (Row 0, Column 0) to R71C71 (Row 71, Column 71).
- The index of the images (Pos0 to Pos143). Each image contains 36 wells, mapping to 144 grids of 6 x 6 wells across the Single-Cell chip.

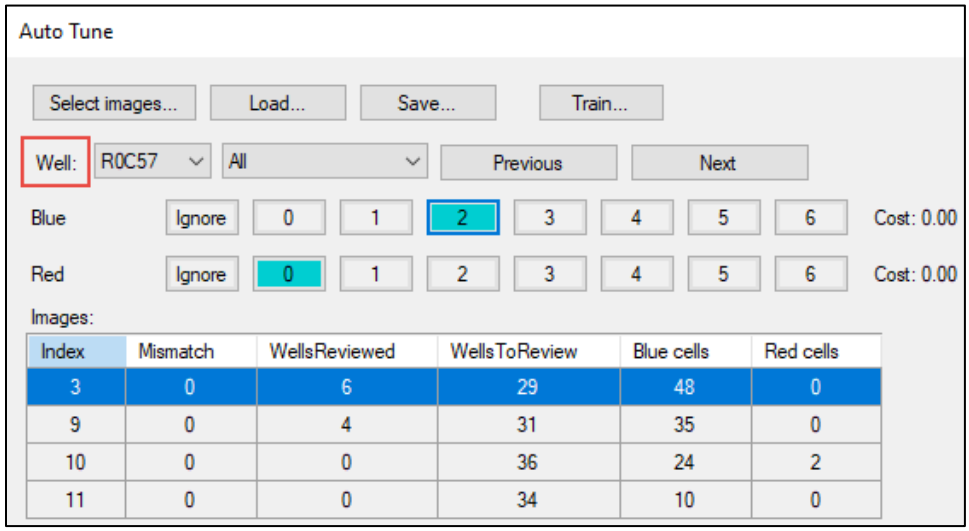


Figure 85. The *Auto Tune* window after images have been selected. The descriptions of these fields are detailed in Table 14. The "Well" row, mentioned just after Table 14, is highlighted by the red box.

Table 14. Descriptions of the *Auto Tune* window buttons and menu options.

Parameter	Purpose
Select images…	Analyze all images and let user decide which images to include in the training set
Load…	Load a previously saved training set.
Save…	Save the current training set parameters. This includes information about user overrides and which nanowells have been reviewed. The save file will have a `.xat` extension by default and is only used within the Tune function. **NOTE:** this is different than the file (`.xml`) saved when quitting out of Tune, documented in Step 3, below.
Train…	Start an iterative training session. For details see below.
Well…	List of nanowells in the current training set (more details below)
Previous	Show the previous nanowell in the filtered list. Filters are set by the "Well" row dropdown menu option
Next	Show the next nanowell in the filtered list.

"Well" row

The dropdown box immediately to the right of the "Well" text represents the list of nanowells in the current training set. The second dropdown, which defaults to 'All' is the nanowell filter, can be used to filter the nanowells to restrict what wells are in the first dropdown box.

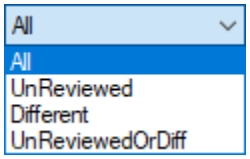


Figure 86. The options of the nanowell filter dropdown menu in the *Auto Tune* window. The descriptions of the drop-down options are described in Table 15.

Table 15. Options and descriptions of the *Auto Tune* window "Well" dropdown menu.

Option	Filter effect
All	Include all nanowells.
UnReviewed	Only list nanowells that have not been reviewed. A nanowell is flagged as 'reviewed' once it has been shown.
Different	Only list nanowells where the user has made an override, including "Ignore".
UnReviewedOrDiff	List nanowells that are not classified as 'UnReviewed' or 'Different'.

Cell count override

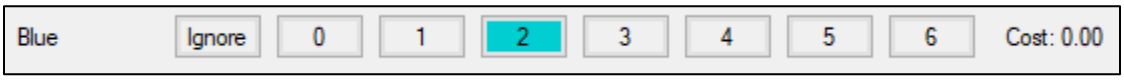


Figure 87. The cell count override section of the *Auto Tune* window.

The cell count override display shows the color channels used in the scan. By default, the button highlighted represents the number of cells that were initially detected by the system in that channel.

Click on any button to override the number of cells. Keep in mind that clusters should be counted as two cells. If the initial number of cells is ≥7, the *Ignore* button will be highlighted and the number buttons in the row will be disabled. This button should be pressed when a well is showing a lot of debris or reflections.

Images

Displays summaries for the image field in the training set. Each row represents all colors at the specified location.

Index	Mismatch	WellsReviewed	WellsToReview	Blue cells	Red cells
3	0	6	29	48	0
9	0	4	31	35	0
10	0	0	36	24	2
11	0	0	34	10	0

Figure 88. The images section of the *Auto Tune* window. The description of each column is documented in Table 16.

Table 16. Column name and descriptions or the Images table of the *Auto Tune* window.

Column	Description
Index	Image field, corresponds to the PosN (index) part of the image filenames.
Mismatch	Number of wells that have a mismatch. Note that a nanowell position can have a mismatch for each color.
WellsReviewed	Number of nanowells that have been seen by the user.
WellsToReview	Number of nanowells that have not been reviewed.
Blue cells Red cells	Number of cells present in the specified color.

Whenever a nanowell is selected in the "Well" dropdown box, the cell number buttons for each wavelength (blue or red) are updated. The button number equaling the number of cells in the nanowell is highlighted in teal.

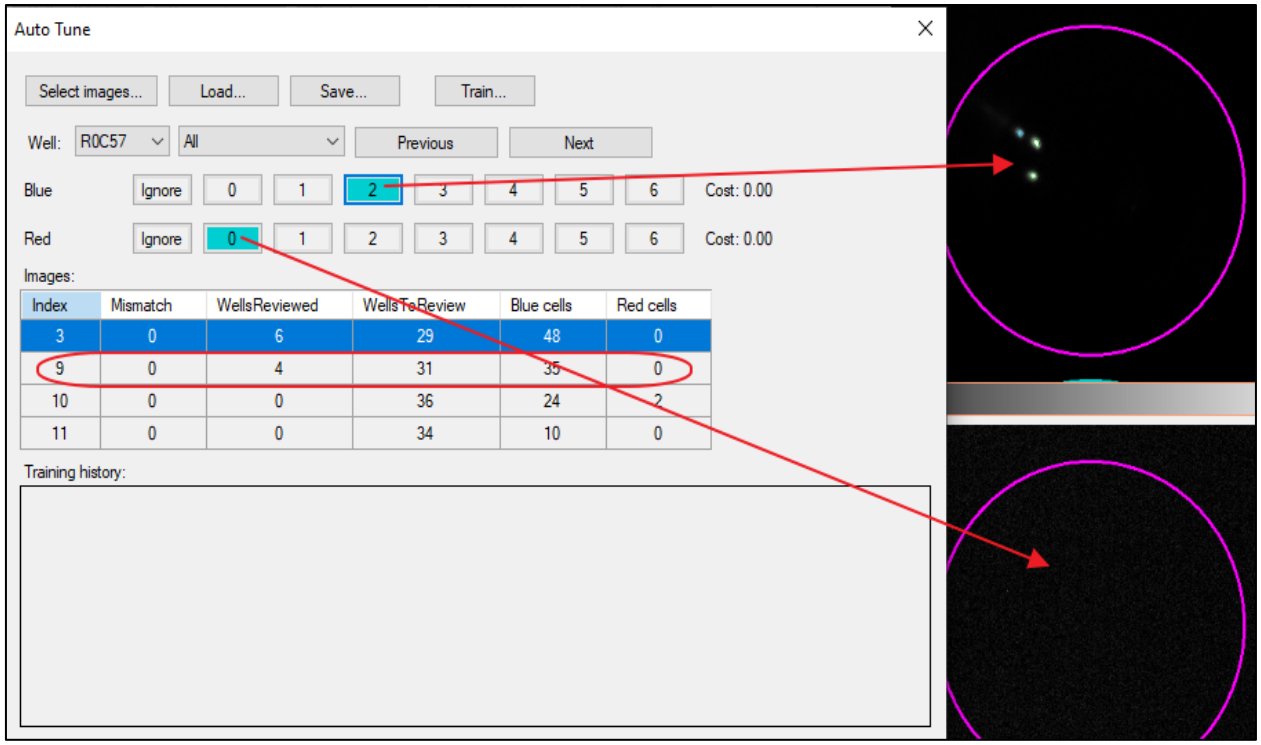


Figure 89. The *Auto Tune* window example when a "Well" is selected.

If you want to select a different value for the cell count override of any wavelength, click on the corresponding button. Figure 90 shows an example of Blue [3] being selected after [2] was initially returned.

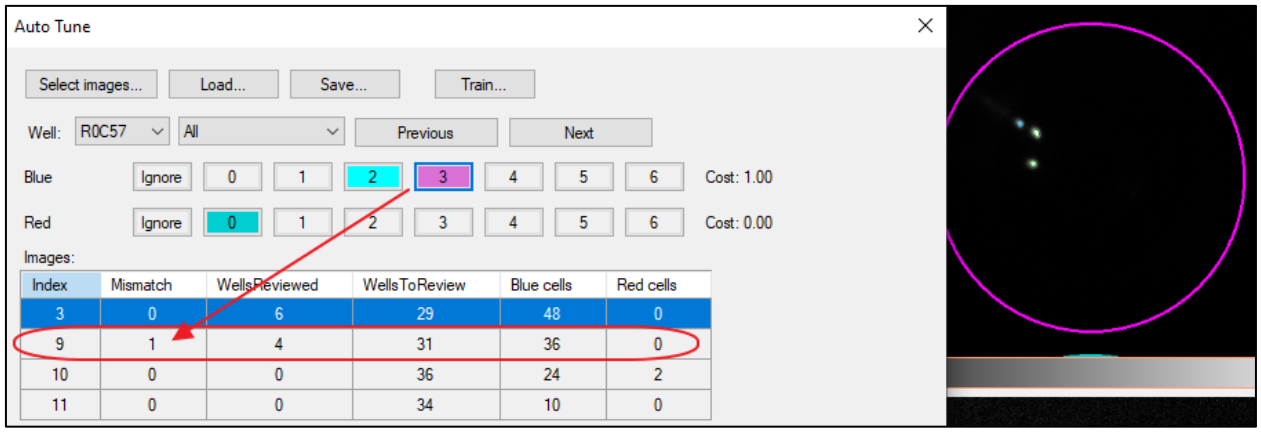


Figure 90. The *Auto Tune* window example when a button is manually selected in the cell count override row. In this figure, button [3] is selected for the blue wavelength.

The selected, override value will have a purple background. At the same time, the number of mismatched nanowells increases for the corresponding image field, thereby increasing the Cost (see formula below).

NOTE: Sufficiently elongated ellipses are considered to be a cluster of two cells by the software. The SuspiciousCircularity parameter (Appendix D.R.3) is determined by these clusters. If your training set does not include cell clusters, the algorithm considers itself to have insufficient information to properly determine the best circularity threshold (see **NOTE** in Step 2, "Train").

In this situation, the user should estimate the suspicious circularity value.

At this point there is a difference between the number of cells that were found by the system and the number of cells the user thinks are present. When training the system, we try to minimize the total cost function which is defines as:

$$Cost = \sum_{wave} \sum_{well} \sqrt{N_{user}(wave, well) - N_{system}(wave, well)}$$

where

- N_{user}(color, well) is the user-defined number of cells in a nanowell for a color
- N_{system}(color, well) is the number of cells that were found by the system using the currently active parameters.

Sometimes the objects in a nanowell are not representative at all. They should not be used for training. In this case select the [Ignore] button. Nanowells that are flagged to ignore do not participate in the cost calculation.

At this point, save your selections by pressing the [Save…] button. You can restore your work at a later point with [Load…].

NOTES:

– Use the Select images button to choose a different set of images that should be processed.

– Make sure to save your work before selecting a completely new set of images, as changes will otherwise be lost.

2. Train

When you press the [Train] button in the *Auto Tune* window, you are presented with a quick summary of the current training set. There may also be a warning about insufficient numbers of cells. In this case, the user should modify the training dataset such that the chosen images contain more cells in the specified color. If this is not possible, auto-tune is not appropriate for the dataset, and the user must set the cell selection thresholds manually.

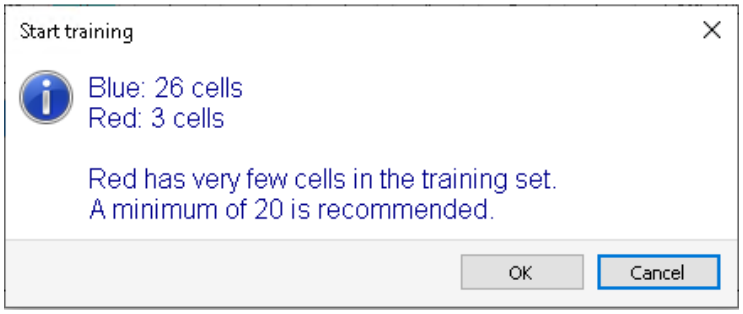


Figure 91. Example of the *Start training* window pop-up after clicking the [Train] button in the *Auto Tune* window.

During the training operation the system calculates cell counts by varying the Thresholds, SecondDerivativeScale, and Circularity values, described in Appendix D. The result of the training operation is the set of parameters where the Cost function is at a minimum.

An iterative training session is started, where the system tries to find the global minimum cost function and stops when no more improvement is possible. This may take several minutes. The required time depends on how far off from the optimal the original set of parameters has been, the number of images and on the speed of the computer. With five training images, the iterative training session should be complete within 10 min. Each iteration produces an entry in the training history table.

Training history

The training history table shows a list of consecutive training results. The cost value should decrease with each iteration.

	Threshold1	Threshold2	SecondDerivScale	Circularity	Cost
▶	40.000	30.000	2.000	0.750	34.171
	120.000	25.733	1.490	0.750	9.828
	258.920	32.056	1.757	0.750	5.000
	258.920	32.056	1.757	0.750	5.000

Figure 92. An example training history table in the automated threshold detection tool.

When using the V3 (or V2) algorithms, the four parameters adjusted by the training process are as shown above (Threshold1, Threshold2, SecondDerivativeScale and Circularity). When using the Cardiomyocytes algorithm, instead of the Circularity parameter, the Elongation parameter is adjusted, as shown below:

Threshold Red	Threshold Blue	SecondDerivScale	Elongation	Cost	CostPerCell
50.000	25.000	2.000	0.880	8.560	
50.000	25.000	2.000	0.815	112.966	0.032
50.000	25.000	2.000	0.773	112.966	0.032
50.000	25.000	2.000	0.747	112.966	0.032

Figure 93. An example training history table with the Cardiomyocytes algorithm

NOTE: It is possible that multiple sets of parameters result in the same cost. This is particularly common when the training set is small (3 images or less) or when a narrow search range is used. In this case, the settings represent the averages of the settings that result in the same cost.

3. Reviewing Wells after Training

After a training iteration it is useful to review the nanowells where the system still does not match the user choice. To speed up this review, you can filter the nanowells to only show the ones that are '*Different*'.

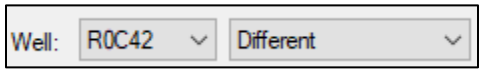


Figure 94. Example selecting the 'Different' option from the "Well" dropdown menu in the *Auto Tune* window.

It often turns out that experiences may be inconsistent during the first pass and this provides an opportunity to correct the questionable calls.

NOTE: If some values are changed, be sure to use the [Save…] button to save the modifications.

When you close the *Auto Tune* dialog you will see a pop-up window similar to Figure 95. Click on 'Save optimized parameters' to save the results and quit the tool.

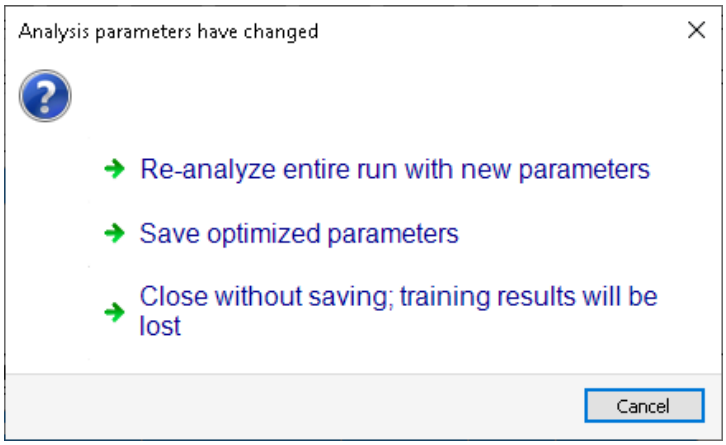


Figure 95. The *Analysis parameters have changed* window when quitting out of the auto-tune tool.

- If you choose 'Re-analyze entire run with new parameters', the full dataset will be re-analyzed with the new optimized analysis parameters.

- Selecting 'Save optimized parameters' will skip re-analyzing the dataset and simply save the optimized analysis threshold parameters to an XML file (`.xml`).

  NOTE: The optimized threshold parameters may also be saved at any time by choosing the **File > Save…** menu item found in the Analysis Settings dialog. This dialog can be accessed via the [Edit…] button located in the *Settings* tab. Refer to Section II.C.4, "Saving Settings to an XML File", for more information.

  This saved XML file can either be utilized as a custom setting file when setting up in an experiment in CELLSTUDIO software (Shasta Single Cell System User Manual, Section VII.D, "Start New Experiment") or within CellSelect software by loading the file (Section II.C.5, "Loading a Saved Settings File").

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