Takara Bio USA, Inc.

ICELL8® cx CellSelect® v2.5 Software User Manual

Cat. No. 640188, 640189, 650002
software v2.5
(102920)

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I. Introduction

A. Welcome to the ICELL8 cx CellSelect v2.5 Software

The **ICELL8 cx Single-Cell System** (Cat. No. 640188, 640189) 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 hundreds 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

ICELL8 cx CellSelect v2.5 Software (referred to as CellSelect Software hereafter) analyzes images generated with ICELL8 cx CELLSTUDIO™ v2.5 Software and provides researchers with the following capabilities:

- Automated or manual image analysis and selection of isolated cells for downstream processing
- 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.

Figure 2 (Section I.D, below) summarizes the dispensing, imaging, and image analysis portions of the ICELL8 cx single-cell analysis workflow.

B. What's New

1. ICELL8 cx CellSelect v2.5 Software

- Automatic threshold settings ("Tune" function, or "auto-tuning") for efficient candidate selection (Section III.J and Appendix F)
- User-interface and file management improvements

2. CellSelect Software version 2.0

ICELL8 cx CELLSTUDIO v2.0 Software, the open architecture software for the ICELL8 cx system, introduced the following features that influenced usage of CellSelect software:

- Apply dispense patterns that only utilize a subsection of the ICELL8 cx chip.
- Choose among three different LED/filter configuration when scanning the chip. The filter names and the corresponding image names are now generic (Red, Green, Blue) instead of using dye-specific names (Texas Red, DAPI, FITC).

Because of the flexibility that is provided, the user needs to specify the rules by which CellSelect Software identifies candidate wells. Refer to Section II.C.

- When a custom application on CELLSTUDIO Software includes barcode dispenses, the appropriate barcodes are forwarded to CellSelect Software.

C. Safety

Refer to safety guidelines in the user manuals for all equipment used in this protocol.

WARNING: Perform all experimental procedures in sterile environments with the proper personal protective equipment (PPE). Use designated UV hoods with proper ventilation for manipulating cells and setting up molecular biology reactions. Decontaminate gloves with nuclease decontamination solution, water, and ethanol. Change gloves routinely.

WARNING: Use of equipment and reagents for cell preparation and isolation with the ICELL8 cx Single-Cell System may cause exposure to toxic or biohazardous chemicals, thereby presenting a hazard. Always wear appropriate personal protective equipment (PPE), which should at minimum include gloves, eye protection, and a lab coat when handling equipment and reagents and operating instruments.

Note and heed all warning labels on the instruments used in this protocol.

D. Workflow Diagram

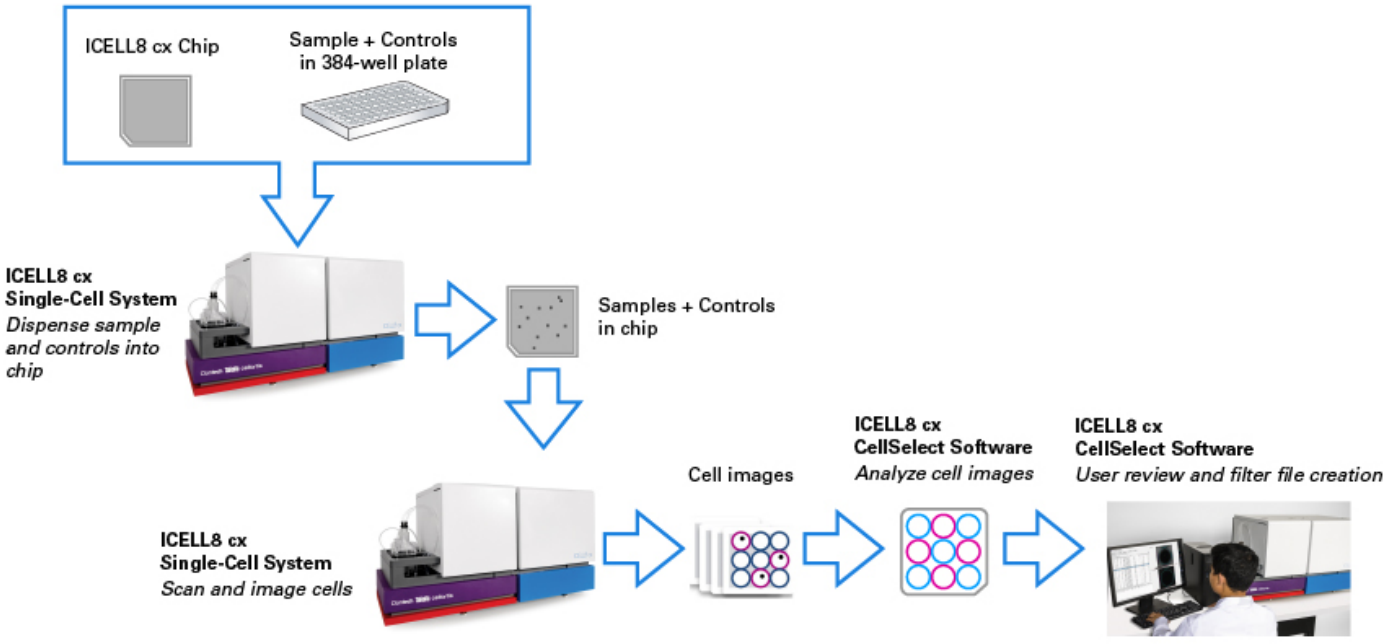


Figure 2. Workflow diagram depicting single-cell isolation, imaging, and image processing steps.

II. Overview: User Interface

The data section of the *Main* screen 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 3, below) can be found in Appendix E.A. The description of the columns of the cell details view (bottom table in Figure 3) can be found in Appendix E.B.

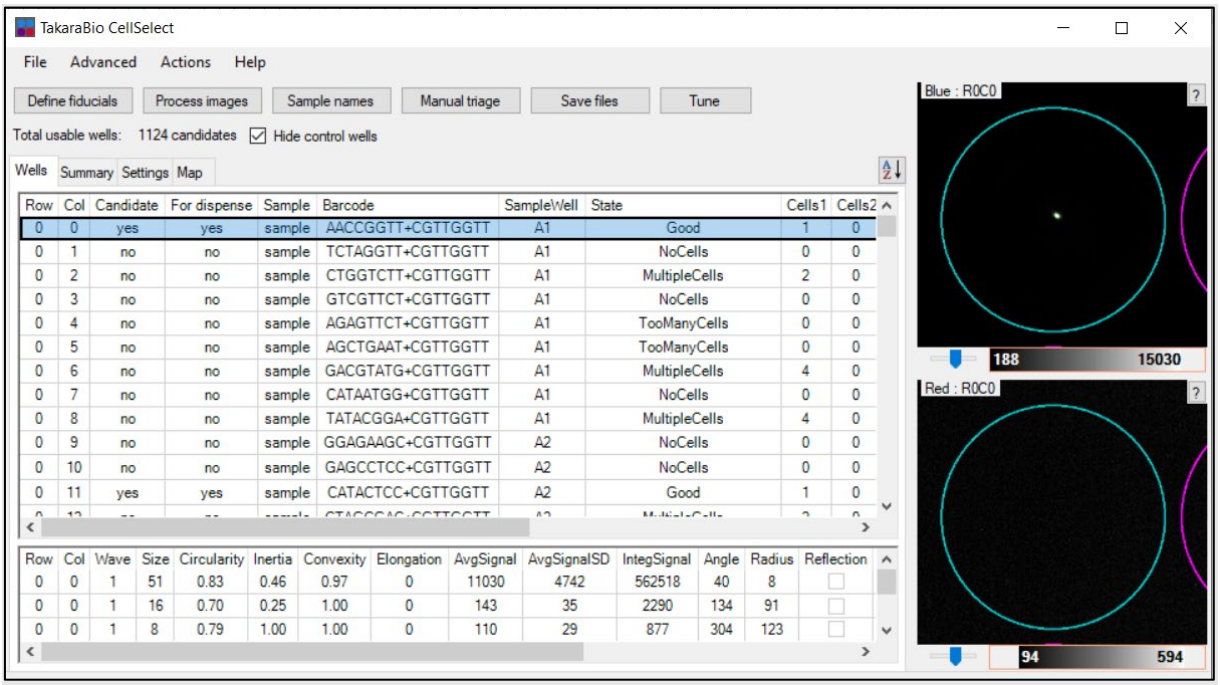


Figure 3. *Wells* data table.

You can view additional data by selecting **Advanced > Switch to advanced user mode**.

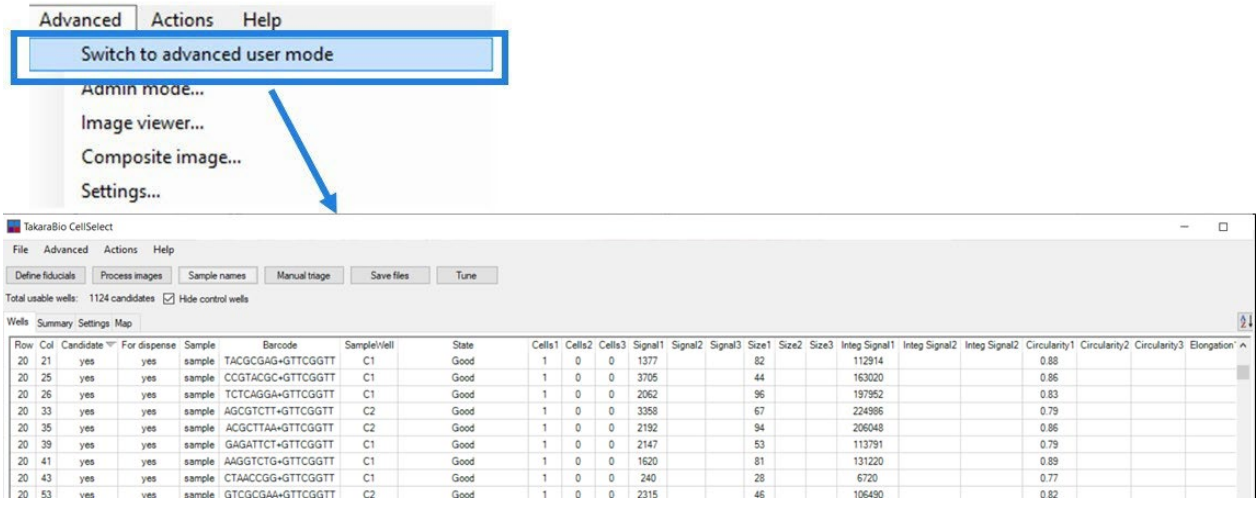
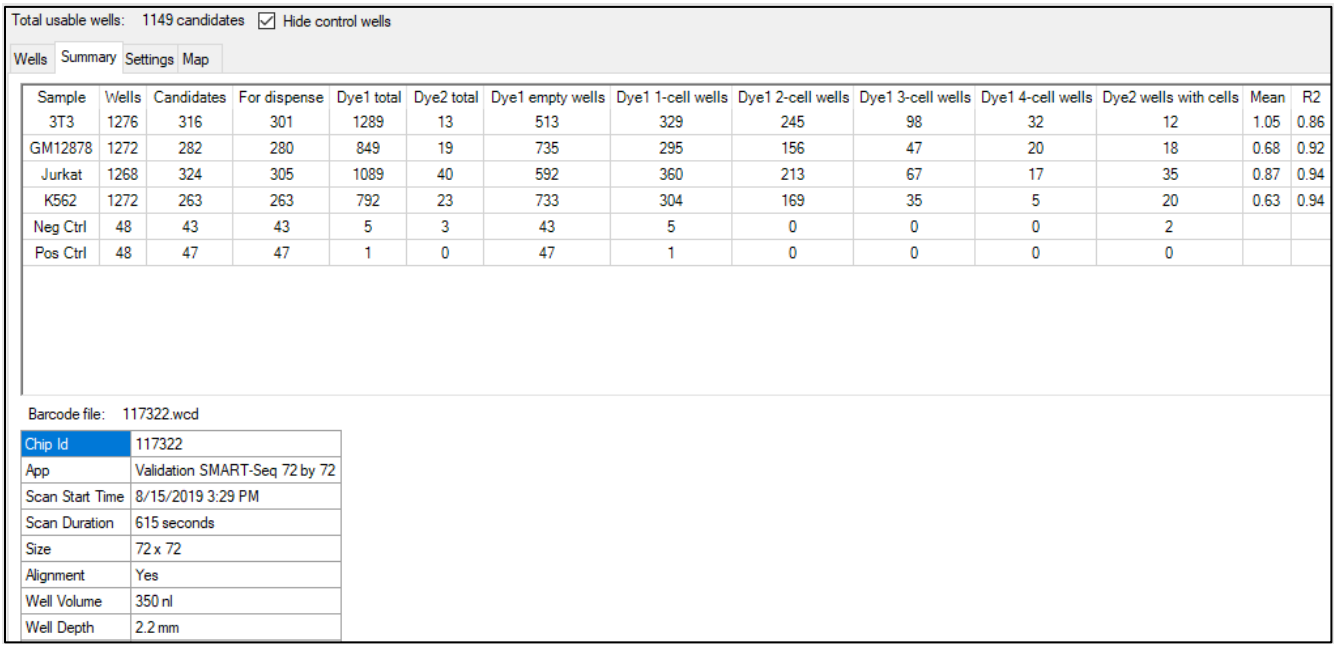


Figure 4. Additional well information in Advanced User Mode.

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 E.C.

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



Sample	Wells	Candidates	For dispense	Dye1 total	Dye2 total	Dye1 empty wells	Dye1 1-cell wells	Dye1 2-cell wells	Dye1 3-cell wells	Dye1 4-cell wells	Dye2 wells with cells	Mean	R2
3T3	1276	316	301	1289	13	513	329	245	98	32	12	1.05	0.86
GM12878	1272	282	280	849	19	735	295	156	47	20	18	0.68	0.92
Jurkat	1268	324	305	1089	40	592	360	213	67	17	35	0.87	0.94
K562	1272	263	263	792	23	733	304	169	35	5	20	0.63	0.94
Neg Ctrl	48	43	43	5	3	43	5	0	0	0	2		
Pos Ctrl	48	47	47	1	0	47	1	0	0	0	0		

Chip Id	117322
App	Validation SMART-Seq 72 by 72
Scan Start Time	8/15/2019 3:29 PM
Scan Duration	615 seconds
Size	72 x 72
Alignment	Yes
Well Volume	350 nl
Well Depth	2.2 mm

Figure 5. *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 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. The default settings have been preconfigured for single-cell analysis.

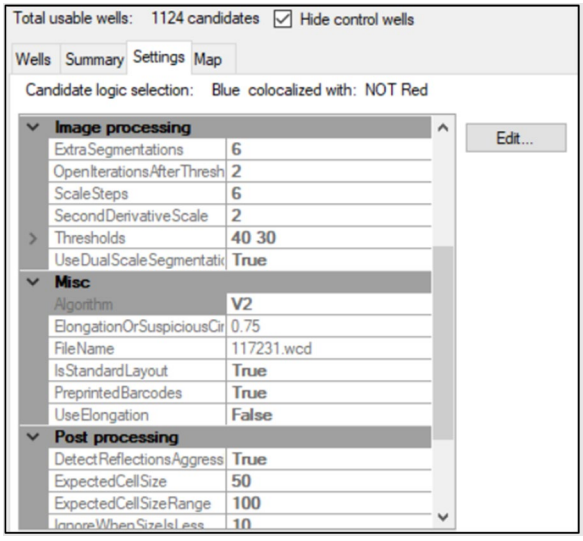


Figure 6. *Settings* tab.

You have the option to edit the settings, either by clicking the [Edit…] button under the *Settings* tab (Figure 6) or through the **Advanced > Settings…** menu (Figure 7).

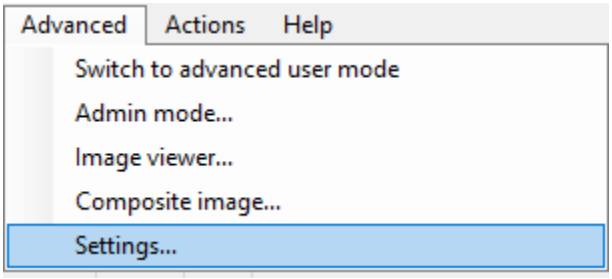


Figure 7. Advanced > Settings… menu.

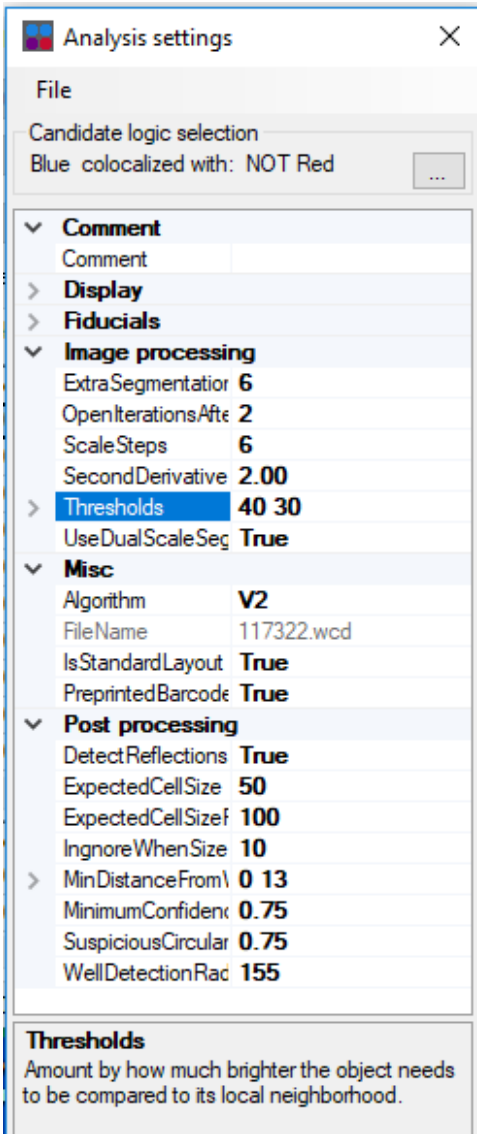


Figure 8. *Analysis settings* window. Setting values can be customized here.

We **strongly** recommend that you become familiar with a parameter before editing it. The most common parameters that might need to be adjusted, "Candidate logic selection" and "Thresholds", are described below. Appendix D lists information about other, less commonly modified parameters.

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 'Master'. Objects that are visible with this dye are considered cells and therefore pass to the next test.
2. Objects in the secondary channel (Dye2) that are colocalized with cells identified in the Master 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 channel that are not colocalized with a cell in the Master channel.

NOTES:

- Wells that have more than one cell in either of the channels are not considered candidates.
- Samples stained with only one dye are excluded from the following logic rules.

To define the rules:

1. Click on the […] button to the right in the "Candidate logic selection" box of the *Analysis settings* window to configure the channels that determine the logic rules.

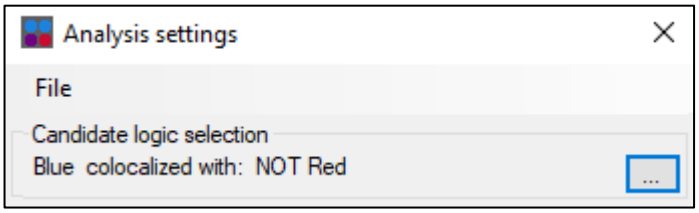


Figure 9. Where to find the "Candidate logic selection" information. The […] is clicked to bring up the window to configure the logic rules.

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

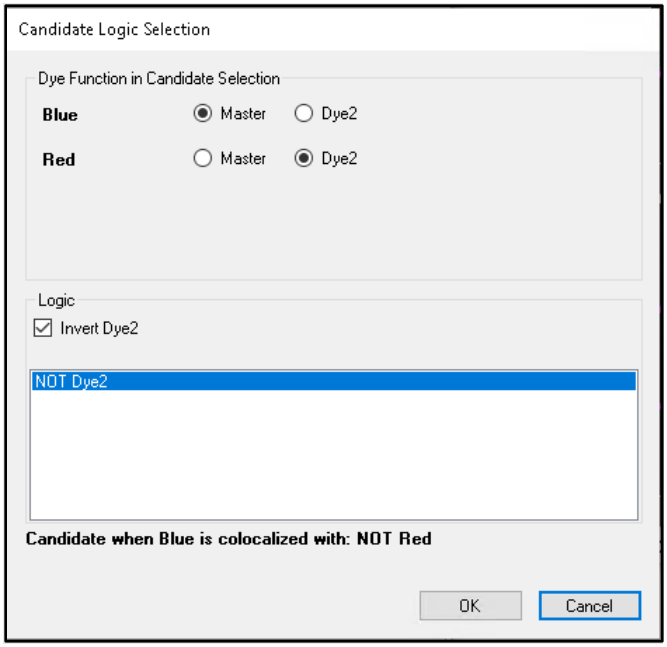


Figure 10. The *Candidate Logic* Selection window. Master/Dye2 Assignment is made here as well as basic logic rules for Dye2.

2. Select the ‘Master’ channel used to identify cells. If two dyes were used, the second dye option will automatically be selected as ‘Dye2’ when the Master dye is designated.
3. If necessary, check the “Invert Dye2” box to indicate that candidate wells should not be visible in the second channel. Checked is the default setting.

   If unchecked, candidate wells need to be present in the second channel.
4. The final rule will display in the bottom box field. The screenshot in Figure 10 (above) looks for objects that are visible in Blue channel but do not show signal in the Red channel.

   Wells with cells in the Master channel that do not pass the Logic test will be flagged as ‘FailedLogic’ in the “Status” column in the *Wells* tab.
5. Click [OK] to accept the logic settings.

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. You might want to adjust this value in cases where, for example, the cells didn’t stain well or stained too well, or if there is high background brightness.

A lower Threshold detects a larger number of cells; however, note that 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”.

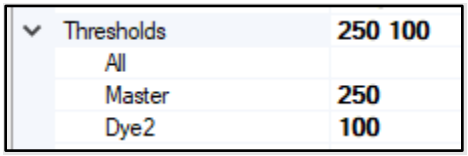


Figure 11. Expanded “Thresholds” option in the *Settings* window.

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

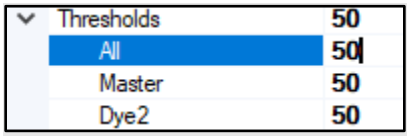


Figure 12. Selecting the “Thresholds” > “All” option in *Settings* window to customize it.

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.

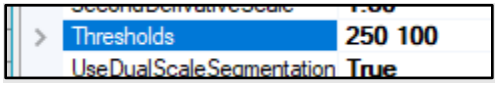


Figure 13. The *Settings* “Threshold” section, displaying multiple sublevel values in the collapsed view.

Restore to Defaults

If you need to reset the parameters in the *Settings* window back to the default, see Appendix D.A.

D. *Map*

The data 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 (see Figure 14, below). The measure options in the drop-down box are the same as the column names under the *Wells* tab; definitions of the options can be found in Table 11 in Appendix E.A.

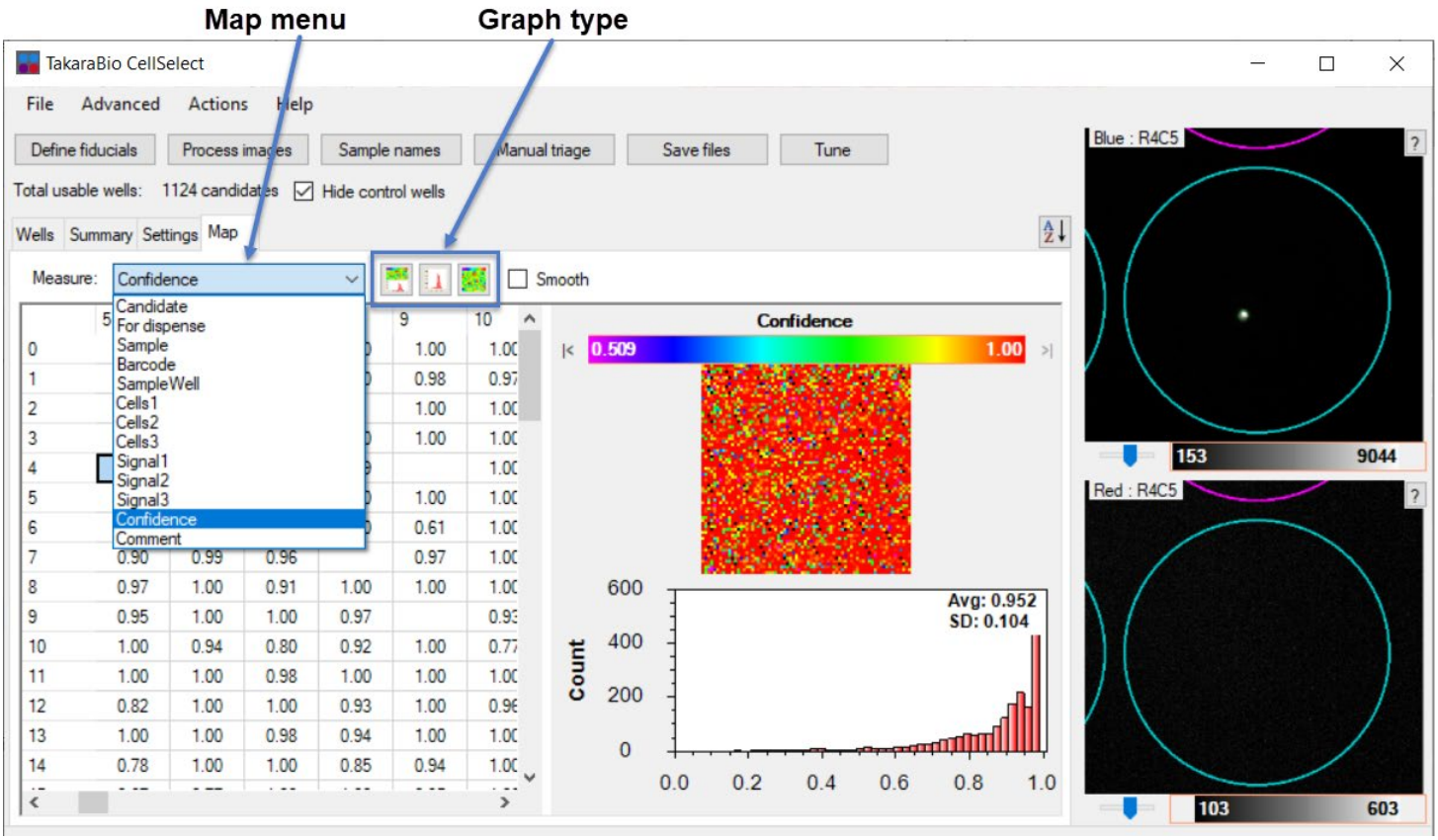


Figure 14. *Map* tab.

III. Procedure

A. Overview

In this procedure, you will select nanowells using CellSelect Software and then generate a filter file to be used for dispensing reagents. The procedure consists of the following steps:

1. **Load Images:** Load images directly from CELLSTUDIO Software after scanning, or later from CellSelect Software (**File** menu).
2. **Load a Different Barcode File (optional):** Barcodes are already selected when you dispense cells in the ICELL8 cx 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 analyzes every well image made during the scan and determines which wells are considered candidates for additional testing. (Candidate selection logic is described in Section II.C, 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 procedure.

Icon for ICELL8 cx CELLSTUDIO v2.5 Software (for dispensing and imaging) 

Icon for ICELL8 cx CellSelect v2.5 Software (for image analysis) 

Figure 15. Icons for the ICELL8 cx CELLSTUDIO v2.5 Software and ICELL8 cx CellSelect v2.5 Software.

B. Load Images

You can open CellSelect Software directly when scanning from the ICELL8 cx Single-Cell System or save a file and open it later from CellSelect Software.

From the ICELL8 cx CellSelect v2.5 Software

1. Launch CellSelect Software by clicking the `CellSelect` icon: 
2. In the *Main* window, navigate the menu **File** > **Open Chip Folder**… item.

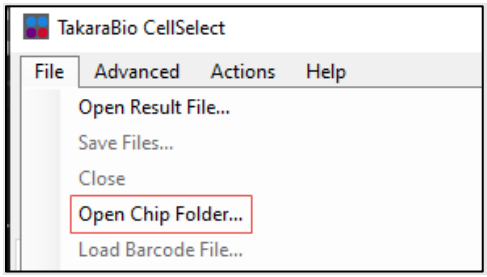


Figure 16. Where to find the File > Open Chip Folder… menu item.

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.

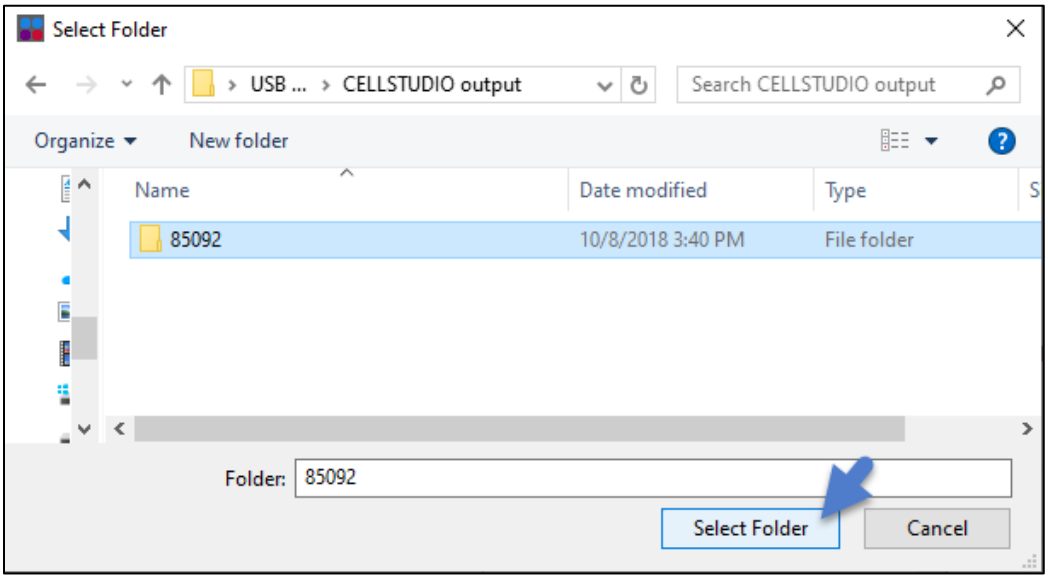


Figure 17. Selecting a chip folder. Do **NOT** double-click and open the folder.

- If there is one `.wcd` file in the folder, that file will be loaded
- If there are more than one analysis file in the folder specified**,** the software will prompt you to select one (Figure 18)

Figure 18. The *Load file* window prompt if multiple `.wcd` analysis files are present in the selected folder.

- If there is no analysis file in the folder, the software will prompt for the chip number and the analysis parameters so you can analyze the images (Figure 19). If using a preprinted chip (e.g., ICELL8 cx 3' DE Chip, Cat. No. 640199, or ICELL8 cx TCR Chip, Cat. No. 640200), you will also have to also select the `AnalysisSetting_250nL_chip.xml` barcode file (Appendix D.A, Table 9).

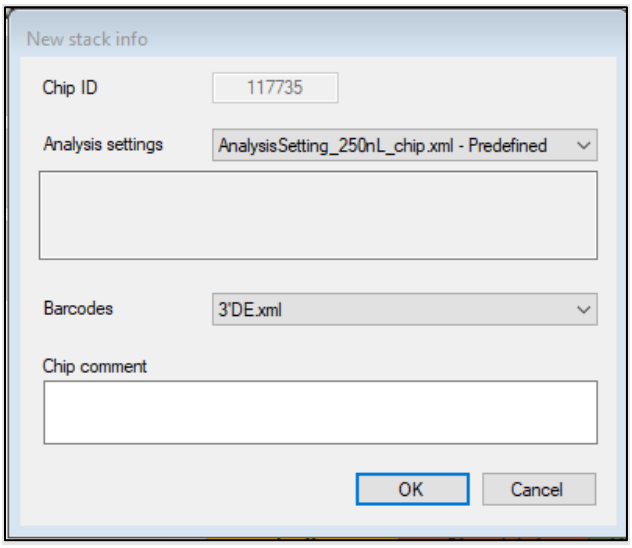


Figure 19. The prompt window if no analysis (`*.wcd`) files are found when attempting to open a chip folder (Figure 18).

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.

Drag and drop onto the CellSelect Software icon

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

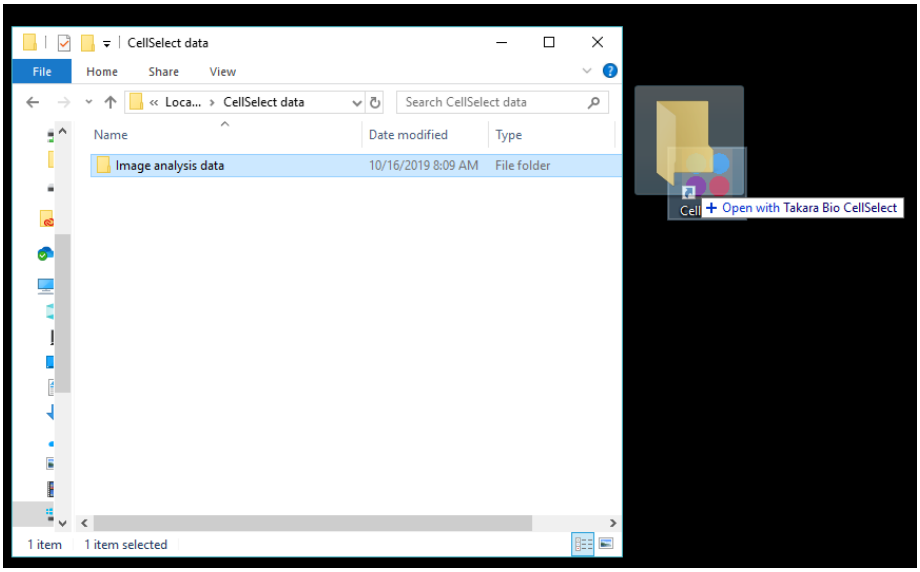


Figure 20. Drag and drop a folder of analysis data onto the CellSelect Software icon to open a results (`*.wcd`) file. If multiple or no `.wcd` files are contained in the folder, it will behave similarly to the cases of Figure 18 and 19, above.

C. Load a Different Barcode File (optional)

Barcodes are already selected when you scan images in the ICELL8 cx system. However, this menu item allows you to associate a different barcode file to the run.

NOTE: If an incorrect index set was selected during sample dispense (ICELL8 cx Single-Cell System User Manual, Section X.C), 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…**.

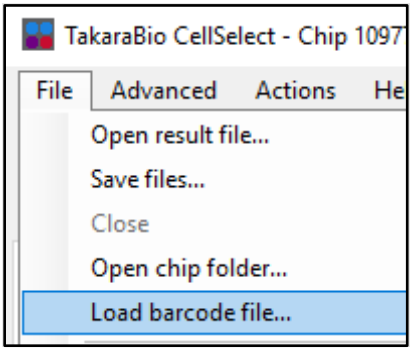


Figure 21. File menu.

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

Select the XML based on whether the indices are provided by Takara Bio 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	Usage
`3'DE.xml`	3' DE	
`ICELL8-cx_SMART-seq_SetA.xml` `ICELL8-cx_SMART-seq_SetB.xml`	SMART-Seq	For use with imaging output files (`*.wcd`) created on an ICELL8 cx system
`ICELL8_SMART-seq_SetA.xml` `ICELL8_SMART-seq_SetB.xml`	SMART-Seq	For use with imaging output files (`*.wcd`) created on the original ICELL8 system (Cat. no. 640000)
`TCR.xml`	TCR	

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

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

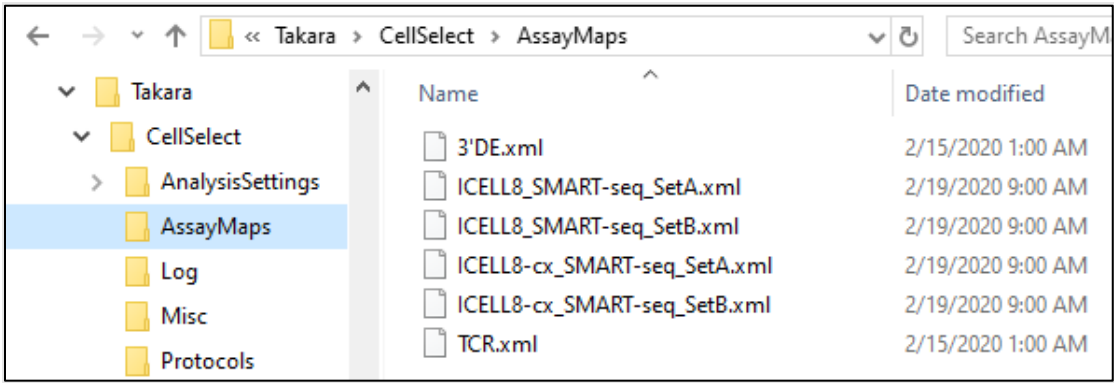


Figure 22. Location of the barcode files preloaded in CellSelect Software.

2. Custom indexes

If custom barcode index files were defined and incorporated into the CELLSTUDIO dispense application (ICELL8 cx Single-Cell System User Manual, Section VII.B), then the barcodes will be imported automatically into CellSelect software.

If, however, you need to apply a different barcode file at this step of the procedure in CellSelect software, an XML file will need to be created by export within CELLSTUDIO software. See the ICELL8 cx Single-Cell System User Manual, Appendix C.C, "Exporting a Custom Barcode File" 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. It is recommended that custom-index XML files be saved in the default folder:

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

to make it easier to locate, but it can be wherever you choose to save it.

D. Process Images

The [Process images] function analyzes all 5,184 nanowell images in the TIFF image files generated by CELLSTUDIO Software during chip imaging.

1. Click [Process images]. The software will analyze the sets of loaded multiwell images, and automatically identify and select all nanowells that contain viable, single cells (i.e., candidates) and controls based on the parameters defined in the *Settings* window. 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)
> - It must be the only one visible in the nanowell
> - It must satisfy defined size/shape parameters
>
> These conditions can be modified in *Settings* window. See Section II.C and Appendix D for more information on the settings parameters.

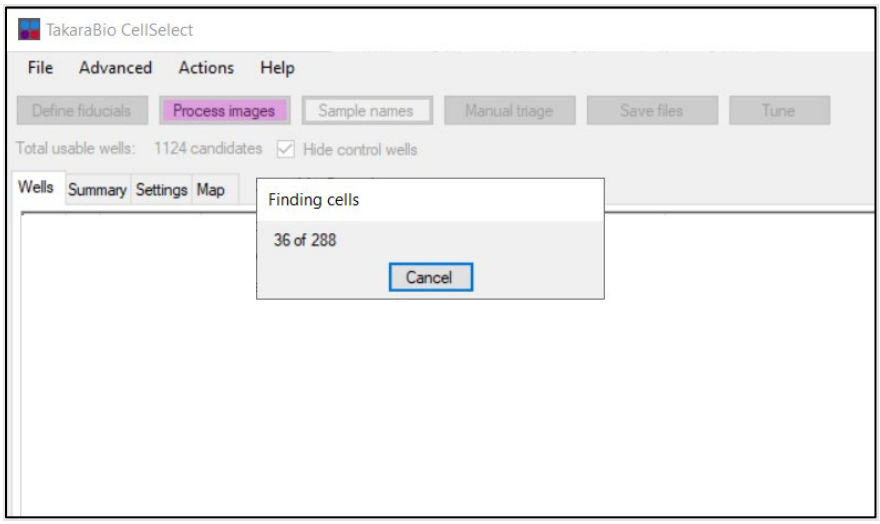


Figure 23. Process images, in progress.

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 of the ICELL8 cx chip, Figure 24) and `<date>` is the date the image was processed.

   Example: `117322_053020.wcd`

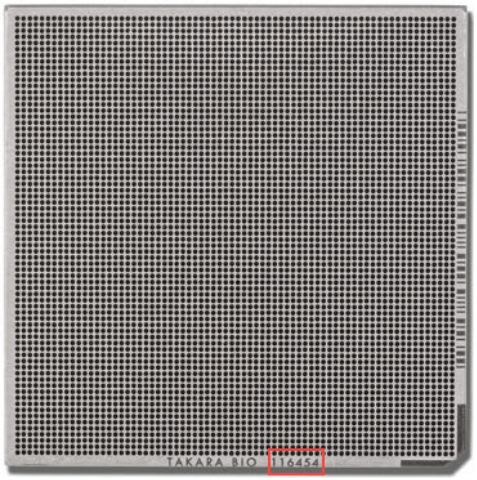


Figure 24. ICELL8 cx chip. The chip ID is located next to the Takara Bio branding, on the bottom when the chamfered edge is on the lower right.

> **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., `117322_053020.wcd`). The results file will contain the selected nanowells and setting parameters.
- Generate the filter file for the dispense of reagents (e.g., `117322_053020_FilterFile.csv`). The selected wells in the filter file will be displayed in the *For Dispense* column.
- Generate a PDF file called `117322_053020_Report.pdf`, which contains a short summary of the results.
- Generate a text file called `117322_053020_WellList.txt`, which contains the content of the *Wells* table in a form that can be read easily by downstream analysis software.

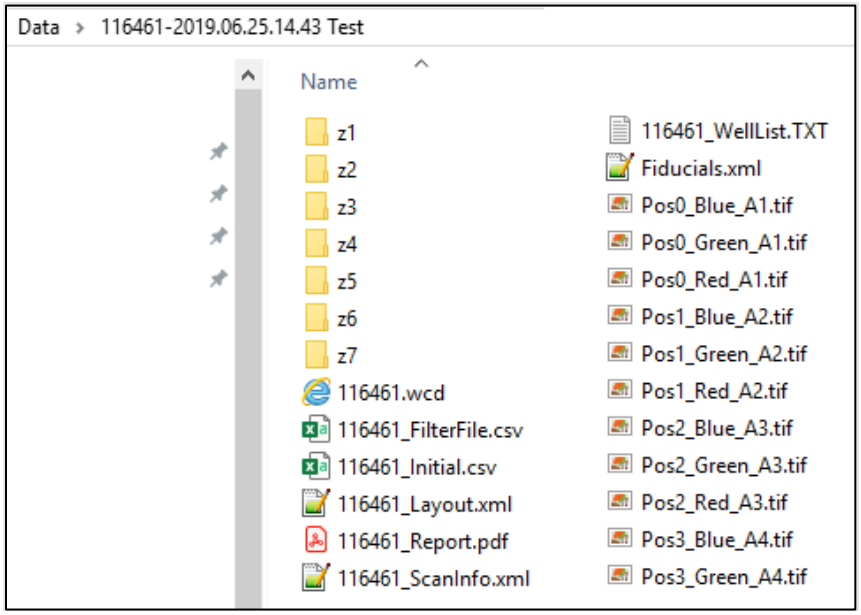


Figure 25. Files generated by CellSelect Software. The chip ID in this example is '116461'. You can load the results along with the settings from the saved `*.wcd` file.

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

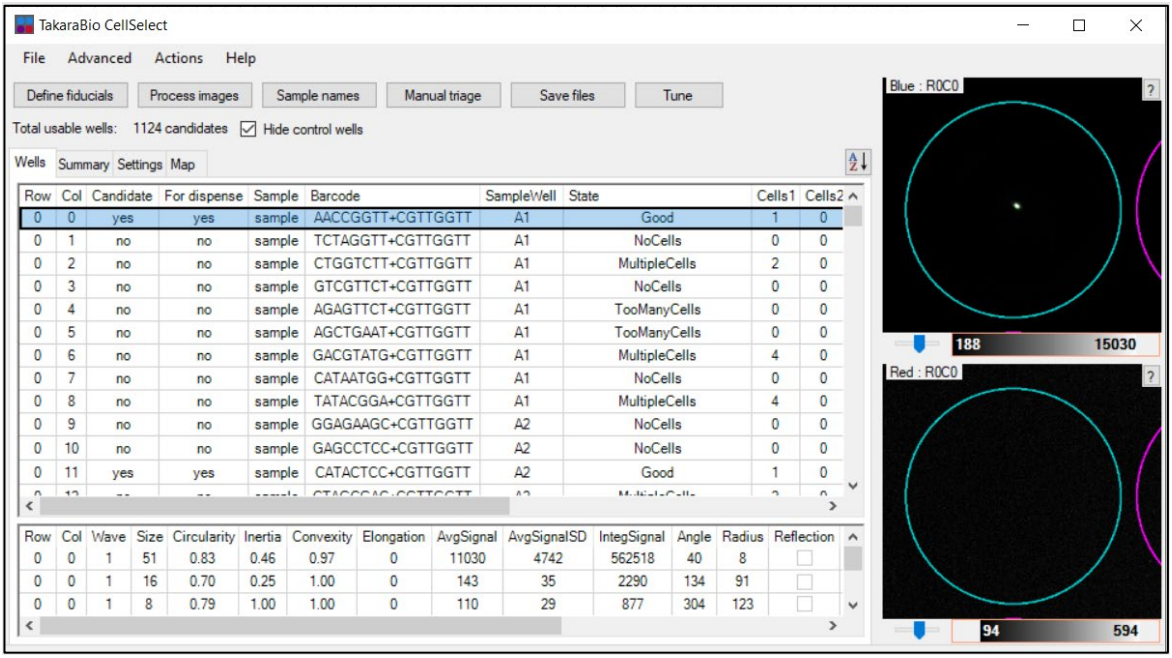


Figure 26. Example of the *Wells* tab with results data post-image process.

NOTE: Images can be reprocessed after changing the analysis settings.

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 whatever you edit it to, e.g., to grid names from the source plate (see Figure 27, below)

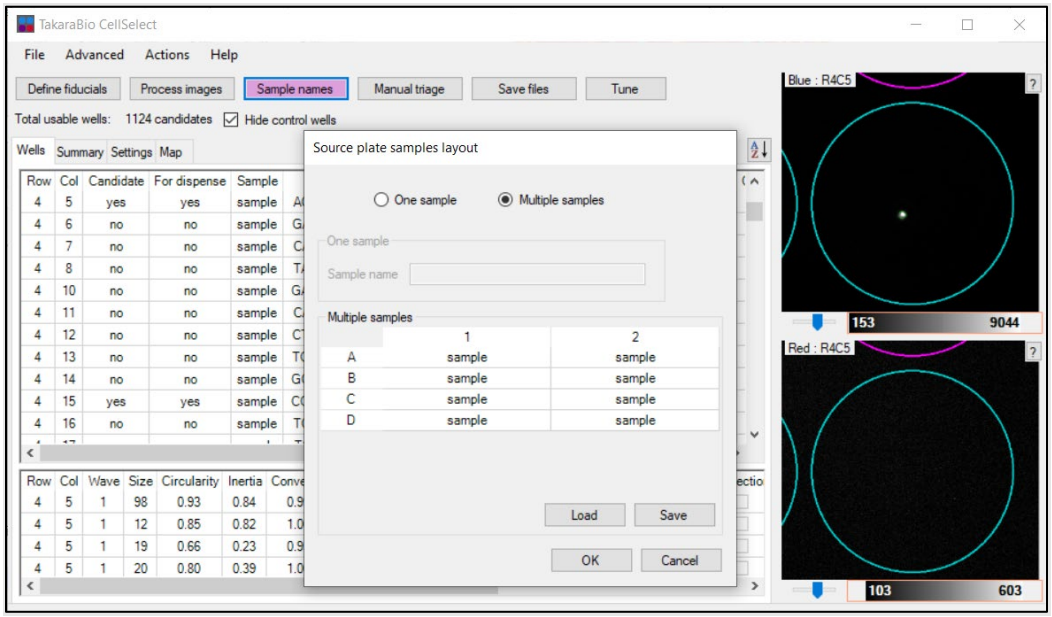
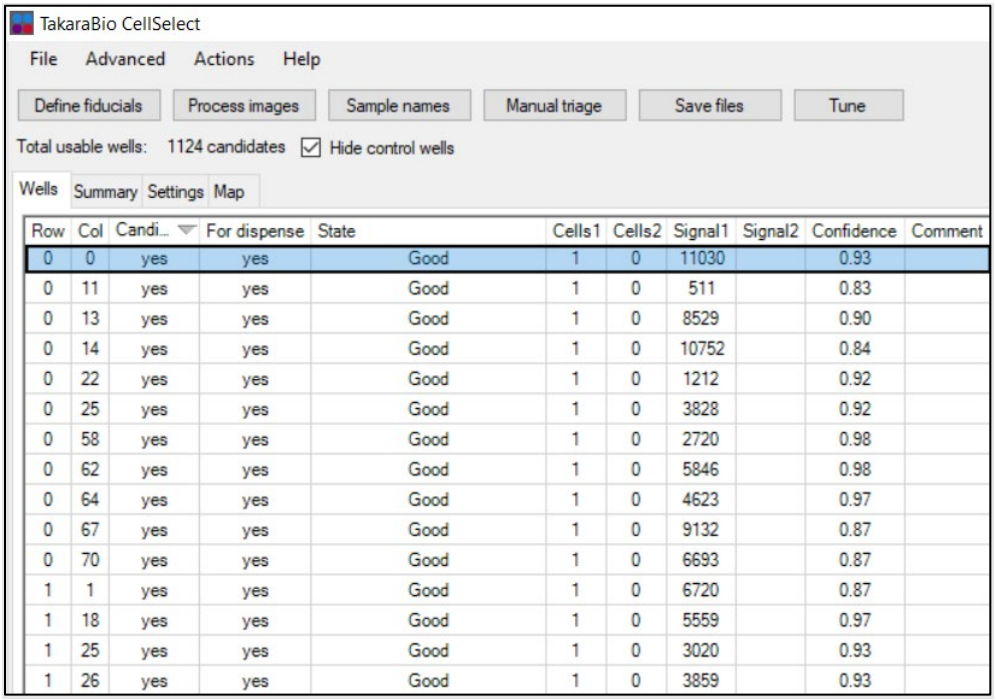


Figure 27. Inputting multiple sample names.

F. Review Images

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 (see Figure 28, below). The software will sort all selected nanowells based on the "Candidate" value.



Row	Col	Candi...	For dispense	State	Cells1	Cells2	Signal1	Signal2	Confidence	Comment
0	0	yes	yes	Good	1	0	11030		0.93	
0	11	yes	yes	Good	1	0	511		0.83	
0	13	yes	yes	Good	1	0	8529		0.90	
0	14	yes	yes	Good	1	0	10752		0.84	
0	22	yes	yes	Good	1	0	1212		0.92	
0	25	yes	yes	Good	1	0	3828		0.92	
0	58	yes	yes	Good	1	0	2720		0.98	
0	62	yes	yes	Good	1	0	5846		0.98	
0	64	yes	yes	Good	1	0	4623		0.97	
0	67	yes	yes	Good	1	0	9132		0.87	
0	70	yes	yes	Good	1	0	6693		0.87	
1	1	yes	yes	Good	1	0	6720		0.87	
1	18	yes	yes	Good	1	0	5559		0.97	
1	25	yes	yes	Good	1	0	3020		0.93	
1	26	yes	yes	Good	1	0	3859		0.93	

Figure 28. 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.

View/Edit Results List

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

1. In the *Main* window, click **Advanced > Image Viewer…** (see Figure 29, below). 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 29. 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, by double-clicking on a well in the *Imager Viewer* the corresponding row in the *Wells* table will be highlighted.

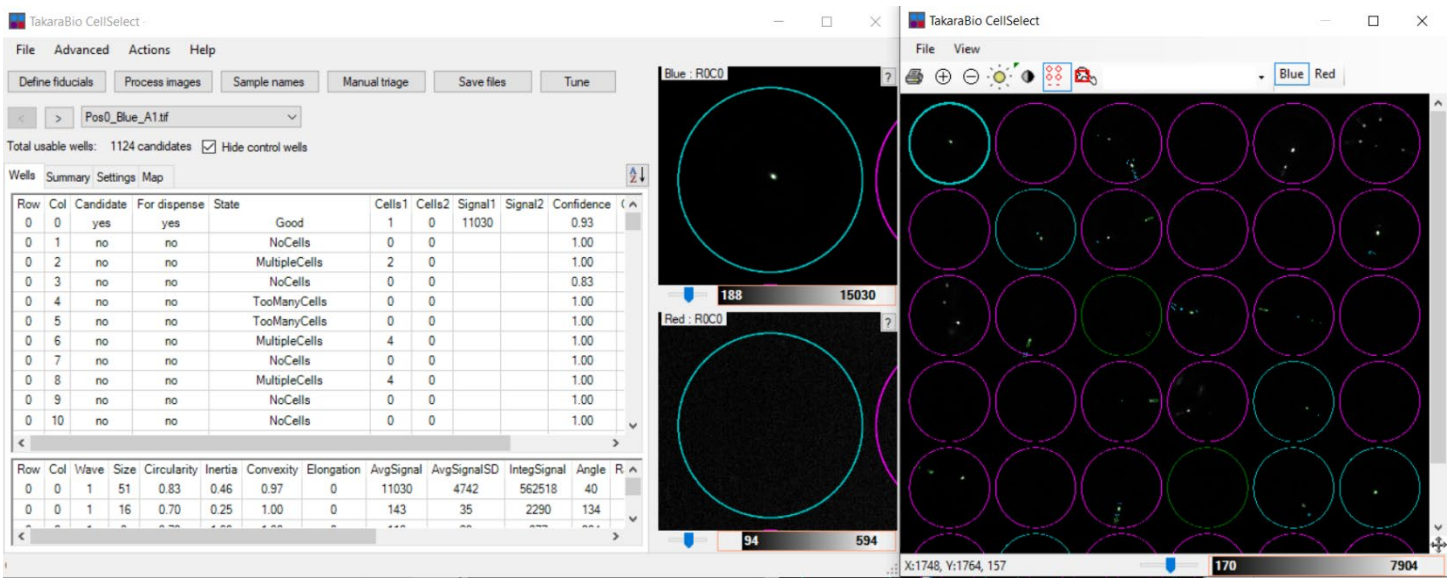


Figure 29. Opening and arranging windows for optimal image viewing. The image on the right is the multiwell image in Image Viewer. The images in the center show a single cell in a nanowell stained by Blue dye (top) and the absence of Red stain in the corresponding position (bottom).

3. Use the tools in the *Image Viewer* toolbar to critically examine the cells (see Appendix B.F 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** (see Figure 30, below). 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 noncandidate 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.

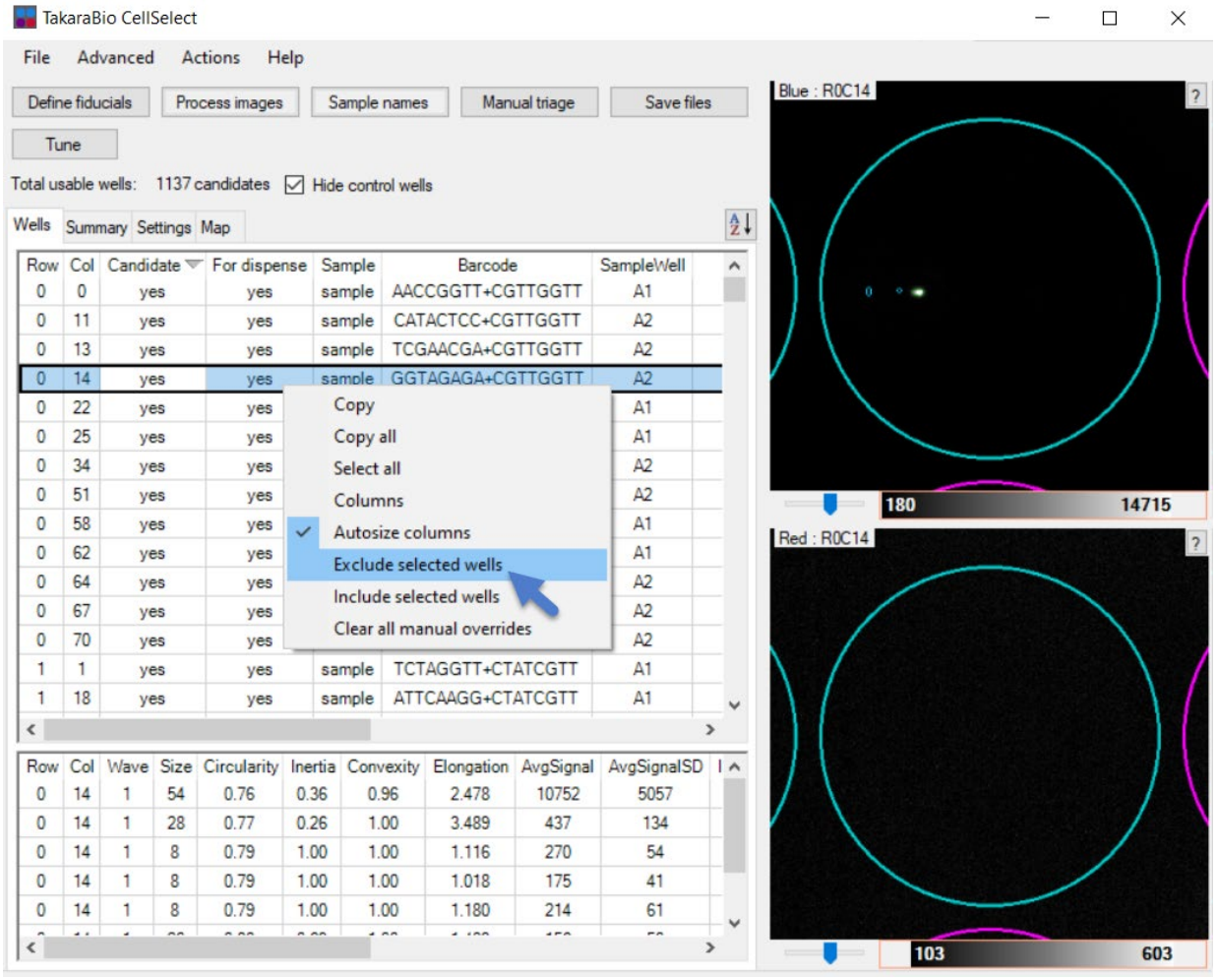


Figure 30. Excluding candidate wells.

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 contents.

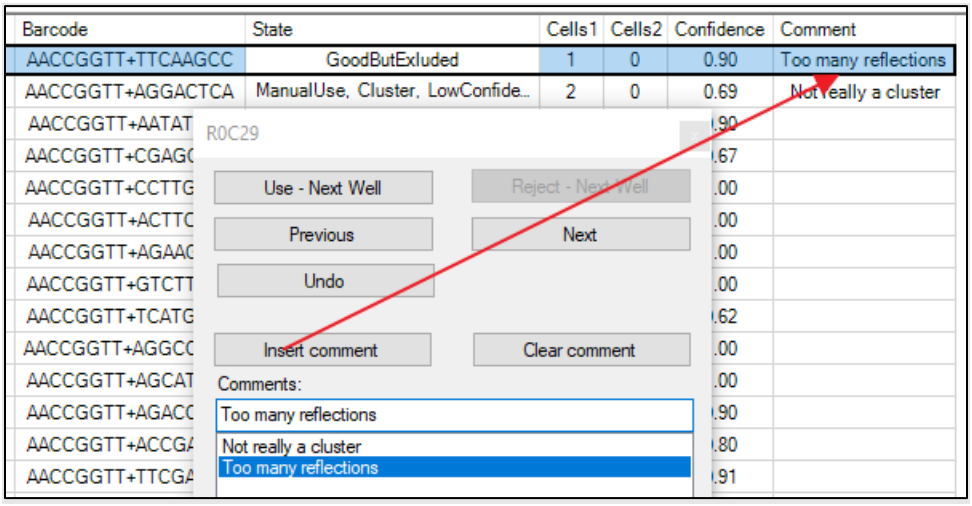


Figure 31. Adding comments to selected nanowells.

Best practice recommendation

Although for some users and in some instances, triaging every nanowell on the ICELL8 cx chip may be desired, others may feel comfortable simply doing a subset.

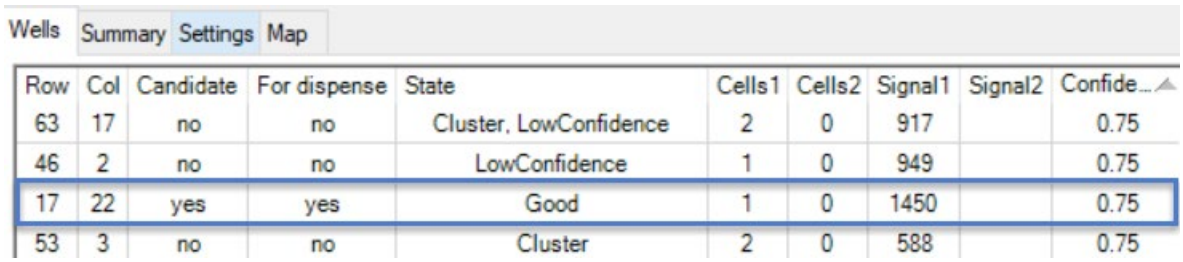
To find wells that may need manual triage:

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.G).
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 32).



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

The wells before and after the one identified in Figure 32 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 33 was encountered. Marked yes, but with a calculated Confidence (Appendix D.C) of 0.77, the image displayed for the blue channel (top) shows indications of being a multiplicate.

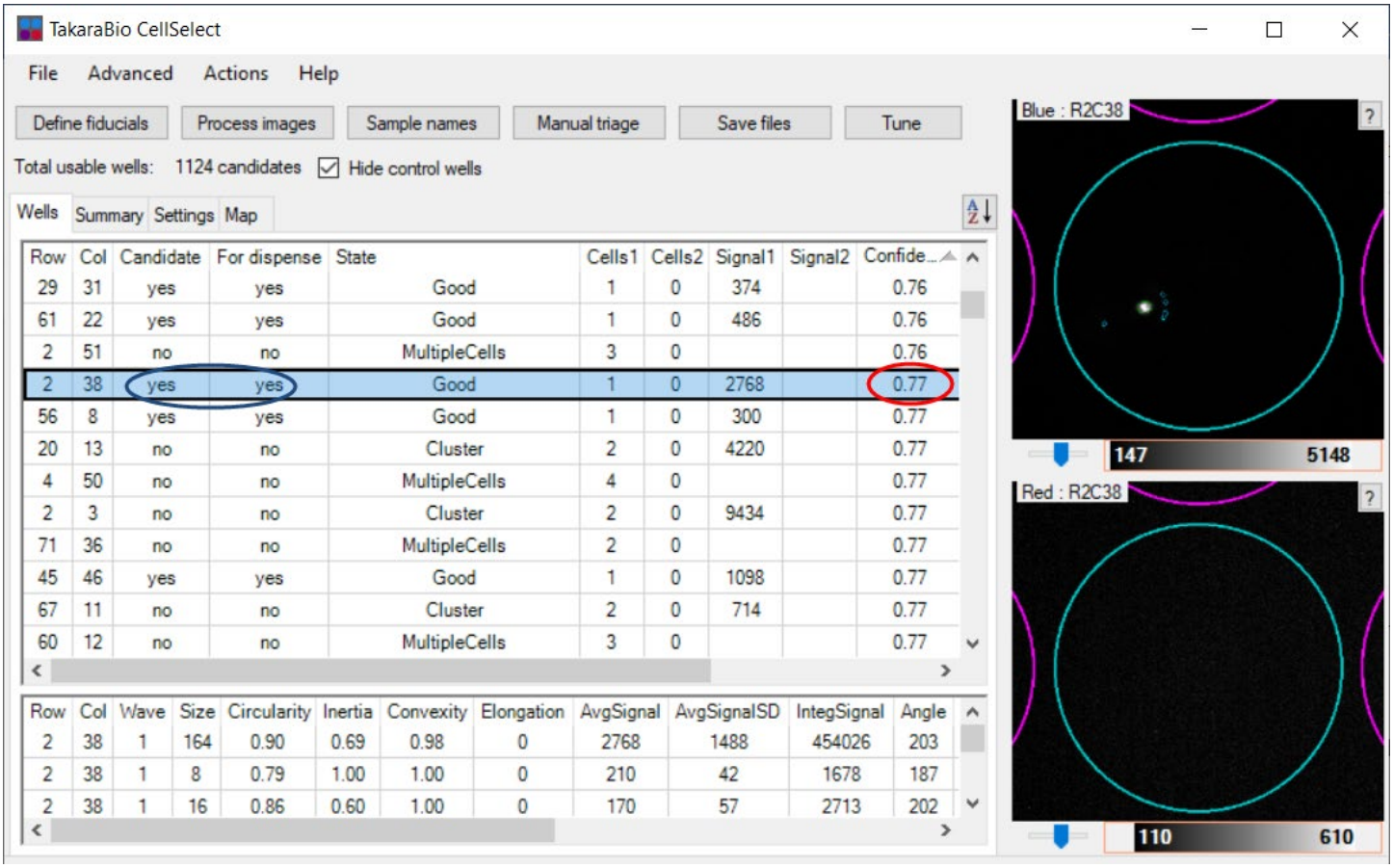


Figure 33. Example: low-confidence well marked as a candidate by the software that was rejected by the user. The image displayed for the blue channel (top) shows multiple objects, an indication that it might be a multiplicate.

In this case, the user decided to not dispense to this nanowell by clicking [Manual triage] > [Reject - Next well] and moved on. Figure 34 shows how the *Wells* information is displayed after this step was taken.

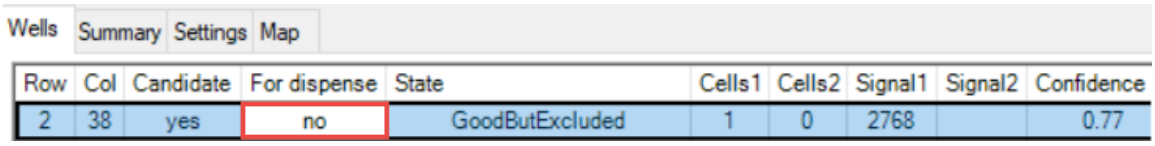


Figure 34. The well information from Figure 33 after the user rejected the well. The "For dispense" field is updated to 'no', meaning it will be excluded from reagent dispense at the next phase of the application workflow, and the "State" has changed to 'GoodButExcluded'.

H. Downselect (optional)

Downselect is used when fewer candidates than possible are selected. This may be done by the user in order to save reagents or to increase the downstream sequencing depth for each individual sample.

Downselection is required when barcodes are duplicated. If you have replicate barcodes, the system may not always be able to match the full number of requested nanowells without selecting duplicate barcodes. If there are repeated barcodes, the software will prompt the user to downselect such that barcodes are unique. The software will automatically pick one candidate per barcode and will try to pick the best candidate based on the *Settings* parameters. In that case, 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**.

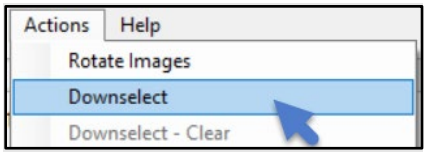


Figure 35. Accessing the Downselect menu item.

2. Enter the desired number of nanowells for controls and sample wells.

> **NOTE:** You can view the number of controls and samples in the *Summary* tab. However, if you enter a number much 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).

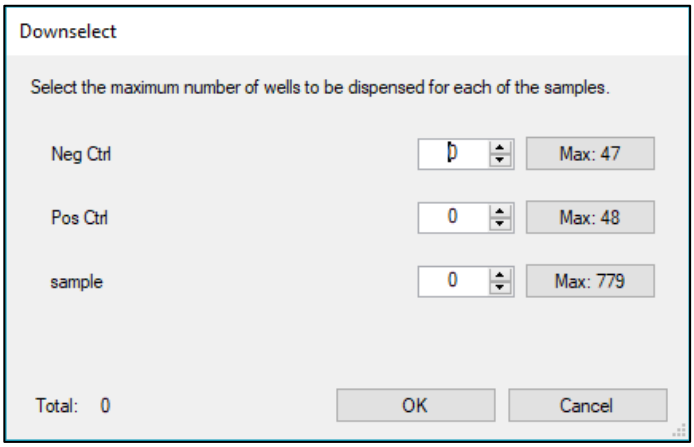


Figure 36. Enter the number of nanowells for controls and samples.

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

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.

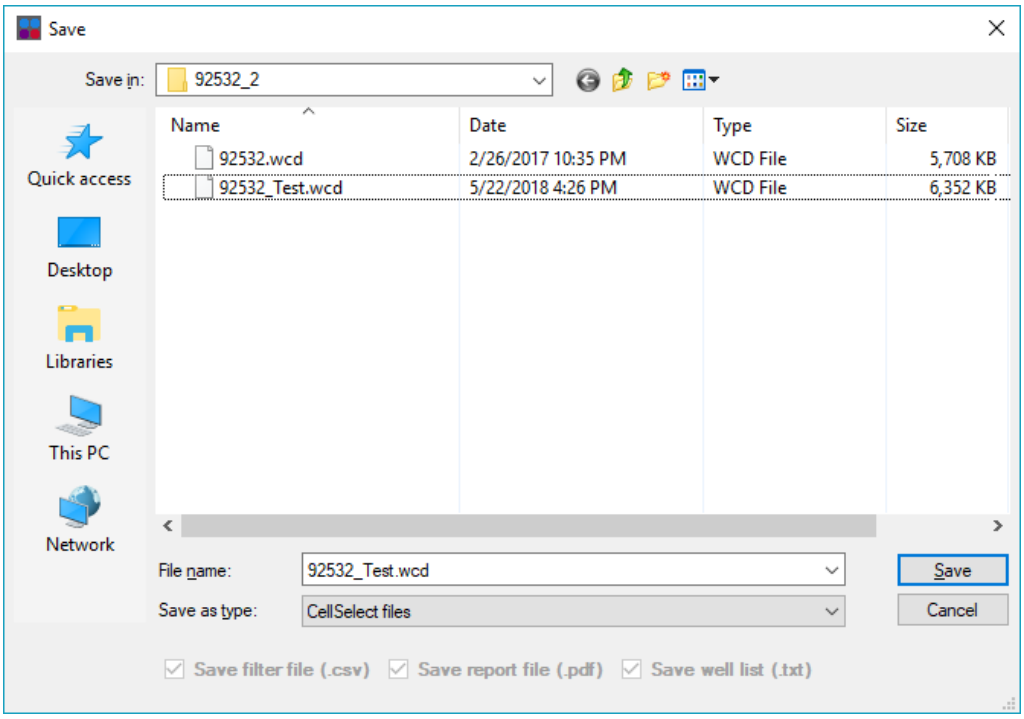


Figure 37. Saving the results file.

J. Tune

Tune can be useful for unique staining protocols or experiments with certain cell types. By default, CellSelect software is programmed with default settings baselined from a combination of K562 or NIH3T3 cell lines stained with Hoechst 33342 and propidium iodide (the procedure described in the ICELL8 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.

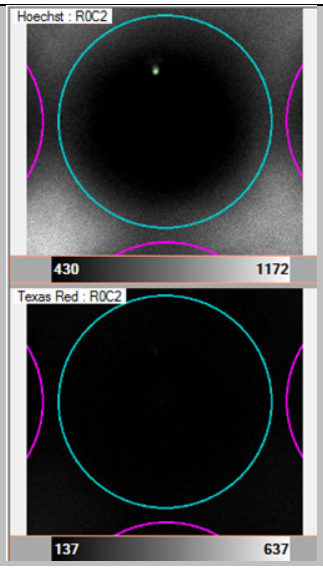
For details and the procedure to use this function, please see Appendix F.

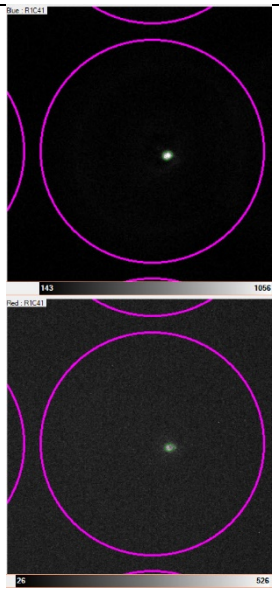
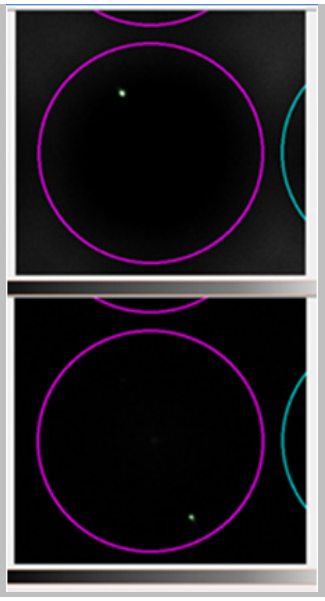
Appendix A: Interpreting the "State" value in the *Wells* tab

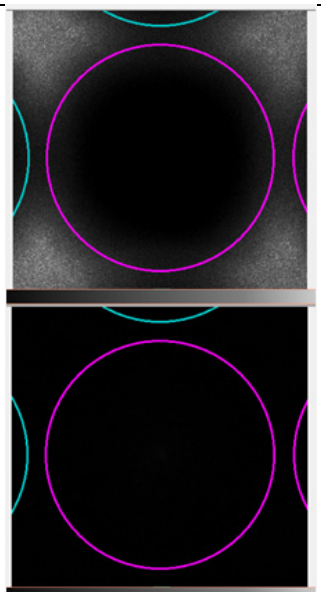
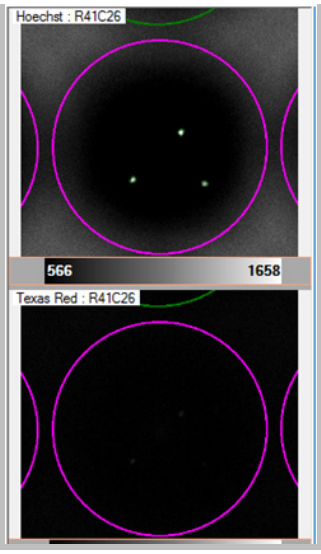
The following parameters apply to the images in Table 2, below. For more information about the "State" parameter, see Table 11 in Appendix E.A.

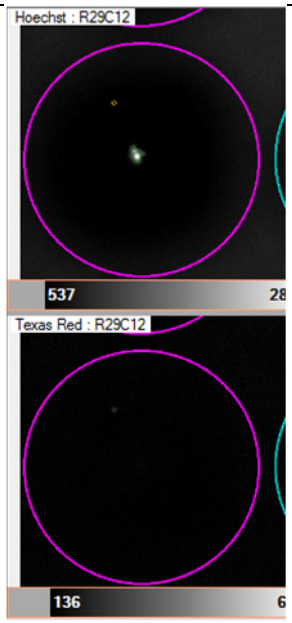
- **Top image:** Blue staining (Hoechst) indicates the presence of a live cell, provided that the object meets established size and shape parameters and does not appear in the bottom image
- **Bottom image:** Red staining (propidium iodide) indicates the presence of a dead cell, a well bottom, or an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

Table 2. Nanowell state descriptions and sample images

State	Conditions (all conditions must be met)	Image example
Good	Single cell that meets the designated parameters of appearing in the top image (Master channel), but not in the bottom image (Dye2 channel)	

State	Conditions (all conditions must be met)	Image example
FailsLogic	Cell appears visible in top image (Master channel) but also has a signal in the bottom image (Dye2 channel)—this combination causes it to not pass the colocalization condition	
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	

State	Conditions (all conditions must be met)	Image example
NoCells	No cells present in either view	
MultipleCells	Multiple cells appear in the top image; no cells appear in the bottom image	

State	Conditions (all conditions must be met)	Image example
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 B: 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.

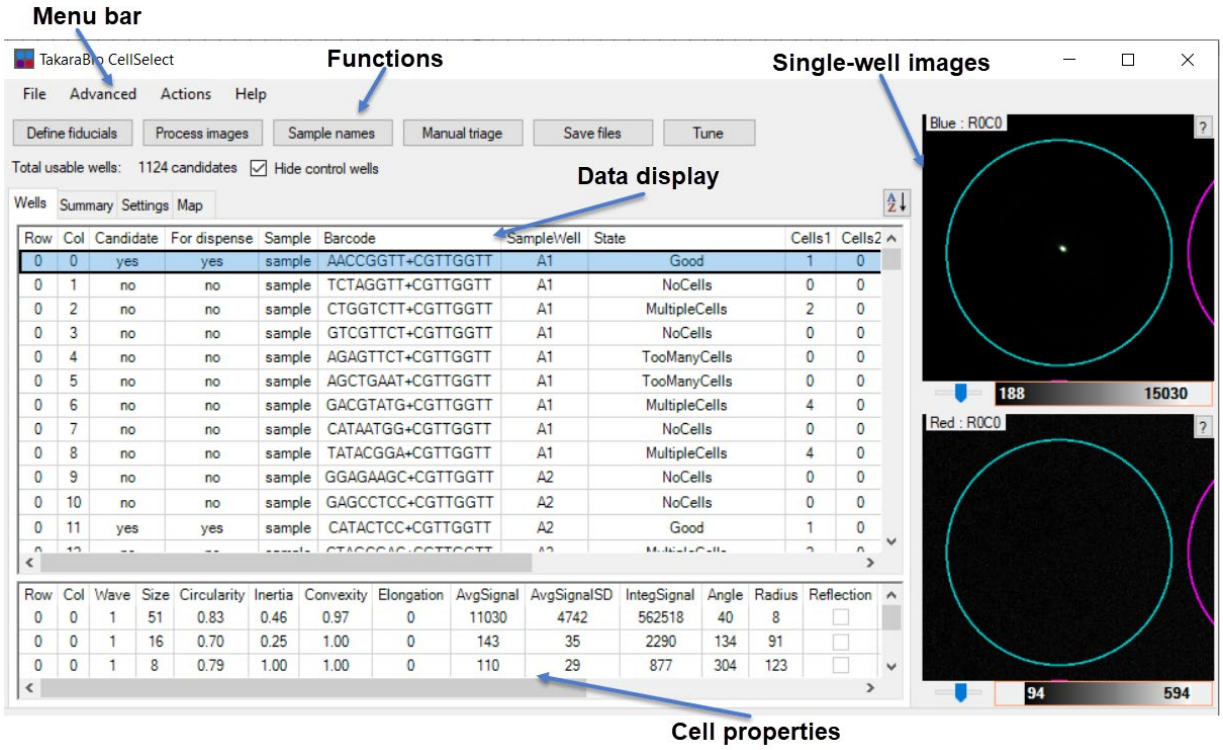


Figure 38. *Main* window.

B. Navigation tips

CellSelect Software uses tools and actions in its graphical user interface that are familiar to most users, thus 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 39 (below), the example menu shown will pop up when right-clicking while hovering over any of the cells with data values.

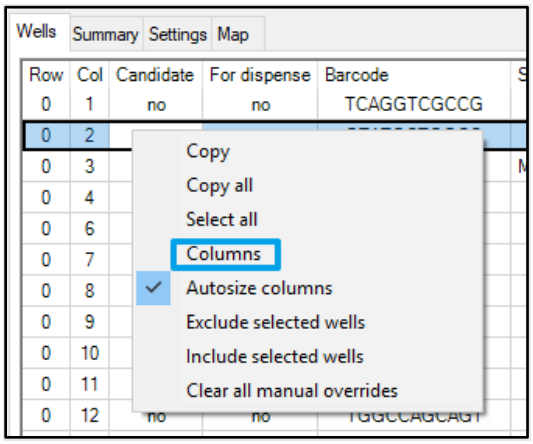


Figure 39. Example right-click menu.

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** (see Figure 39, above). This action will open a *Selected fields* window with checkboxes next to the information categories (see Figure 40, below). 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.

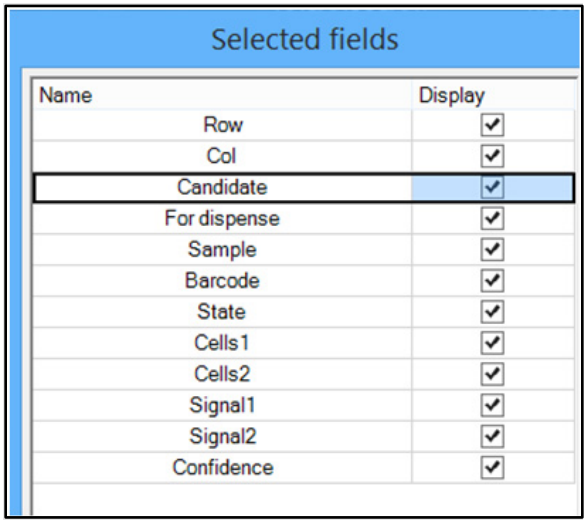


Figure 40. Column heading options.

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 (see Figure 41, below).

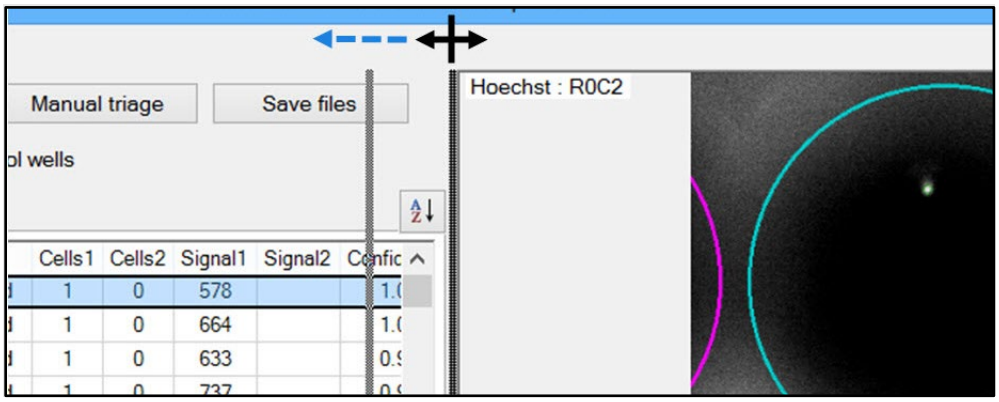


Figure 41. Adjusting the width of the *Image Viewer* window.

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 3. 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 will automatically adjust 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 B.F), you can press the **[Ctrl]** key on the keyboard while moving the scroll wheel to zoom.

C. Menu Bar

Table 4. ***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	Displays additional technical attributes of each well.
	Admin Mode…	Password protected. This section is for use in troubleshooting by TBUSA FAS and/or Technical Support.
	Image Viewer…	Adjusts image size, brightness, contrast, and well overlay. See Appendix B.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 technical support before taking this action.)
	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).
	3D – stack	Opens a 3-D viewer to inspect images from individual z-planes (Appendix B.G).
	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.
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

CellSelect Software analyzes images of cells taken with any combination of two of the three possible filters: Blue, Red, and Green. For example, the Blue filter can be used to detect the presence of cells (Master channel, e.g., stained with Hoechst 33342), while the Red filter (Dye2) can be used to detect dead cells (e.g., stained with propidium iodide). In this example, if a single cell is visible in the Blue filter but

not in the Red filter AND meets all the other criteria defined in the "Candidate selection logic" criteria under the *Settings* tab (Section II.C), the cell is considered a good candidate.

To make wells containing candidate cells easier to spot, the software overlays these wells with a teal-colored circle in both the single- and multiwell images. Wells containing noncandidate cells (such as dead cells, see the Table 2 in Appendix A) 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 42, below).

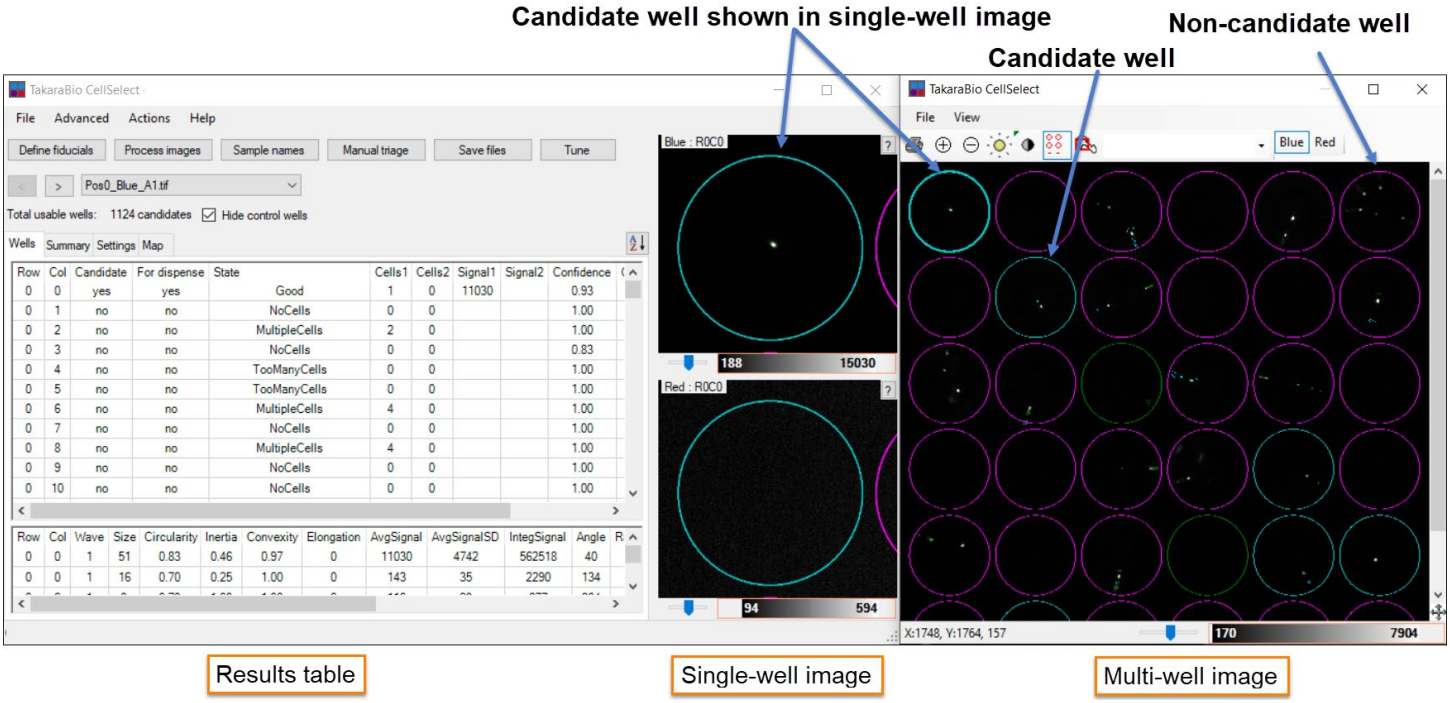


Figure 42. Understanding nanowell color codes. The differing brightness of the selected well (first column, first well down) can be noticed when compared to the nonselected candidate well down one row and one column to the right of it.

If you look closely at the cells, you will also see other color indicators:

- **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 rare reflection artifact

To understand how the software determines the status of each nanowell, refer to Table 2 in Appendix A.

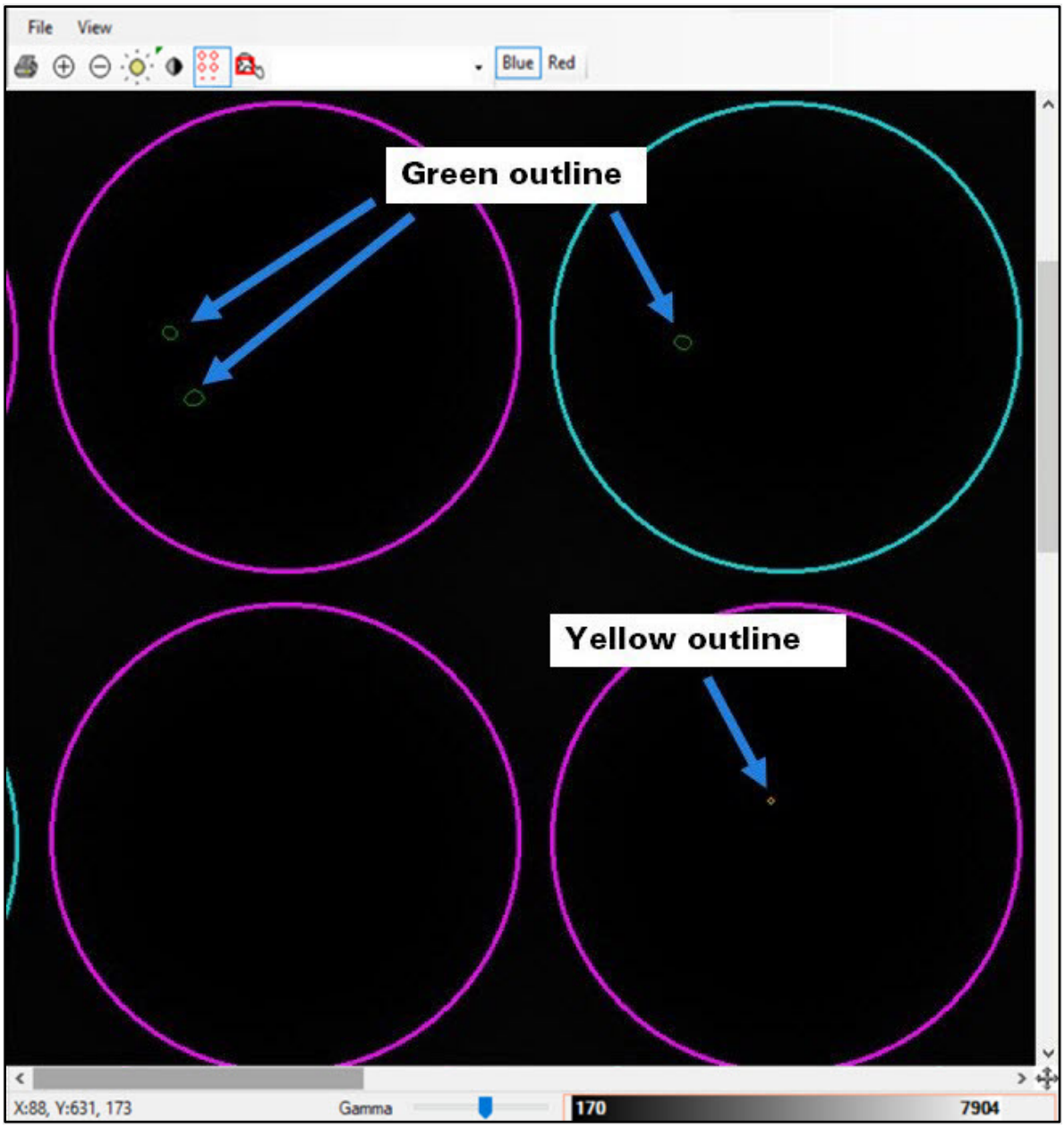


Figure 43. Closeup of wells, showing cell outlines. Green outlines are highlighted in the top wells, while a yellow outline is exampled in the well on the bottom right.

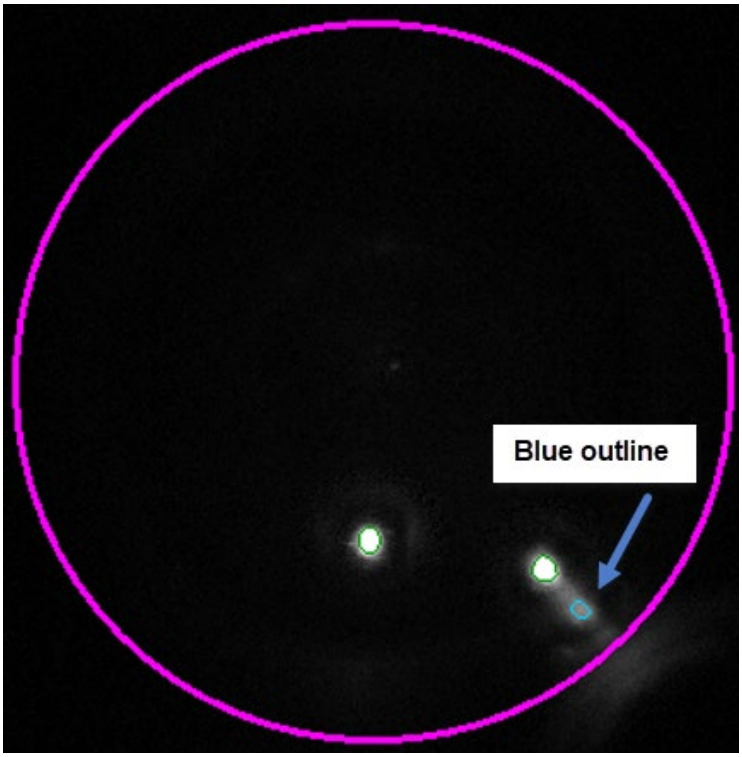


Figure 44. Closeup of single well, showing cell outlines. A blue outline is exampled in this image.

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 were scanned with the Blue and Red channels, two images will be shown, with the Master dye 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.

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

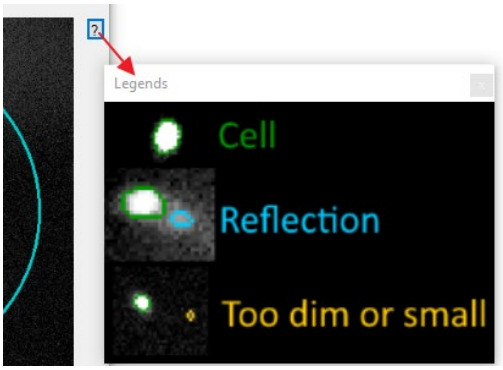


Figure 45. How to access the Legends window. Click on the [?] icon in the single-well image window to pop it up and help interpret the object outlines in image viewer. The green outline indicates the detection of a cell, light blue indicates a reflection that is not considered a cell but reduces the 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.H).

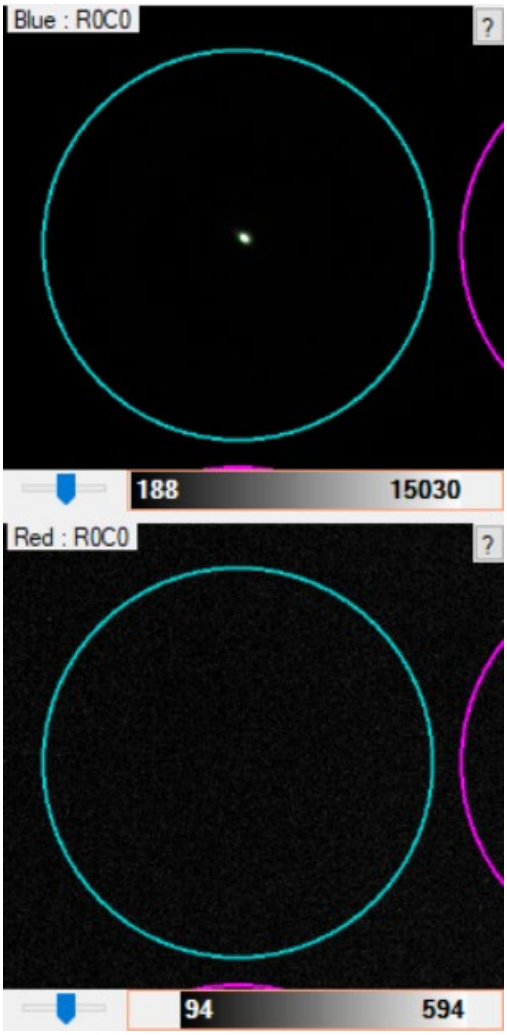


Figure 46. Single-well image from a two-color chip scan of cells stained with Hoechst (Blue channel) and propidium iodide (Red channel). The top image shows cells that have absorbed Hoechst, which indicates the presence of one or more live cells. The bottom image would show cells that have absorbed the propidium iodide, which occurs if a cell is dead; a live cell is not expected to absorb propidium iodide and, therefore, should not appear in the bottom image. In this example, the well matches the designated candidate logic selection parameters (see Section II.C) 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 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).

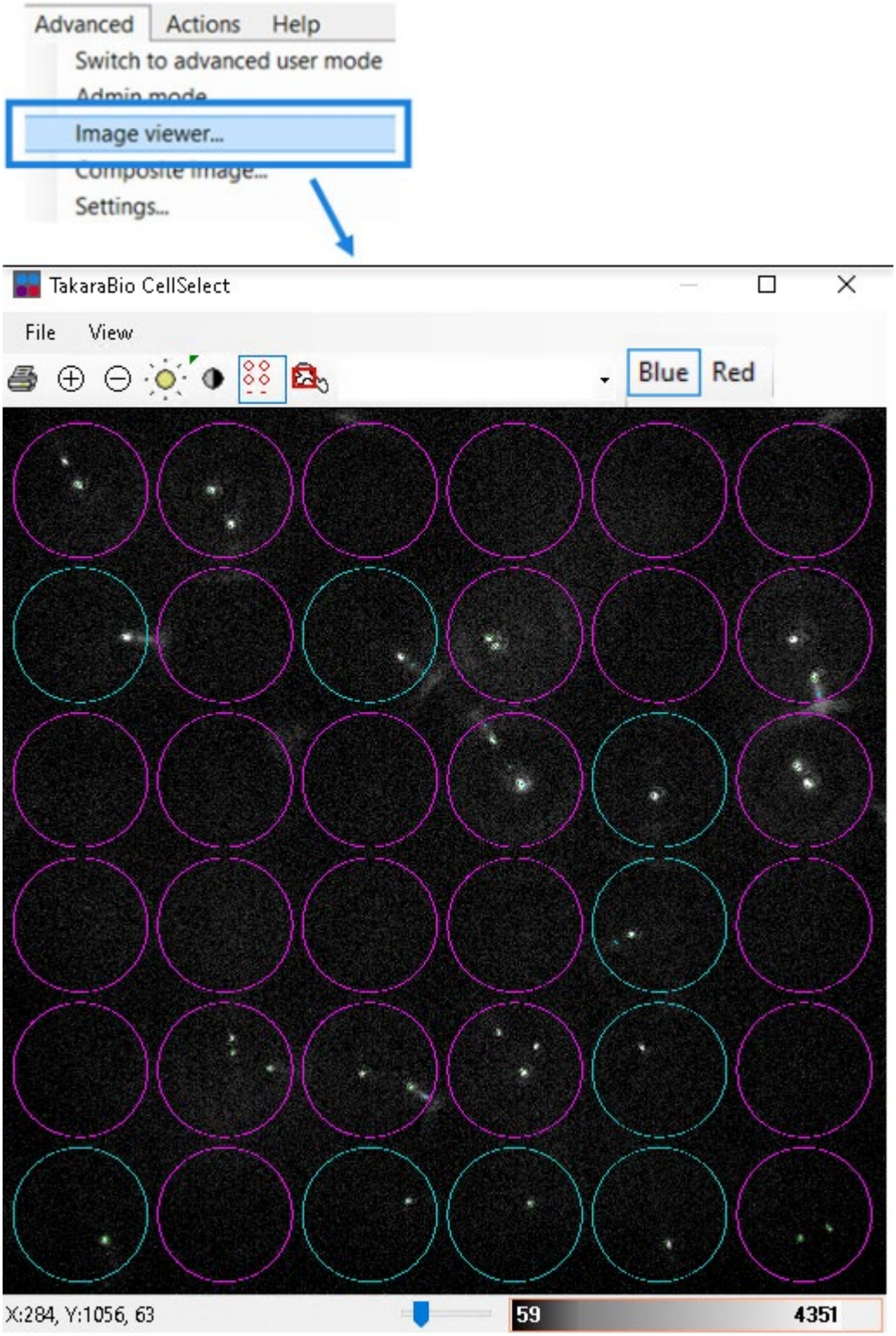


Figure 47. *Image Viewer* window.

Image Viewer Toolbar Icons

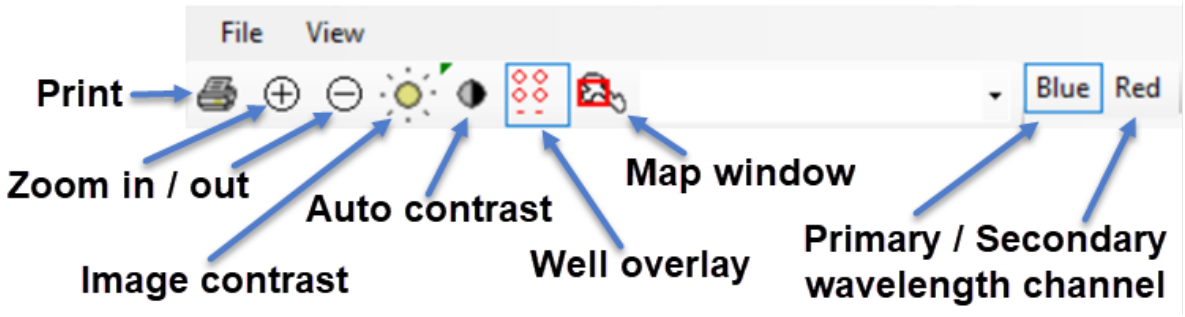


Figure 48. *Image Viewer* toolbar icons.

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 ICELL8 cx instrument 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 (see Figure 49, below). 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.

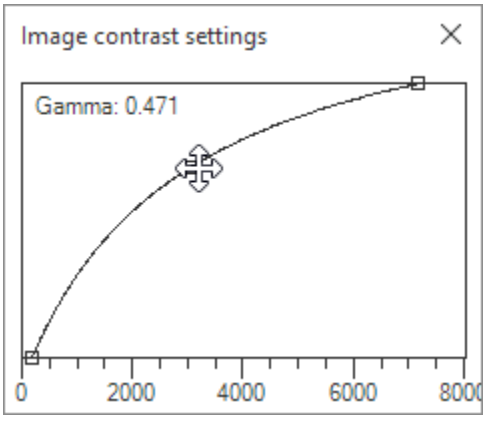


Figure 49. *Image contrast settings* window.

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

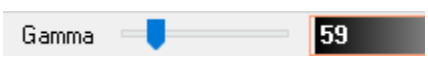


Figure 50. Gamma trackbar slider on the *Image Viewer*.

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 (see Figure 51, below). 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 50). 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.

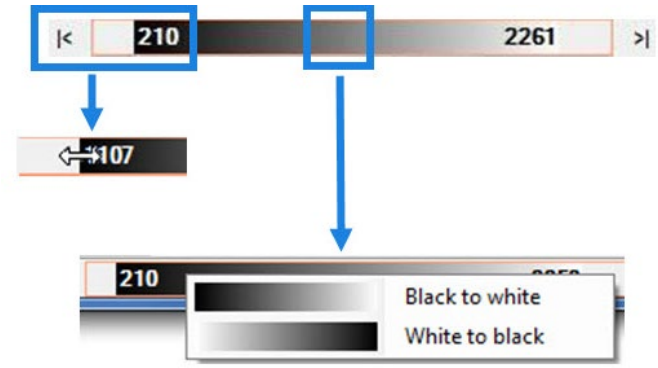


Figure 51. Adjusting image contrast using the slider.

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…**).

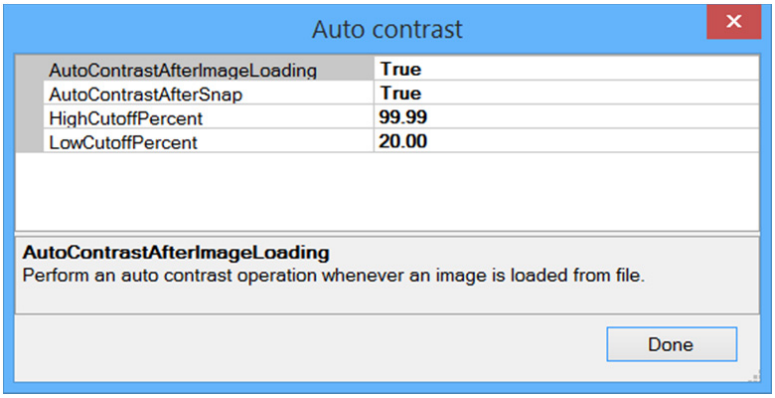


Figure 52. *Auto contrast settings* window. The description of the parameters are summarized in Table 5 (below).

Table 5. 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.

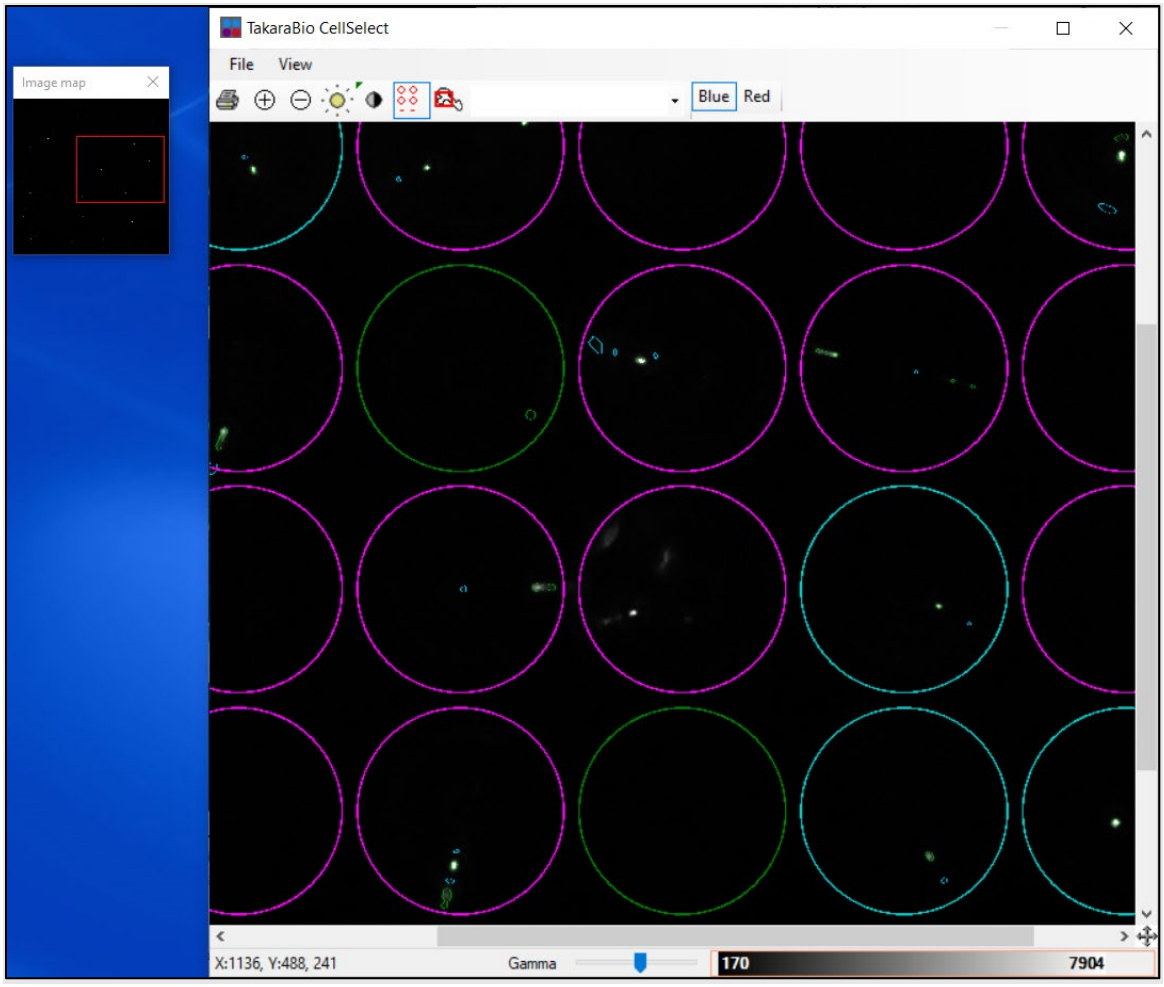


Figure 53. Viewing a subsection of an image using the Map window icon and *Image map* window. The red rectangle mentioned in the description can be seen in the small box in the upper left.

G. 3-D Stack Control

When scanning a chip, the ICELL8 cx system acquires images in different z-planes to capture cells that may not be at the bottom of the well. The plane labeled 'z1' starts at the bottom of the chip, with sequential z-planes moving up the well. The number of z-planes captured is dependent on the depth of the chip and automatically adjusted by the imaging function of CELLSTUDIO Software.

CELLSTUDIO Software combines (flattens) the images from the various z-planes to create a single image that will be further analyzed. If you wish to know the z-location of the cells, you may want to see the images at the various z-positions.

1. Enter the 3-D Stack Control from the **Action > 3D - stack** menu to review the images from the individual z-planes.

Figure 54. Actions menu location in the CellSelect user interface.

2. You will be prompted to specify the parent folder that contains the z-plane images subfolders. In most cases, this should be the chip folder.

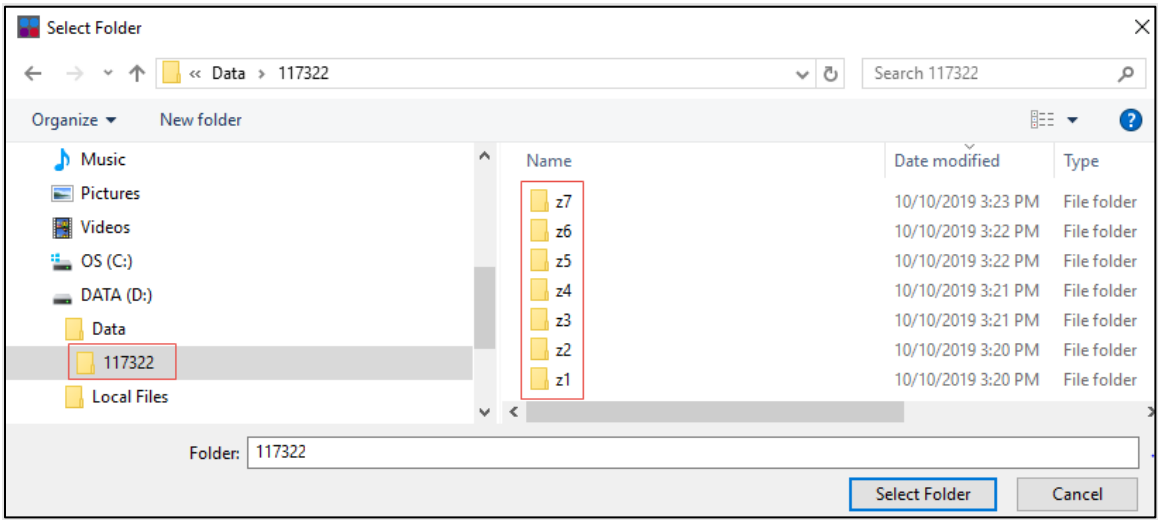


Figure 55. Example of the location of the z-plane images subfolders, shown in Windows Explorer. 117322 is the ID of the chip and, therefore, the name of the parent chip folder; folders `z1–z7` are the z-plane image subfolders.

3. Next, you will be asked to select the z-planes that you want to examine. It is recommended that you select all z-planes, but you can check or uncheck the folders to include and/or exclude certain folders, if needed (Figure 56).

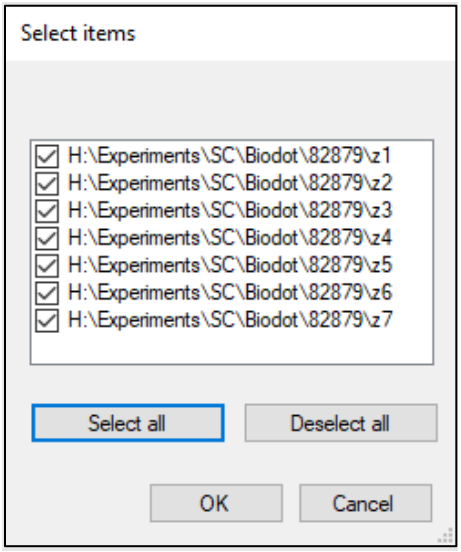


Figure 56. Selection window for inclusion/exclusion of the z-plane images subfolders.

4. Click [OK] to proceed; the *3D Stack Control* dialogue window will pop up.

3D Stack Control Dialogue Window

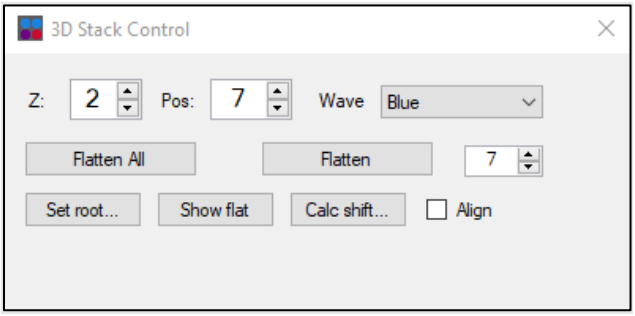


Figure 57. Close-up of the *3D Stack Control* dialogue window. The parameters are described in Table 6.

Table 6. *3D Stack Control* dialogue window options

Option	Function
Z	Value indicates which z-plane image file to display.
Pos	Specifies the position (i.e., well) image to display.
Wave	View the Master or Dye2 channels, “W1” or “W2”.
Flatten All	Flattens all images and save the resulting images in the parent (root) folder. **IMPORTANT:** Existing images in the root folder will be overwritten.
Flatten	Flattens the z-plane images for the current position (“Pos”) and display the resulting image. On a full 72 x 72 scan, there is a total of 144 scan location and images per dye, so “Pos” is a number from 0 to 143. See Figure 58.
Set root…	Reloads the root (parent) folder and returns to the z-folder selection screen (step 3, above).
Show flat	Shows the flattened image that exists in the root folder.
Calc shift…	See the “Image shift” section, below.
Align	See the “Image shift” section, below.

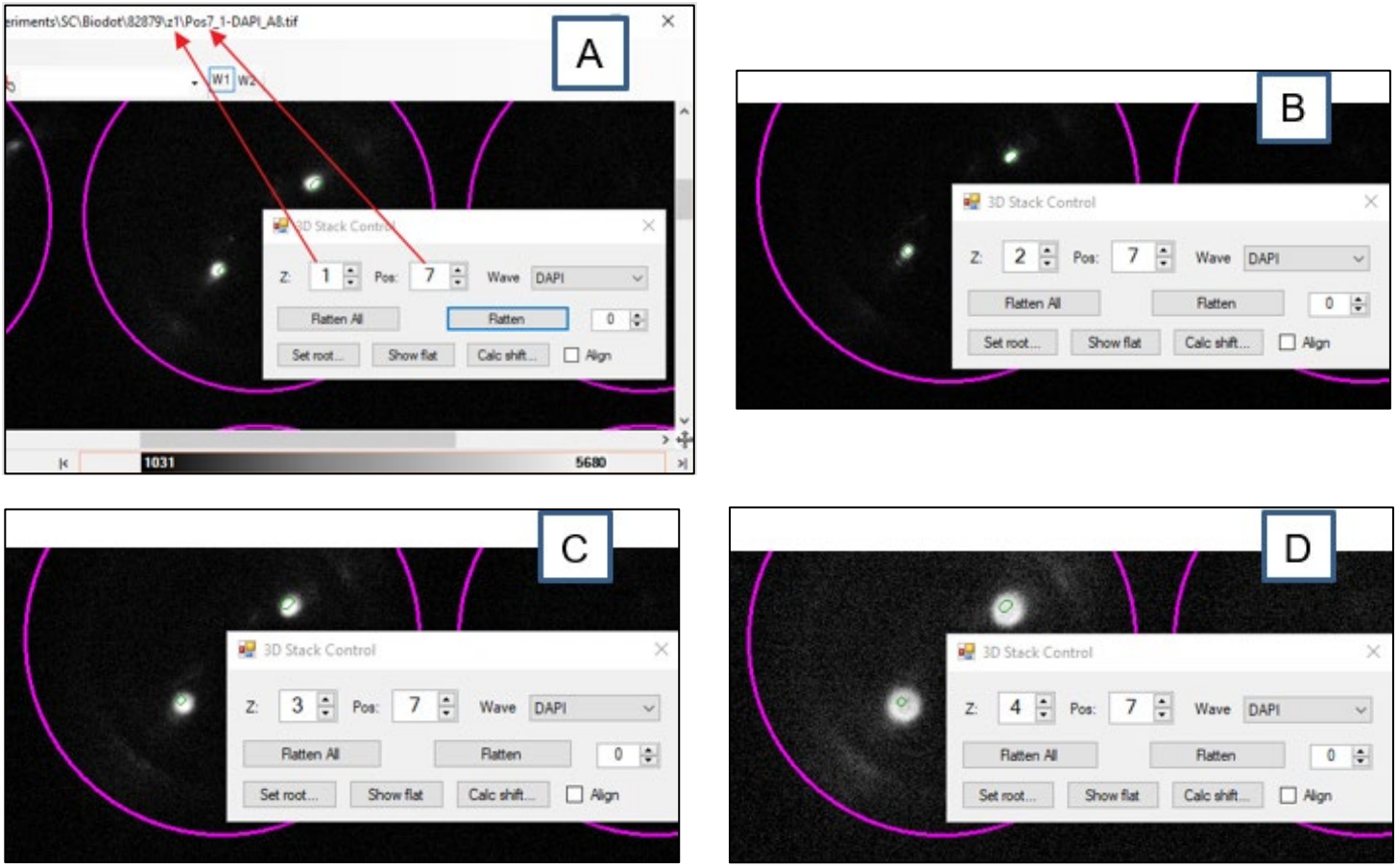


Figure 58. Example of viewing the same well through its z-planes. From A–D, the image cycles through z-planes 1–4, showing a different view at each layer.

Image shift

A problem sometimes occurs that, due to vibrations, individual images in a z-stack are not perfectly aligned, but instead are shifted by one or more pixels. On rare occasions, when a single cell is equally in focus in two z-planes, but it is shifted by a large amount on the x- and y-axes, the resulting flat image would appear to contain two separate cells.

The [Calc shift…] button in the *3D Stack Control* dialogue window initiates an alignment procedure between the image in the current z-plane and the one above it; the estimated shift between the two images is reported.

To fix the shift problem, the "Align*"* option corrects for the shift during flattening.

! **IMPORTANT:** If the "Align" button toggle is changed, you will need to click [Flatten All] to regenerate the flattened images.

NOTE: During a chip scan, the images are flattened by CELLSTUDIO Software, not CellSelect Software. Therefore, the "AlignImagesBeforeFlattening" option is present in CELLSTUDIO Software. For the SMART-Seq® full-length application, it is on ('True') by default. For the 3′ DE and TCR applications, it is set to off ('False').

If you've upgraded from a previous version of the CellSelect Software (pre-2.0), the option can be turned off (set 'False') to maintain consistency with previous scan results.

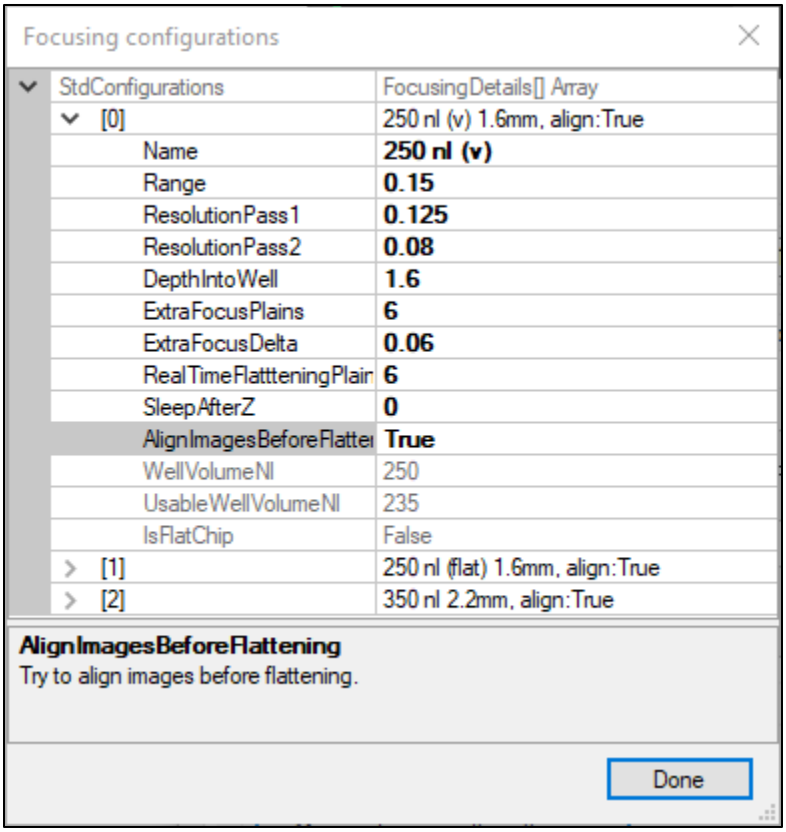


Figure 59. The "AlignImagesBeforeFlattening" focus option in CELLSTUDIO Software.

Appendix C: Comparing result files

With new assays, adjusting the analysis parameters in *Settings* will probably be necessary so that the algorithm correctly produces candidates. Often it is not easy to predict how a particular change in a parameter will affect the outcome across the entire chip—for example, lowering a detection threshold is expected to result in more cells being detected. If all newly detected objects are cells, this is a good change. However, if the threshold is too low, the system may end up classifying small debris or random image artifacts as cells, which is not desirable. The goal is to find settings that strike a good balance between false positives and false negatives.

To help researchers determine the optimal parameters, CellSelect Software includes a result file comparison feature. To use it, you would process the images with one set of parameters and save the result file (`*.wcd`).

1. Enter the Settings dialogue window in **Advanced > Settings**.
2. Modify one or more parameters.
3. Reanalyze the images.
4. Select **Actions > Compare with Existing Result File**.
5. In the *Open chip results* dialog window, select the previously saved `*.wcd` file.

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

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

In the two tables, many items are labeled A and B. ‘A’ refers to results from the run that is currently loaded, and ‘B’ refers to the results in the `*.wcd` file on disk.

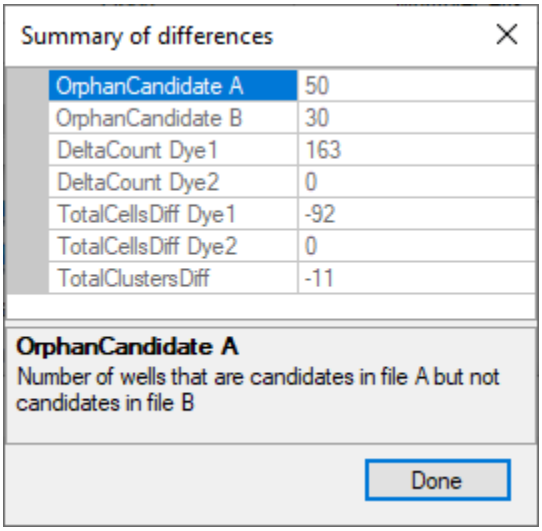


Figure 60. Example of the Summary of differences table. The descriptions of the parameters are described in Table 7.

Table 7. Description of the fields of the Summary of differences table.

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 (Master dye) are different
DeltaCount Dye2	Number of wells where cell counts for Dye2 are different
TotalCellsDiff Dye1	Difference in total number of cells for Dye1 (Master dye)
TotalCellsDiff Dye2	Difference in total number of cells for Dye2
TotalClustersDiff	Difference in total number of clusters

The example in Figure 60 is the result of only changing the Master dye Threshold from ‘40’ to ‘45’. The immediate effect is that the total number of cells for Dye1 (Master dye) 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 60, 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:

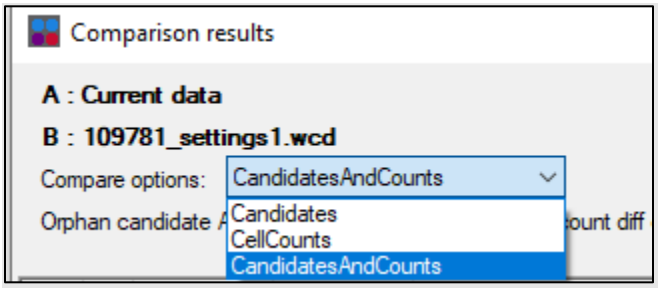


Figure 61. “Compare options” drop-down menu in the *Comparison results* window. The descriptions of the drop-down options are described in Table 8.

Table 8. 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 (Master dye) or for Dye2 is different
CandidatesAndCellCounts	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 62 (below).

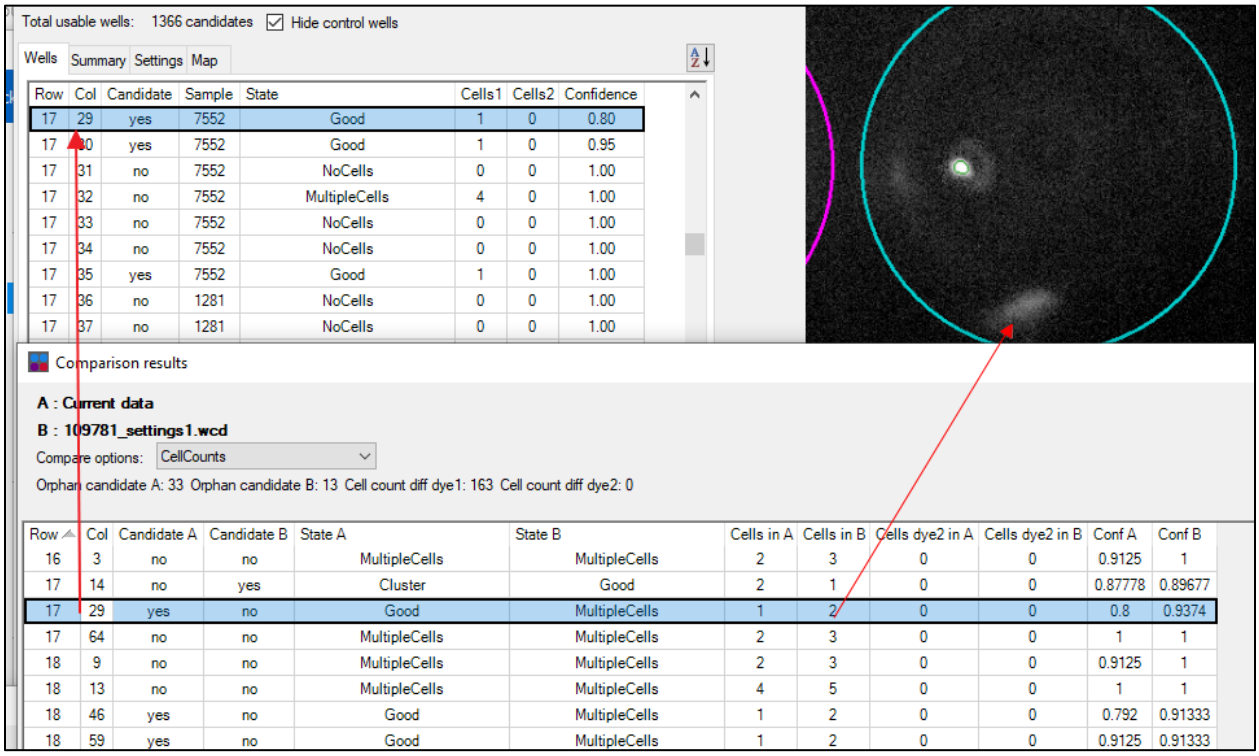


Figure 62. An example of the *Comparison results* window and how to read the information on it. The highlighted row in *Comparison results* corresponds to the highlighted row under the *Wells* tab. The arrow from the “Cells in B” column of the report relates to the dim image the arrow points to in the 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 the parameters some more.

A few things to keep in mind:

- Changing parameters will frequently produce better outcomes for some wells but worse for others. There usually is not one set of settings that works best in all situations.
- Sometimes, it is also difficult for the operator to decide which call is accurate. Sometimes, different operators 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 D: Advanced settings

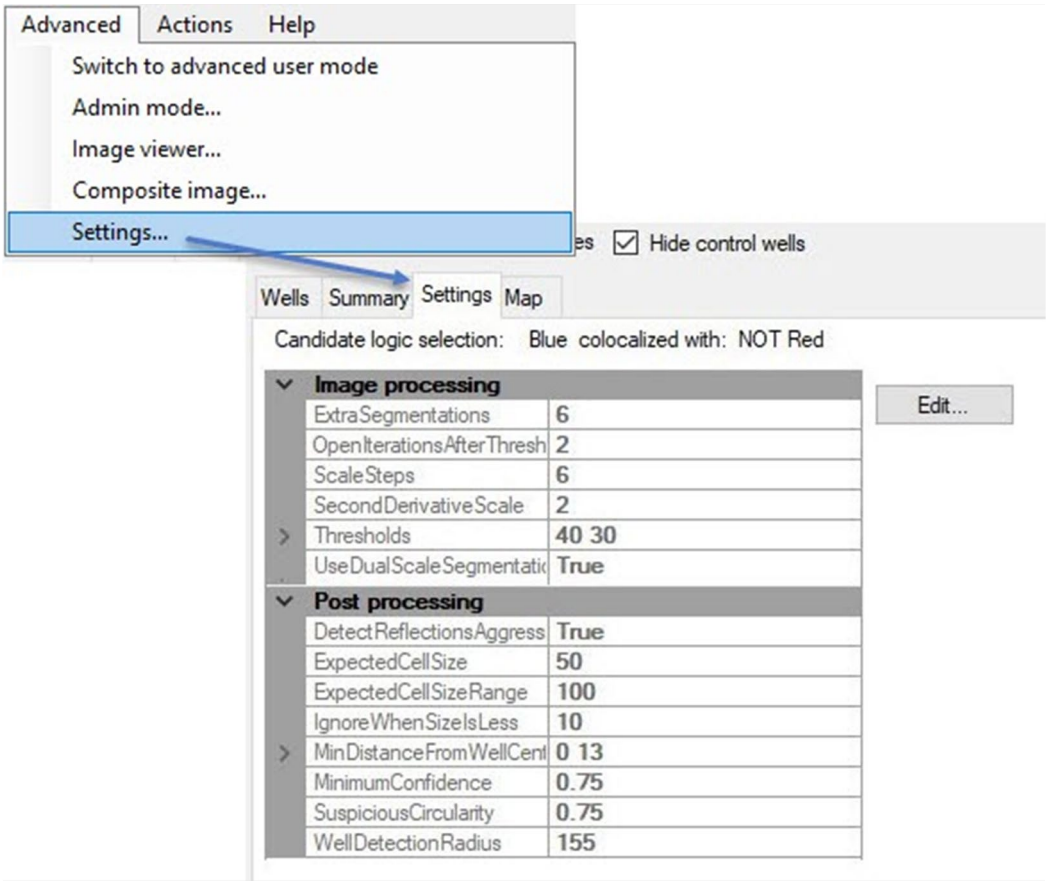


Figure 63. *Settings* tab.

The sections below discuss several of the options listed in the *Settings* tab. For each section, the section name corresponds to the setting option name. Section A describes how to restore all the settings to the defaults, if you need to override the customizations or start over.

A. Restore to Defaults

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

1. Select **File** > **Load…** (see Figure 64, below).
2. Select the appropriate XML file for the type of ICELL8 chip being used.

Table 9. Chip XML file selections.

Chip type	Cat. No.	XML file name
ICELL8 cx 3' DE Chip	640199	`AnalysisSetting_250nL_chip.xml`
ICELL8 cx TCR Chip	640200	`AnalysisSetting_250nL_chip.xml`
ICELL8 350v Chip	640019	`AnalysisSetting_350nL_chip.xml`

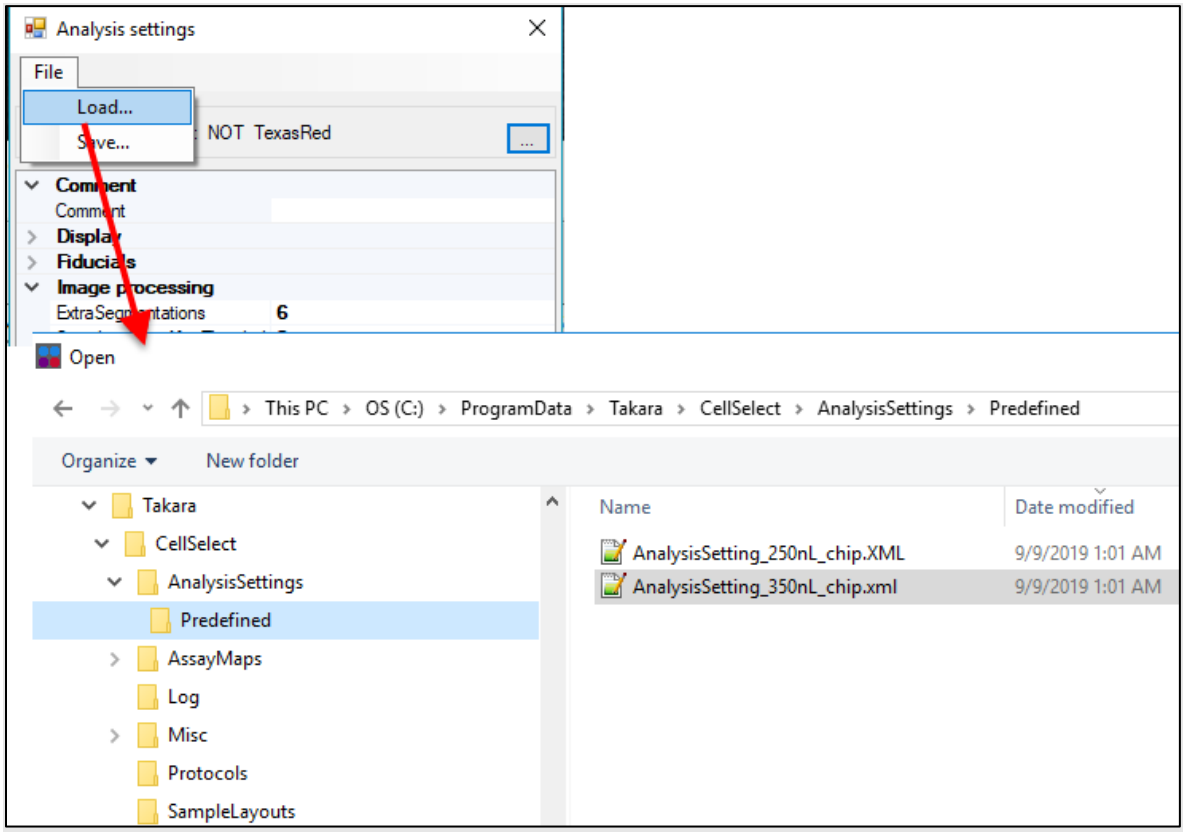


Figure 64. Loading/reloading the settings file for single-cell analysis.

B. Algorithm

There are two options for this setting, V1 or V2.

- The V2 algorithm is the algorithm used for new applications and during the tune process
- V1 is only used for 3′ DE and TCR applications on a 250 nl chip

C. Confidence

In order 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, e.g., no cells in the well, dead cells, or more than one cell.

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 *WellDetectionRadius* (Appendix D.K)

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).

D. ExpectedCellSize and ExpectedCellSizeRange

Default parameters were defined based on cultured cells. For cells that are larger or smaller than this, there are two parameters, “ExpectedCellSize” and “ExpectedCellSizeRange” that may be modified in order for your cells of interest to be considered.

“ExpectedCellSize” and all size measurements are in units of image pixels (area). One pixel has a width of 1.6 µm; however, cells shown in the image usually appear larger than the actual cells. If a cell of area ‘A’ pixels is round, the diameter ‘D’ of the cell (in micrometers) is determined by:

$$D = 1.6\sqrt{\frac{4A}{\pi}} \approx 1.8\sqrt{A}\ \mu m$$

Because of artifacts in fluorescence imaging and image flattening (Appendix B.G), as well as uncertainty in segmentation, the measured size might not accurately reflect the cell size.

“ExpectedCellSizeRange” is the ratio of maximum acceptable size over the minimum acceptable size.

$$Max = \text{ExpectedCellSize} * \sqrt{\text{ExpectedCellSizeRange}}$$

$$Min = \text{ExpectedCellSize} / \sqrt{\text{ExpectedCellSizeRange}}$$

When a cell’s size exceeds the Min / Max limits, the well confidence (Appendix D.C) will be reduced. Substantial reduction in confidence may result in a sample not being marked as a candidate.

E. IgnoreWhenSizeIsLess

Any artifact with a size less than this threshold value 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.

F. MinDistanceFromWellCenter

With v-bottom ICELL8 cx chips, debris sometimes accumulates at the bottom (center) of the wells. If the "Threshold" parameter (Appendix D.J) is lowered, the signal of this debris might be interpreted as a dim cell, although it does not impact confidence. If the value of "MinDistanceFromWellCenter" 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.

G. MinimumConfidence

If the confidence of a well falls below the threshold defined by this 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.

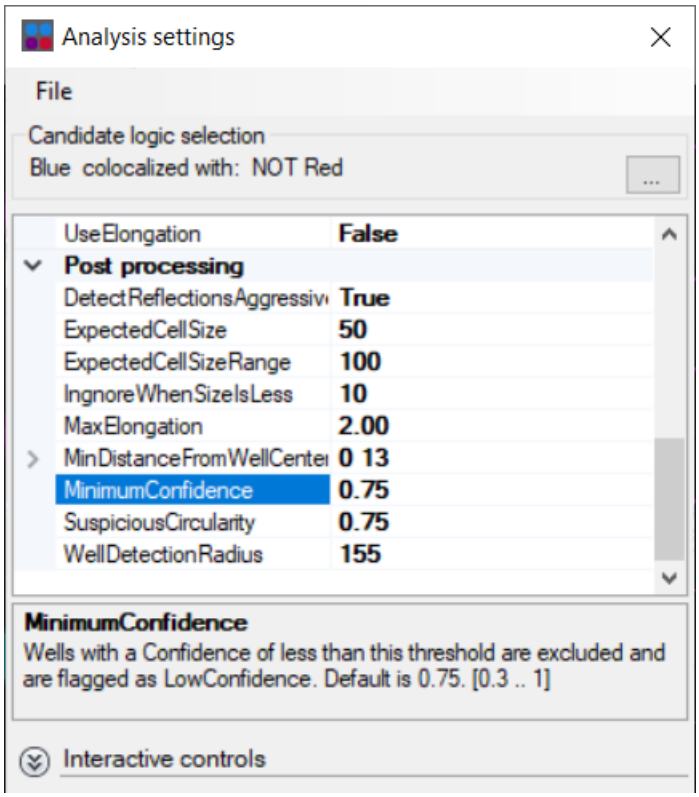


Figure 65. MinimumConfidence option in *Settings*.

H. PaintOutlines

In order to better see objects in single-well images (Appendix B.E), "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.

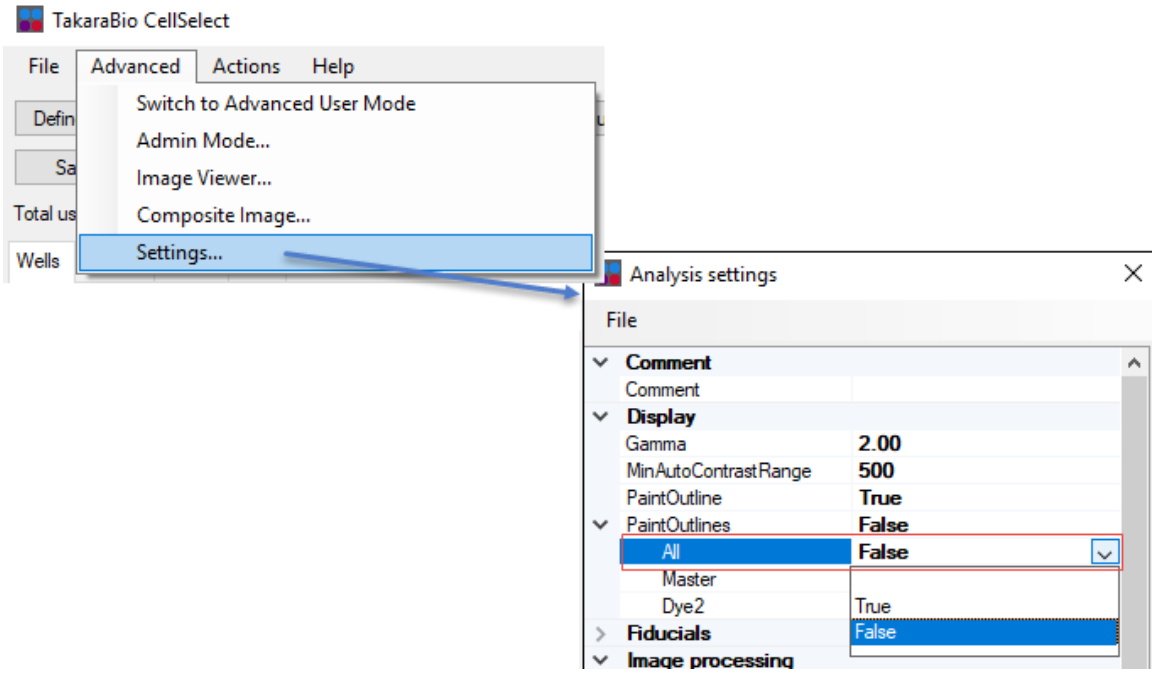


Figure 66. Accessing the "PaintOutlines" option in the *Analysis settings* window.

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 both.

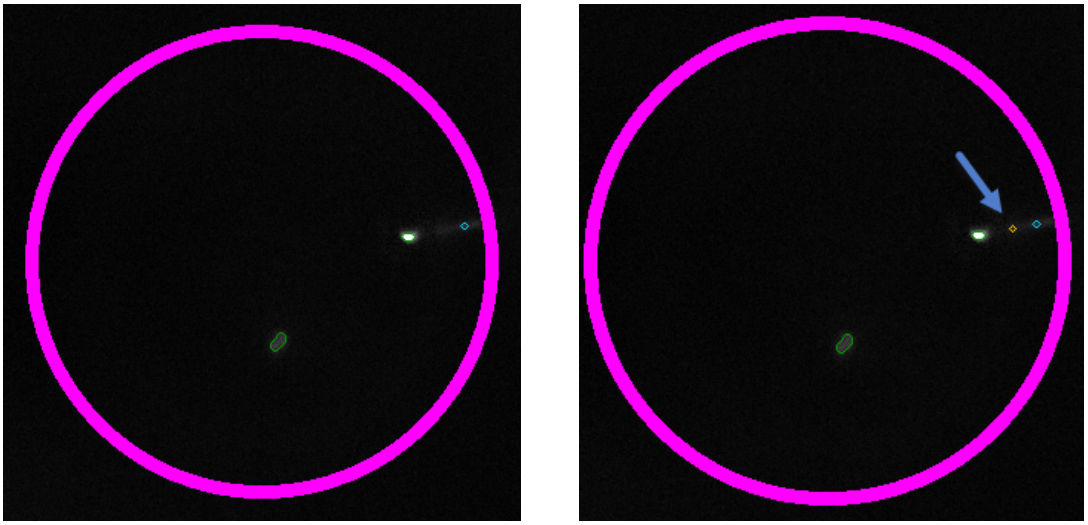


Figure 67. The same noncandidate well with "PaintOutlines" disabled and enabled. (Left) disabled is the default mode. **(Right)** enabled, a yellow-outlined object, highlighted by the arrow, displays.

I. 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.

J. Thresholds

Thresholds are the 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. You might want to adjust this value in cases where, for example, the cells didn't stain well or stained too well, or if there is high background brightness.

A lower threshold detects a larger number of cells; however, if the thresholds are too low, the software may identify wells as the 'TooManyCells' status and report a very low number of candidates.

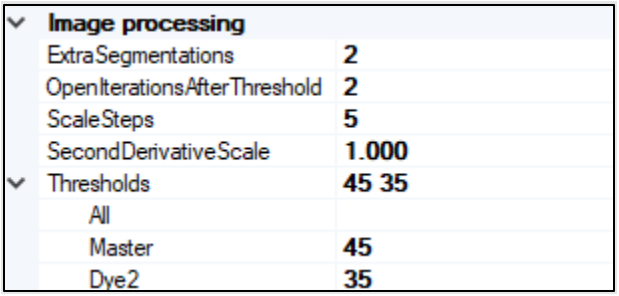


Figure 68. The "Thresholds" section under Advanced > Settings.

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

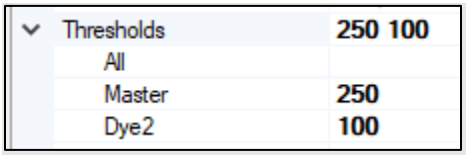


Figure 69. Expanded "Thresholds" option in the *Settings* window.

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

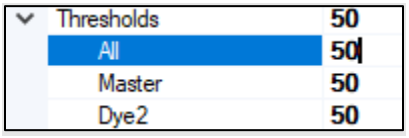


Figure 70. Selecting the *Settings* >"Thresholds" > "All" option to customize it.

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.

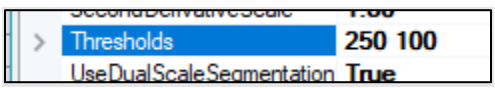


Figure 71. The *Settings* > "Threshold" section, displaying multiple sub-level values in the collapsed view.

K. WellDetectionRadius

This setting is the maximum distance of the well from the expected well center, measured in units of pixels (one pixel = 1.6 µm). Objects inside the circle defined by this radius setting are considered cells; outside objects are ignored.

L. ScaleSteps and SecondDerivativeScale (V2 algorithm only)

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.

The scale-space approach (https://en.wikipedia.org/wiki/Scale_space) 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:

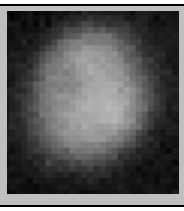
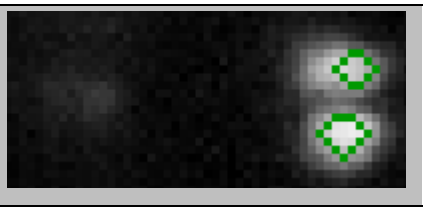
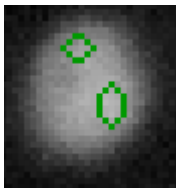
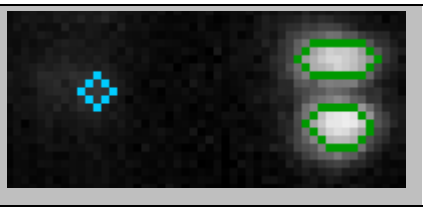
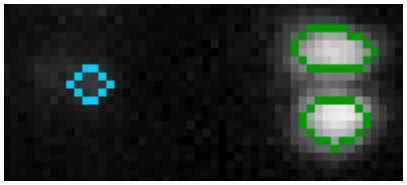
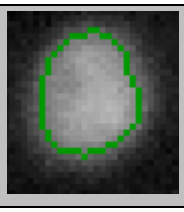
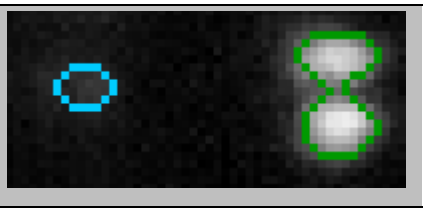
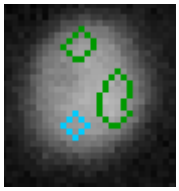
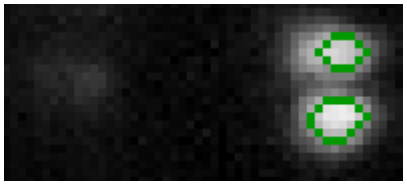
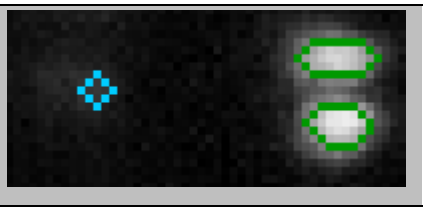
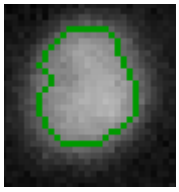
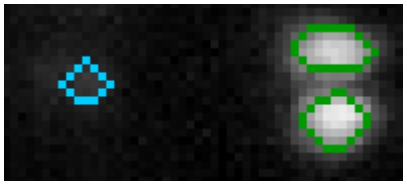
- Small values of "Scale" detect small objects, even when they are close together, but larger objects may be oversegmented
- Large values of "Scale" are better at detecting large objects, but they also tend to blur smaller objects together

Table 10 (below) shows the effect of changing the setting values for "ScaleSteps" and "SecondDerivativeScale".

Example

In the last row of Table 10, 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.

Table 10. Effects of changing ScaleSteps and SecondDerivatveScale. Objects outlined in blue are interpreted as a reflection.

Scale steps	SecondDerivativeScale	Example images		
1	0.75			
1	1.0			
1	1.5			
1	2			
1	2.5			
2	0.75			
3	0.75			
4	0.75			

M. Interactive controls: Fast Image Analysis

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

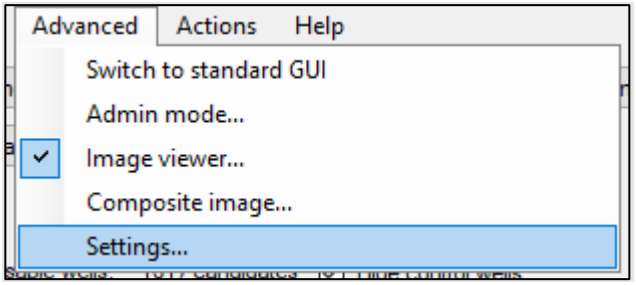


Figure 72. Selecting Settings… under the Advanced menu view.

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

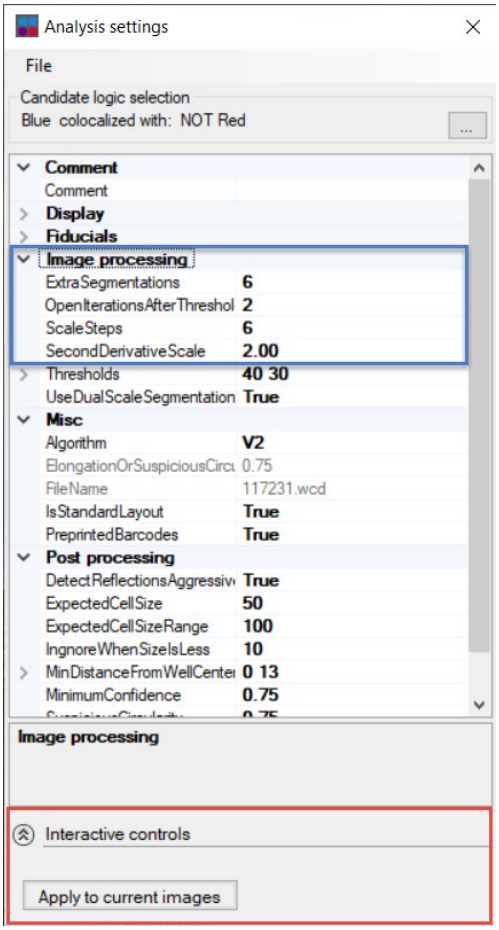


Figure 73. Example *Analysis settings* window. The interactive controls section is highlighted by the red rectangle and the Image processing section is outlined in blue.

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: User interface table column descriptions

A. Wells data table

Table 11. 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
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 12. Additional column names in the *Wells* tab when in Advanced User Mode (Section II.A)

Column name	Field description
Size1	Size of cell (area) in channel 1, in pixels.
Size2	Size of cell (area) in channel 2, in pixels.
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 has circularity 1.
Circularity2	A perfectly circular cell has circularity 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.

Column name	Field description
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 13. 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 (pixels)
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 "IgnoreWhenSizeIsLess" (Appendix D.E)
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 "MinDistanceFromWellCenter" (Appendix D.F)

C. Summary table

Table 14. 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 (Master dye)
Dye2 total	Total number of cells that were detected in channel 2 (Dye2)
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 cells	Number of wells that have 1 or more cells in channel 2
Mean	Estimated Poisson lambda parameter
R2	Comparison value of the observed sample distribution with the expected probability distribution (Poisson goodness of fit)

Appendix F: Automated threshold detection

A. Quick Start Guide

1. Open the Auto-tune Dialog

Open the desired dataset with the CellSelect application. Press the [Tune] button to access the Auto-tune dialog:

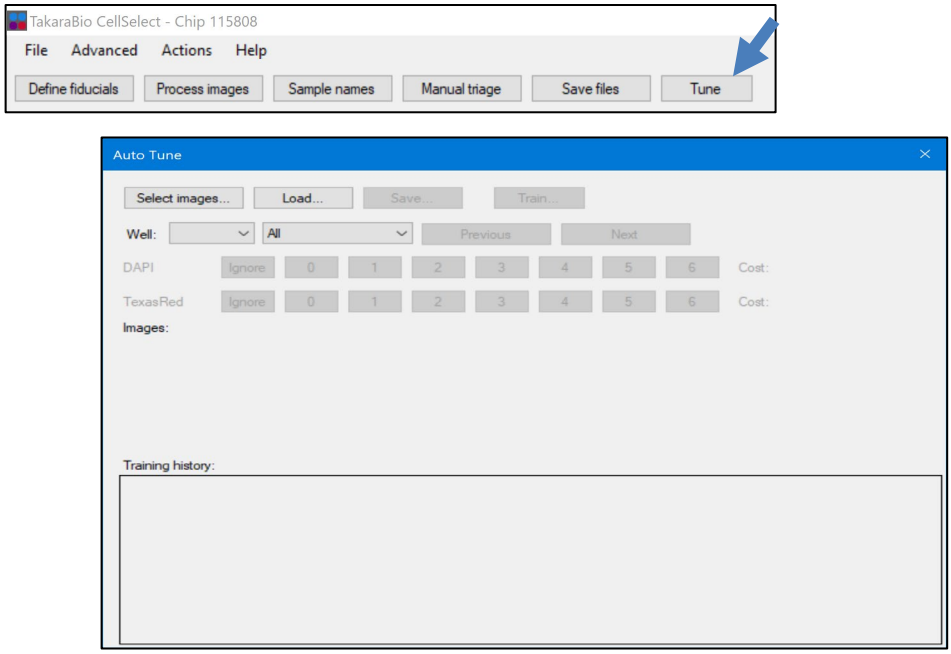


Figure 74. Auto-tune Dialog

2. Select Images (6x6 wells) for Training

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

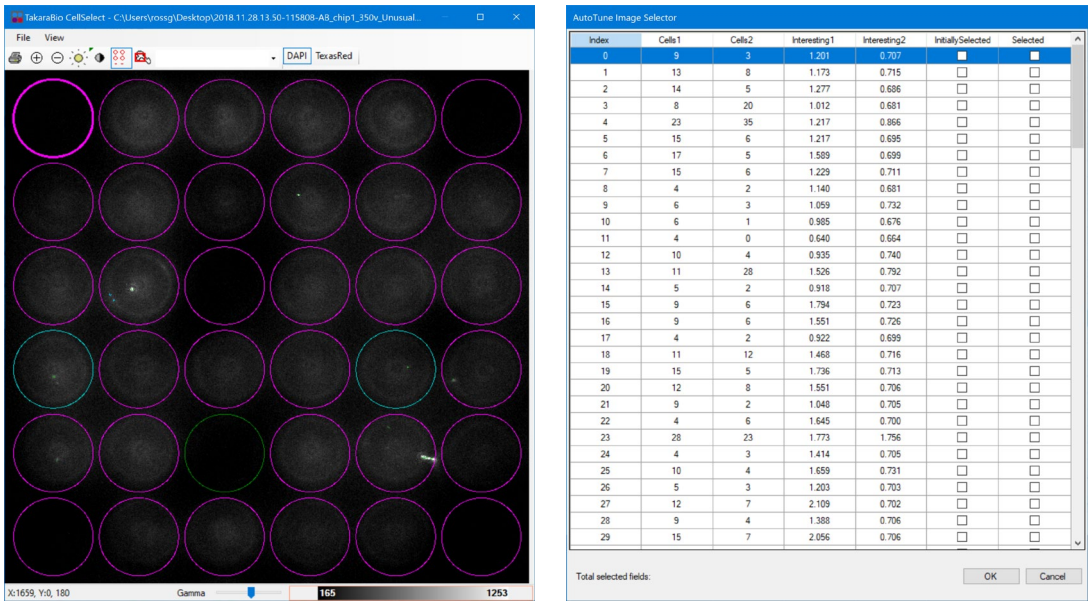


Figure 75. Image Viewer and Image Selector Dialogs

3. Choose five optimal images

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

AutoTune Image Selector

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	☐	☐

Figure 76. 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:

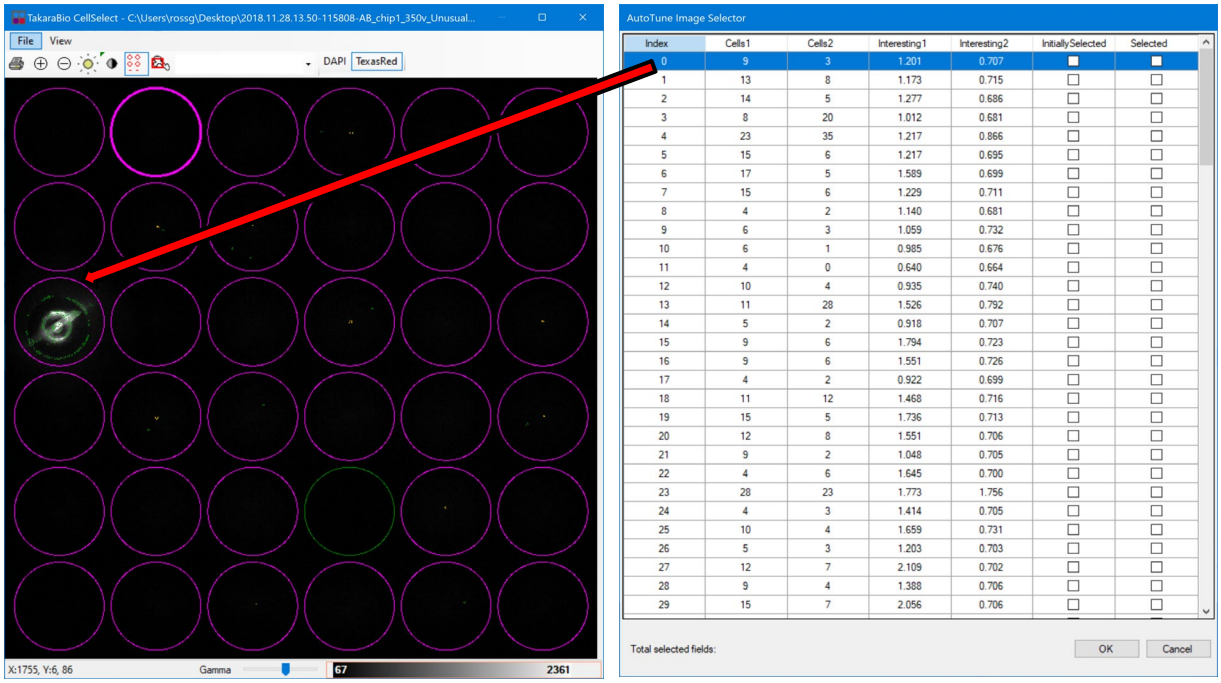


Figure 77. Image Viewer showing a well with debris and reflections

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

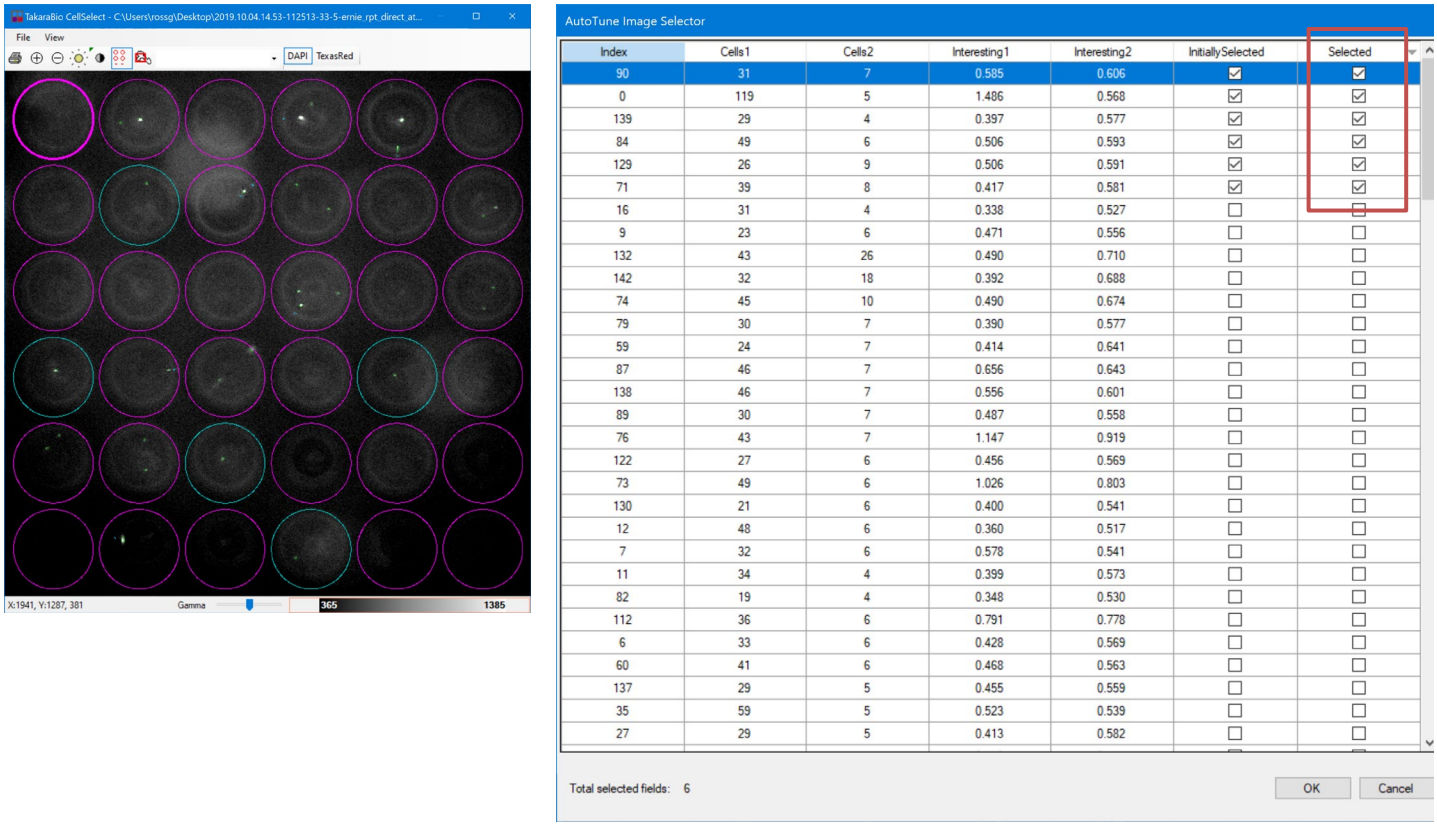


Figure 78. Selected images shown in Image Selector dialog

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 Image Selector dialogs, you will be left with the Auto-tune dialog:

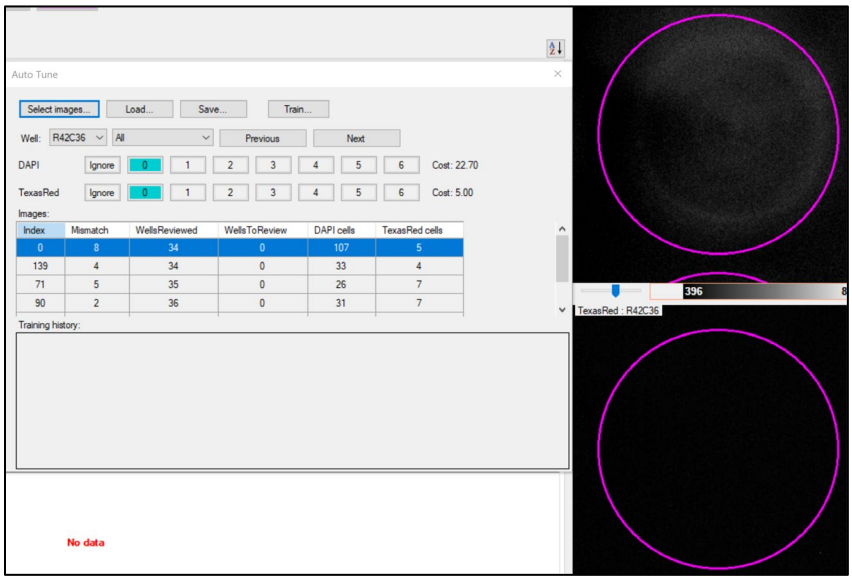


Figure 79. Auto-tune dialog with well selection shown in Main window

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 dialog.
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.

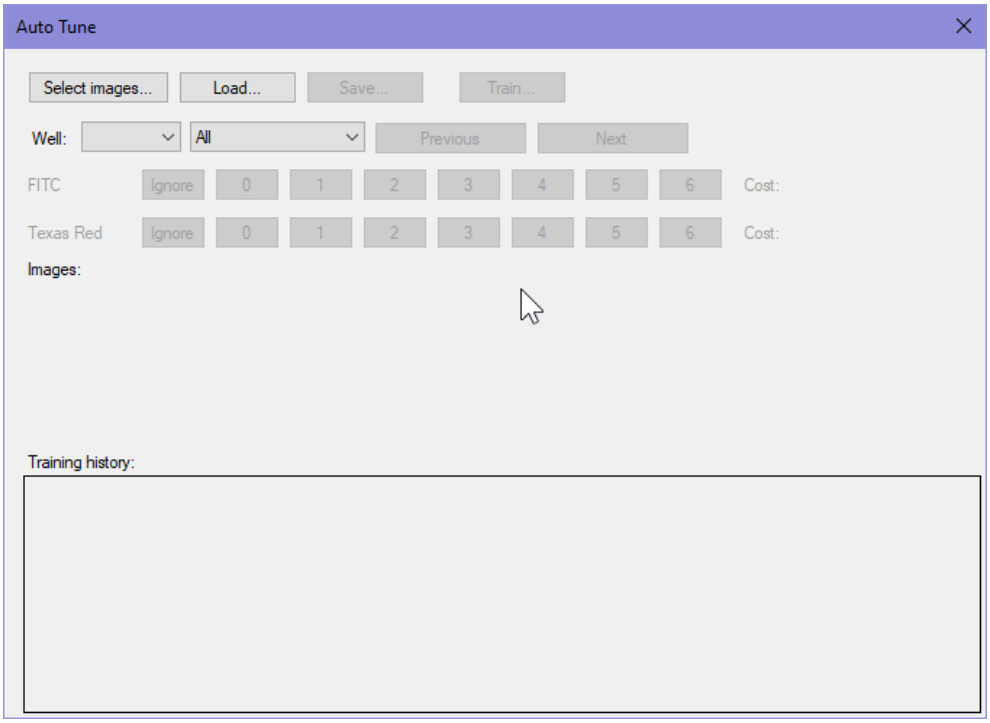


Figure 80. The *Auto Tune* dialog window.

When the *Auto Tune* dialog is first opened, the only actions available are *Load* and *Select images. 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 5 optimal images" step above (Appendix F.A, Step 3), the system analyzes all images to locate interesting ones and displays the results in a table:

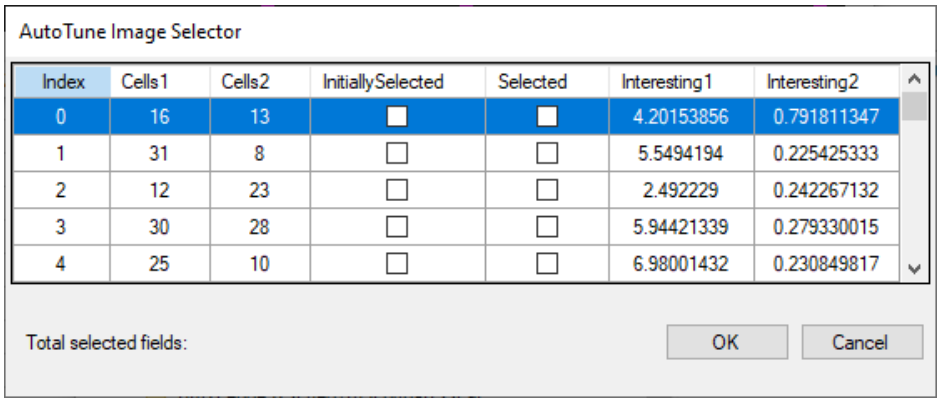


Figure 81. The *Auto Tune Image Selector* dialog window.

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 Master 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 *ImageViewer* toolbar. Figure 82 uses a red square to highlight where to find the channel color buttons, while 'Blue' is the selected value.

Figure 82. The *ImageViewer* 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.

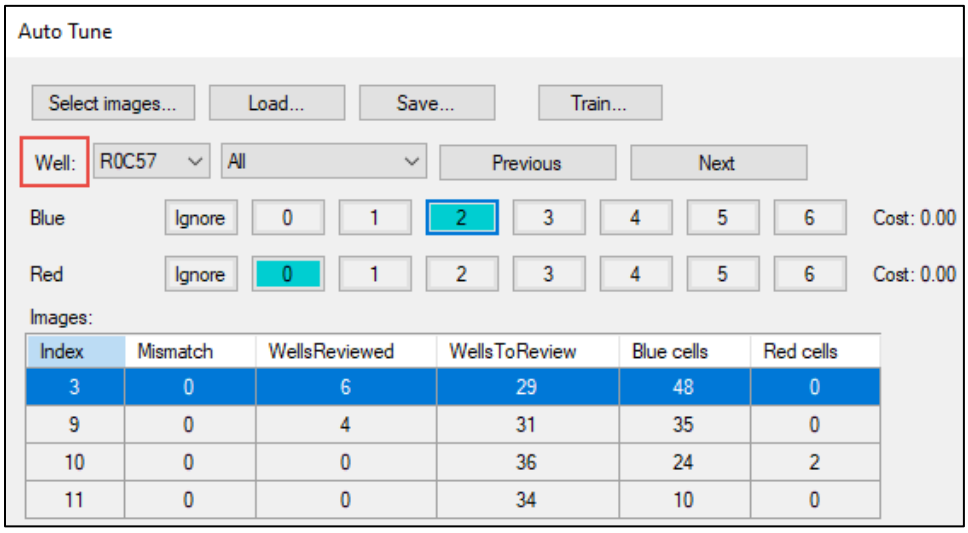


Figure 83. The Auto Tune window after images have been selected. The descriptions of these fields are detailed in Table 15. The "Well" row, mentioned just after Table 15, is highlighted by the red box.

Table 15. 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…	Starts 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.

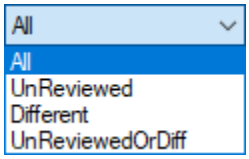


Figure 84. The options of the nanowell filter dropdown menu in the *Auto Tune* window. The descriptions of the drop-down options are described in Table 8.

Table 16. 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

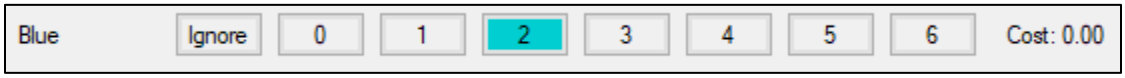


Figure 85. The cell count override section of the *Auto Tune* window.

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 86. The images section of the *Auto Tune* window. The description of each column is documented in Table 17.

Table 17. 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.

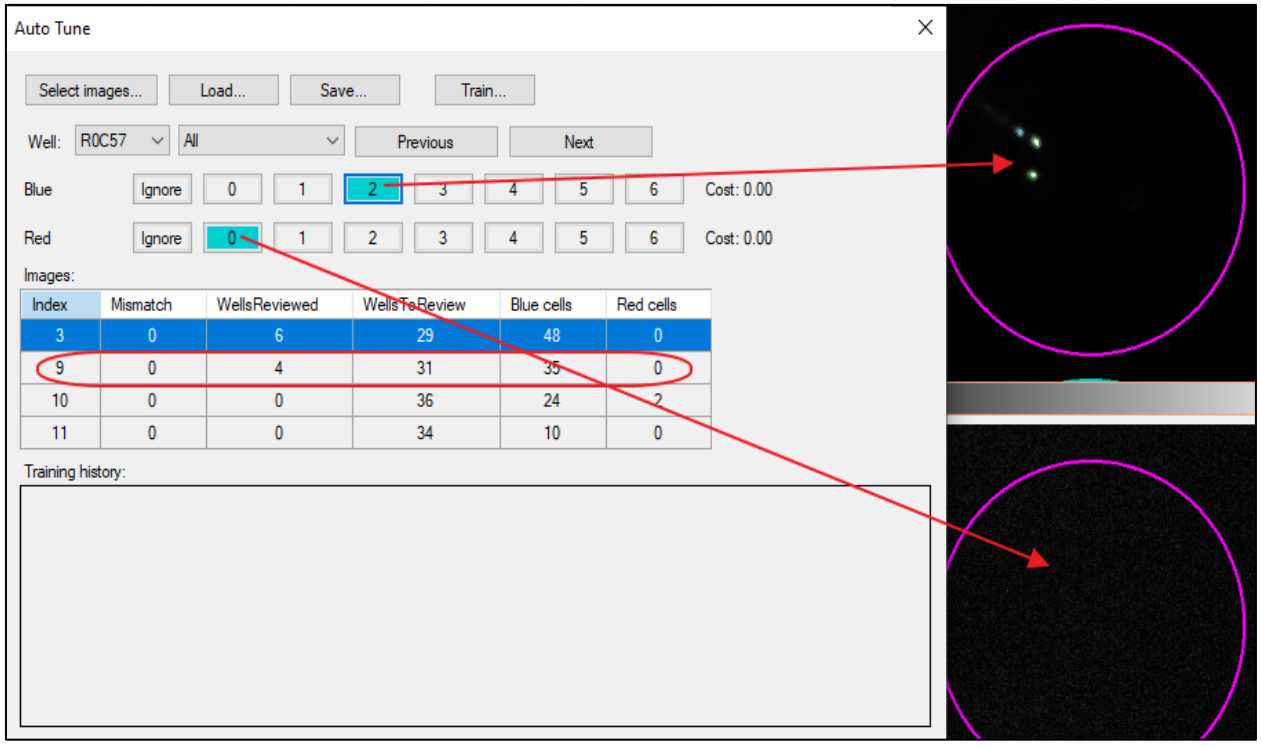


Figure 87. The *Auto Tune* window example when a "Well" is selected.

If you want to select a different value for the cell count override of either wavelength, click on the corresponding button. Figure 88 shows an example of Blue [3] being selected after [2] was initially returned.

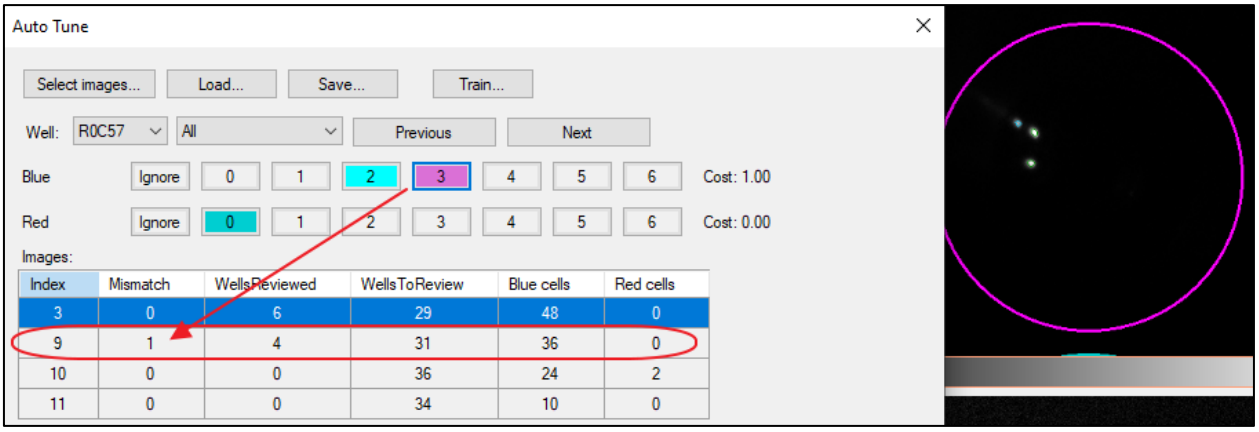


Figure 88. The *Auto Tune* window example when the [3] button is manually selected in the cell count override row for the blue wavelength.

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 suspicious circularity parameter (Appendix D.I) 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 1, 'Select images').

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.

– You can 'lose' your work if you do not save first and you select a completely new set of images.

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.

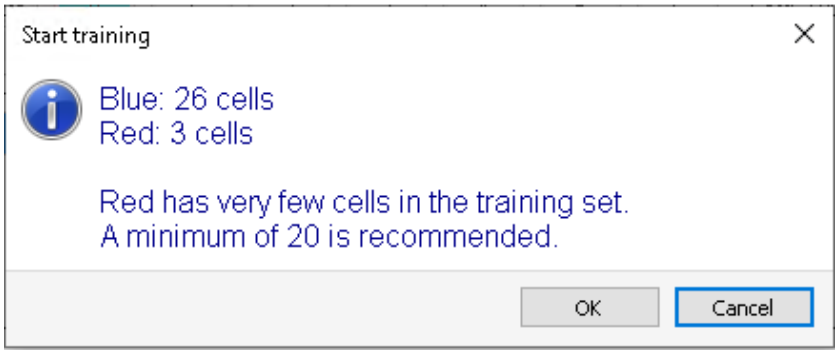


Figure 89. An example of the *Start training* window pop-up seen after clicking the [Train] button in the *Auto Tune* window.

During the training operation the system calculates cell counts by varying the Threshold, Scale, 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 time also depends on the number of images and, obviously, on the speed of the computer. With 5 training images, the iterative training session should be complete within 10 minutes. 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 90. An example training history table in the automated threshold detection tool.

NOTE: It is entirely 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*'.

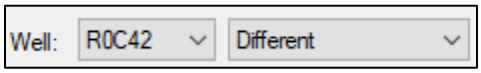


Figure 91. Example selecting the 'Different' option from the "Well" dropdown menu in the *Auto Tune* window.

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 92. Click on 'Save optimized parameters' to save the results and quit out of the tool.

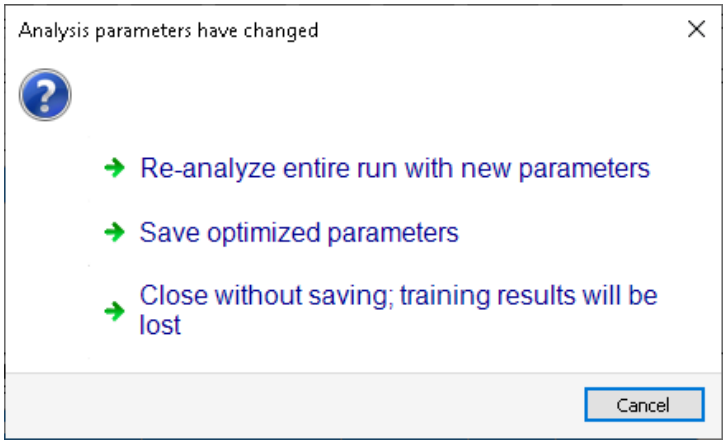


Figure 92. The *Analysis parameters have changed* window when quitting out of the auto-tune tool.

- 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.

This saved XML file can be called up later to run analysis on an imaging results file (`.wcd`) by the following steps:

a. From the main CellSelect interface, choose the *Settings* tab
b. Click the [Edit] button to bring up the *Analysis settings* window
c. Go to **File > Load…**

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