Takara Bio USA, Inc.

ICELL8® CellSelect® Software User Manual

Cat. Nos. 640198, 640000, 640188, 640189
software v1.5
(110519)

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I. Introduction

A. Welcome to the ICELL8 CellSelect 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 forward your research.

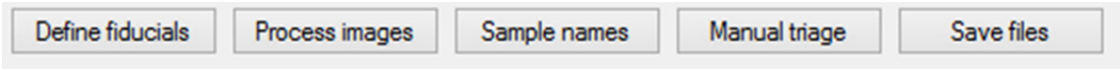
- **Power**—isolate thousands of cells of any size and process multiple chips per day.
- **Control**—choose which cells to process using image analysis software.
- **Insight**—process up to eight different samples per chip and leverage experimental flexibility for greater biological insight.

ICELL8 CellSelect Software analyzes images generated with the ICELL8 cx instrument 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).
- 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:



The diagram on the following page (Figure 1) summarizes the dispensing, imaging, and image analysis portions of the ICELL8 cx single-cell analysis workflow.

B. 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 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.

C. Workflow Diagram

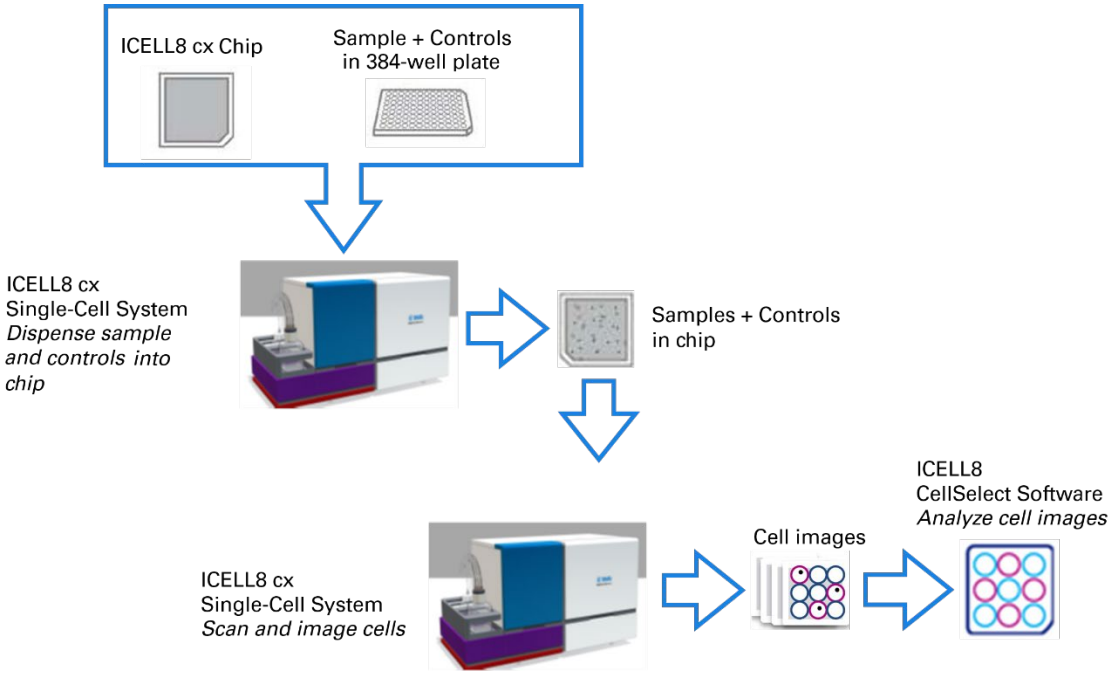


Figure 1. Workflow diagram depicting single-cell isolation, imaging, and image processing steps.

II. Procedure

A. Overview

In this procedure you will select nanowells using ICELL8 CellSelect Software and then generate a filter file to be used for dispensing RT mix. The procedure consists of the following steps:

1. **Load Images:** Load images directly from the ICELL8 cx Software when scanning, or later from the ICELL8 CellSelect Software (**File** menu).
2. **Load a Different Barcode File (optional):** Barcodes are already selected when you scan images from the ICELL8 cx system. However, this menu item allows you to associate a different barcode file to the run for analyzing 5,184 nanowells.
3. **Process Images:** Start image processing. The software analyzes every well image in 288 image files and determines the best candidates for future processing.
4. **Save Files:** Save details of all well analyses as well as the filter file for dispensing RT mix to selected candidate wells.

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

Icon for ICELL8 cx Software (for dispensing) 

Icon for ICELL8 CellSelect Software (for image analysis) 

Figure 2. Icons for the ICELL8 cx System software and ICELL8 CellSelect Software.

B. Load Images

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

From the ICELL8 CellSelect Software

1. Launch the ICELL8 CellSelect software by clicking the `CellSelect` icon:

2. In the *Main* window, click the **File** tab and select **Open chip folder**.

Figure 3. Opening the chip folder from the Main window.

Alternatively, you can drag and drop a folder from Windows File Manager into the application

3. If the images in that folder were not previously analyzed, you will be prompted to supply the necessary information.
    a. If there is one `.wcd` file in the folder, that file will be loaded.
    b. If there are multiple `.wcd` files in the folder, you will be prompted to select the file you want to load.

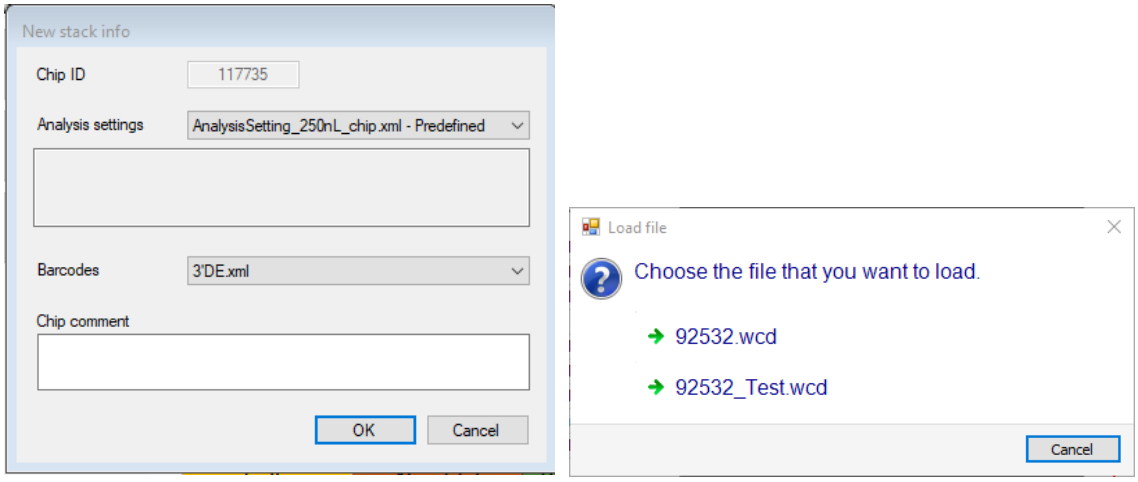


Figure 4. Loading the settings file.

C. Load a Different Barcode File (optional)

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

1. In the *Main* window, click the **File** tab and select **Load barcode file**.

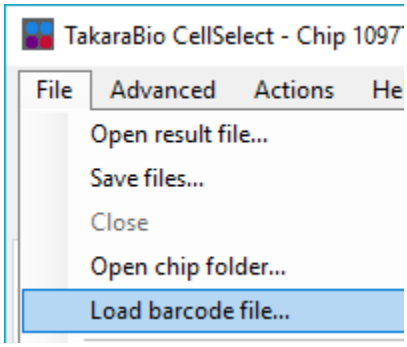


Figure 5. File menu.

2. Select one of the preloaded XML files: `3'DE.XML`, `Smart-seq_SetA.XML`, or `Smart-seq_SetB.XML`. These XML files contain nanowell barcode sequences for each preprinted nanowell location. The default folder is:

```
C:\ProgramData\Wafergen\SingleCell\AssayMaps\
```

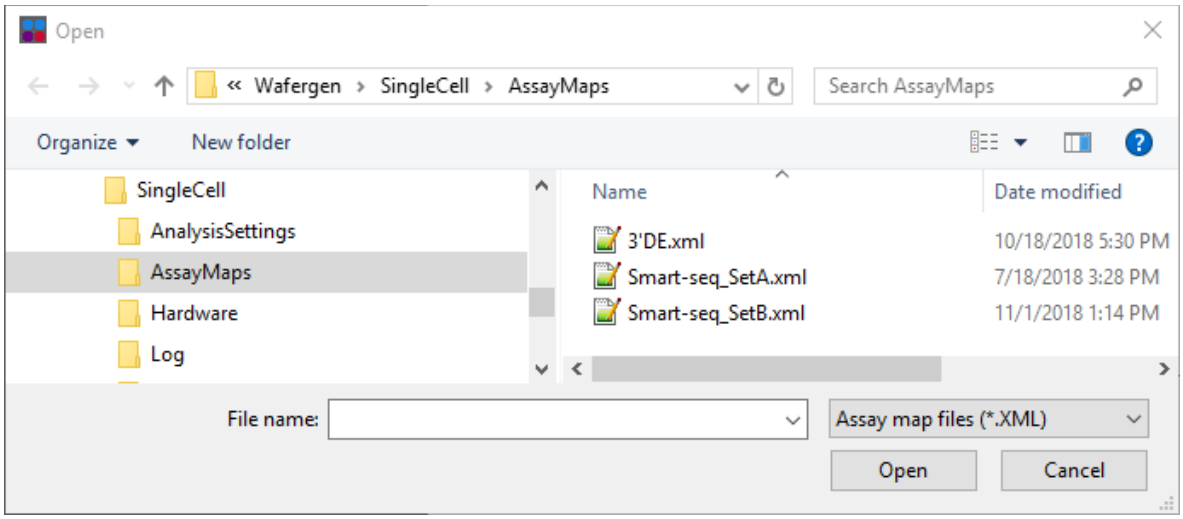


Figure 6. Loading the barcode file for the preprinted chip.

D. Process Images

1. Click **Process images**. The software will analyze two sets of 144 multi-well images taken using DAPI and Texas Red filters, respectively, and automatically identify and select all nanowells that contain viable, single cells (i.e., "candidates") and controls based on the parameters defined in *Settings*.

NOTE: For each image, the following criteria must be satisfied for the software to identify a cell as being a candidate for downstream analysis (please refer to the Appendix, below, for more details):

- The cell must appear in the DAPI channel and NOT in the Texas Red channel.
- It must be the only one visible in the well.
- It must satisfy the size/shape parameters specified in *Settings*.

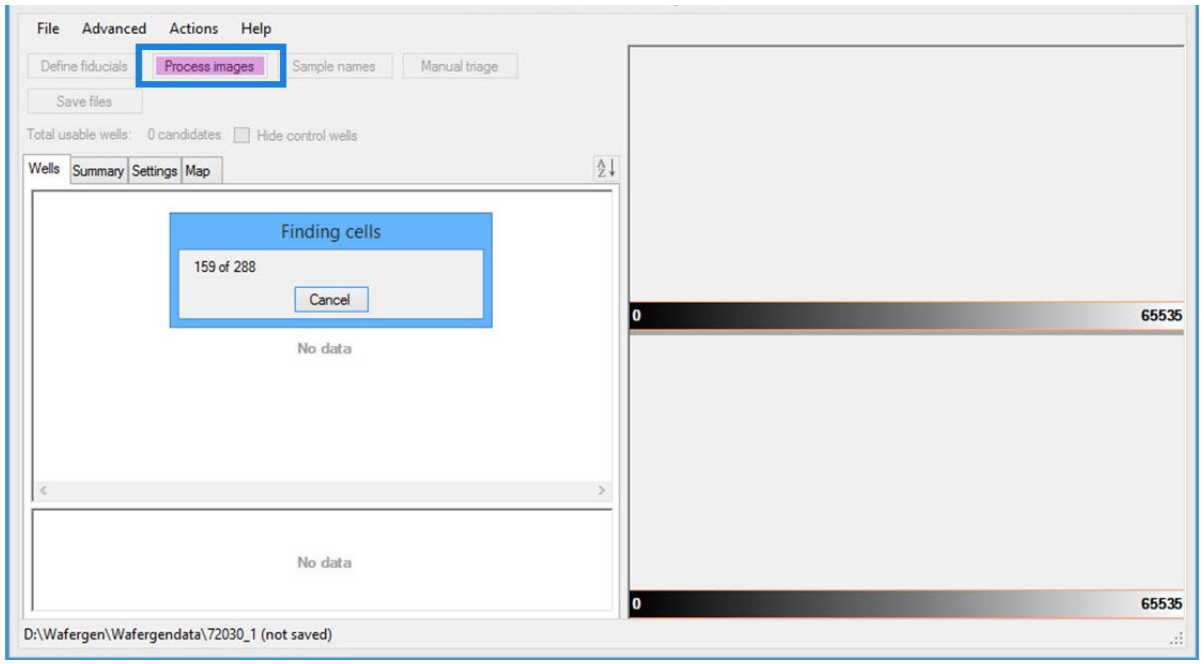


Figure 7. Having the software find cells based on parameters defined in *Settings*.

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 (e.g., `<Chip ID>_<Date>.wcd` or `72030_09282015.wcd`). The software will then do the following:
   - Select wells among all those that are checked under **Candidate** according to the best parameters defined in the *Settings* tab.
   - Save the result file under the specified name (e.g., `72030_09282015.wcd`). The result file will contain the selected nanowells and setting parameters.
   - Automatically generate the filter file for the dispense of RT reagents (e.g., `72030_09282015_FilterFile.csv`). The selected wells in the filter file will be displayed in the *For Dispense* column.
   - The software will also generate a PDF file called `72030_09282015_Report.pdf` which contains a short summary of the results.
   - Also, a file `72030_09282015_WellList.txt` is generated which contains the content of the *Wells* table in a form that can be read easily by downstream analysis software.

3. Each preprinted chip contains a total of 5,184 unique nanowell barcodes. You can load the results along with the settings from the saved WCD file. The software uses the following legend when examining nanowells:
    - **Green** = a cell
    - **Yellow** = ignored
    - **Blue** = a reflection

    See "Understanding the Software Color Code" in Section II.F (below) for more information.

> **NOTE:** The user can re-process images after changing the analysis settings.

E. Change Sample Names

1. Click **Sample names** and enter a single sample name or use the source plate configuration layout for ICELL8 cx dispensation.
    - The **One sample** option changes ALL names to the name you enter (the default name is "Sample").
    - The **Multiple samples** option changes the default name ("Sample") to grid names from the source plate—or you may edit the grid name (see Figure 8, below).

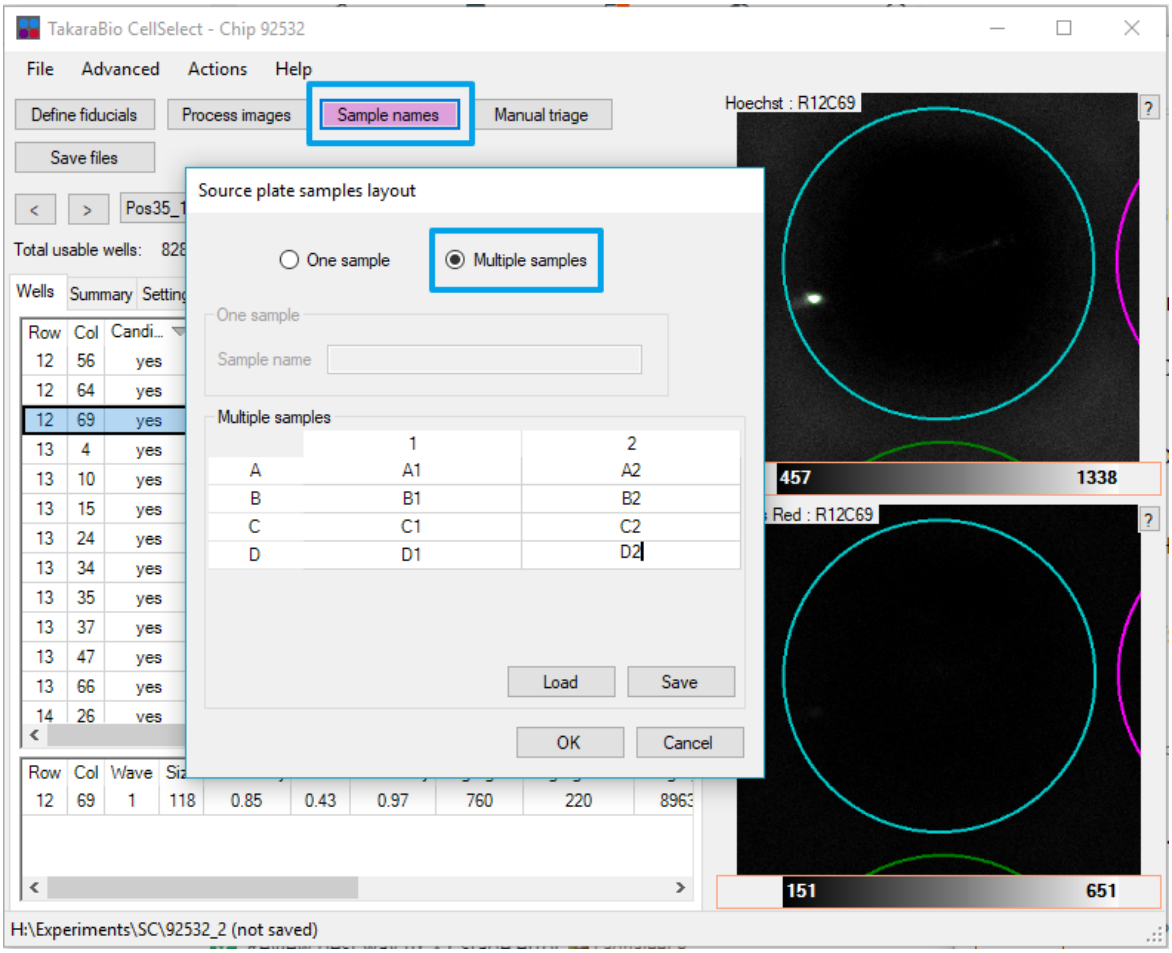


Figure 8. Inputting multiple sample names and automatically identifying control- and fiducial-containing wells.

F. Review Images

Consolidate All Candidate Wells to the Top

1. Click the **Wells** tab and sort the nanowell statistics in the Candidate column by clicking the **Candidate** heading (see Figure 9, below). The software will sort and consolidate all selected nanowells to the top of the table.

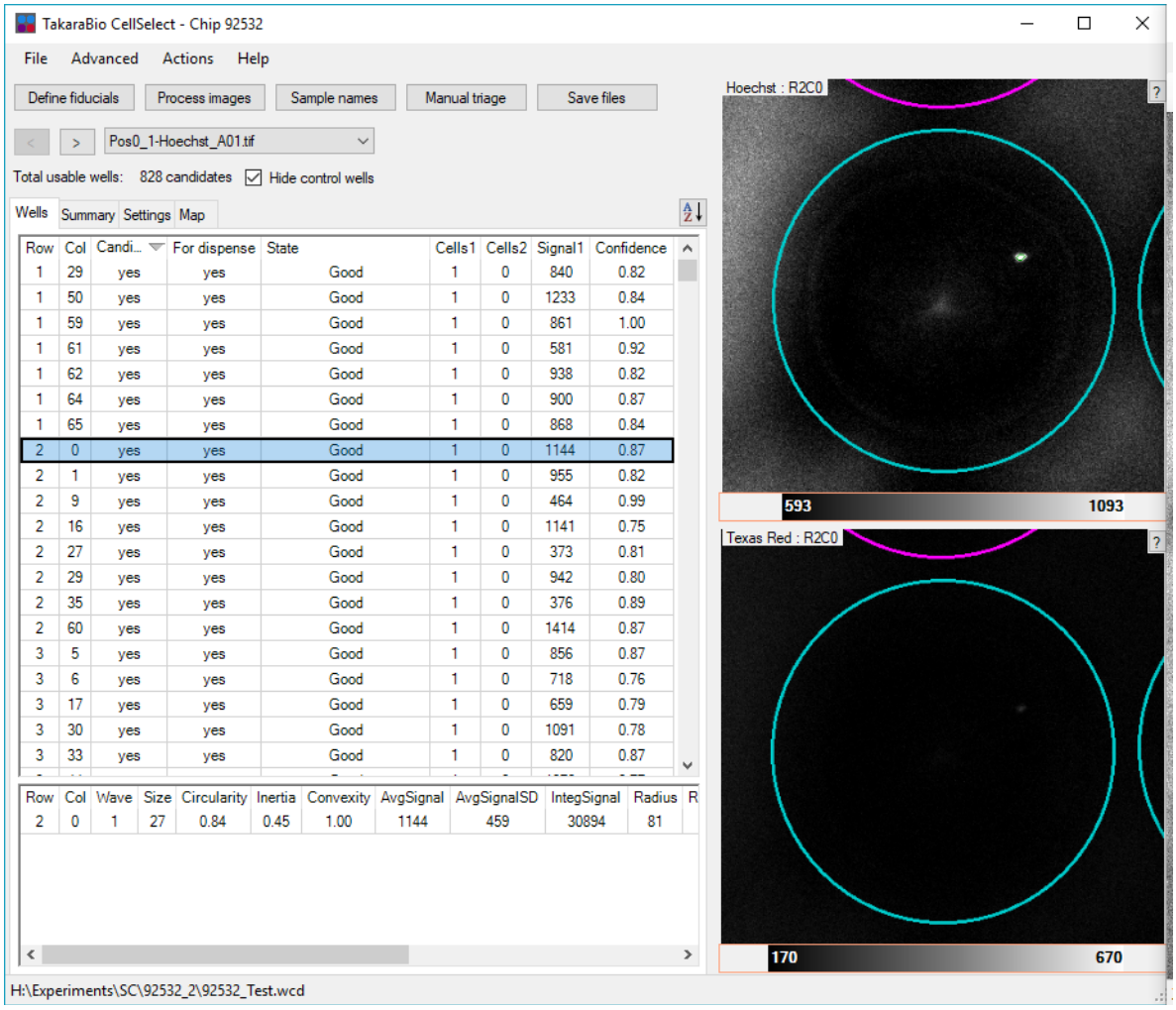


Figure 9. Sorting the Candidate column to consolidate all selected nanowells to the top of the table.

2. Click any row for closeup views of the selected nanowell using DAPI (top) and Texas Red (bottom) filters. In Figure 9 (above), the selected nanowell is located in Row 2/Column 0 of the imaged chip.

View/Edit Results List

If desired, you can examine any selected nanowell in the Candidate column and view it as a single-well image as well as a multi-well image.

1. In the *Main* window, click the **Advanced** tab and select **Image viewer** (see Figure 10, below). A multi-well image highlighting the nanowell selected in the results table (on the *Wells* tab) will appear.
2. Arrange the windows as demonstrated in Figure 10 (below). When you click on any of the Candidate rows in the results table (on the *Wells* tab), the software will present corresponding single-well images, and display the corresponding multi-well image in the *Image Viewer* window. Similarly, by double-clicking on a well in the *Imager Viewer* the corresponding row in the *Wells* table will be highlighted.

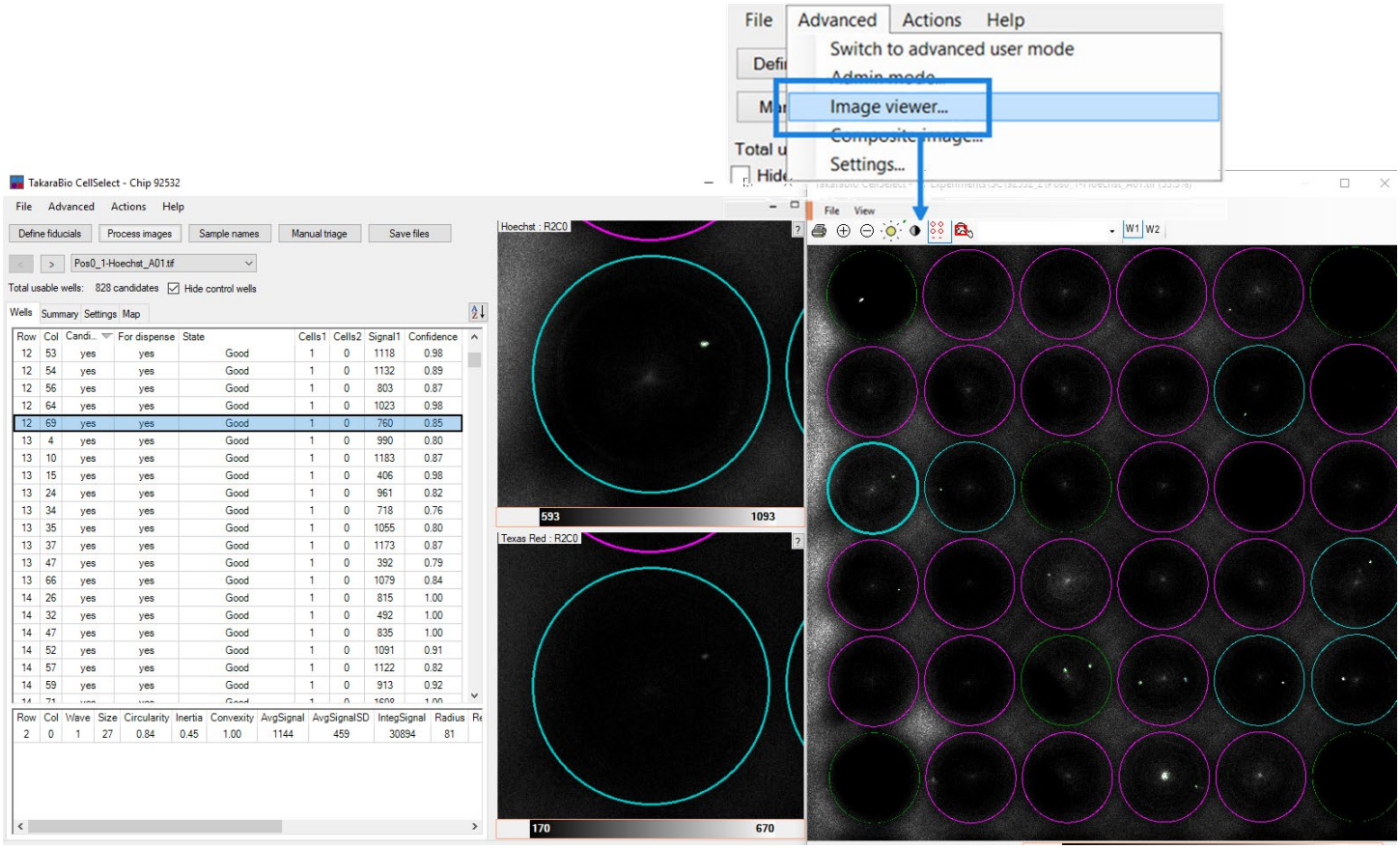


Figure 10. Opening and arranging windows for optimal image viewing. The image on the far right is the multi-well image. The horizontally split images in the center show a single cell in a nanowell stained by Hoechst 33342 (top) and the absence of propidium iodide stain in the corresponding position (dead-cell stain; bottom). In the bottom image, the green dot in the center (if seen) marks the bottom of the nanowell and is not from a dead cell because it does not match the location of the Hoechst-stained cell.

3. Use the tools in the *Image Viewer* toolbar to critically examine the cells (see Figure 10, above, for a legend describing the toolbar icons).

4. To manually exclude one or more candidate wells, right-click the highlighted row(s) and select **Exclude selected wells** (see Figure 11, below). To exclude several candidate wells, consider using the **Manual triage** function described in the section below (Section II.G).

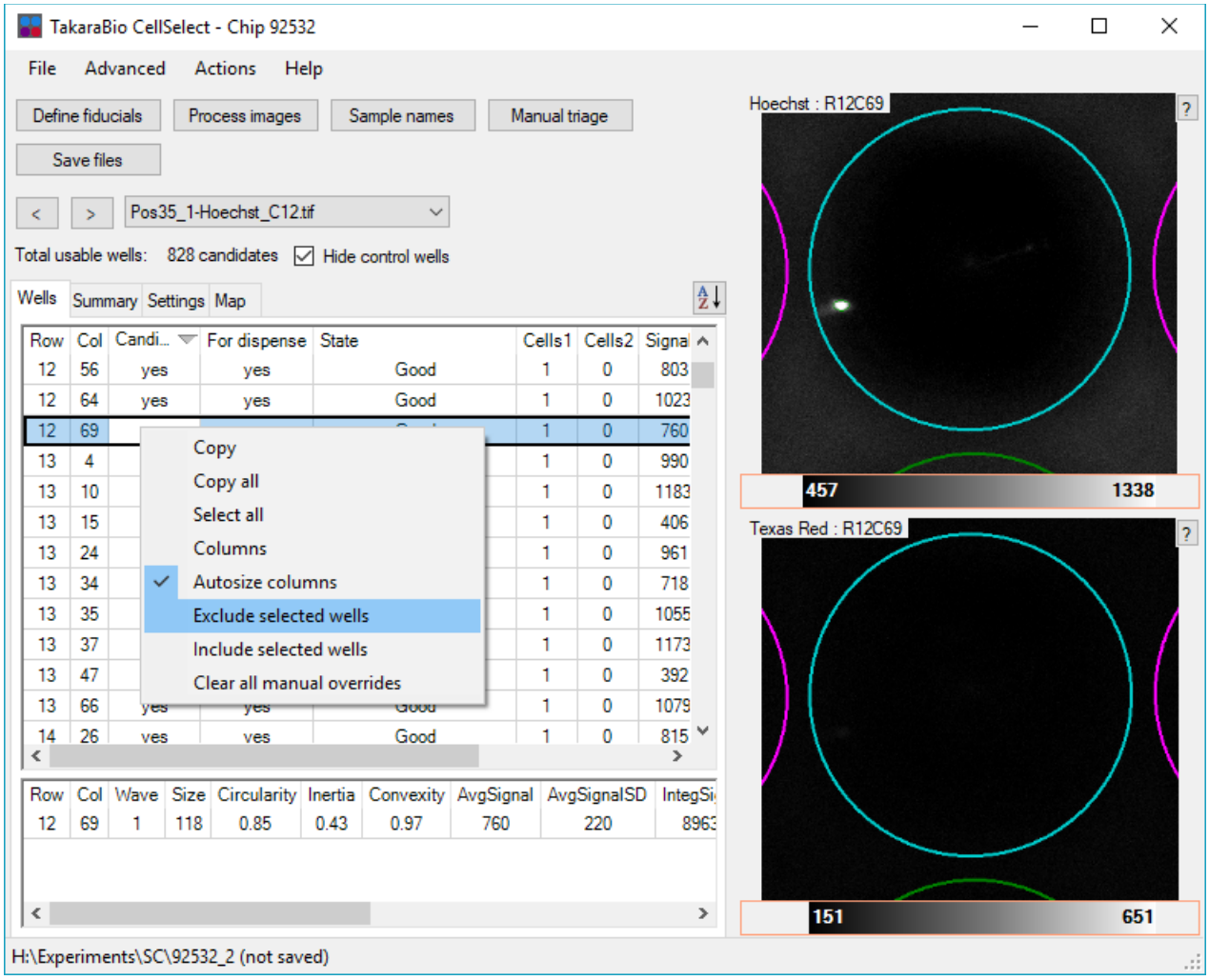


Figure 11. Excluding candidate wells.

Understanding the Software Color Code

CellSelect software analyzes images of cells taken with both DAPI and Texas Red filters. The DAPI filter is used to detect live cells (stained with Hoechst 33342), while the Texas Red filter is used to detect dead cells (stained with propidium iodide). If a single cell is visible in the DAPI filter but not in the Texas Red filter AND meets the size and shape criteria programmed in the settings, 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 multi-well images, while wells containing non-candidate cells (such as dead cells, see the Table VI in the Appendix, below) are marked with pink-colored circles. A well currently displayed in the single-well image is marked with a brighter overlay than surrounding wells in the multi-well image (see example in Figure 12, below).

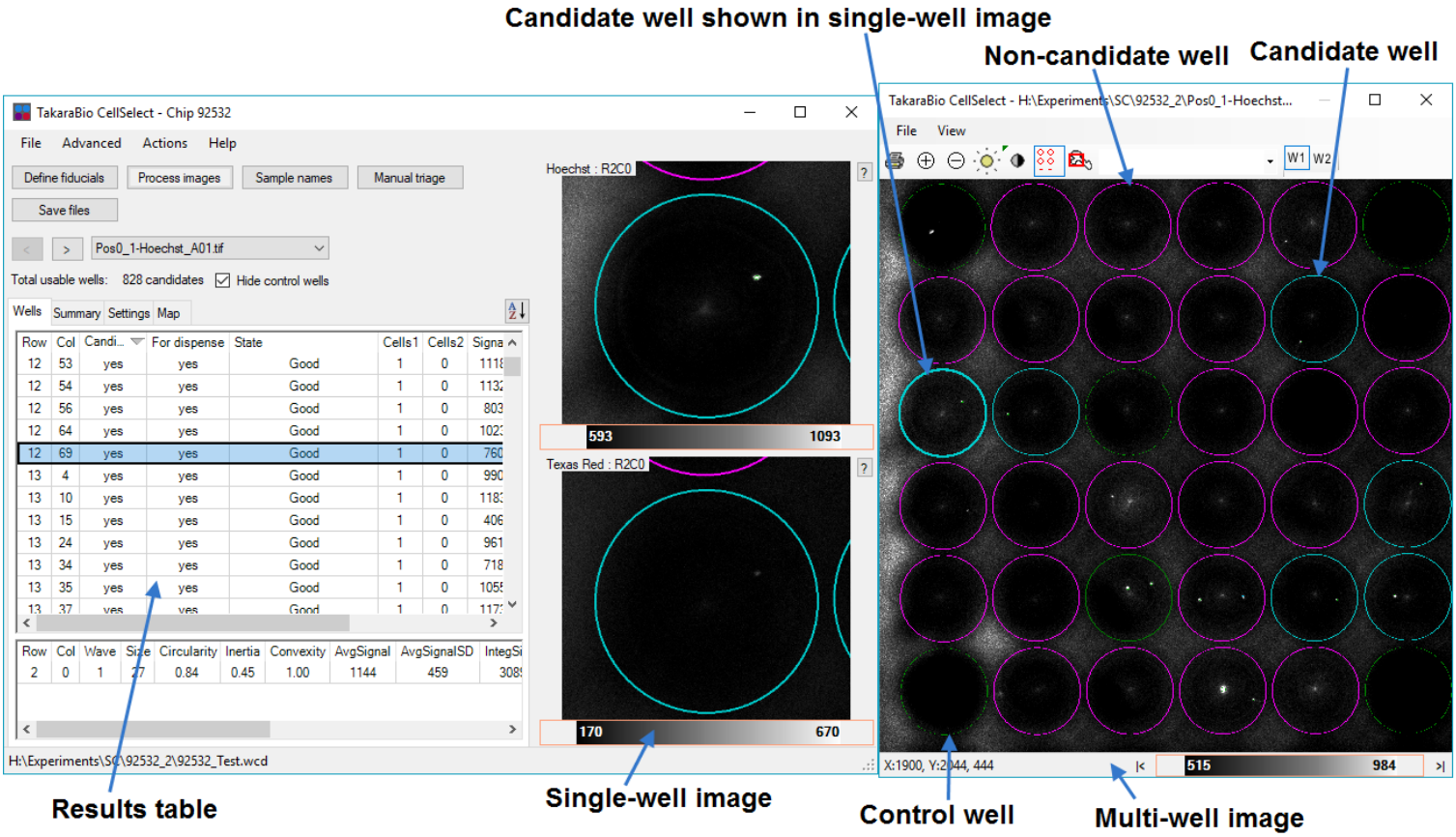


Figure 12. Understanding nanowell color codes.

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 the Table VI in the Appendix (below).

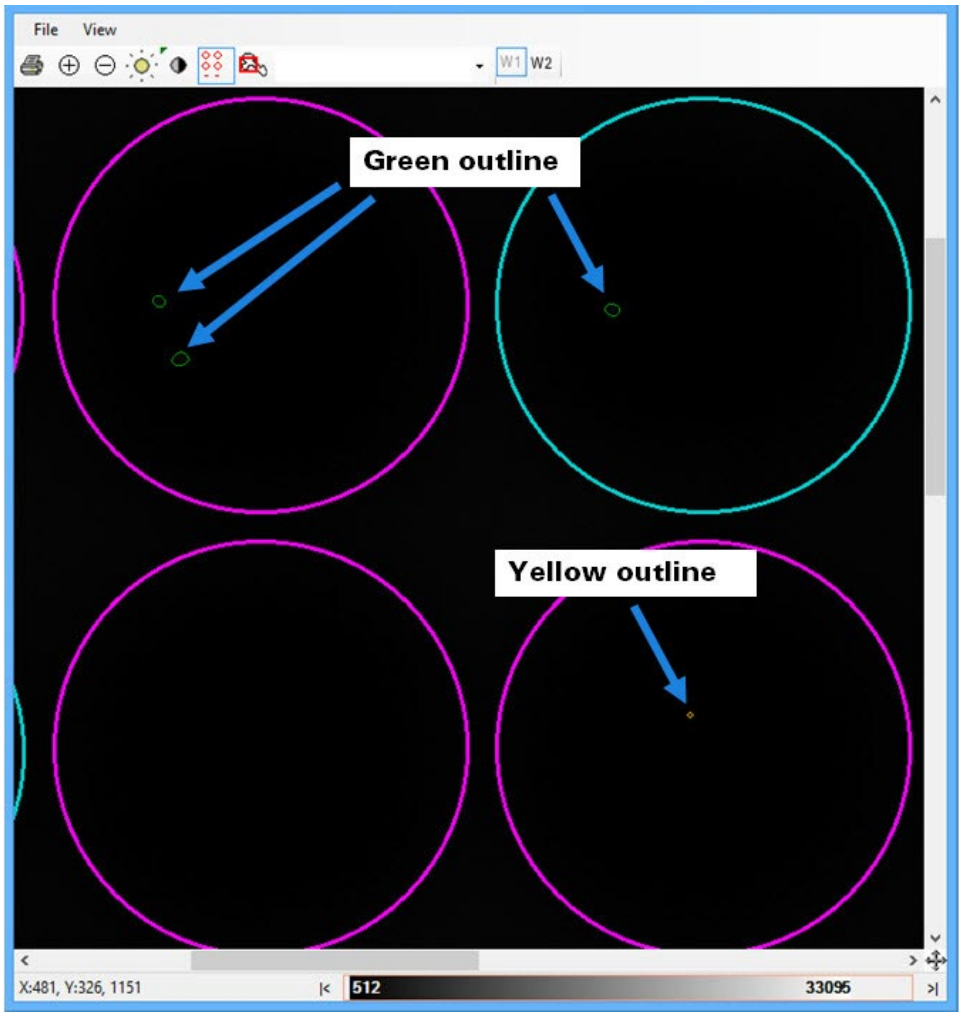


Figure 13. Closeup of wells, showing cell outlines.

G. Use Manual Triage

The **Manual triage** function opens a dialog box to quickly review and reject (or bypass) consecutive wells down the *Wells* table (after sorting good candidates to the top of the table).

1. Click **Manual triage** to open the corresponding prompt box.
2. Examine each consecutive well image and click [Reject - Next Well] to exclude a candidate well and move on to the next candidate well, or click [Next] to retain a candidate well and move on to the next candidate well.
3. Add a comment to the selected well by typing it into the "Comment" field and pressing [Enter]. You can also double-click on one of the already defined comments in the list to edit the contents.

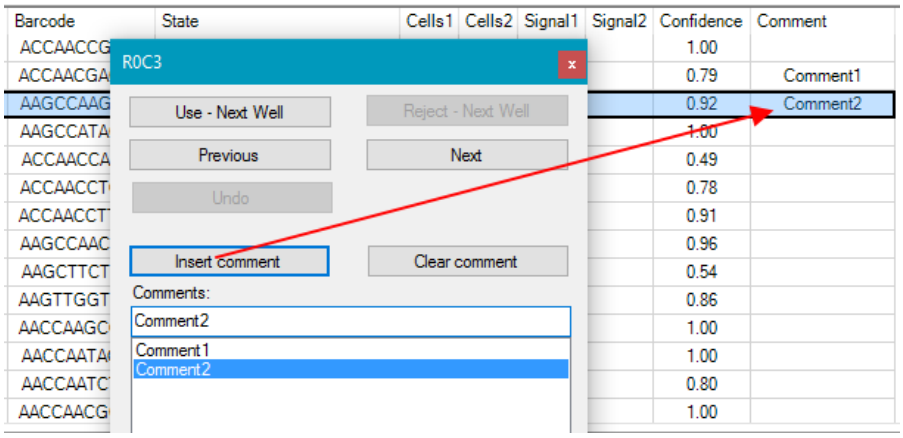


Figure 14. Adding comments to selected wells.

H. Save Files

1. Click **Save Files** and enter a new file name to save any changes you have made to the results file. We strongly recommend using a new file name to avoid overwriting the original results file. The file is saved with the `.wcd` file extension. **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.

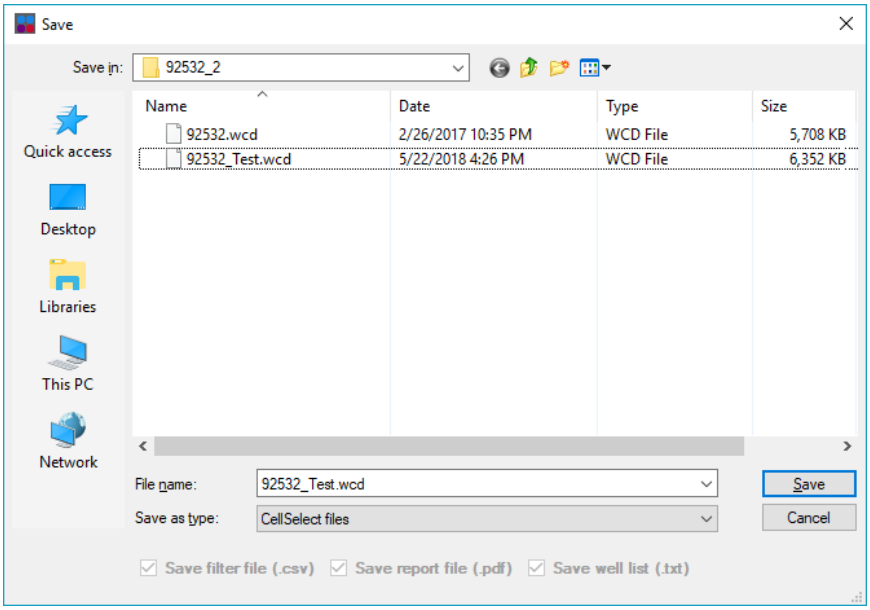


Figure 15. Saving the results file.

2. If you would like to downselect nanowells, in the *Main* window, click the **Actions** tab and select **Downselect**.

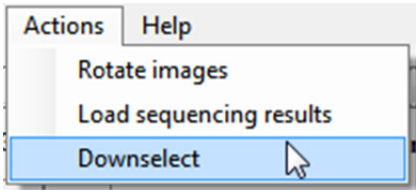


Figure 16. Saving the filter file.

3. 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).

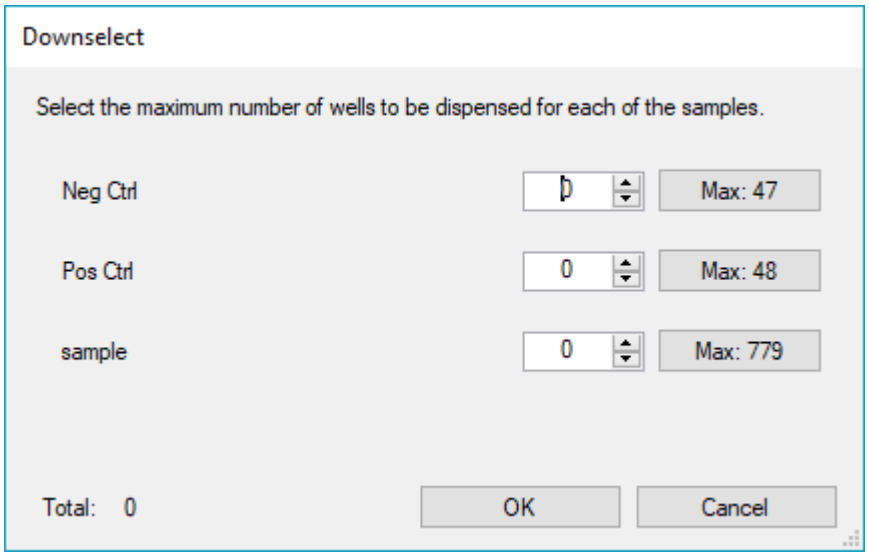


Figure 17. Enter the number of nanowells for controls and samples.

4. Save the filter file after downselecting the number of wells. A 72 x 72 grid of the entire chip will be saved as a `.csv` file automatically named as `<Chip ID>_FilterFile.csv`, in which nanowells to be included in subsequent dispensations are marked with a 1, and nanowells to be excluded are marked with a 0.
5. Use the filter file (`.csv`) for dispensing the RT reaction mix on the ICELL8 cx unit.

III. 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.

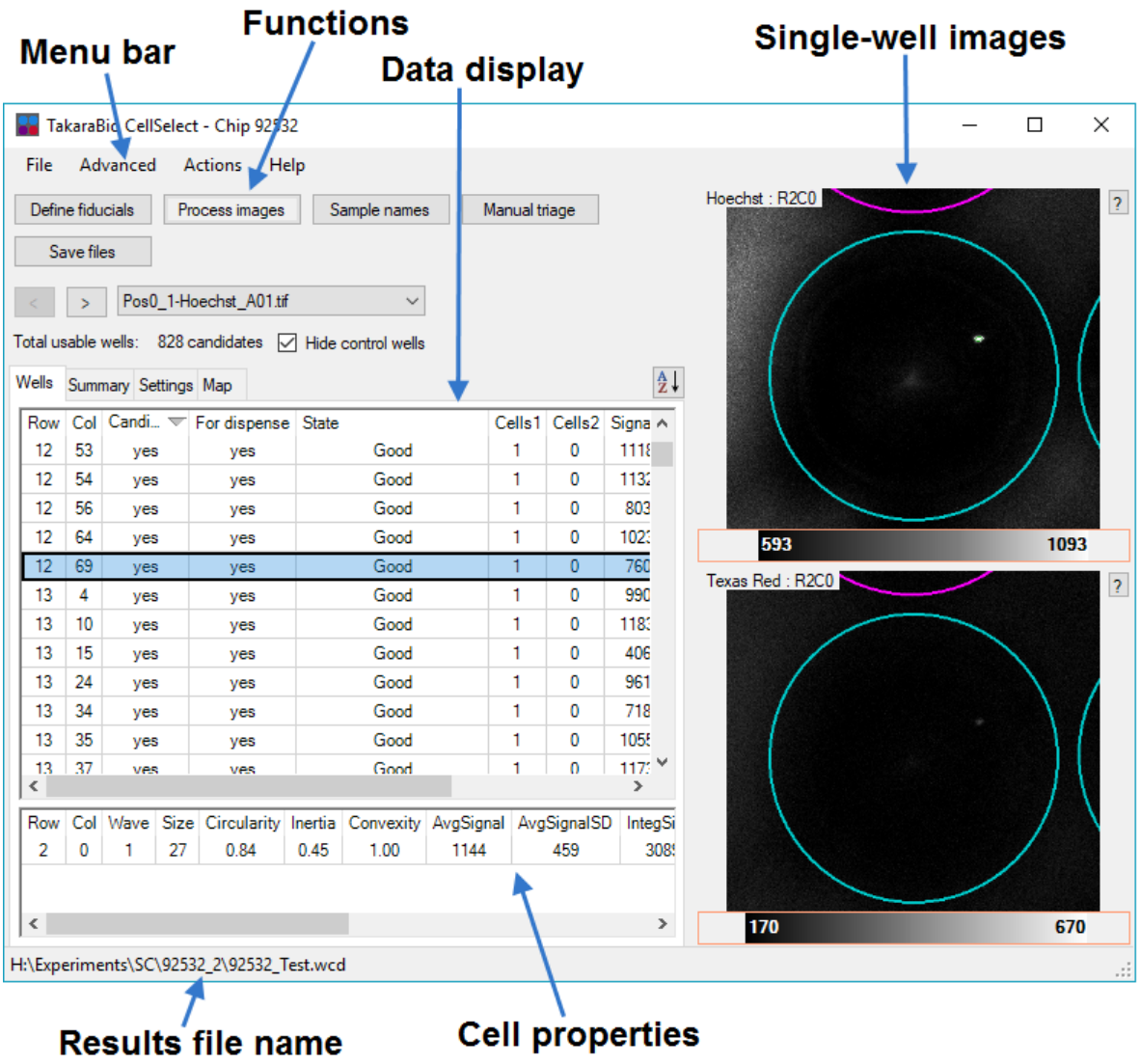


Figure 18. ***Main*** **window.**

B. User Interface

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. Users are encouraged to try right-clicking while using the software to access numerous features.

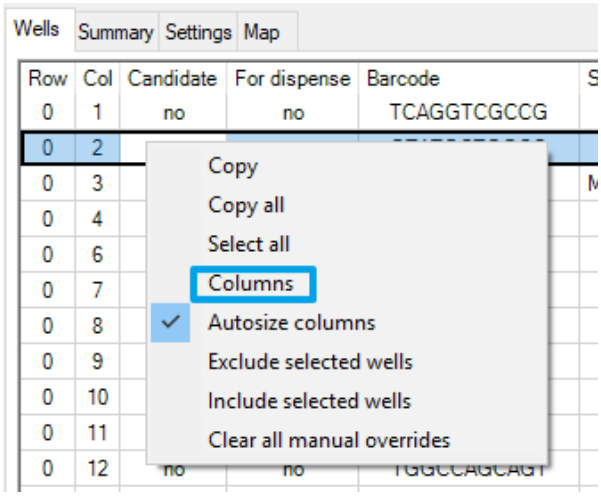


Figure 19. Example right-click menu.

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 19, above). This action will open a *Selected fields* dialog box with checkboxes next to the information categories (see Figure 20, 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.

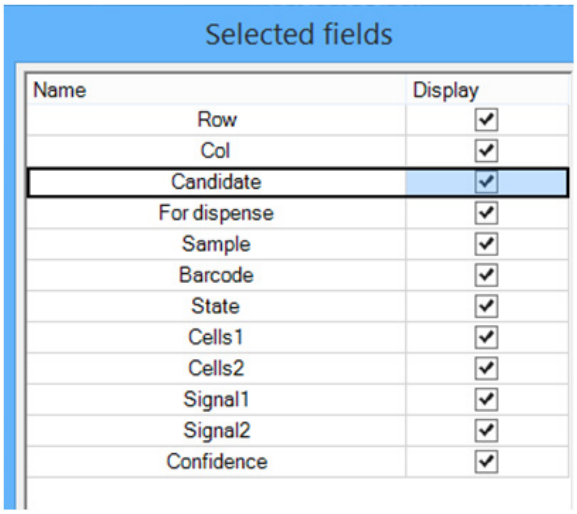


Figure 20. Column heading options.

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 21, below).

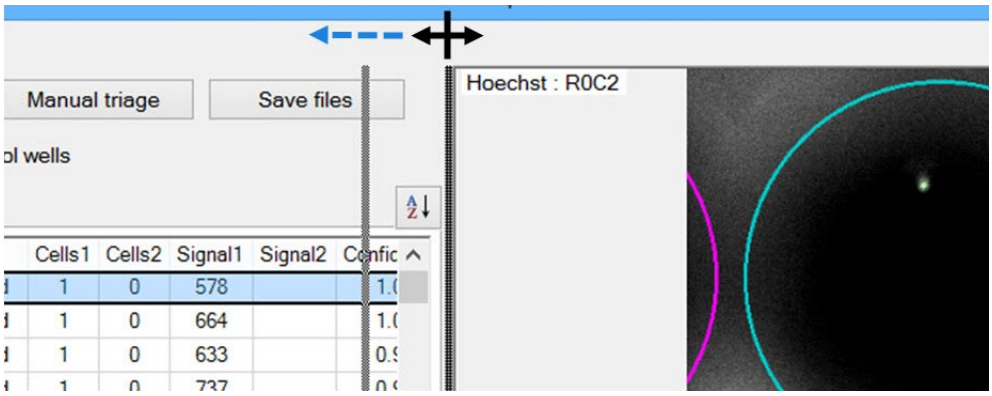


Figure 21. Adjusting the width of the *Image Viewer* window.

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 1. 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 some screens you may need to press the **Ctrl** key on the keyboard while moving the scroll wheel.

C. Menu Bar

Table 2. *Main* Window Menu Items.

Menu Item	Option	Function
File	**Open result file…**	Opens a previously saved result file (`.wcd`).
	Save files…	Processes and saves the results for the current chip*.
	Open chip folder…	Opens the chip folder containing images created by the Micro-Manager software.
	Load barcode file…	Loads the configuration file that identifies each well in the chip with a barcode.
Advanced	**Switch to advanced user mode**	Displays additional technical attributes of each well.
	Admin mode…	Password protected.
	Image viewer…	Adjusts image size, brightness, contrast, and well overlay. See Section III.G (below) for more details.
	Composite image…	Displays the multi-well image.
	Settings…	Displays the preconfigured settings for single-cell analysis.
Actions	**Downselect**	Selects the number of wells to be dispensed for each of the samples.
	Downselect – clear	Removes existing Downselect settings.
	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 at technical_support@takarabio.com before taking this action.)
	3D – stack	Opens a 3-D viewer to inspect images from individual z-planes.
Help	**About**	Displays the software version and End User License Agreement (EULA).
	Open user manual…	Opens this manual.

*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. Process Images

The **Process images** function analyzes all 5,184 nanowell images in the 288 TIFF image files generated for a chip.

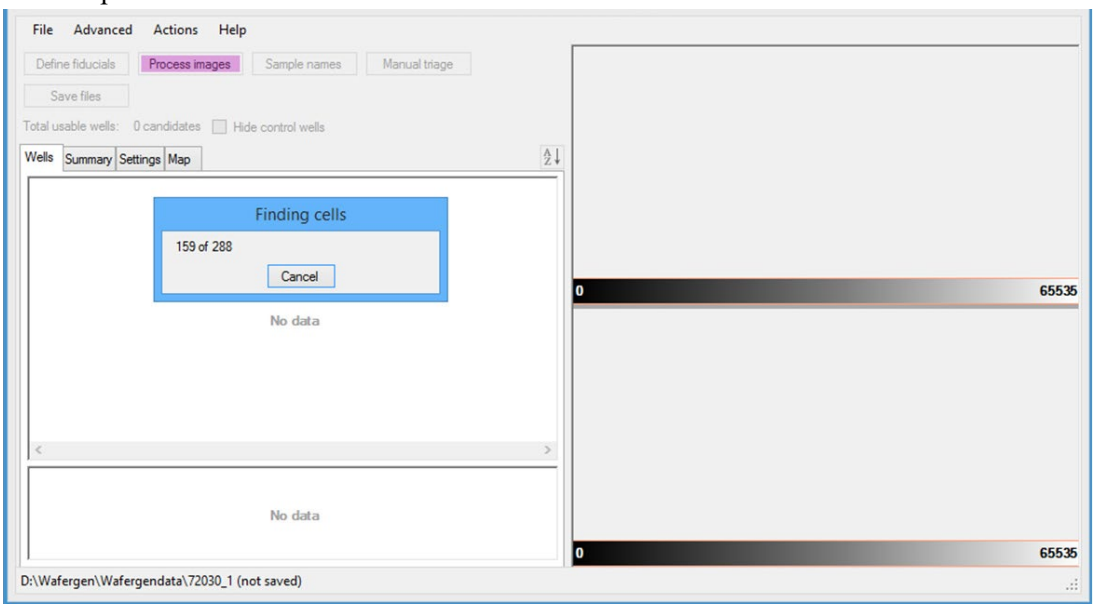


Figure 22. The software analyzes well images.

After the software finishes the analysis, it prompts you to save the results file. When you click [Yes] the software will prompt you to enter a file name and will then do the following:

- Select wells among all those that are checked in the Candidate column (*Wells* tab) according to the parameters defined on the *Settings* tab. See "Settings" in Section III.G.
- Save the result file. The result file will contain the selected nanowells and setting parameters under a new file name (e.g., `72030_10202015.wcd`), as demonstrated in Figure 23 (below). The results file contains all the data shown on the *Wells* tab.
- Automatically generate the filter file for dispensing RT reagents (e.g., `72030_10202015_FilterFile.csv`). The selected wells in the filter file will be checked in the "For dispense" column in the results on the *Wells* tab. The filter file is a simple map of all well positions in the chip. Each well position receives a "1" if it is marked as a candidate in the results file and a "0" if it is not a good candidate.
- The software will also generate a PDF file called `72030_09282015_Report.pdf` that contains a short summary of the results.
- Also, a file `72030_09282015_WellList.txt` is generated that contains the content of the Wells table in a form that can be read easily by downstream analysis software.

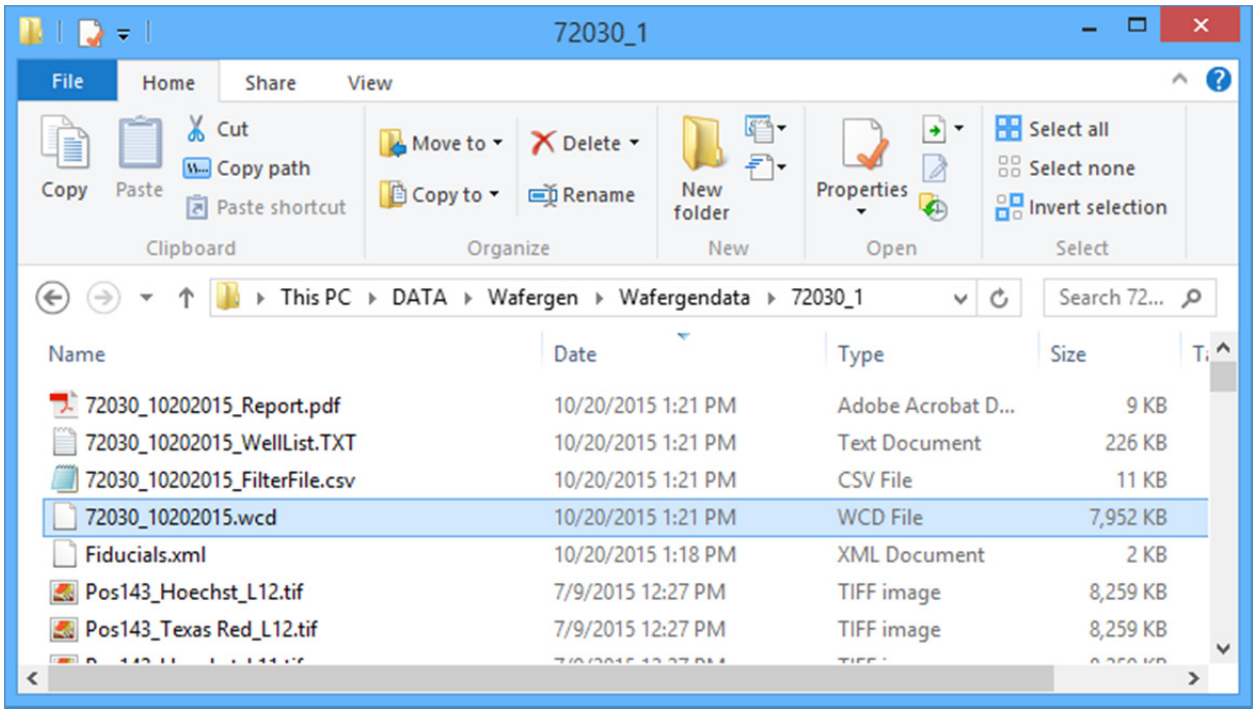


Figure 23. Files generated by ICELL8 CellSelect Software. Each preprinted chip contains a total of 5,184 unique nanowell barcodes. You can load the results along with the settings from the saved WCD file.

E. Data Display

The data section of the *Main* screen includes the following tabs: *Wells*, *Summary*, *Settings*, and *Map*.

Wells

The data table on the *Wells* tab lists all the wells in the chip and statuses of any cells found.

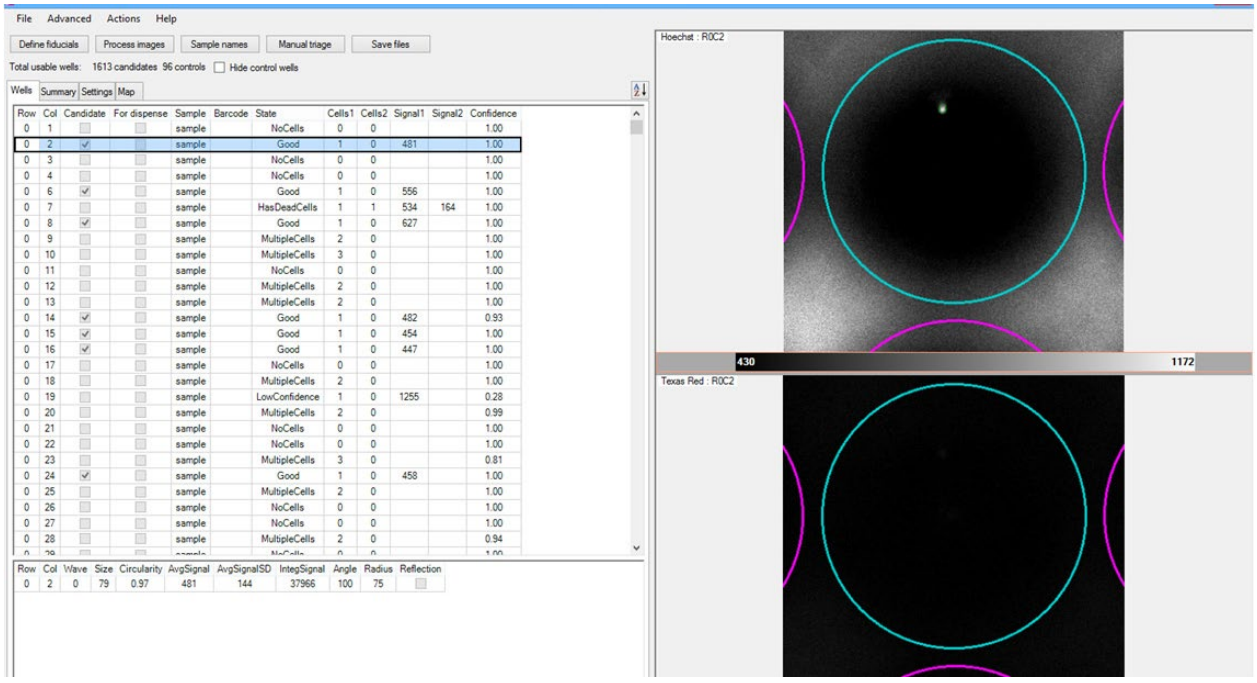


Figure 24. *Wells* data table.

You can view additional data by selecting **Switch to advanced user mode** from the **Advanced** menu.

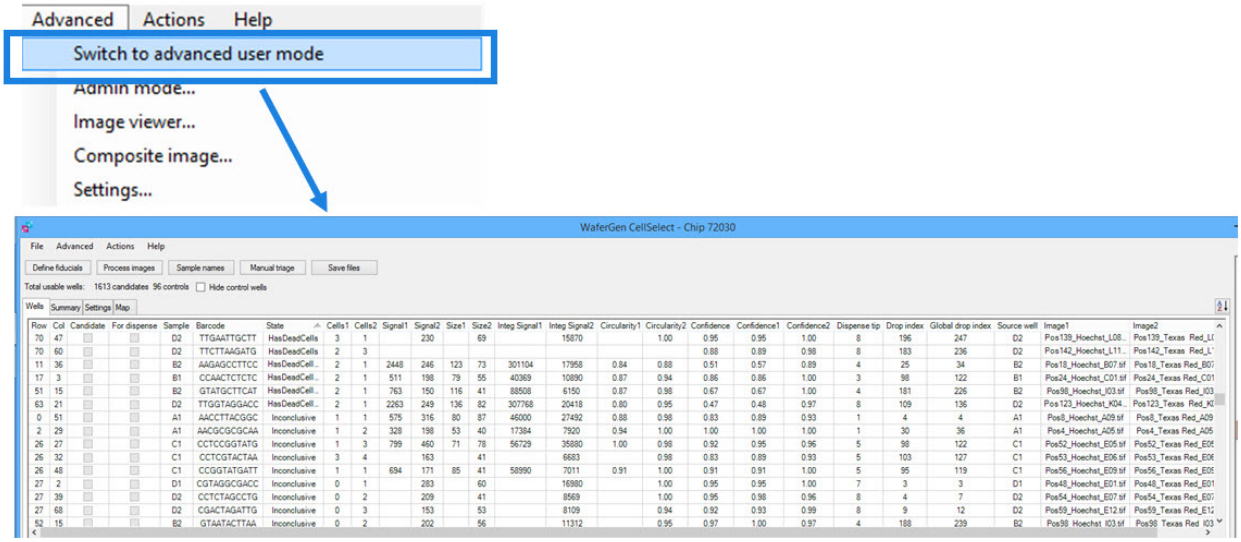


Figure 25. Additional well information in Advanced User Mode.

Summary

The data table on the *Summary* tab displays tallies for controls, samples, and all categories in the results table.

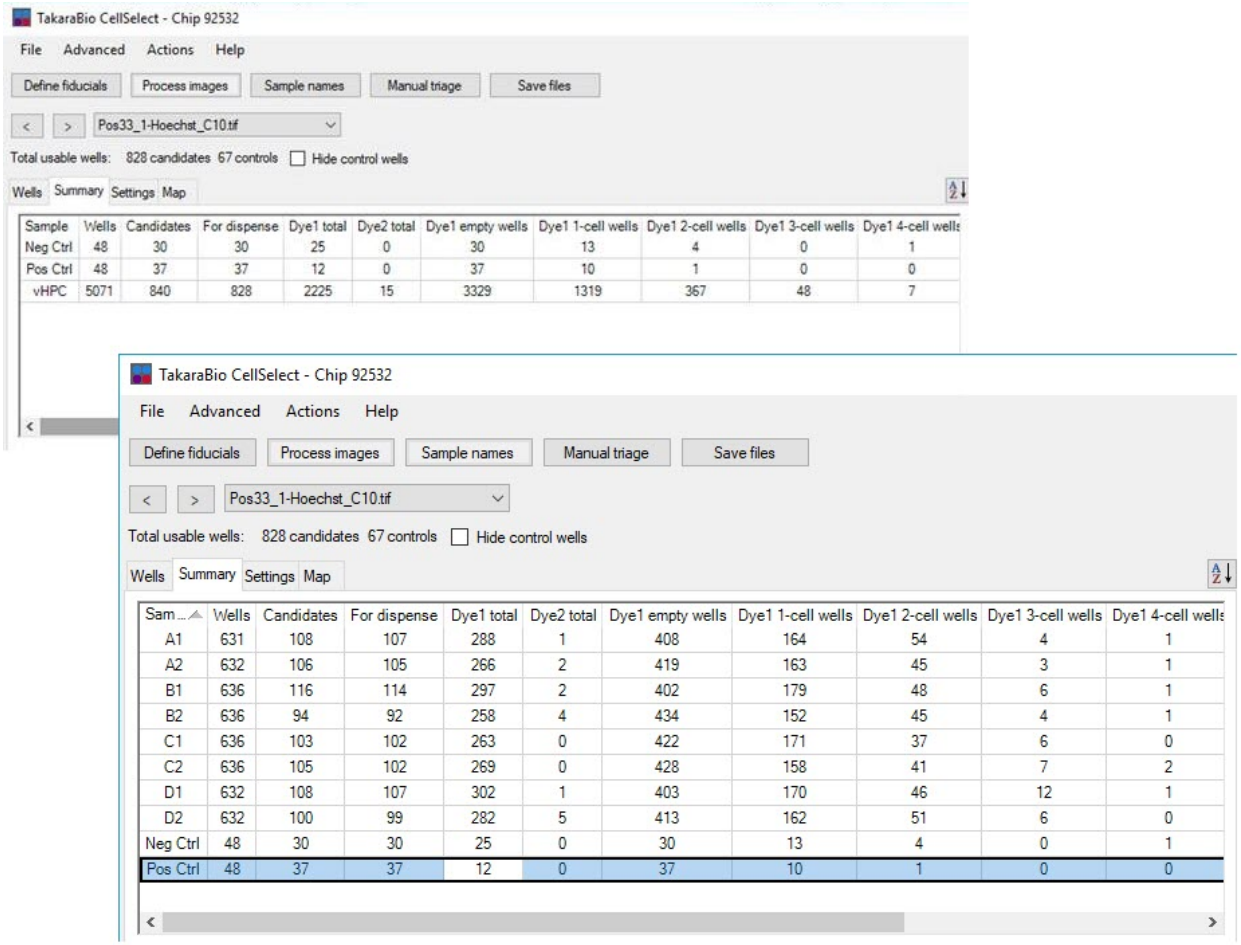


Figure 26. *Summary* data table.

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

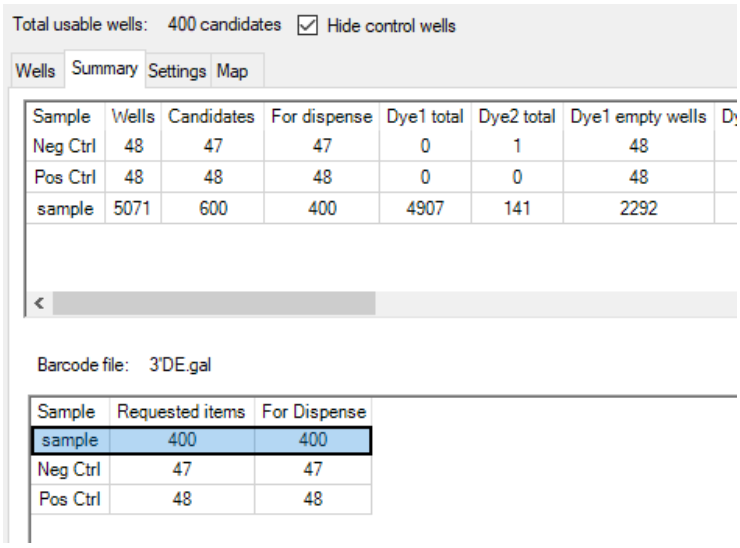


Figure 27. *Summary* data table with barcode filenames and downselect information.

If you have to replicate barcodes, the system may not always be able to satisfy the full number of requested wells without selecting duplicate barcodes. In that case, the number of wells for dispense may be less than what was requested.

NOTE: The Downselect table is only visible if downselect is in effect.

Settings

The following settings have been preconfigured for single-cell analysis. You DO have the option to edit the settings; however, we strongly recommend that you become very familiar with all the setting parameters before editing the file.

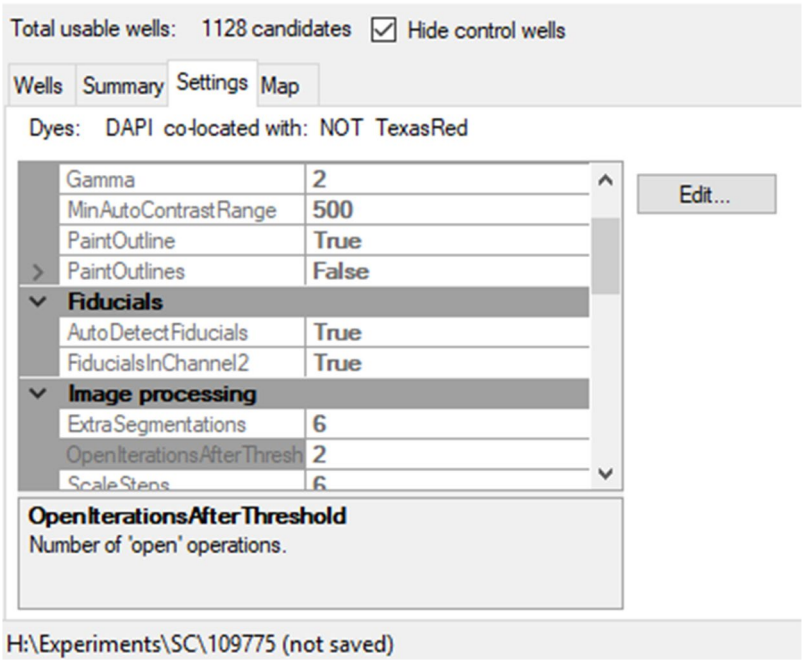


Figure 28. *Settings* tab.

If you edit the Settings file and wish to return to the standard settings for single-cell analysis:

1. Select **File** > **Load** (see Figure 29, below).
2. Select the appropriate XML file for the type of ICELL8 chip being used.

Table 3. Chip XML file selections.

Chip type	Cat. No.	XML file name
ICELL8 250v Chip	640183	`AnalysisSetting_250nL_chip.xml`
ICELL8 350v Chip	640019	`AnalysisSetting_350nL_chip.xml`

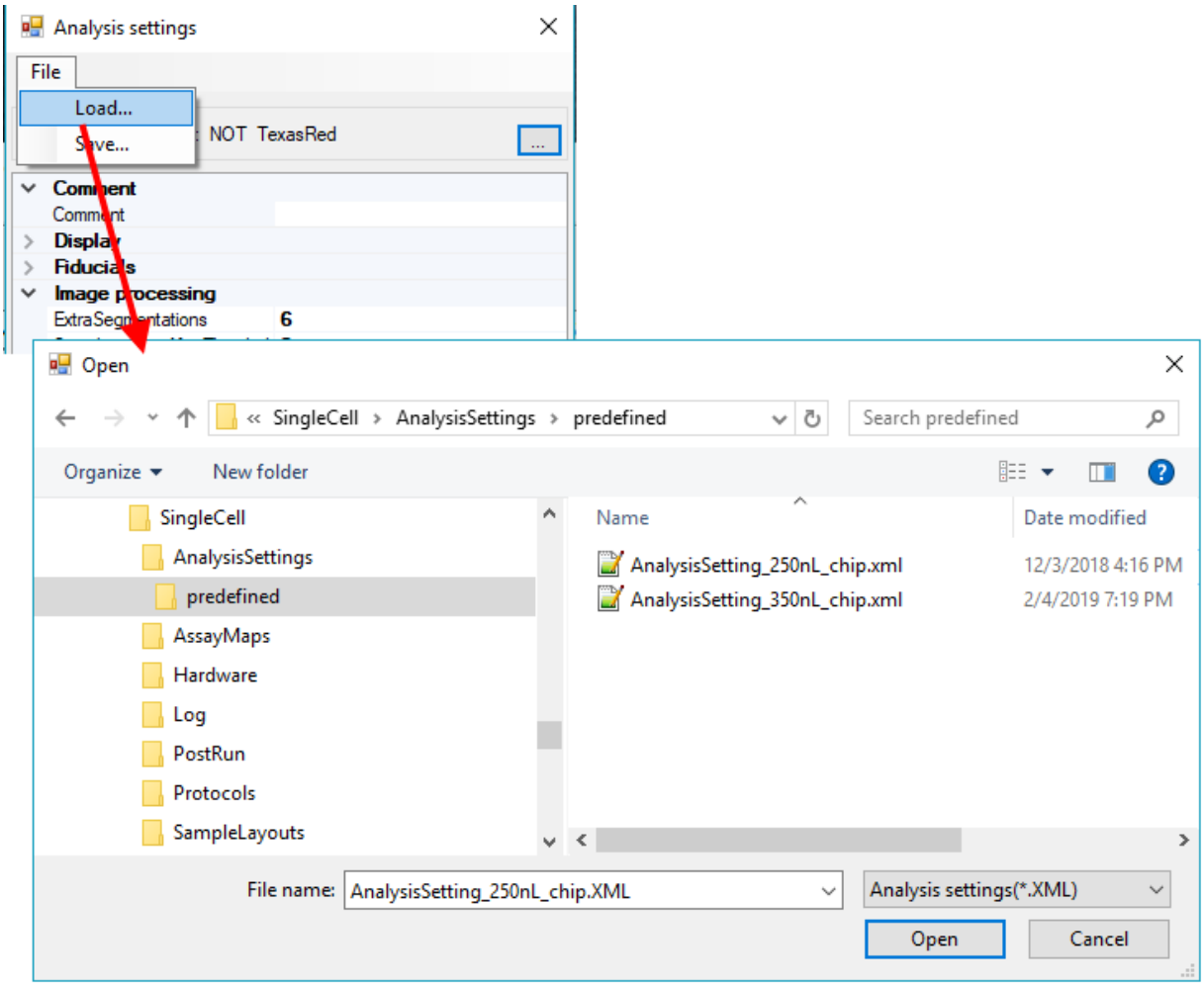


Figure 29. Loading/reloading the settings file for single-cell analysis.

The distinction between live and dead cells is defined in the Dye Assignment dialogue box. Here you select what constitutes a "good" cell.

1. Select the **Master** dye used to identify cells.
2. If necessary, check the **Invert Dye2** check box to indicate that candidate cells should not be visible in the second dye.
3. The bottom of the box will display your final rule. The screenshot below (Figure 30) indicates that we want objects that are visible in FITC, but invisible in Texas Red.
4. Click [OK].

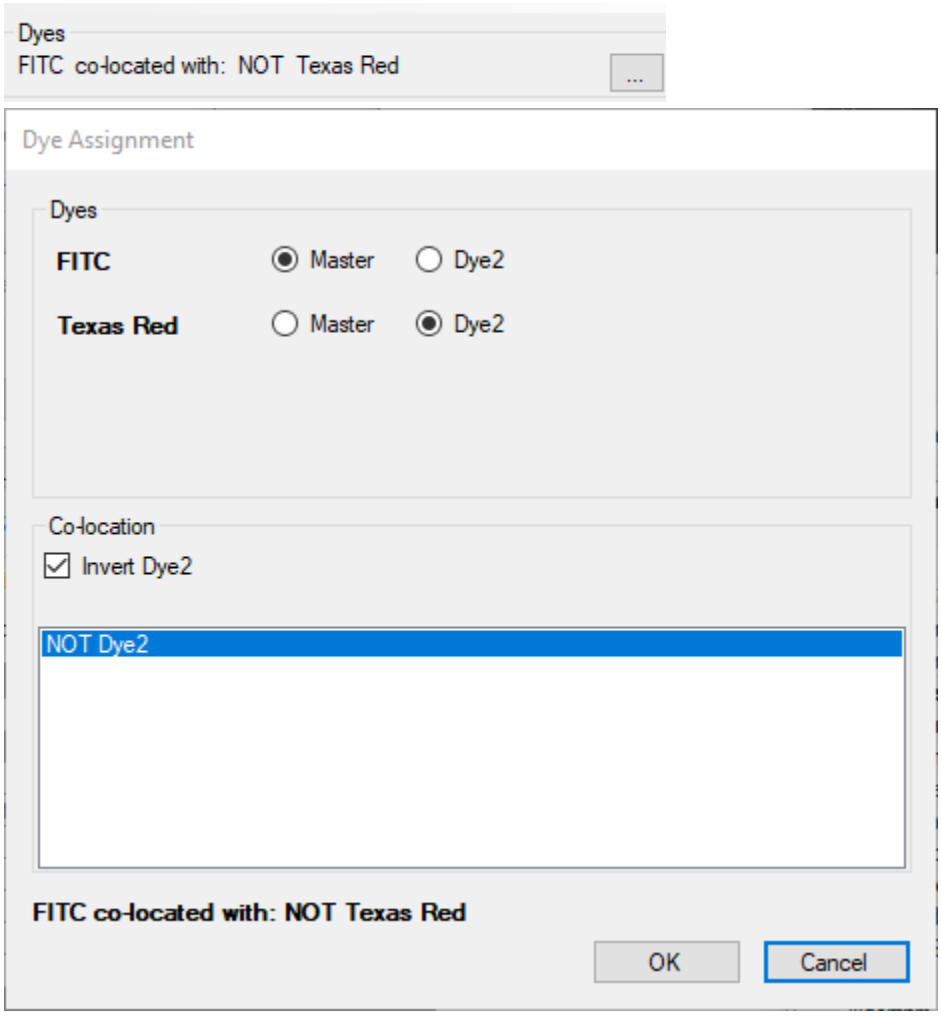


Figure 30. Dye Assignment dialogue box.

Back in the *Settings* window, you can set PaintOutlines, Thresholds, and MinDistanceFromWellCenter for the two dyes.

1. Expand the item to edit the individual values.

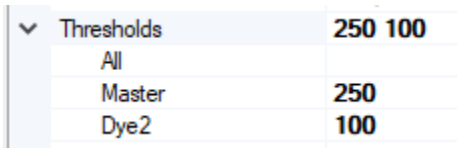


Figure 31. Expanded "Thresholds" option in the *Settings* window.

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

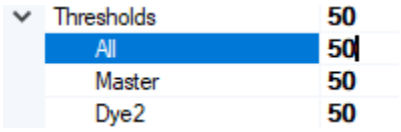


Figure 32. Selecting the *Settings,* "Thresholds", "All" option to customize it.

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

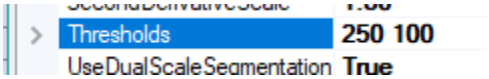


Figure 33. The *Settings* "Threshold" section, displaying multiple sub-level values in the collapsed view.

You can also access settings for **ScaleSteps** and **SecondDerivativeScale**.

1. Cells of significantly different sizes can be detected using the scale space approach (https://en.wikipedia.org/wiki/Scale_space).
2. The **SecondDerivativeScale** value indicates the filter kernel size for the first scale level.
3. For each additional **ScaleStep**, the image is blurred with a Gaussian kernel of radius sqrt(2).

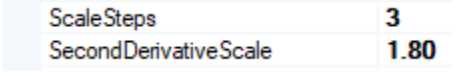


Figure 34. The "ScaleSteps" and "SecondDerivativeScale" options in *Settings*.

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 35, below).

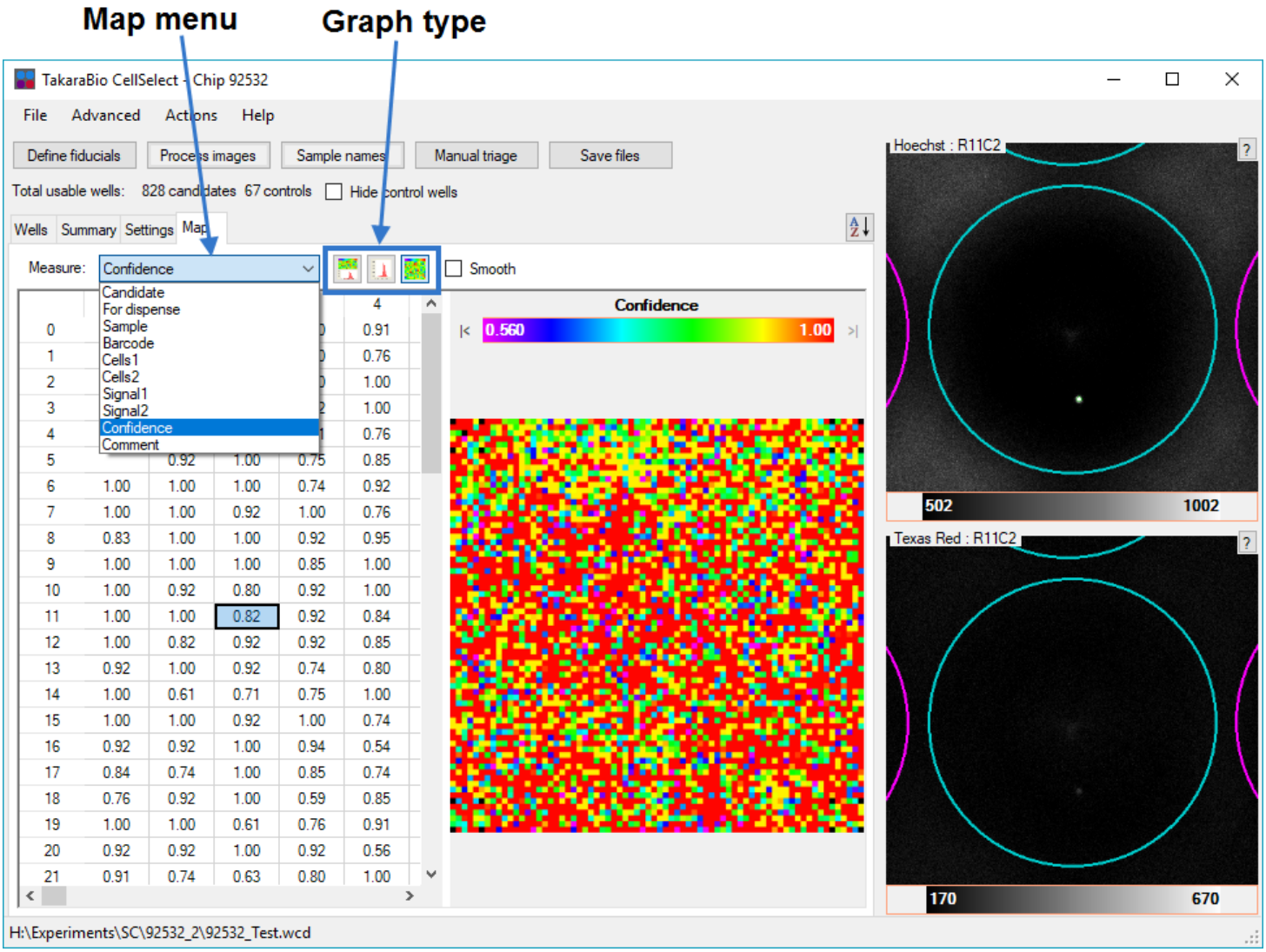


Figure 35. *Map* tab.

F. Well Images

Top view: The top view shows cells that have absorbed Hoechst stain, which indicates the presence of one or more live cells—given that the cell(s) match the designated parameters. A live cell will not absorb propidium iodide and therefore will not appear in the bottom view. A good candidate is a single cell that fits the shape and size parameters of a live cell and appears in the top view only.

Bottom view: The bottom view shows cells that have absorbed propidium iodide, which occurs if a cell is dead. Cells that appear in the bottom view, or both the top and bottom views, are either dead or an artifact (considered inconclusive).

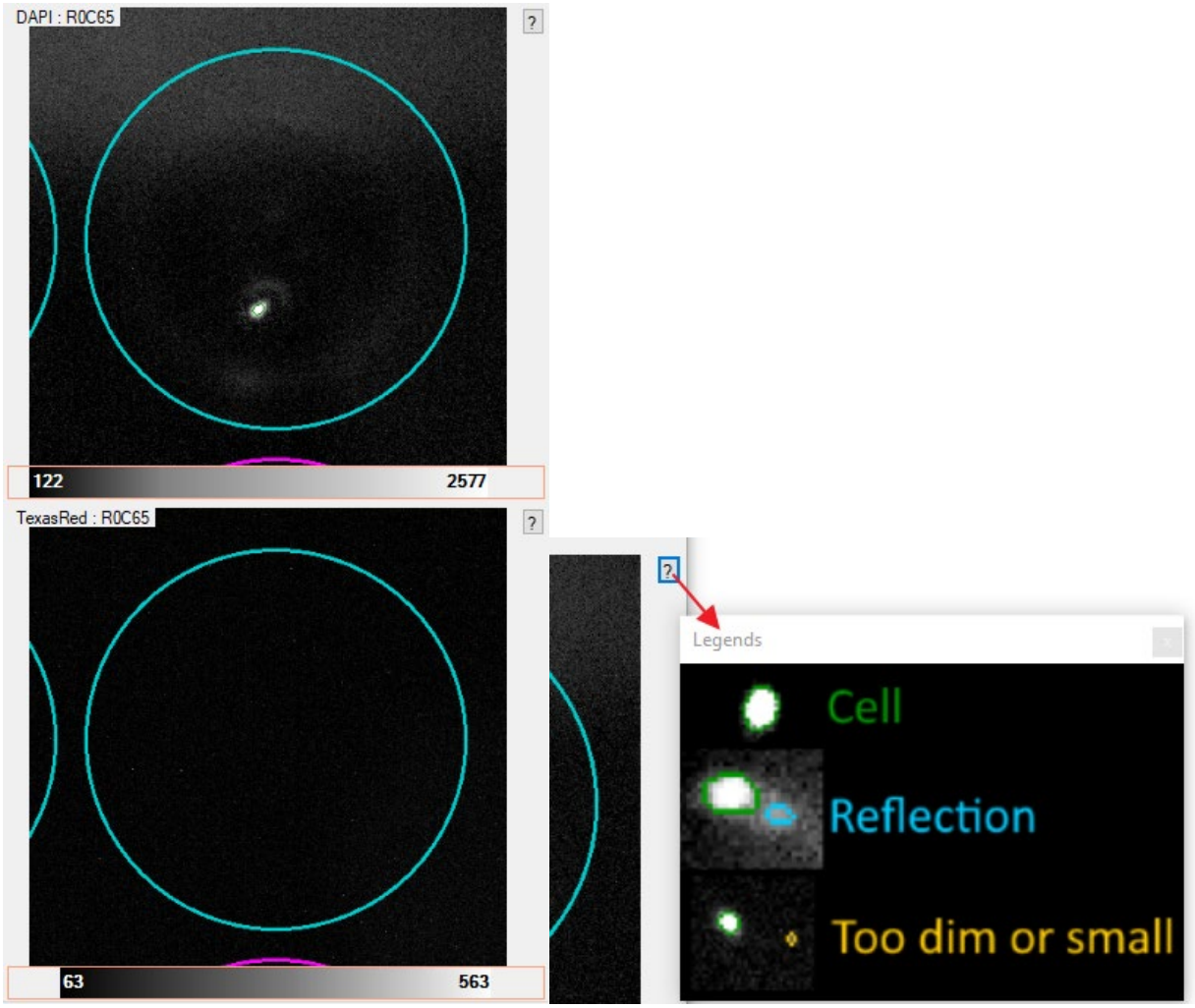


Figure 36. Single-well image.

G. Image Viewer

The *Image Viewer* window is accessible via the **Advanced** dropdown menu in Analysis mode and displays multi-well images (6 x 6 nanowells).

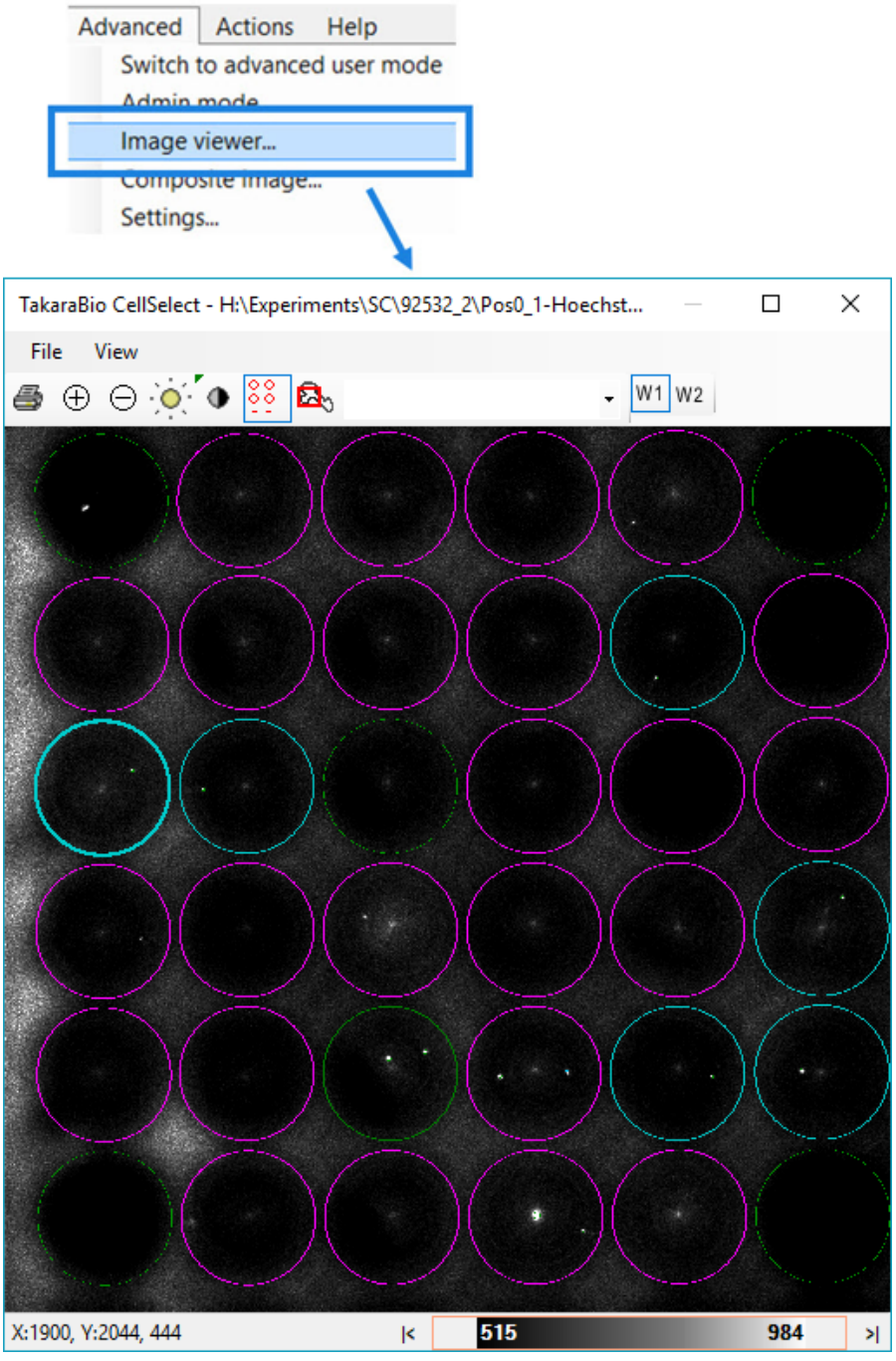


Figure 37. ***Image Viewer*** **window.**

Image Viewer Toolbar Icons

Figure 38. *Image Viewer* toolbar icons.

Zoom Icons

The **Zoom** icons are for increasing or decreasing the size of the multi-well image. Clicking the **Zoom in** icon 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 resize the image is to press the **Ctrl** key while turning the scroll wheel on the mouse.

Image Contrast Icon

Fluorescence images generated by the 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 39, below). Drag the control points with the mouse to adjust the mapping.

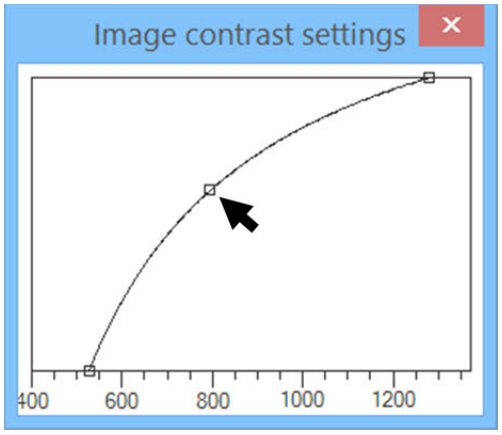


Figure 39. *Image contrast settings* window.

Image Contrast Slider

In addition to the **Contrast** icon on the *Image Viewer* toolbar, the slider at the bottom of each single- and multi-well image can be used to adjust image contrast (see Figure 40, 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. 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.

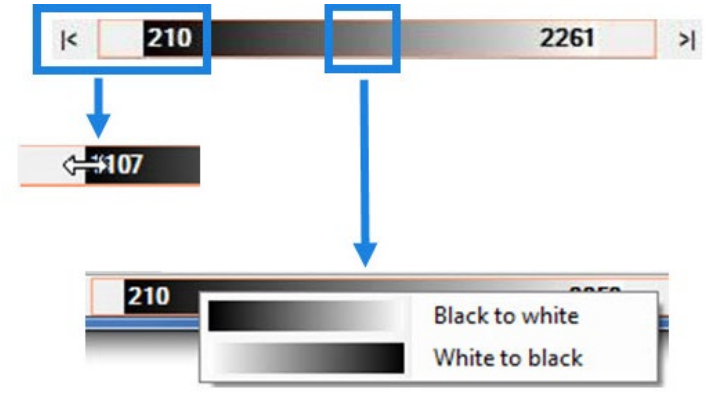


Figure 40. Adjusting image contrast using the slider.

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.

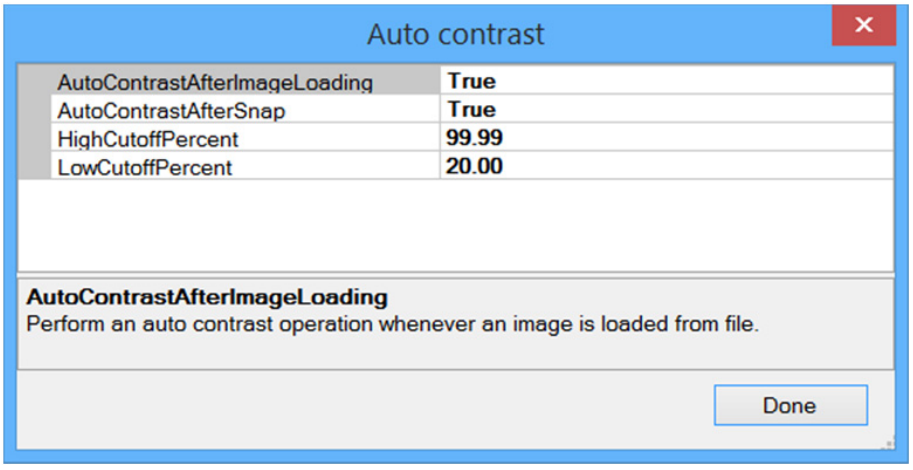


Figure 41. *Auto contrast settings* window.

Table 4. Auto Contrast Settings.

Option	Function
AutoContrastAfterImageLoading	Perform an auto contrast operation whenever an image is loaded from a file.
AutoContrastAfterSnap	Perform 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.

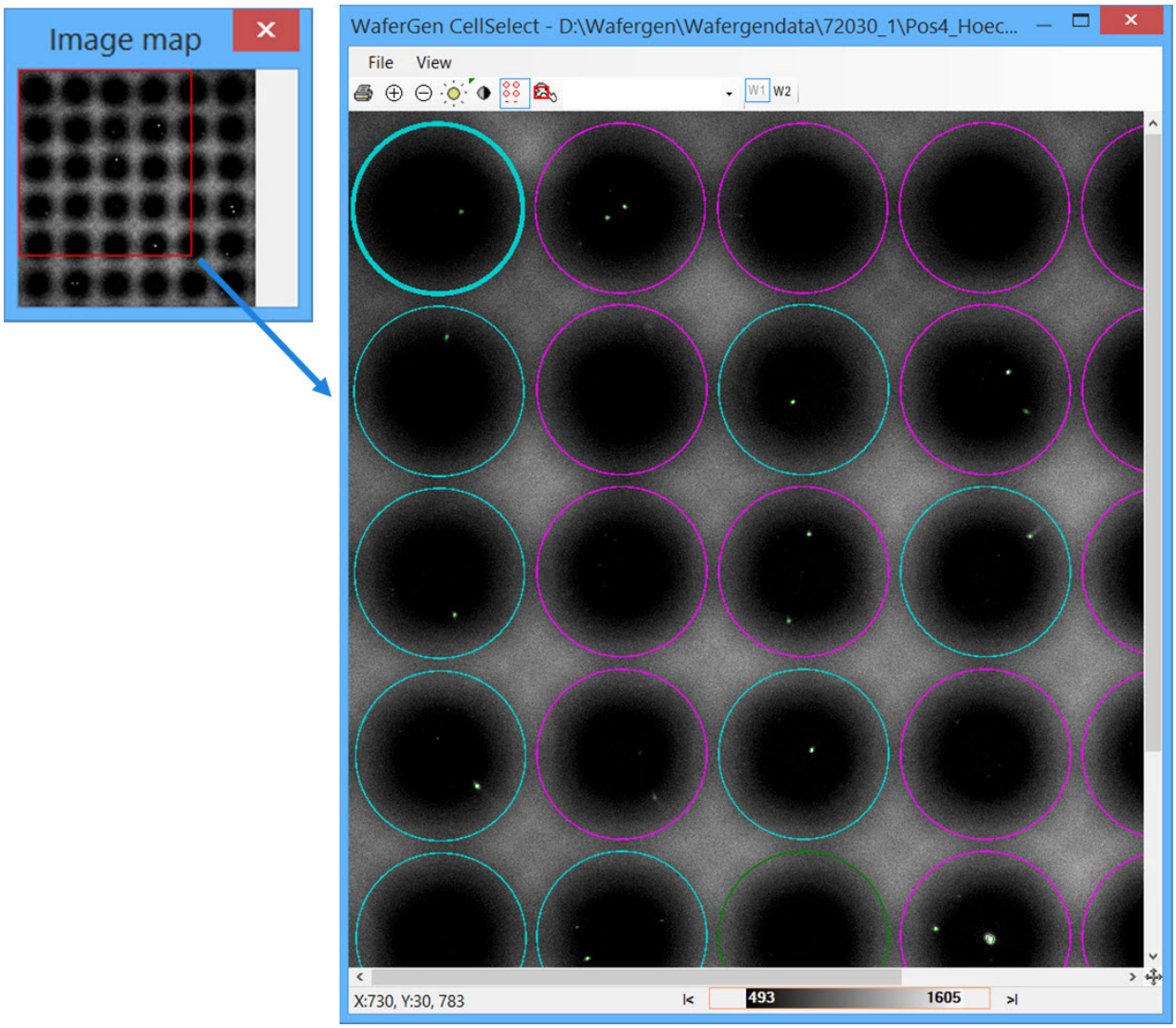


Figure 42. Viewing a subsection of an image using the Map window icon and *Image map* window.

H. 3-D Stack Control

When scanning a chip, the ICELL8 cx system acquires images at different z-planes to capture cells that may not be at the bottom of the well. The cx System software combines (flattens) the images from the various z-planes to create a single image that will be further analyzed.

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

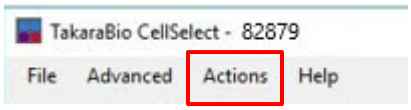


Figure 43. Actions menu location in the CellSelect user interface.

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

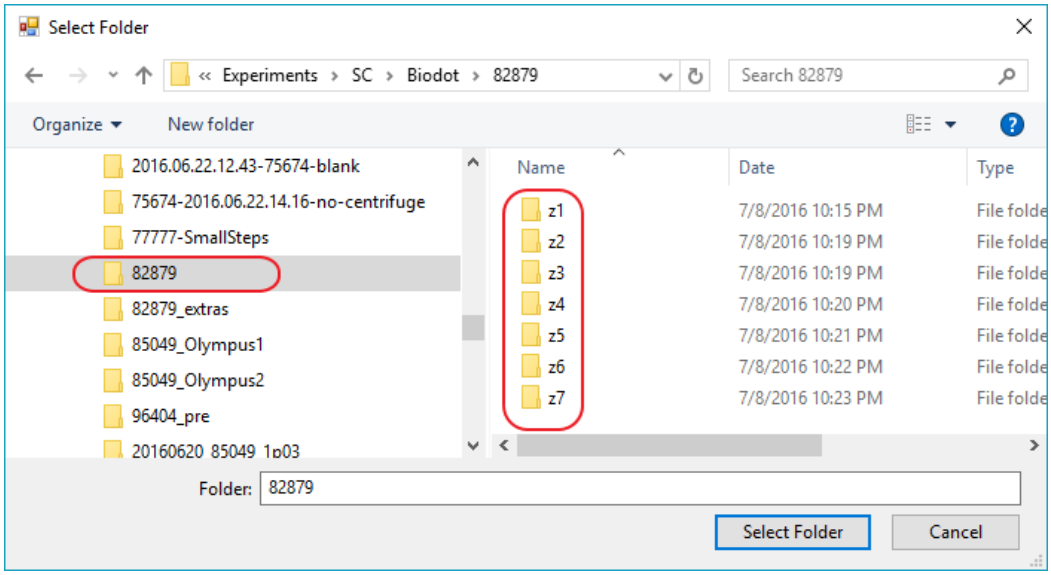


Figure 44. Example of the location of the z-plane images sub-folders, shown in Windows Explorer. 82879 is the ID of the chip and therefore the chip folder; folders z1–z7 are the image sub-folders.

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, if needed. Click [Ok] to proceed; the *3D Stack Control* dialogue window will pop up.

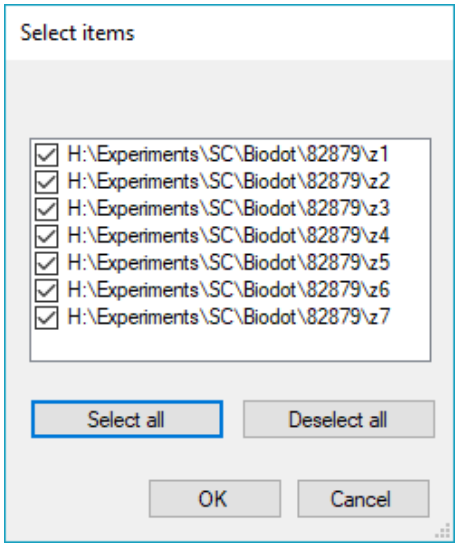


Figure 45. Selection window for inclusion/exclusion of the z-plane images sub-folders.

3D Stack Control dialogue window

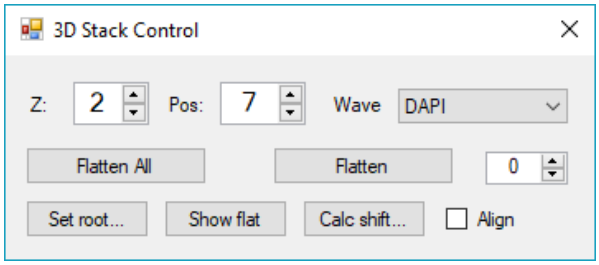


Figure 46. Close-up of the *3D Stack Control* dialogue window.

Table 5. *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 DAPI or Texas Red channels.
Flatten All	Flatten all images and save the resulting images in the parent (root) folder. **IMPORTANT:** Existing images in the root folder will be overwritten.
Flatten	Flatten the z-plane images for the current position (“Pos”) and display the resulting image.
Set root…	Re-loads 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.

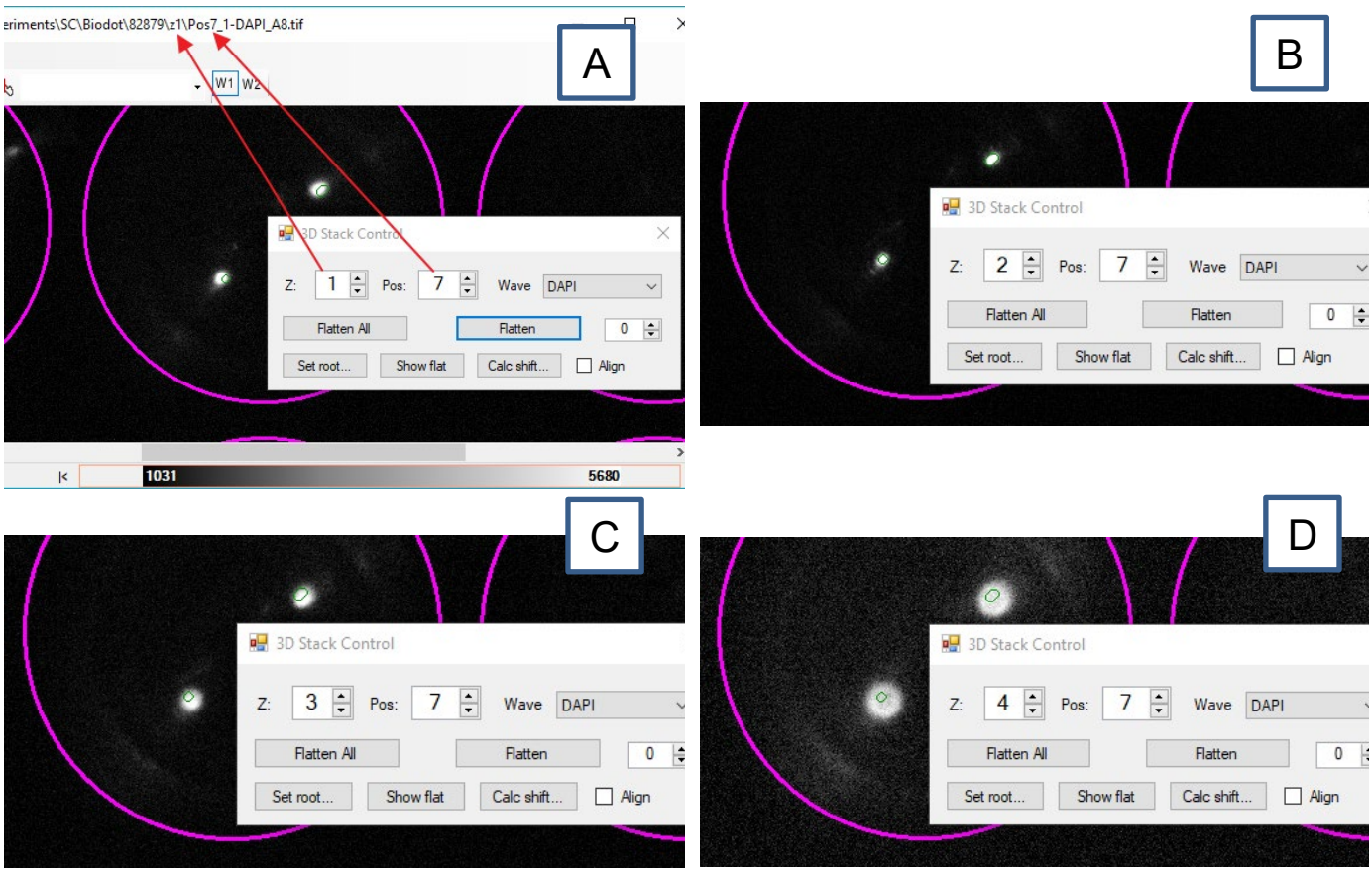


Figure 47. Example of viewing the same well through its z-planes. From A–D, the image cycles through layers 1–4, showing a different view at each layer.

Image shift

A problem that sometimes occurs is 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.

NOTE: During a chip scan, the images are flattened by the cx instrument software, not CellSelect. Therefore, the "AlignImagesBeforeFlattening" option is also present in the cx software.

If you've upgraded from a previous version of the CellSelect cx software, the option can be turned off (set 'False') to maintain consistency with previous scan results. For the 3' DE / TCR application, it is off by default.

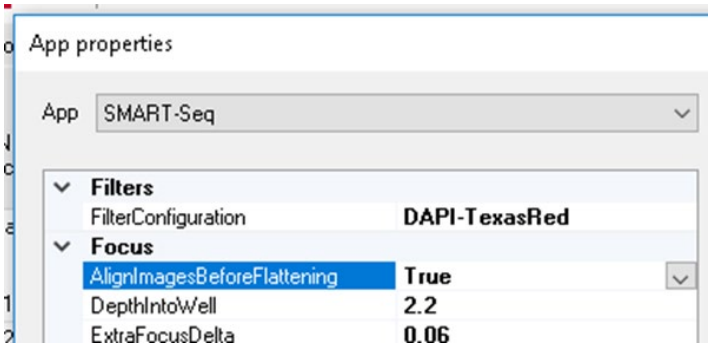


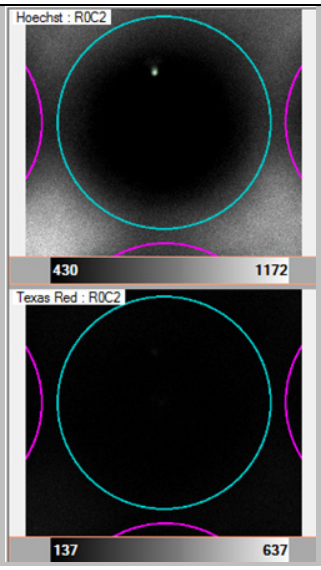
Figure 48. The "AlignImagesBeforeFlattening" Focus option in the ICELL8 cx System software.

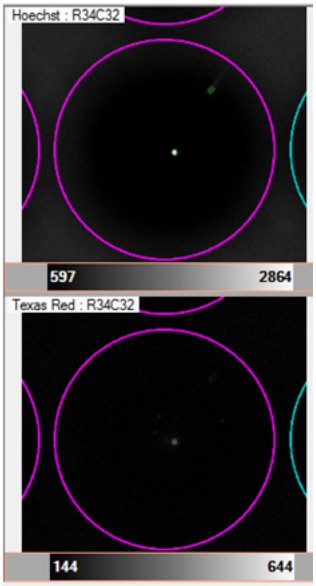
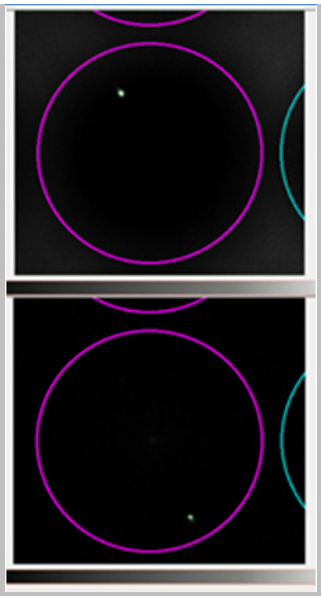
IMPORTANT: If the "Align" button toggle is changed, you will need to click [Flatten All] to re-generate the flattened images.

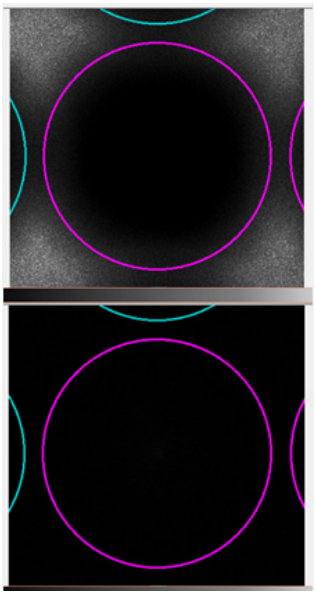
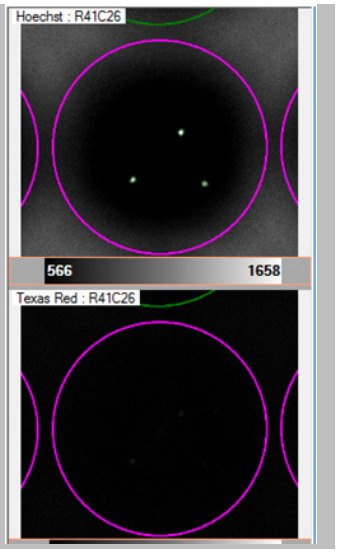
Appendix: Status Table

Top view: Hoechst staining indicates the presence of a live cell, provided that the object meets established size and shape parameters and does not appear in the bottom view.
Bottom view: Propidium iodide staining indicates the presence of a dead cell, a well bottom, or an artifact.

Table 6. Nanowell Status Descriptions and Sample Images.

State	Conditions (all conditions must be met)	Image Example
Good	Single cell that meets the designated parameters appears in the top view (DAPI filter), but not in the bottom view (Texas Red filter).	

Inconclusive and Has Dead Cells	Cell appears viable in top view but is also visible in bottom view.	
Inconclusive	One cell appears in the top view, one or more cells appear in the bottom view. No cells occupy the same location in both views.	

NoCells	No cells present in either view.	
MultipleCells	Multiple cells appear in the top view, no cells appear in the bottom view.	
Has Dead Cells	At least one cell in the top view has a colocalized signal in bottom view.	

Low Confidence Cell is not clear.

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