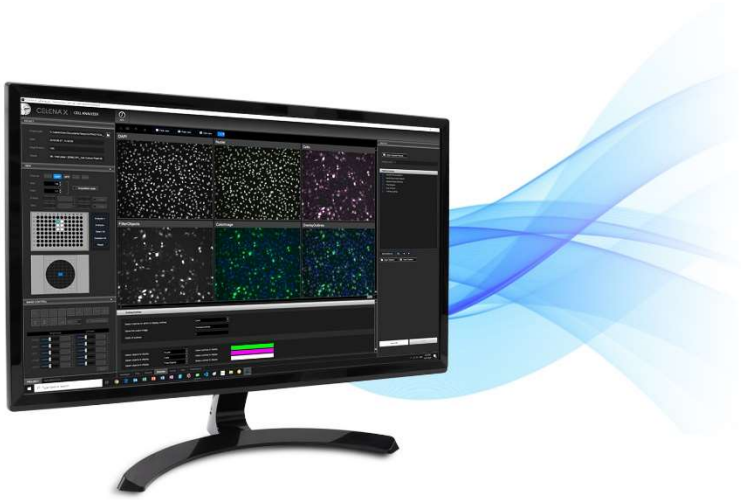
logos biosystems

CELENA® X

CELL ANALYZER



USER MANUAL

PUBLISHING DETAILS
CELENA® X Cell Analyzer, User manual
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DISCLAIMER
The contents of this document are subject to change without notice.
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Table of Contents

1. General

1.1 About this user manual

This user manual provides the information necessary to operate the CELENA® Cell analyzer. **PLEASE READ THIS USER MANUAL BEFORE OPERATING THE SYSTEM.** If any part of this user manual is not clear, please contact Customer Support for assistance.

1.2 Explanation of symbols

Safety notes

Safety notes in this user manual are introduced with a symbol and a signal word to describe the severity of the hazard.
Follow these safety notes and proceed with caution to avoid accidents, personal injury, damage to property

This symbol and the word “WARNING” indicate a possible hazardous situation that, if not avoided, could cause death or serious injury.

This symbol and the word “CATUION” indicate a possible hazardous situation that, if not avoided, could result in minor or moderate injury.

This symbol and the word “NOTICE” indicate a possible hazardous situation that, if not avoid, could result in damage to property or the environment.

Hints, recommendations

This symbol emphasizes useful tips and recommendations as well as information for the efficient and trouble-fee use of the product.

2. Safety information

2. Symbols

2. Safety symbol

Symbol	Description
	Follow instructions for use

General Safety

⚠ WARNING

CELENA® X Cell Analyzer does not include functions for diagnosing diseases by analyzing acquired images. Do not use beyond the intended use of Cell Analyzer.

Do not use beyond the intended use of CELENA® X Cell Analyzer. Cell Analyzer is intended to show quantitative analysis results from the acquired images to the user. It does not include a function for diagnosing a disease.

⚠ CAUTION

Do not modify CELENA® X Cell Analyzer without authorization of the manufacturer. Modification can result in performance degradation.

Be aware that the computer is susceptible to malware, viruses, data corruption, and privacy breaches. Work with your IT administrators to protect your computer by following your institution's password and security policies.

Install CELENA® X Cell Analyzer on the computer which meets the system requirement discussed in the 4.3 Installing Cell Analyzer.

Note

CELENA® X Cell Analyzer is distributed in the form of an executable installer like CELENA® X Cell Analyzer Installer(ver.x.x.x).exe. To update or reinstall the software, get the newest installer from Logos Biosystems or local distributors and install it following the same procedures with the installation guide.

CELENA® X Cell Analyzer can be freely installed in user's computers through the executable installer supplied by your distributor or Logos Biosystems, Inc. if it meets the system requirements, but running the software needs a verification key. The verification key must be plugged into the computer on which CELENA® X Cell Analyzer has been installed.
If customers want to purchase additional verification keys, please contact Logos Biosystems or local distributors.

Recommend that you regularly backup or purge your database. A huge data set stored in the computer can affect the performance of your computer.
Logos Biosystems does not provide a guarantee and/or a warranty against data loss on any storage of any computer under any circumstances. Logos Biosystems disclaims any and all liabilities for emotional distress and/or physical and/or monetary damages or losses of any kind caused by loss and/or restoration of the data stored on the storage. Be sure to backup important data at all times.

If you get analysis results that are not what you expected, please contact Logos Biosystems.

Personal safety

Safety guidelines

Read the user manual thoroughly before using the CELENA® X Cell Analyzer.
Keep the user manual in a safe and easily accessible place for future reference.

3. Getting Started

3.1 Product contents

Your CELENA® X Cell Analyzer is composed of the following components:

CELENA® X Cell Analyzer (Software)

Accessories

- Verification Key

3.2 Product description

CELENA® X Cell Analyzer

CELENA® X Cell Analyzer is used to process and analyze images to quantify numerous cellular phenotypes simultaneously. CELENA® X Cell Analyzer also provides tools to edit and annotate images as well as create videos.

CELENA® X Cell Analyzer

CELENA® X Cell Analyzer can load and analyze images only acquired from CELENA® X Explorer.

3.3 Installing Cell Analyzer

Installation prerequisites

Users should log in Windows operating system as an administrator.

System requirements

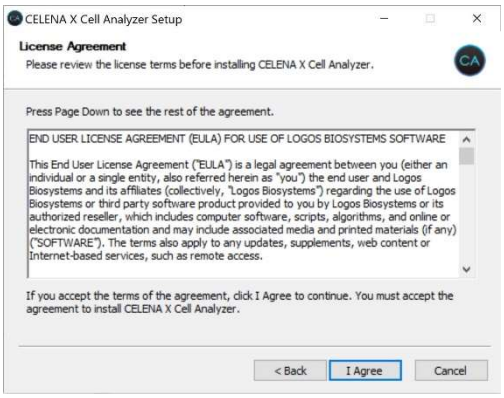
Operating system: Microsoft Windows 10
RAM: 8 Gb or more
CPU: Intel i7 or higher recommended
Hard disk space: at least 10 GB available space
Monitor: 4K UHD resolution (3840 x 2160) required

Installation

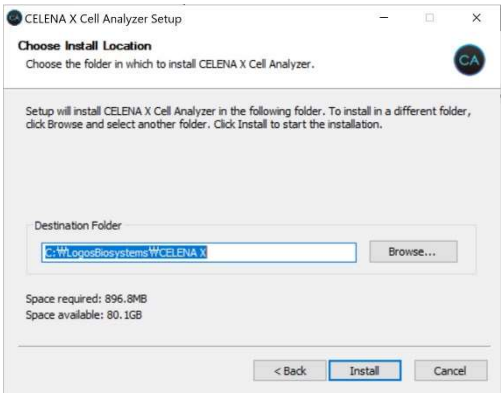
To install the software, follow the steps:

① Execute latest CELENA® X Cell Analyzer Installer(ver.x.x.x) .exe supplied by your distributor or Logos Biosystems, Inc.
② Follow the instructions on your monitor.
③ Click <Next>.

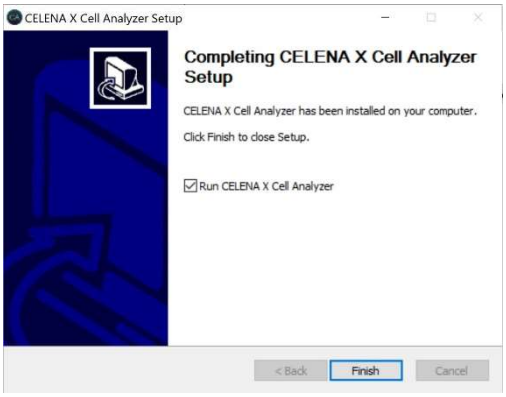
④ Click <I Agree> if you agree the license agreement.



⑤ Click <Install>.



⑥ Click <Finish>.



Remove CELENA® X Cell Analyzer

To remove the CELENA® X Cell Analyzer from your computer, execute "Uninstall Cell Analyzer" in the installed folder or in "Apps & features".

Execute CELENA® X Cell Analyzer

To execute the CELENA® X Cell Analyzer, double-click the icon or select All Programs > CELENA® X Cell Analyzer > Cell Analyzer.

Upgrade CELENA® X Cell Analyzer

Uninstall the previous version of CELENA® X Cell Analyzer.

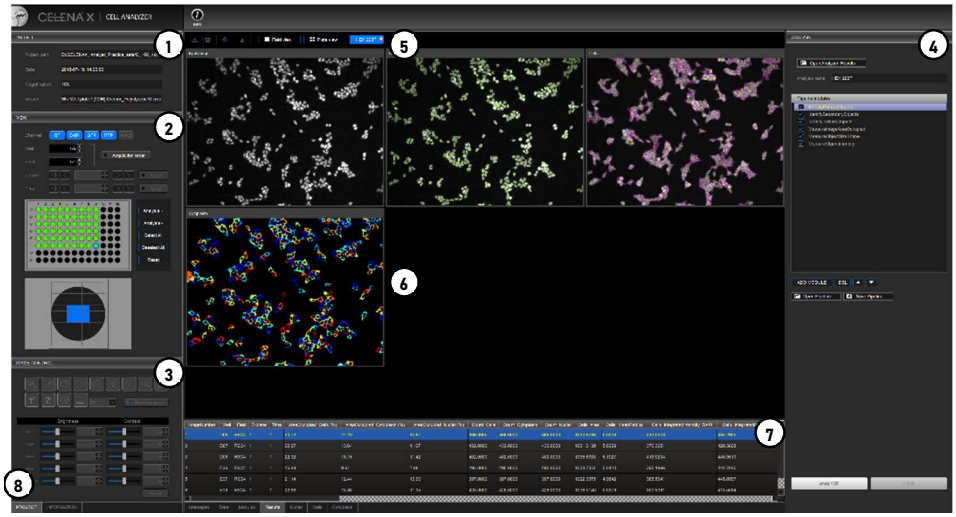
Check if the folder CellAnalyzer_nolock or CellAnalyzer remains.
You can find the folder at Logosbiosystems > Logosbiosystems > CELENA X.
If either of them is left, delete the folder.

Install the new version as instructed above.

4. Overview

4.1 Front page

CELENA® X Cell Analyzer can set up automated image analysis sequences to batch processed images captured on the CELENA® X. CELENA® X Cell Analyzer also provides tools to edit and annotate images as well as create videos. The CELENA® X Cell Analyzer Verification Key must be plugged in to use CELENA® X Cell Analyzer.



CELENA® X Cell Analyzer

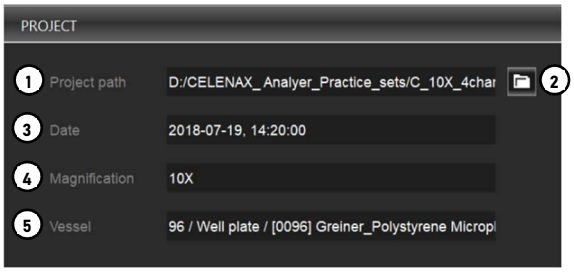
① **PROJECT:** Allows you to load a project for analysis and see project details.
② **VIEW**: Allows you view captured images and select wells for analysis.
③ **IMAGE CONTROL:** Allows you to edit images, add annotations, and make simple measurements.
④ **ANALYSIS:** Allows you to set up, edit, and run analysis pipelines.
⑤ **Toolbar:** Has tools to export images, create videos, and visualize images.
⑥ **Viewing area:** Shows captured and analyzed images.
⑦ **Messages:** Displays system messages, annotation measurement data, module details, and analysis results.

At the bottom of the window(⑧), there is a PROJECT tab and INFORMATION tab.

- **PROJECT:** Shows the PROJECT, VIEW, and IMAGE CONTROL panels.
- **INFORMATION:** Shows a detailed description of the project imaging details.

4.2 Loading a Project

This panel is used to load a project for analysis and displays project details.



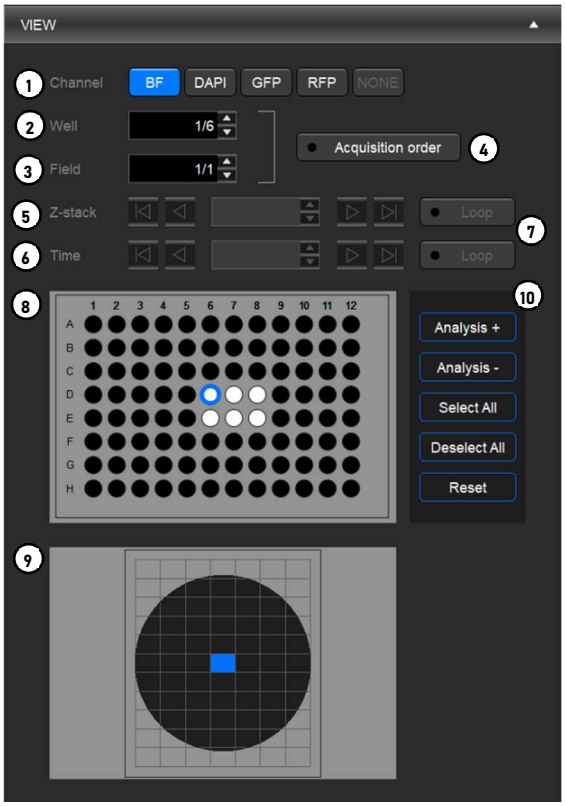
① **Project path:** Shows where the project file and images are located.
② **Folder icon:** Allows you to load a project for analysis.
③ **Date:** Displays the date and time the project was captured.
④ **Magnification:** Displays the objective magnification used for imaging.
⑤ **Vessel:** Displays the sample vessel used.

Note

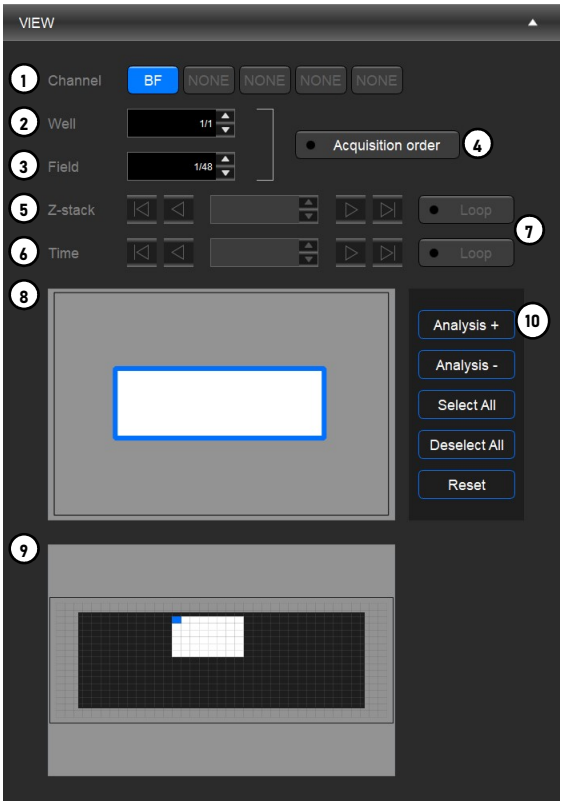
CELENA® X Cell Analyzer can load and analyze images only acquired from CELENA® X Explorer.

4.3 View

This panel allows you to view the captured images and select wells to analyze for microplates.



This panel allows you to view the captured images and to select a slide to analyze.



① **Channel:** Allows you to select which channels to display.
② **Well:** Allows you to select a well or a slide to view.
③ **Field:** Allows you to select a field in the selected well to view.
④ **Acquisition order:** Shows the images in the order they were captured.
⑤ **Z-stack:** Allows you to go through the captured Z-planes (if applicable).
⑥ **Time:** Allows you to go through the sequence of time lapse images (if applicable).
⑦ **Loop:** Sets the images in a loop so images can be cycled through continuously without stopping at the end of the sequence.
⑧ **Vessel map:** Represents the imaged vessel.
⑨ **Well map:** Shows the imaged fields within each well or a slide.
⑩ **Analysis buttons:** Allows you to select wells or a slide for analysis.

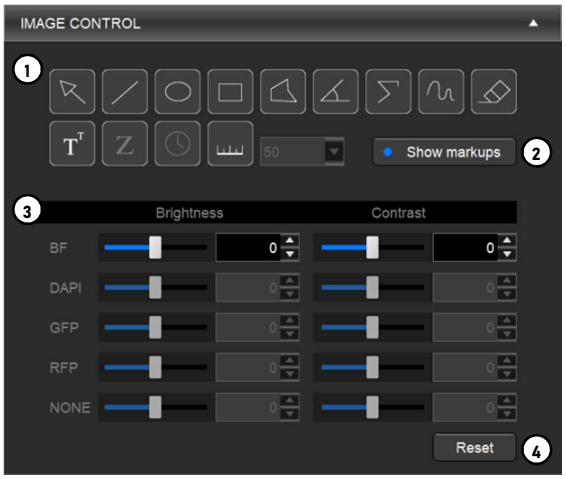
Click a well or a slide and fields to view their corresponding images. The currently displayed well/slide is rimmed in blue and the displayed field is filled with blue.

Analysis buttons (⑩)

- **Analysis +**: Adds wells or a slide to the list of wells to be analyzed.
  Wells to be analyzed will be filled with yellow.
- **Analysis –**: Removes wells or a slide from the list to be analyzed.
  Imaged wells or a slide that are not set to be analyzed will be filled with white.
- **Select All**: Selects all imaged wells or a slide. Selected wells or a slide will be rimmed in blue.
  This only selects the wells or a slide. To add to the analysis list, you must click **Analysis +**.
- **Deselect All**: Deselects all wells or a slide. This only deselects wells or a slide. To remove from the analysis list, you must select the desired well(s) or a slide and click **Analysis -**.
- **Reset**: Clears the list of wells or a slide to be analyzed.

4.4 Image Control

This panel allows you to edit images, add annotations, and make simple measurements.



① **Annotation tools:** Allows you to mark and measure specific areas of interest.
② **Show markups:** Shows or hides annotations.
③ **Editing tools:** Allows you to adjust the brightness and contrast of each channel
④ **Reset:** Resets all image adjustments.

Annotation tools

Select	Line	Ellipse	Rectangle
Polygon	Angle	Segmented line	Freehand
Eraser	Text	Z-position	Time
Scale bar			

Use the select tool to select and manipulate annotations.

Right-click on an annotation to change properties such as color and size as well as to copy, paste, and delete the annotation.

Double-click to deselect the annotation.

Editing tools
Adjust the brightness and contrast of each channel using the respective sliders or text boxes.

To select or deselect channels, use the channel buttons in the VIEW panel.

4.5 Toolbar

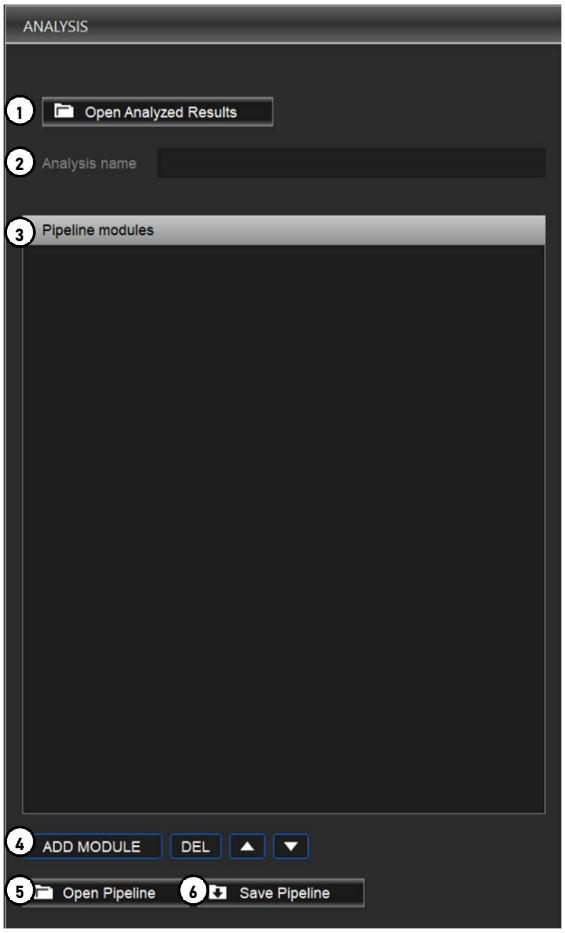
The toolbar has tools to export images, create videos, and visualize images

Export images		Create a video	
Pseudocolor		Histogram / line profile	
Field view		Plate view	

- **Export images:** Allows the export of annotated or edited images.
- **Create a video:** Allows the creation of a video of time lapse or Z-stack images.
- **Pseudocolor:** Shows each channel in its designated pseudocolor.
- **Histogram/line profile:** Displays tonal values of the whole image or a specific annotation.
- **Field view:** Shows a single field in the viewing area.
- **Plate view:** Shows the captured fields laid out according to their location in the vessel in the viewing area.

4.6 Analysis

This panel allows you to set up, edit, and run analysis pipelines.



① **Open analyzed results:** Allows you open a previously analyzed project (*.cxasis*).
② **Analysis name:** Shows you the analysis name.
③ **Pipeline modules:** Shows you the modules in the pipeline.
④ **Module buttons:** Allows you to add, delete, or rearrange pipeline modules.
⑤ **Open pipeline:** Allows you to select a previously saved pipeline.
⑥ **Save pipeline:** Allows you to save a newly created or edited pipeline.

4.7 Messages

This panel is used to display system messages, annotation measurement data, module details, and analysis results. You can resize the message panel by dragging the top border.

There are four tabs: Messages, Data, Modules, and Results. Upon analysis, additional tabs will appear for each analyzed object.

Messages

This tab shows the analysis process.

The text can be copied for troubleshooting.

- To copy all the messages, right-click inside the message panel and click **Select All** from the context menu. Right-click the selection and select **Copy** from the context menu. The selection is copied and can be pasted as desired.
- To copy a specific message, select the desired message and right-click the selection. Select **Copy** from the context menu. The selection is copied and can be pasted as desired.

Data

This tab shows the values for measurements made with the annotation tools in the IMAGE CONTROL panel.

Data will appear in this tab as you mark specific areas of interest with the annotation tools.

- To see the location of an annotation, select the annotation from the list.
- To delete a specific measurement, select it and right-click to select **Clear**.
- To delete all data, right-click and select **Clear All**.
- To export measurement data as a CSV file, select the data to export and right-click to select **Export CSV**.

Modules

This tab allows you to adjust pipeline modules as needed.

Select a module in the pipeline module list in the ANALAYSIS panel to show module parameters.

For a complete list of available modules, go to 7.3 Pipeline module reference.

Results

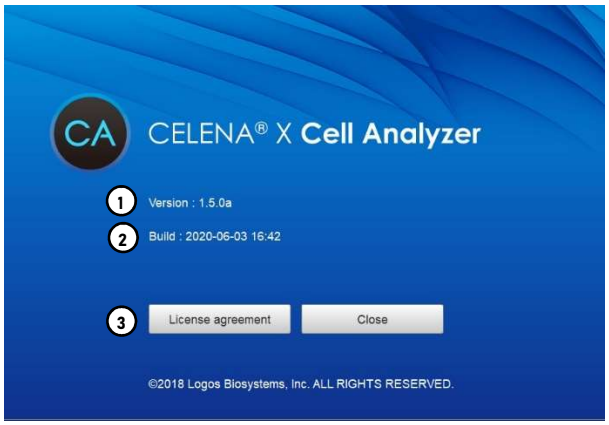
This tab allows you to examine analysis results.

4.8 Information

This section contains information about software version and date, the end user license agreement (EULA).

Click the INFO icon 

at the top of the screen to display the About window as below.



① **Software version:** Shows the current software version.
② **Build:** Shows the date and the time of build.
③ **License agreement:** Shows the end user license agreement (EULA).

5. Annotation and Measurement

5.1 Workflow

Annotate images and make simple measurements

1. **Open project**

   Make sure the project folder created by CELENA® X Explorer is on your computer.

   Click the folder icon next to Project path.

   Select a *.cxproj* file. This loads the project file, which contains a list of the project image files, the file locations, and the associated metadata.

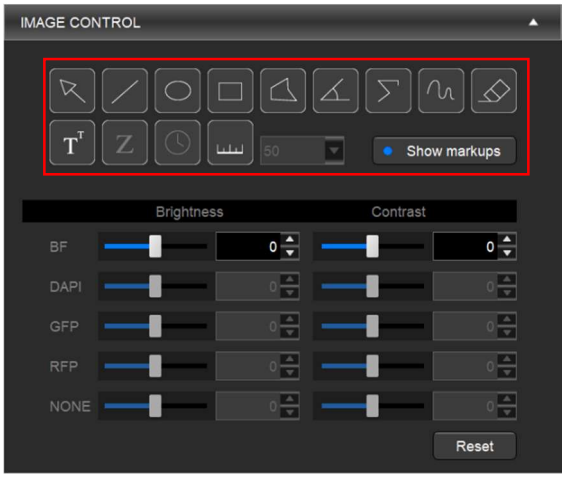
2. **Select image**

   Use the VIEW panel to go through images captured.

   Select the desired image.

3. **Add annotations**

   Use the annotation tools in the IMAGE CONTROL panel to add annotations and make simple measurements.



   Click the Data tab in the messages panel to show the data panel. There will be a table that displays all the measurements related to each annotation.

4. **Export**

   (Optional) To export measurement data, select the desired measurement(s), right-click, and click **Export CSV**.

   (Optional) To save the annotated image, click the export images icon in the toolbar.

Edit images

1. **Open project**

   Make sure the project folder created by CELENA® X Explorer is on your computer.

   Click the folder icon next to Project path.

   Select a *.cxproj* file. This loads the project file, which contains a list of the project image files, the file locations, and the associated metadata. The project images and metadata will be loaded.

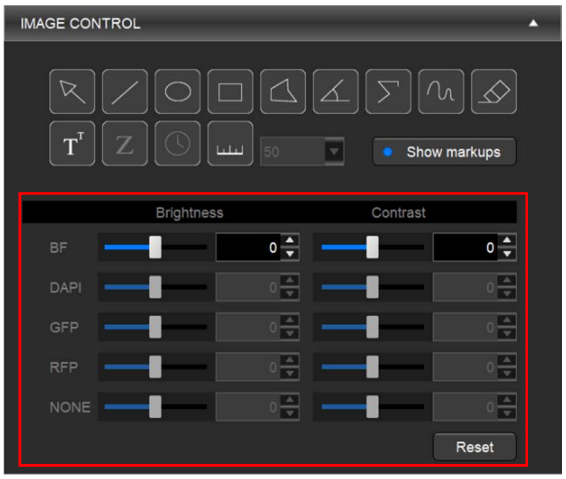
2. **Select image**

   Use the VIEW panel to go through images captured.

   Select the desired image.

3. **Edit image**

   Use the IMAGE CONTROL panel to adjust the brightness and contrast of each channel.



In the VIEW panel, select the desired channels to display.

Adjust the brightness and the contrast of each channel using the respective sliders or the text boxes.

To undo image adjustments, click **Reset**.

To save the edited images, click the export images icon in the toolbar.

4. Export

(Optional) To save the annotated image, click the export images icon in the toolbar.

5.2 Annotation tools

Cell Analyzer supplies several annotation tools as below.

Annotation tools

Select	Line	Ellipse	Rectangle
Polygon	Angle	Segmented line	Freehand
Eraser	Text	Z-position	Time
Scale bar			

Use the Select tool to select and manipulate annotations.
Double-click outside the annotation to deselect the annotation.

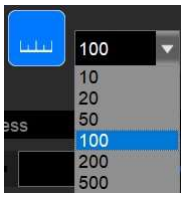
Right-click on an annotation to change properties such as color and size as well as to copy, paste, and delete the annotation.

Use text icons like , or to add a text message, a timestamp or a relative position on Z-axis.

Show or hide annotations in the image by selecting or deselecting “Show markups” button.

5.3 Scale bar

Select scale bar icon to add a scale bar in the image and change its size by selecting the scale in the combo box as below.

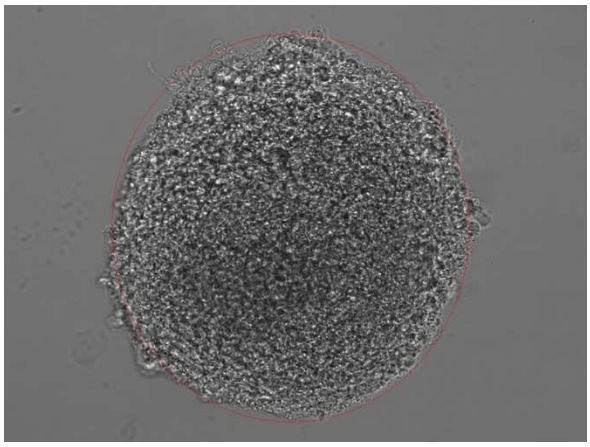


Right-click on a scale bar to change properties such as color, scale, font size, line width and position.

Show or hide a scale bar in the image by selecting or deselecting the scale bar icon.

5.4 Measurement

To add an annotation tool and measure values select one of annotation tools in the IMAGE CONTROL box and drag and drop the tool in the image.



The measured results are shown in Data tab as below.

	Tool	Length (µm)	Area (µm²)	Angle (°)	Pixels	Min (BF)	Max (BF)	Mean (BF)	Std (BF)
1	Ellipse	1739.7	241020.4	-	701873	22.0	255.0	97.6	33.4
2	Line	547.6	-	-	934	28.0	238.0	86.6	33.3

In this example of Ellipse the Length means the peripheral length of the ellipse, the Area means the inner area of the ellipse, the Pixels are the number of pixels of the inner ellipse, Min(BF), Max(BF), Mean(BF) and Std(BF) are the minimum intensity, the maximum intensity, the mean intensity and the standard deviation of the intensity in BF channel, respectively.

The highlight in blue indicates the measurement result of the currently selected annotation tool.

Right-click on the Results tab to delete one or all measured results or to export data as a CSV file.

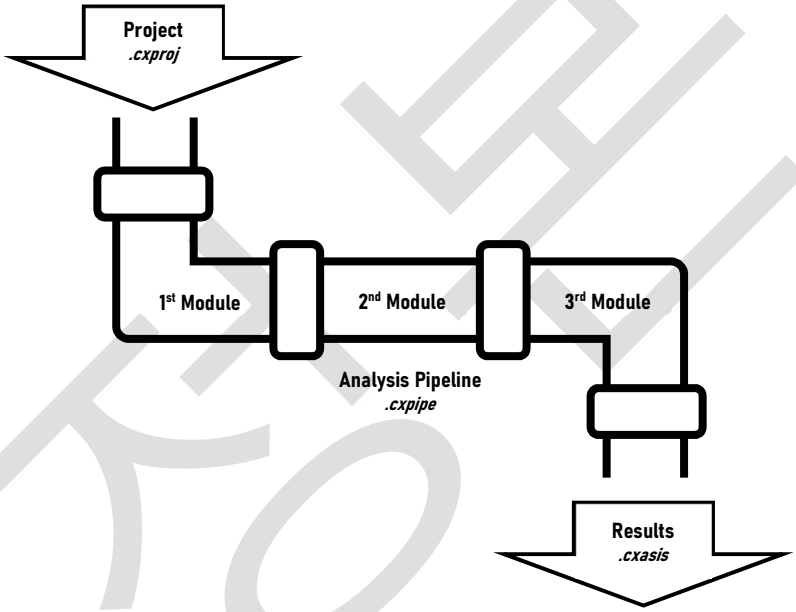
6. Analysis Modules

6.1 Workflow

Set up project analysis

Overview

Using CELENA® X Cell Analyzer, users can create an image analysis pipeline, which is a sequence of modules that each performs a specific image processing task. This allows the quantitative analysis of multiple cellular features from images. Modules can be mixed, matched, and adjusted to measure phenotypes of interest quantitatively. Once a pipeline has been established, it can analyze subsequent projects.

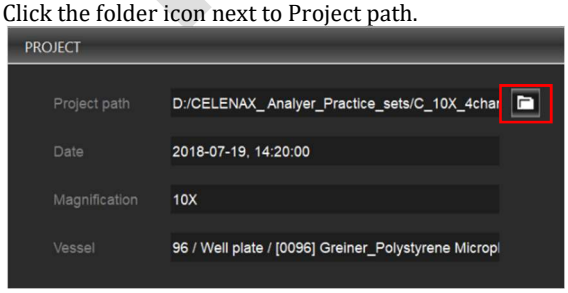


Open project.
▼
Select wells to analyze.
▼
Open/create/modify pipeline.
▼
Analyze images.

1. Open project

Make sure the project folder created by CELENA® X Explorer is on your computer.

Click the folder icon next to Project path.



Select a *.cxproj* file. This loads the project file, which contains a list of the project image files, the file locations, and the associated metadata. The project images and metadata will be loaded.

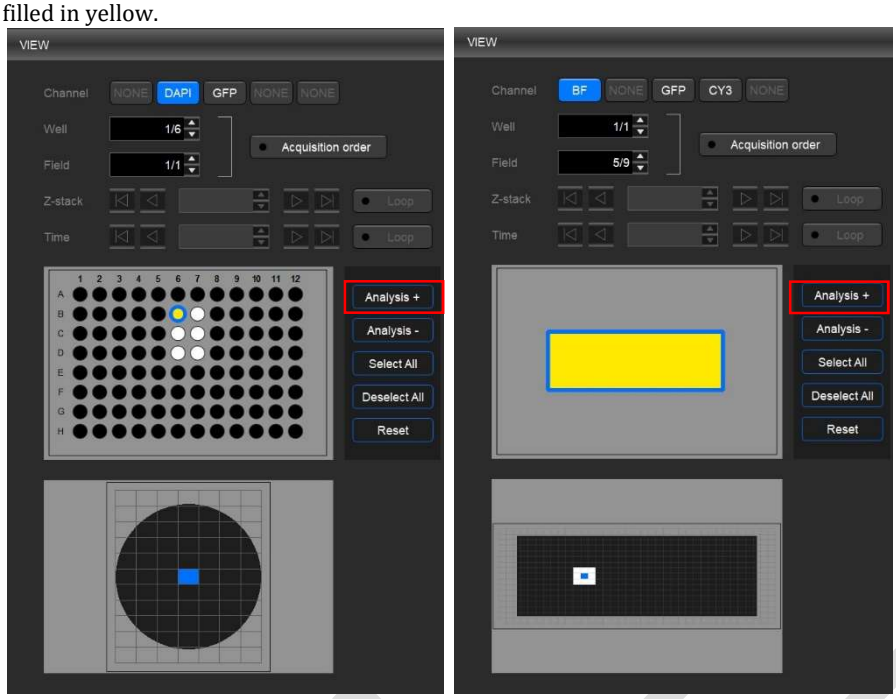
Note

Make sure the images were captured with the monochrome camera or covert color images to grayscale by using ColorToGray module. Pipelines to analyze images require them to be in grayscale.

2. **Select wells to analyze**

Use the VIEW panel to go through images captured.

Select the wells or a slide to be analyzed and click **Analysis +**. Wells programmed for analysis will be filled in yellow.

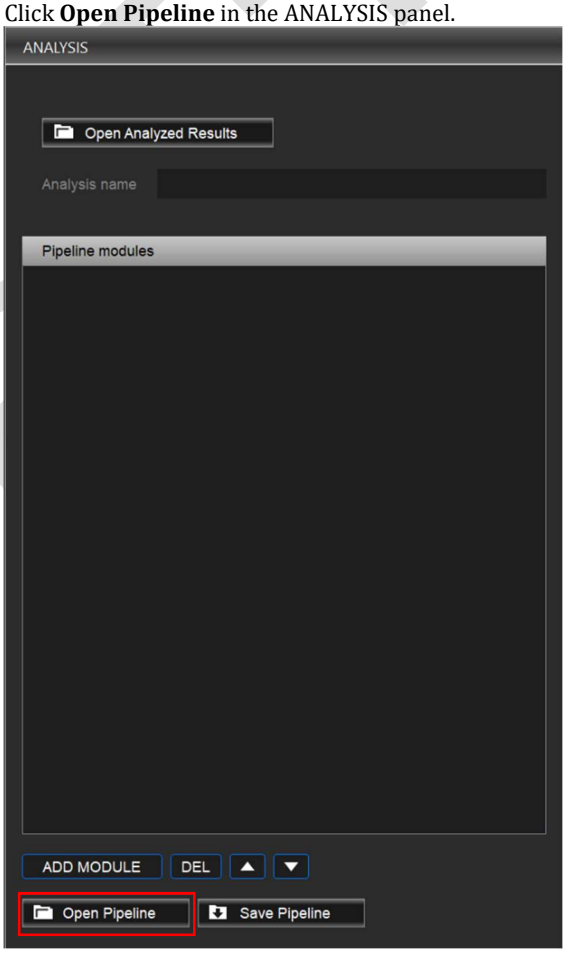


3. **Select a pipeline**

Select a previously saved pipeline or create a new pipeline by using the modules located in the pipeline window.

Open a pipeline

- Click **Open Pipeline** in the ANALYSIS panel.

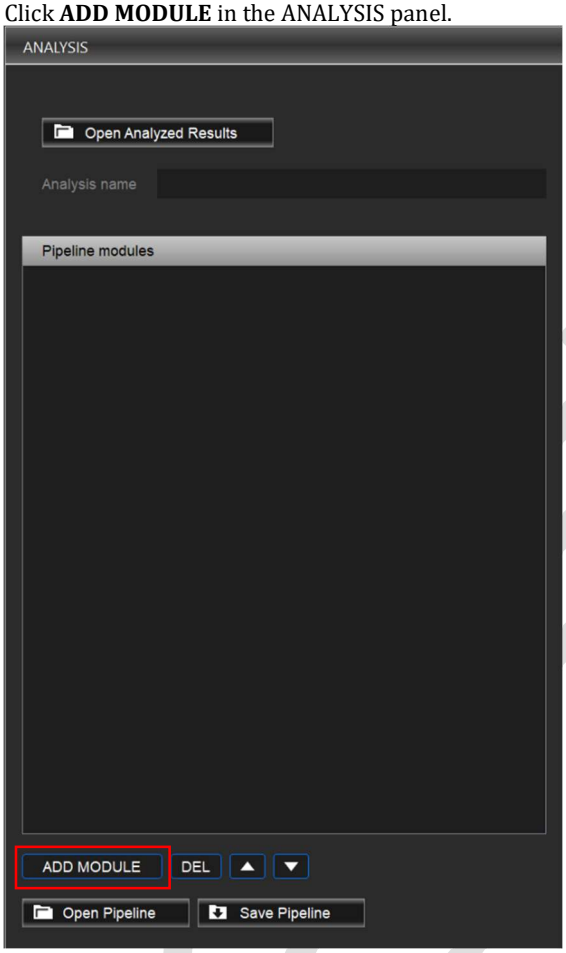


- Select a *.cxpipe* file. This loads the pipeline file and the pipeline modules with the saved settings will appear.

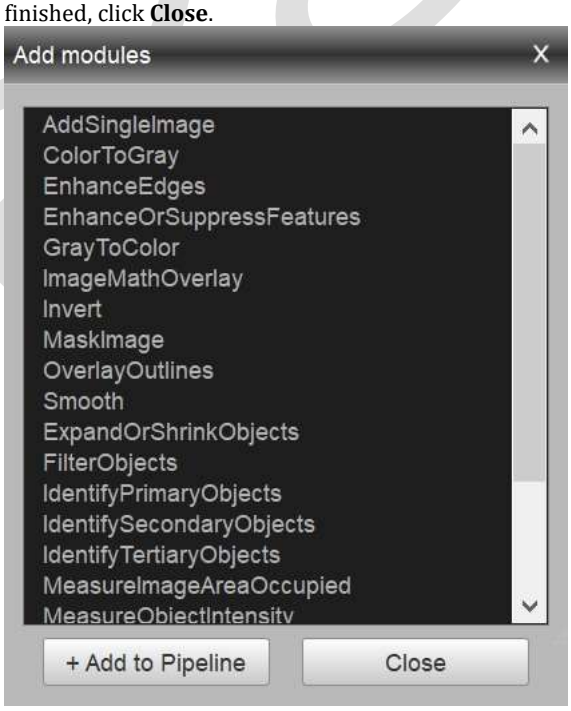
- Select a module in the pipeline to see its settings in the Modules tab. Adjust the settings for each module as needed.

Create a pipeline

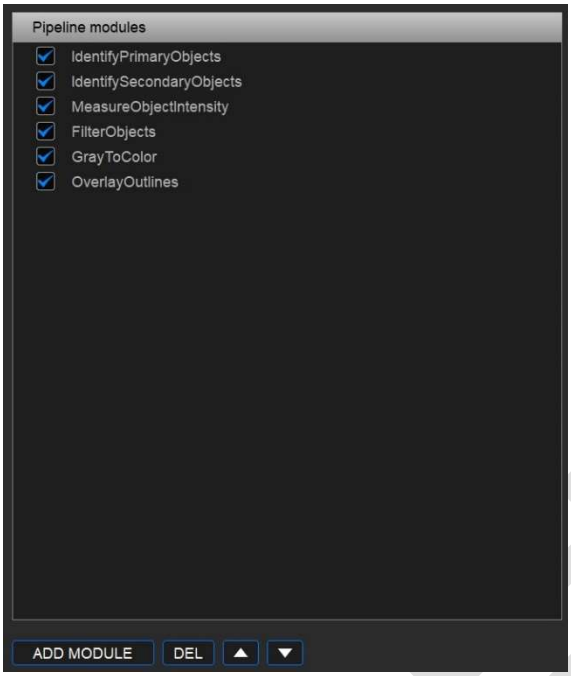
- Click **ADD MODULE** in the ANALYSIS panel.



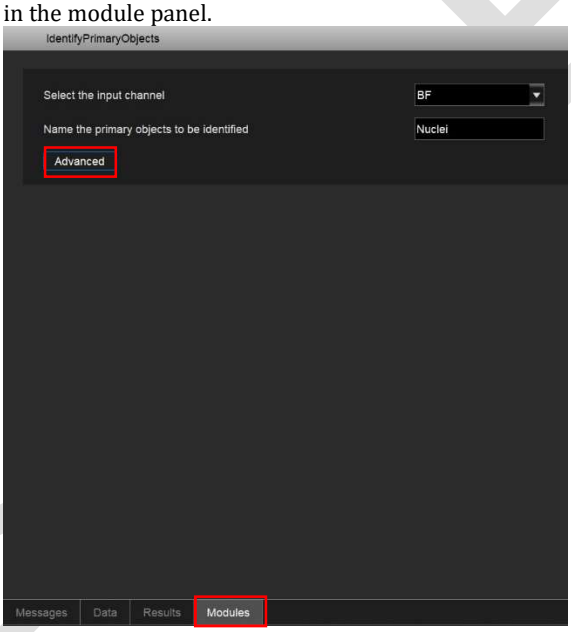
- Select the module(s) you want to use from the modules box and click **Add to Pipeline**. When finished, click **Close**.



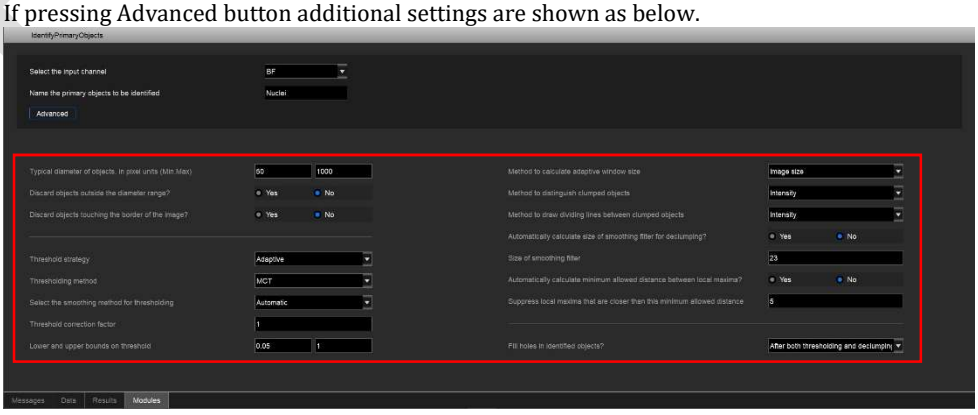
- Modules are processed in the order specified. Adjust the sequence by using the ▲ and ▼ buttons. Delete selected module(s) from the pipeline using the **DEL** button.



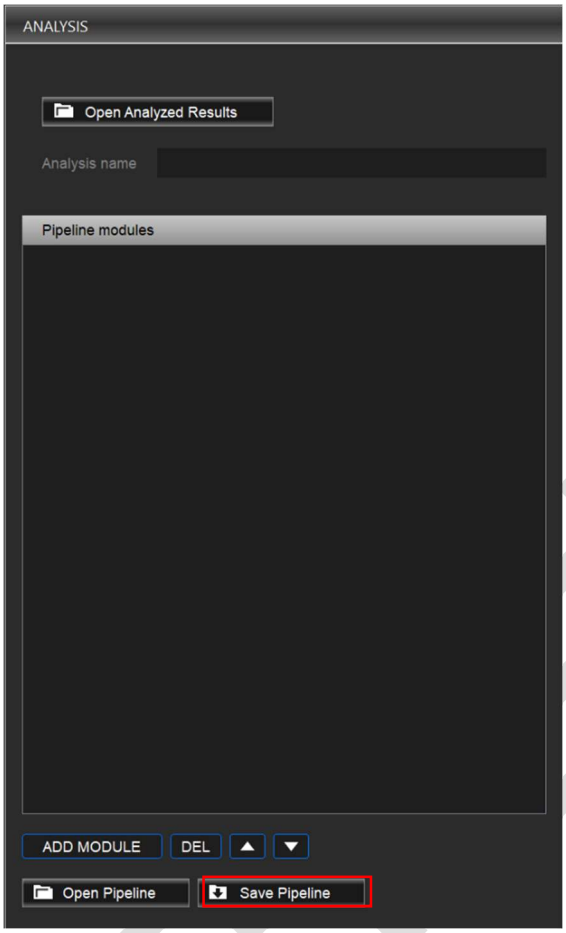
- Adjust the settings for each module as needed. Click a module in the pipeline to see its settings in the module panel.



If pressing Advanced button additional settings are shown as below.



- (Optional) Click **Save Pipeline** to save.



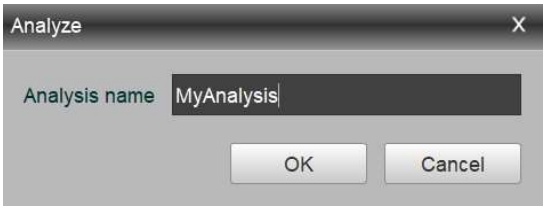
For more detailed information on pipeline modules, see 7.3 Pipeline module reference.

Note

Pipelines are automatically saved to the analysis folder once analysis is run.

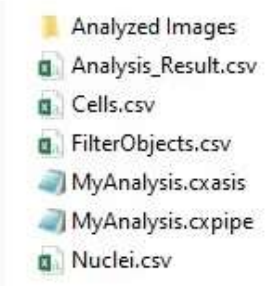
4. **Analyze images** Click **ANALYZE** at the bottom of the ANALAYSIS panel.

Name the analysis to create a *.cxasis* file and begin image analysis.



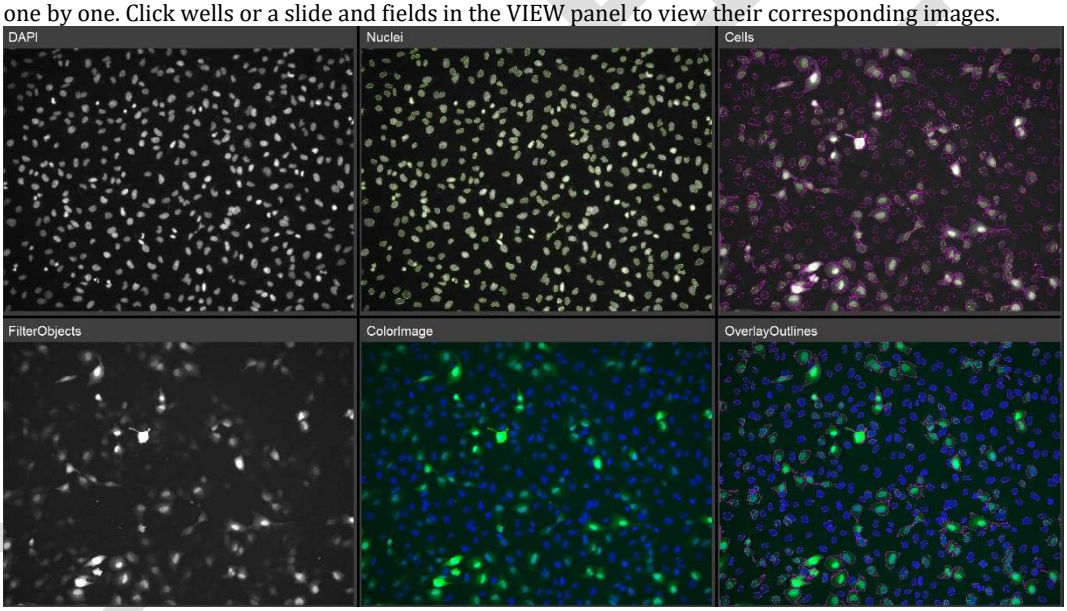
The following files will be saved to the project folder:

- Analyzed images (*.tif*)
- Analysis results (*.csv*)
- Analysis file (*.cxasis*)
- Pipeline file (*.cxpipe*)



5. View data

During analysis the analyzed output images as specified in the added modules are displayed on screen one by one. Click wells or a slide and fields in the VIEW panel to view their corresponding images.



Once analysis is complete, you can see a summary of the analysis results onscreen.
Click the **Results** tab in the messages panel to show the results panel. There will be a table that displays the results of all analyzed images. One line of measured data is generated for each Well, Filed, Z-plane and Time.

ImageNumber	Well	Field	Z-plane	Time	Count_Cells	Count_FilterObjects	Count_Nuclei	Cells_IntegratedIntensity_GFP
1	B06	R6C4	1	1	454.0	1.0	454.0	248.7575
2	B07	R6C4	1	1	427.0	0.0	427.0	74.0220
3	C06	R6C4	1	1	437.0	0.0	437.0	308.8152
4	C07	R6C4	1	1	378.0	2.0	378.0	56.6687
5	D06	R6C4	1	1	412.0	0.0	412.0	292.9185
6	D07	R6C4	1	1	570.0	1.0	570.0	56.9785

Messages | Data | Modules | Results | Nuclei | Cells | FilterObjects

Results panel

Additional tabs will appear for each analyzed object. Click on these tabs to view object measurements. There will be a table that displays the results of all analyzed images.

ImageNumber	ObjectNumber	Intensity_IntegratedIntensity_GFP	Location_Center_X	Location_Center_Y
1	1	449.9294	749.8932	17.7654
1	2	0.2156	245.0	0.5
1	3	19.8823	330.6395	3.3720
1	4	84.3843	675.0202	9.3002
1	5	54.4470	1155.7985	6.9320
1	6	22.5019	1215.1515	3.7196
1	7	54.2823	1428.6657	6.0869
1	8	282.9019	1308.7606	10.6383
1	9	96.8823	624.4209	12.2557
1	10	51.1058	238.6970	11.2118
1	11	878.3882	835.5861	24.3212
1	12	562.8117	176.2244	30.5857
1	13	52.3647	1286.2893	26.4212
1	14	92.9137	569.2343	32.5387
1	15	61.2745	246.7669	35.6587
1	16	66.5450	1062.3608	38.1227
1	17	61.9372	1193.8505	47.1417
1	18	62.0470	1569.96	43.9777
1	19	143.7529	1442.4480	45.2733
1	20	253.0588	111.0501	50.5096

Messages | Data | Results | Modules | Nuclei | Cells | FilterObjects

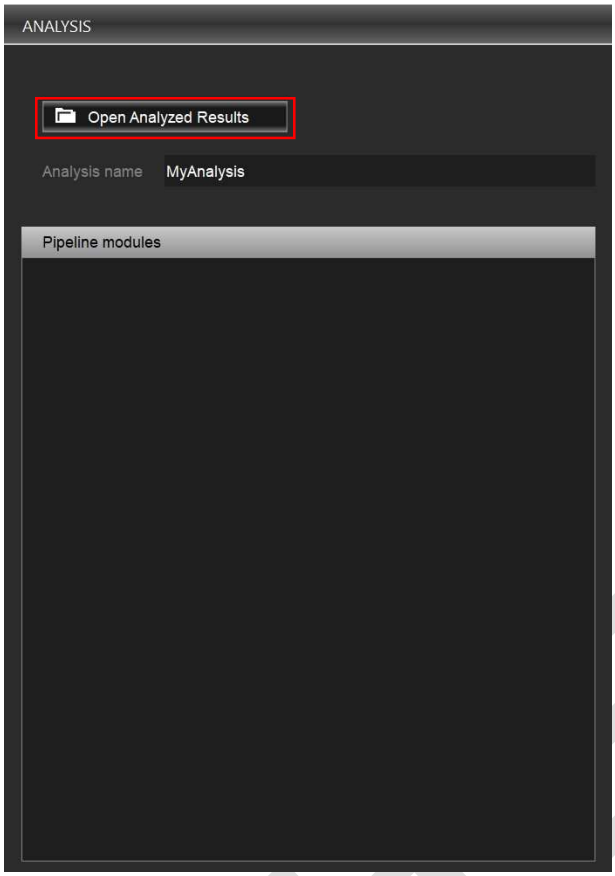
Objects (ex. Nuclei, Cells or FilterObjects) panel

Location_Center_X and Location_Center_Y show the position of each object in the corresponding image. The measured data like Intensity_IntegratedIntenisty GFP is also displayed in the table for each object. The ImageNumber is the same as the ImageNumber in the **Results** panel. If the ImageNumber is 1 as shown above, it represents the image at Well of B6, Field of R6C4, Z-plane of 1 and Time of 1. Fields of ImageNumber, ObjectNumber, Location_Center_X and Location_Center_Y are automatically generated but the other fields like Intensity_IntegratedIntenisty_GFP should be inserted by users using measurement modules like MeasureObjectIntensity or MeasureObjectSizeShape.

6.2 Load previously analyzed images

Previously analyzed projects can be reviewed in CELENA® X Cell Analyzer.

Click **Open Analyzed Results** in the ANALYSIS panel.



Select a *.cxasis* file. This will load the analyzed images, applied pipeline, measurement data, analysis results, and respective metadata.

Click the Results tab in the messages panel to show the results panel. There will be a table that displays the results of all analyzed wells and fields. Additional tabs will appear for each analyzed object. Click on these tabs to view object measurements.

Click wells and fields in the VIEW panel to view their corresponding images.

6.3 Pipeline module reference

Overview

Pipeline modules can be divided into the following categories:

1) **Image processing**
    a. AddSingleImage
    b. ColorToGray
    c. EnhanceEdges
    d. EnhanceOrSuppressFeatures
    e. FilterObjects
    f. GrayToColor
    g. ImageMathOverlay
    h. Invert
    i. MaskImage
    j. OverlayOutlines
    k. Smooth
2) **Object identification**
    a. IdentifyPrimaryObject
    b. IdentifySecondaryObject
    c. IdentifyTertiaryObject
3) **Measurements**
    a. MeasureImageAreaOccupied
    b. MeasureObjectIntensity
    c. MeasureObjectSizeShape
4) **Standalone**
    a. MergFocus

b. StitchImages

Image processing

AddSingleImage

The AddSingleImage module selects an image and makes it as a mask to other images. Multiple images can be used as masks.

Module settings:

1. Select the input image to be used as a mask.
2. Assign a name it
3. Add another image if necessary
4. Remove an image if necessary

ColorToGray

The ColorToGray module converts RGB color images to grayscale images. Multiple channels can be merged into one grayscale image or converted into individual grayscale images.

Module settings:

1. Select the input image.
2. Select to:
    a. Combine multiple channels into one grayscale image or
    b. Split each channel to create individual grayscale images.
3. If 2a, name the output image.
   If 2b, select which channels to convert to gray and name the output image(s).
4. If 2a, the relative weights will adjust the contribution of the colors relative to each other. If necessary, adjust as needed.

EnhanceEdges

The EnhanceEdges module enhances or identifies edges in an image for downstream image processing and/or object identification. This can be used to enhance cell boundaries for effective determination of cell areas.

Module settings:

1. Select the input channel.
2. Name the output image.
3. Select an edge-finding method. Choose from the following:
    a. Sobel
    b. Prewitt
    c. Roberts
    d. LoG
    e. Canny
    f. Kirsch
4. If 3a or 3b, select edge direction to enhance.
   If 3d or 3e, select whether or not to calculate Gaussian's sigma automatically. If not, enter the Guassian's sigma value.
   If 3e, select whether or not to automatically calculate the threshold. If not, enter the absolute threshold value.
   If 3e, select whether or not to automatically calculate the value for low threshold. If not, enter the low threshold value.
   If 3e, enter the threshold adjustment factor.

Tips:

- All edge-finding methods besides Canny produce grayscale images on which Identify modules can be used downstream. The Canny method produces a black and white mask image of the edge pixels.

EnhanceOrSuppressFeatures

The EnhanceOrSuppressFeatures module enhances or suppress specific features in an image to improve downstream object identification.

Module settings:

1. Select the input channel.
2. Name the output image.
3. Select to:
    a. Enhance or
    b. Suppress features.
4. If 3a, select a feature type to enhance. Choose from the following:
    a. Speckles
    b. Neurites
    c. Dark holes
    d. Circles
    e. Texture

f. DIC

If 3b, select the feature size.

5. If 4a, select the speed and accuracy, and enter the feature size.
   If 4b, select the enhancement method and smoothing scale.
   If 4c, enter the range of hole sizes.
   If 4d, enter the feature size.
   If 4e, enter the smoothing scale.
   If 4f, enter the smoothing scale, shear angle, and decay.

FilterObjects

The FilterObjects module eliminates select identified objects based on certain measurements produced by another module. Objects can be also be filtered based on whether or not they touch image borders.

Module settings:

1. Select objects to filter.
2. Name the output objects.
3. Select the filtering mode. Choose from the following:
   a. *Measurements*: Specify a per-object measurement made by an upstream module in the pipeline.
   b. *Image or mask border*: Remove objects touching the border of the image and/or the edges of an image mask.
4. If 3a, select the filtering method. Choose from the following:
   a. *Minimal*: Keep the object with the minimum value for the measurement of interest. If multiple objects share a minimal value, retain one object selected arbitrarily per image.
   b. *Maximal*: Keep the object with the maximum value for the measurement of interest. If multiple objects share a maximal value, retain one object selected arbitrarily per image.
   c. *Minimal per object*: This option requires you to choose a parent object. The parent object might contain several child objects of choice. Only the child object whose measurements equal the minimal child-measurement value among that set of child objects will be kept.
   d. *Maximal per object*: Same as Maximal per object, except filtering is based on the maximum value.
   e. *Limits*: Keep an object if its measurement value falls within a range you specify.
5. If 4c or 4d, child object can overlap two parent objects and can have the maximal/minimal measurement of all child objects in both parents. Select to which parent to assign the overlapping child. Choose from the following:
   a. *Both parents*: The child will be assigned to both parents and all other children of both parents will be filtered.
   b. *Parent with most overlap*: The child will be assigned to the parent with the most overlap and a child with a less maximal/minimal measurement, if available, will be assigned to other parents.
6. If 5b, select the objects that contain the filtered objects.
7. Select whether or not to retain outlines of the identified objects.
   - Yes: Will retain the outlines of new objects for downstream modules.
   - No: Will not retain the outlines of new objects for downstream modules.

Tips:

- Any objects that are filtered are considered a new object, so the measurements associated with the original objects do not carry over to the new objects. For measurements on the new objects, make the measurements downstream.

Generated measurements:

- Count: The number of objects remaining after filtering.
- Parent: The identity of the input object associated with each filtered (remaining) object.
- Location_Center_X: The X coordinate of the center of mass of the filtered object.
- Location_Center_Y: The Y coordinate of the center of mass of the filtered object.

GrayToColor

The GrayToColor module converts grayscale images to color images.

Module settings:

1. Name the output image.
2. Select the images to convert.
3. Assign their respective colors.
4. Adjust the brightness of each color by using relative weights.

ImageMathOverlay

The ImageMathOverlay module multiplies image intensities.

Module settings:

1. Name the output image.
2. Select the image(s) to convert.
3. Enter how much to multiply each selected image by.

Invert

The Invert module inverts images.

Module settings:

1. Select the input channel.
2. Name the output image.

MaskImage

The MaskImage module hides specific areas in an image (based on objects identified upstream or a binary image) so they are ignored by downstream mask-respecting modules in the pipeline.

This module masks an image so you can use the mask downstream in the pipeline. The masked image is based on the original image and the masking object or image that is selected. If using a masking image, the mask is composed of the foreground (white portions); if using a masking object, the mask is composed of the area within the object. Note that the image created by this module for further processing downstream is grayscale. If a binary mask is desired in subsequent modules, use the Threshold module instead of MaskImage.

Module settings:

1. Select the input image.
2. Name the output image.
3. Select to:
    a. Use objects or
    b. An image as a mask.
4. If 3b, select the image.
5. Select whether or not to invert the mask.

OverlayOutlines

The OverlayOutlines module outlines objects in images.

Module settings:

1. Select the channel on which to display outlines.
2. Name the output image.
3. Enter the width of outlines.
4. Select objects to display.
5. Select outlines to display.

RelateObjects

The RelateObjects module assigns relationships between a parent object (e.g., nucleus) and children objects (e.g., speckles) within a parent object. This is useful for counting the number of children associated with each parent.

Module settings:

1. Select the input child objects.
2. Select the input parent objects.

Smooth

The Smooth module smooths or blurs images to remove small artifacts.

Module settings:

1. Select the input channel.
2. Name the output image.

Object identification

Pipelines will depend on identifying the objects in the image. In Cell Analyzer, you will identify primary, secondary, or tertiary objects.

IdentifyPrimaryObject

The IdentifyPrimaryObject module identifies primary objects from grayscale images.

A primary object is an object that can be identified in an image without needing another object or image as a reference. Nuclei are good candidates for primary object identification as they are uniform in shape, have a high contrast relative to its background once stained, and are well-spaced apart from adjacent nuclei.

Module settings:

1. Select the input channel.
2. Name the primary objects to be identified.

Tips:

- Images must be grayscale.
- The regions of interest must be lighter than the background – if they are dark on a light background, invert the images using the **Invert** module upstream.
- If the images are phase or brightfield images, process the images using the **EnhanceOrSuppressFeatures** module upstream.

Generated measurements:

- Count: The number of primary objects identified.
- Location_Center_X: The X coordinate of the center of mass of the primary object.
- Location_Center_Y: The Y coordinate of the center of mass of the primary object.

IdentifySecondaryObject

The IdentifySecondaryObject module identifies secondary objects from grayscale images by using the primary object as a reference.

A secondary object is an object that can be identified in an image using another as a reference. Cells are challenging to identify without a reference as their borders are usually overlapping especially in the case of a confluent monolayer and are lower contrast due to diffuse staining. Cells are good candidates for secondary object identification as they need a previously identified primary object such as nuclei as a reference to detect cell borders.

Module settings:

1. Select the input channel.
2. Select the input objects. The input objects will be identified from a prior module. Although it is usually from the **IdentifyPrimaryObjects** module, it can be any an object identified by any other module.
3. Name the objects to be identified.

Tips

- Images must be grayscale.
- Primary objects must be completely contained within a secondary object. Secondary objects must be larger than or equal in size to primary objects.

Generated measurements:

- Count: The number of secondary objects identified.
- Location_Center_X: The X coordinate of the center of mass of the secondary object.
- Location_Center_Y: The Y coordinate of the center of mass of the secondary object.

IdentifyTertiaryObject

The IdentifyTertiaryObject module identifies tertiary objects from grayscale images by using the primary and secondary object as a reference.

A tertiary object is an object that can be identified in an image by removing primary objects from the larger secondary objects. For example, cytoplasm is an object that is outside the nuclei but contained within the cell boundaries. This means that it can be identified by subtracting nuclei (smaller identified objects) from cells (larger identified objects).

Module settings:

1. Select the larger identified objects. This will be identified from a prior module. Although it is usually from the **IdentifySecondaryObjects** module, it can be any object identified by any other module.
2. Select the smaller identified objects. This will be identified from a prior module. Although it is usually from the **IdentifyPrimaryObjects** module, it can be any object identified by any other module.
3. Name the objects to be identified.

Tips:

- Images must be grayscale.
- The regions of interest must be lighter than the background – if they are dark on a light background, invert the images using the **Invert** module upstream.
- Primary objects must be completely contained within a secondary object. Secondary objects must be larger than or equal in size to primary objects.

Generated measurements:

- Count: The number of tertiary objects identified.
- Location_Center_X: The X coordinate of the center of mass of the tertiary object.
- Location_Center_Y: The Y coordinate of the center of mass of the tertiary object.

Measurements

MeasureImageAreaOccupied

The MeasureImageAreaOccupied module measures the total area occupied by identified objects within an image.

Module settings

1. Select objects to measure.

Generated measurements:

- AreaOccupied: The total area occupied by the input objects.

MeasureObjectIntensity

The MeasureObjectIntensity module measures the intensity of identified objects.

Module settings:

1. Select a channel.
2. Select objects to measure.
3. Select measurements to export.

Tips:

- Microscopes are not calibrated to an absolute scale, so when using intensity measurements in publications, the units of intensity can be called, "intensity units" or "arbitrary intensity units". Moreover, specify which intensity unit you are referring to (e.g. integrated intensity units, mean intensity units, etc.).

Generated measurements:

- IntegratedIntensity: The sum of the pixel intensities within an object.
- IntegratedIntensityEdge: The sum of the edge pixel intensities of an object.
- LowerQuartileIntensity: The intensity value of the pixel for which 25% of the pixels in the object have lower values.
- MADIntensity: The median absolute deviation (MAD) value of the intensities within the object. The MAD is defined as the median($|x_i$ - median(x)|).
- MassDisplacement: The distance between the centers of gravity in the gray-level representation of the object and the binary representation of the object.
- MaxIntensity: The maximal pixel intensity within an object.
- MaxIntensityEdge: The maximal edge pixel intensity of an object.
- MeanIntensity: The average pixel intensity within an object.
- MeanIntensityEdge: The average edge pixel intensity of an object.
- MedianIntensity: The median intensity value within the object.
- MinIntensity: The minimal pixel intensity within an object.
- MinIntensityEdge: The minimal edge pixel intensity of an object.
- StdIntensity: The standard deviation of the pixel intensities within an object.
- StdIntensityEdge: The standard deviation of the edge pixel intensities of an object.
- UpperQuartileIntensity: The intensity value of the pixel for which 75% of the pixels in the object have lower values.

MeasureObjectSizeShape

The MeasureObjectSizeShape module measures the area and shape of identified objects.

Module settings:

1. Select objects to measure.
2. Select measurements to export.

Tips:

- This module is only reliable for objects that are completely inside an image. If there are objects that touch the image borders, process images using the **IdentifyPrimaryObjects** module advanced settings upstream or the **FilterObjects** module downstream.

Generated measurements:

- Area: The number of pixels in the region.
- Center: The X, Y coordinates of the point farthest away from any object edge (the centroid). This is not the same as the Location-X and -Y measurements produced by the Identify modules.
- Compactness: The mean squared distance of the object's pixels from the centroid divided by the area. A filled circle will have a compactness of 1, with irregular objects or objects with holes having a value greater than 1.
- Eccentricity: The eccentricity of the ellipse that has the same second-moments as the region. The eccentricity is the ratio of the distance between the foci of the ellipse and its major axis length. The value is between 0 and 1. (0 and 1 are degenerate cases; an ellipse with an eccentricity of 0 is a circle, while an ellipse with an eccentricity of 1 is a line.)
- EulerNumber: The number of objects in the region minus the number of holes in those objects, assuming 8-connectivity.
- Extent: The proportion of the in the bounding box that are also in the region. Computed as the area/volume of the object divided by the area/volume of the bounding box.
- FormFactor: Calculated as 4*π*Area/Perimeter2. Equals 1 for a perfectly circular object.
- MajorAxisLength: The length (in pixels) of the major axis of the ellipse that has the same normalized second central moments as the region.
- MinFeretDiameter, MaxFeretDiameter: The Feret diameter is the distance between two parallel lines tangent on either side of the object (imagine taking a caliper and measuring the object at various angles). The minimum and maximum Feret diameters are the smallest and largest possible diameters, rotating the calipers along all possible angles.
- MaximumRadius: The maximum distance of any pixel in the object to the closest pixel outside of the object. For skinny objects, this is 1/2 of the maximum width of the object.
- MeanRadius: The mean distance of any pixel in the object to the closest pixel outside of the object.
- MedianRadius: The median distance of any pixel in the object to the closest pixel outside of the object.
- MinorAxisLength: The length (in pixels) of the minor axis of the ellipse that has the same normalized second central moments as the region.
- Orientation: The angle (in degrees ranging from -90° to 90°) between the x-axis and the major axis of the ellipse that has the same second-moments as the region.
- Perimeter: The total number of pixels around the boundary of each region in the image.
- Solidity: The proportion of the pixels in the convex hull that are also in the object.

Standalone

Overview

Standalone modules work independently. They cannot be combined or mixed with other modules and stored in a pipeline because they don't use Cell Profiler engine.
If you want to use other modules with standalone modules analyze images following the steps below.

1. Open an original project.
2. Add a standalone module to be used in a pipeline
3. Adjust parameters in the module
4. Set the output folder name
5. Analyze images with the standalone module
6. Open a project in the output folder
7. Build a pipeline with modules
8. Analyze the output images with the pipeline

MergeFocus

The MergeFocus module merges multiple focal planes on Z-axis acquired by Z-Stack imaging. Acquired images in Z-stack should have the same brightness, otherwise this module will generate artificial edges.

Module settings:

1. Adjust the mesh size.
2. Select the first and the last Z-planes.
3. Select input channels to be merged and select the merging methods.

Tips:

- This module only supports 8-bit grayscale images. Do not try to merge color or 16-bit images.
- There are two types of merging methods. StackFocuser merges images according to image contrast. MaxValue merges images based on the maximum intensity of each plane. StackFocuser is suitable for brightfield images, and MaxValue is suitable for fluorescence images.
- The mesh size depends on the size of objects. The larger the objects in the images, the larger the mesh size should be.

Generated outputs:

- Output folder: User-defined folder
- Output project: The project has only one plane on Z-axis. The other parameters are the same with those of the original project.
- Images: Merged images are stored in "Images" folder which is located in the output folder.

StitchImages

The StitchImages module stitches multiple overlapping images on the XY-plane acquired by CELENA® X Explorer.

Module settings:

1. Overlap (%) means the overlap region of adjacent images. This value is automatically set to the same value acquired in Explorer, and it is highly recommended to maintain the original value.
2. Search range (%) is the searching range to find exact matching. It is the ratio by overlap. For example, if the original image resolution is 1600 x 1200, the overlap is 10% and search range is 50% the overlap is 1600 x 10% = 160 pixels and the search range is 160 pixels x 50% = 80 pixels. Normally the search range of 50% is the optimal choice as the first trial. If the matching position seems to locate outside the search range increase the value expensing processing time.

Tips:

- Images must be 8-bit grayscale or color images. This module does not support 16-bit images.
- The first channel is the principal channel for stitching. It is beneficial to select a imaging channel having many features such as grains, stripes and/or textures. Normally brightfield channel is a good choice because brightfield images normally have larger contrast than fluorescence images.
- StitchImages module does not perform matching algorithm to match adjacent images from the second channel but it uses the calculated positions of the first channel to stitch images from the second channel. This method makes perfect overlay images after stitching.
- If the original project has no overlap and the overlap is set to zero StitchImages module combines images in theoretical positions to create a stitch image.

Generated outputs:

- Output folder: User-defined folder
- Output project: The project has only one field per each well or a slide. The other parameters are the same with those of the original project.
- Images: Stitched images are stored in "Images" folder which is located in the output folder.

7. Immunohistochemistry

Immunohistochemistry (IHC) is the most popular application in the diagnosis of cancerous cells in properly stained tissues. Tissues taken from patients are stained specific molecular markers and examined under a microscope by pathologists. The immunohistochemical examination traditionally depends on pathologists and the results are rather qualitative than quantitative, but recent advances in automated microscopy and image analysis software help them to easily examine abnormal tissues more quantitatively. The following examples show how to analyze IHC slides with CELENA® X Cell Analyzer.

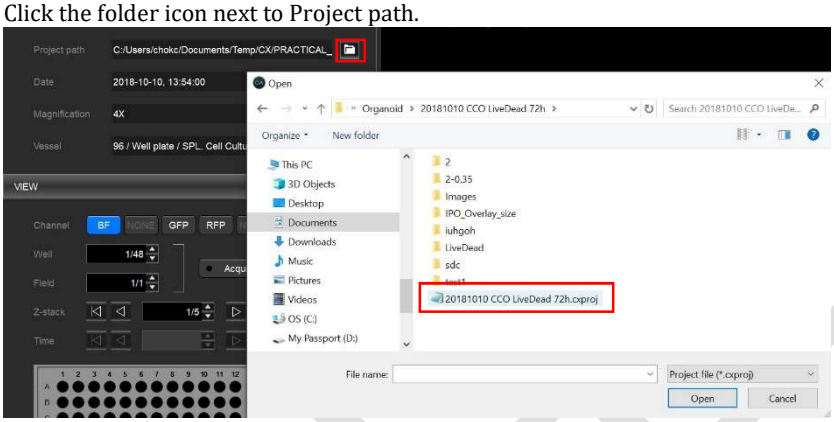
7.1 Nuclear recognition of IHC slides

1. Purpose

Finding nuclei and counting them in IHC slides.

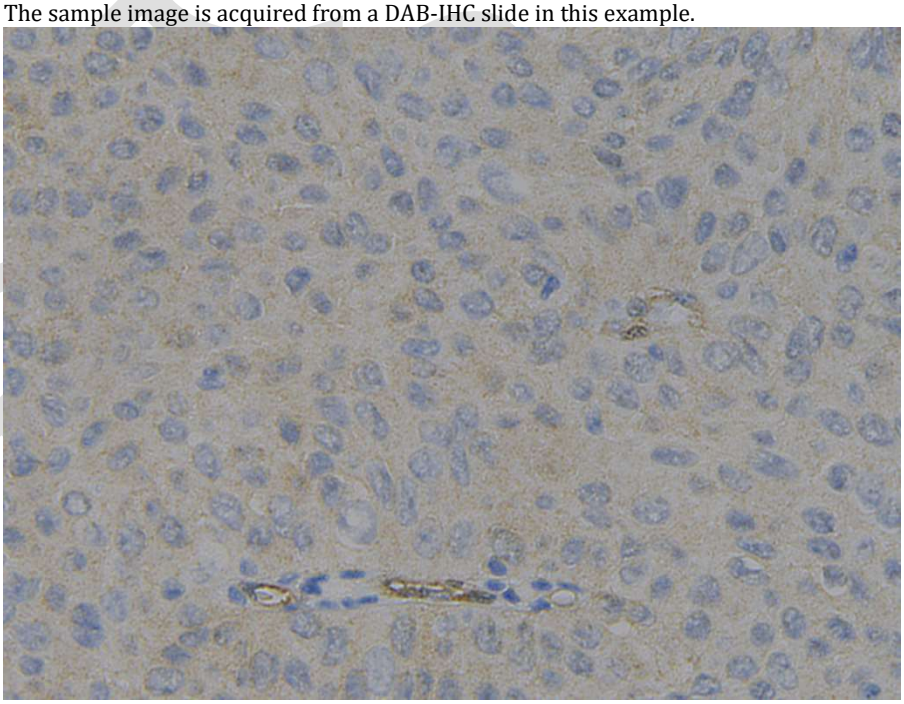
2. Open project

Click the folder icon next to Project path.



Select a *.cxproj* file. This loads the project file, which contains a list of the project image files, the file locations, and the associated metadata.

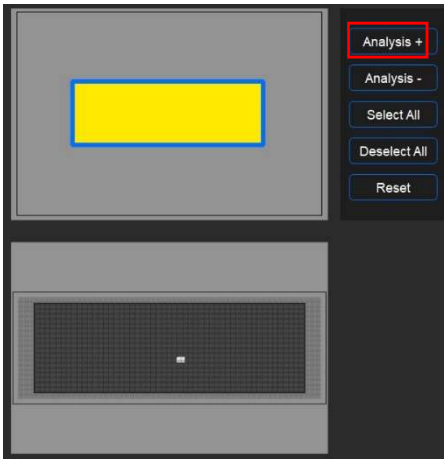
The sample image is acquired from a DAB-IHC slide in this example.



DAB-IHC slide (Courtesy to Asan Medical Center)

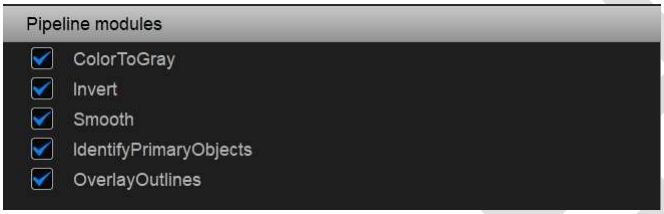
3. Add Analysis

Select wells or a slide and click **Analysis +** button. The selected wells or a slide will turn yellow.



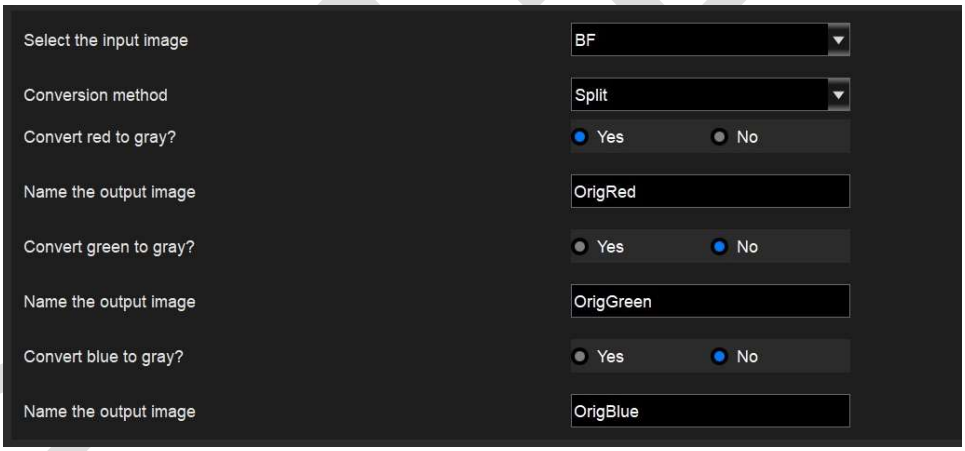
4. Add modules

Add modules as below.



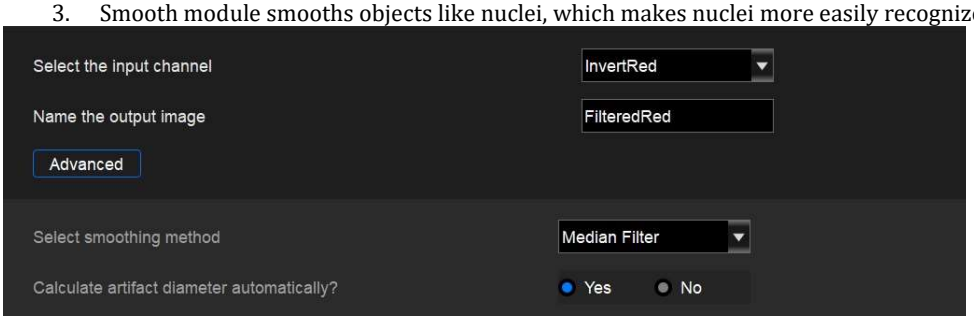
Set parameters for each module as below.

1. ColorToGray module converts color images to grayscale images for next analysis and splits RGB channels. In this example only red channel is used to recognize blue-colored nuclei.

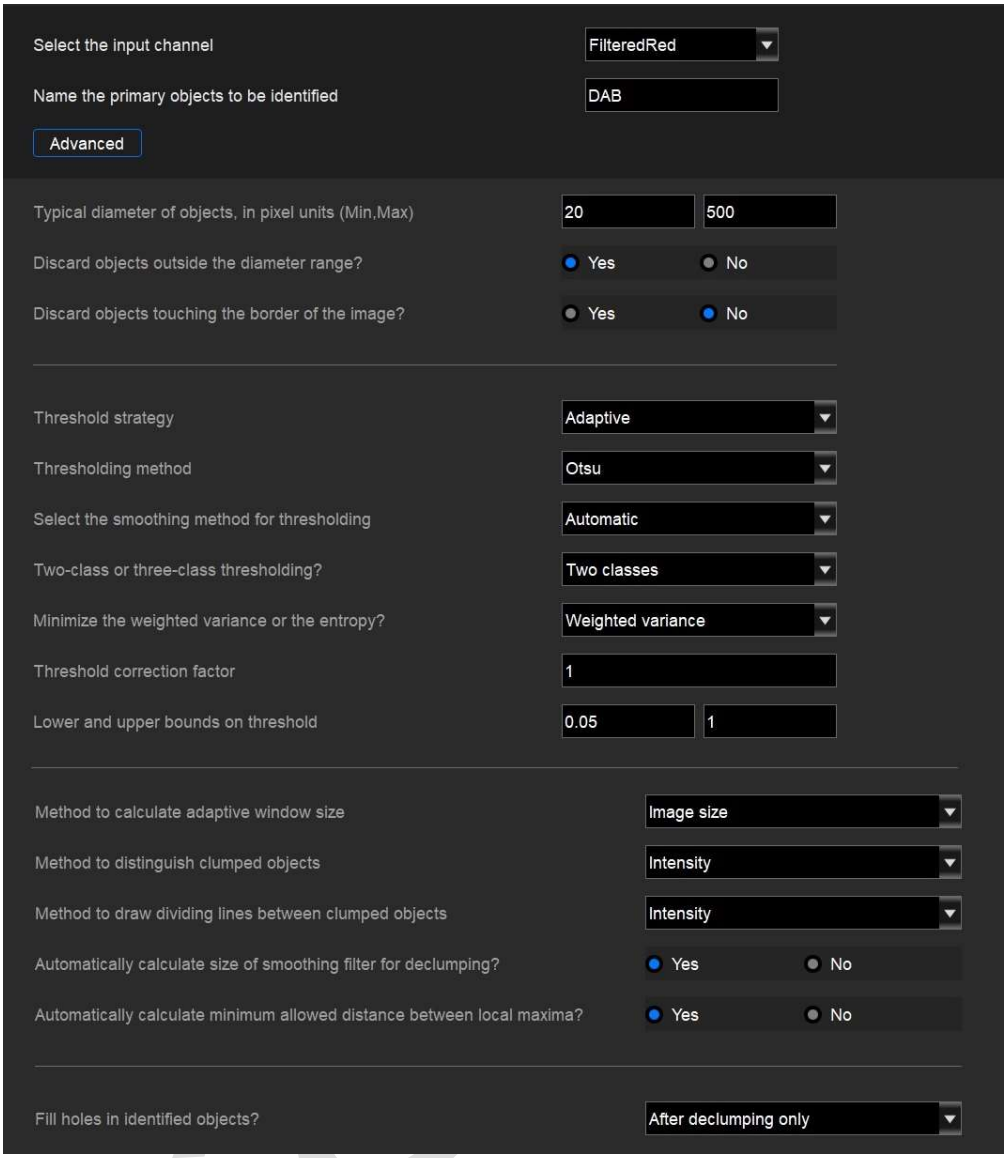


2. Invert module inverts the intensity of grayscale images. which makes dark nuclei bright and bright background dark.

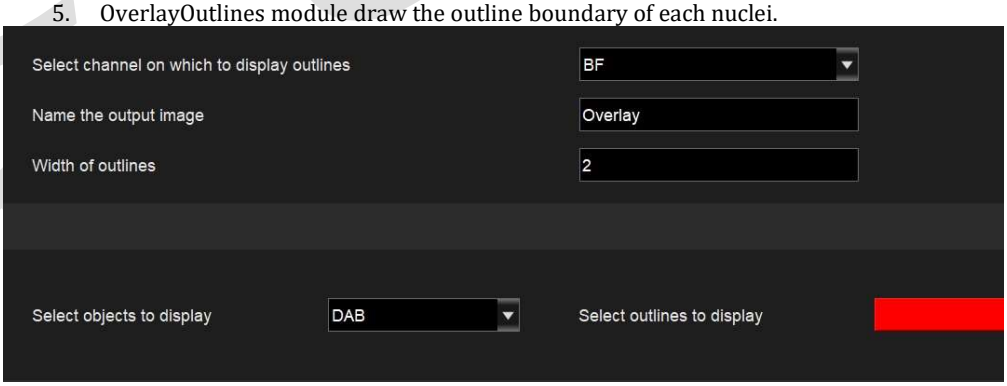
3. Smooth module smooths objects like nuclei, which makes nuclei more easily recognized.



4. IdentifyPrimaryObjects module segments objects and recognizes nuclei.

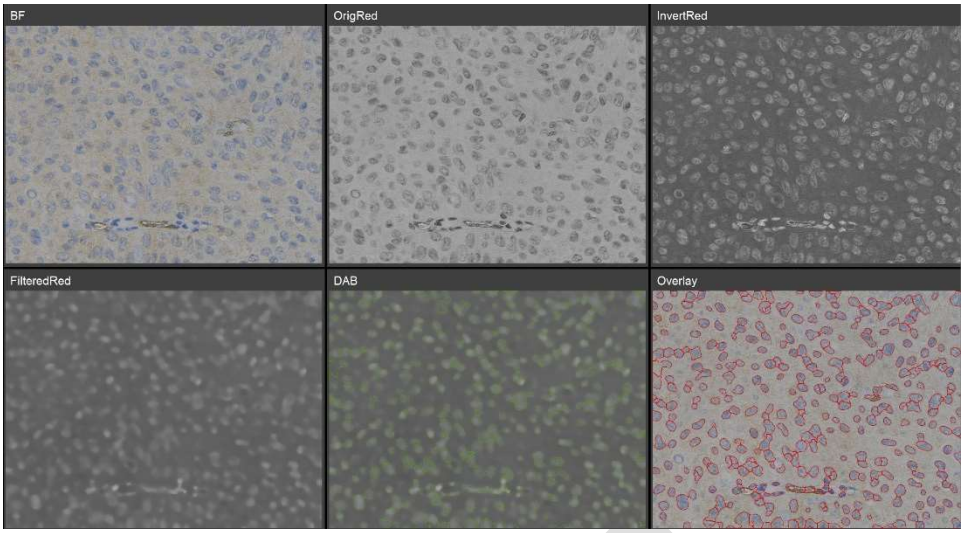


5. OverlayOutlines module draw the outline boundary of each nuclei.

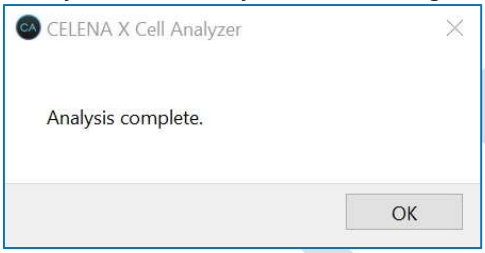


5. Run ANALYSIS

Click **ANALYZE** button and write down **Analysis name**. Click OK to analyze images. During analysis the result images will be displayed on screen.

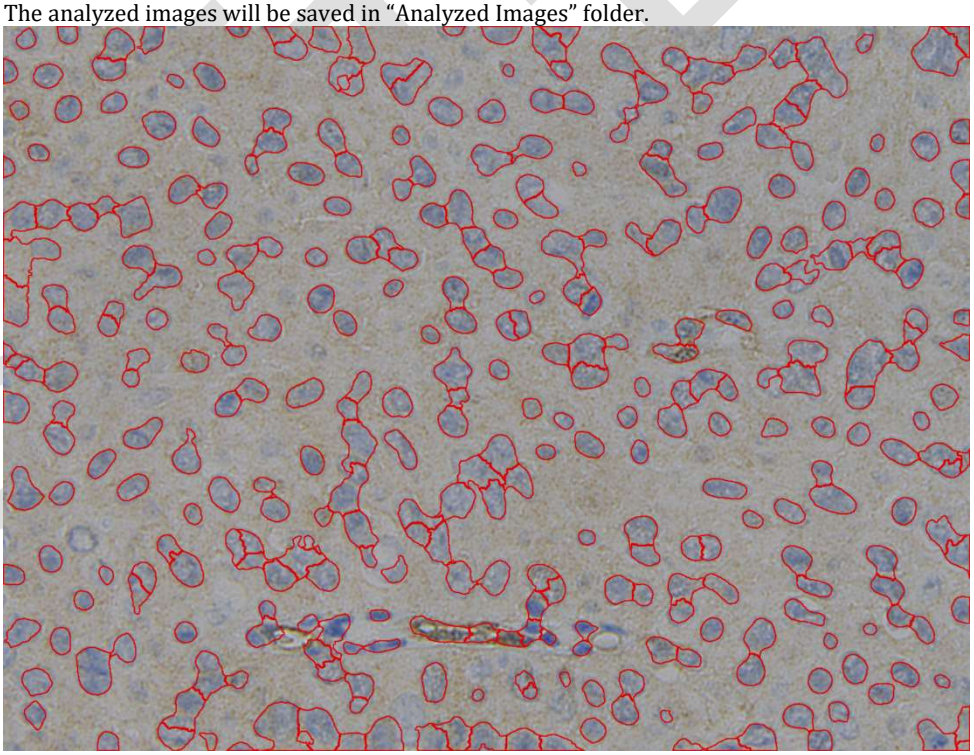


If analysis is successfully finished a message window of “Analysis complete.” will be displayed.

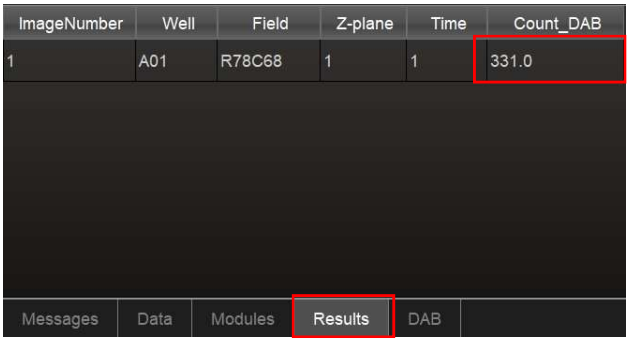


6. Results

The analyzed images will be saved in “Analyzed Images” folder.



The number of nuclei is displayed in **Results** panel.



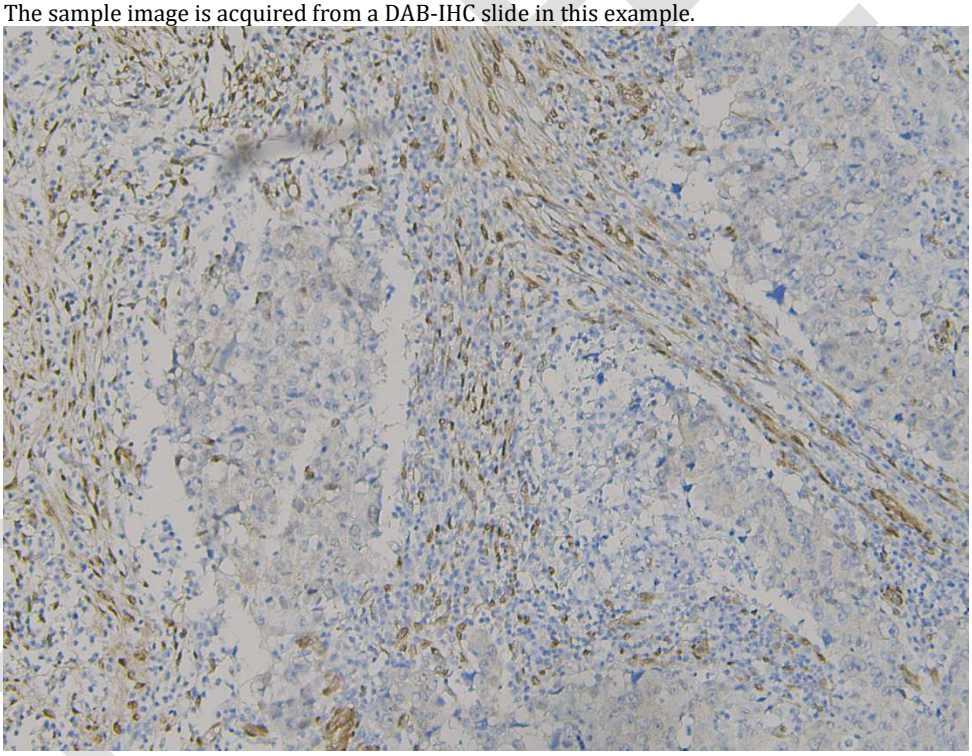
7.2 Color recognition of IHC slides

1. **Purpose** — Separating different colors and calculating occupied area of each color in IHC slides

2. **Open project** — Click the folder icon next to Project path.

   Select a *.cxproj* file. This loads the project file, which contains a list of the project image files, the file locations, and the associated metadata.

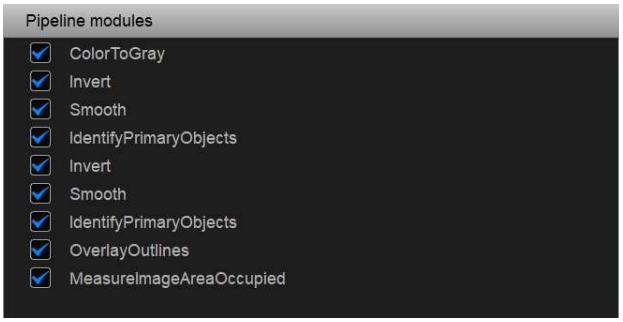
   The sample image is acquired from a DAB-IHC slide in this example.



DAB-IHC slide (Courtesy to Asan Medical Center)

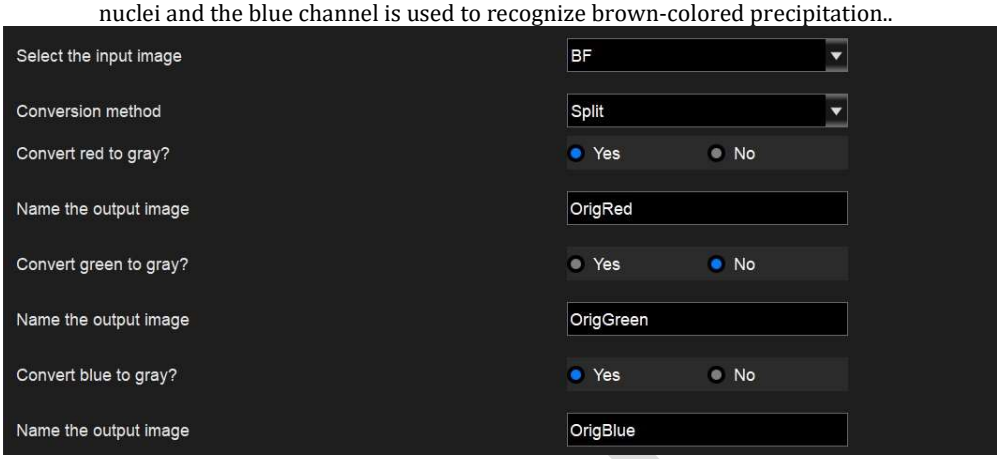
3. **Add Analysis** — Select wells or a slide and click **Analysis +** button. The selected wells or a slide will turn yellow.

4. **Add modules** — Add modules as below.



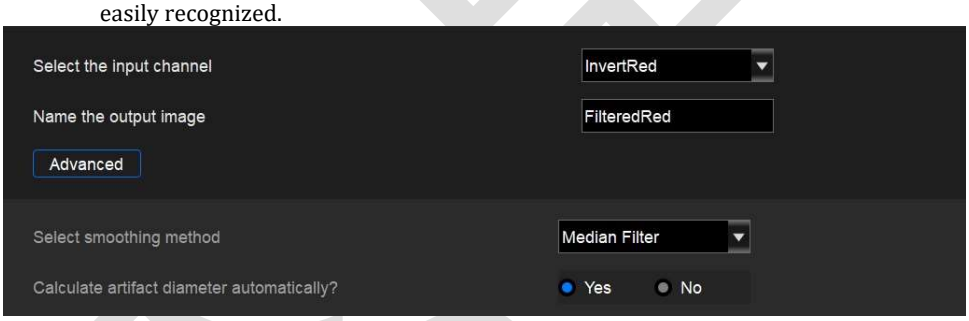
   Set parameters for each module as below.

1. ColorToGray module converts color images to grayscale images for next analysis and splits RGB channels. In this example the red channel is used to recognize blue-colored nuclei and the blue channel is used to recognize brown-colored precipitation..



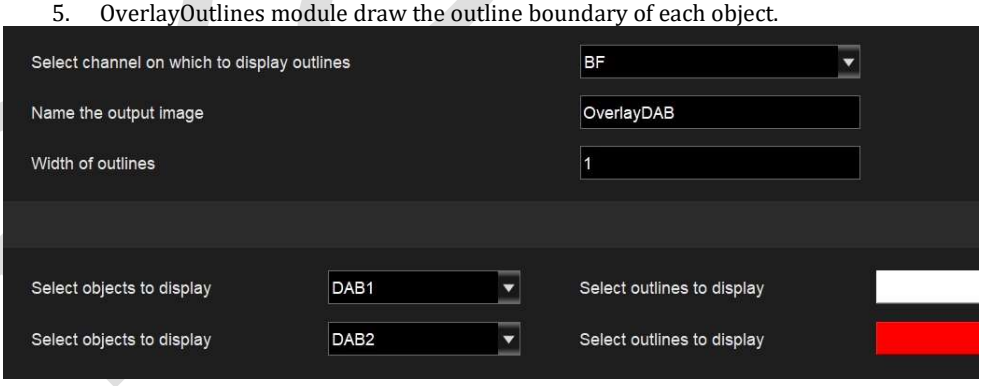
2. The first and the second Invert modules invert the intensity of grayscale images of the red channel and the blue channel, respectively.

3. The first and the second Smooth modules smooth objects, which makes nuclei more easily recognized.

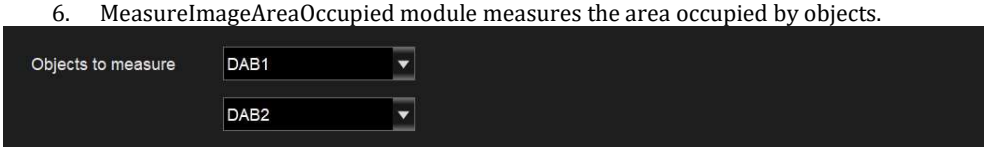


4. The first and the second IdentifyPrimaryObjects modules segments blue-colored objects and brown-colored objects, respectively. The parameters depend on images and optimization is necessary.

5. OverlayOutlines module draw the outline boundary of each object.

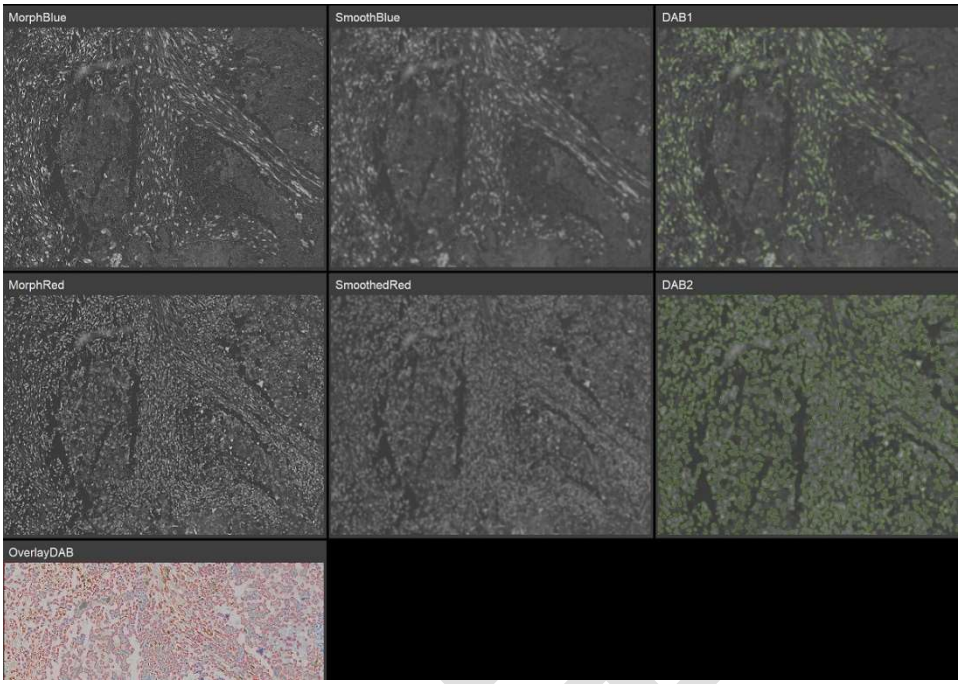


6. MeasureImageAreaOccupied module measures the area occupied by objects.



5. Run ANALYSIS

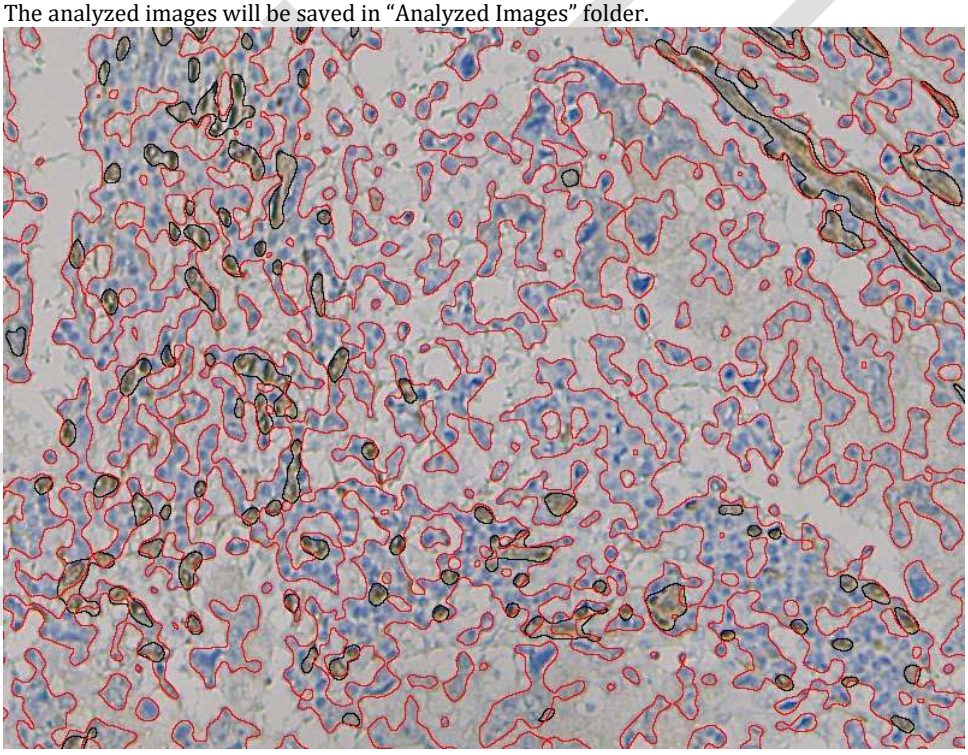
Click **ANALYZE** button and write down **Analysis name**. Click OK to analyze images. During analysis the result images will be displayed on screen.



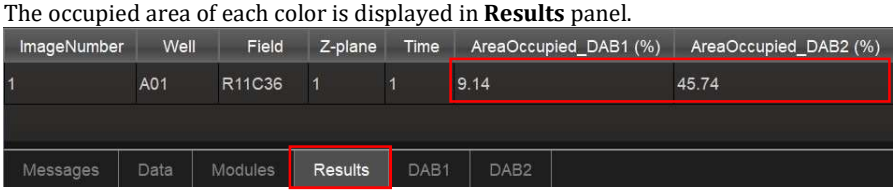
If analysis is successfully finished a message window of “Analysis complete.” will be displayed.

6. Results

The analyzed images will be saved in “Analyzed Images” folder.



The occupied area of each color is displayed in **Results** panel.



ImageNumber	Well	Field	Z-plane	Time	AreaOccupied_DAB1 (%)	AreaOccupied_DAB2 (%)
1	A01	R11C36	1	1	9.14	45.74

Messages | Data | Modules | Results | DAB1 | DAB2

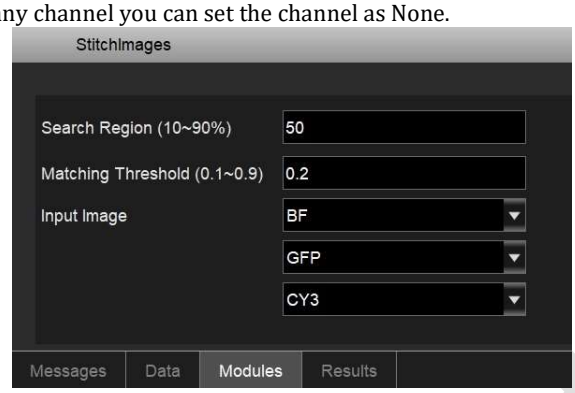
In this example the cells stained in blue (DAB1) has a red outline and its occupied area in this image is 9.14%, and the cells stained in brown (DAB2) has a black outline and its occupied area is 45.74%, respectively.

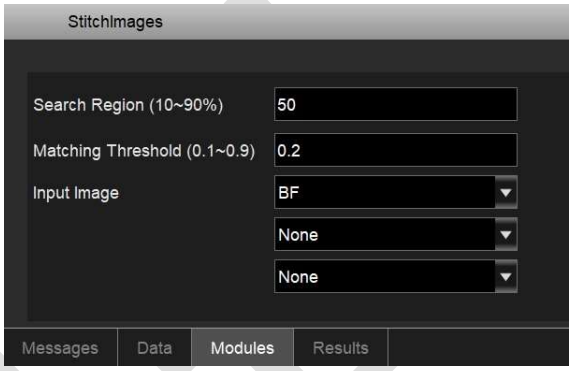
8. Stitch Images

8.1 Overview

Image stitching is a useful image manipulation tool to combine multiple fields to a single image. The StitchImages module in CELENA® X Cell Analyzer performs stitching by matching adjacent images in an overlapped region. To properly stitch images they should be acquired by overlapping them at least 10% in CELENA® X Explorer to find matching features in the overlapped region. For this reason, blank images or recursive patterns are not stitched well. The Overlap value is automatically set as acquired by CELENA® X Explorer. If images are acquired without overlap, the value will be set to zero. It is not recommended to change the value even though you can change it in the parameter setting window because the stitching performance is very poor when using an incorrect value.

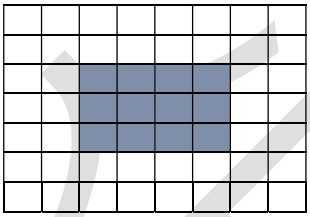
This module supports multi-channel stitching, too even though most stitching applications focus on brightfield imaging. If multiple channels are selected as below the StitchImages module stitches images in the selected channel. If you do not want to stitch any channel you can set the channel as None.



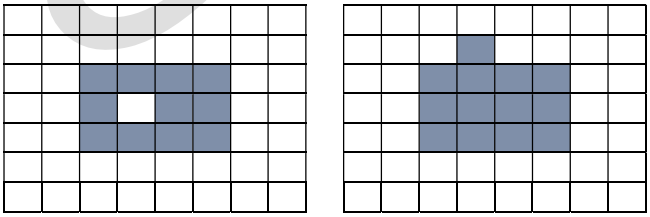


The first channel is always the reference channel. The StitchImages module searches matching features in an overlapped region in the first channel and combines images acquired in other channels in the calculated position from the first channel. This approach makes overlay images have the same pixel position at each channel after stitching. Therefore it is recommended to select a channel as the first channel having many features in the corresponding images, which is helpful for exact stitching. Normally brightfield images have more features compared to fluorescence images.

Images without overlap can be combined to a single image with this module. The StitchImages module does not search matching features and combine images in a theoretical position if the overlap is set to zero in the parameter setting window.
The arrangement of fields which is possible in stitching is only rectangular. If there are vacant fields inside the rectangular arrangement or there are imaged fields outside it stitching is not possible.



proper arrangement

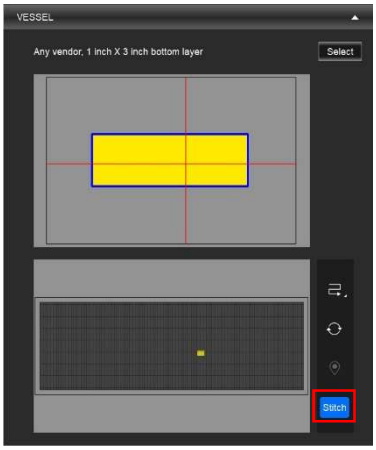


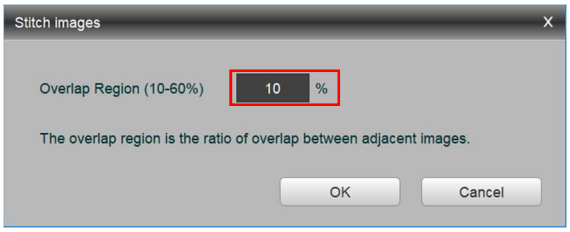
improper arrangement

The maximum image size of the stitched output image is about 32,000 x 32,000 pixels. If it exceeds this value reduce the number of acquired images in CELENA® X Explorer, and try again.

8.2 Performing image stitching

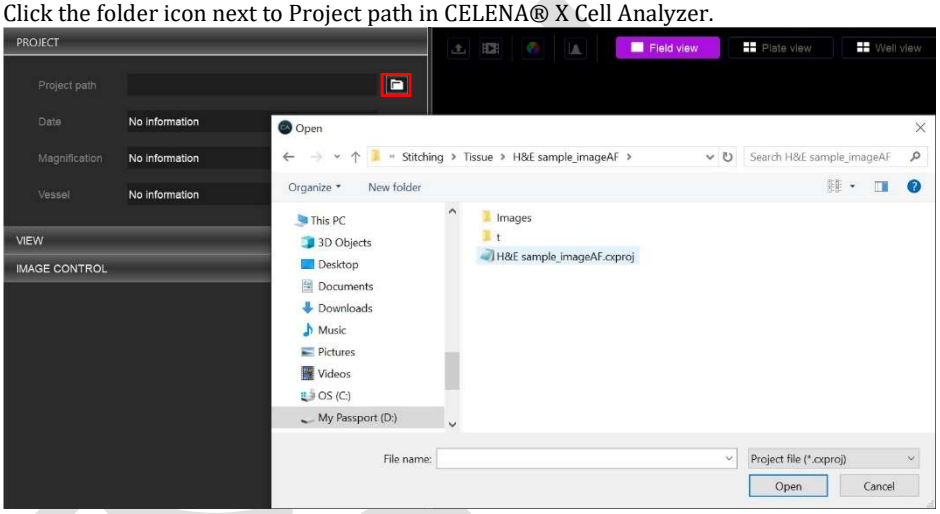
1. **Acquire images** Acquire images in CELENA® X Explorer with turning on Stitch button and setting overlap.





2. **Open project**

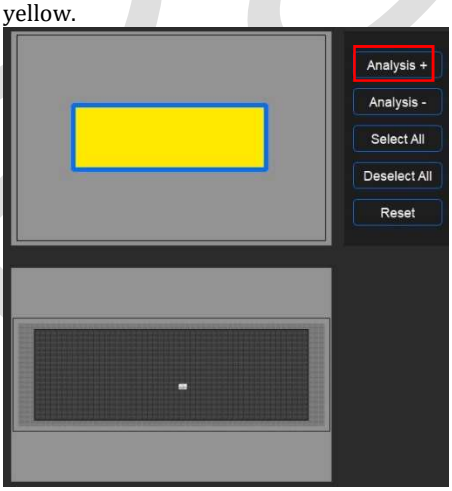
Click the folder icon next to Project path in CELENA® X Cell Analyzer.



Select a *.cxproj* file. This loads the project file, which contains a list of the project image files, the file locations, and the associated metadata.

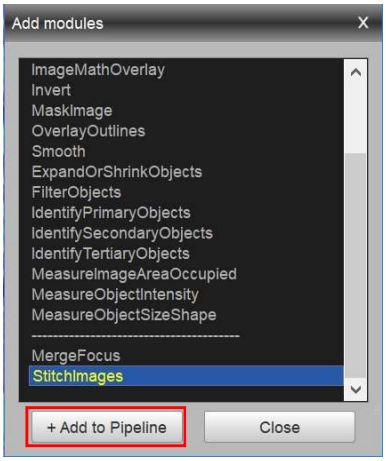
3. **Add Analysis**

Select wells or a slide and click **Analysis +** button. The selected wells or a slide will turn yellow.

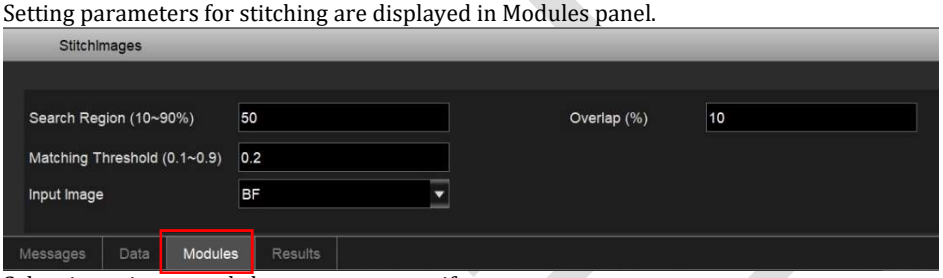


4. **Add StitchImages module**

Click **ADD MODULE** and double-click the module name or click **StitchImages** and **+ Add to Pipeline**.



Setting parameters for stitching are displayed in Modules panel.

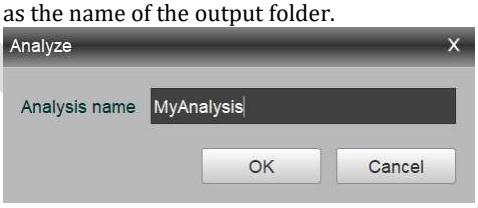


Select input image and change parameters if necessary.
Overlap shows the overlapped ratio set in CELENA® X Explorer, and Search Region represents the searching region for finding matching points in the overlapped region. In this example Overlap of 10% means 160 pixels in width if the original image size is 1,600 pixels in width, and the searching region is 80 pixels in width because it is 50% of the overlapped region.
The mismatch between adjacent images is generated by stage inaccuracy, vibration and so on. This effect is greater in higher magnification. Therefore the Search Region is recommended to be set to a higher value at higher magnification. Search Region of 50% is a good choice as a first trial.
The StitchImages module calculates the matching score between two adjacent images and determines that the matching between these images fails if the score is below the matching threshold. If the matching threshold is too high matching failure frequently occurs even though the matching is successful. If the matching threshold is too low the StitchImages module can determine these image successfully match even though they abnormally match. The matching threshold of 0.2 is a default value.

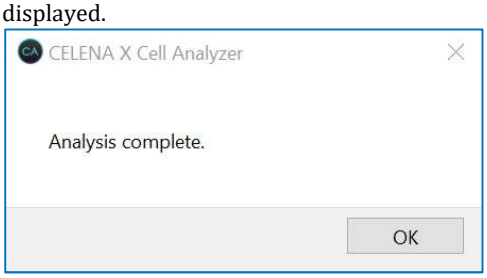
Caution: The StitchImages module is a standalone module. Do not try to add another modules.

5. Run StitchImages

Click **ANALYZE** button and write down **Analysis name**. The analysis name will be the same as the name of the output folder.



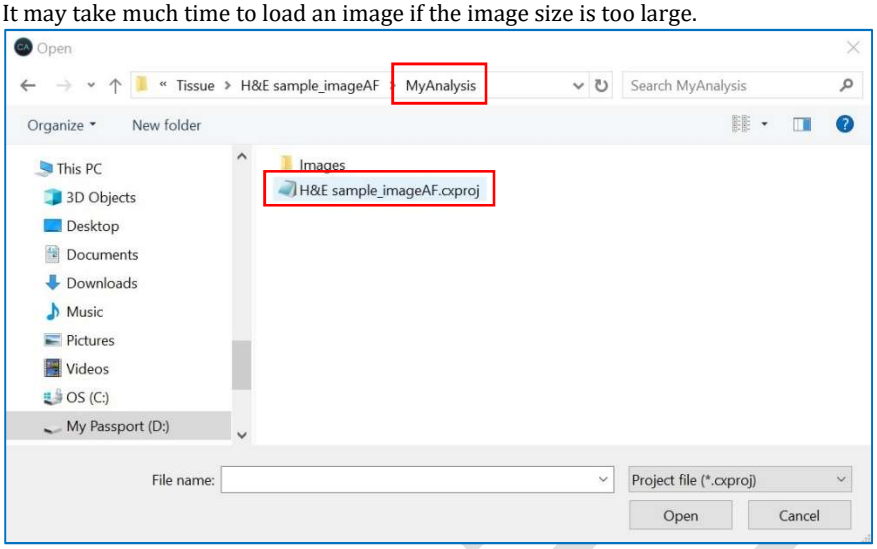
Click OK to run StitchImages.
The StitchImages module load all images to be stitched, search matching points between adjacent images, and save the stitched image with an output project.
If stitching is successfully finished a message window of “Analysis complete.” will be displayed.



8.3 Viewing stitched image

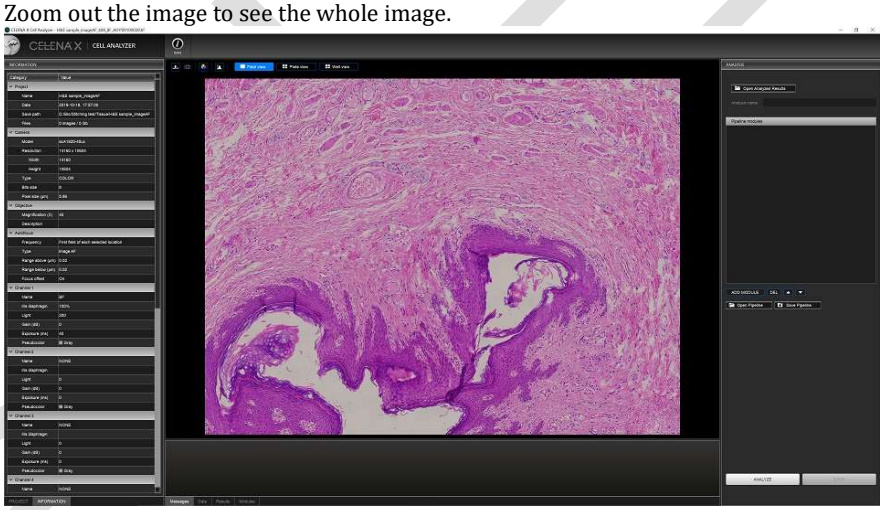
1. **Open output project**

Open the output project in the output folder which is located in the original folder.
The name of the output project is the same as the name of the original project.
It may take much time to load an image if the image size is too large.



2. **View whole image**

Zoom out the image to see the whole image.



You can see the information for the stitched image in **INFORMATION** panel as below.

Project	
Name	H&E sample_imageAF
Date	2019-10-18, 17:57:00
Save path	D:/Bio/Stitching test/Tissue/H&E sample_imageAF
Files	0 images / 0 Gb
Camera	
Model	acA1920-40uc
Resolution	14160 x 10604
Width	14160
Height	10604
Type	COLOR
Bits size	8
Pixel size (μm)	5.86

Adjust the brightness/contrast if necessary.

8.4 Analyzing stitched image

1. **Annotations**

Adding annotations in the stitched image

- Add annotation tools like line, circle, rectangle, etc.
- Add text messages
- Add a scale bar.

2. **Histogram**

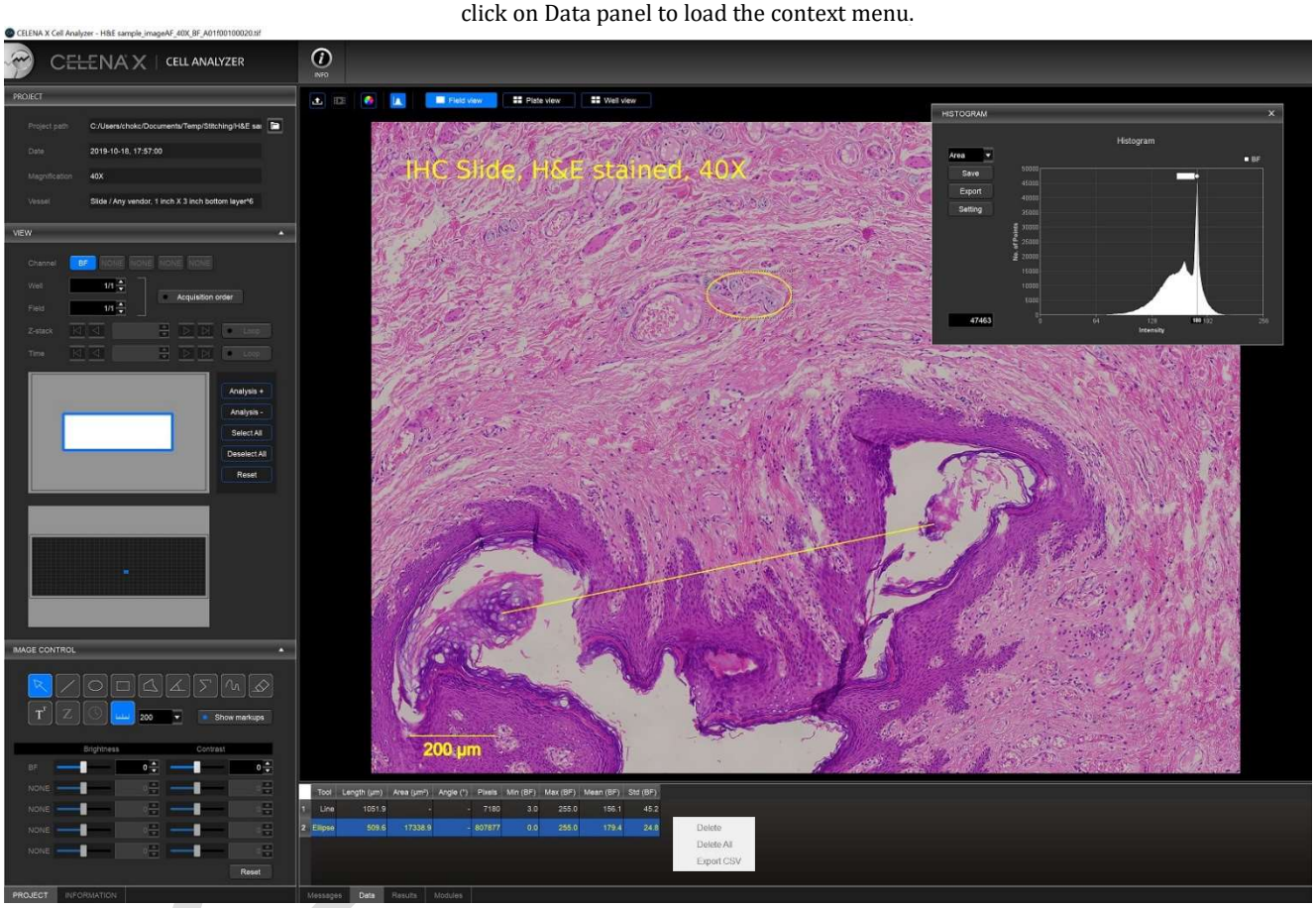
Displaying line profile or histogram of intensity

- The generated line profile or histogram can be stored as an image or a CSV file.
- The chart shows the intensity value at a specified point on mouse cursor in real time.

3. **Measurement**

The measured values are displayed on Data panel.

- Line annotation shows the length and the intensity on the line.
- Area annotation like a circle or a rectangle shows the peripheral length, the occupied area and the intensity inside the annotation.
- The measured results can also be saved as a CSV file using a context menu. Right-click on Data panel to load the context menu.



H&E stained IHC slide, 14160 x 10604 pixels (Courtesy to Asan Medical Center)

Tool	Length (um)	Area (um^2)	Angle (deg)	Pixels	Min (BF)	Max (BF)	Mean (BF)	Std (BF)
Line	1051.86	-	-	7179.94	3	255	156.086	45.2048
Ellipse	509.565	17338.9	-	807877	0	255	179.36	24.7684

Exported data as a CSV file format

9. Merge Focus

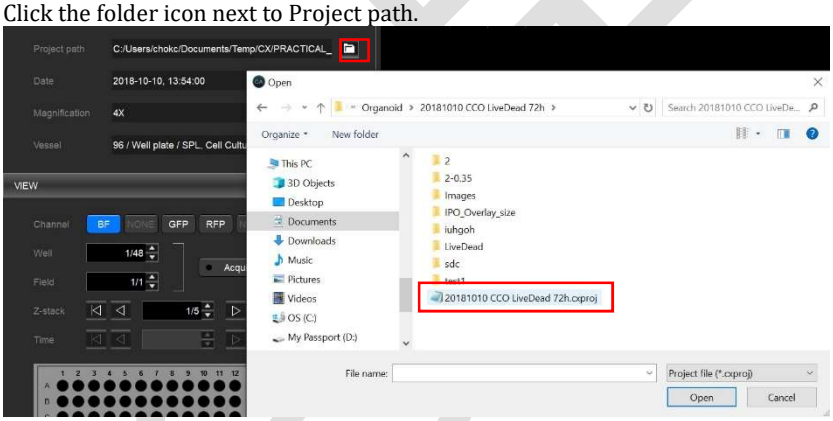
9.1 Overview

There exist multiple focal planes in thick samples like tissues, 3D culture and organoids/spheroids. Z-Stack imaging acquires images with multiple focal planes, but these images are not adequate for image analysis in many cases. First, there are too many images for analysis and it needs much time to analyze all images. Second, images in adjacent focal planes have almost the same biological/clinical information and the analyzed results are superimposed. The MergeFocus module compresses images with multiple focal planes to a single image which looks like with only a single focal plane.
This module supplies two kinds of merging algorithms. StackFocuser combines images gathering pixels with the highest contrast. MaxValue combines images gathering pixels with the maximum intensity. StackFocuser is proper in brightfield images because the contrast increases if the focus matches. MaxValue is proper in fluorescence images because the fluorescence intensity of each pixel increases if the focus matches.
The output project and the output images have only a single plane on Z-axis, and they can be analyzed like normal ones, which reduces analysis time dramatically and makes analysis easier and more accurate.

9.2 Performing MergeFocus

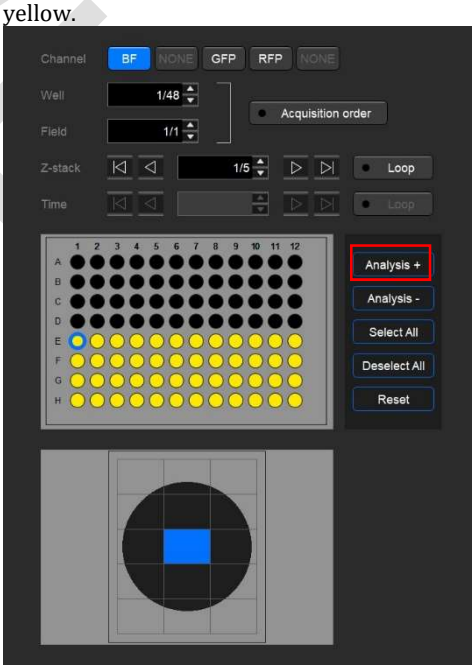
1. **Acquire images** — Acquire images in CELENA® X Explorer with Z-stack imaging.

2. **Open project** — Click the folder icon next to Project path.

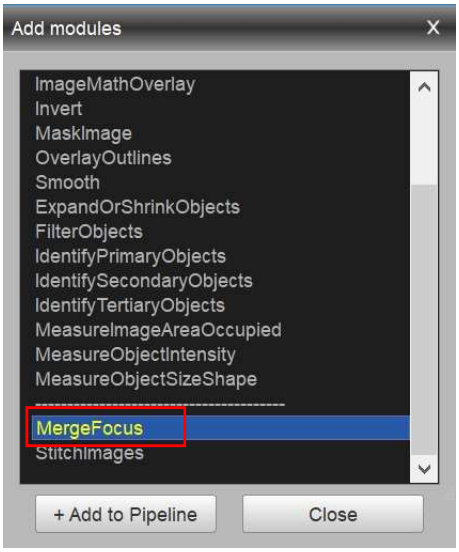


Select a *.cxproj* file. This loads the project file, which contains a list of the project image files, the file locations, and the associated metadata.

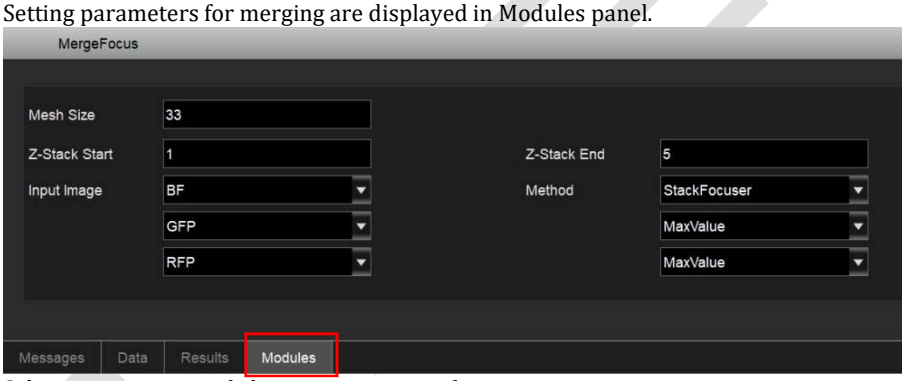
3. **Add Analysis** — Select wells or a slide and click **Analysis +** button. The selected wells or a slide will turn yellow.



4. **Add MergeFocus module** — Click **ADD MODULE** and double-click the module name or click **MergeFocus** and **+ Add to Pipeline**.



Setting parameters for merging are displayed in Modules panel.

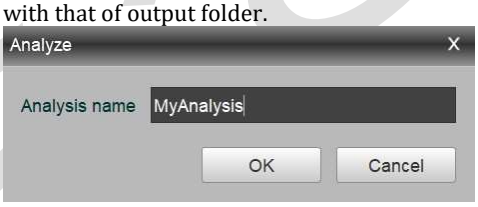


Select input image and change parameters if necessary.
For information of MergeFocus module, go to 7.3 Pipeline module reference.

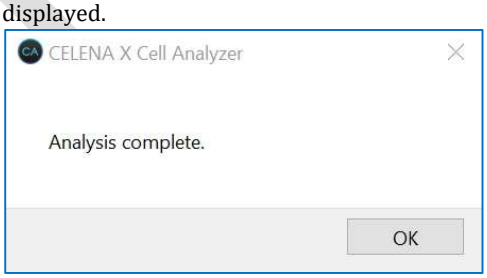
Caution: The MergeFocus module is a standalone module. Do not try to add another modules.

5. Run MergeFocus

Click **ANALYZE** button and write down **Analysis name**. The analysis name will be the same with that of output folder.



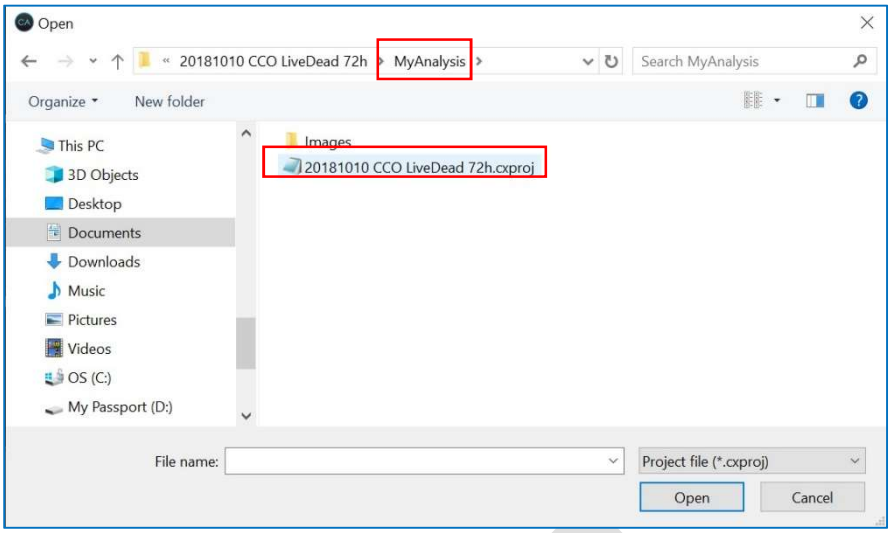
Click OK to run MergeFocus.
If merging is successfully finished a message window of “Analysis complete.” will be displayed.



9.3 Viewing merged images

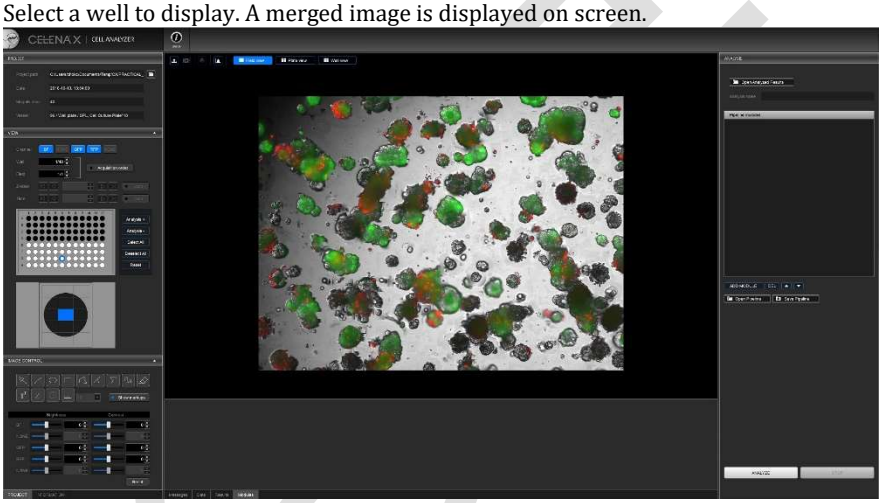
1. Open output project

Open the output project in the output folder which is located in the original folder.
The name of the output project is the same with that of the original project.



2. View merged image

Select a well to display. A merged image is displayed on screen.



You can see the information for the merged image in **INFORMATION** panel as below.

Project	
Name	20181010 CCO LiveDead 72h
Date	2018-10-10, 13:54:00
Save path	D:/MFLCI-CCO test 72h
Files	720 images / 1160 Gb
Camera	
Objective	
Autofocus	
Channel 1	
Channel 2	
Channel 3	
Channel 4	
Channel 5	
Vessel	
Name	SPL, Cell Culture Plate^10
Type	Well plate
Wells	96
Rows	8
Columns	12
Imaged wells	48
Imaged fields	1
Acquisition order	Horizontal, zigzag
Z-stack	
On/Off	On
Distance (µm)	800.00
Interval (µm)	200.00
Steps	5

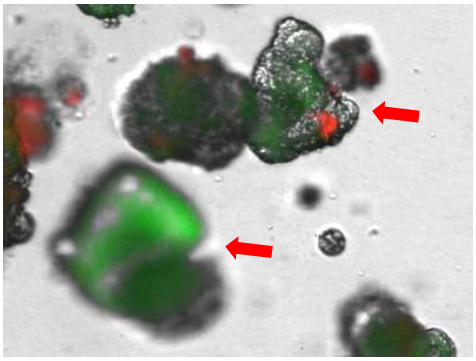
Before merging (original project)

Project	
Name	20181010 CCO LiveDead 72h
Date	2018-10-10, 13:54:00
Save path	D:/MFLCI-CCO test 72h
Files	720 images / 1160 Gb
Camera	
Objective	
Autofocus	
Channel 1	
Channel 2	
Channel 3	
Channel 4	
Channel 5	
Vessel	
Name	SPL, Cell Culture Plate^10
Type	Well plate
Wells	96
Rows	8
Columns	12
Imaged wells	48
Imaged fields	1
Acquisition order	Horizontal, zigzag
Z-stack	
On/Off	Off
Distance (µm)	0.00
Interval (µm)	0.00
Steps	0

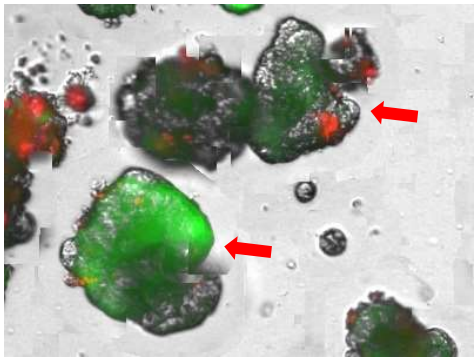
After merging (merged project)

Z-stack is On state before merging, but it is changed to Off state after merging.

3. **Merged results**



Before merging



After merging

9.4 Analyzing merged images with image tools

1. **Annotations**

Adding annotations in the merged image

- Add annotation tools like line, circle, rectangle, etc.
- Add text messages
- Add a scale bar.

2. **Histogram**

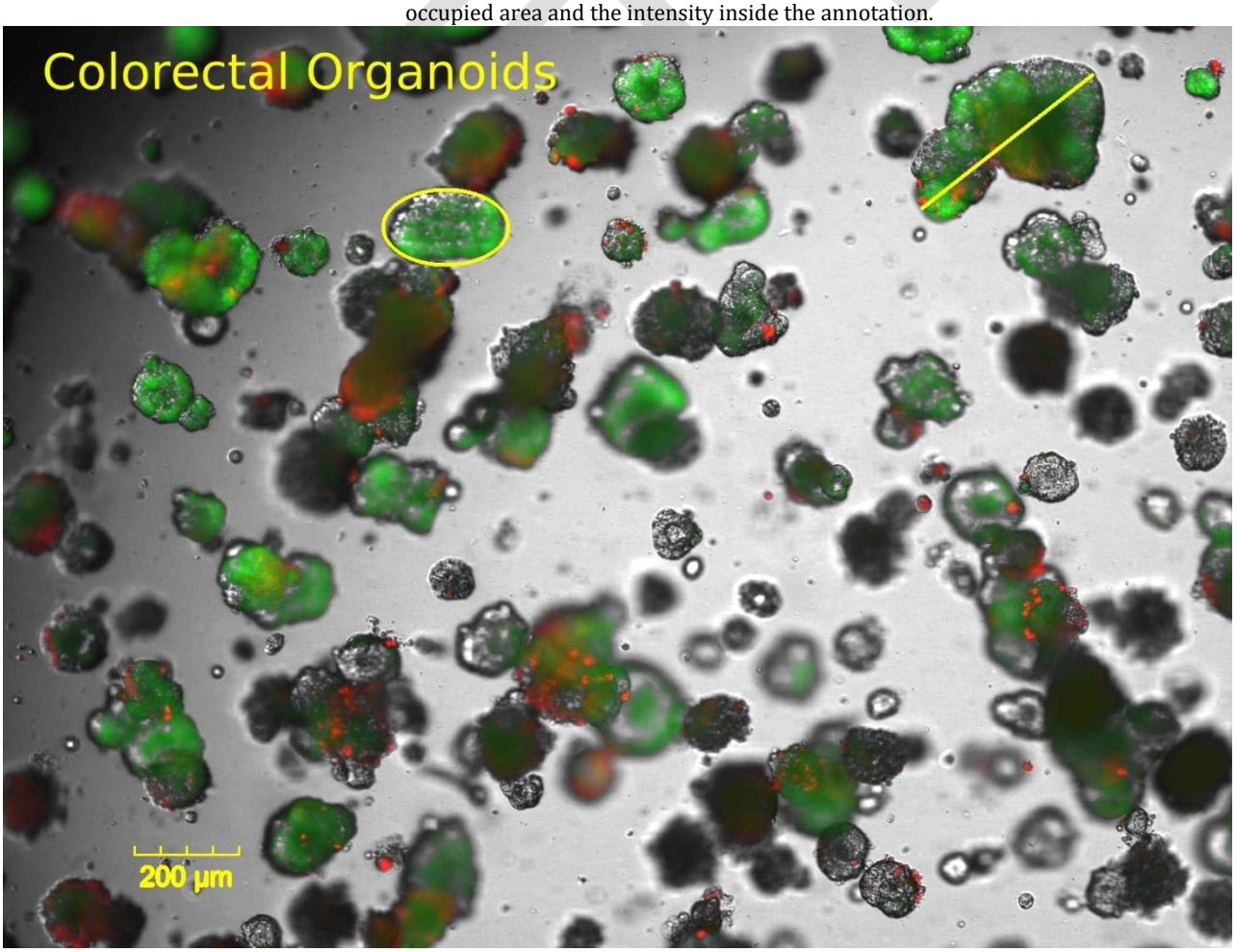
Displaying line profile or histogram of intensity

- The generated line profile or histogram can be stored as an image or a CSV file.
- The chart shows the intensity value at a specified point on mouse cursor in real time.

3. **Measurement**

The measured values are displayed on Data panel.

- Line annotation shows the length and the intensity on the line.
- Area annotation like a circle or a rectangle shows the peripheral length, the occupied area and the intensity inside the annotation.



Tool	Length (μm)	Area (μm²)	Angle (°)	Pixels	Min (BF)	Max (BF)	Mean (BF)	Std (BF)	Min (GFP)	Max (GFP)	Mean (GFP)	Std (GFP)	Min (RFP)	Max (RFP)	Mean (RFP)	Std (RFP)
Line	411.2	-	-	281	12.0	197.0	43.6	32.2	54.0	217.0	101.1	35.9	14.0	179.0	41.8	24.4
Ellipse	619.0	27102.5	-	12628	19.0	255.0	98.8	51.6	3.0	233.0	104.5	53.8	1.0	120.0	20.4	9.8

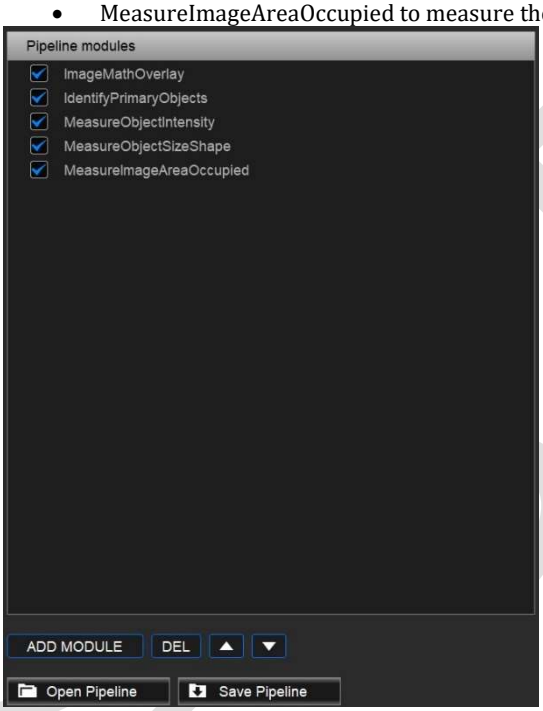
Patient-derived colorectal organoids treated by Fluorouracil (5-FU) for 3 days and stained by ethidium homodimer-1 and propidium iodide (Courtesy to Asan Medical Center)

9.5 Analyzing merged images with analysis modules

1. Add modules

Adding modules to analyze images or loading a predefined pipeline.
In this example, the following modules are added.

- ImageMathOverlay to add GFP and RFP channel
- IdentifyPrimaryObjects to find objects
- MeasureObjectIntensity to measure the fluorescence intensity of the channels
- MeasureObjectSizeShape to measure the area of the objects
- MeasureImageAreaOccupied to measure the total area occupied by the objects

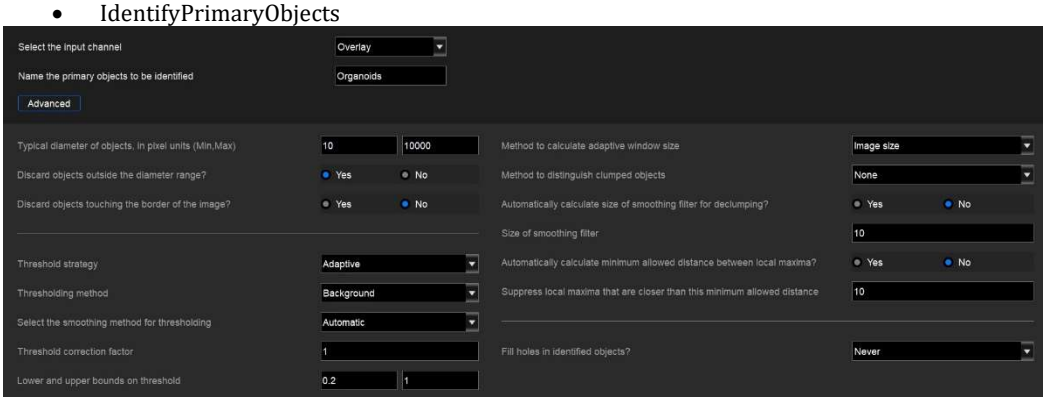


2. Adjust parameters

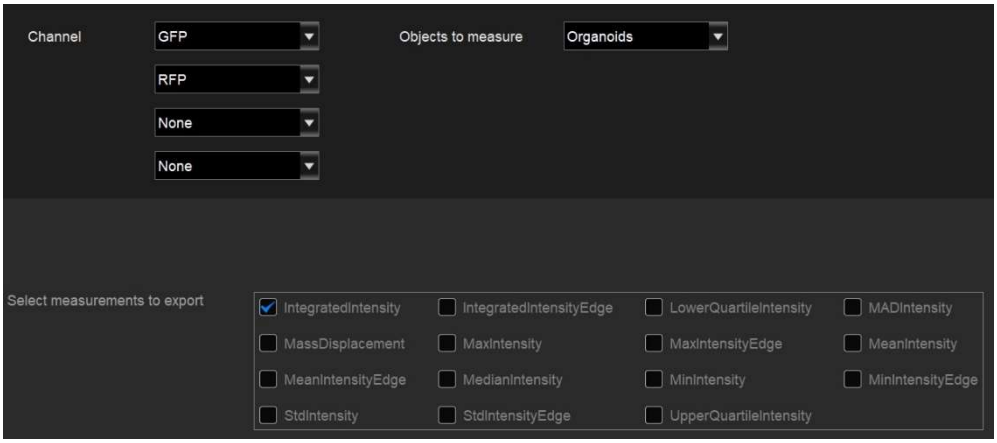
Adjusting parameters to optimize analysis

- ImageMathOverlay

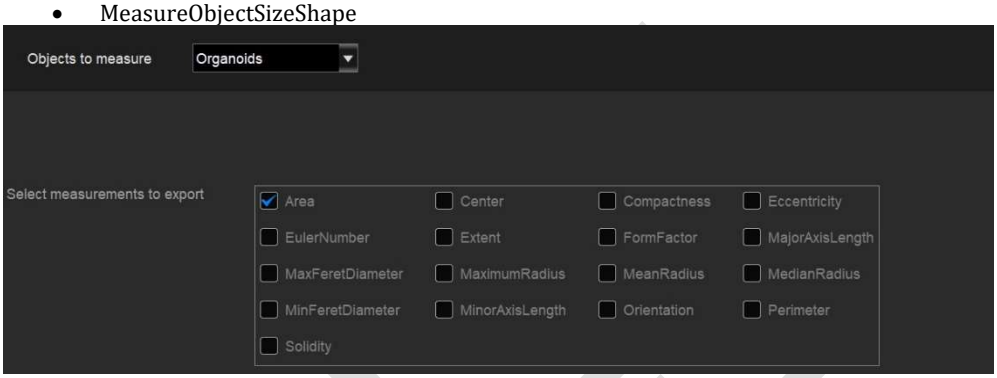
- IdentifyPrimaryObjects



- MeasureObjectIntensity



- MeasureObjectSizeShape



- MeasureImageAreaOccupied

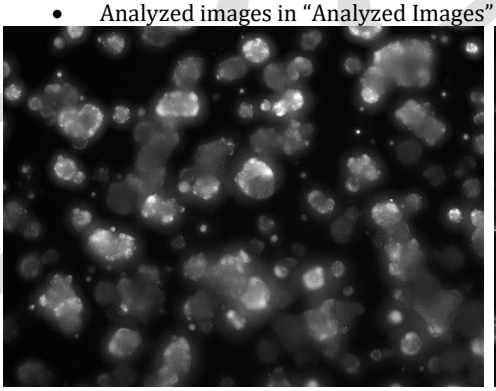
3. Run analysis

Select wells to be analyzed in VIEW panel.
Clicking **Analysis +** and add wells in an analysis list.
Click **ANALYZE** to run analysis in ANALYSIS panel.
Input Analysis name to save results and click OK.

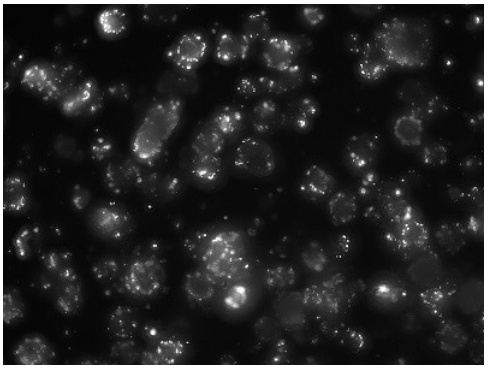
4. Analyzed results

The measured results are as below.

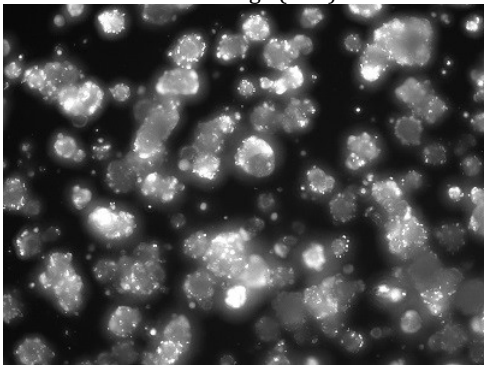
- Analyzed images in “Analyzed Images” folder



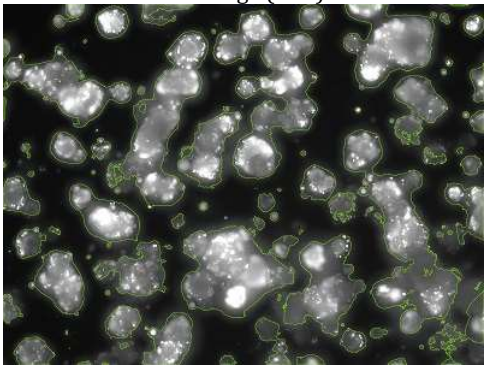
Raw image (GFP)



Raw image (RFP)



Overlay (GFP + RFP)



Organoids

- Object (Organoids) data

ImageNumber	ObjectNumber	AreaShape_Area	Intensity_IntegratedIntensity_GFP	Intensity_IntegratedIntensity_RFP	Location_Center_X	Location_Center_Y
30	2	5751	1521.3647	565.7333	524.1650	26.7812
30	3	5546	1164.5843	1191.2549	1024.4706	41.8503
30	4	55689	20477.0318	9222.4551	1280.6953	129.9402
30	5	332	63.6196	24.5176	1515.7168	2.5391
30	6	9155	2077.7177	3510.2432	362.9962	59.6892
30	7	95117	32324.6087	17218.1181	885.5643	280.4938
30	8	346	88.5019	18.1490	1432.2254	35.8034
30	9	900	196.0078	94.0666	1469.5077	47.4222
30	10	3544	1305.4588	566.6274	1566.5849	70.7260
30	11	176	11.6431	45.9686	700.5284	90.6818
30	12	77218	24057.2006	15901.1533	531.9669	395.5970
30	13	1116	389.1215	74.9019	9.4865	145.6621
30	14	98	2.6666	25.3529	272.0612	123.6020

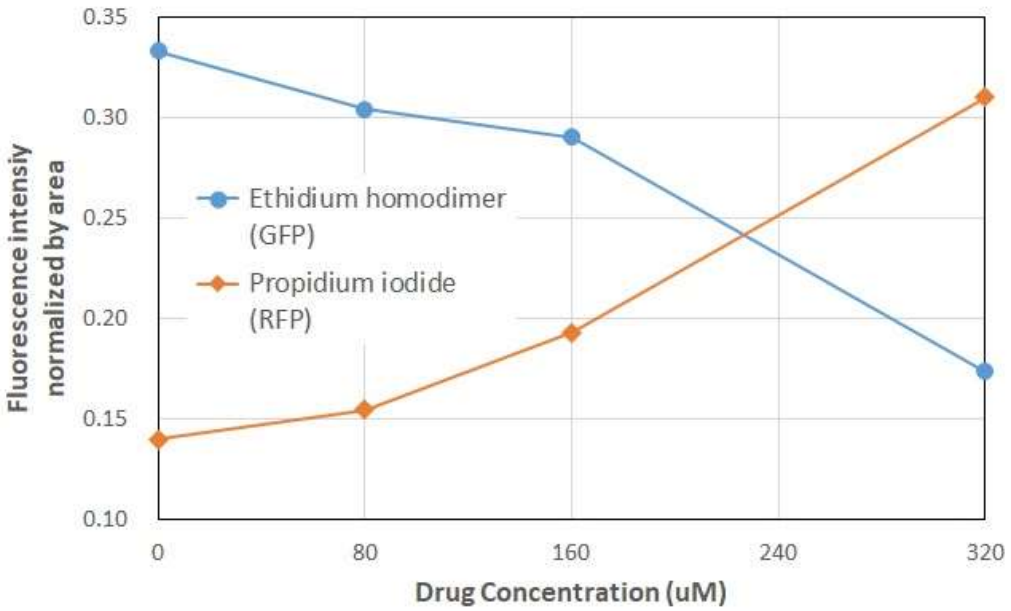
- Overall result

ImageNumber	Well	Field	Z-plane	Time	ed_Org	Count_Organoids	Organoids_Area	Organoids_IntegratedIntensity_GFP	Organoids_IntegratedIntensity_RFP
30	G06	R3C2	1	1	56.43	55.0	19700.8	4932.4446	3360.0675
31	G07	R3C2	1	1	58.80	62.0	18208.1612	4366.1574	2711.4523
32	G08	R3C2	1	1	55.67	47.0	22742.6808	5889.4017	3372.3494
33	G09	R3C2	1	1	57.58	43.0	25710.7209	6187.1464	3811.2355
34	G10	R3C2	1	1	54.60	43.0	24379.8372	6358.5040	3830.8382
35	G11	R3C2	1	1	50.05	52.0	18479.0769	4661.1445	2814.0205
36	G12	R3C2	1	1	43.37	42.0	19828.3571	6149.0628	2955.7807
37	H01	R3C2	1	1	16.14	38.0	8155.1052	2719.9836	888.9534
38	H02	R3C2	1	1	52.28	52.0	19301.5961	5211.7882	2707.2441
39	H03	R3C2	1	1	56.56	40.0	27147.325	6373.5885	3787.8447
40	H04	R3C2	1	1	56.20	40.0	26977.55	4214.5269	6660.8057

Messages | Data | Modules | **Results** | Organoids

5. Further analysis

The exported CSV file can be used for further analysis as below.



In this example ethidium homodimer stains live cells and propidium iodide stains dead cells, respectively. This graph shows live cells decrease and dead cells increase as the drug concentration increases

Appendix A: Troubleshooting

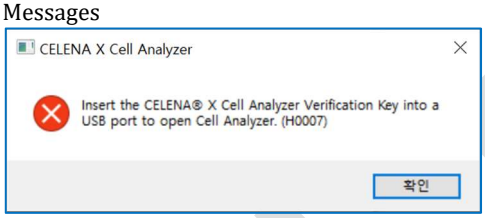
A.1 Troubleshooting

Performance

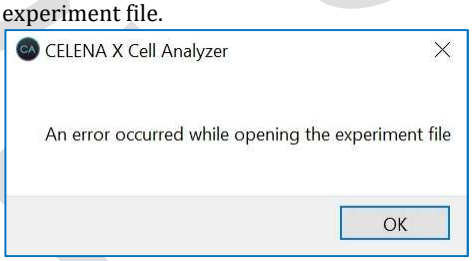
	Recommended actions
Monitor	The monitor should support 4K UHD resolution. If not CELENA® X Cell Analyzer does not properly operate on screen.
Folder and file name	CELENA X® Cell Analyzer properly recognizes only English characters. Do not use other character like French, Germany, Spanish, Japanese and Korean characters in both folder and file names.

A.2 Device Messages

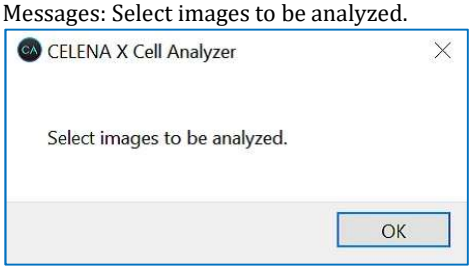
Security

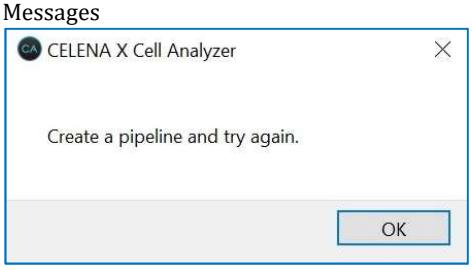
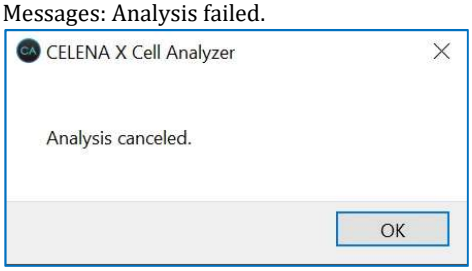
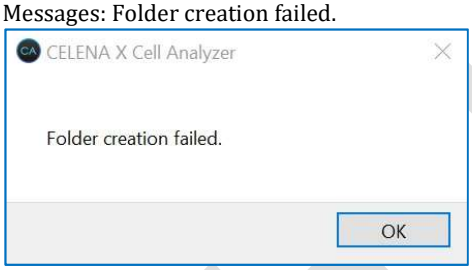
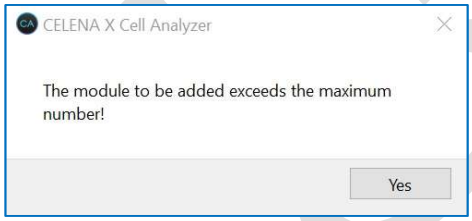
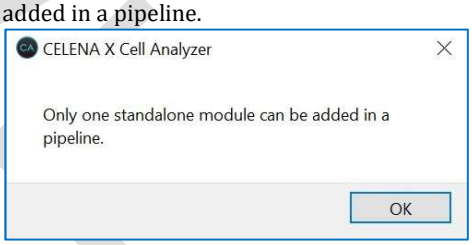
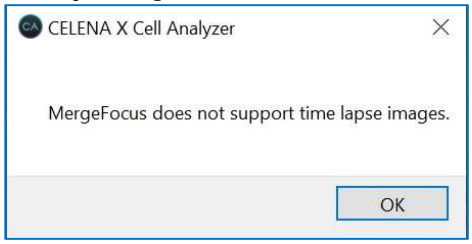
	Messages	Cause and recommended action(s)
Verification key		The verification key is not installed in your computer. Plug in the verification key in any USB port in your computer. 

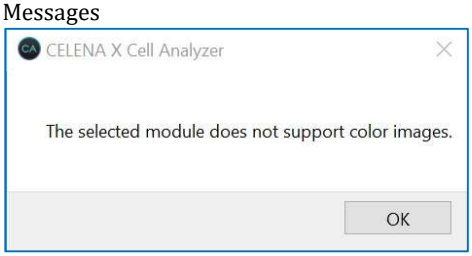
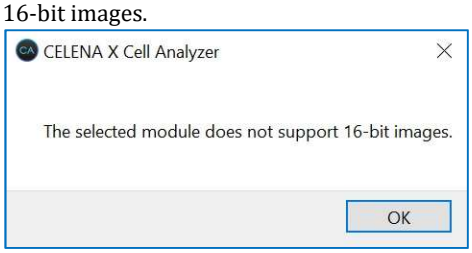
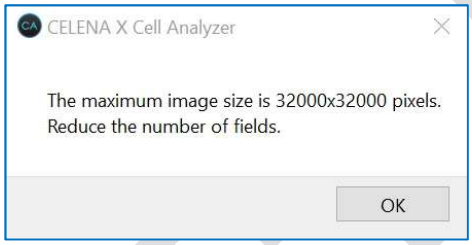
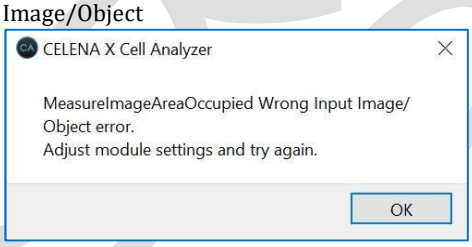
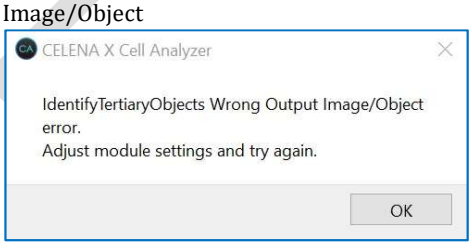
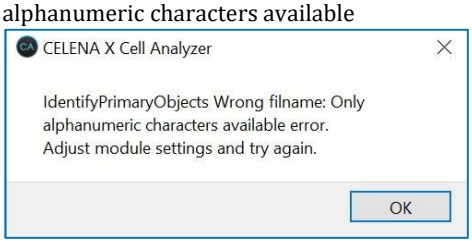
Project

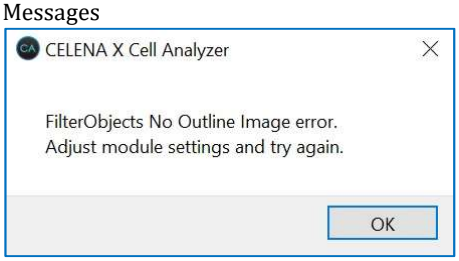
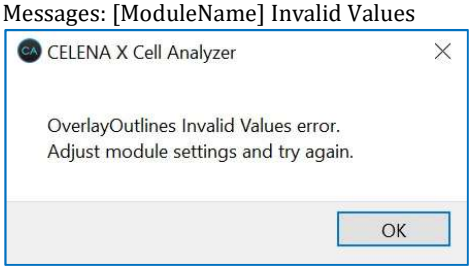
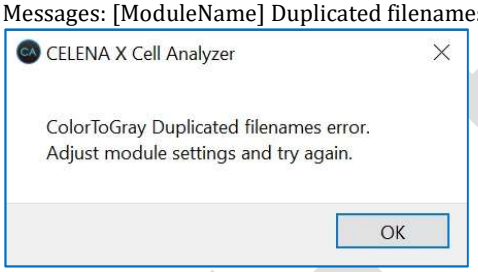
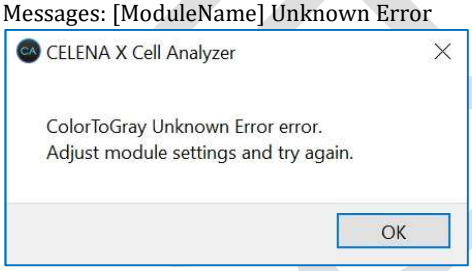
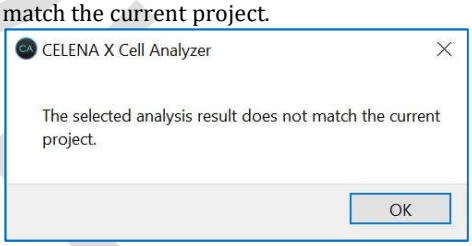
	Messages	Cause and recommended action(s)
Opening project	Messages: An error occurred while opening the experiment file. 	The information in the project file is abnormal. Select a proper project file and open it again.

Analysis

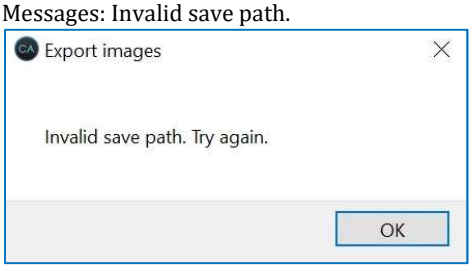
	Messages	Cause and recommended action(s)
Selecting wells or a slide	Messages: Select images to be analyzed. 	Any wells or a slide are not selected. Select wells or a slide and click **Analysis +** to add images to be analyzed.
Creating a pipeline	Messages: Create a pipeline and try again.	There is no module in a pipeline. Add modules in the pipeline.

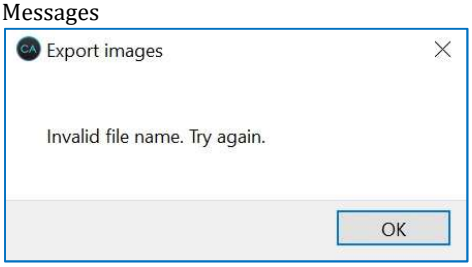
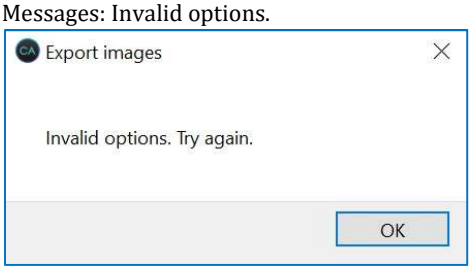
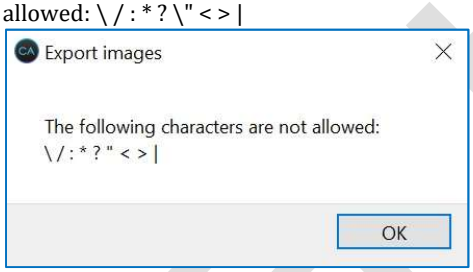
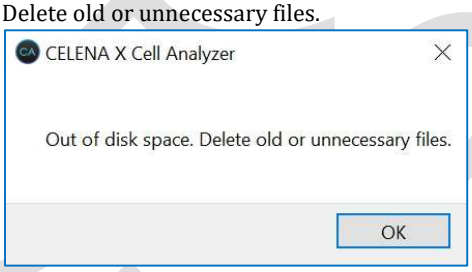
	Messages	Cause and recommended action(s)
		
Analysis failure	Messages: Analysis failed. 	Analysis has been failed because of any reason. Verify if parameters in each module are properly set and try again.
Folder creation error	Messages: Folder creation failed. 	The output folder cannot be generated because of any reason. If an image or a CSV file in the output folder currently opens the output folder cannot be generated. Close all the files and try again.
Limitation of module numbers	Messages: The module to be added exceeds the maximum number! 	The maximum number of modules should be less than 100. Reduce the number of modules in the pipeline and try again.
Standalone module	Messages: Only one standalone module can be added in a pipeline. 	A standalone module like MergeFocus and StitchImages operates independently and it cannot be used with other modules. Remove other modules in the pipeline.
MergeFocus	Messages: MergeFocus does not support time lapse images. 	Images acquired by Time Lapse in CELENA® X Explorer cannot be analyzed with this module.
	Messages: The selected module does not support color images.	Color images cannot be analyzed with this module.

	Messages	Cause and recommended action(s)
		
StitchImages	Messages: The selected module does not support 16-bit images. 	16-bit images cannot be analyzed with this module.
	Messages: The maximum image size is 32,000 x 32,000 pixels. Reduce the number of fields. 	The maximum image size of the stitched output image is smaller than the above size. Reduce the number of fields and acquire images again in CELENA® X Explorer, and try again.
Module setting error	Messages: [ModuleName] Wrong Input Image/Object 	Abnormal input image or input object is selected in the [ModuleName] module. Set a proper input image or input object.
	Messages: [ModuleName] Wrong Output Image/Object 	Abnormal output image or output object is selected in the [ModuleName] module. Set a proper output image or output object.
	Messages: [ModuleName] Wrong filname: Only alphanumeric characters available 	An image or object name should have only alphanumeric characters. Remove other characters like special characters in the [ModuleName] module.
	Messages: [ModuleName] No Outline Image	No outline image is designated. Assign an outline image name in the [ModuleName] module.

	Messages	Cause and recommended action(s)
		
	Messages: [ModuleName] Invalid Values	Invalid values are set in the [ModuleName] module. Set a proper value.
		
	Messages: [ModuleName] Duplicated filenames	There are same names in image or object names. Change the name in the [ModuleName] module.
		
	Messages: [ModuleName] Unknown Error	There is an unknown error in the [ModuleName] module. Examine the parameters.
		
Analyzed images	Messages: The selected analysis result does not match the current project.	The analysis result should match the currently loaded project because the analysis result uses the information in the project. Open a proper project and an analysis result.
		

Saving images or videos

	Messages	Cause and recommended action(s)
Exporting images	Messages: Invalid save path.	The folder name is improper. Set a proper name.
		
	Messages: Invalid file name.	The file name is improper. Set a proper name.

	Messages	Cause and recommended action(s)
		
	Messages: Invalid options. 	The image options are improper. (No option is selected.) Set a proper options.
	Messages: The following characters are not allowed: \ / : * ? \" < > | 	Special characters cannot be included in the file name. Remove these characters.
Deficient disk space	Messages: You are out of disk space. Delete old or unnecessary files. 	Disk space is deficient.

Appendix B: Specifications & Requirements

CELENA® X Cell Analyzer

Imaging modes	4-channel fluorescence and brightfield
Image inputs	Monochrome: 8-/16-bit (12-bit dynamic range) TIF Color: 24-bit color TIF
Image outputs	Monochrome: 8-/16-bit (12-bit dynamic range) TIF, PNG, or JPG Color: 24-bit color TIF, PNG, or JPG Movies: MP4
Computer requirements	External PC running Windows™ 10 RAM: 8 Gb or more CPU: Intel i7 or higher recommended Hard disk space: at least 10 GB available space
Monitor requirement	4K UHD monitor

Appendix C: Ordering Information

Accessories

Cat #	Product
CX31002	CELENA® X Cell Analyzer Verification Key

Appendix D: Purchaser Notification

Limited use label license

Research use only

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CellProfiler

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