IN Carta

Image Analysis Software

VoluMetrics User Guide

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September 2021

MOLECULAR DEVICES

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Chapter 1: IN Carta Image Analysis Software

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The IN Carta® Image Analysis Software provides powerful analytics combined with an intuitive interface to simplify workflows for advanced phenotypic classification and 3D image analysis.

Faster Data

- Intuitive design makes complex analysis accessible with minimal training.
- Shorten analysis time with true parallel processing.

Reliable Data

- Sophisticated algorithms generate reliable data with minimal user input.
- Improved segmentation algorithms represent cellular structures more accurately.

Results that Matter

- See real results quickly—from populations to single cells— using integrated data visualization tools.
- User-friendly interface guides you through your discoveries with continual updates that grow with your needs.

Terminology

The following table defines the terms and abbreviations used in this guide.

Term	Definition
3D Stack	Series of images taken in different Z-planes of a specimen. Combines images to provide a composite image with a greater depth of field than the individual source images.
FOV	Field of view.
GUI	Graphical User Interface.
Mask	Image showing object segmentation.
ROI	Region of interest.
Voxel	A voxel represents a point within a 3D dataset similarly to how a pixel represents a point in a 2D image.
Z-plane / Z-slice	One of a set of 2D images (XY planes) that compose a 3D dataset, representing a single focal plane.
Binning	A method to combine a cluster of pixels/voxels into a single pixel/voxel. Binning operation makes image smaller in size. For example, 2x2 binning adds up pixel intensities within a block of 2x2 pixels and assign the resulting value to a single pixel.

Obtaining Support

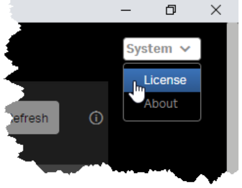
Molecular Devices is a leading worldwide manufacturer and distributor of analytical instrumentation, software, and reagents. We are committed to the quality of our products and to fully supporting our customers with the highest level of technical service.

Our Support website—www.moleculardevices.com/service-support—describes the support options offered by Molecular Devices, including service plans and professional services. It also has a link to the Molecular Devices Knowledge Base, which contains documentation, technical notes, software upgrades, safety data sheets, and other resources. If you still need assistance, you can submit a request to Molecular Devices Technical Support.

Technical Support

To contact Molecular Devices Technical Support, submit a support request through the Molecular Devices Knowledge Base at support.moleculardevices.com.

You can also submit a support request by phone. For regional support contact information, go to www.moleculardevices.com/contact.



To expedite support, please be prepared to provide the software version and the license ID.

- To display the software version, in the top right corner of the IN Carta window, click **System > About**.
- To display the license ID, in the top right corner of the IN Carta window, click **System > License**.

Documentation

Review the product documentation on the Molecular Devices Knowledge Base at support.moleculardevices.com. In addition, online Help is available within the IN Carta software.

Additional Resources

Web-based microscopy courses:

- www.microscopyu.com
- www.ibiology.org/ibioeducation/taking-courses/ibiology-microscopy-short-course.html

The *Molecular Probes Handbook* offers advice on fluorescent probes and can help you determine if there are better stains available for your analysis:

- www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook.html

The *Assay Guidance Manual* details state-of-the-art approaches to high-content screening (HCS) and discussed challenges specific to HCS. It serves as a good introduction for new HCS practitioners.

- www.ncbi.nlm.nih.gov/books/NBK100913

About This Guide

This guide is intended for the scientist using the IN Carta software. It describes how to use IN Carta VoluMetrics module.

The information in this guide is valid for IN Carta software version 1.15 and is subject to change without notice. We recommend that you review the guide on the Molecular Devices Knowledge Base at support.moleculardevices.com for the most up-to-date information.

Chapter 2: IN Carta VoluMetrics

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IN Carta VoluMetrics module is a set of image analysis algorithms to segment biological objects and extract measurements in three dimensions (3D). These algorithms operate on voxels to segment objects and extract relevant measures throughout the depth of an object. Since IN Carta VoluMetrics analyzes voxels, datasets must have at least four Z-slices.

This module requires an additional license (Product numbers: 29418664, 29418665, or 29418666).

Chapter 3: Algorithms

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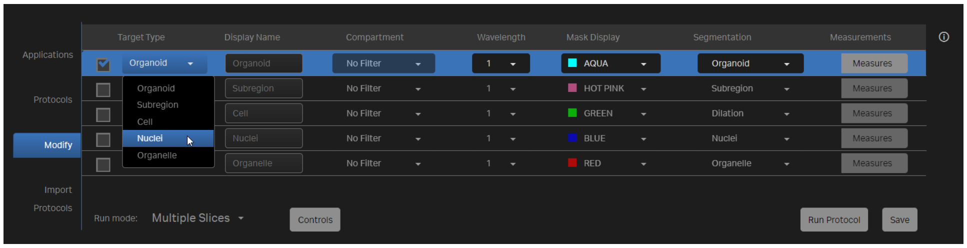
This chapter describes the available segmentation algorithms and segmentation parameters.

- Segmentation Algorithms, see below
- Segmentation Parameters, see page 10
- Organoid and Subregion Segmentation, see page 14
- Nuclei and Organelle Segmentation, see page 14
- Cell Segmentation, see page 15

Segmentation Algorithms



	Target Type	Display Name	Compartment	Wavelength	Mask Display	Segmentation	Measurements
☑	Organoid	Organoid	No Filter	1	AQUA	Organoid	Measures
☐		Subregion	No Filter	1	HOT PINK	Subregion	Measures
☐		Cell	No Filter	1	GREEN	Dilation	Measures
☐		Nuclei	No Filter	1	BLUE	Nuclei	Measures
☐		Organelle	No Filter	1	RED	Organelle	Measures

Run mode: Multiple Slices

The following table describes the five segmentation algorithms:

Algorithm	Function
Organoid	Segmentation of large structures like spheroids or organoids.
Subregion	Segmentation of smaller regions within an organoid.
Nuclei	Segmentation of nuclei.
Cell	There are two options: • **Dilation**: Dilation of the nuclear mask for a defined distance. **Cell Dilation** is a simpler algorithm that does not account for intensities within a target channel and performs dilation of a nuclear mask. • **Fast**: Segmentation of cells based on nuclear mask and intensity distribution within a target channel. **Cell Fast** is a more complex algorithm which traces cells' edges and splits touching cells. Algorithms are optimized to segment cells in 3D and require a previously segmented nuclear mask.
Organelle	Segmentation of subcellular structures.

Each algorithm, except for **Cell Dilation**, has optional pre- and post-processing steps that can be applied as needed.

Segmentation Parameters

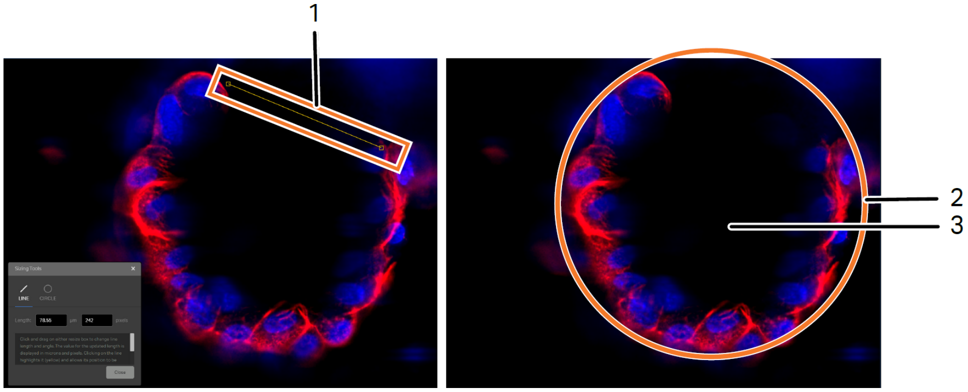
There are various fields that can be used to adjust segmentation. Not all fields are available in all algorithms. For more information regarding which field can be used for each algorithm, see the algorithm-specific sections below.

Field	Function	Algorithm
Background	Defines the intensity value for the background in the surrounding area of the organoid or subregion.	• Organoid • Subregion
Intensity Sensitivity	Defines detection sensitivity [0-100]. Higher sensitivity values increase the number of segmented objects and/or their volume.	• Nuclei • Organelle
Split Sensitivity	Controls how clumped nuclei or organelles are separated into individual objects. Recommended to start with high values (>95) and reduce it if objects are overly split.	• Nuclei • Organelle
Min. Volume	Defines the minimum volume of an organoid or subregion set (in μm^3).	• Organoid • Subregion
Min. Diameter	Defines the approximate minimum nuclei or organelle diameter (in µm) used to identify a nuclei or organelle. This value is not a size threshold for segmented objects.	• Nuclei • Organelle
Max. Diameter	Defines the approximate maximum nuclei or organelle diameter size (in µm) used to identify a nuclei or organelle. This value is not a size threshold for segmented objects.	• Nuclei • Organelle
Allow Holes	Controls whether nuclei or organelles are allowed to have hollow parts within their 3D masks. Holes are excluded from the object’s volume when calculating measures.	• Nuclei • Organelle
Smoothing	Controls whether or not the software accounts for the difference in intensity values within nuclei or organelles and segment as one object if variations of intensity values do not exceed the input value for **Intensity Contrast**.	• Nuclei • Organelle
Gap Size	Defines the maximum gap (in µm) allowed between objects included in an organoid or subregion that are not connected to each other. For more information, see Gap Size on page 12.	• Organoid • Subregion
Nuclei Seed	The Nuclear mask to be used as a seed for cell segmentation. It is chosen from the drop-down list. The dilation is applied in 3D by a given **Growing Radius**. For more information, see Nuclei Seed on page 12.	• Cell Dilation • Cell Fast
Growing Radius	Defines the distance (in µm) that the nuclear mask is increased (dilated). Dilation occurs evenly in X, Y, and Z-dimensions until the radius is reached, or the mask reaches the mask of an adjacent cell. Masks of neighboring cells never overlap.	• Cell Dilation

Field	Function	Algorithm
Fine Dilation	Selecting the check box provides more accurate cell outlines. This is more apparent when large values for **Growing Radius** are set or in case of high magnification datasets. **Note:** Selecting **Fine Dilation** might result in slower segmentation	• Cell Dilation
Threshold	Minimum intensity value to separate cells from background.	• Cell Fast
Low Contrast Borders	Select this check box if cells are clumped and have no clear border between each other.	• Cell Fast
Scaling Factor X,Y	Controls the binning of an image by reducing the image size in X and Y while maintaining the aspect ratio of the image. **Scaling Factor X,Y** increases the speed of the analysis processes, but reduces the lateral resolution within the image which can decrease the segmentation accuracy. For more information, see Scaling Factor X,Y on page 13.	• Organoid • Subregion • Nuclei • Organelle • Cell Dilation • Cell Fast
Scaling Factor Z	Controls the binning of a 3D stack in Z by reducing the final number of Z-slices analyzed. This speeds up analysis processes but reduces axial resolution within the image, which can decrease the segmentation accuracy. For more information , see Scaling Factor Z on page 13.	• Organoid • Subregion • Nuclei • Organelle • Cell Dilation • Cell Fast

Gap Size

Defines the maximum gap (in µm) allowed between objects included in an organoid or subregion, which are not connected to each other. The gap can be estimated using the **Line tool**, as shown below.

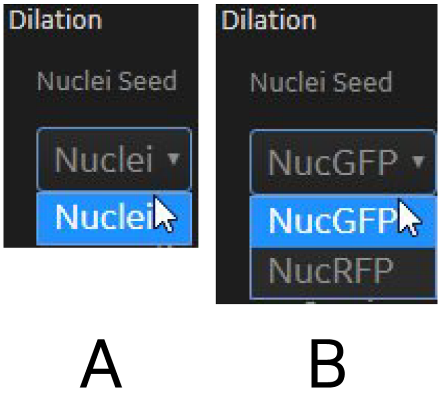


Part	Description
1	Gap
2	Organoid
3	Background value of organoid

Nuclei Seed

When a protocol has more than one nuclear segmentation mask available, it is important to specify which nuclear mask should be used as the seed for cell segmentation.

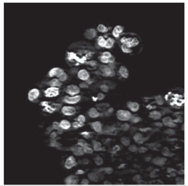
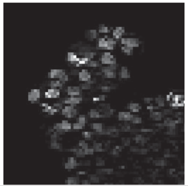
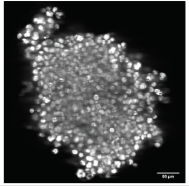
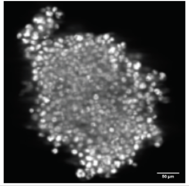
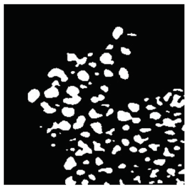
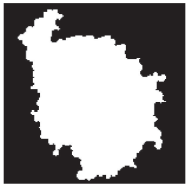
The following illustration shows nuclei seed selection with single (A) or multiple (B) nuclei targets.



Scaling Factor X,Y

To select a value for **Scaling Factor X,Y**, consider the size of the objects and make sure an appropriate binned image pixel size is selected. A value of 1 maintains the original size of the image. Adjusting the scaling factor does not change the output size of the segmented objects.

The following illustration shows the effect of **Scaling Factor X,Y**.

Structures	Nuclei and organelles		Organoids and subregions	
Scaling Factor X,Y	1	0.25	1	0.25
Original/ Binned image				
Resulting mask				

Tip: Reducing **Scaling Factor X,Y** is more applicable for larger biological structures, such as spheroids or organoids. Using a lower **Scaling Factor X,Y** for nuclei or organelle segmentation can produce biologically irrelevant results.

The following table shows an example of how the image size changes based on the input **Scaling Factor X,Y**.

Scaling Factor X,Y	Stack Size (X x Y x Z)
1	2040 x 2040 x 100
0.25	510 x 510 x 100

Scaling Factor Z

To select a value **Scaling Factor Z**, consider the size of the objects and make sure an appropriate binned image voxel size is selected. A value of 1 maintains the original number of Z-slices. Reducing **Scaling Factor Z** results in binning of the 3D stack along the Z-axis. Adjusting the scaling factor does not change the output size of the segmented objects.

The following table shows an example of how the image size changes based on the input **Scaling Factor Z**.

Scaling Factor Z	Dataset Size (Voxels)
1	2040 x 2040 x 100
0.5	2040 x 2040 x 50

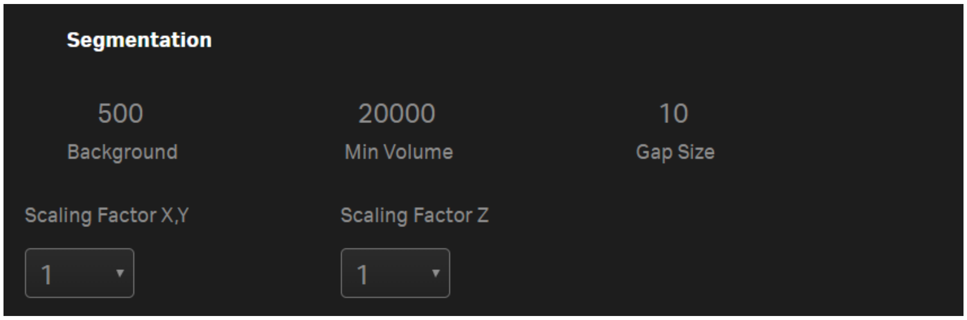
Organoid and Subregion Segmentation

The organoid algorithm is optimized for segmentation of large 3D structures.

The subregion algorithm is optimized to perform segmentation of smaller regions within an organoid.

The parameters that control segmentation are the same for these two algorithms.

The illustration below shows the parameters for the two algorithms.



Segmentation

500
Background

20000
Min Volume

10
Gap Size

Scaling Factor X,Y
1

Scaling Factor Z
1

For details on the functions of available parameters for the segmentation of organoids and/or subregions, see Segmentation Parameters on page 10.

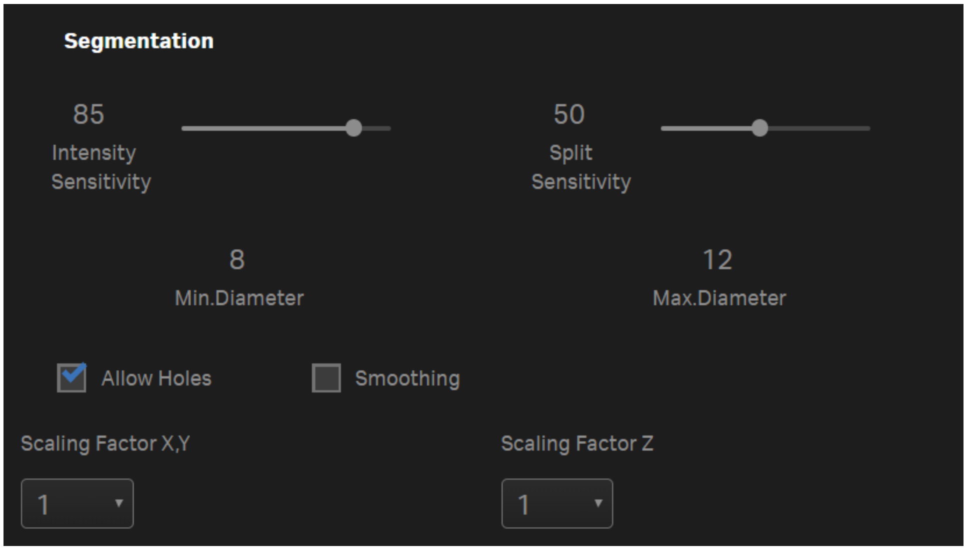
Nuclei and Organelle Segmentation

The nuclei algorithm is optimized to perform the detection of nuclei in a 3D stack.

The organelle algorithm is optimized to perform the detection of subcellular organelles in a 3D stack.

The parameters that control segmentation are the same for these two algorithms.

The illustration below shows the parameters for the two algorithms.



Segmentation

85
Intensity Sensitivity

50
Split Sensitivity

8
Min.Diameter

12
Max.Diameter

☑ Allow Holes
☐ Smoothing

Scaling Factor X,Y
1

Scaling Factor Z
1

For details on the functions of available parameters for the segmentation of nuclei and organelles, see Segmentation Parameters on page 10.

Cell Segmentation

Cell Dilation

Cell Dilation segments cells by dilating the nuclear mask for a defined distance: the **Growing Radius**.

The illustration below shows the parameters for the algorithm.

Segmentation

Nuclei Seed

Nuclei

☐ Fine Dilation

2
Growing Radius

Scaling Factor X,Y
1

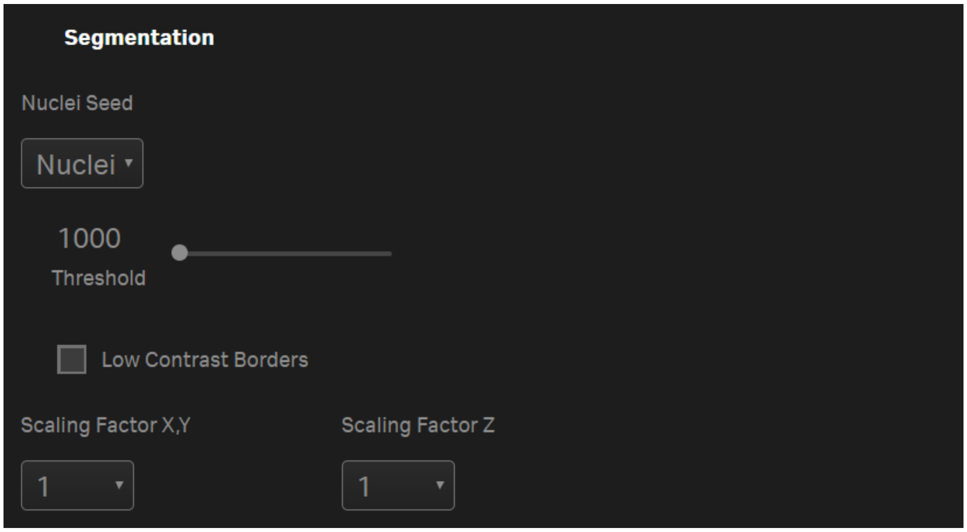
Scaling Factor Z
1

For details on the functions of available parameters for the segmentation of cells during **Cell Dilation**, see Segmentation Parameters on page 10.

Cell Fast

This algorithm detects cells based on patterns characteristic for cellular stains and selected nuclear mask.

The illustration below shows the parameters for the algorithm.



Segmentation

Nuclei Seed

Nuclei

1000
Threshold

☐ Low Contrast Borders

Scaling Factor X,Y
1

Scaling Factor Z
1

For details on the functions of available parameters for the segmentation of cells during **Cell Fast**, see Segmentation Parameters on page 10.

Chapter 4: Pre-Processing

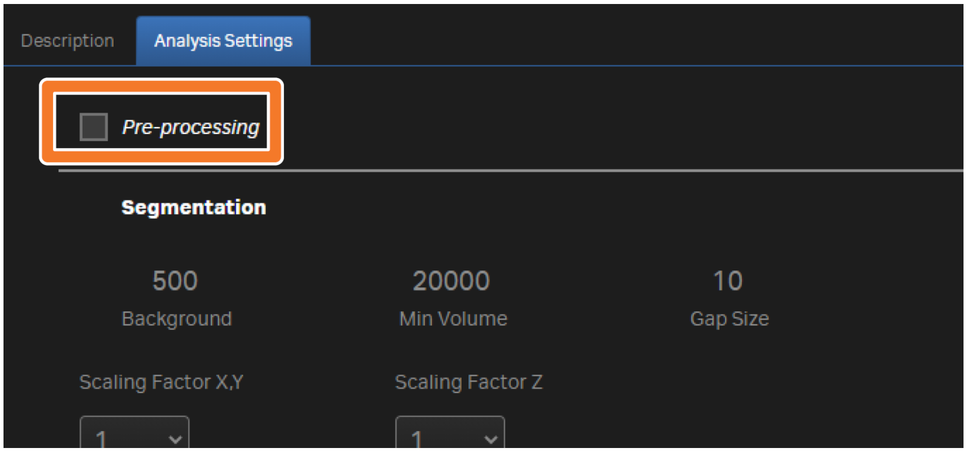
For all targets except **Cell Dilation**, you can apply optional pre-processing filters to images prior to the segmentation step. These filters only affect segmentation results; they do not alter raw images.

The section describes the following pre-processing filters:

- Denoising, see below
- Local Normalization, see page 20
- Global Normalization, see page 21

Accessing and Previewing Pre-Processing Filters

Select the **Pre-processing** check box to activate the pre-processing filters.

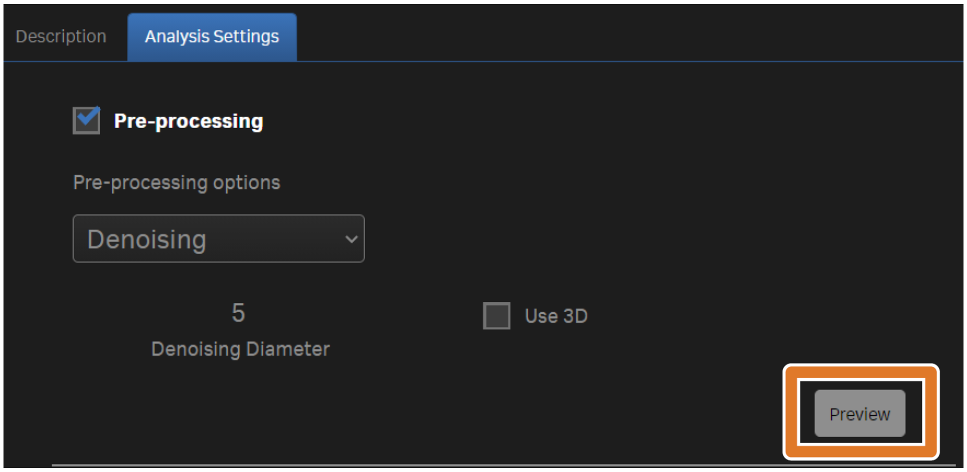


☐ Pre-processing

Segmentation

Background: 500
Min Volume: 20000
Gap Size: 10
Scaling Factor X,Y: 1
Scaling Factor Z: 1

Click **Preview** to display the current Z-slice with the pre-processing filters activated.



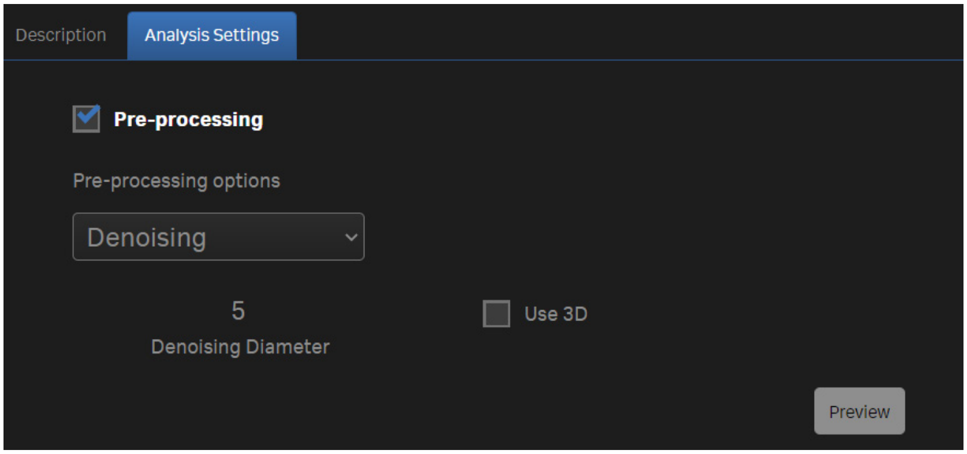
☑ Pre-processing

Pre-processing options: Denoising

Denoising Diameter: 5
☐ Use 3D

Denoising

Denoising is a mathematical transformation of a stack. It is an image filter that removes local aberrations in image pixel values and minimizes noise in a 3D stack. This means that denoising replaces the intensity value of each voxel in an image stack with a new value. This is achieved by averaging intensity values within a local neighborhood. This operation is performed one voxel at a time. You can define the size of the neighborhood surrounding each voxel that is used for calculations. This method is useful to eliminate segmentation artifacts caused by bright small debris or general signal fluctuations. As a result, intensity variation within neighboring pixels is reduced and the image appears smoother.



☑ Pre-processing

Pre-processing options

Denoising

5

Denoising Diameter

☐ Use 3D

Denoising Diameter

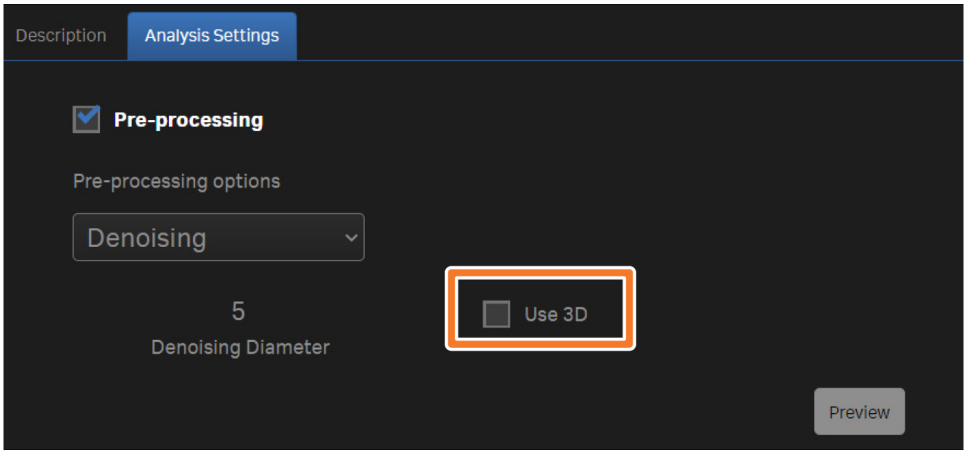
A rolling block (set in µm) defines the smoothing neighborhood. A larger diameter results in a larger number of neighboring pixels being used in the calculation and more pronounced smoothing effect.

Note: High denoising diameter values can result in the loss of not only the background noise, but also of the target-defining details, and it can lead to less effective segmentation. The value can be set between 1 and 100.

3D Denoising

Denoising is performed on each Z-slice independently when the **Use 3D** check box is cleared.

Selecting the **Use 3D** check box performs denoising within a defined 3D neighborhood. Performing 3D-based denoising is recommended when the axial resolution is no more than two times larger than the lateral resolution.



☑ **Pre-processing**

Pre-processing options: Denoising

5 Denoising Diameter

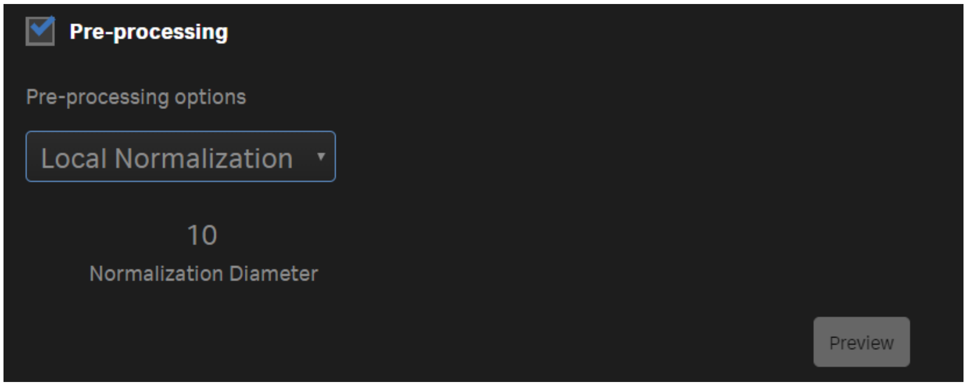
☐ Use 3D

Images were acquired using 20x objective (pixel size 0.325 µm). Z-step during acquisition must be set to 0.65 µm or lower in order to gain better results with 3D-denoising.

Note: Denoising runs significantly faster in 2D and is suitable for most data sets.

Local Normalization

Local Normalization is a mathematical transformation of a stack. This filter is helpful to improve contrast of dim fluorescent structures and reduce the background. **Local Normalization** performs normalization of intensity values in an image within a user-defined, local neighborhood. This means that **Local Normalization** replaces the intensity value of each voxel in an image stack with a new value. The resulting value depends on the intensities of the neighboring voxels. This operation is performed one voxel at a time. You can define the size of the neighborhood surrounding each voxel that is used for calculations.


☑ Pre-processing

Pre-processing options: Local Normalization

Normalization Diameter: 10

Normalization Diameter

The **Normalization Diameter** value corresponds to the neighborhood size (set in µm). This parameter controls the diameter of the neighborhood to which the normalization is applied. The diameter should be comparable with the size of the biological structure of interest.

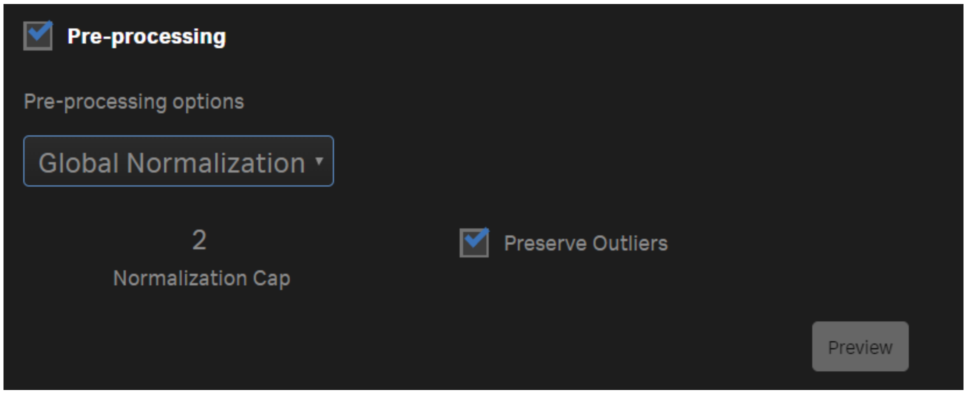
Note: High values of the normalization diameter can result in the loss of target-defining details and lead to a less effective segmentation. The value can be set between 1 and 100.

Global Normalization

Global Normalization is a filter that depends on intensity values in entire 3D stack or an ROI selected for analysis. **Global Normalization** helps to reset intensity values based on the mean (**Mean**) and standard deviation (**Std**) of pixel intensity values within a 3D stack, as well as the user-defined normalization cap (**Cp**). All the pixel values in the 3D stack are recalculated such that:

- Pixel values with intensities less than or equal to **Mean - Std * Cp** are set to 0 (minimum of 16-bit scale).
- Pixel values with intensities greater than or equal to **Mean + Std * Cp** are set to 65,535 (maximum of 16-bit scale).
- Pixel values with intensities between **Mean - Std * Cp** and **Mean + Std * Cp** are scaled linearly.

Increasing the **Normalization Cap** sets contrast in the pre-processed image closer to the original image. This method is helpful to equalize intensity values across all the wells in a plate during segmentation. At the same time, this method can generate artificially bright results in cases when the FOV does not contain any fluorescent structures and only has background signal.



☑ Pre-processing

Pre-processing options: Global Normalization

Normalization Cap: 2

☑ Preserve Outliers

Global Normalization Parameters

The following table shows the functions of parameters for **Global Normalization**.

Parameters	Function
Normalization Cap	Sets the extent of dynamic range expansion. Smaller values result in more pronounced changes in the pre-processed images. Recommended values are between 1 and 10.
Preserve Outliers	Selecting the **Preserve Outliers** check box means the first and the last 5% of the recalculated intensity range are kept for outliers.

Chapter 5: Post-Processing

This chapter describes the analysis settings that control post-segmentation improvement of the object masks.

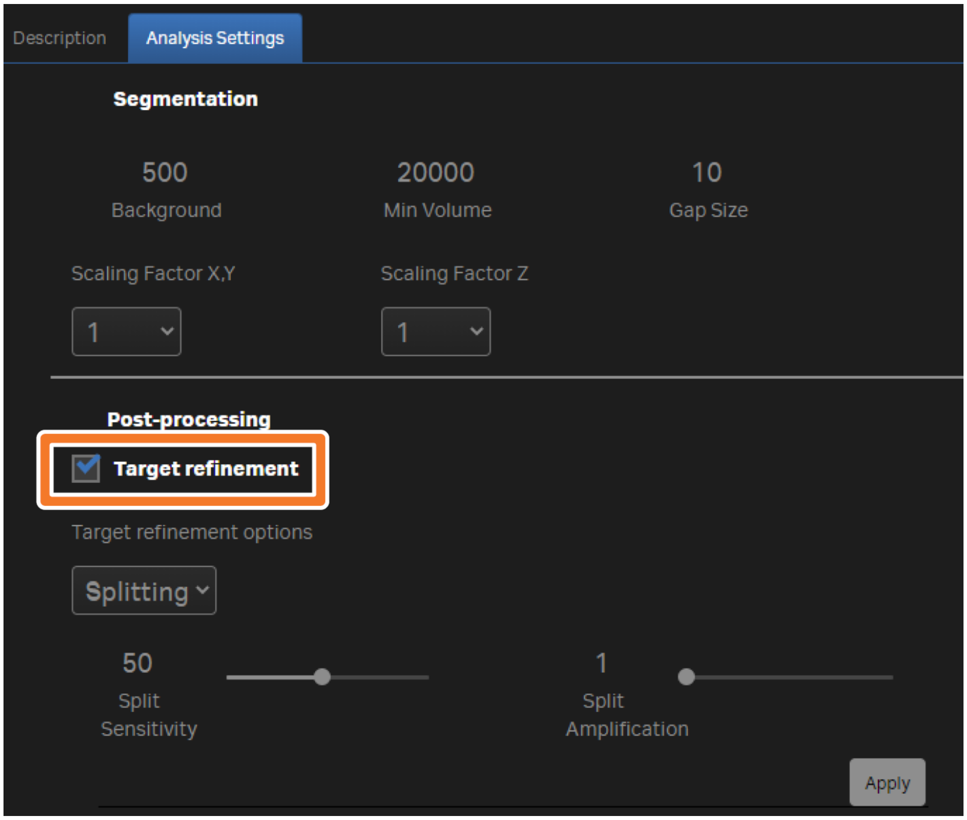
- Target Refinement, see below
- Filters, see page 25

Target Refinement

For all targets except **Cell Dilation**, you can apply optional post-processing operations.

Selecting Target Refinement

Select the **Target Refinement** check box to activate the target refinement filters.



Segmentation

500 Background

20000 Min Volume

10 Gap Size

Scaling Factor X,Y: 1

Scaling Factor Z: 1

Post-processing

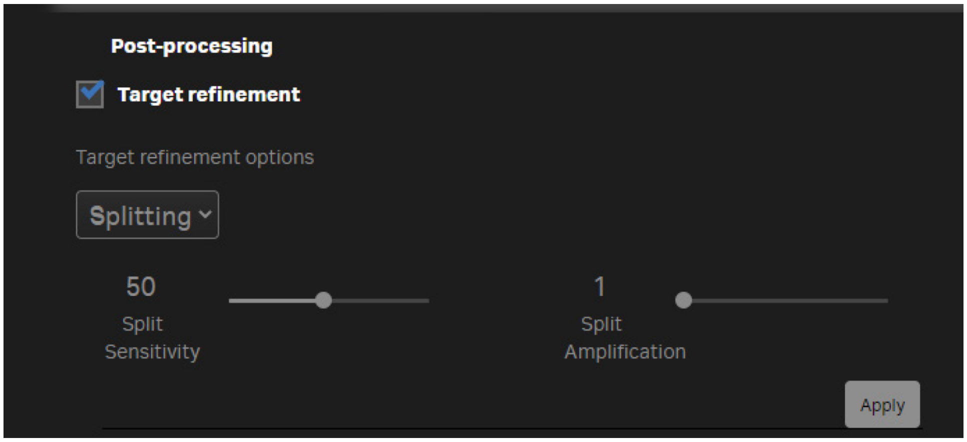
☑ **Target refinement**

Target refinement options: Splitting

50 Split Sensitivity

1 Split Amplification

The only available option for the refinement is **Splitting**, which performs splitting of the touching objects to improve the accuracy of the segmentation mask.



Post-processing

☑ **Target refinement**

Target refinement options

Splitting

50 Split Sensitivity

1 Split Amplification

Splitting Parameter

The table below shows the settings available during **Splitting**.

Filters	Description
Split Sensitivity	Controls the strength of splitting. The higher the value, the higher the stringency of the splitting algorithm and the more objects are split. Value must be between 1 and 100.
Split Amplification	Adjusts the dynamic range of Split Sensitivity. Higher values have higher impact on Split Sensitivity, resulting in more objects being split. Value must be between 1 and 100.

Filters

Selecting Filters

Select the **Filters** check box to activate the filters.

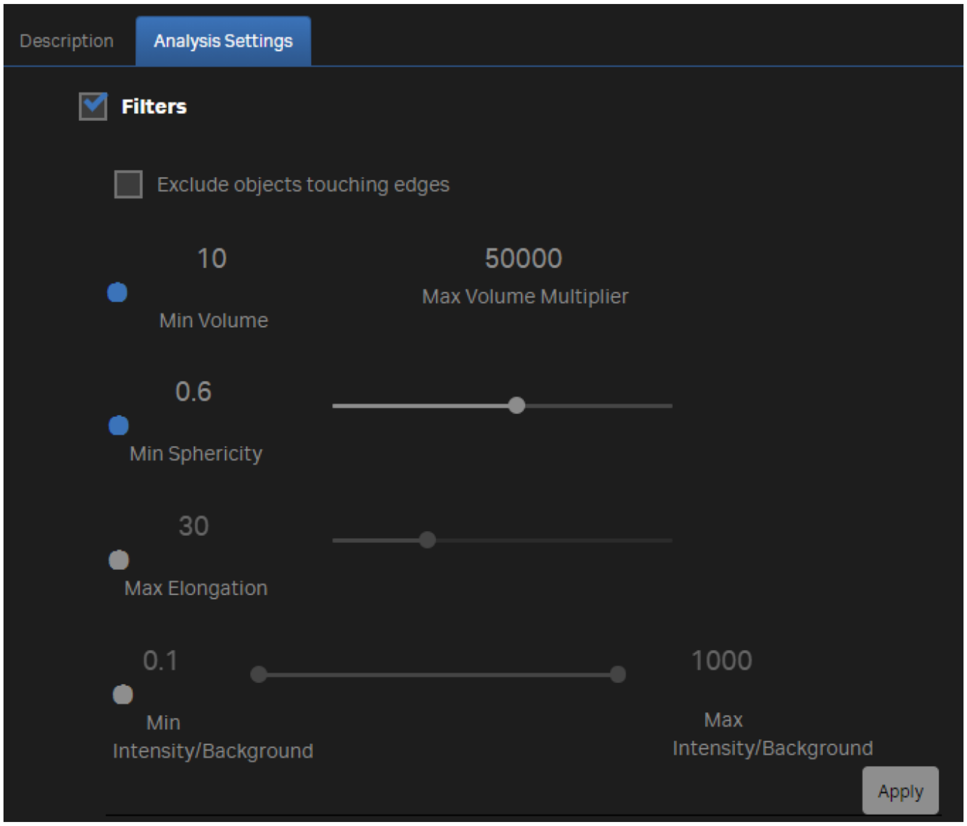
☑ Filters

☐ Exclude objects touching edges

10

50000
Max Volume Multiplier

All algorithms share a similar set of post-processing filters.



☑ Filters

☐ Exclude objects touching edges

Min Volume: 10

Max Volume Multiplier: 50000

Min Sphericity: 0.6

Max Elongation: 30

Min Intensity/Background: 0.1

Max Intensity/Background: 1000

Tip: Each filter can be selected or cleared individually.

Filter Parameters

The following table describes the filter parameters.

Filter	Description
Exclude objects touching edges	Removes objects touching the edges of an image at any given Z-slice. When **ROI** is selected, objects touching borders of an **ROI** are removed from the displayed segmentation.
Volume	Retain only those objects within the defined minimum and maximum volume limits. **Min Volume** refers to the minimum threshold for the object volume. Multiply **Min Volume** by the **Max Volume Multiplier** to obtain the maximum volume threshold of the objects to be included. For example: If **Min Volume** is set to 10,000 µm^3 and **Max Volume Multiplier** is set to 10,000, the maximum threshold is 100,000,000 µm^3.
Min Sphericity	Estimates how close the object is to a perfect sphere based on its surface area. The sphericity value cannot be above 1, which is equivalent to a perfect sphere. Activating this filter only keeps objects with a sphericity above the defined threshold.
Max Elongation	Calculates the ratio of the strongest and weakest principal moments of inertia for an object, and allows for the exclusion of overly elongated objects. Elongation cannot be below 1, which is equivalent to the elongation of a perfect sphere. The higher the value, the more elongated the object. Activating this filter only retains objects with an elongation below the defined threshold.
Intensity	This measurement is a normalized intensity ratio of the mean object intensity compared to global background. Activating this filter only retains objects with normalized intensities within the defined limits.

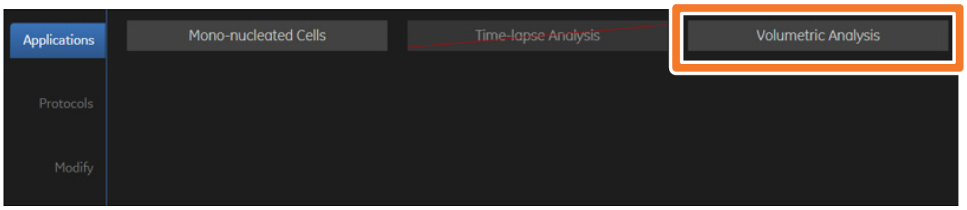
Note: When the **Filters** check box is cleared, only the **Exclude objects touching edges** filter is available.

- Analysis Setup with IN Carta VoluMetrics, see below
- Multiple Targets, see page 28
- Defining an ROI in the FOV, see page 29

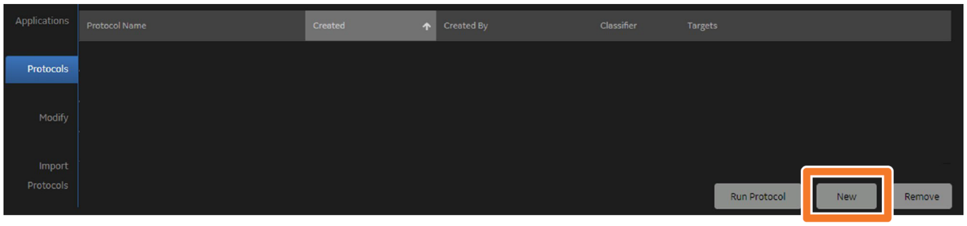
Analysis Setup with IN Carta VoluMetrics

To set up an analysis in IN Carta VoluMetrics:

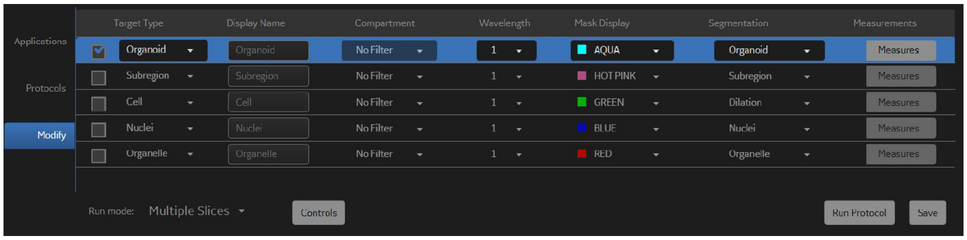
1. Use the **Worklist** to select and load a three-dimensional data set.
2. Click the **Analyze** tab.
3. From the **Applications** tab, select **Volumetric Analysis**.



4. In the **Protocols** tab, click **New** to create a new analysis protocol.



All available targets for IN Carta VoluMetrics are displayed by default and can be selected or cleared as needed.



	Target Type	Display Name	Compartment	Wavelength	Mask Display	Segmentation	Measurements
☑	Organoid	Organoid	No Filter	1	AQUA	Organoid	Measures
☐	Subregion	Subregion	No Filter	1	HOT PINK	Subregion	Measures
☐	Cell	Cell	No Filter	1	GREEN	Dilation	Measures
☐	Nuclei	Nuclei	No Filter	1	BLUE	Nuclei	Measures
☐	Organelle	Organelle	No Filter	1	RED	Organelle	Measures

Run mode: Multiple Slices

Note: Only selected target types are segmented using analysis settings for a specific algorithm. Once a target type is cleared, analysis settings for that target are reset to the default.

Multiple Targets

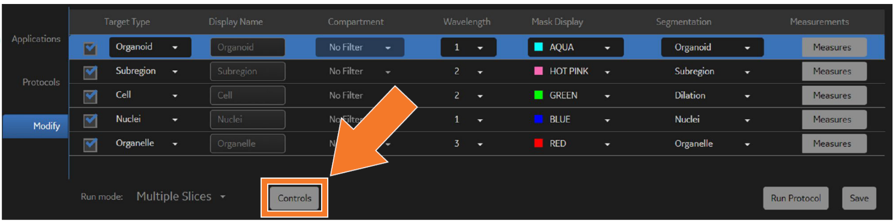
A single analysis protocol can include multiple targets of the same type, as shown below.

	Target Type	Display Name	Compartment	Wavelength	Mask Display	Segmentation	Measurements
☑	Organoid	Spheroid GFP	No Filter	1	🟩 GREEN	Organoid	Measures
☑	Organoid	Spheroid DAPI	No Filter	2	🟦 AQUA	Organoid	Measures

For example, a protocol can include two to five organoid targets, provided that unique display names are used and different wavelengths are selected for segmentation.

To select a subset of Z-slices, click **Controls** (A) to display the **Multiple Slices Controls** dialog (B) and select at least four Z-slices.

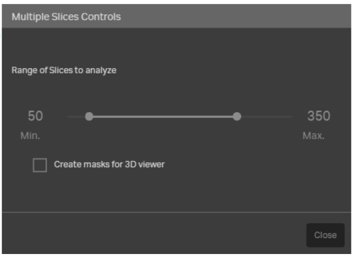
A



	Target Type	Display Name	Compartment	Wavelength	Mask Display	Segmentation	Measurements
☑	Organoid	Organoid	No Filter	1	🟦 AQUA	Organoid	Measures
☑	Subregion	Subregion	No Filter	2	🟪 HOT PINK	Subregion	Measures
☑	Cell	Cell	No Filter	2	🟩 GREEN	Dilation	Measures
☑	Nuclei	Nuclei	No Filter	1	🟦 BLUE	Nuclei	Measures
☑	Organelle	Organelle	N	3	🟥 RED	Organelle	Measures

Run mode: Multiple Slices

B



Multiple Slices Controls

Range of Slices to analyze

50 Min.

350 Max.

☐ Create masks for 3D viewer

The current Z-slice selection is saved in the protocol settings.

Note: Once protocol optimization is complete, adjust the range of Z-slices to include all Z-slices that require analysis.

Subsets of Z-Slices

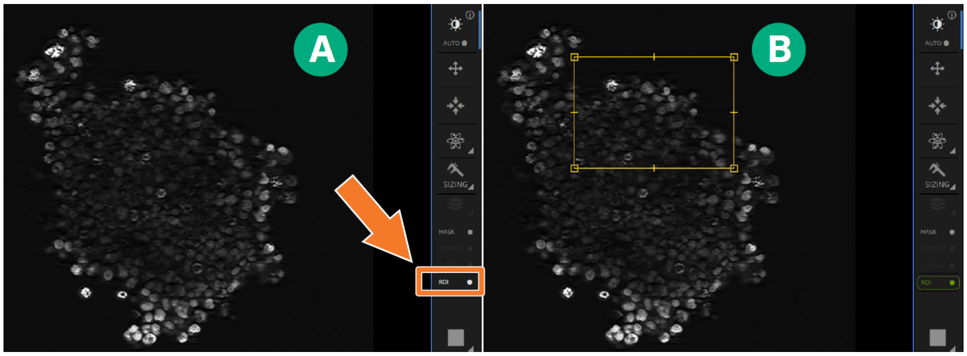
The protocol editor allows a subset of Z-slices to be selected for analysis. This expedites analysis during protocol optimization.

Defining an ROI in the FOV

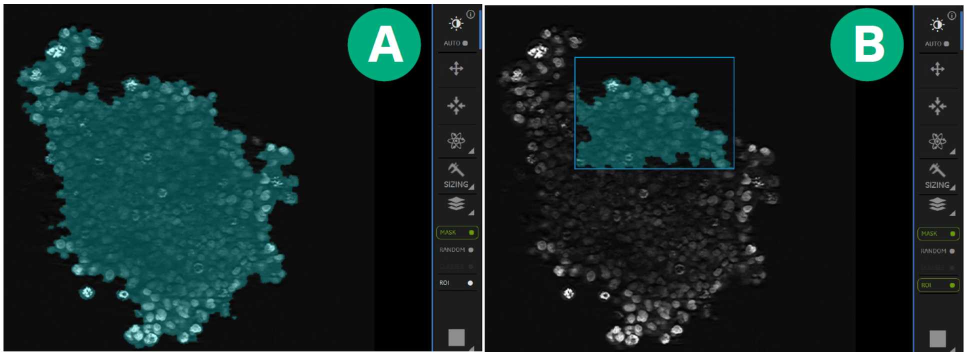
The **ROI** feature allows a smaller XY region to be defined and analyzed within the larger FOV. This increases the speed of segmentation testing during set up of parameters.

To define an **ROI** within the larger FOV:

1. Do one of the following to access the **ROI** bounding box:
    - Click the **ROI** check box (A) and a **ROI** bounding box appears in the image window (B).



    - Click the **Mask overlay** check box (A) and a **ROI** bounding box appears in the image window (B).



2. Adjust the boundaries of the **ROI** by dragging the corners using the mouse.

Chapter 7: Segmentation Workflows and Analysis

7

This chapter describes the workflow instructions for performing segmentation and running an analysis.

- Organoid or Subregion Segmentation Workflow, see below
- Nuclei or Organelle Segmentation Workflow, see page 35
- Cell Segmentation Workflow, see page 39
- Selecting Measures and Running Analysis, see page 40

Organoid or Subregion Segmentation Workflow

The segmentation workflow allows you to perform the following adjustments on organoids or subregions:

- Adjust organoid or subregion segmentation parameters.
- Add and adjust pre-processing step, if needed.
- Select a minimum set of measures.
- Run preliminary analysis.
- Identify which post-processing filters to apply during segmentation.
- Run final analysis with measures of interest.

Reducing Image Data Set Size

Reducing the size of the image data set is recommended, but not required.

To reduce the size of the image data set:

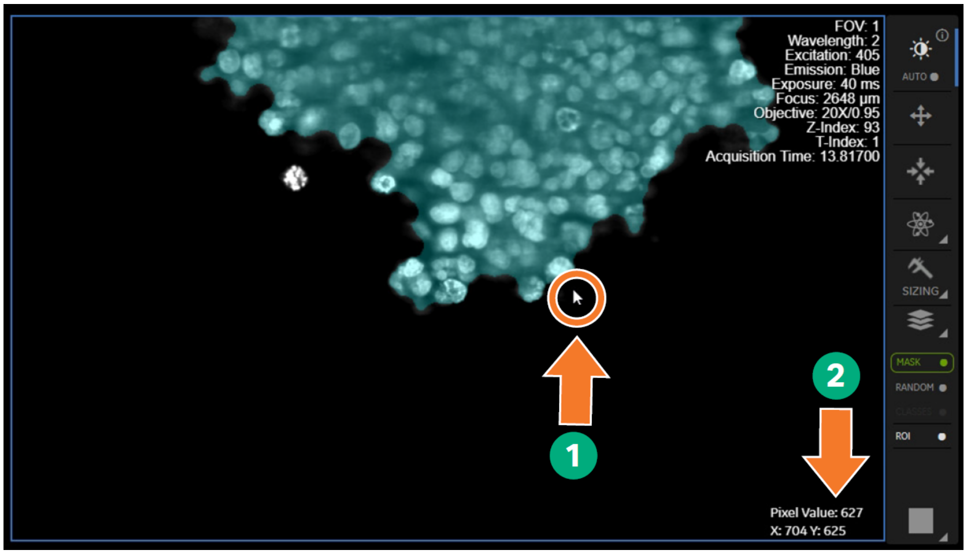
1. Select the Z-slice range by clicking **Controls** and selecting at least four Z-slices.
2. Activate and define **ROI**, if needed. See Defining an ROI in the FOV on page 29 for details.

Note: Nuclei or organelle segmentation algorithms calculate segmentation thresholds based on the intensity distribution for a 3D stack. When an **ROI** is selected for analysis, the 3D stack submitted for segmentation is cropped in X, Y, and Z. Therefore, threshold values might be different when compared to values for the entire 3D stack. For this reason, when selecting an **ROI**, make sure the selected region is representative of the entire 3D stack. The same applies when selecting a subset of Z-slices for analysis. It is important to verify segmentation parameters on an uncropped stack (meaning the **ROI** feature is disabled and all Z-slices are included) prior to running analysis.

Selecting Background Intensity

To enter the estimated background intensity:

1. Hover the mouse over the image in the viewport (1) to assess background intensity.



FOV: 1
Wavelength: 2
Excitation: 405
Emission: Blue
Exposure: 40 ms
Focus: 2648 µm
Objective: 20X/0.95
Z-Index: 93
T-Index: 1
Acquisition Time: 13.81700

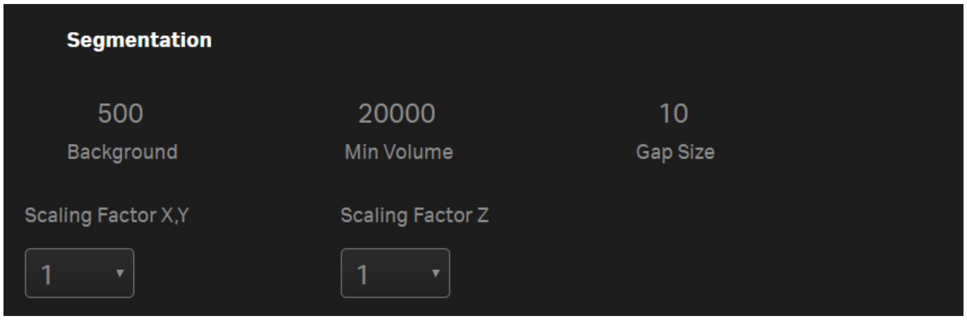
Pixel Value: 627
X: 704 Y: 625

   Pixel coordinates and intensity values for the current cursor position displays in the bottom right corner (2).

2. Make a note of the pixel coordinates and intensity values displayed at the bottom right (2).
3. Enter the estimated background intensity (either for noise or to exclude organoids or subregions from the analysis) in the **Background** field.

Excluding Small Organoids or Subregions

To exclude small organoids or subregions, enter the minimum volume threshold in the **Min Volume** field.



Segmentation

Background: 500
Min Volume: 20000
Gap Size: 10
Scaling Factor X,Y: 1
Scaling Factor Z: 1

Note: When using an **ROI** and/or defining a Z-slice selection, the overall image volume used for segmentation is reduced, which makes the segmented objects smaller than they do when all Z-slices are selected.

Measuring Internal Gaps

To measure the internal gaps in organoids or subregions:

1. Click **Sizing** in the **Viewport** to activate measurement tools.
2. Use the **Line tool** to measure an internal gap. Adjust the **Line tool** to fit the gap by clicking and dragging with the mouse.

   Note: Click the **i** symbol for details on using **Sizing tools**.

3. Adjust the **Gap value** to change the gap size.

Adjusting Scaling Factor X,Y

To select the **Scaling Factor X,Y**:

1. Adjust the **Scaling Factor X,Y** to 0.5 or 0.25 to increase the speed of analysis and performance.

   Note: To maximize the resolution and segmentation accuracy, set **Scaling Factor X,Y** to 1.

2. Verify the segmentation accuracy is still acceptable.
3. Click **Apply** and check the segmentation quality.

Separate Objects That Are Clumped Together

To separate any objects that are clumped together:

1. If objects of interest are clumped together, use the **Target refinement** filters. See Target Refinement on page 23
2. Adjust **Splitting** settings as needed to declump objects.

Optimizing Segmentation Settings

Adjust the segmentation settings as needed to obtain optimal segmentation results.

Note: During initial testing of the segmentation parameters, it can be useful to skip the post-processing filters (clear the check box). However, you can select the filtering parameters if they are known in advance. See Filters on page 25.

To verify segmentation accuracy, perform the following actions:

- Use a different **ROI**.
- Use a different Z-slice selection.
- Perform the segmentation within control wells in the plate.
- Run analysis on a limited set of wells (for example, one to two wells for each control type) to ensure the correct parameters are selected and to decide whether post-processing filters are needed.

Tip: If the segmentation accuracy varies from well to well, try using one of the pre-processing filters to make segmentation more robust. See Pre-Processing on page 17.

Once all target type segmentation parameters are specified, check that all Z-slices to be processed are selected.

Troubleshooting Segmentation

If the applied segmentation settings do not remove debris or unwanted objects, select one or more post-processing filters and then re-run the analysis. See Filters on page 25 and the following table.

Measure Selection	Filter	Purpose
Exclude objects touching edges	---	Eliminate objects that may reach beyond the borders of current FOV
Morphology I **Volume**	**Volume**	Size-based filter
Morphology I **Sphericity**	**Min Sphericity**	Shape-based filter
Morphology I **Elongation**	**Max Elongation**	Shape-based filter
Intensity I **Mean Int by Global Bkg**	**Intensity** or **Background**	Intensity-based filter

Nuclei or Organelle Segmentation Workflow

The segmentation workflow allows you to perform the following adjustments for nuclei or organelles:

- Adjust nuclei or organelle segmentation parameters.
- Add and adjust pre-processing step, if needed.
- Select a minimum set of measures.
- Run preliminary analysis.
- Identify which post-processing filters to apply during segmentation.
- Run final analysis with measures of interest.

Reducing Image Data Set Size

Reducing the size of the image data set is recommended, but not required.

To reduce the size of the image data set:

1. Select the Z-slice range by clicking **Controls** button and selecting at least four Z-slices.
2. Activate and define **ROI**, if needed. See Defining an ROI in the FOV on page 29 for details.

Note: Since the segmented object is limited to the selected region, the minimum volume threshold should be lowered when using a defined ROI.

Setting the Diameter Size

To measure the range of diameters for nuclei in the data set:

1. Use the **Sizing** tool to measure the range of diameters for nuclei in the data set.
2. Adjust the **Min Diameter** and **Max Diameter** settings based on the above measurements.

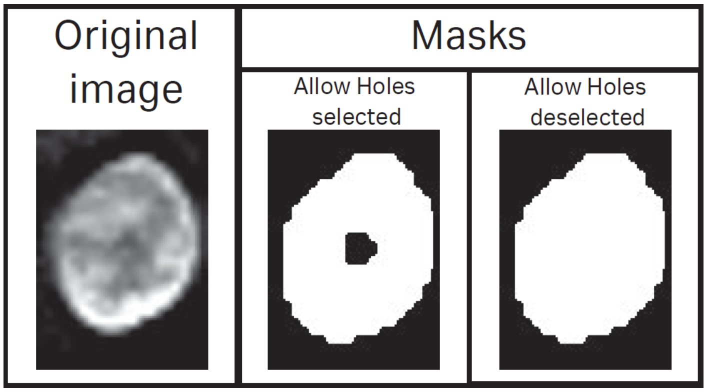
Note: These parameters do not act as size thresholds. You can define exclusion of small or large objects using post-processing parameters.

Selecting Segmentation Fields

To select segmentation fields for nuclei and organelles:

1. Set the **Intensity Sensitivity**.
2. Set the **Split Sensitivity**.
3. Do one of the following:
    - Clear the **Allow Holes** check box for nuclei targets.
    - Select the **Allow Holes** check box for organelle targets.

Tip:
For example, the following illustration shows segmentation on a cropped image of a DAPI-stained sample.



4. Set **Scaling Factor X,Y** and **Scaling Factor Z** to 1, then click **Apply**.

Note: If nuclei or organelle object size is relatively small, reducing the scaling factor to less than 1 may result in overly smoothed segmentation and poor object separation.

5. To review segmentation results, select **Random** mask (turn on) to check nuclei separation.
6. Once all target type segmentation parameters are specified, verify that all Z-slices to be processed are selected.
7. Run analysis on a limited set of wells (one to two for each control type) to make sure the correct parameters are selected and to decide whether post-processing filters are needed.
8. If the applied segmentation settings do not remove debris or unwanted objects, select one or more post-processing filters. See Filters on page 25 and Removing Debris or Unwanted Objects on page 38.
9. Re-run analysis.
10. After the analysis is complete, do the following to review the results:
    a. Locate unwanted objects, such as debris, included in the segmentation.
    b. Optimize the post-processing filter settings to exclude unwanted objects.
    c. Make sure that objects of interest are not excluded by the post-processing filter settings.
11. Make sure the **Post-processing** filter settings are relevant for additional wells.
12. Select the measurements of interest for final analysis. Filters selected in step 8 can be deselected if they are not required to be output within the results. See List of Available Measures on page 65 for details on each measure.
13. Run analysis across all the wells of interest.

Reviewing Segmentation

To review segmentation:

1. Select **Random** mask (turn on) to check nuclei separation.
2. Select all Z-slices to be processed once all target type segmentation parameters are specified.
3. Run analysis on a limited set of wells (one to two for each control type) to make sure the correct parameters are selected, and to decide whether post-processing filters are needed.
4. If the applied segmentation settings do not remove debris or unwanted objects, select one or more post-processing filters. See Filters on page 25 and Removing Debris or Unwanted Objects on page 38.
5. Re-run analysis.
6. After the analysis is complete, do the following to review the results:
    a. Locate unwanted objects, such as debris, included in the segmentation.
    b. Optimize the post-processing filter settings to exclude unwanted objects.
    c. Make sure that objects of interest are not excluded by the post-processing filter settings.

Finalizing Segmentation

To optimize and finalize segmentation:

1. Select the measurements of interest for final analysis. Deselect measures if they are not required to be output within the results. See List of Available Measures on page 65 for details on each measure.
2. Run analysis across all the wells of interest.

Troubleshooting Nuclei or Organelle Segmentation Results

Nuclei Segmentation Troubleshooting

The following table describes the recommended action when nuclei or organelle segmentation results need to be improved.

Result	Recommended Action	Reason
Nuclei are not sufficiently split	Increase the **Split Sensitivity** value from the initial setting of 50. Increments of 10 allow parameters to be set faster.	Higher values result in more splits.
Nuclei are over segmented	Select **Pre-processing** and use the **Denoising** or **Local Normalization** option (see Pre-Processing on page 17).	Uneven intensity distribution within the individual nuclei.

Removing Debris or Unwanted Objects

The following table describes post-processing filters that can help to remove debris or unwanted objects.

Measure Selection	Filter	Purpose
Exclude objects touching edges	---	Eliminate objects that may reach beyond the borders of current FOV
Morphology | **Volume**	**Volume**	Size-based filter
Morphology | **Sphericity**	**Min Sphericity**	Shape-based filter
Morphology | **Elongation**	**Max Elongation**	Shape-based filter
Intensity | **Mean Int by Global Bkg**	**Intensity** or **Background**	Intensity-based filter

Cell Segmentation Workflow

Select a cell segmentation method based on the required segmentation accuracy. Use **Cell Fast** segmentation if higher segmentation accuracy needs to be achieved and there is a dye that stains the cell body.

Note: It is not recommended to use **Target Refinement Splitting** for **Cell Target** as it might generate cell objects that are not linked to any of the nuclei.

Setting up Cell Dilation Segmentation Parameters

To set up Cell Dilation segmentation parameters:

1. Select **Cell Target** and **Dilation** as a segmentation method.
2. Select a **Target Type** of **Nuclei** to be used as a seed for cell segmentation.
3. Adjust **Growing Radius** value to control the nuclear mask dilation (in µm).

   Note: The algorithm converts this value to pixels, rounding up to the nearest whole number.

4. Set **Scaling Factor X,Y** and **Scaling Factor Z** to 1.
5. Select the measurements of interest. See List of Available Measures on page 65 for details on each measure.

Setting Up Cell Fast Segmentation Parameters

To set up Cell Fast segmentation parameters:

1. Select **Cell Target** and **Fast** as a segmentation method.

   Note: This is for **Cell Fast** segmentation only.

2. Select a **Target Type** of **Nuclei** to be used as a seed for cell segmentation.
3. Enter the estimated background intensity (either for noise or to exclude unrelated fluorescent objects from the analysis) in the **Threshold** field. To assess background intensity, hover the mouse over the image in the viewport.
4. If cell segmentation is not robust enough, do one of the following:
   - Activate and adjust pre-processing options (**Denoising** or **Local Normalization**).
   - Select **Low Contrast Borders** check box.
   - A combination of pre-processing and active **Low Contrast Borders**.
5. To increase the speed of analysis and performance, adjust the **Scaling Factor X,Y** to 0.5 or 0.25. Verify the segmentation accuracy is still acceptable.

   Note: To maximize the resolution and segmentation accuracy, set **Scaling Factor X,Y** to 1.

Selecting Measures and Running Analysis

To select measures and run an analysis:

1. Check that the post-processing filter settings for all selected segmentation parameters are relevant for the remaining wells.
2. Select the measurements of interest. See List of Available Measures on page 65 for details on each measure.
3. If a subset of slices was selected during segmentation setup, adjust the range of Z-slices to include all Z-slices that need to be analyzed.
4. Run final analysis on all wells of interest.

Chapter 8: Results Visualization and Export

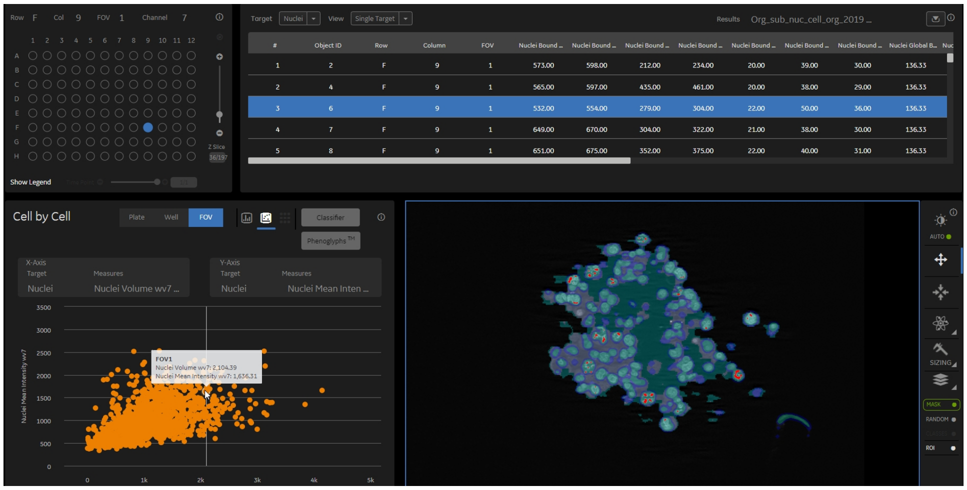
8

This chapter describes how to view and export results.

- Viewing Results, see below
- Single-Target Data, see below
- Reviewing Single-Target Data, see page 42
- Data Export, see page 42

Viewing Results

Results for each target can be reviewed in the **Data** tab. The **Target** drop-down list allows you to select which target data to display. The **View** drop-down list allows you to switch between single target data, a summary by FOV, or a summary by well.



Target: Nuclei
View: Single Target
Results: Org_sub_nuc_cell_org_2019 ...

#	Object ID	Row	Column	FOV	Nuclei Bound ...	Nuclei Bound ...	Nuclei Bound ...	Nuclei Bound ...	Nuclei Bound ...	Nuclei Bound ...	Nuclei Bound ...	Nuclei Global B...
1	2	F	9	1	573.00	598.00	212.00	234.00	20.00	39.00	30.00	136.33
2	4	F	9	1	565.00	597.00	435.00	461.00	20.00	38.00	29.00	136.33
3	6	F	9	1	532.00	554.00	279.00	304.00	22.00	50.00	36.00	136.33
4	7	F	9	1	649.00	670.00	304.00	322.00	21.00	38.00	30.00	136.33
5	8	F	9	1	651.00	675.00	352.00	375.00	22.00	40.00	31.00	136.33

X-Axis Target: Nuclei, Measures: Nuclei Volume wv7 ...
Y-Axis Target: Nuclei, Measures: Nuclei Mean Inten ...

Single-Target Data

Single-target data is interactively linked between a Z-plane in the image viewport, **FOV Chart Dashboard**, and **Results Analysis** table.

Action	Result
Click on any data point in the scatter plot	• The row in the Results Analysis table that corresponds to the selected object is highlighted. • The Z-slice is set to the middle Z-slice for the selected object. • The object in the viewport is highlighted (makes a mask white).
Click on any of the rows in the **Results Analysis** table	• The data point in the scatter plot is highlighted • The object in the viewport is highlighted (makes a mask white) • The Z-slice is set to the middle Z-slice for the selected object.
Click on an object in the viewport	• Makes a mask for the selected object white. • The data point in the scatter plot is highlighted. • The row in the **Results Analysis** table is highlighted corresponding to the selected object.

Reviewing Single-Target Data

To review single-target data:

1. Select **Single Target** view in **Results Analysis** table.
2. Select **FOV** in the **Chart Dashboard**.
3. Set the X-axis and Y-Axis target for the same target type as in the **Results Analysis** table, choosing measures of interest.
4. Link the single-target data between a Z-plane in the image viewport, **FOV Chart Dashboard**, and **Results Analysis** table. For details, see Single-Target Data on page 41.

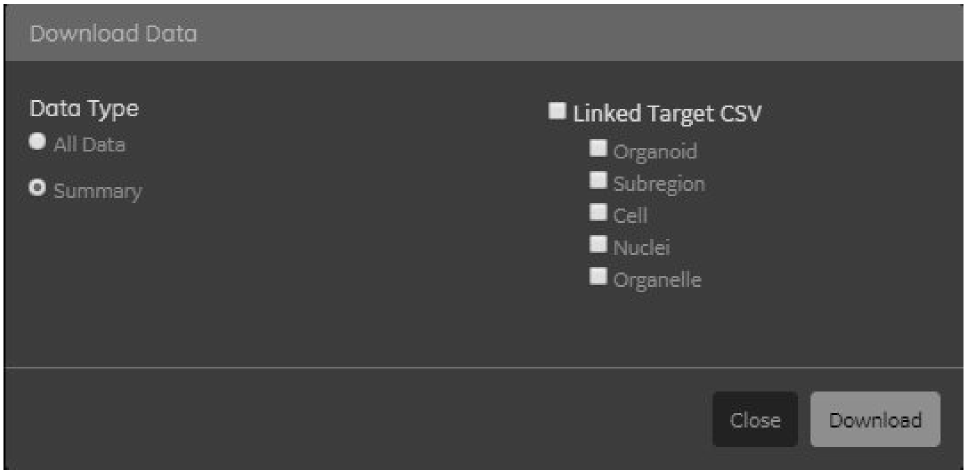
Data Export

Results are saved automatically in the **Results** folder within a subfolder (named with the protocol name and the date/time). They can also be downloaded from **GUI/Results Analysis** table.

#	Object ID	Row	Column	FOV	Nuclei Centroi...	Nuclei Centroi...	Nuclei Centroi...	Nuclei Weight...	Nuclei Weight...	Nuclei Weight...	Nuclei Bound ...	Nuclei Bound ...	Nu
1	1	A	1	1	234.26	270.87	4.34	234.31	270.91	4.34	1436.00	1450.00	
2	2	A	1	1	201.39	18.30	4.21	201.43	18.35	4.22	1214.00	1257.00	
3	3	A	1	1	187.21	24.99	4.97	187.19	24.90	5.01	1129.00	1176.00	
4	4	A	1	1	266.56	25.61	3.51	266.59	25.65	3.57	1627.00	1653.00	
5	5	A	1	1	36.00	309.46	3.88	36.03	309.41	3.87	202.00	241.00	

The download can include:

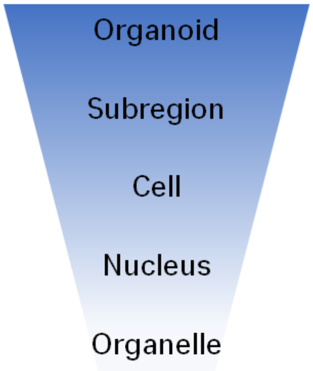
- Separate .csv files with **Summary Data** only for well and FOVs (**Summary**).
- Separate .csv files with **Summary** and **Single Target** data (**All Data**).
- (Optional) Separate .csv files for each selected target type, showing linked target data (**Linked Target CSV**).


Download Data

Data Type
○ All Data
● Summary

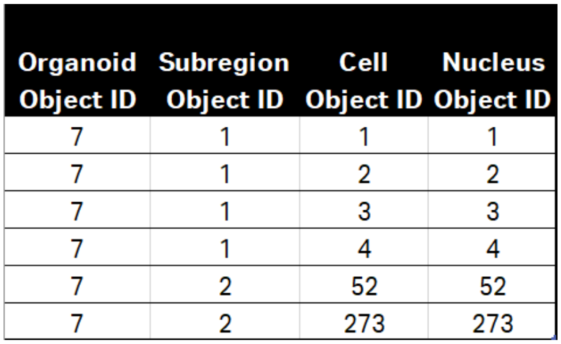
☐ Linked Target CSV
  ☐ Organoid
  ☐ Subregion
  ☐ Cell
  ☐ Nuclei
  ☐ Organelle

Linked Target CSV files contain information on inclusion of the targets within other targets according to the following hierarchical structure:



For example:

- Organoid, subregion, cell, and nucleus were segmented within analysis.
- **Linked Target CSV** for organelle was selected for download.
- The .csv file lists object IDs of parent objects (nuclei, cells, subregions, and organoids) based on the hierarchy, if there is a parent object available. An example of a .csv file is seen in the illustration below.



Organoid Object ID	Subregion Object ID	Cell Object ID	Nucleus Object ID
7	1	1	1
7	1	2	2
7	1	3	3
7	1	4	4
7	2	52	52
7	2	273	273

Chapter 9: 3D Viewer

9

This chapter describes how to use of **3D Viewer**.

3D viewer performs the following functions:

- Enables visualization of single-channel and multi-channel 3D image stacks as a volume rendering.
- Enables mask generation in IN Carta VoluMetrics application with raw images.
- Enables inspection of 3D stacks, assessing segmentation accuracy and obtaining biologically relevant visualization of 3D samples.

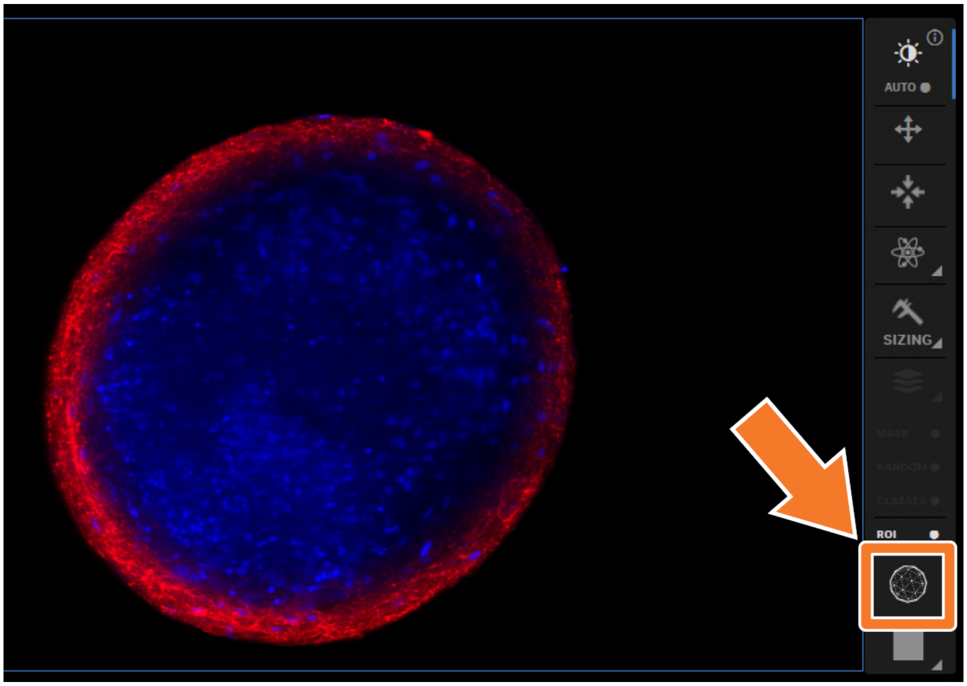
A separate license is required to use 3D Viewer. The **Launch 3D Viewer** icon is inactive if a valid license is not available or has not been selected from the **System/Licenses** dialog.

Launching 3D Viewer

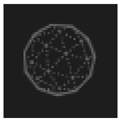
Analyze Tab (Volume Rendering Only)

To run the analysis with volume rendering only:

1. Create a new IN Carta VoluMetrics protocol as described in Analysis Setup with IN Carta VoluMetrics on page 27 or open an existing one.
2. Click the 3D viewer icon as shown in the image below.



Note: If valid 3D viewer license is not available or network license was not checked, then the icon is inactive.



Analyze Tab (Volume Rendering with Optional Masks)

Mesh-like masks for 3D viewer (3D masks) are generated on demand using a separate algorithm once regular segmentation masks are created. These masks can be generated automatically during protocol execution, if **Create 3D masks** check box in **Controls** dialog is selected. It is recommended to select the check box, when segmentation settings are roughly optimized for a given 3D stack.

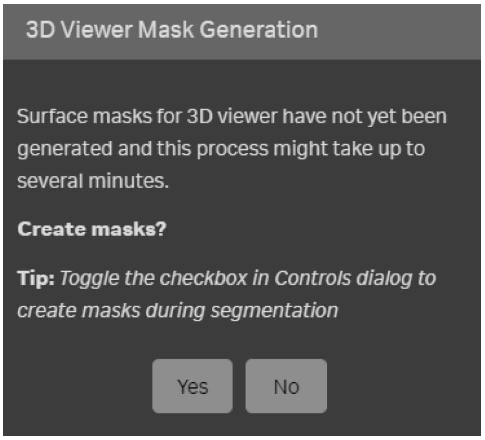
To run the analysis with additional masks:

1. Create a new IN Carta VoluMetrics protocol as described in Analysis Setup with IN Carta VoluMetrics on page 27 or open an existing one.
2. Select target(s) of interest and adjust analysis settings.
3. Click **Apply** to create segmentation masks for selected targets.
4. (Optional) Select a subset of Z-slices in **Controls** dialog to analyze a subset of z-slices and load them into 3D viewer. (For more information, see Multiple Targets on page 28.)

   Note: By default, all Z-slices from a current stack are rendered in 3D viewer.

5. Click **3D Viewer** icon to launch the 3D viewer.

   Note: If the **Create 3D Masks** check box was not selected, then the dialog shown in the image below, appears.

   3D Viewer Mask Generation

   Surface masks for 3D viewer have not yet been generated and this process might take up to several minutes.

   Create masks?

   Tip: *Toggle the checkbox in Controls dialog to create masks during segmentation*

   Click **Yes** to open the 3D viewer opens after generation of 3D masks is completed.

   Click **No** to open the 3D viewer without loading 3D masks.

Data Tab (Volume Rendering with Masks)

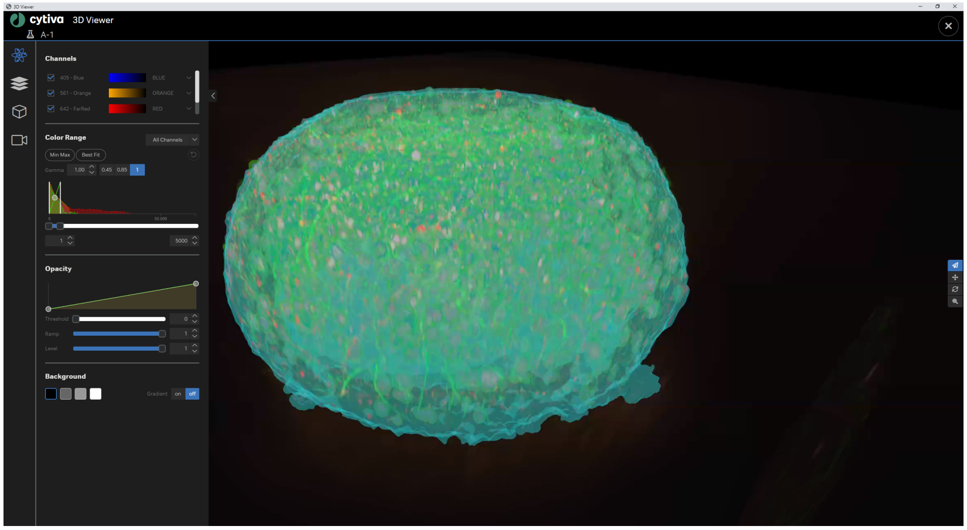
3D Viewer can be opened for datasets previously analyzed using IN Carta VoluMetrics application. If **Create 3D Masks** check box is selected as a part of the protocol, **Volume Renderings** opens together with 3D masks. Alternatively, a dialog will appear as described in the previous section.

Note: If subset of Z-slices have been specified upon saving of a protocol, then only those Z-slices are rendered in **3D Viewer**.

3D Viewer Navigation Controls

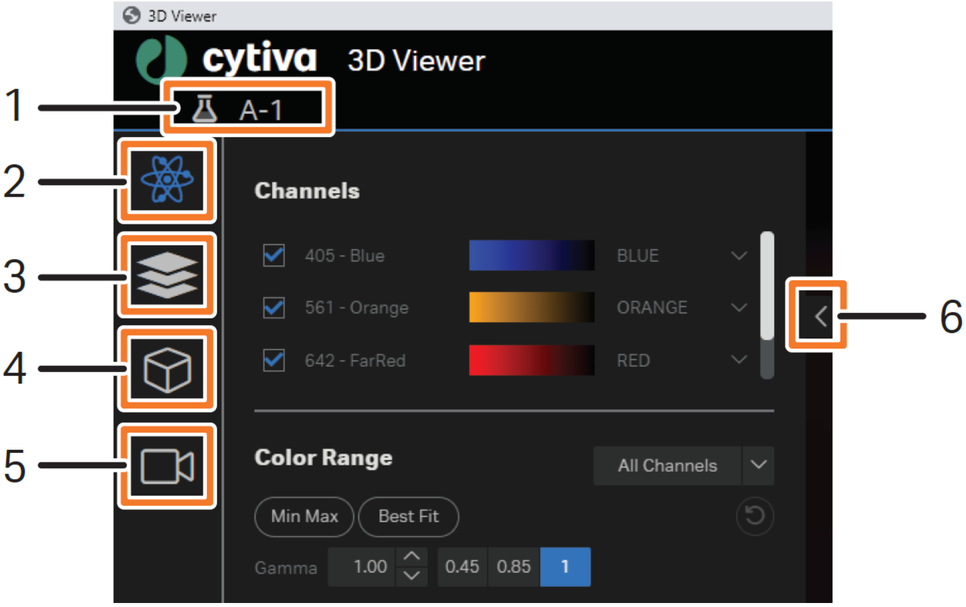
Upon launch of 3D Viewer, the selected 3D stack is rendered with or without **3D masks** depending their availability.

The following illustration shows the **3D Viewer**.



Note: Click on X in the top right corner to close 3D viewer to return to main IN Carta window. IN Carta cannot be used while 3D viewer is open.

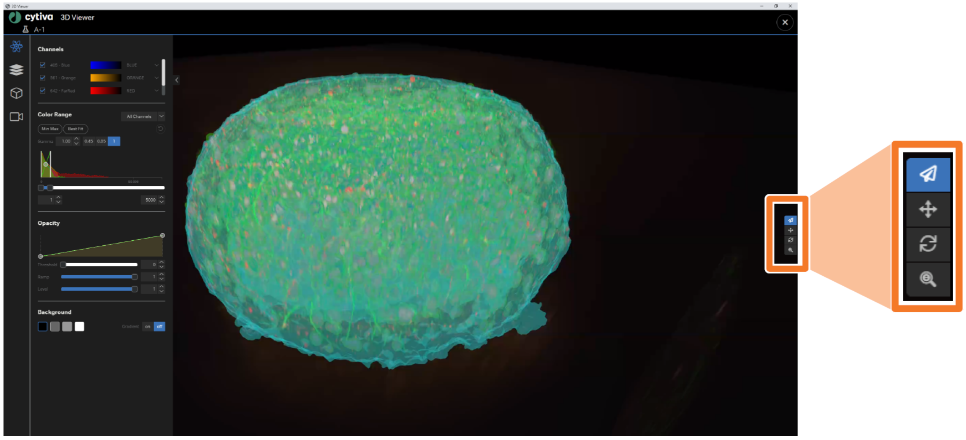
Tools Panel



Part	Description
1	A well for the loaded FOV is displayed.
2	Channel control. This is the default active tab.
3	Target mask control.
4	3D settings.
5	Export tools.
6	Panel can be collapsed and expanded. Tools panel is shown expanded by default.

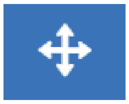
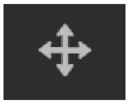
Navigation Controls

Navigation tools, show in the image below, can be used to rotate, pan and zoom the volume and masks rendered in the **3D Viewer**.



The examine tool is the universal tool. Additional tools can be selected to have more control and perform a specific manipulation with the rendering.

The table below describes the navigation tools used in **3D Viewer**:

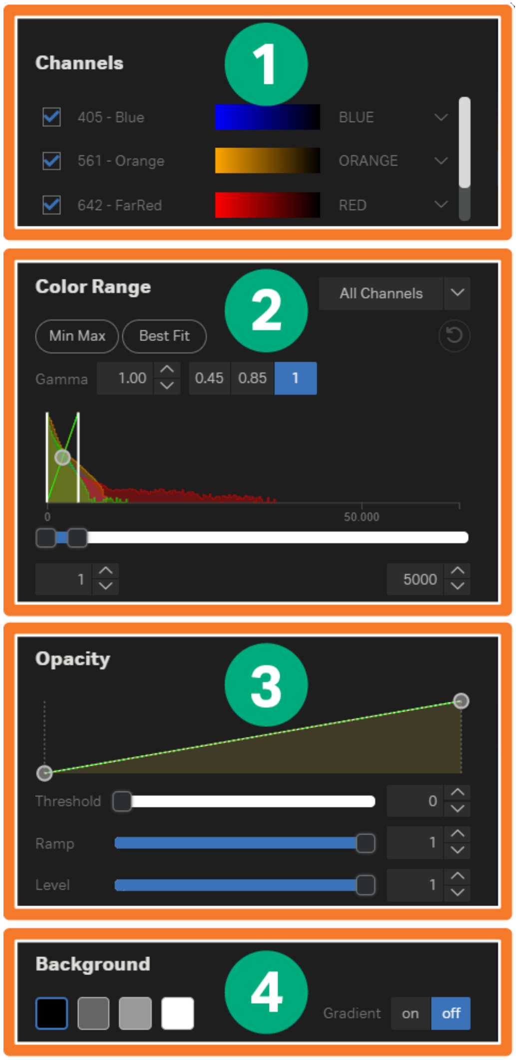
Tool	Active	Inactive	Description
Examine			Examine tool combines Pan, Rotate, and Zoom into one tool. Its icon is a paper plane. • To rotate view: Click the left mouse button and drag the mouse around. • For Pan image: Press and hold the Shift key and drag the mouse. • To zoom in: Scroll mouse wheel up, or press and hold the **CTRL** key and drag the mouse to the right or up. • To zoom out: Scroll mouse wheel down OR press and hold the **CTRL** key and drag the mouse to the left or down.
Rotate			Use this tool to rotate the volume rendering in **3D Viewer**. • To rotate the view: Click the left mouse button and drag the mouse around.
Pan			Use this tool to move the volume rendering around in **3D Viewer**. • To move image data and targets in the view area: Click the left mouse button and drag the mouse around.
Zoom			Use this tool to zoom in or out of the volume rendering in the **3D Viewer**. • To zoom in: Click the left mouse button and drag the mouse to the right or up. • To zoom out: Click the left mouse button and drag the mouse to the left or down.

Note: Only one of the tools can be active at a time. The tools are activated by clicking on the tool icon.

Channel Controls

The channel controls allows to adjust display of raw image data (volume).

The table below describes the available channel controls.

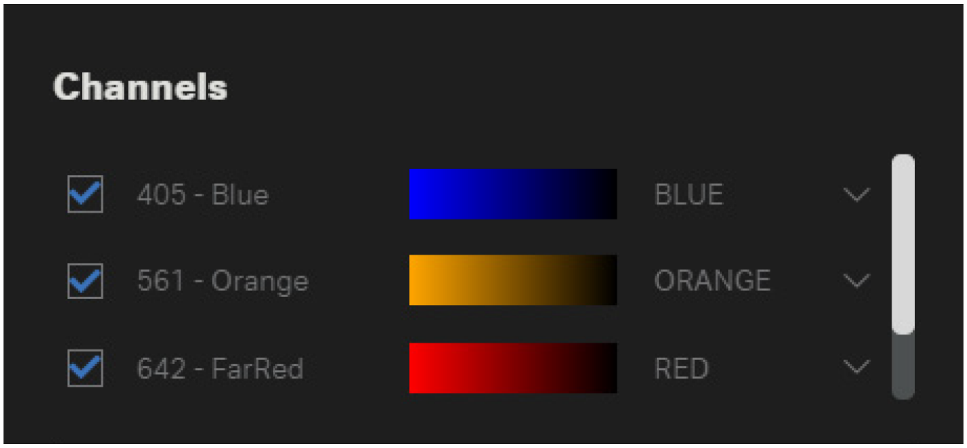


Part	Description
1	**Channels** selects channel color.
2	**Color range** selects channel contrast.
3	**Opacity** controls lower intensity values.
4	**Background** controls background display.

Note: Setting the contrast or highlight of lower intensity values can be done for a selected channel or all channels simultaneously.

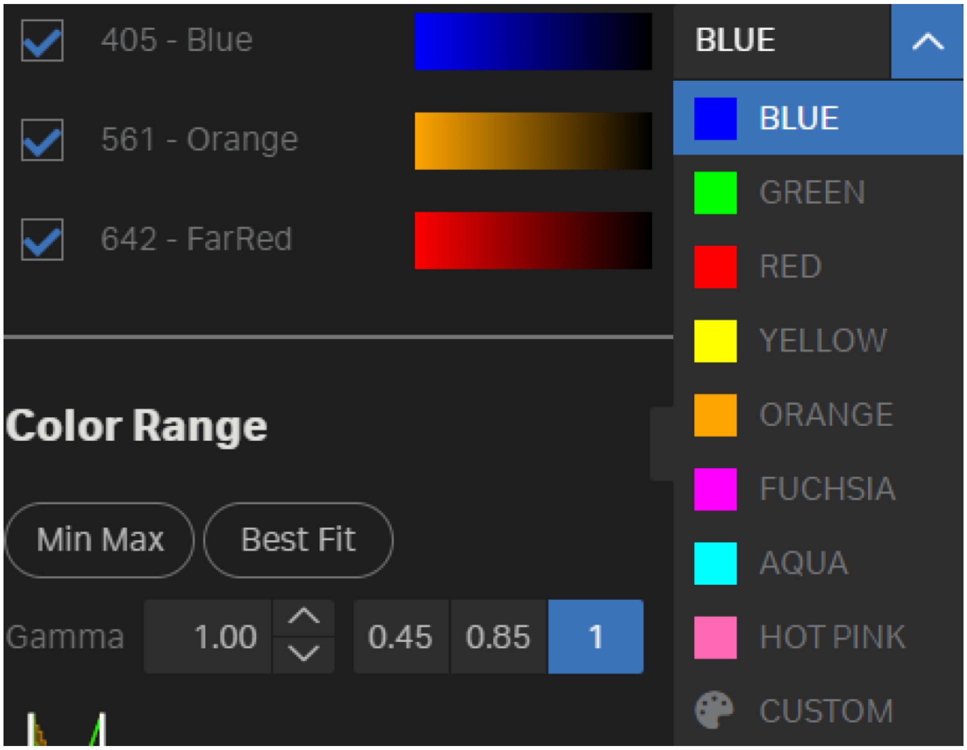
Channels

Used to show/hide channels displayed by selecting or deselecting a check box next to each channel, as seen in the image below.



Up to three channels are shown on the display. The scroll bar can be used to access additional channels (if available). Colors for all channels are set based on the color selections made in the main IN Carta window. Color for each channel can be modified in 3D viewer from a dropdown menu.

You can define a new color which is not listed by selecting **Custom**. A new dialog appears to set a color by setting positions of control circles, seen in the image below. Alternatively, you can specify a color by entering an RGB or a Hex code.



Color Range

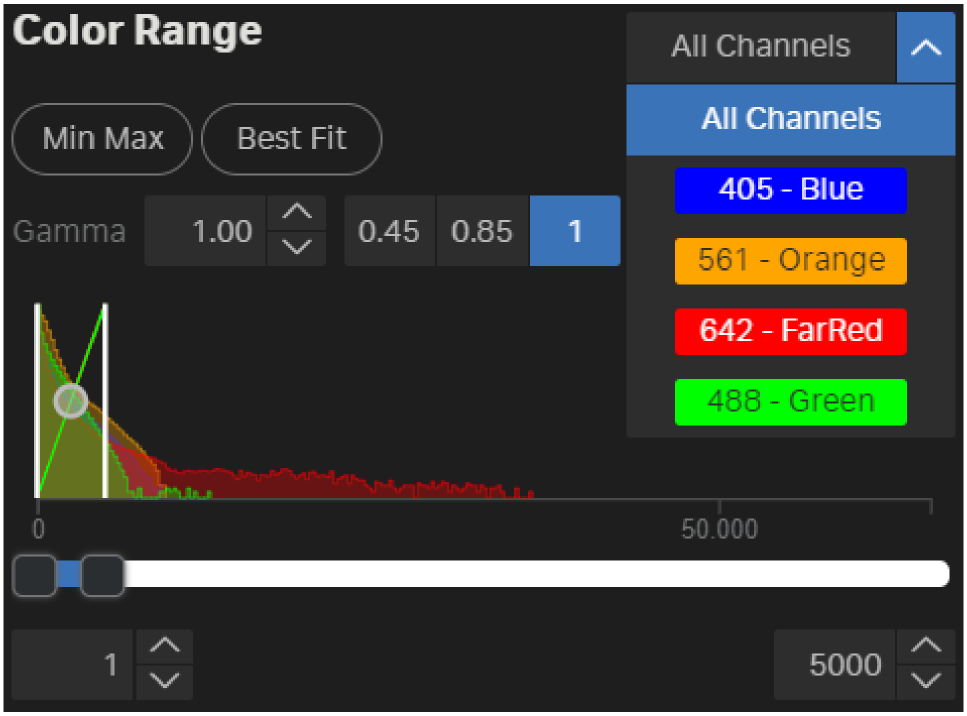
The color range is used to adjust optimal contrast across all the channels displayed in 3D viewer.

Minimum (**Min**) and maximum (**Max**) display values are set to 1 and 5,000 respectively on load of the viewer. All the voxels with intensities below **Min** are hidden from display and shown as black, while all the voxels with intensities above **Max** are displayed as white (or another selected color).

The **Min** value should be increased above the background or any other dim structures.

The **Max** value should be increased so that the rendering is bright enough to view all the biological structures of interest.

By default, display adjustments are applied to all channels. Specific channel can be selected from the dropdown menu to set the display settings for an individual channel, as seen in the below illustration.



Adjusting Min and Max Display Values

Min and **Max** display values are shown at the bottom of the section and can be adjusted in several ways, highlighted in the below illustration.

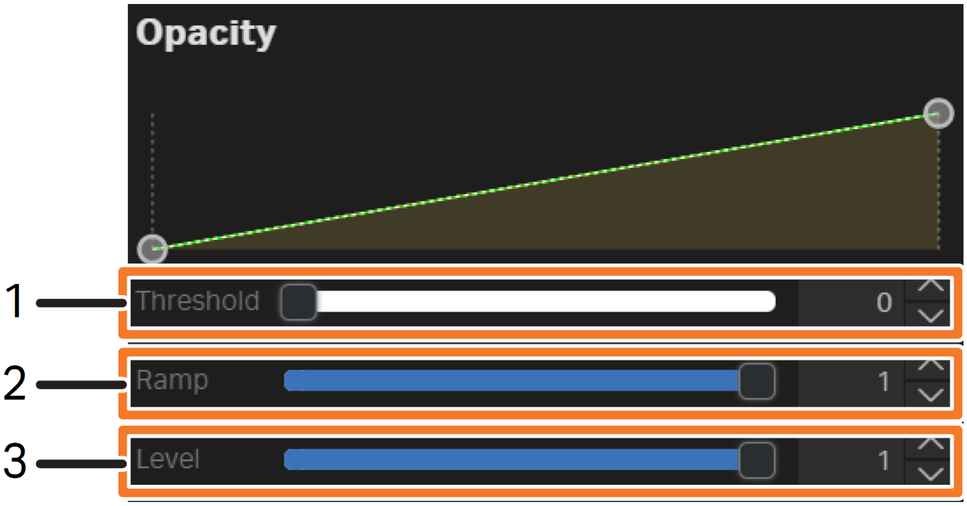
Part	Adjustment Description
1	Click **Min Max** to adjusts display values such that Min and Max are set to minimum and maximum voxel intensities of an active channel. Click **Best Fit** to adjust optimal display values based on the histogram of an active channel.
2	Adjustments of **Gamma** can help highlight or hide low intensity voxels. Decreasing **Gamma** values below 1 highlights dim voxels while **Gamma** values above 1 makes bright voxels look even brighter thus hiding dim voxels. **Note:** The two **Gamma** controllers are synchronized. Adjusting 2 changes 4, and vice versa.
3	Move the white bars left and right to adjust the **Min** and **Max** values, respectively.
4	Drag the **Gamma** control up or down. Dragging it down decreases gamma while dragging it up increases gamma values.
5	Move the left and right sliders to adjust the **Min** and **Max** values, respectively.
6	Enter values to specify **Min** (left) and **Max** (right) display values.
7	Click **Reset** to restore initial display Min and Max values back to default (1 and 5,000 respectively).

Opacity

Opacity control is required to achieve optimal display settings across the channels.

Note: By default, opacity settings are applied to all the channels. Specific channel can be selected in **Color Range** section to adjust opacity for a single channel.

The table below describes the available **Opacity** settings.



Part	Description
1	Controls which voxels are hidden based on their intensity values. Increasing **Threshold** hides more low intensity signal, which is similar to Min in **Color Range** section. **Threshold** can be adjusted by moving either the **Threshold** slider or left control circle.
2	Controls the contrast of a rendering. Decreasing the ramp increases the contrast. Ramp can be adjusted by moving either the **Ramp** slider or right control circle left/right.
3	Controls the brightness of the displayed rendering. Decreasing the level results in decreased brightness. Levels for channels can be adjusted by either moving the **Level** slider or by moving right control circle up/down.

Tip: It is possible to adjust **Ramp** and **Level** simultaneously by moving the right control circle diagonally.

Background

Background section allows for adjustments of background color and whether it is shaded or has a solid color.

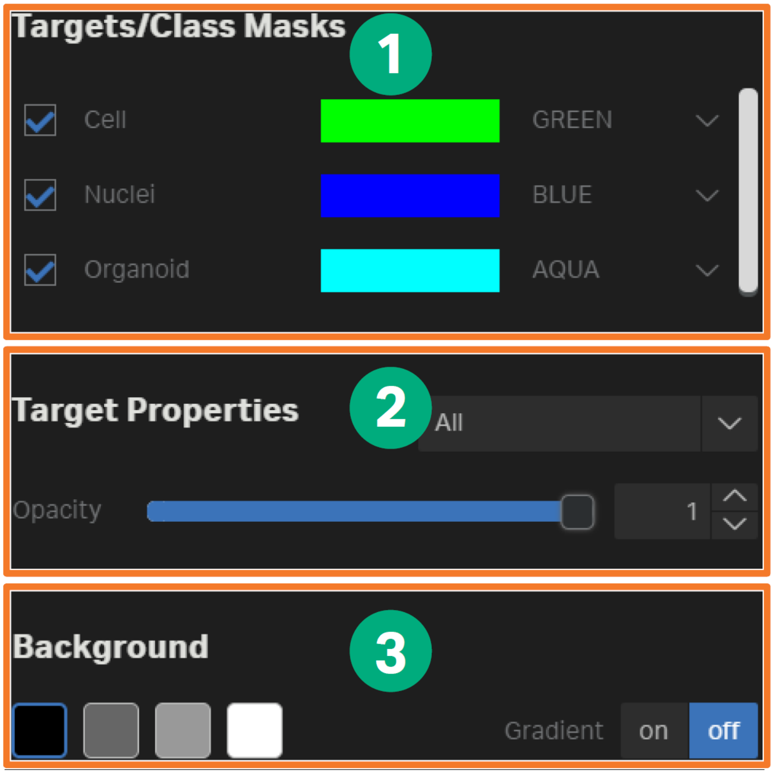
Note: By default, background color is set to black with shading (**Gradient** is **on**). Turning **Gradient** to **off** helps achieve better contrast.

Tip: Setting the background color to white can improve visualization of transmitted imaging modalities, for example phase contrast or DIC.

Target Mask Control

Target mask control provides control options for visibility of target masks generated in IN Carta VoluMetrics.

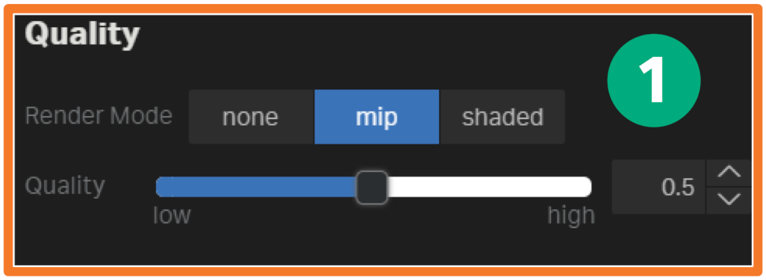
The table below describes the available target mask controls.



Part	Description
1	**Target/Class Masks** controls visibility and color assignments for target masks. Color for each target mask can be assigned similarly to how it is performed for the channels (see Channels on page 53). Besides selection of a preset channel or user-defined color, you can also randomize color assignment across all objects for a given target mask. This option is helpful to evaluate splitting of adjacent objects.
2	**Target Properties** controls the transparency of target masks. Transparency of target masks can be controlled by moving the slider or entering a value from 0 to 1. By default, opacity is controlled for all the target masks and can be applied to an individual target when it is selected from the dropdown list.
3	**Background** color can be set similarly to how it was described for the Channel tab. Background settings are shared between Channels and Target Mask tabs, when color or gradient are modified in one of the tabs, change is reflected in the other tab.

3D Viewer Settings

Current tab allows to adjust general display settings in 3D Viewer and visualize cross-sectional view of the rendering.



Render Mode: mip

Quality: 0.5

on

Presets: YZ

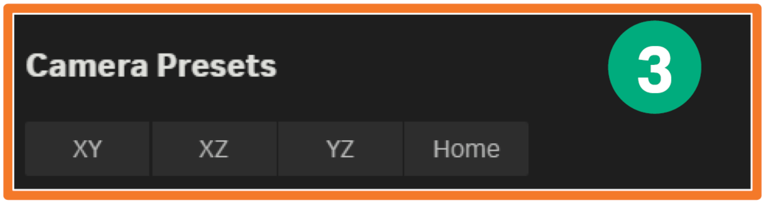
Position: 0

Angle 1: 0

Angle 2: 0

Clip: Target

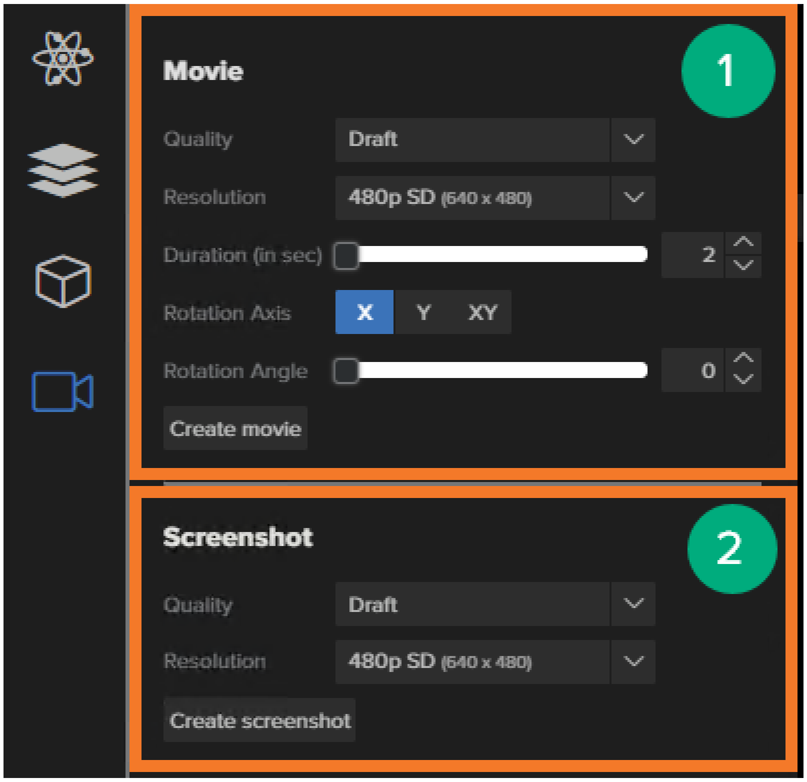
Opacity: 0.5



Part	Description
1	**Quality** provides options to change the render mode of channels and display quality. **Render Mode** includes the following options: • **none**: Hides channels (volume) and displays only target masks. This can be used as a quick way to visualize masks without individually deselecting the channels tabs. • **mip**: Maximum intensity projection rendering, which is a default option and is optimal for most visualizations. • **shaded**: True volumetric rendering with shading, however it can show excessive levels of details. The **Quality** slider controls the level of details in 3D rendering. Higher settings for **Quality** requires an advanced model GPU, and can result in a lagging visualization while working with the rendering.
2	**Clipping** allows for adjustments of a plane to generate an interactive cross-sectional view of channels and/or target masks. It is possible to adjust clipping plane in the following ways: • Set to one of 3 pre-defined positions (**XY**, **XZ**, **YZ**). **YZ** is the default option. • Move across the rendering using the **Position** slider. • Tilt using two **Angle 1** / **Angle 2** sliders. • Set to clip **Targets** (target masks only), **Volume** (channels only) or **Both** (channels and target masks). • Adjust for optimal **Opacity**. • Set any user-defined **Color** of the clipping plane.
3	**Camera Presets** section provides shortcuts to several pre-defined positions of 3D rendering in the viewer, rotating it to the following selected options: • **XY**: Equivalent to view from the top. • **XZ**: Orthogonal (sideways) view along XZ plane. • **YZ**: Orthogonal (sideways) view along YZ plane. • **Home**: Default view of the rendering when **3D Viewer** is open.

3D Viewer Export Tools

Export tools allows you to export still and animated renderings of the 3D Viewer display.



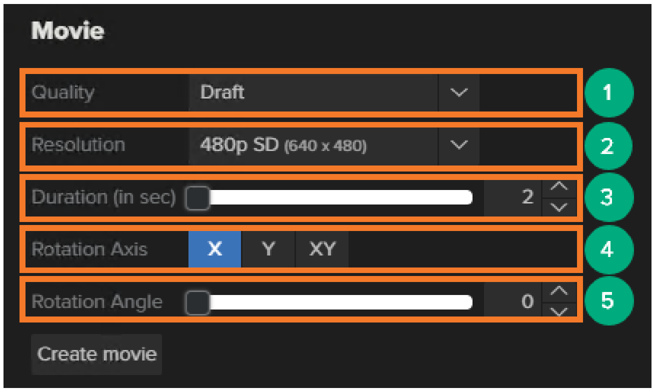
Quality: Draft
Resolution: 480p SD (640 x 480)
Duration (in sec): 2
Rotation Axis: X Y XY
Rotation Angle: 0

Quality: Draft
Resolution: 480p SD (640 x 480)

Part	Description
1	**Movie** exports an animated (rotated) visualization of single- and multi-channel 3D image stacks as an MP4 file with or without 3D target masks as a volume rendering.
2	**Screenshot** exports a still image as a JPEG file. You can specify the quality and size of exported image.

Exporting a Movie

You can export an animated (rotated) visualization of single- and multi-channel 3D image stacks as an MP4 file with or without 3D target masks as a volume rendering. Use the following controls to specify the quality, size, duration, and rotation axis and angle of exported movie.



Movie

Quality: Draft

Resolution: 480p SD (640 x 480)

Duration (in sec): 2

Rotation Axis: X Y XY

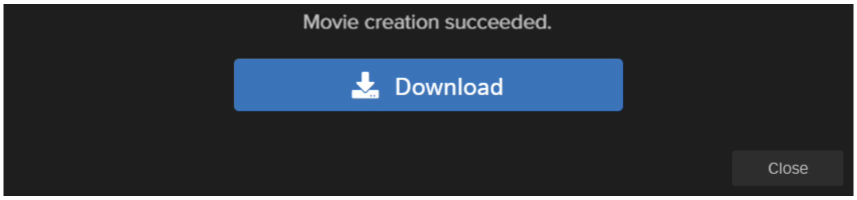
Rotation Angle: 0

Part	Description
1	**Quality** selects the quality of the export image. Better quality provides greater detail in the exported movie, but requires more time to generate. The following options are available: - **Draft** - **Low** - **Medium** - **High** - **Ultra**
2	**Resolution** selects the size of the export image. The following options are available: - **SD** (640 × 480) - **HD** (1280 × 720) - **Full HD** (1920 × 1080) - **QHD** (2560 × 1440)
3	**Duration (in sec)** specifies the length of the video—that is, the amount of time it will take for the image to rotate to the specified angle). The available range is from 2 to 60 seconds. In a longer movie, the image rotates more slowly, allowing you to observe greater detail. However, a longer movie requires more time to generate and has a larger file size.
4	**Rotation Axis** selects the axis around which the image will rotate. Select the axis that achieves the desired perspective during the rotation. The following options are available: - **X** - **Y** - **XY**
5	**Rotation Angle** specifies the distance for the rotation during the specified duration. The available range is from 0° to 360°.

To export a movie:

1. Use the **Movie** controls to specify the movie settings.
2. Click **Create movie** to start the export.

   A progress bar displays. When the image is ready for download, the following message appears:

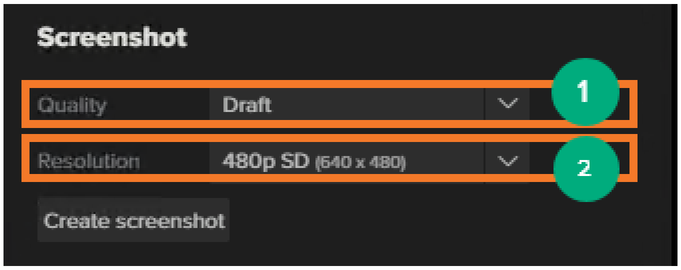
   

   Movie creation succeeded.

3. Click **Download** to save the screenshot.

Exporting a Screenshot

You can export a still image of the 3D Viewer display as a JPEG file. Use the following controls to specify the quality and size of exported image.



Screenshot

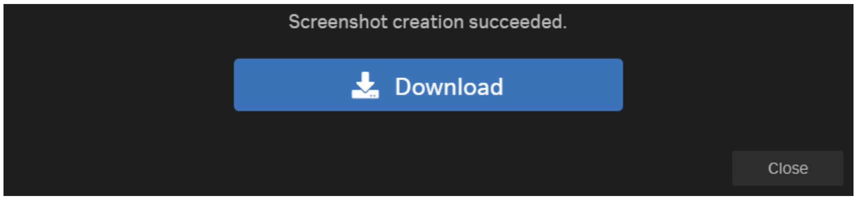
Quality: Draft

Resolution: 480p SD (640 x 480)

Part	Description
1	**Quality** selects the quality of the export image. Better quality provides greater detail in the exported movie, but requires more time to generate. The following **Quality** options are available: • Draft • Low • Medium • High • Ultra
2	**Resolution** selects the size of the export image. The following output options are available: • **SD** (640 × 480) • **HD** (1280 × 720) • **Full HD** (1920 × 1080) • **QHD** (2560 × 1440)

To export a screenshot:

1. Use the **Screenshot** controls to specify the image settings.
2. Click **Create screenshot** to start the export.
   A progress bar displays. When the image is ready for download, the following message appears:



Screenshot creation succeeded.

3. Click **Download** to save the screenshot.

Chapter 10: List of Available Measures

This chapter provides information about the available measures.

Morphological Measures

The following table describes the morphological measures.

Morphological Measures	Description
Volume	The physical volume of the object $V(L) = \int_L dp$.
Surface Area	The physical area A(L) of the surface S(L).
Surface Area by Volume	The ratio of the surface area by volume $A(L)/V(L)$
Elongation	The ratio of the objects' strongest principal moment of inertia I of by its weakest principal moment I. With this ratio, a perfect sphere would have a ratio of 1, while elongated objects have a ratio greater than 1.
Sphericity	Ratio relating surface area of a perfect sphere with the volume of the object and the surface area of the object: $$sphericity = \frac{S_{sphere}}{S(L)} = \frac{4\pi * \left(\frac{3 * V(L)}{4\pi}\right)^{\frac{2}{3}}}{A(L)}$$ With the sphericity ratio, a perfect sphere would have a ratio of 1, while less spherical objects are between 0 and 1. This measure requires significant computational resources and therefore increase the time required for analysis.
Gyration Radius	Gyration radius is an average radius of a shape: $r_G\ (L) = \sqrt{\int_L \lVert p - C(L) \rVert^2\ dp}$ This measure requires significant computational resources and therefore increase the time required for analysis.
Projection Area	The area of the object projected onto the xy, xz, and yz planes.
XY Projection Area	A_{xy} - area of projected onto XY plane $\int_{L_{xy}} dp$ of within xy plane.
XZ Projection Area	A_{xz} - area of projected onto XZ plane $\int_{L_{xz}} dp$ of within xz plane.

Morphological Measures	Description
YZ Projection Area	A_{yz} - area of projected onto YZ plane $\int_{L_{yz}} dp$ of within yz plane.
Projection Perimeter	The perimeter of the object projected onto the xy, xz, and yz planes.
XY Projection Perimeter	P_{xy} perimeter length of L projected onto XY plane.
XZ Projection Perimeter	P_{xz} perimeter length of L projected onto XZ plane.
YZ Projection Perimeter	P_{yz} perimeter length of L projected onto YZ plane.
Projection Roundness	The roundness of the object projected onto the xy, xz, and yz planes.
XY Projection Roundness	Roundness of L projected onto XY plane. Ratio relating perimeter length of a circle with the same area as A_{xy} and $P_{xy} \quad \frac{2\sqrt{\pi A_{xy}}}{P_{xy}}$ Roundness of 1 refers to perfect circle.
XZ Projection Roundness	Roundness of L projected onto XZ plane. Ratio relating perimeter length of a circle with the same area as A_{xz} and $P_{xz} \quad \frac{2\sqrt{\pi A_{xz}}}{P_{xz}}$ Roundness of 1 refers to perfect circle.
YZ Projection Roundness	Roundness of L projected onto YZ plane. Ratio relating perimeter length of a circle with the same area as A_{yz} and $P_{yz} \quad \frac{2\sqrt{\pi A_{yz}}}{P_{yz}}$ Roundness of 1 refers to perfect circle.

Spatial Measures

The following table describes the spatial measures.

Spatial Measures	Description
Center of Mass (Centroid)	Coordinates of physical center point of L defined according to: $C(L) = \frac{\int_L p\,dp}{V(L)}$
Centroid X	X coordinate of a physical center point.
Centroid Y	Y coordinate of a physical center point.
Centroid Z	Z coordinate of a physical center point.
Intensity Weighted Center of Mass	Coordinates of weighted center of mass of L, defined according to: $C_m(L) = \frac{\int_L i(p) \cdot p\,dp}{V(L)}$
Weighted Center of Mass X	X coordinate of weighted center of mass of L.
Weighted Center of Mass Y	Y coordinate of weighted center of mass of L.
Weighted Center of Mass Z	Z coordinate of weighted center of mass of L.
Mass Displacement	This is the distance (in µm) between the **Center of Mass** and the **Weighted Center of mass**. This is one way to measure intensity spread across an object (others are intensity spreading, **SD**, and **CV**). It is measured by mass displacement: distance between centroids: $\|C(L) - Cm(L)\|$
Oriented Bounding Box	The smallest cuboid that encompasses the object following rotation of the cuboid in all dimensions. Selecting the oriented bounding box measures in IN Carta returns coordinates of the cuboid for more precise characterization of object's dimensions in 3D. If oriented bounding box is required at any steps of the downstream analysis, be certain to check all relevant boxes. $B(L) = \{b + ul + vm + ws \mid 0 \leq u, v, w \leq 1\}$ bounding L where l, m, s are orthogonal vectors:
Bound Box Oriented X1	X-coordinate of the origin point b of the oriented bounding box.
Bound Box Oriented Y1	Y-coordinate of the origin point b of the oriented bounding box.
Bound Box Oriented Z1	Z-coordinate of the origin point b of the oriented bounding box.

Spatial Measures	Description
Bound Box Oriented Ax1_X	X-coordinate of the longest orthogonal vector (l) of the oriented bounding box.
Bound Box Oriented Ax1_Y	Y-coordinate of the longest orthogonal vector (l) of the oriented bounding box.
Bound Box Oriented Ax1_Z	Z-coordinate of the longest orthogonal vector (l) of the oriented bounding box.
Bound Box Oriented Ax2_X	X-coordinate of the middle orthogonal vector (m) of the oriented bounding box.
Bound Box Oriented Ax2_Y	Y-coordinate of the middle orthogonal vector (m) of the oriented bounding box.
Bound Box Oriented Ax2_Z	Z-coordinate of the middle orthogonal vector (m) of the oriented bounding box.
Bound Box Oriented Ax3_X	X-coordinate of the short orthogonal vector (s) of the oriented bounding box.
Bound Box Oriented Ax3_Y	Y-coordinate of the short orthogonal vector (s) of the oriented bounding box.
Bound Box Oriented Ax3_Z	Z-coordinate of the short orthogonal vector (s) of the oriented bounding box.

Intensity Measures

The following table describes the intensity measures.

Intensity Measures	Description
Total Intensity	The sum of pixel values within an object. Sum over L pixel intensities $I(L) = \int_L i(p)dp$
Mean Intensity	The total intensity divided by the total number of pixels. average intensity $I_{\emptyset}(L) = \frac{I(L)}{V(L)}$
Global Background	Global background takes all pixels outside of the segmented objects and calculates the mean intensity of those values. Mean intensity of the background $I_G = \frac{\int_G i(p)\,dp}{\int_G p\,dp}$ where background includes all the pixels outside of segmented objects.
Local Background	For the local background calculation, the segmented objects are dilated by 5 pixels and all the pixels values within that dilated shell are averaged for each object. Mean of local background $I_G(L) = \frac{\int_{G(L)} i(p)\,dp}{\int_{G(L)} p\,dp}$ where local background includes 5-pixel wide spherical shell around L.
Total Int by Global Bkg	Total intensity normalized by local background $\frac{I(L)}{I_G}$
Total Int Minus Global Bkg	Local background subtracted from total intensity $I(L) - I_G$
Total Int by Local Bkg	Total intensity normalized by local background $\frac{I(L)}{I_G(L)}$
Total Int Minus Local Bkg	Local background subtracted from total intensity $I(L) - I_G(L)$

Intensity Measures	Description
Mean Int by Global Bkg	Mean intensity normalized by global background $\frac{I_{\emptyset}(L)}{I_G}$
Mean Int Minus Global Bkg	Global background subtracted from mean intensity $I_{\emptyset}(L) - I_G$
Mean Int by Local Bkg	Mean intensity normalized by local background $\frac{I_{\emptyset}(L)}{I_G(L)}$
Mean Int Minus Local Bkg	Local background subtracted from mean intensity $I_{\emptyset}(L) - I_G(L)$
Median Intensity	Median pixel intensity value within L.
Max Intensity	Maximum pixel intensity values within L.
Min Intensity	Minimum pixel intensity values within L.
Intensity SD	Standard deviation of pixel intensity values within L(SD(L))
Intensity SNR	Signal-to-noise ratio, defined by ratio of mean intensity to intensity SD $\frac{I_{\emptyset}(L)}{\mathrm{SD}(\mathrm{L})}$
Intensity CV	Intensity variation coefficient is defined by ratio of intensity SD to mean intensity. $\frac{\mathrm{SD}(\mathrm{L})}{I_{\emptyset}(L)}$
Intensity Spreading	Measure for uniformity of pixel intensity values with an object. This measure requires significant computational resources and therefore increases the time required for analysis. $\frac{\int_L i(p) \cdot \lVert p - C(L) \rVert^2 dp}{I(L) \cdot r_G(L)^2}$

Texture Measures

Texture measures reflect the local variation in pixel intensity within an object. Run-length measures are quantitative measurements of similarity of pixel intensity in any given direction. Even though these measures are hard to interpret from the qualitative perspective, they can be useful in downstream object classification.

These measures are computationally intensive and including them in the measures list can significantly increase processing time.

The table below describes the texture measures.

Texture Measure	Description
Non-uniform	Run length non-uniformity measure calculated for pixel values within an object, quantized into 32 grayscale bins.
Run-length, non-uniform, gray	Run length gray level non-uniformity measure calculated for pixel values within L, quantized into 32 grayscale bins.
Run-length, non-uniform, gray, high	Run length high gray level run emphasis measure calculated for pixel values within L, quantized into 32 grayscale bins.
Run-length, non-uniform, gray, low	Run length low gray level run emphasis measure calculated for pixel values within L, quantized into 32 grayscale bins.

See itk.org/Doxygen/html/classitk_1_1Statistics_1_1HistogramToRunLengthFeaturesFilter.html for details.

Timing Considerations for Measure Calculations

This table is a quick reference to guide the selection of measures with relation to the amount of time required to perform the calculations.

	Target Type	Nuclei	Organoid	Organoid
Stack information	Dimensions (X x Y x Z)	2040 x 2040 x 22	2040 x 2040 x 71	2040 x 2040 x 5
	Number of objects segmented	892	1	1
		Time to calculate (ms) per field of view		
Measure	Volume	<1	<1	<1
	Bound box oriented X1	32	<1	<1
	YZ projection area	250	375	<1
	Projection Y roundness	234	447	<1
	Projection X roundness	267	283	<1
	Gyration radius	4168	73623	284
	Sphericity	2132	38416	156
	Mean intensity	<1	<1	<1
	Intensity SNR	16	<1	<1
	Intensity SD	<1	<1	<1
	Intensity spreading	2217	37805	156
	Run length non-uniform	7605	139202	618

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