MOLECULAR DEVICES

IN Carta Image Analysis Software

QUICK REFERENCE GUIDE

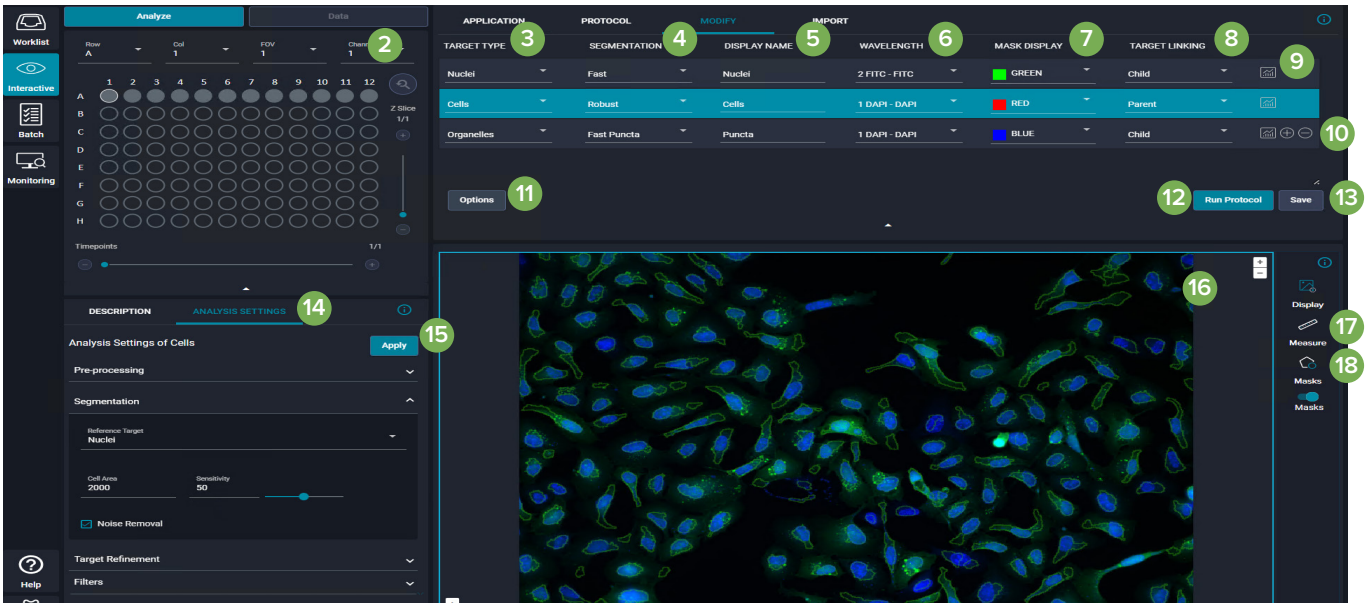
Protocol Design (Flexi-Protocol)

Creating a Protocol

1. Open an experiment on the **Interactive: Analyze** page.
2. In the **Protocol Editor Table** pane, on the **Application** tab, click **Flexi-Protocol** (1).

3. On the **Protocol** tab, click **New** to create a new protocol.
4. On the **Modify** tab, click **Add a new target** (10) to add targets to the protocol as needed.
5. For each target (that is, each row) in the protocol:
   a. Select the **Target Type** (3) for the target.
   b. Select the **Segmentation** (4) method. See “Segmentation Method Descriptions” on page 2 of this guide for details.
   c. Enter a unique **Display Name** (5) (or use the default name).
   d. Select a **Wavelength** (6) and **Mask Display** (7) color.
   e. Select a **Target Linking** (8) value to indicate if a target is a ***Parent***, ***Child***, or ***Non-linked***.
   f. Click **Measures** (9) and select/deselect measurements for each target. Measures are available for ***Morphology***, ***Intensity***, ***Spatial***, ***Texture***, and ***Colocalization***. Custom measures are also available.

      The ***Intensity***, ***Texture***, and ***Colocalization*** categories contain a Wavelength selector, which enables you to perform intensity-relevant measurements on wavelengths other than the one used for segmentation. For example, segment nuclei in the DAPI channel and measure intensity within a nuclear mask in FITC channel.
6. Click **Options** (11) to define execution options for data sets with multiple Z slices and/or multiple time points.
   - For Z stack experiments, options include **Middle**, **Current**, **Best focus**, **Process all**, and **Custom Z selection**.
   - For timelapse experiments, options include **Current**, **First**, **Last**, **Process all**, and **Custom T selection**.



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Optimizing Segmentation Parameters

1. In the **Analysis Settings** pane (14), set the analysis parameters for each target in the protocol. See "Analysis Setting Parameter Descriptions" on pages 3 and 4 of this guide for details.
2. Click **Apply** (15) to view preliminary segmentation results overlaid on the images in the Image Viewer.
3. In the **Image Viewer** pane (16), use one or more of the following tools to check the segmentation results for each target:
   - Click  **Measure** (17) to measure the length and area of objects.
   - Click  **Masks** (18) to toggle individual mask displays on/off and change mask type, color, and opacity.
4. Repeat these steps until segmentation results are satisfactory.
5. In the **Specimen Navigation** pane (2), select another well and verify that the **Analysis Settings** are appropriate across the plate.
6. In the **Protocol Editor Table** pane, click ***Save*** (13).

Running a Protocol

1. Click **Run Protocol** (12).
2. Select wells using by clicking and dragging the mouse or by clicking **Select All**. Selected wells appear in blue. Click Reset to clear a selected well. Hold CTRL

Segmentation Method Descriptions

Nuclei	Fast	• Provides results quickly, but detection of nuclei may be less accurate than with Robust segmentation. • Recommended for total intensity assays or when morphology information is not critical.
	Robust	• Provides the most accurate detection of nuclei, but detection is slower than with Fast segmentation.
	SINAP	• Provides nuclei segmentation based on deep convolutional neural networks (DCNN).
Cell	Fast	• Provides results quickly, but detection of cell edges and boundaries between adjacent cells may be less accurate than with Robust segmentation. • Does not require a nuclear seed. • Recommended for well-separated, reasonably large cells.
	Collar	• Collar segmentation creates an approximate cell boundary by defining a virtual cytoplasm compartment based on a previously segmented target. • Uses the outer edge of the Nuclei Target mask as the inner boundary seed; the radius determines the distance in µm to the cell edge. • Recommended when there is no cellular staining, when identifying positive/negative staining, or when precise cell segmentation is not required.
	Robust	• Provides the most accurate detection of cell edges and boundaries between adjacent cells based on a previously segmented target, but detection is slower than with Fast segmentation. • Uses a watershed algorithm to very accurately define the cell boundary. • Recommended when morphology or identification of objects within the cell boundary is critical.
	SINAP	• Provides cellular segmentation based on deep convolutional neural networks (DCNN).
Organelle	Fast Puncta	• Provides results quickly, but detection of puncta may be less accurate than with Robust Puncta segmentation. • Uses a multi-scale top hat algorithm. • Recommended for identifying puncta of varying size.
	Robust Puncta	• Provides the most accurate detection of puncta, but detection is slower than with Fast Puncta segmentation. • Recommended for identifying puncta of varying intensity.
	Networks	• Provides detection of intracellular networks, such as mitochondria or Golgi. • Note that this segmentation is not optimized to detect individual fibers for cytoskeletal analysis.
	Membrane	• Provides detection of a membrane compartment within a previously segmented target. • Recommended to segment a membrane compartment or create a new compartment within the cell.
	SINAP	• Provides object segmentation based on deep convolutional neural networks (DCNN).
User-Defined	Binary	• Provides object segmentation based on two existing targets and a Boolean operator (AND, OR, NOT, XOR).

Analysis Setting Parameter Descriptions

Segmentation Parameters

Setting	Target (Segmentation)	Description
Advanced Sensitivity Range	Nuclei (Fast) Cells (Fast) Organelles (Fast Puncta)	When Expand Sensitivity is selected, specifies a value to change upper limits of the local contrast range. The contrast scale is nonlinear. Smaller values increase overall sensitivity; larger values reduce overall sensitivity.
Cell Area	Cells (Fast, Robust)	Specifies the typical cell area in µm² (between 1 and 50,000).
Collar Radius	Cells (Collar)	Specifies the distance in µm to dilate the reference target. Useful if a cell stain is not available.
Expand Sensitivity	Nuclei (Fast) Cells (Fast) Organelles (Fast Puncta)	Enables the Advanced Sensitivity Range.
Low Background	Nuclei (Fast) Cells (Fast) Organelles (Fast Puncta)	Automatically scales image intensity for a low background signal image, which improves the performance of the algorithm.
Membrane Thickness	Organelles (Membrane)	Sets the distance in µm (between 0.1 and 5) to erode reference target
Min / Max Area	Organelles (Fast Puncta)	Specifies the upper and lower limits for puncta area in µm² (between 0.1 and 1 0).
Min / Max Diameter	Organelles (Robust Puncta)	Specifies the upper and lower limits for puncta diameter in µm (between 0.1 and 1 0).
Noise Removal	Nuclei (Robust) Cells (Robust) Organelles (Robust Puncta)	Attenuates noise to reduce false-positive detection when few objects are present in the images or when image contrast is low. (Note that the efficiency of this parameter depends on a set object diameter.)
Nuclei Area	Nuclei (Fast)	Specifies the minimum area in µm² of a segmented nucleus.
Nuclei Diameter	Nuclei (Robust)	Specifies a value in µm for the typical diameter of a nucleus calculated as the average of short and long axes of an elliptical nuclear mask.
Precise Mask	Nuclei (Fast) Cells (Fast) Organelles (Fast Puncta)	Provides a more accurate mask to improve segmentation of small objects, such as nuclei and organelles.
Pre-trained Model	Nuclei (SINAP) Cells (SINAP) Organelles (SINAP)	Specifies the SINAP deep-learning model for the analysis.
Reference Target	Cells (Collar) Organelles (Membrane)	Specifies the seed target for segmentation. For Collar segmentation, the reference target is typically a nuclei target. For Membrane segmentation, the reference target is typically a cell target.
#Scales	Organelles (Fast Puncta)	Specifies the number of scales to use. Scales are size classes that the detection algorithm uses for granule segmentation and quantitation. The Min Area and Max Area are the size range boundaries. • If 1, detects only objects that are approximately equal to the specified Min Area. • If 2, detects granules that are approximately equal to the specified Min Area and Max Area. • If more than 2, also detects intermediate-sized granules.
Sensitivity	Nuclei (Fast, Robust) Cells (Fast, Robust) Organelles (Fast Puncta, Robust Puncta, Networks)	Specifies a percentage (between 1 and 100) to alter the segmentation threshold. Increase the sensitivity value if the source image is dim and not all targets are detected. Decrease the sensitivity value if the source image is bright, of high contrast, or includes excessive background noise.
Shape Detection	Cells (Fast) Organelles (Fast Puncta)	Specifies a shape criterion (Any, Peak, and Ridge) for objects to be included in the segmentation result.
Supplement Splitting	Nuclei (SINAP) Cells (SINAP) Organelles (SINAP)	Applies an algorithm that splits objects from the SINAP segmentation to improve segmentation of tightly packed objects.
Thickness	Organelles (Networks)	Specifies the typical thickness in µm of network structures.

Pre-Processing Parameters

Setting	Description
Prefilter	Emphasizes targets of the specified Object Area in µm² by smoothing the image and removing small objects.
Remove Background	Emphasizes targets of the specified Radius value in µm by subtracting background intensity.
Normalize	Adjusts the image to a more uniform intensity based on the specified Reference Target.

Target Refinement Parameters

Setting	Description
Fill Holes	Fills empty spaces inside the target.
Includes Target	Includes only targets with the specified Reference Target, which is the seed target for refinement.
Splitting	Divides the target using the specified Reference Target. For example, a Nuclei reference target can help separate segmented cells.

Filter Parameters

Setting	Description
Min / Max Area	Specifies the upper and lower limits for target area in µm². Targets outside this range are excluded.
Wavelength	Specifies the wavelength used for Min / Max Intensity filter.
Min / Max Intensity	Specifies the upper and lower limits for target intensity. Targets outside this range are excluded.
Exclude Overexposed Target	Excludes objects with one or more overexposed pixels within the mask.
Exclude Objects Touching Edges	Excludes objects at the edge of an image.
Distance from Border	When Exclude Objects Touching Edges is selected, specifies the distance from the border in µm at which targets are excluded.

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