MOLECULAR DEVICES

IN Carta Image Analysis Software

QUICK REFERENCE GUIDE

Protocol Design (Mono-Nucleated Cells)

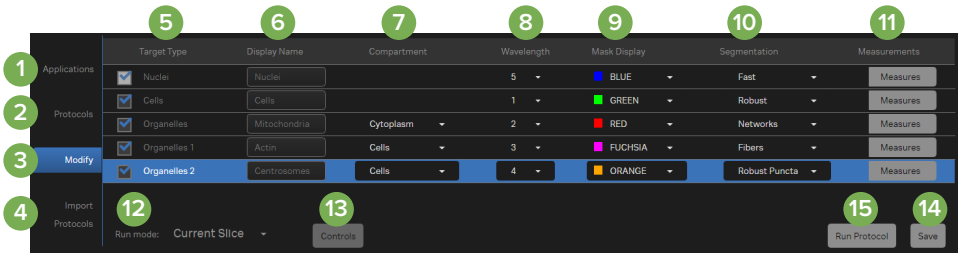
Setting Up a Protocol

1. Select the ***Analyze*** tab.
2. In the ***Applications*** (1) tab, select ***Mono-nucleated Cells***.
3. In the ***Protocols*** (2) tab, click the protocol you would like to run and select the ***Modify*** (3) tab. Alternatively, click ***New*** to design a new protocol.
4. Check the ***Target Type*** (5) boxes to activate the correct targets.
5. For each ***Target Type***:
   a. Enter a unique ***Display Name*** (6), if desired.
   b. For organelles, specify the ***Compartment*** (7) in which they should be identified.
   c. Select ***Wavelength*** (8) and a ***Mask Display*** (9) color.
   d. Select ***Segmentation*** (10) method. Table on next page provides details on the various methods.
   e. Click the ***Measures*** (11) button and select/deselect measurements for each ***Target Type*** as needed. See "Using the Measurements Menu" below for more information.
6. For data sets with multiple z slices, it is possible to define a ***Run mode*** (12). It can be ***Multiple Slices***, ***Current Slice***, or ***Best Focus Z***. In order to specify a custom range of z slices, select ***Multiple Slices*** and click ***Controls*** (13). Select ***Best Focus Z*** to allow the IN Carta™ software automatically determine the slice to be analyzed via image contrast analysis, for a selected channel. Click ***Controls*** (13) to specify a channel used for select best focus z-slice.
7. Only one time point can be analyzed in ***Mono-nucleated Cells*** application. The first time point is selected by default, and another time point selected for analysis in specimen navigation panel. Switch to the ***Time-lapse Analysis*** application when analysis of multiple time points is required.
8. Select the ***Import Protocols*** (4) tab to load previously created analysis protocols or protocols shared by other users.

Optimizing Segmentation Parameters

1. Use the ***Analysis Settings Panel*** to edit available parameters for each ***Target Type***.

   Note: See the table on the next page or click the ***Information*** ⓘ icon for more details on parameters.
2. Click ***Apply*** to see preliminary segmentation results overlaid on the images.
3. Check segmentation results for each target using one or more of the following strategies:
   - Use the ***Mask*** MASK tool to toggle mask display on/off and to access the ***Target Mask Properties*** menu.
   - Measure object length and area to refine size and area estimates using the ***Sizing*** SIZING tool.
   - Apply an ***Area*** or ***Intensity Filter*** as needed.
   - Select the ***Results Review*** tab to display plots of the measured data to refine estimates for algorithm parameters.



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4. Update ***Analysis Settings*** as necessary and click ***Apply***. Repeat steps 2 and 3 until segmentation results are satisfactory.
5. Select another well and repeat steps 2 and 3 to verify that ***Analysis Settings*** are appropriate across the plate.
6. Click ***Save*** (14).

Using the Measurements Menu

- ***Measures*** are divided into ***Count***, ***Spatial***, ***Intensity***, ***Position***, ***Coordination***, ***Texture***, and ***Colocalization*** categories.
- Each category has some default measurements selected. Other available measures can be selected as needed.
- A channel selection is located in the ***Intensity***, ***Texture***, and ***Colocalization*** categories. It allows 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.

Running a Protocol

1. Click ***Run Protocol*** (15).
2. Click and drag or click ***Select All*** to select wells for analysis.

   Note: Press ***Ctrl*** during selection to select non-adjacent wells.
3. Click ***Run***.

   Note: See Using Batch Analysis to run more than one protocol at a time.

Using Batch Analysis

1. Click ***Batch***  .
2. Select ***New Batch***.
3. Enter a name for the ***Batch Container*** and click ***OK***.
4. With the ***Batch Container*** selected in the ***Batch Containers panel***, select multiple data sets from the Acquisition Experiments pane, and select one or multiple analysis protocol. Click ***Add Selected***.
5. Click ***Submit for Processing***. Results are saved to each image stack location.
6. Click the ***Monitoring*** icon to see the status of the batch analysis.

Target type	Segmentation Method	Description
Nuclei	Fast	• Faster than Robust method, but not as accurate. • Use for total intensity assays or when morphology information is not critical.
	Robust	• Slower than Fast method, but will provide more accurate nuclear segmentation results.
Cell	Fast	• Faster than Robust method, but not as accurate. • Use for well-separated, reasonably large cells.
	Collar	• Nuclear dilation method. • Use when there is no cellular staining, when identifying positive/negative staining, or when precise cell segmentation is not required.
	Robust	• Watershed algorithm that very accurately defines cell boundary. • Use when morphology or identification of objects within the cell boundary is important.
	Soma	• Use with Neurites segmentation. • Modified Robust Cell segmentation algorithm to segment soma.
Organelle	Fast Puncta	• Multi-scale top hat algorithm. • Good at identifying puncta of varying size.
	Robust Puncta	• Good at identifying puncta of varying intensity.
	Networks	• Segments network like organelles within the cell (e.g., endoplasmic reticulum, mitochondria, golgi).
	Membrane	• Creates an artificial boundary within the cell. • Use to segment a membrane compartment or to create a new compartment within the cell, for example mitochondria.
	Fibers	• Detects filamentous structures including individual and collapsed fibers.
	Neurites	• Segments neurite outgrowth and performs its tracing.

Parameter	Nuclei	Cells	Organelles	Description	Tips
Area filter	✓	✓	✓	Post segmentation step to remove large or small objects from final data	• Use to remove objects with an area outside the range selected (that is, small segmented background noise or large artifacts).
Background suppression			✓	Advanced parameter for neurite segmentation to correct for low contrast	• Default value is a good starting point and should be changed only when changing sensitivity and width parameters don't improve segmentation. • Higher values reduce noise in the segmentation mask.
Cell area		✓		Average cell area (μm^2)	• Use size tools to establish appropriate value. • 2 × the nuclear area is a good estimate for monolayer cells. • After initial segmentation use ***Results Review*** plot to refine.
Collar radius		✓		Distance (µm) to be dilated from the edges of the nuclear mask	• Use to generate an artificial cell boundary when there is not a sufficient cell marker.
Connected component segmentation			✓	Advanced parameter for neurite segmantation, which triggers the use of an algorithm to segment broken neurites	• Turn on by default and turned off only when changing senstivity and width parameters dont improve segmentation.
Diameter	✓			Typical target diameter (µm)	• Use size tools to establish appropriate value. • After initial segmentation, use ***Results Review*** plot to refine.
Enhance image			✓	Advanced parameter for neurite target to improve image contrast prior to segmentation	• Turned off by default and should be turned on only when changing sensitivity and width parameters don't improve segmentation.
Intensity filter	✓	✓	✓	A post segmentation step to remove bright or dim objects from final data	• Use to exclude objects with a mean intensity outside the selected range.
Min. length			✓	Approximate length (μm^2) of the shortest fiber to be segmented	• Larger values will detect longer continuous objects; smaller values will detect small fragments or break up incorrectly joint objects. • This value is not a filter so the algorithm may still identify objects smaller than the specified length.
Minimum target area	✓			Approximate area (μm^2) of smallest nucleus (Used for optimization of other algorithm functions. This does not mean that smaller nuclei cannot be segmented)	• Use size tools to establish appropriate value. • Default value is a good starting point. • After initial segmentation, use ***Results Review*** plot to refine.
Minimum/maximum target area or target diameter			✓	Approximate area (μm^2) or diameter (µm) of the smallest and largest organelles to be segmented	• These values adjust the steps of the algorithm to favor objects of a specified size.
Minimum/maximum width			✓	Approximate thickness (µm) of the smallest and largest fibers and neurites to be segmented	• To segment individual fibers, use very small min value since actin filament width is typically close to the resolution limit. • Increase Min/Max width for lower magnification images when segmenting individual fibers/neurites isn't possible.
Noise removal	✓	✓	✓	Noise removal filter to improve segmentation results for samples with few cells and/or low contrast	• Use if background is high, illumination is uneven, or if there is debris in the field-of-view (FOV).

Parameter	Nuclei	Cells	Organelles	Description	Tips
Ratio		✓		Controls the size of soma required for downstream neurite segmentation	• Similar values result in larger soma. • Decrease sensitivity first if soma is over-segmented. If soma mask still expands beyond cell borders then increase ratio value.
Scales			✓	Number of distinct sizes of objects to be identified	• Increasing the number of scales allows for detection of a larger range of organelle sizes. • When scales equal: **1:** Only the minimum area is considered **2:** Both minimum and maximum values are used **3 or more:** Values between minimum and maximum are used
Sensitivity	✓	✓	✓	Determines whether pixels are assigned to objects or background based on intensity relative to local background	• Lower contrast images typically require higher sensitivity. • If object borders are not well defined, adjust sensitivity. • If too many small false positives are found in background, use the area filter to remove.
Sensitivity threshold			✓	Noise suppression filter	• Increase if too many organelles are identified with *Sensitivity* slider at lowest value. • Decrease if too few organelles are identified with *Sensitivity* slider at maximum value. • Increase or decrease by increments of 0.25.
Thickness (membrane)			✓	Distance (µm) to erode cell boundary to create membrane compartment	• After membrane is defined, it can be used as a compartment for subsequent organelles.
Thickness (network)			✓	Thickness (µm) of individual structures of the network	• Default value is a good starting point. • Increase to identify thicker structures, decrease to identify thinner structures.
Tubeness			✓	Advanced parameter for neurite target to obtain continuous segmentation mask	• Default value is a good starting point and should be changed only when changing sensitivity and width parameters do not improve segmentation. • Higher values result in smoother segmentation masks.

Contact Us

Phone: +1.800.635.5577
Web: www.moleculardevices.com
Email: info@moldev.com
Check our website for a current listing of worldwide distributors.

Regional Offices

USA and Canada	+1.800.635.5577	Taiwan/Hong Kong	+886.2.2656.7585
United Kingdom	+44.118.944.8000	Japan	+81.3.6362.9109
Europe*	00800.665.32860	South Korea	+82.2.3471.9531
China	+86.4008203586		

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