# IN Carta Image Analysis Software

# Protocol design (Mono-nucleated Cells application)

Set up 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 measurements menu" 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* button (13). Select *Best Focus Z* to allow IN Carta™ software automatically determine the slice to be analyzed via image contrast anaysis, for a selected channel. Click *Controls* button (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 point is selected by default, and another time-point selected for analysis in specimen navigation panel. Switch to *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.

Optimize 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* ❶ 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 any or all of the strategies below:

   • Use the *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* 🔨 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.
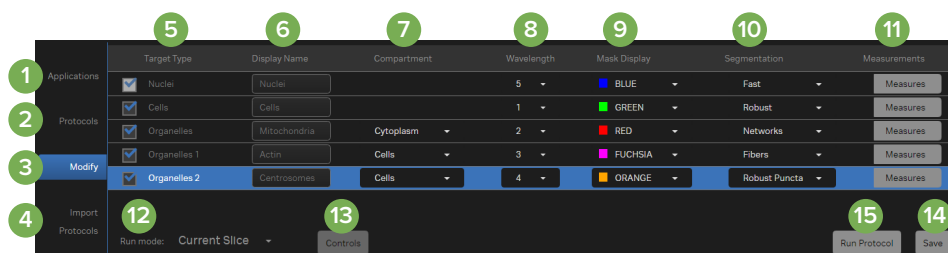
4. Update **Analysis Settings** as necessary and click **Apply**. Repeat steps 2 and 3 until segmentation results are satisfactory.

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 measurements menu

- **Measures** are divided into **Count**, **Spatial**, **Intensity**, **Position**, **Coordination**, **Texture** and **Colocalization** categories.

- Each category has some default measurements selected and other available measure 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.

Run 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 panel 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	Explanation	Tips
Area filter	✓	✓	✓	A post segmentation step to remove large or small objects from final data	• Use to remove objects with an area outside the range selected (i.e., 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 while 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	Explanation	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 don't improve segmentation • Higher values result in smoother segmentation masks