Kinetic Image Cytometry®

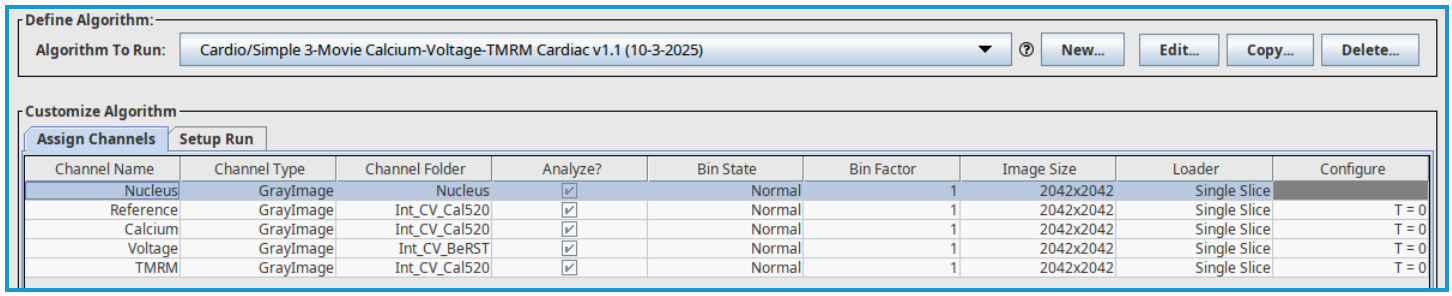
Simple 3-Movie Calcium-Voltage-TMRM Algorithm

This algorithm identifies cardiomyocyte nuclei in each well from a single nucleus image. It then approximates cell boundaries by expanding out from each nucleus by a user-defined radius. For each cell, the algorithm measures the average pixel intensity for each frame in up to three time series channels (calcium, voltage, and/or TMRM). The algorithm can also measure single-cell contractile motion based on the displacement of cellular features in the same channels. This document describes the settings needed to run this algorithm and the measurements it performs.

Assign Channels Tab

Use this tab to specify the Channel Folders from the KIC® scan the algorithm will use to identify cardiomyocyte nuclei (Nucleus Channel) and perform intensity and/or contraction measurements (Calcium, Voltage, and/or TMRM Channels). If possible, the Reference Channel should be set to either the calcium or the voltage Channel Folder. The algorithm will use this channel to identify a reference time series frame from which to measure contractile motion.

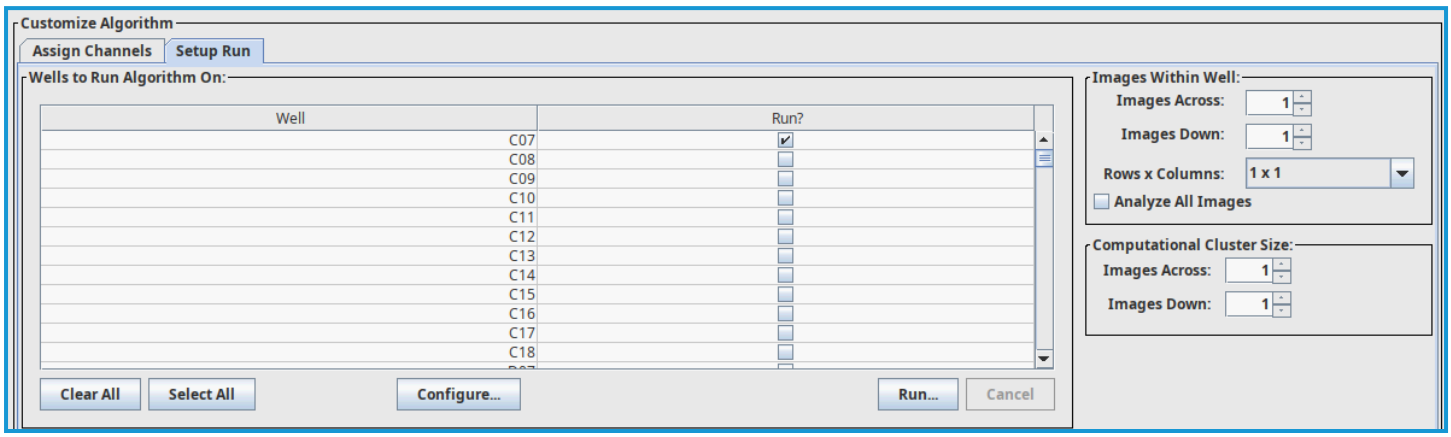
Only channels from which intensity and/or contraction measurements are desired need to be assigned to an appropriate Channel Folder. If less than three channels were scanned or need to be analyzed, the Channel Folder assignment, algorithm settings, and measurements for any remaining channels can be ignored. The settings in the remaining columns of the Assign Channels tab can be left at their default values.



Setup Run Tab

Use this tab to view and edit the algorithm user inputs and to begin analysis. The checked boxes in the “Run?” column indicate which of the scanned wells the algorithm will analyze. Click the “Configure…” button to open a new window in which to set the user inputs and to evaluate the Nucleus and Cell masks. See the following page for information about this window. Click the “Run…” button to confirm user inputs and to begin analysis.

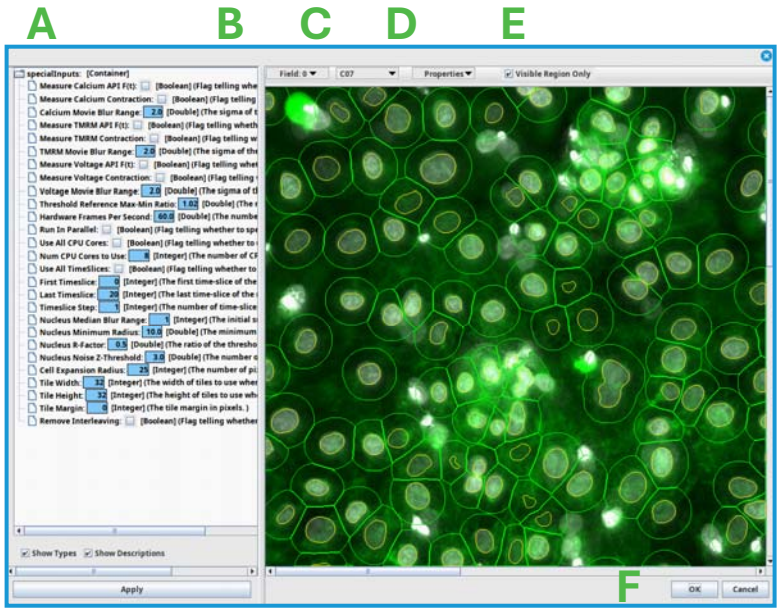
If the scan folder has a single field of view per well, which is typical for KIC® scans, leave the settings in the “Images Within Well” and “Computational Cluster Size” boxes at the default values, as shown in the example below. Otherwise, use the “Images Within Well” box to select how many rows and/or columns of images to analyze. Use the “Computational Cluster Size” box to indicate whether the algorithm should analyze each field independently (1x1 cluster size) or analyze two or more images together.



Configure Window

This window displays editable user inputs along with the first acquired time series image overlaid with the corresponding nucleus image. After the Configure window opens, CyteSeer® will begin to analyze the well, as indicated by the "Running Algorithm…" message under the image. When the analysis is complete, this message will disappear, and the window will display the resulting Nucleus and Cell LabeledMasks. The Configure window can be used to assess the masks in multiple wells and to modify the user inputs as necessary. See the User Input Glossary below for descriptions of each input.

Below is an example of a Configure window for iPSC-cardiomyocyte nuclei (gray) and calcium dye (green). The Nucleus LabeledMask is outlined in yellow, and the Cell LabeledMask is outlined in green. Important features of the Configure window are indicated with green letters.



A This panel shows all user inputs that can be changed to modify how CyteSeer® runs the algorithm. Only the inputs near the bottom of the list with names beginning with "Nucleus" or "Cell" affect the Nucleus or Cell LabeledMasks. To test how the inputs affect the masks, change one or more inputs and click "Apply". CyteSeer® will then rerun the algorithm using the new inputs and change the masks accordingly. To speed up Configure window mask creation, uncheck all Boolean boxes to skip time series analysis.

B If the scan folder contains more than one field of view per well, use this drop-down menu to select another field in which to test the masks.

C Use this drop-down menu to select other wells in the scan folder in which to test the masks.

D Use this drop-down menu to hide or display individual channels and masks, to change image intensities, and to change image and mask colors.

E By default, the Configure window will only generate masks for the section of the image visible within the window. Uncheck the "Visible Region Only" box to tell CyteSeer® to generate masks for the full image the next time you click Apply. Generating masks for the full image will take longer but may be more accurate if background intensity and noise vary across the Nucleus image.

F After identifying acceptable user inputs, click "OK" at the bottom of the window to apply the changes. Otherwise, click "Cancel" to revert to the previous settings.

To start full analysis, return to the Setup Run tab and click the "Run…" button to open a separate window from which you can view and edit the user inputs. Check that all settings are input correctly, including the Boolean check boxes indicating which channels to analyze and how many time slices to use. Click "OK" to tell CyteSeer® to begin analysis of the specified wells. Otherwise, click "Cancel" to cancel analysis and close the window.

User Input Glossary

Input	Description
Measure Calcium/Voltage/TMRM API F(t)	If checked, the algorithm will measure the average pixel intensity (API) over the Cell LabeledMask for each movie frame of the selected channel. The algorithm will also measure the API of the full image for each movie frame. Both time series measurements will be output as functions of time.
Measure Calcium/Voltage/TMRM Contraction	If checked, the algorithm will measure the cellular motion of each cell as detected from the selected movie channel. The contractile motion is output as a function of time called Stretch F(t). The algorithm will also measure the stretch averaged across the full images. Both time series measurements will be output as functions of time.
Calcium/Voltage/TMRM Movie Blur Range	Used to decrease imaging noise in the time series images before detecting motion. If this value is too low, not enough noise will be removed, and the Stretch function(s) may include small fluctuations that do not correspond to cardiomyocyte contraction. If this value is too high, the cellular features in the images will be over-blurred, making it difficult to detect cellular motion. ***Recommended value: 2.0***

Table continued on the following page.

Input	Description
Threshold Reference Max-Min Ratio	The minimum required max-min intensity ratio required for a peak in the Reference API F(t) function to be classified as a full transient. The algorithm will set the reference frame for contraction analysis at a local minimum before a full transient. This ratio may need to be decreased if the reference movie transients have low amplitudes. It may need to be increased if the Reference F(t) function contains noise that exceeds the default ratio. If the algorithm does not identify any full transients, the first movie frame will be used as the reference frame. ***Recommended value: 1.02***
Hardware Frames Per Second	The number of frames per second acquired by the camera. This value specifies the scale on the x-axis for each of the functions of time. For interleaved movies, this value should reflect the total number of frames per second acquired across all channels.
Run In Parallel	If checked, CyteSeer will speed up calculations by analyzing different movie frames within each well in parallel. To control parallel analysis of different wells, go to the top menu in CyteSeer and select View > Preferences. Use the “Analysis # of Threads” box to specify how many wells to run in parallel.
Use All CPU Cores	If Run in Parallel is checked, tells CyteSeer whether to use all available CPU cores for parallelized analysis of movie frames in each well. Do not check this box if the number of threads for well analysis in the Preferences menu is greater than 1.
Num CPU Cores to Use	If Run In Parallel is checked but Use All CPU Cores is unchecked, this value tells CyteSeer how many of the available cores to use. This number should not be greater than the number of available CPU cores divided by the number of threads for well analysis specified in Preferences. Depending on the hard drives and memory available, reducing the number of CPUs may decrease analysis time by preventing memory overload. To optimize analysis speed, test single-well analysis times using different numbers of CPU cores.
Use All Time Slices	If checked, CyteSeer will use all the acquired frames across all movie channels for analysis and will ignore the values for First Timeslice, Last Timeslice, and Timeslice Step.
First Timeslice	If Use All Timeslices is unchecked, this value specifies the first time series frame to use for analysis. Frames are indexed starting at 0. For a movie with N frames, this value can be set to any number between 0 (first image) and N-1 (last image).
Last Timeslice	If Use All Timeslices is unchecked, this value specifies the last time series frame to use for analysis. Frames are indexed starting at 0. For example, if the movie has 300 frames, the final frame will be frame 299.
Timeslice Step	The step between timeslices to use in analyzing each movie. If this value is set to 1, no frames will be skipped.
Nucleus Median Blur Range	The initial smoothing radius in pixels to use to smooth out the nucleus image before detecting nuclei. Smoothing lessens the influence of background and noise in the Nucleus Image on Nucleus LabeledMask. Too much smoothing may blur the image and interfere with the algorithm’s ability to detect nucleus edges. ***Recommended value: 1***
Nucleus Minimum Radius	The minimum acceptable radius in pixels of a live cardiomyocyte nucleus. Objects segmented from the Nucleus Image with a smaller mean diameter will be excluded from the Nucleus LabeledMask. The pixel size varies with the magnification of acquired images: 10x = 0.65 µm, 20x = 0.325 µm, 40x = 0.1625 µm, and 60x = 0.108 µm. ***Recommended value for 20x scans: 10***
Nucleus R-Factor	The R-Factor sets the fractional amount of the local Nucleus Image peak intensity to use as a threshold for segmenting each nucleus to generate the Nucleus LabeledMask. If the R-Factor is set to 0.5, all pixels above 50 percent of each nucleus peak intensity will be included in the mask for that nucleus. This is true whether the nucleus is bright or dim. Larger values will exclude more pixels of low relative intensities in each identified nucleus, resulting in smaller nucleus areas and improving separation of nuclei that are close together. Smaller values will include more pixels of low relative intensities in each identified nucleus, resulting in larger nucleus areas and preventing separation of individual nuclei into multiple objects. ***Recommended value: 0.5***
Nucleus Noise Z-Threshold	The number of multiples of the noise to subtract from the Nucleus Image when doing noise suppression before segmenting nuclei. Larger values will exclude more noise but may also exclude real signal. Smaller values will include more real signal but may also include more noise. ***Recommended value: 3.0***
Cell Expansion Radius	The number of pixels to expand out from the boundary of each nucleus to define the Cell LabeledMask. Expansion will stop at either the Cell Expansion Radius or where boundaries of neighboring cells collide so that cell masks will not overlap. ***Recommended value for 20x scans: 25***

Table continued on the following page.

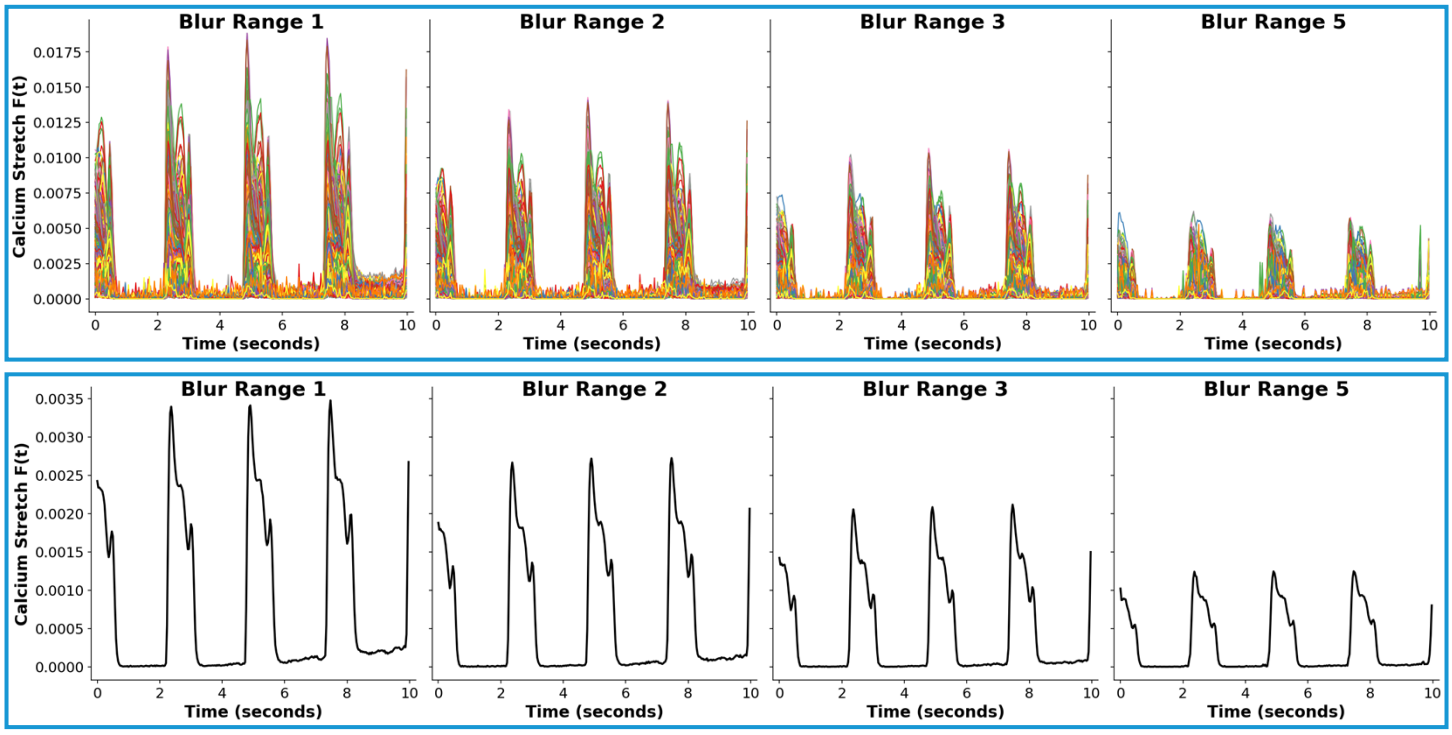
Input	Description
Tile Width/Height	Dimensions in pixels of tiles to use when computing the local distortion field on each tile of an image. The tile width and height should not be significantly larger than a typical cell diameter. Decreasing the tile dimensions improves the resolution of motion detection, but it may decrease the signal to noise ratio. Increasing the tile dimensions may improve the signal to noise ratio of motion detection, but it worsens the resolution. The optimal tile dimensions for motion detection can vary based on the cell type, the magnitude of contractile motion, the imaging parameters, and the dye used to detect motion. ***Recommended value for 20x scans: 32***
Tile Margin	The number of pixels to overlap each tile with its neighbors during motion detection. For example, a Tile Margin of 8 would add 8 pixels to both ends of the Tile Width and Height for a total increase of 16 pixels to both the Width and Height. The contractile motion detected across the larger tile will be attributed to the smaller tile with the original width and height. Increasing the Tile Margin increases the signal-to-noise ratio of contractile motion without a large decrease in resolution. However, increasing the Tile Margin will significantly increase analysis time. ***Recommended value: 0***
Remove Interleaving	If checked, the algorithm will remove interpolated points in each interleaved function before saving. This will significantly reduce the file size of the resulting DataTables. ***Recommended value: True***

User Input Examples

This section describes how key user inputs affect the Stretch F(t) functions and the Nucleus and Cell LabeledMasks.

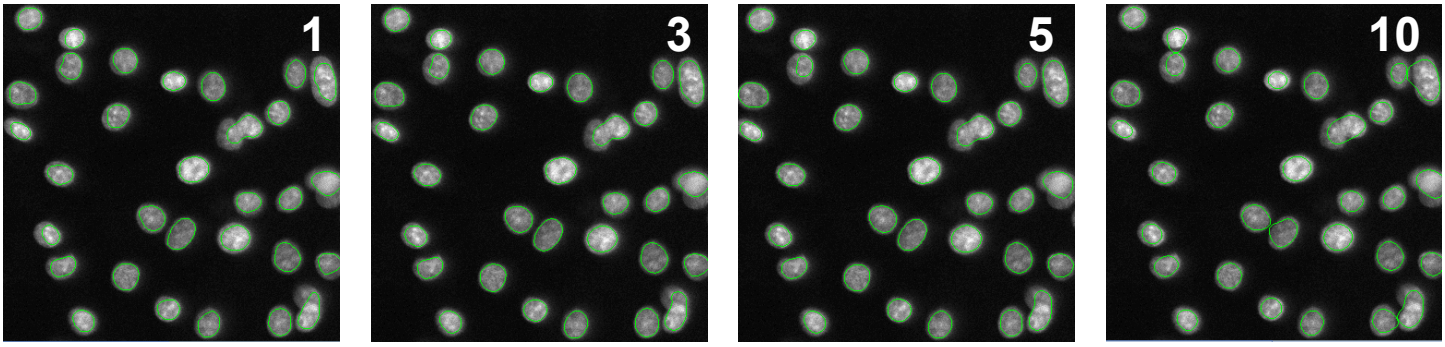
Movie Blur Range

The input value for the Calcium, Voltage, or TMRM Movie Blur Range affects the noise levels in the resulting Stretch functions. The plots below show Calcium Stretch F(t) functions from one well analyzed with the indicated values for the Calcium Movie Blur Range. The top plot shows single-cell functions, and the bottom plot shows the well averages. When the blur range is 1, a significant amount of noise affects the function baseline, especially near the end of the recording period. Increasing the blur range removes more noise and produces a smoother, more level baseline. Although the larger blur ranges 2 and 3 decrease stretch transient amplitudes, they do not significantly affect the transients' other kinetic features. Smaller amplitudes will not affect the ability to detect changes in contractile motion from the stretch functions. When the blur range is 5, smaller features of the stretch transients begin to be smoothed out, when may interfere with detection of differences in contractile motion.



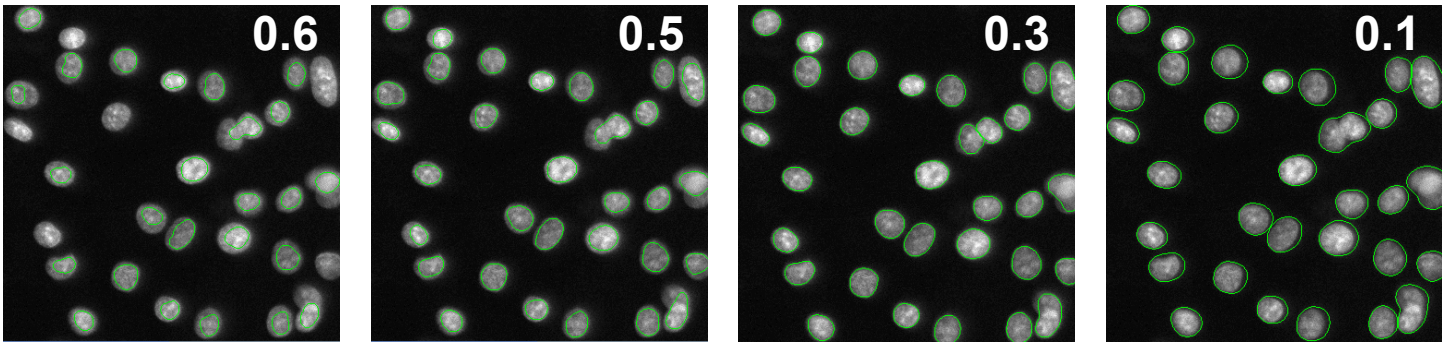
Nucleus Median Blur Range

Increasing the range to 3 or 5 can increase the algorithm's ability to detect the edges of most nuclei. The large amount of blurring with range 10 can decrease the accuracy of edge detection.



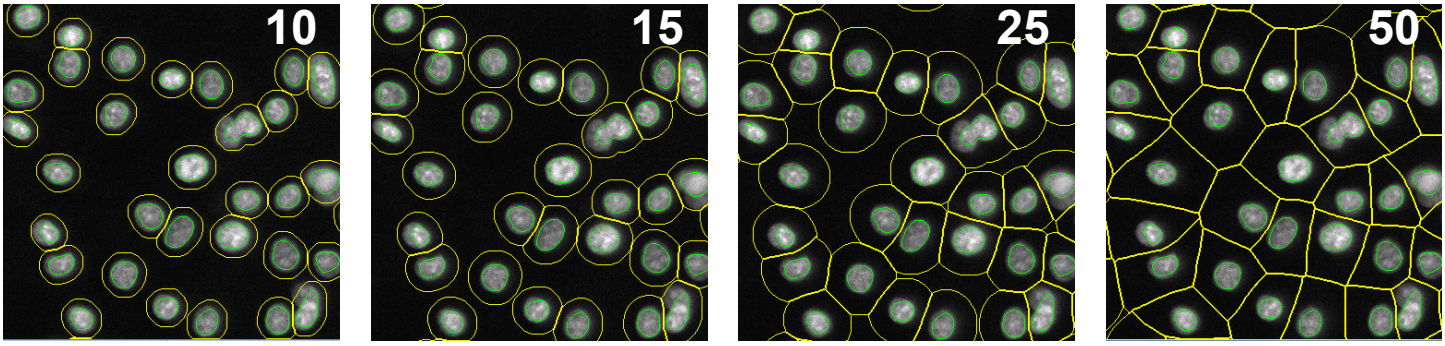
Nucleus R-Factor

Changing the R-Factor can change the range of nuclear stain intensities incorporated into the nucleus mask. An R-Factor of 0.6 is too large for the nuclei in this example, and several of the nuclei are not detected at all. With R-Factors of 0.5 and 0.3, most of the nuclei are detected, and 0.3 has slightly improved edge detection. An R-Factor of 0.1 is too low; areas around the edges are included and some masks include more than 1 nucleus.



Cell Expansion Radius

For 20x images, an expansion radius of 10 will include a small area around each nucleus in the Cell LabeledMask. Increasing the radius to 15 or 25 will include more of the cytoplasm from each cell and therefore capture more of each cell's voltage, calcium, and/or TMRM signal and the contractile motion detected from those channels. Increasing the radius to 50 or more may include parts of the cytoplasm of multiple cells and areas outside of cells in a single mask, which may reduce the accuracy of average pixel intensity or contraction measurements.



Primary Data Table

This data table reports cell and nucleus size measurements for each cell identified in each well. It also reports the average pixel intensity (API) and contraction F(t) functions of time for each channel selected in the Configure window.

Measurement	Description
Cell Area	Area of the Cell LabeledMask in square pixels, including the area that is also part of the Nucleus LabeledMask.
Cell Perimeter	Perimeter of the Cell LabeledMask in pixels.
Nucleus Area	Area of the Nucleus LabeledMask in square pixels.
Nucleus Perimeter	Perimeter of the Nucleus LabeledMask in pixels.
Calcium/Voltage/TMRM API F(t)	The average pixel intensity (API) over the Cell LabeledMask for each time series frame of the indicated channel.
Calcium/Voltage/TMRM Dx F(t) and Dy F(t)	The cellular motion in the horizontal x-direction and vertical y-direction over the Cell LabeledMask as detected from the indicated movie channel. An F(t) value of 0 represents the cell's x/y-position in the reference frame. Positive F(t) values indicate cellular displacement towards the right or top side of the field of view, while negative values indicate displacement towards the left or bottom side. The slope of this function indicates the speed of displacement in either direction.
Calcium/Voltage/TMRM Stretch F(t)	The Stretch function is computed from Dx F(t) and Dy F(t). Stretch measures the average displacement over the whole cell from its position in the reference frame. Stretch is expressed as a fraction, meaning that if the Stretch for a cell is 0.01 at a certain time point, it has displayed an average of 1% deformation from the reference frame. Because Stretch is a whole-cell average, parts of the cell may have moved further than 1% while others may have remained stationary. Stretch is a scala value and not a vector, and it does not reflect the direction of contractile motion.

Reference Measurements Data Table

Measurement	Description
Calibration Reference Frame	The index of a movie frame in which the cardiomyocytes are at rest, with no voltage, calcium, or contractile activity. The algorithm will set the reference frame for contraction analysis at a local minimum before a full transient in the Reference API F(t) function. If the algorithm fails to identify a full transient, the reference frame will be set to the first movie frame.
Reference API F(t)	The average pixel intensity over the full image for the scan folder assigned to the Reference Channel for each time series frame. This function is used to identify the Calibration Reference frame, from which the algorithm will measure contractile motion.

Whole-Image Measurements Data Table

Measurement	Description
Nucleus Noise Level	The amount of imaging noise in the Nucleus Image for each well. Large or small outliers for this value may indicate wells with imaging problems and/or significant cell death.
Whole-Image Calcium/Voltage/TMRM API F(t)	The average pixel intensity (API) over the full image for each movie frame of the indicated channel. Wells with synchronous cardiomyocyte activity will have large, smooth transients in the Whole-Image Calcium and Voltage F(t) functions.
Whole-Image Calcium/Voltage/TMRM Stretch F(t)	The cellular displacement over time from the Calibration Reference Frame averaged over the full image as opposed to single cells.