

Guidelines for the IncuCyte® Cell-by-Cell Analysis Software Module

Our novel, patent-pending image analysis algorithm enables identification of individual cells by segmenting IncuCyte® HD phase-contrast images. This permits (1) label-free true cell counting (2) extraction of basic morphological features of individual cells (e.g. size, shape), and (3) measurement of fluorescence intensity that originates from within each cell (fluorescence within a phase boundary). IncuCyte live-cell analysis fluorescence reagents can be used including apoptosis probes (Caspase 3/7, Annexin V), live/dead markers (e.g. IncuCyte Cytotox Green) and GFP/RFP labels. Identification of cell subsets in heterogeneous cultures may be achieved by using antibodies conjugated to the IncuCyte FabFluor-488 reagent to monitor surface protein expression.

The purpose-built IncuCyte software tools allow visualization of cell populations in either density plots or histograms, and classification into subsets using simple gates. Importantly, changes in the population and different subsets over time can be explored via the interface and linked back to the raw images with masks that are color-coded to reflect classes. Together, the tool set enables researchers to easily observe and analyze subsets of living cells over time based on morphological, surface marker, cell health and/or functional properties.

This guideline covers the following topics for defining Cell-by-Cell analysis parameters:

- [Acquiring images using the IncuCyte® S3](#)
- [Segmentation of phase objects using the IncuCyte® Cell-by-Cell Analysis Software Module](#)
- [Classification of cell populations based on phase and/or fluorescent parameters](#)

The following procedures are for example purposes only and are designed to provide a frame of reference for defining the Cell-by-Cell Analysis Parameters within the Analysis Guided Interface.

For new IncuCyte users, it is recommended to review Sections 1, 2 and 3 of the IncuCyte S3 User Manual or have experience scheduling and acquiring scans, viewing images, and performing image analysis and visualizing results prior to reviewing this technical note.

Acquiring images for subsequent analysis using IncuCyte® Cell-by-Cell Analysis Software Module


This module enables analysis of HD phase, and up to two fluorescence channel images acquired using Standard acquisition mode. Analysis is designed for cells growing in 96-well plates and flasks. Cell-by-Cell Analysis utilizes different acquisition and analysis parameters depending on whether your cell types are adherent or non-adherent. For adherent cell types, Cell-by-Cell analysis can be performed only on images that are captured at 10x, and only on images captured with version 2019A or newer. For non-adherent cell types, Cell-by-Cell Analysis can be performed only on images captured at 20x, and that are captured with 2019A or any previous software version. Please see the IncuCyte S3 User Manual Section 1 for a detailed description of how to log in to the IncuCyte and launch the Acquisition Window. Follow instructions for scheduling a scan as described in the User Manual until the “Scan Type” Window is displayed.

1. In the Scan Type Window, select Standard.
2. Select from the Standard Scan Type radio buttons dependent on the cell type you wish to analyze.
 - a. For data not requiring Cell-by-Cell analyses select None.
 - b. For all Adherent Cell-by-Cell analyses, predefined acquisition is at 10x.
 - c. For all Non-adherent Cell-by-Cell analyses, predefined acquisition is at 20x.
3. In the Scan Settings Window, Phase Image Channel will be selected as default. Additionally, select Green and/or Red depending on your chosen reagents.
4. Proceed through the remaining windows referencing the IncuCyte S3 User Manual as needed.

Non-adherent cell analysis: segmenting phase images using IncuCyte® Cell-by-Cell Analysis Software Module

The following section will guide you through creating a new analysis definition to accurately mask non-adherent phase objects, in order to produce kinetic data tracking of label-free individual objects using the Cell-by-Cell Software Analysis Module. Creation of a Cell-by-Cell phase mask will also automatically generate metrics on fluorescence within this boundary (if fluorescence images were acquired). Using this method, separate fluorescence masks are not required.

For adherent cell analysis see page 7-10.

1. From an open Vessel View, launch the Analysis Wizard by clicking on the icon  (described in the S3 User’s Manual on page 90 in Section 3, Chapter 1.)
2. Click Next until the Analysis Type window is presented. Select Cell-by-Cell to continue. Phase image channel is automatically selected, also select fluorescence channels if you wish these to be included in the analysis.

3. Perform Image Set Selection, using your mouse hover over images you wish to select and click the site. See below for further information on Image Set Selection or refer to the S3 User's Manual. Click Next to present you with the Analysis Definition Window.
4. If desired, enter the name of the object(s) into the Object name field that are being analyzed (Step 1). [See Figure 1](#)



For easier identification of the analysis definition, you might want to name the object the same as the cell type that was used in the assay, for example, Jurkat.

5. Object diameter is set here, this should be the average diameter of objects you are looking to mask (Step 2). To measure cell diameter, you can use the measurement tool on the right-hand panel.
6. Click Preview Current or All (Step 3). [See Figure 1](#)



The best way to begin setting up the Analysis Definition is to use the preset Segmentation Sensitivity and Cleanup values already contained within the Analysis Definition Editor.

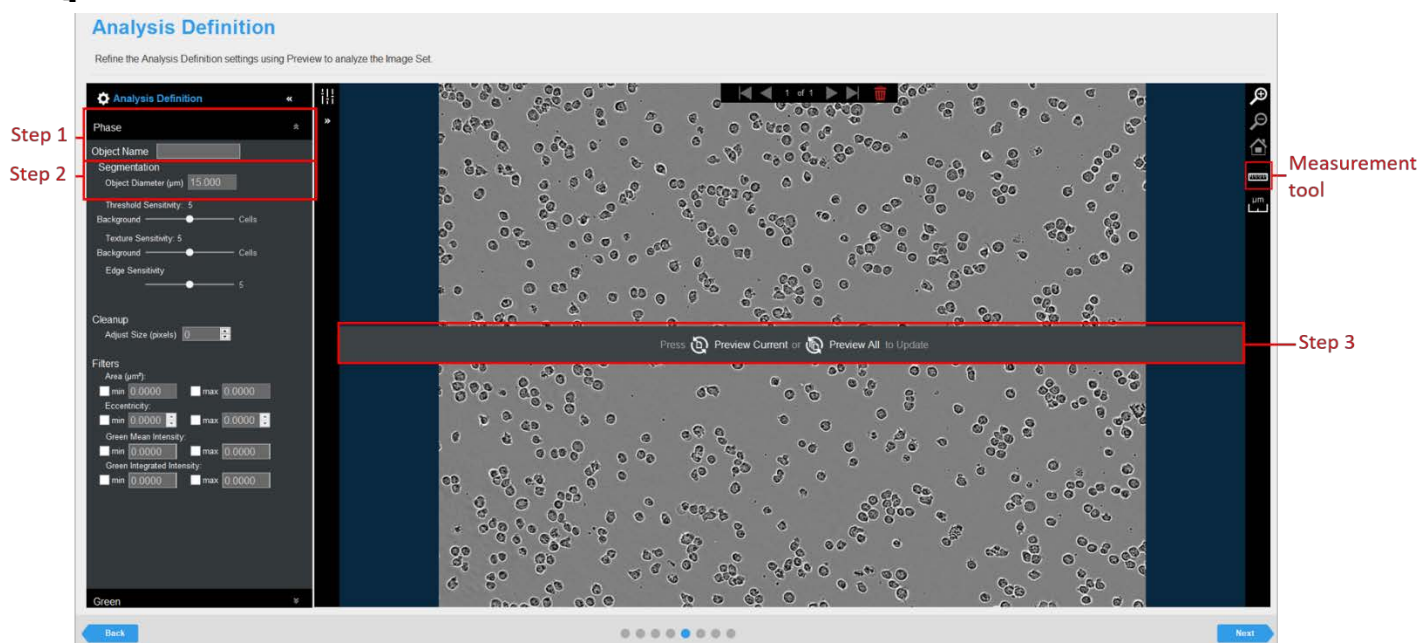
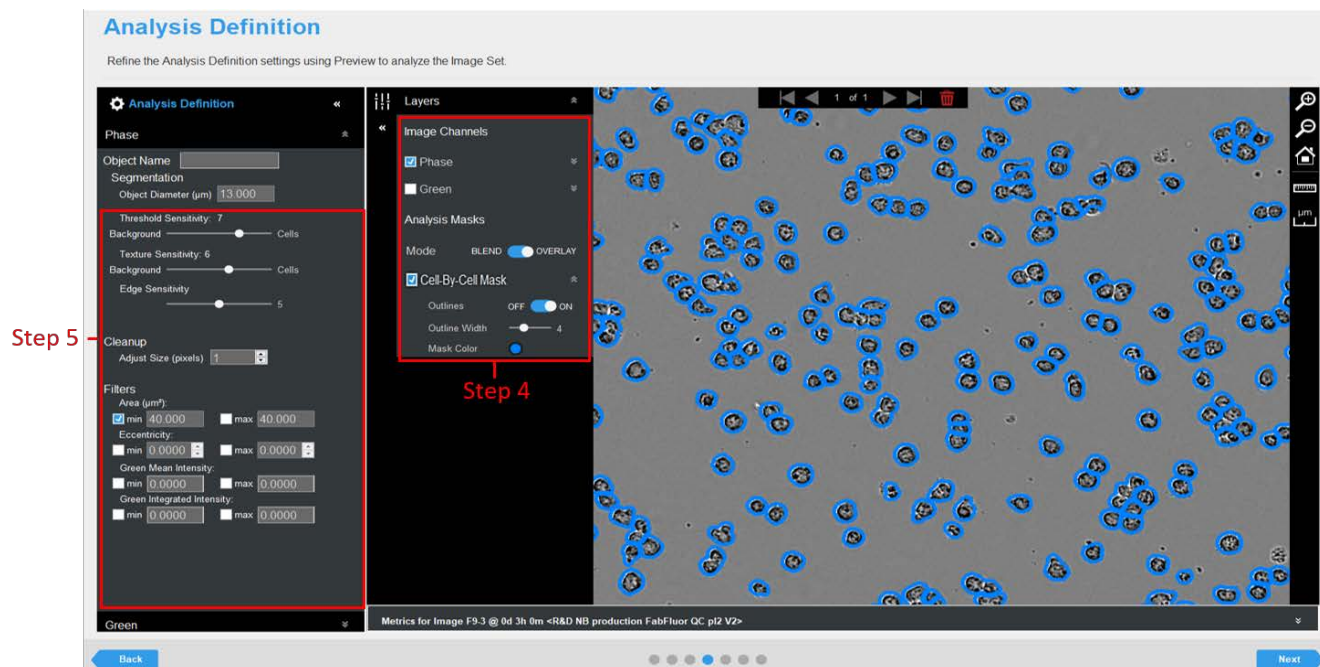


Figure 1. Cell-by-Cell Analyzer Image Preview

7. Evaluate the Cell-by-Cell Mask and refine the parameters accordingly. [See Figure 2 and Table 1](#).
 1. Assess the Mask using the Blend or Overlay Mode (Step 4). Mask Outline, has a slider to adjust the Outline Width, and Color selection options aid in evaluating the Analysis Mask. Changing these parameters will affect the visualization only and not affect the analysis definition.
 2. Modify only a single analysis definition parameter at a time (Step 5). After you define the value for a parameter, click Preview Current to apply and view the change for the

image that is currently displayed in the Image pane.



3. Once you are happy with the parameters set, click Preview All to ensure these are appropriate for other time points or treatments selected.

Figure 2. Parameter Refinement with Cell-by-Cell Mask

Table 1: Phase Image Channel Analysis Definition Options

| Option | Description |
|--|---|
| Parameters | |
| Object diameter (µm) | Average diameter of objects you aim to mask. You can use the measurement tool located in the right panel. |
| Threshold sensitivity Background/Cells | <p>Use the slider bar to adjust the mask.</p> <ul style="list-style-type: none"> For high contrast non-adherent cells the slider can usually be left in the middle, for lower contrast non-adherent cells the slider can be moved to the right (towards cell) for more optimal masking. <p>Adjust the slider bar as needed to accurately mask the cells.</p> |
| Texture sensitivity Background/cells | <p>Use the slider bar to adjust the mask.</p> <ul style="list-style-type: none"> Moving the slider further to the right (towards cells) will result in higher texture objects being masked. Sliding the bar further to the left (towards background) will result in less textured objects being masked. <p>Adjust the slider bar as needed to accurately mask the cells.</p> |
| Edge sensitivity | <p>Use the slider bar to adjust the mask.</p> <ul style="list-style-type: none"> Moving the slider further to the right will result in objects being split into multiple smaller objects. Moving the slider further to the left will result in the objects being joined together. <p>Adjust the slider bar as needed to accurately segment the cells.</p> |
| Cleanup | |
| Adjust Size | <p>If set to a positive value, this will enlarge the mask by the specified number of pixels. If set to a negative value, the mask will shrink by the specified number of pixels.</p> <ul style="list-style-type: none"> Depending on the reagents used, the fluorescence can fall at the limits of the phase boundary (e.g. FabFluor-488) therefore expanding the mask ensures the signal is captured within the mask. |
| Filters - Used to remove any masked objects that are not cells (e.g. cell debris) | |
| Area | Defines a range of sizes (in µm ²) for the object and eliminates objects that fall outside this range, e.g., in order to exclude cell debris. Once a Cell-by-Cell Mask has been set use the tip tool to identify an approximate area for debris. |
| Eccentricity | Defines a range of roundness for the object and eliminates objects that fall outside this range. Eccentricity ranges from 0 to 1 with a perfect circle having a value of 0. |
| Green/Red Mean Intensity | Defines a range of green/red mean intensity to further refine the Cell-by-Cell Mask boundary if the green/red channel is enabled. |
| Green/Red Integrated Intensity | Defines a range of green/red integrated intensity to further refine the Cell-by-Cell Mask boundary if the green/red channel is enabled. |
| Green/Red – No fluorescent mask is required | |
| Top-Hat – Radius (µm ²) | <p>This allows background fluorescence to be subtracted from object properties.</p> <ul style="list-style-type: none"> The radius size should be larger than any object or cluster of objects. This can be measured using the measurement tool on the right panel. |

8. It is not necessary to create a mask for the Green or Red channels, since the fluorescence signal within the phase boundary is automatically analyzed. The default setting of Top-Hat No Mask for Green or Red channels will enable background subtraction without generation of a mask. Ensure that the Top-Hat radius is set to a value higher than the radius of the larger clusters to avoid excess background subtraction (Step 6). See [Figure 3](#)

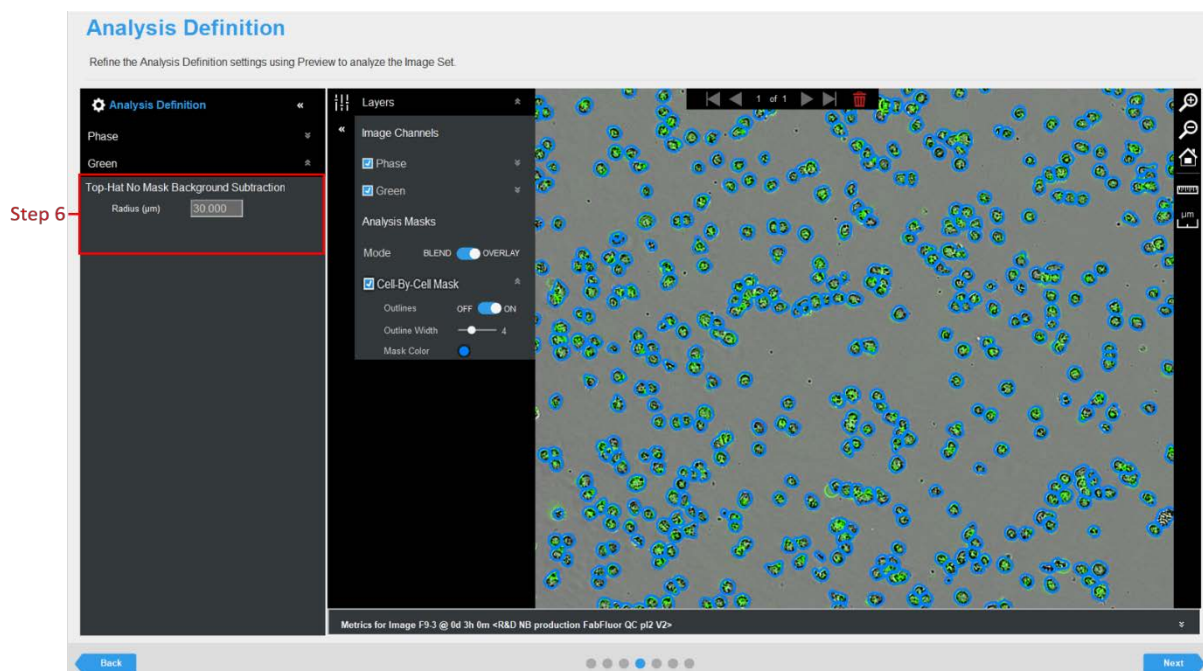



Figure 3. Fluorescence Parameter Refinement within the Cell-by-Cell Mask

9. Once you have previewed all of the images within the wizard image set and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and wells to be analyzed, as well as assigning an analysis definition name. Note that if your experiment is in progress you will have an option to check “Analyze Future Scans” to perform real-time analysis (please see IncuCyte User Manual Section 3 Chapter 1 for more information on Defining Image Analysis).

Adherent cell analysis: segmenting phase images using the IncuCyte® Cell-by-Cell Analysis Software Module

The following section will guide you through creating a new analysis definition to accurately mask adherent phase objects, in order to produce kinetic data for label-free individual objects using the Cell-by-Cell Analysis Software Module. This analysis will generate three masks; the Cell Boundary Mask defines the boundary of the cells, the Seed Mask identifies each individual cell, and then each “seed” will “grow” out to the boundary and generate the third mask which is called the Cell-by-Cell Mask. The Cell-by-Cell mask will be used for generating metrics such as cell counts, and, if desired, classification into subsets. Creation of a Cell-by-Cell Mask will also automatically generate metrics on green or red fluorescence within this boundary (if fluorescence images were acquired). Using this method, separate fluorescence masks are not required.

For non- adherent cell analysis see page 2-6.

1. From an open Vessel View, launch the Analysis Wizard by clicking on the icon  (described in the S3 User’s Manual on page 90 in Section 3, Chapter 1.)
2. Click Next until the Analysis Type window is presented. Select Adherent Cell-by-Cell to continue. Phase image channel is automatically selected, also select fluorescence channels if you wish these to be included in the analysis.
3. Perform Image Set Selection. Using your mouse hover over images you wish to select and click the site. For further information on Image Set Selection refer to the S3 User’s Manual. Click Next to present you with the Analysis Definition Window. Selecting an appropriate training set is vital in creating a good Cell-by-Cell Mask.
 1. Select images with 50% confluence if cells are medium/high contrast (e.g. A549/HT1080).
 2. If cells are low contrast (e.g. SKOV-3) select ~4 images that have low confluence (~20%) along with ~2 images at higher confluence.
 3. If debris and plate texture is high in wells (e.g. drug treatment) select ~4 images that have high debris.
4. Click Preview Current or All. [See Figure 4](#)
5. Evaluate your Cell Boundary Mask first (Step 1) and refine the parameters accordingly. [See Figure 4 and Table 2](#). This mask defines the cell boundaries and must be set appropriately.
 1. Assess the Analysis Mask using the Blend or Overlay Mode. Mask Outline, is automatically selected, this has a slider to adjust the Outline Width, and Color selection options aid in evaluating the Analysis Mask. Changing these parameters will affect the visualization only and not affect the analysis definition.
 2. Click Preview Current or All to assess changes to mask.

Step 1

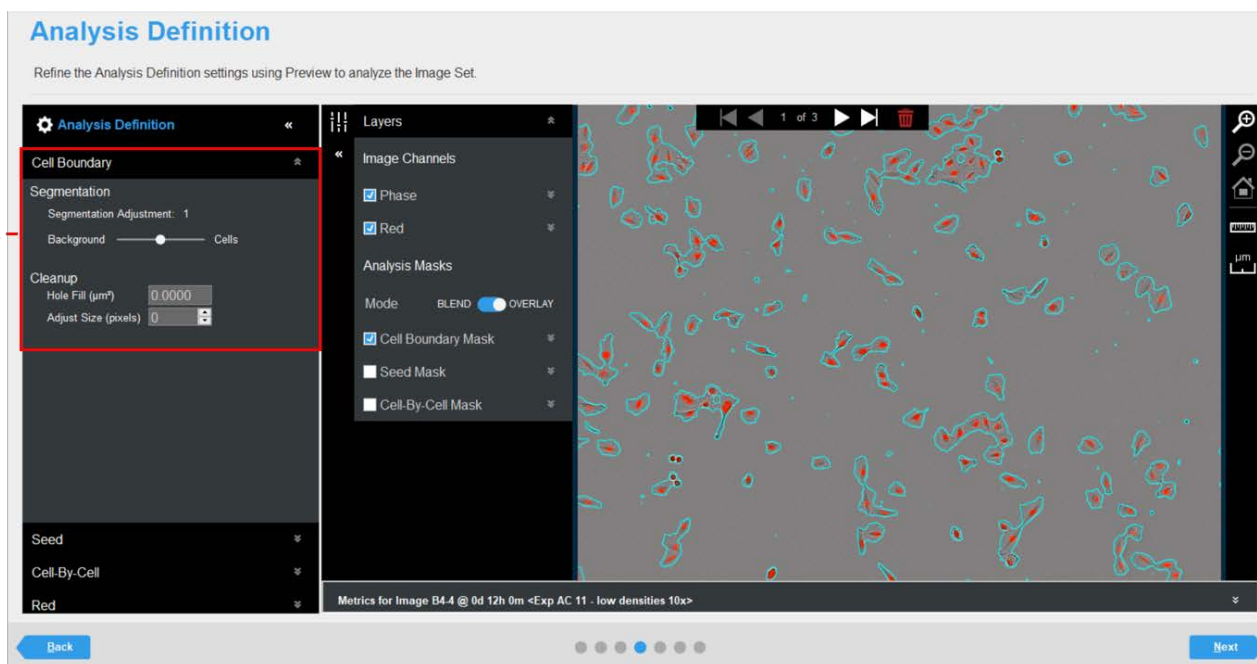


Figure 4. Cell-by-Cell Analyzer Image Preview

6. Next open the Seed drop down menu. Evaluate your Seed and Cell-by-Cell Mask simultaneously (Step 2) and refine parameters accordingly. For accurate Cell-by-Cell masking one seed per cell is required. See Figure 5. Also see Table 2 for further tips about how to select parameter settings.
 1. Adjust the Cell Detection Sensitivity slider (low = small and fewer seeds, high = larger more seeds) as required.
 2. Assess the cells you are analyzing and set the Cell Contrast slider appropriately (1 = low contrast and 3 = high contrast).
 3. Adjust the Cell Morphology slider accordingly.
 4. Click Preview Current or All.



One seed per cell is required for an accurate Cell-by-Cell Mask.

Any objects without seeds will not be included in the Cell-by-Cell Mask, irrespective of whether the object is included in the Cell Boundary Mask.

7. Open the Cell-by-Cell drop down menu.
 1. In the Object Name field, enter the name of the object(s) that are being analyzed (Step 3). See Figure 6.
 2. The filters apply only to the Cell-by-Cell Mask (Step 4). As any objects that do not have a seed will not be included within the Cell-by-Cell Mask it is not necessary to filter out debris captured in the Cell Boundary Mask.



For easier identification of the analysis definition, you might want to name the object the same as the cell type that was used in the assay, for example, A549. This will also change the name of the Cell-by-Cell Mask to the chosen text.

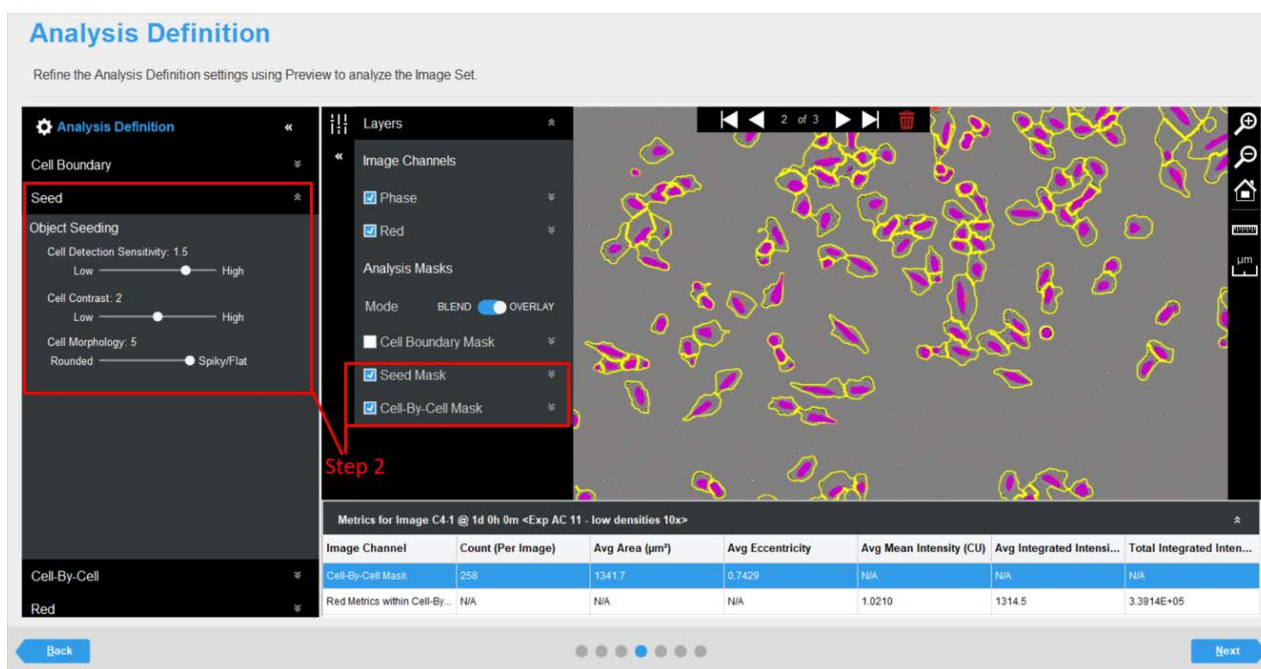


Figure 5. Parameter Refinement for Cell-by-Cell and Seed Mask
Figure 6. Refinement using filters for Cell-by-Cell Mask

8. Once you are happy with the parameters set, click Preview All to ensure these are appropriate for other time points or treatments selected.
9. It is not necessary to create a mask for the Green or Red channels, since the fluorescence signal within the Cell-by-Cell boundary is automatically analyzed. The default setting of Top-Hat No Mask for Green or Red channels will enable background subtraction without generation of a mask. Ensure that the Top-Hat radius is set to a value higher than the radius of the larger clusters to avoid excess background subtraction.
10. Once you have previewed all of the images within the wizard image set and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and wells to be analyzed, as well as assigning an analysis definition name. Note that if your experiment is in progress you will have an option to check “Analyze Future Scans” to perform real-time analysis (please see IncuCyte User Manual Section 3 Chapter 1 for more information on Defining Image Analysis).
11. An existing Cell-by-Cell analysis definition can be re-used on additional plates. If the Copy Existing Analysis Definition feature is selected you will be prompted about which Phase Training Model to use. Click Yes to use the existing phase training model from the selected definition, this will not force any retraining of the mask. Click No to build a new phase training model based on the images selected for the new definition.

Table 2: Image Channel Analysis Definition Options

| Option | Description |
|---|---|
| Cell Boundary/Segmentation | |
| Segmentation adjustment Background/Cells | <p>Use the slider bar to adjust the mask.</p> <ul style="list-style-type: none"> This slider affects the Cell Boundary Mask only. To increase the amount of cells captured within the Cell boundary Mask, move the slider to the right (towards cells.) To reduce the amount of background captured in the Cell Boundary Mask move the slider to the left (towards background). <p>Adjust the slider bar as needed to accurately mask the cells.</p> |
| Cleanup | |
| Hole Fill | <p>This will fill in holes within the Cell Boundary Mask.</p> <ul style="list-style-type: none"> Required in low contrast cells (SKOV-3) to improve boundary masking. |
| Adjust Size | <p>If set to a positive value, this will enlarge the mask by the specified number of pixels. If set to a negative value, the mask will shrink by the specified number of pixels.</p> <ul style="list-style-type: none"> Depending on the reagents used, the fluorescence can fall at the limits of the cell boundary (e.g. FabFluor-488) therefore expanding the mask ensures the signal is captured within the mask. |
| Seed | |
| Cell Detection Sensitivity Background/cells | <p>Use the slider bar to adjust the mask, this affects the Seed Mask and overall segmentation.</p> <ul style="list-style-type: none"> Moving the slider further to the right (towards high) will cause larger and more seeds to be deposited, this can result in more cells being captured within the mask. Moving the slider further to the right (towards low) will cause seeds to shrink and be removed, this results in smaller cells being split appropriately. <p>Adjust the slider bar as needed to accurately mask the cells.</p> |
| Cell contrast | <p>Use the slider bar to adjust the mask.</p> <ul style="list-style-type: none"> 1 – low contrast cells (e.g. SKOV-3) 2 – medium contrast or heterogeneously contrast cells (e.g. A549) 3 – high contrast cells (e.g. HT1080) <p>Adjust the slider bar as needed to accurately segment the cells.</p> |
| Cell Morphology | <p>Use the slider bar to adjust the mask for overall cell morphology.</p> <ul style="list-style-type: none"> For larger, flat or spiky cells with protrusions move the slider to the right. This will cause removal of seeds on high contrast protrusions and will join seeds together. For rounded or smaller cells move the slider to the left. This will cause more seeds on high contrast portions and allow the separation of seeds joined together. <p>Adjust the slider bar as needed to accurately segment the cells.</p> |
| Cell-by-Cell Filters - Used to remove objects from the Cell-by-Cell Mask that are not cells (e.g. cell debris) | |
| Area | Defines a range of sizes (in μm^2) for the object and eliminates objects that fall outside this range, e.g., in order to exclude cell debris. Once a Cell-by-Cell Mask has been set use the tip tool to identify an approximate area for debris. |
| Eccentricity | Defines a range of roundness for the object and eliminates objects that fall outside this range. Eccentricity ranges from 0 to 1 with a perfect circle having a value of 0. |
| Green/Red Mean Intensity | Defines a range of green/red mean intensity to further refine the Cell-by-Cell Mask if the green/red channel is enabled. |
| Green/Red Integrated Intensity | Defines a range of green/red integrated intensity to further refine the Cell-by-Cell Mask if the green/red channel is enabled. |
| Green/Red – No fluorescent mask is required | |
| Top-Hat – Radius (μm^2) | <p>This allows background fluorescence to be subtracted from object properties.</p> <ul style="list-style-type: none"> The radius size should be larger than any object or cluster of objects. This can be measured using the measurement tool on the right panel. |

Visualization of results for both non-adherent and adherent analysis.

Visualization of Analysis Results is described in Section 3, Chapter 2 of the IncuCyte S3 User's Manual.

After the vessel images have been analyzed using Cell-by-Cell Analysis, the analysis definition can be opened. The following set of metrics are automatically provided within the IncuCyte software:

Table 3: Automatic metrics list

| Phase Metric | Description |
|--|---|
| Count (Per image) | The number of objects per image. |
| Avg Area (μm^2) | The average area of the phase objects in the image. |
| Green (or Red) Metrics within Phase Object Boundary | |
| Avg Green (or Red) Mean Intensity (CU) | The Green (or Red) Mean Intensities of the objects defined by the Phase Mask. The mean intensity is calculated as the sum of pixel fluorescence (within the Object Mask) divided by the total area of the object in pixels. |
| Total Green (or Red) Integrated Intensity (CU x $\mu\text{m}^2/\text{image}$) | The total Green (or Red) Integrated Intensity within the boundary defined by the Phase Mask. The integrated intensity is the sum of all green or red fluorescence intensity values within the M multiplied by the pixel area. |

10. It is also possible to add additional metrics and normalized metrics at this stage. By pressing the “+” you can open the metric interface. Here you are able to select the metrics you wish to add to the User Defined Metrics using the drop-down lists to view choices. It is also possible to create new normalized metrics using the metric interface, this can also be completed using the drop-down lists. See Figure 7.

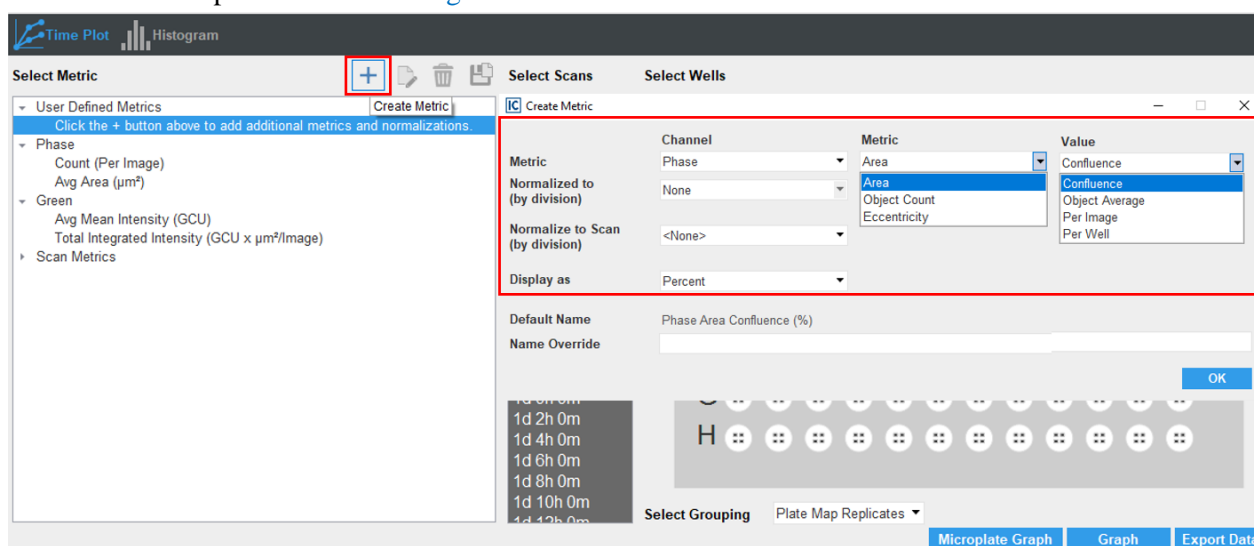



Figure 7. Creating a new metric using the metric interface

11. At this stage there is the ability to export data for individual objects, at individual time points in the form of CSV files. By selecting this icon,  a guided interface will open, allowing you to

select the wells and time points of interest to be exported.

Classification of cell populations based on label-free or fluorescence parameters.

Using IncuCyte Cell-by-Cell Analysis, it is possible to classify each individual cell at each time-point using either one or two parameters, thus classifying into two or four classes or subsets). The division into classes is performed by setting a “gate”, cells with values above the gate fall into the “high” class, cells below fall into the “low” class. To perform classification, you must first have performed an analysis job as described above, and then you will need to define and run a classification job as described below. The following steps are applicable to both non-adherent and adherent cell analysis.

1. To apply classification to an analysis, open the analysis job you wish to classify and press the Launch Cell-by-Cell Classification button on the left panel. This will open Well Selection, allowing you to select wells for review. There is also a Plate Map selection method, allowing the selection of replicate wells. See Figure 8.



The classification definitions are specific to each plate and will not be automatically applied if the same analysis definition is reused on a different plate.

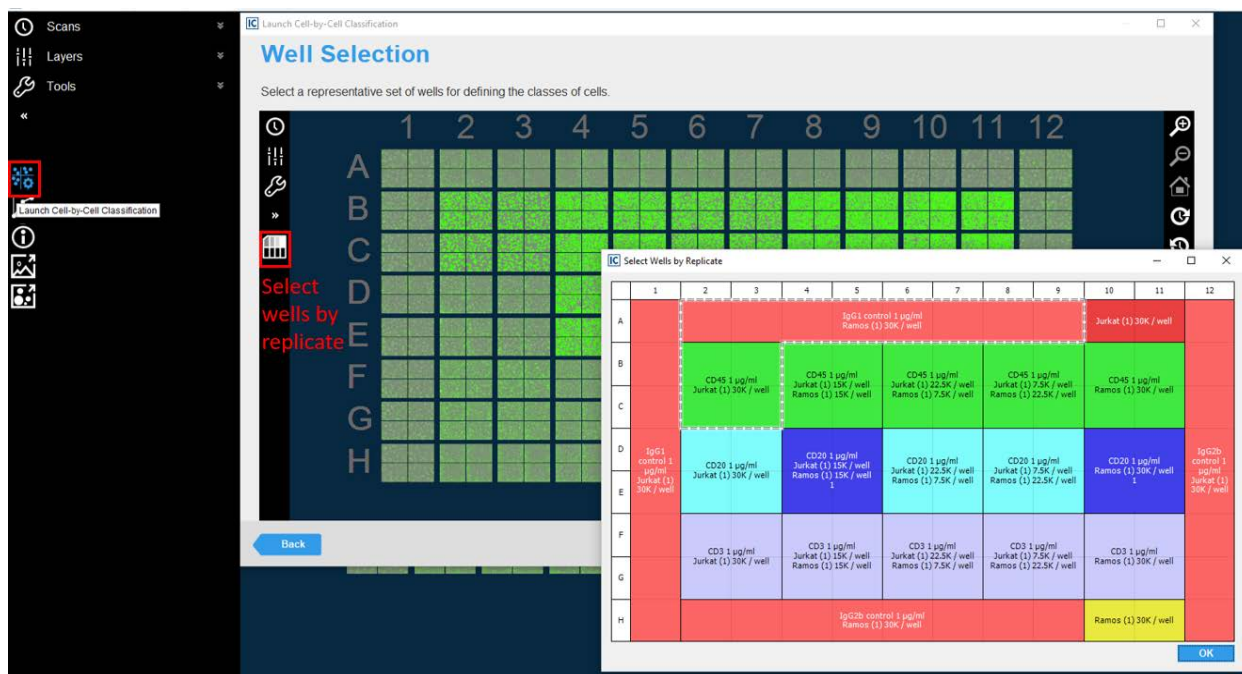


Figure 8. Selecting classification and well selection using plate map view

2. Select OK to advance to the next screen.
3. Defining Classification Metric Selection requires a selection of classifying criteria. Here you can select which metric/s you wish to gate on: Phase Object Area (μm^2), Phase Object Eccentricity and Fluorescence Mean Intensity (CU). You have the option to re-name the low and high classification, to allow simpler metric use later. Selecting single criteria will create a histogram plot, while double criteria selection produces a 2D dot plot alongside the

corresponding histograms.

4. Gating Values may be set using one of two methods. The first allows you to drag the gates to a position you think appropriate, the second allows you to input values directly into the Gate Value input field (Step 1).
 1. Once gates are defined it is possible to scroll through the time points, to visualize how your data changes over time, and whether the gates are still appropriate at later time points (Step 2).
 2. Auto scale is applied to these plots at each time point; however, this can be turned off or manually changed if required using the check boxes in the Graph Settings drop down. A summary of the data can be seen on the right of the Gating Definition page, this provides data on the percentage of cells within each half and quadrant regardless of replicate group (Step 3). Using the drop-down selection menu, it is possible to separate chosen groups from the “Summary” into specific data on replicates. [See Figure 9.](#)



At this stage it is possible to go back and view a different set of replicates to assess if the gates are appropriate here too. The gates you have set will be remembered when going back.

5. Once you have previewed the gates at various time points within the interface, and are satisfied with the parameters, complete the Launch Cell-By-Cell Classification workflow by selecting the Scan Times and wells to be analyzed, as well as assigning an analysis definition name. Note that if your experiment is in progress you will have an option to check “Analyze Future Scans” to perform real-time analysis (please see IncuCyte User Manual Section 3 Chapter 1 for more information on Defining Image Analysis).



These gates can be applied to separate analysis jobs by using the Copy Classification option when applying a gate.

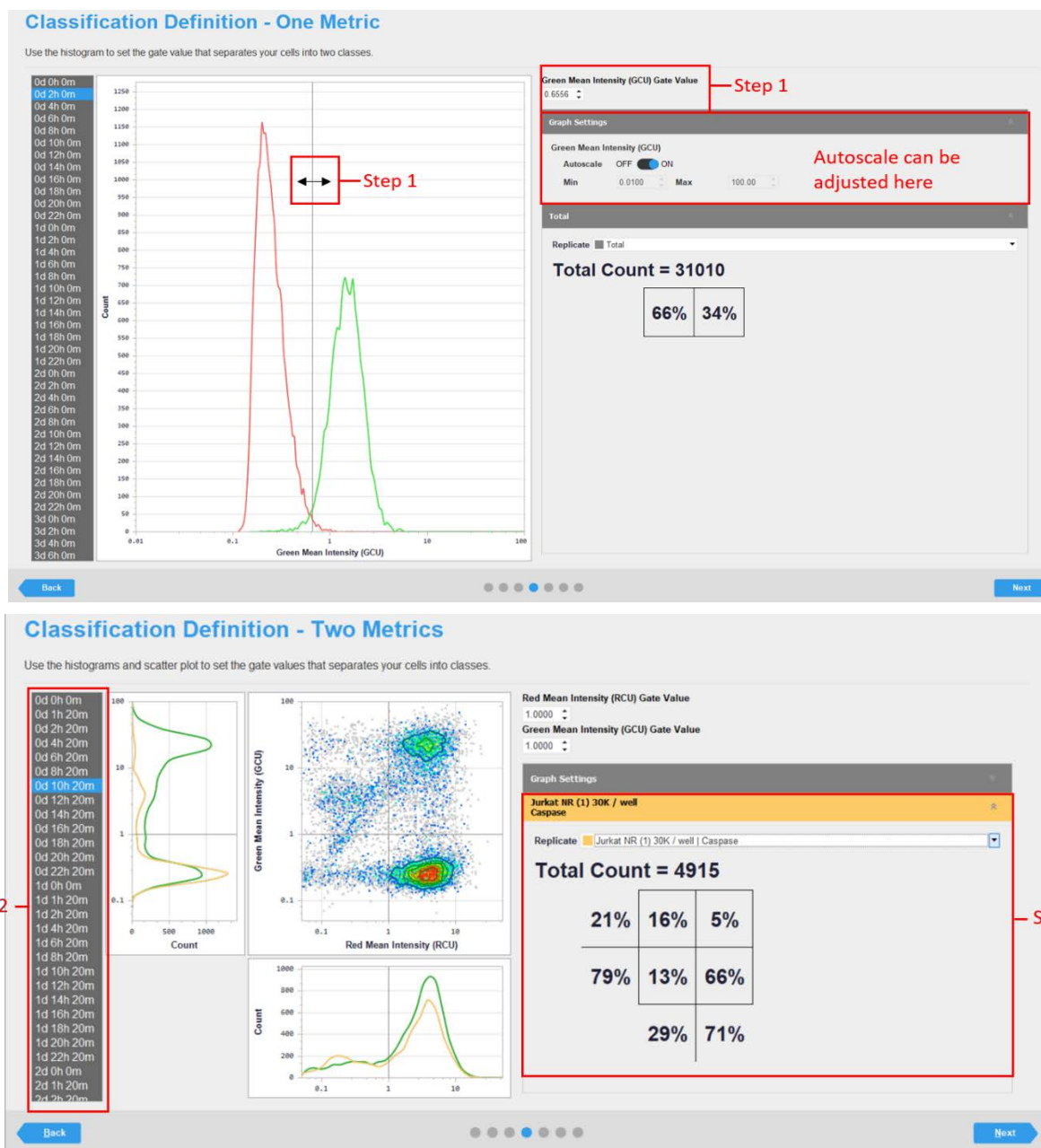


Figure 9. Classification definition – setting gates with one or two metrics.

- On the View Page, the Classification can be viewed as a sub-analysis under the analysis definition, this will be accessible once classification has been applied to all selected wells/scans.
- After the classification has been applied to the Cell-by-Cell analysis definition, the classification job can be opened and the following set of metrics are provided:

| Pre-defined metrics | Description |
|----------------------|--|
| Cell count per Image | The total number of cells per image. It is possible to view this metric within each individual quadrant of your classification analysis, including a summary of all quadrants. |
| % of Total cells | The objects within a quadrant as a percentage of the total population of cells. |

8. At this stage as before it is possible to create metrics using the metric interface. We can also now create metrics regarding the individual halves/quadrants of classified cells. See [Figure 10](#).

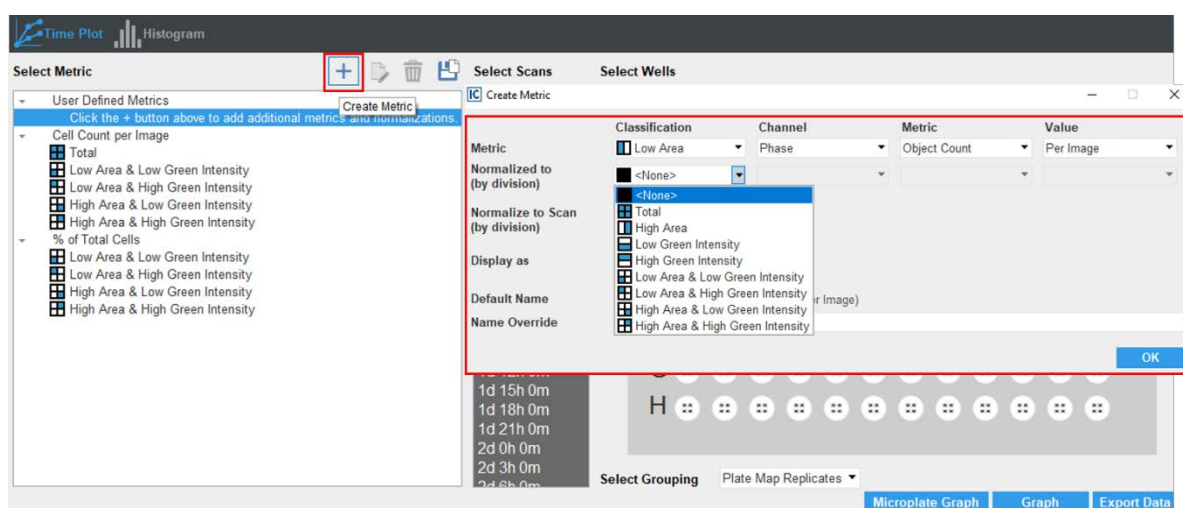


Figure 10. Creating metrics using the metric interface.

Visualization of classification of cell subsets using label-free or fluorescent readouts.

1. Once a classification job has run, the subsets can be visualized using color-coded masked images. Each classification or subset can be given a user-defined color code (halves/quadrants). To do this, choose the quadrant/s you wish to visualize (Step 1) and turn on outlines (optionally adjust color), and view how the distribution into classes changes over time (Step 2). See Figure 11.
2. It is also possible to view histograms per well at individual time points by right-clicking on an image and selecting view histogram. See Figure 11.

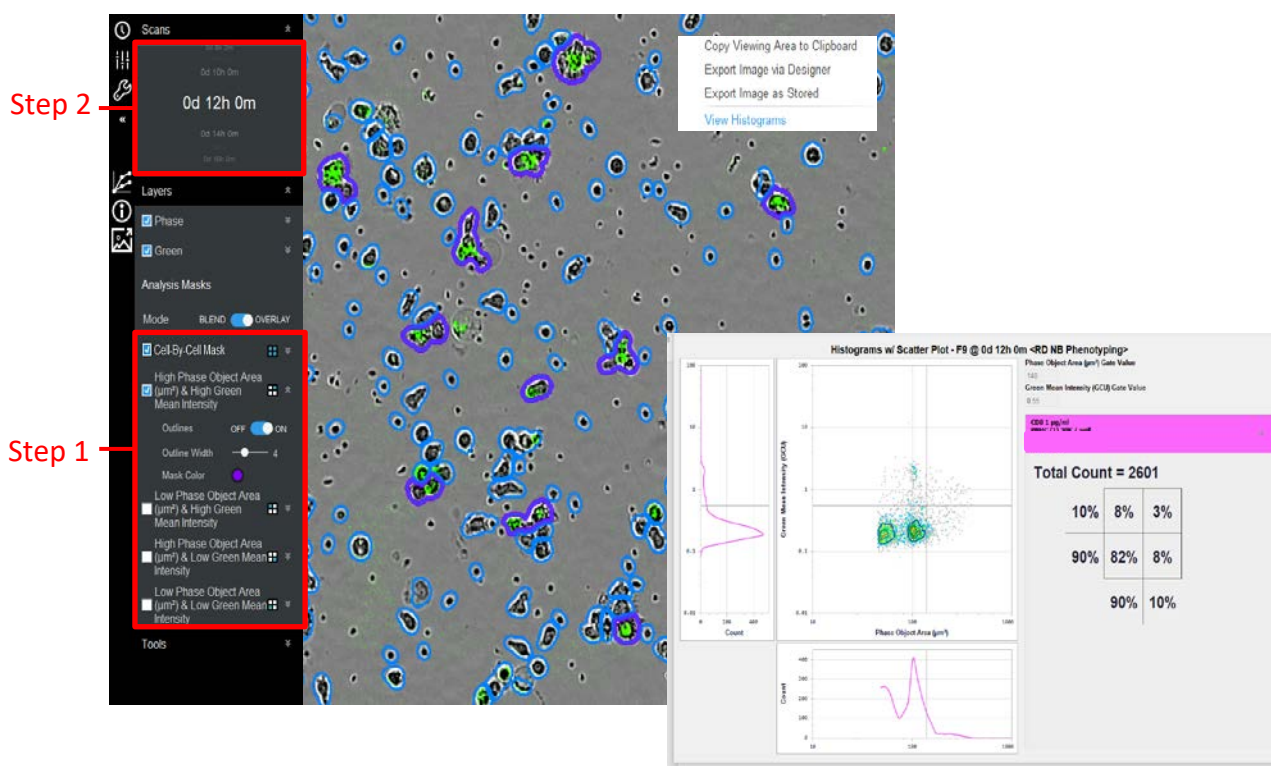


Figure 11. Visualizing changing populations within each image over time.