Guidelines for the Incucyte® AI Cell Health Software Module

Our novel, artificial intelligence (AI)-driven image analysis algorithm enables segmentation and label-free Live/Dead classification of Incucyte® HD phase-contrast images. The Incucyte® AI Cell Health Analysis Software Module is available to purchase for all S-series instruments and requires a GPU co-processor installed as a drop-in hardware upgrade to the controller.

The purpose-built Incucyte® software tool performs cell segmentation and label-free classification of live versus dead cells with a simple workflow using pre-trained neural networks. Segmentation and classification are contained within a single analysis job, requiring minimal user input.

This guideline covers the following topics for defining AI Cell Health Analysis parameters and subsequent classification (Figure 1):

- Acquiring images using the Incucyte® AI Cell Health Analysis Software Module
- Cell segmentation and classification of cells as live or dead using the Incucyte® AI Cell Health Analysis Software Module
- (Optional) Further classification of cell populations based on label-free or fluorescence parameters

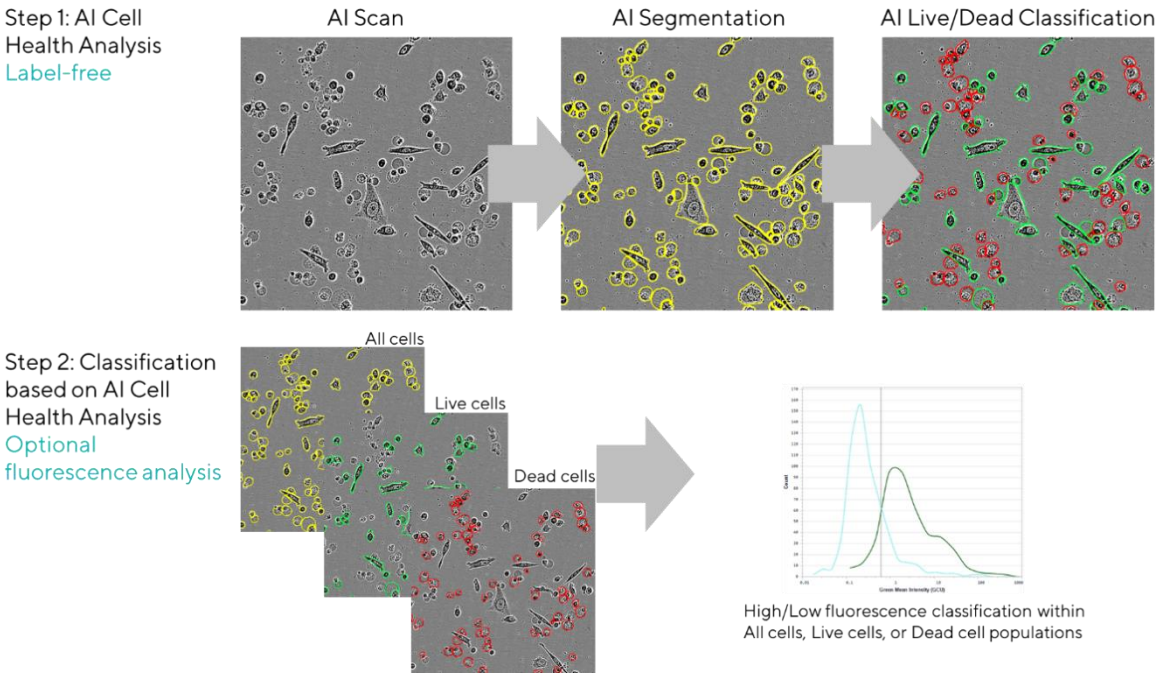

Figure 1. AI Cell Health Analysis (Step 1) and optional classification using the resulting populations (All cells, Live cells only, Dead cells only; Step 2).

The following procedures are for example purposes only and are designed to provide a frame of reference for defining the AI Cell Health Analysis Parameters within the Analysis Wizard.

For new Incucyte® users, it is recommended to review Sections 1, 2 and 3 of the Incucyte® Live-Cell Analysis Systems User Manual (Incucyte® SX1, S3 and SX5) or have experience scheduling and acquiring scans, viewing images, performing image analysis, and visualizing results prior to reviewing this technical note.

Acquiring images using Incucyte® AI Cell Health Analysis Software Module

This module enables analysis of HD phase-contrast and fluorescence channel images acquired using a new AI Scan acquisition mode. Analysis is designed for investigation of cytotoxicity. Adherent and non-adherent cell types can be analyzed using images acquired at 10x or 20x. Please see the Incucyte® Live-Cell Analysis Systems 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 AI Scan.
2. In the Scan Settings Window, Phase Image Channel will be selected as default. Additionally, select Fluorescence channels depending on your chosen reagents.
3. Proceed through the remaining windows referencing the Incucyte® Live-Cell Analysis Systems User Manual (Incucyte® SX1, S3 and SX5) as needed.

Cell segmentation and classification of cells as live or dead using the Incucyte® AI Cell Health Analysis Software Module

The following section will guide you through the process of creating a new Incucyte® AI Cell Health Analysis definition for segmentation of individual cells and Live/Dead classification, all within a single analysis.

1. From an open VesselView, launch the analysis wizard by clicking on the icon 
2. Click Next until the Analysis Type window is presented. Select AI Cell Health to continue. The Phase image channel is automatically selected – also select fluorescence channels if you would like these to be included within the analysis.
3. Perform Image Set Selection. In this window, select representative images to preview analysis results. Use your mouse to hover over images you would like to select and click the site. We recommend selecting images which represent live, healthy cells as well as dead cells. Click Next to present you with the Analysis Definition window (Figure 2).
4. If desired, enter the name of the object(s) that are being analyzed into the Object Name field.

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.

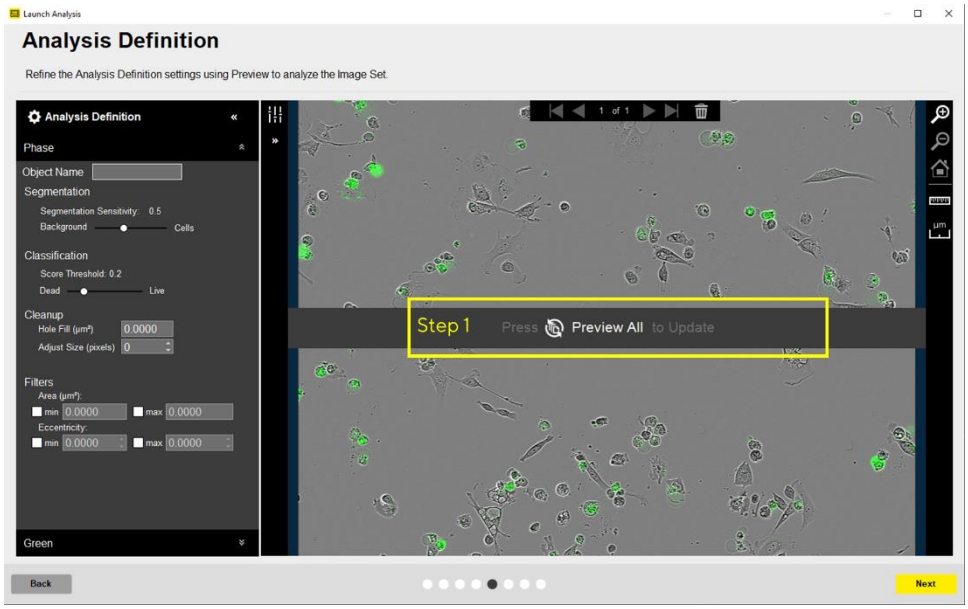

Figure 2. AI Cell Health image preview.

5. Preview the Segmentation and Classification using default values by selecting Preview All (Figure 2, Step 1).
6. Optimize the cell segmentation by moving the Segmentation Sensitivity slider (Figure 3, Step 2).
    - Moving towards Background reduces the detection sensitivity and can result in fewer objects being segmented.
    - Moving towards Cells increases the detection sensitivity and can result in more objects being segmented.
7. For each image, click Preview All to evaluate the AI Cell Mask and move the slider accordingly. Mask Outline visualization mode has a slider to adjust the outline width and can aid in evaluation of the analysis mask.
8. The AI Cell Segmentation can be optimized using cleanup settings, for example hole fill or area, eccentricity filters.
9. It is not necessary to create a mask for fluorescence channels since fluorescence intensity within the phase boundary is automatically analyzed.

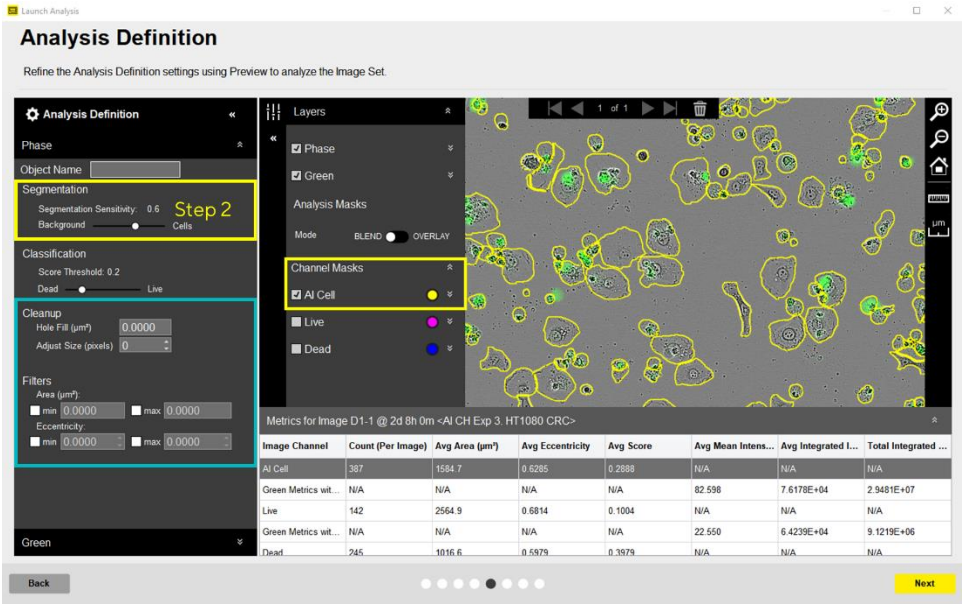

Figure 3. AI Cell Segmentation preview. Move the Segmentation Sensitivity slider to adjust the AI Cell segmentation mask (yellow boxes). Adjust the Cleanup options and Filters accordingly to remove any non-cell objects (teal box and Table 1).

10. All segmented cells will additionally be automatically classified as Live or Dead.
11. Assess the classification results by clicking on the Live and Dead segmentation masks (Figure 4). Outline visualization mode has a slider to adjust the outline width and can aid in evaluation of the classification mask. The default classification mask colors can be changed to assist visualization.
12. Optimize the Live/Dead classification by moving the Score Threshold slider (Figure 4, Step 3). Cell death is a process that occurs over time and can be captured at all stages with live-cell imaging. The observed morphology as cells die exists on a spectrum with live, healthy cells at one end and dead cells at the other. The Score Threshold slider allows users to alter this threshold between live and dead cells.
    - Moving towards Dead classifies more cells as Dead.
    - Moving towards Live classifies more cells as Live.
13. Once you have previewed all the images within the wizard image set and are satisfied with the parameters, complete the Launch Analysis wizard 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.

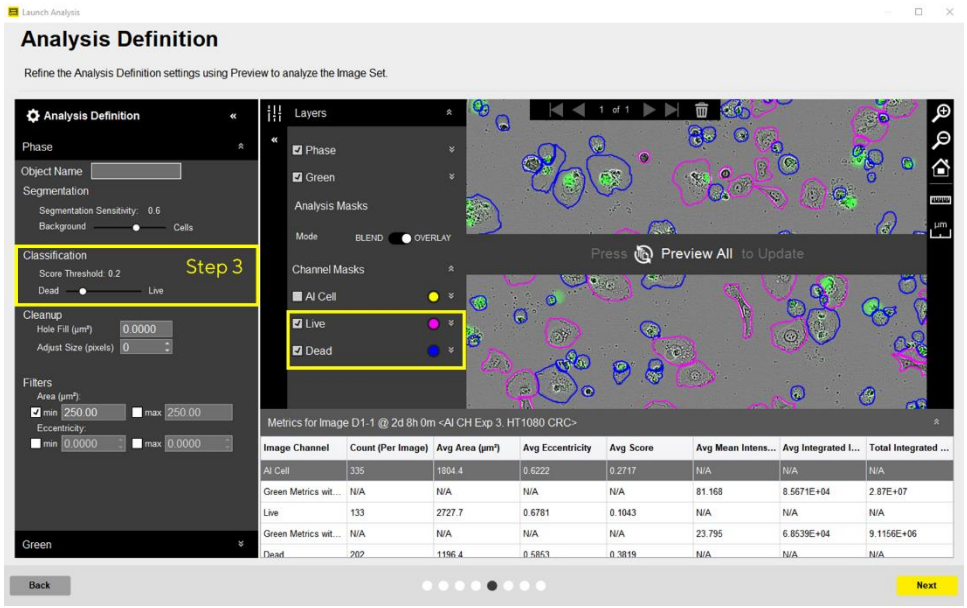

Figure 4. AI Live/Dead classification preview. Adjust the Score Threshold to preview Live/Dead classification results indicated by the masks (yellow box).

Table 1: AI Cell Health Analysis definition options

Option	Description
Parameters	
Segmentation Sensitivity Background/Cells	Use the slider to adjust the AI Cell segmentation mask ▪ Moving towards Background reduces the detection sensitivity and can result in fewer objects being segmented. ▪ Moving towards Cells increases the detection sensitivity and can result in more objects being segmented.
Score Threshold Dead/Live	Use the slider to adjust the Live/Dead classification of segmented cells. The Score Threshold slider allows users to alter the threshold between live and dead cells. ▪ Moving towards Dead classifies more cells as Dead. ▪ Moving towards Live classifies more cells as Live.
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. Hover over a segmented object to identify its area.
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 have a value of 0.

Visualization of results

Visualization of Analysis Results is described in Section 3, Chapter 2 of the Incucyte® Live-Cell Analysis Systems User Manual.

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

Table 2: Automatic metrics list

Option	Description
Phase object metrics	
Phase Count (per image)	Total count of segmented cells within the image
Average Area (μm^2)	Average area of segmented cells within the image
Average Mean Fluorescence Intensity (CU)	The Fluorescence 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 Integrated Intensity (CU x μm^2/image)	The total Fluorescence 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 object multiplied by the pixel area.
Classified cell metrics	
Live/Dead cell count (per image)	Count of cells classified as Live or Dead within the image
Live/Dead cell %	The percentage of total cells within the image which have been classified as Live or Dead

It is also possible to add additional metrics and new normalized metrics. By pressing the "+" you can open the metric interface where you are able to select the metrics you would like to add to the User Defined Metrics, using the drop-down lists to view choices.

Further classification of cell populations based on label-free or fluorescence parameters

Using the AI Cell Health Analysis, it is possible to classify cells based on one or two parameters (thus forming two or four subpopulations). 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.

1. To apply classification to an analysis, open the AI Cell Health Analysis job you would like 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 (Figure 5).
2. Select OK to advance to the next screen.

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.

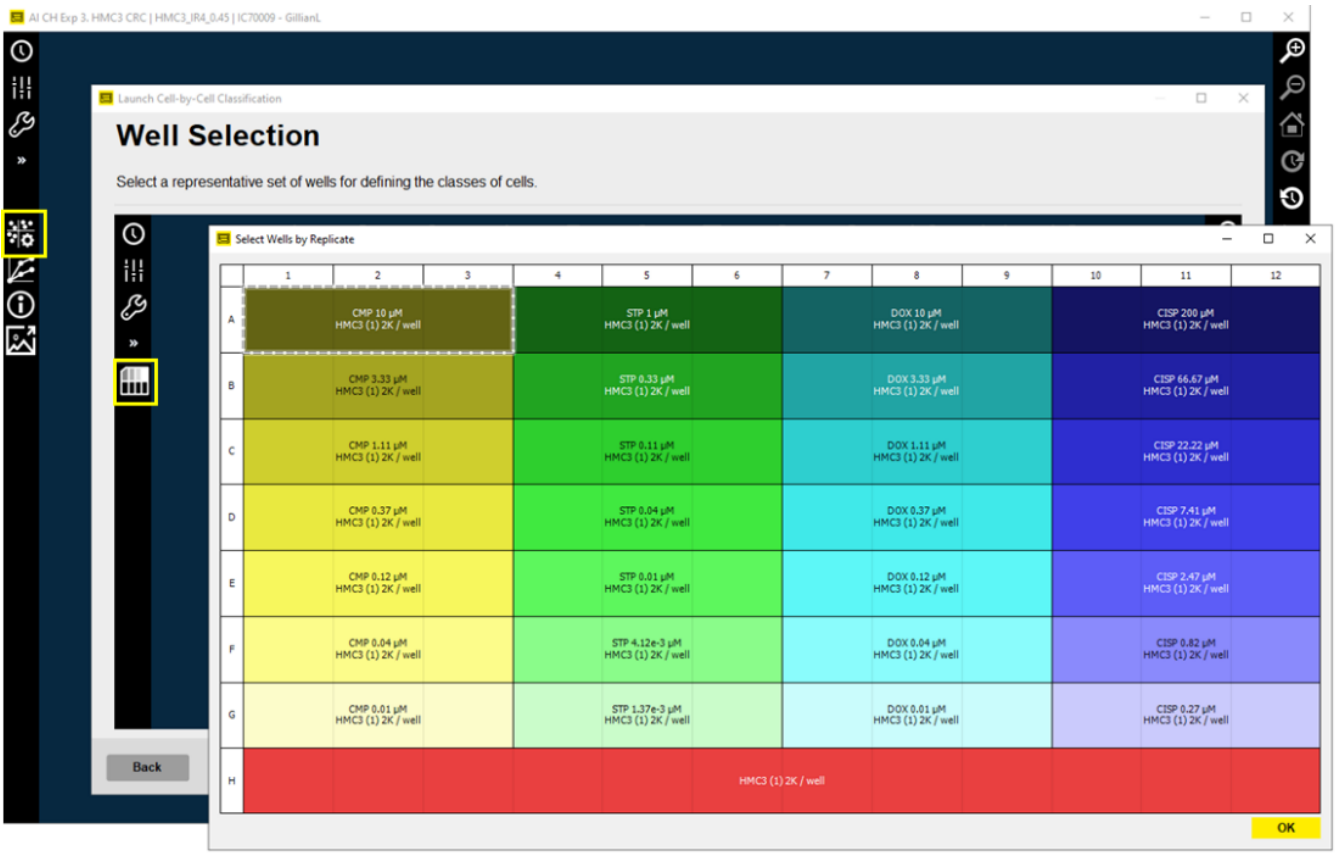

Figure 5. Selection of replicate wells for classification.

3. Classification can be performed using all segmented cells, or only within those groups identified as Live or Dead by the AI Cell Health Analysis. Select your group of interest using the first drop-down menu (Figure 6, Step 1).
4. Defining Classification Metric Selection requires a selection of classifying criteria (Figure 6, Step 2). Here you can select which metric/s you would like 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 for ease of identification.

Selecting single criteria will create a histogram plot, while double criteria selection produces a 2D dot plot alongside the corresponding histograms.

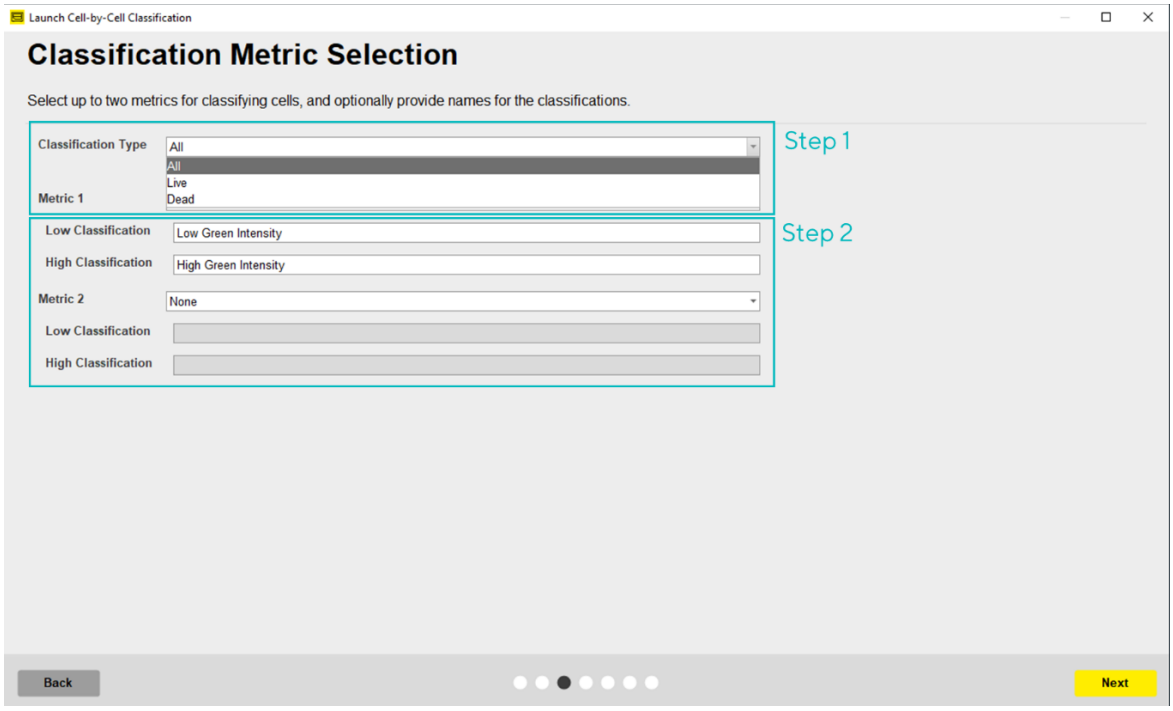

Figure 6. Select cells of interest - all cells, or those identified as Live or Dead (Step 1). The selected cells can then be classified according to the chosen metrics (Step 2).

5. Gating Values may be set using one of two methods (Figure 7). 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).

    a. 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).
    b. Autoscale 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 and 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.

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.

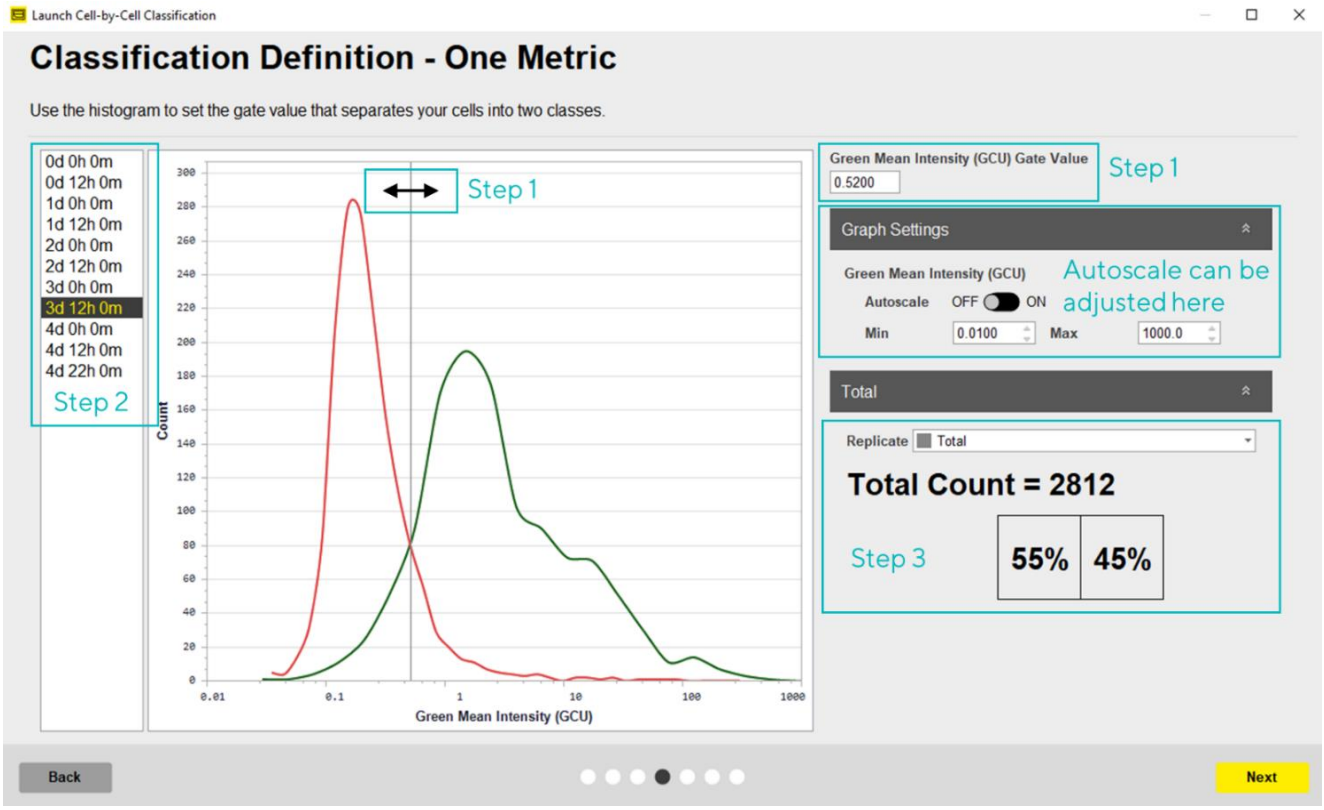

Figure 7. Classification definition – setting gates with one metric.

6. 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.
7. 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.
8. 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:

Table 3: Pre-defined metrics list

Pre-defined metrics	Description
Cell count (per image)	Total number of cells per image. It is possible to view this metric within each individual quadrant of your classification analysis.
% of Total cells	The objects within a quadrant as a percentage

9. At this stage it is possible to create metrics using the metric interface. We can also now create metrics regarding the individual halves/quadrants of classified cells (Figure 8).

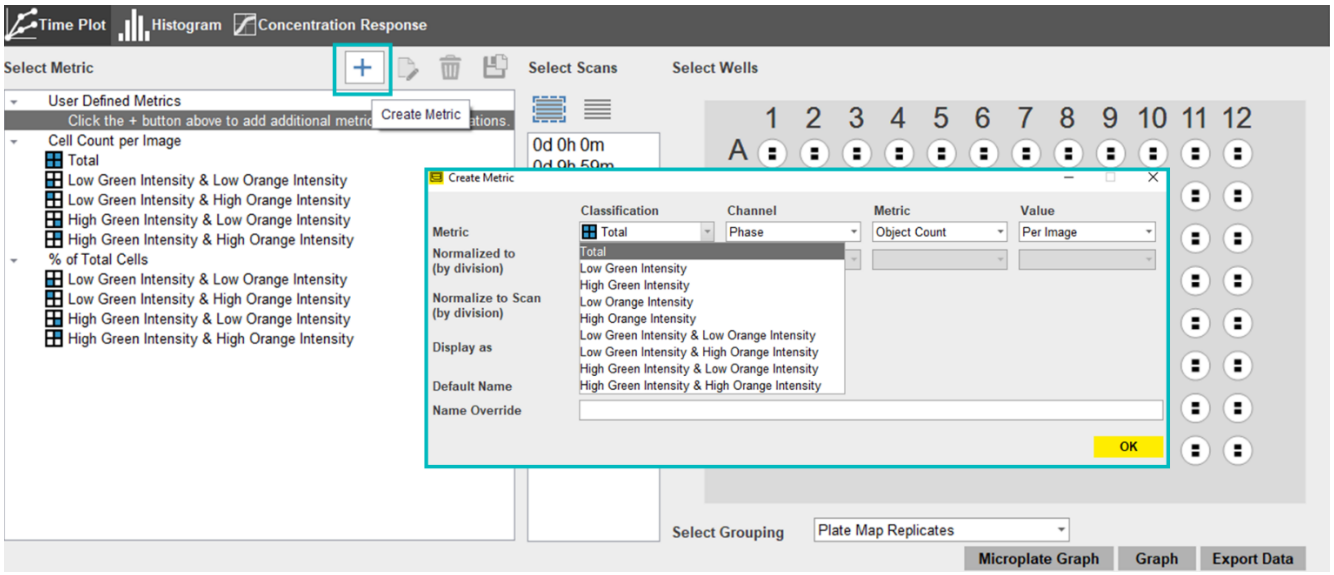

Figure 8. Creating metrics using the metric interface.

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

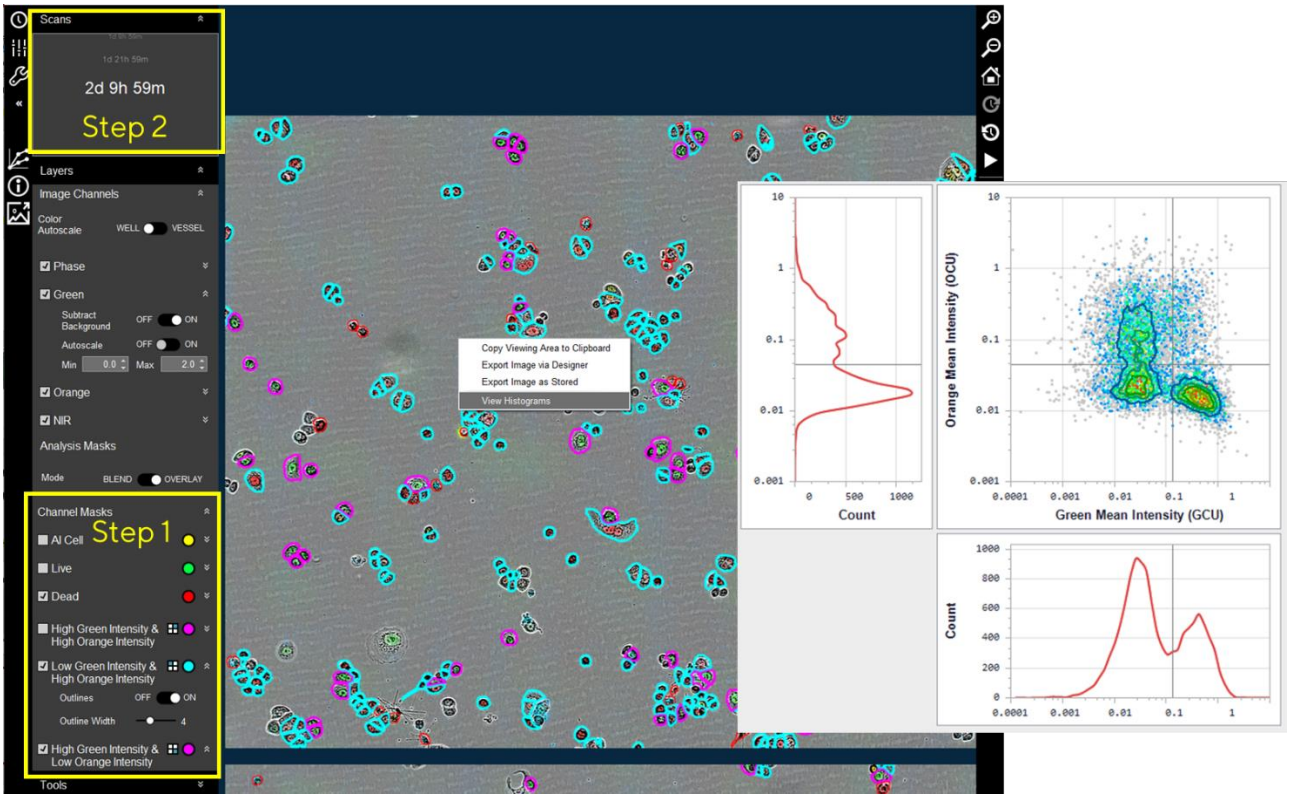

Figure 9. Visualization of changing populations within each image over time.

1. Once a classification job has run, the subsets can be visualized using color-coded masked images (Figure 9). Each classification or subset can be given a user-defined color code (halves/quadrants). To do this, choose the quadrant/s you would like to visualize (Step 1) and turn on outlines (optionally adjust color), and view how the distribution into classes changes over time (Step 2).
2. It is also possible to view histograms per well at individual time points by right-clicking on an image and selecting view histogram (Figure 9).

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