Guidelines for the Incucyte® Advanced Label-Free Classification Analysis Software Module

Our novel, patent-pending image analysis algorithm enables classification of individual cells using segmented Incucyte® HD phase-contrast images. The Incucyte® Advanced Label-Free Classification Analysis Software Module is an add-on to the Incucyte® Cell-by-Cell Analysis Software Module enabling adherent cells to be classified into two user-specified groups based on their total morphology.

The purpose-built Incucyte® software tool enables classification into subsets using a simple interface. 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 morphology.

This guideline covers the following topics for defining Advanced Label-Free Classification analysis parameters:

- Classification of cell populations based on selected control wells (the training set)
- Refinement of the training set where images contain cells with heterogeneous morphology

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

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

Prior to Advanced Label-Free Classification, users must segment cells using the Incucyte® Cell-by-Cell Analysis Software Module.

Classification of cell populations based on morphology

Using Incucyte® Cell-by-Cell Analysis with Advanced Label-Free Classification, it is possible to classify each individual cell at each time-point into one of two classes. The division into classes is performed by identifying images which provide a clear example of the morphologies of interest. To perform classification, you must first have performed an Adherent Cell-by-Cell analysis job as described in the Cell-by-Cell Analysis Guide, then you will need to define and run a classification job as described below. The following steps are applicable only to adherent cell analysis.

1. To apply classification to an analysis, open the analysis job you wish to classify and press the Launch Advanced Label-Free Classification button on the left panel. Create a new classification definition and click Next. This will open Classification Names, allowing you to label your classes of interest – for example, Live and Dead.

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

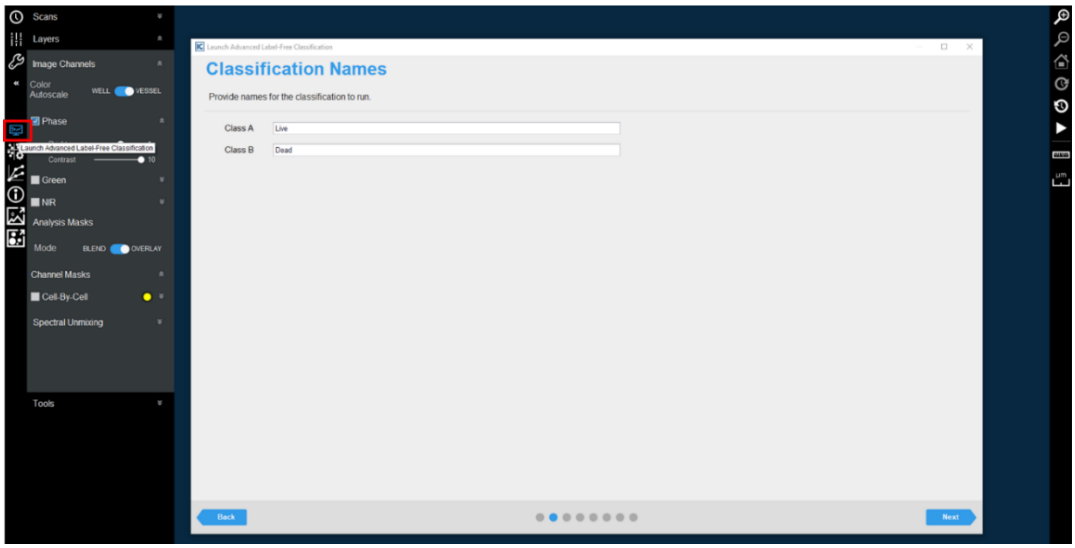

Figure 1. Naming the two classes of interest.

2. Select Next to advance to the next screen, which allows you to select control wells and timepoints containing cells that best represent the two classes of interest. The two classes must be defined separately using the tabs at the top of the screen.

Cells can display slightly different morphologies at various confluence values, therefore we recommend that under conditions where cells are growing (such as vehicle control wells), a wide range of timepoints are selected for the training set.

Figure 2. Selection of wells and timepoints which represent the morphology of the first class.

Wells can be selected individually or via the interface showing the plate map.

3. At the top of the selection box, change tabs to select wells and timepoints which represent the second class of interest. The chosen images must be different to those selected for the first class. Smart training algorithms automatically remove outlier cells and selects the best cells to use for identifying the morphology of the two classes.

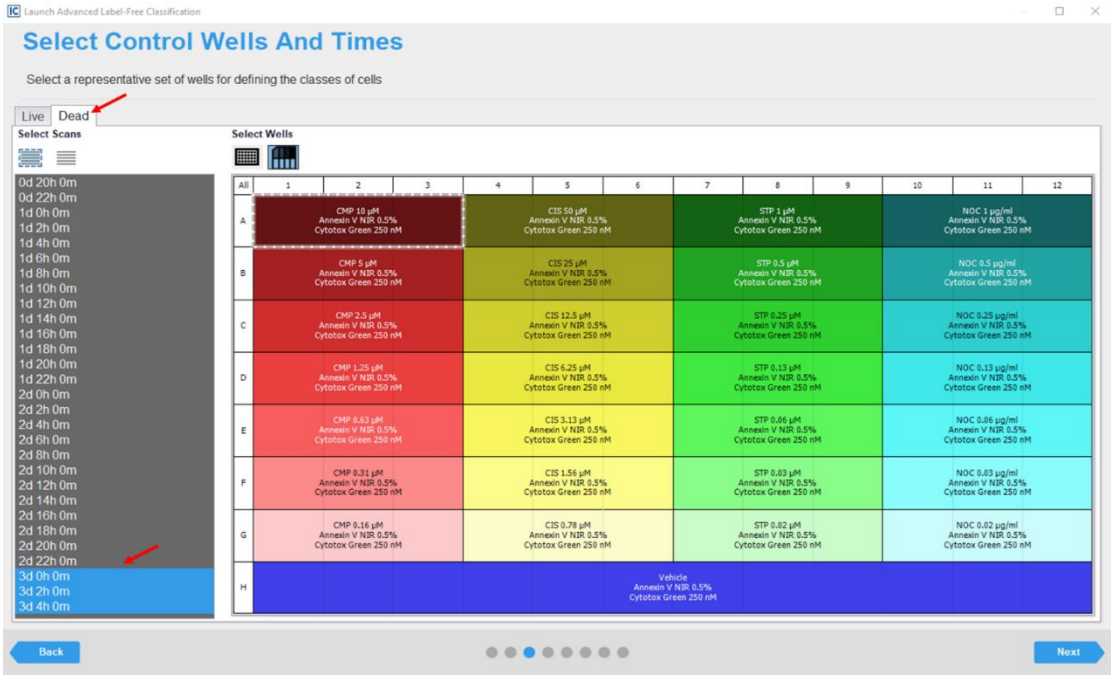

Figure 3. Selection of wells and timepoints which represent the morphology of the second class.

4. Select Next to advance to the next screen, which allows you to select images to preview the classification results. Choose images which were not included in the training set to help you visualize how the analysis definition performs with your data.

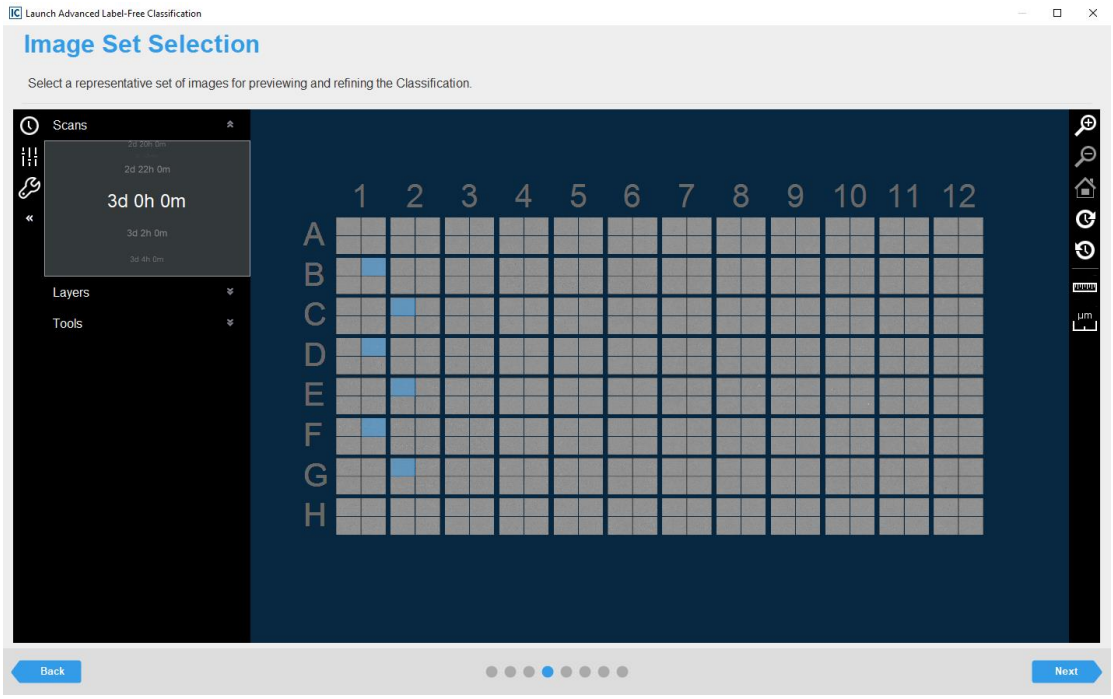

Figure 4. Selection of images to preview the classification results.

5. Select Next to advance to the next screen, where the classification results of the chosen images can be previewed by selecting Preview All. The analysis definition can be refined by changing the Class Threshold slider.

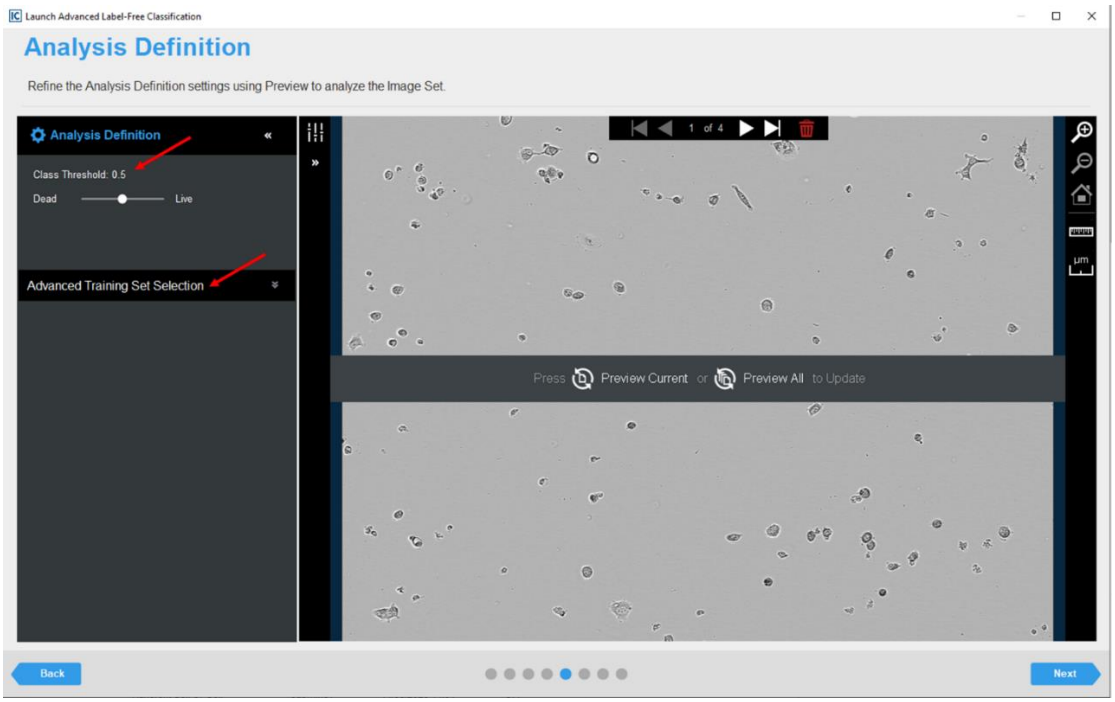

Figure 5. Visualize the classification results on the chosen preview images and change the Class Threshold.

If cell morphology in the training images is heterogeneous, the training set can be refined by selecting the blue refinement option. This enables individual cells to be added and removed. For more detailed instructions, see section below "Refinement of control datasets".

Cell-by-Cell and Classification masks will appear on each cell allowing the results to be visualized. The class names will be automatically populated and the appearance (color, outlines) of each mask can be altered as required. A box underneath displays metric data for the image.

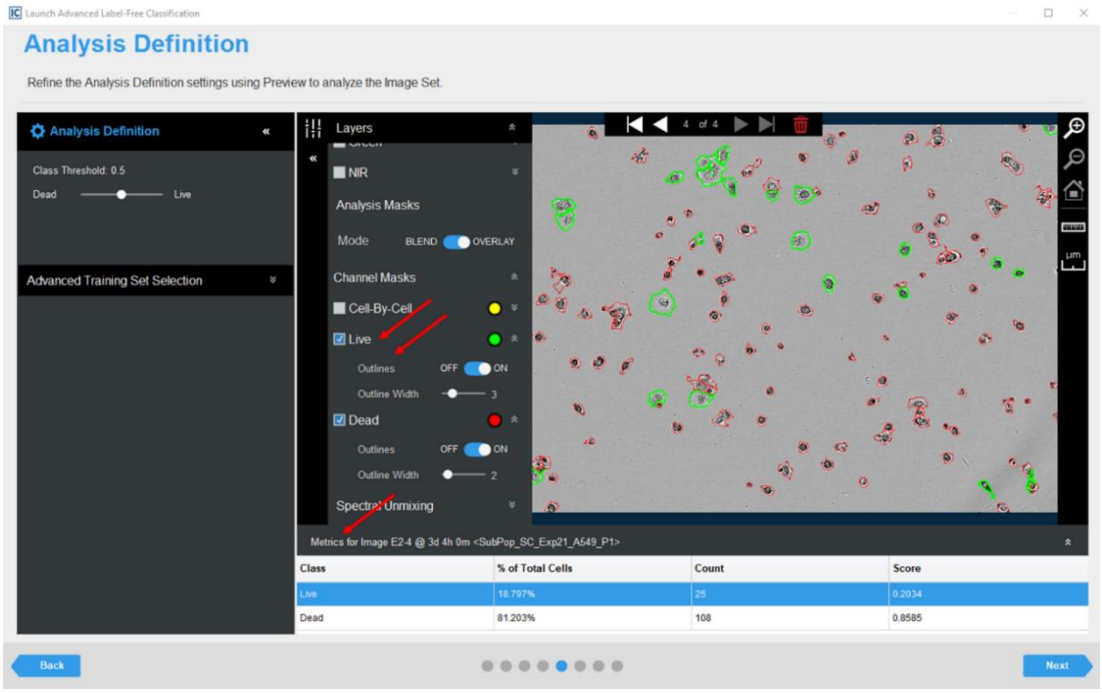

Figure 6. Previewing the analysis definition on chosen images. Colored masks indicate the classification of each cell and metrics show the count, score and % of cells in each class.

At this stage it is possible to go back and view a different set of images to assess if the analysis is appropriate here too.

6. The classification generates a score value for each cell, which is on a scale from 0 to 1 where control class A cells are equal to 0, and control class B cells are equal to 1. Altering the Class Threshold changes the gate between these classes, enabling the user to choose an appropriate threshold based on the displayed score values.

7. Once you have adjusted the Threshold value using various images within the interface and are satisfied with the preview classification results, complete the Launch Advanced Label-Free Classification workflow by selecting the Scan Times and wells to be analyzed, as well as assigning an analysis definition name.

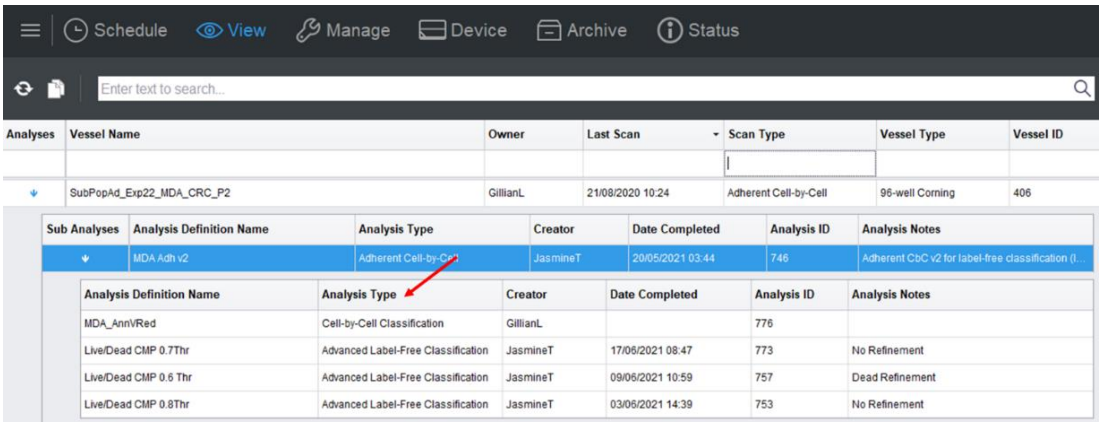

Figure 7. Classification jobs appear as sub-analyses under the Cell-by-Cell analysis definition

8. On the View Page, the Advanced Label-Free 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.

9. 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 1. Phase analysis metrics

Pre-defined metrics	Description
Cell count per Image	The total number of cells per image; the total number of cells in each class per image.
% of Total cells	The objects within a class as a percentage of the total population of cells.

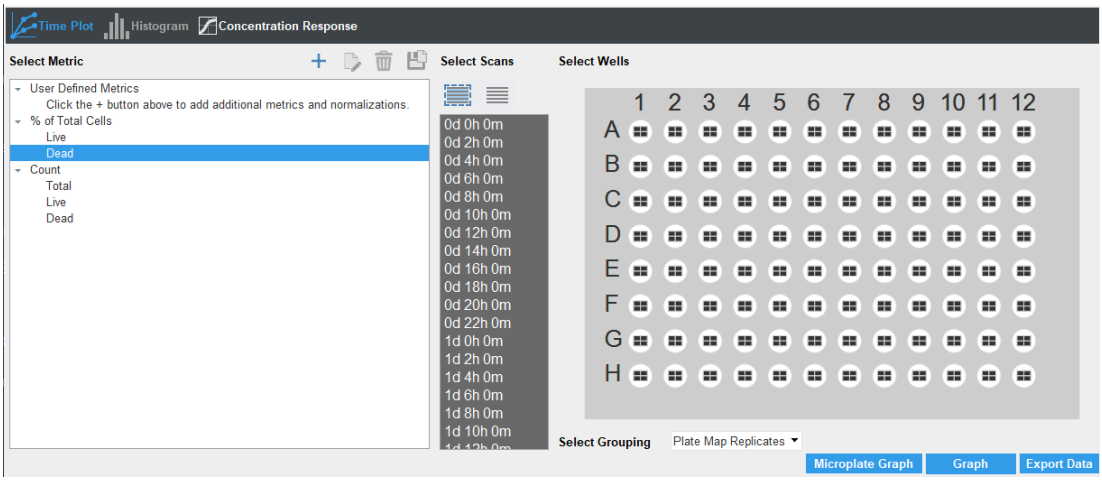

Figure 8. Metrics displayed for Advanced Label-Free Classification. Classes are named according to user input when the Classification Analysis is set up (see Figure 1). Class object count and percentage of total object number are available.

10. Custom metrics and normalizations can be set up. Select the + button to open a box which enables normalization to metrics or to scan times.

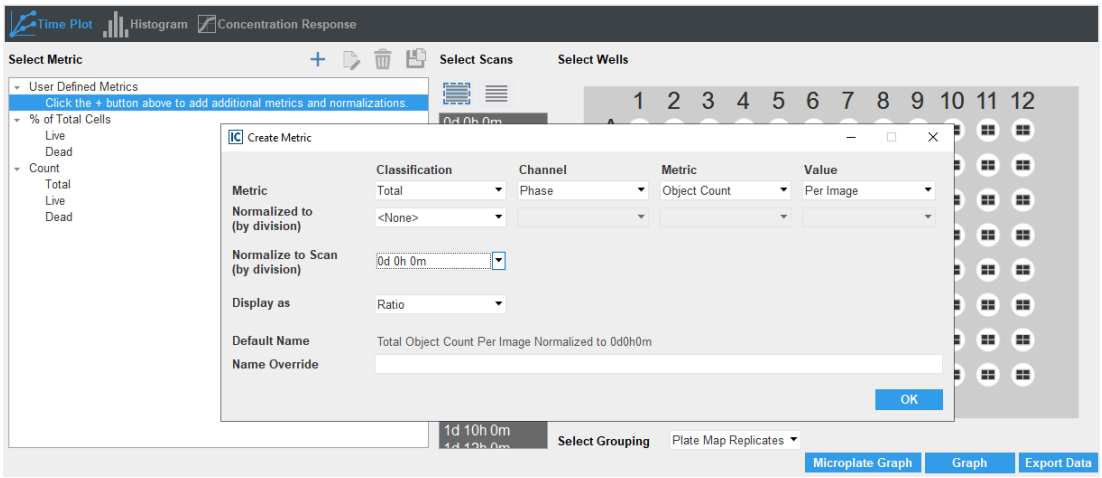

Figure 9. Selection of custom metrics.

Refinement of control training sets (optional)

In some biological models, the control images may contain cells with a heterogeneous morphologies – for example, differentiation of monocytes to mature dendritic cells may result in a mix of monocytes (typically small, round) and mature dendritic cells (larger cells with long extended area). To use images such as these for Advanced Label-Free Classification, a subset of cells of interest within the image must be selected.

After selection of control wells and timepoints, enter advanced refinement mode by selecting "Advanced Training Set Selection" and choosing the class to be refined (e.g. Dendritic cells). This opens the refinement window.

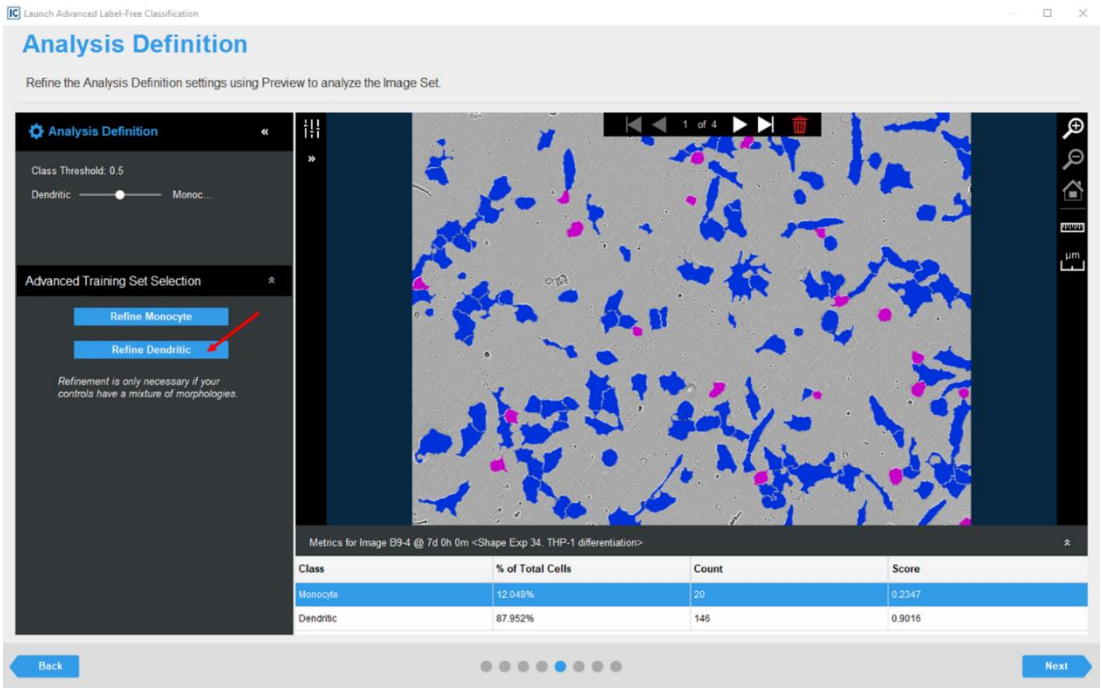

Figure 10: Enter Advanced Training Set Selection (Refinement) mode

Navigate through the images using the list of wells and timepoints on the left side of the screen. On first entering the refinement, the display will show the cells selected by the smart training algorithm from the wells and timepoints selected in Step 4.

To change the cells selected, identify a cell displaying the morphology of interest, right-click on it and select "Set Representative Cell". A warning will appear "By setting a representative cell, you will be resetting all changes within this class. Are you sure you want to continue?"

This indicates that selecting a representative cell will override all previous refinement,

as only one representative cell can be chosen at any one time.

Click OK to continue, and the cells that display morphology most like the representative cell will automatically be included in the selection. To change the % similar cells, move the "Cells to include" slider. Decreasing the "% cells to include" will retain only the cells which are most similar to the representative cell. Set the slider showing "Cells to include" to a percentage value that indicates the % of cells which represent the morphology of interest – for example if around half of the cells have differentiated, set the slider to 50%.

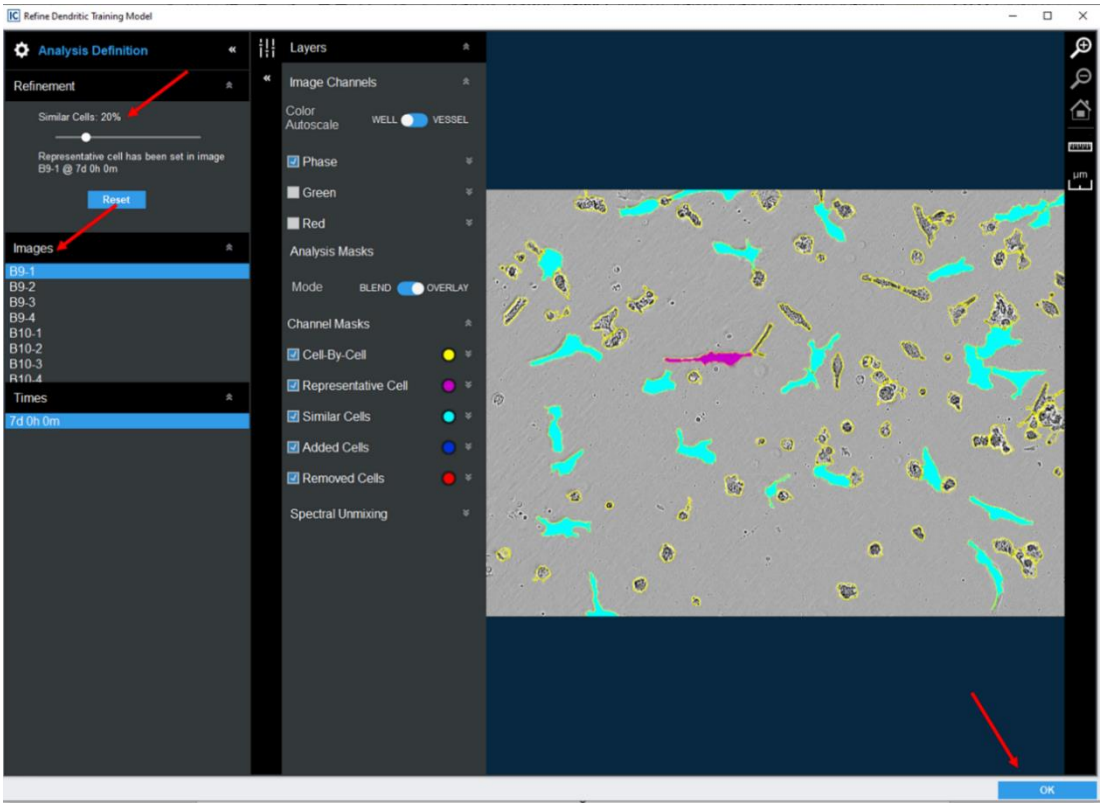

Figure 11: Advanced Training Set Selection (Refinement) mode enables a subset of cells within control images to be used for training

The selected cells can be further refined by:

- Resetting the % cells to include slider – note that this applies across all images in the training set
- Manual addition of cells of interest – right-click and "Add cell" or hover over a cell until the + symbol appears and left-click
- Manual removal of unwanted cells – right-click on a selected cell and "Remove

cell" or hover over a cell until the x symbol appears and left-click.

When satisfied with the final selection, click OK to return to the Analysis Definition window and click Preview All to examine the classification results on selected preview images. Click Next to select the wells and timepoints to run the classification job.

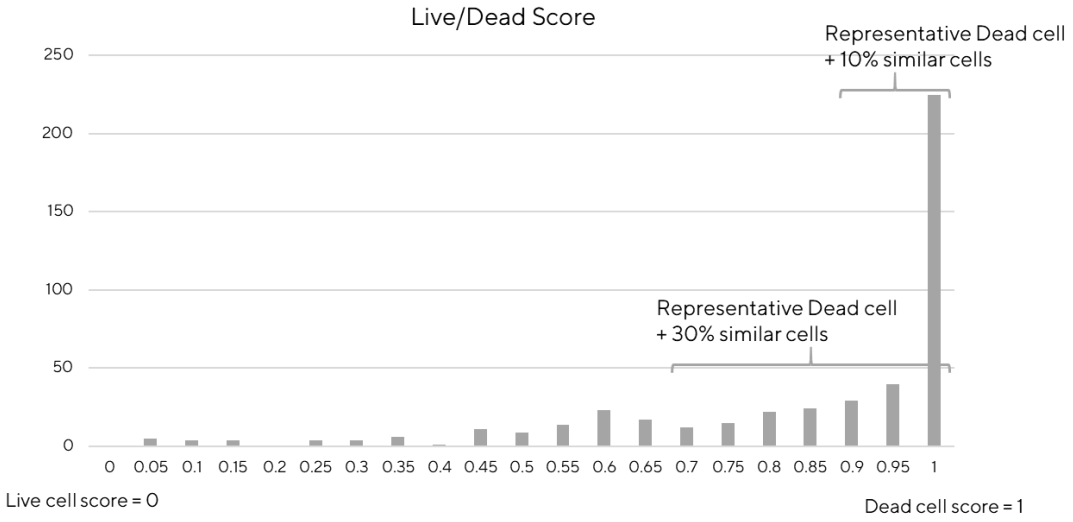

Figure 12: A histogram showing score values of all cells within a single image. In Advanced Refinement mode, a representative Dead cell (Class B) will have a score value equal or close to 1. Cells with morphology similar to this will also have values close to 1, whereas Live cells will have a score closer to 0. Increasing the % cells to include value from 10% to 30% will increase the number of cells included within the "similar cells" mask, however these will be less similar to the representative cell (i.e. have a score value further from 1).

Table 2. Options for Advanced Label-Free Classification definitions

Advanced Label-free Classification	
Classification names	• Naming your morphological classes of interest (e.g. Live and Dead) ensures that your data will be labelled appropriately
Class Threshold slider	• This is the threshold which divides your cells into two classes • Each cell will have a score from 0 to 1 (where true Live is 0 and true Dead is 1) and a threshold of 0.5 will classify cells <0.5 as Live and >0.5 as Dead • Adjust the slider so that cells in preview image are appropriately classified
Metrics: % of Total Cells	• The percentage of total cells within the displayed image which have been identified as belonging to each Class
Metrics: Count	• The number of cells within the displayed image which have been identified as belonging to each Class
Metrics: Score	• The average score of all cells within each class
Advanced Training Set Selection (Refinement) *Enables user to specify which cells within an image are used for training the classification model - this is recommended if control images contain cells with a mixture of morphologies*	
Representative Cells Channel Mask	• This mask Indicates which cell has been chosen by the user as the best representation of the class • To choose a representative cell, right-click and select "Set Representative Cell" • Only one representative cell can be chosen
Similar Cells Channel Mask	• This mask indicates which cells have been automatically identified as having similar morphology to the chosen representative cell • To change the percentage of similar cells, move the Similar Cells slider
Similar cells slider	• This is used to set the percentage of cells which are similar to the chosen representative cell • Text underneath the slider will remind the user which image contains the chosen representative cell
Added Cells Channel Mask	• This is used to indicate which cells have been manually added to the training set by the user • Hover over a cell and if the cursor changes to a blue +, click to add this cell to the training set
Removed Cells Channel Mask	• This is used to indicate which cells have been manually removed from the training set by the user • Hover over a cell and if the cursor changes to a red x, click to remove this cell from the training set

Visualization of classification of cell subsets using label-free morphology analysis.

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. To do this, choose the classes you wish to visualize and turn on outlines (optionally adjust color).

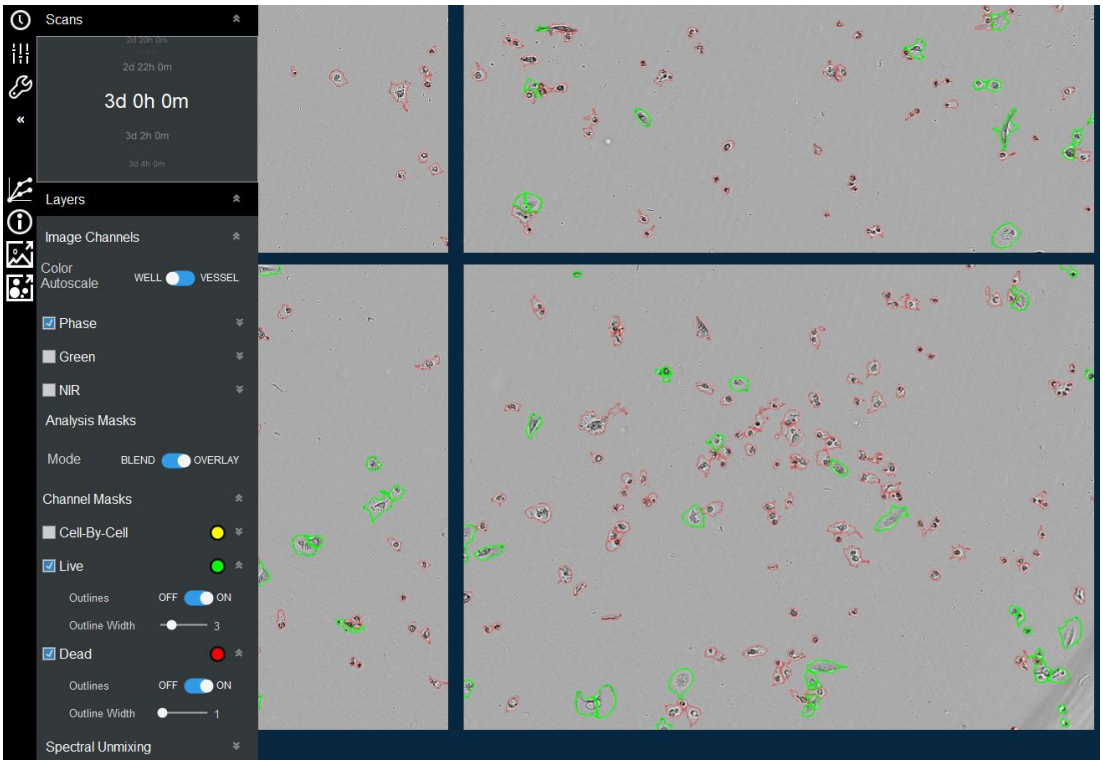

Figure 13. Visualization of classes using segmentation masks. Colors and outlines can optionally be altered.

2. Time-plots, histograms and concentration response data can be visualized within the Incucyte® software. Select the Plot Data button to open the interface which enables selected metrics to be graphed. The wells and timepoints of interest can be selected for simple data visualization or exported to an external program.

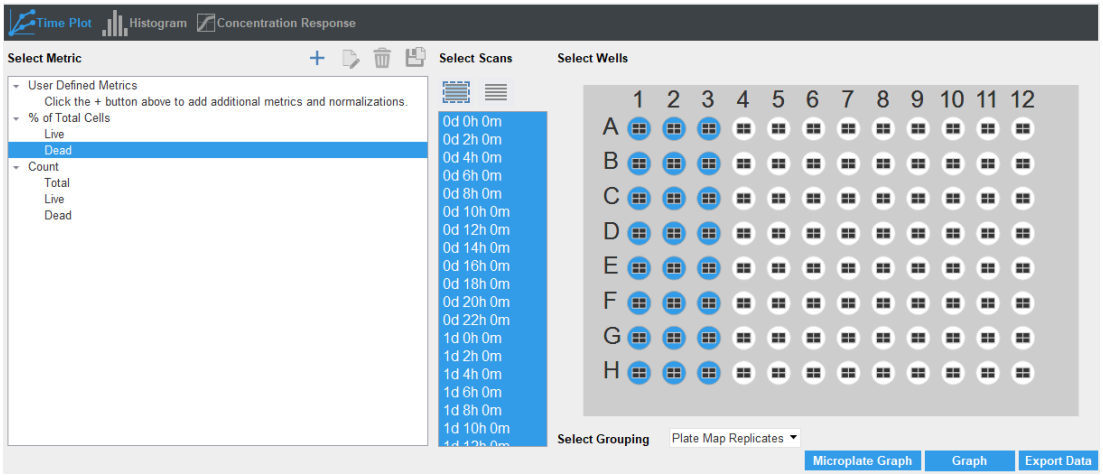

Figure 14. Visualization of results through Incucyte® graphing interface. Change plot type by switching between tabs at the top of the box.

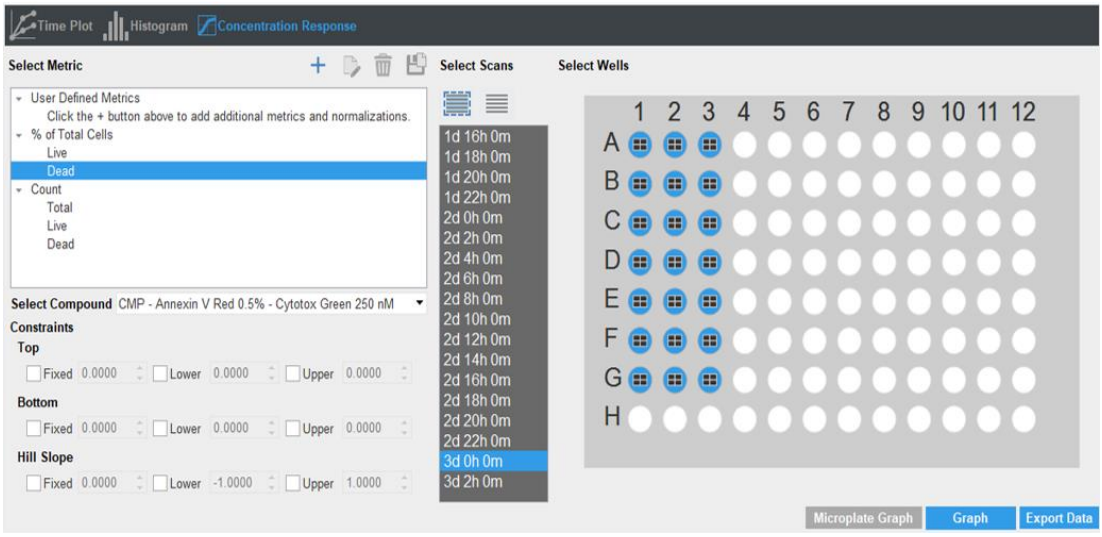

Figure 15. Plotting a concentration response curve using the Incucyte® graphing interface.

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