

Incucyte® Advanced Label-Free Classification

For the Quantification of Cells Using Morphology

This protocol provides a working example for label-free quantification of live and dead cells utilizing the Incucyte® Live-Cell Analysis System and Incucyte® Advanced Label-Free Classification Software Module.

Note: Advanced Label-Free Classification can be performed on any images containing two distinct morphologies, for example, to monitor the differentiation of monocytes to macrophages.

Required Materials

- 96-well flat bottom TC-treated microplate (e.g., Corning Cat. No. 3595)
- Adherent cells of interest (e.g., A549 lung adenocarcinoma) with required medium and supplements
- Cytotoxic compound of interest such as camptothecin (10 mM stock in DMSO)
- Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031) with Advanced Label-Free Classification Analysis Software Module Add-On (Cat. No. BA-04867), including and post version 2021B

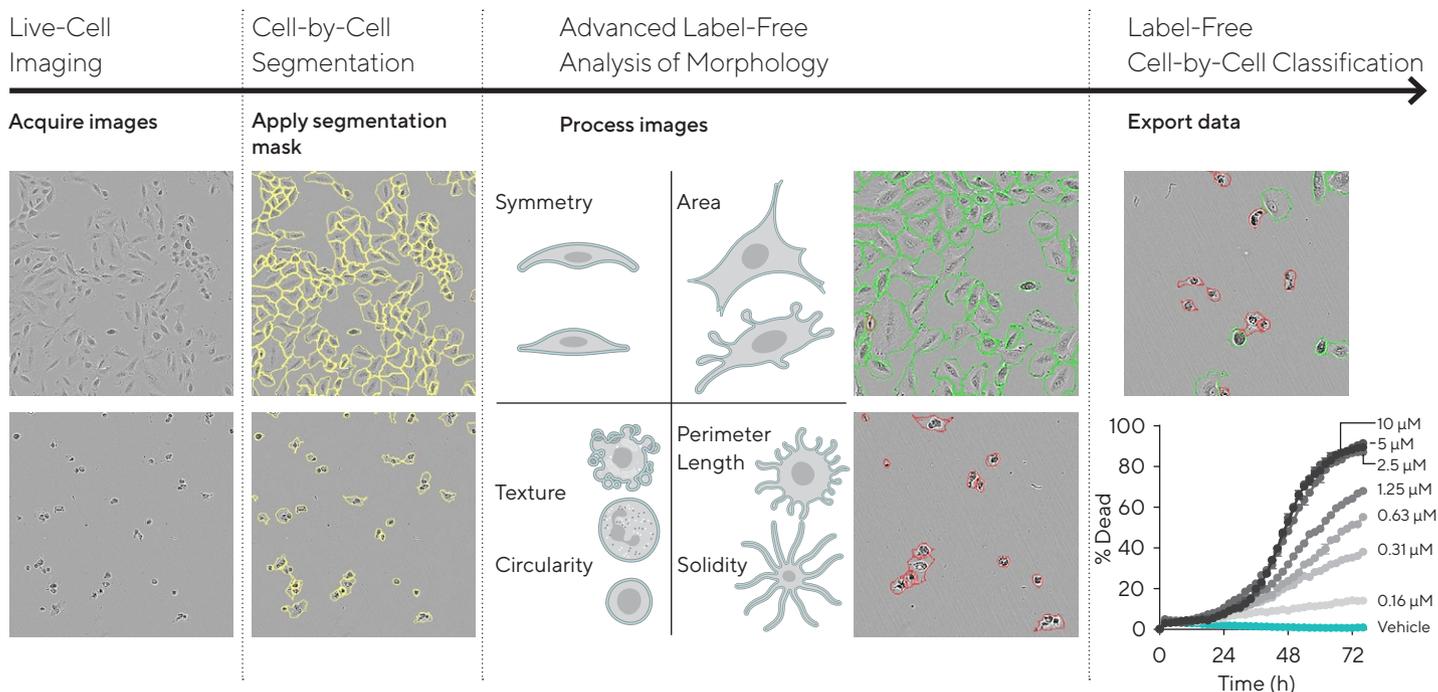
Optional Materials

- Incucyte® Cell Health Reagents

General Guidelines

- 96-well plates should contain control wells which represent the two morphologies of interest. For example, a label-free live | dead assay should have replicate vehicle control wells which represent the diverse morphology of live cells across time and confluence and replicate wells containing a cytotoxic agent known to induce cell death (e.g., camptothecin, CMP).
- When setting up Advanced Label-Free Classification analysis, the representative images chosen will be:
 - Live control wells—full time course where cells are live and Cell-by-Cell segmentation remains appropriate
 - Dead control wells—late time-points only when maximal cell death is observed
- Scans must be acquired using adherent Cell-by-Cell acquisition at 10X or 20X objectives.

Figure 1
Quick Guide



Note. Quick guide shows the steps required to run a label-free live | dead assay. Images are automatically acquired and segmented using the Incucyte® Cell-by-Cell Analysis Software Module. With the Advanced Label-Free Classification Add-On, morphological features are obtained, and a classifier is trained using live and dead control wells. Classifier is then applied to all wells and time-points to achieve number and % live and dead cells per image.

Protocol

Label-Free Live | Dead Assay

- 1.1 Grow cells (e.g., A549) to ~ 70% confluence.
- 1.2 Carefully aspirate medium and wash cells with PBS. Detach cells from flask with Trypsin or other detachment reagent.
- 1.3 Harvest cells by centrifugation and count; resuspend cells to a density of 2×10^4 cells/mL in fresh growth medium. Seed cells into a 96-well plate, 100 μ L/well to achieve a density of 2×10^3 cells/well.

Optional: To compare label-free and fluorescence classification data, seed cells in medium containing 2 x final assay concentration (FAC) cell health reagent, such as Incucyte® Annexin V Dye, and acquire fluorescence scans in the corresponding channel.

- 1.4 Allow cells to settle for 30 minutes at room temperature prior to placing into a 37° C incubator overnight.

Note: Cells must be left to adhere until they have regained their expected morphology. Acquire an image in the Incucyte® Live-Cell Analysis System prior to treatment and examine cell morphology.

- 1.5 Prepare cytotoxic compounds at 2 x FAC in growth medium.

Important: Include replicate control wells containing cytotoxic compound (e.g., CMP, FAC 10 μ M) and vehicle control (e.g., 0.1% DMSO), these are designed to generate images to train the label-free classification job.

- 1.6 Add compound treatments to cell plate at 100 μ L/well. Remove bubbles from the plate using a bottle containing 70-100% ethanol, with the inner straw removed, to gently blow vapor over the surface of each well.
- 1.7 Place plate into Incucyte® Live-Cell Analysis System. Allow plate to warm for 30 minutes prior to imaging.
- 1.8 Acquire scans in Adherent Cell-by-Cell mode every 2 h until control wells indicate maximal cell death.

Figure 2
Example Plate Map for a Label-Free Live / Dead Assay

	All	1	2	3	4	5	6	7	8	9	10	11	12
Dead control images: High CMP treatment (10 μ M), late time-point (~ 72 h)	A	CMP 10 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 2, 50 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 3, 1 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 4, 1 μ g/mL Annexin V 0.5% A549-WT (1) 2K/well		
Remaining wells in this plate contain a concentration range of CMP and 3 additional compounds	B	CMP 5 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 2, 25 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 3, 0.5 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 4, 0.5 μ g/mL Annexin V 0.5% A549-WT (1) 2K/well		
	C	CMP 2.5 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 2, 12.5 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 3, 0.25 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 4, 0.25 μ g/mL Annexin V 0.5% A549-WT (1) 2K/well		
	D	CMP 1.25 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 2, 6.25 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 3, 0.13 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 4, 0.13 μ g/mL Annexin V 0.5% A549-WT (1) 2K/well		
	E	CMP 0.63 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 2, 3.13 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 3, 0.06 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 4, 0.06 μ g/mL Annexin V 0.5% A549-WT (1) 2K/well		
	F	CMP 0.31 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 2, 1.56 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 3, 0.03 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 4, 0.03 μ g/mL Annexin V 0.5% A549-WT (1) 2K/well		
	G	CMP 0.16 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 2, 0.78 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 3, 0.02 μ M Annexin V 0.5% A549-WT (1) 2K/well			Compound 4, 0.02 μ g/mL Annexin V 0.5% A549-WT (1) 2K/well		
Live control images: Vehicle, range of time-points until confluent	H	Annexin V 0.5% A549-WT (1) 2K/well Vehicle											

Note. Plate includes control wells (high CMP known to induce cell death, and vehicle control wells) to train classification model to distinguish live and dead cells. Classifier can then be applied to the whole plate to quantify the concentration response of each compound and obtain EC_{50} values.

Analysis Guidelines

1. Create a New Cell-By-Cell Analysis Definition

Note: It is recommended to create a segmentation mask at the end of the experiment when images of dead cells are available to include in the analysis definition. For detailed information on this process, see Cell-by-Cell Analysis Guidelines document.

- In the Analysis Wizard window, select 'Adherent Cell-by-Cell' Analysis Type.
- Select a set of representative images, ensuring that all morphologies of interest are included.
- Adjust the sliders to create a segmentation mask that appropriately outlines both live and dead cells.
- Once satisfied with all parameters, complete the Launch Wizard analysis by selecting the scan times and wells to be analyzed.

2. Select Images or Cells Representing Two Classes of Interest

Once the Analysis Job is complete, open it and enter Launch Label-Free Classification Wizard. For detailed information on this process, see Advanced Label-Free Classification Guidelines document.

- Rename Classes A and B as Live and Dead.
- Select wells and time-points that represent Live cells, i.e., vehicle wells across a range of time-points and confluences where the Cell-by-Cell segmentation is appropriate. Do not include over-confluent wells where cells have died.
- In the next tab, select wells and time-points that represent dead cells, i.e., CMP 10 μ M late time-points only (usually ~ 72 h). The same images cannot be present in both classes.
- If dead control images contain over 90% dead cells, proceed without refinement to Step 3.
- If the dead control wells contain less than 90% dead cells, the training set may need to be manually refined. Choose a maximum of 12 images.
- Select images to preview the classification results and assist with setting a threshold. Choose at least one image that has partial cell death.
- Select "Preview All" to update the classification definition. If control images are homogeneous (e.g., > 90% cell death), proceed to Step 3.

h. If control images require refinement (where morphology is heterogeneous (e.g., < 90% cell death) in the control), enter refinement mode for the Dead cell class. Refinement is used to select a subpopulation of cells within an image to train a Classification definition. For detailed information on manual refinement, see section “Refinement of control training sets” within Advanced Label-Free Classification Guidelines document.

Note: Some cells may resist cell death induced by compounds with particular mechanisms of action. If images contain a mixture of morphologies (a mixture of normal live and dead cells; or a mixture of dead cells and cells with drug-resistant morphological change), use Advanced Training Set Selection to choose which cells within an image are used to define the Classification algorithm.

3. Create a New Advanced Label-Free Classification

- a. Select Preview All to update the Classification definition.
- b. Adjust the threshold so that the classification of both live and dead cells is appropriate. Once satisfied with the threshold, complete the Launch Wizard analysis by selecting the scan times and wells to be analyzed.

4. Data Interpretation

Once the Classification job is complete, the following metrics are provided:

- a. Time plots:
 - i. % of Total cells classified as Live or Dead per image
 - ii. Count: The total number of segmented cells per image; the number of cells classified as Live per image; the number of cells classified as Dead per image
- b. Concentration response

Tips & Tricks

1. Include Control Compounds on Plate Map

Control wells are intended to generate images with cells that display your morphologies of interest. For a Label-Free Live | Dead assay, the Dead controls should include a compound known to induce cell death—we have suggested CMP but any known cytotoxic agent can be used. Live controls should contain an appropriate vehicle such as DMSO.

2. Good Cell-By-Cell Segmentation is Key

Cell morphology features are derived from the Cell-by-Cell segmentation mask, so it is very important that the cell segmentation outlines cells appropriately. This can be challenging where a mix of very different morphologies are present, but it is important to use representative images and spend time to achieve the best segmentation possible. Refer to the Cell-by-Cell Analysis Guidelines.

3. Check Control Images

Before proceeding with the label-free classification, visually inspect the control images. For dead control images, note which time-points contain the highest percentage of dead cells. Ideally, this should be as close to 100% as possible. For live control images, note which time-points contain growing cells which are well segmented—for example, do not train the classifier using overly confluent images where cells begin to die due to lack of nutrients or where the Cell-by-Cell segmentation breaks down. For growing cells, select

control images across a range of time-points or confluence values to ensure that the full variation of morphologies is included in the training set.

4. Refinement

- a. If the control images contain cells with homogeneous morphology (e.g., close to 100% dead cells), there is no need to enter the advanced refinement mode. Use the whole image to train the classification job, and adjust the threshold as needed.
- b. If control images contain cells with a mixture of morphologies, enter refinement mode to select individual cells within the control images.
- c. Since these images will be manually refined, choose a small number of control images (up to 12).
- d. Using the slider, indicate the percentage of cells that represent the morphology of interest—for example, if around half of the cells possess the morphology of interest, set the slider to 50%.
- e. Move through images and select a representative cell (right-click, set representative cell). Cells which have similar morphology to the representative cell will be automatically selected.
- f. The number of selected cells will depend on the slider position. Decreasing the slider position will choose fewer cells which have a score closer to that of the chosen representative cell. For more information, refer to the “Refinement of Control Training Sets” section of the Advanced Label-Free Classification Analysis Guidelines, in particular Figure 12.

A complete suite of cell health applications is available to fit your experimental needs.
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