Application Note

Cell Analysis

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AI-Powered, Label-Free Cell Segmentation, Counting, and Fluorescence-Based Classification

Using the Agilent xCELLigence RTCA eSight system

Authors

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Abstract

Accurate and reproducible cell segmentation is essential to extract meaningful biological insights from microscopy images, particularly in high-throughput drug discovery and cell biology research. Traditional segmentation methods often rely on fluorescent labeling, which can perturb cellular physiology and require complex workflows. Here we present an AI-powered, label-free cell segmentation solution integrated into the Agilent xCELLigence RTCA eSight system. Powered by deep learning, the software delivers robust, automated single-cell segmentation from brightfield images, enabling reliable cell-level quantification across a wide range of cell morphologies without invasive labeling or manual parameter tuning. The capabilities of the AI-powered cell segmentation feature are demonstrated in complementary application scenarios. First, the software consistently recognizes and segments a series of cell lines with diverse morphologies, supporting accurate cell counting and proliferation analysis. Second, when combined with fluorescence signals, AI-powered cell segmentation enables subpopulation classification. For example, it can identify phagocytic versus nonphagocytic macrophages when Raw 264.7 macrophages are exposed to pHrodo Red E. coli Bioparticles, and it supports live/dead subpopulation analysis when A549 cells are treated with camptothecin. Importantly, AI-based brightfield cell counts show close agreement with fluorescence cell counts in drug response studies, validating label-free quantification for compound evaluation. Finally, this solution is compatible with 384-well plates, simplifying analysis of heterogeneous morphological changes across multiple treatments and enabling efficient, high-throughput compound screening. Overall, the xCELLigence RTCA eSight software AI-powered cell segmentation feature provides a robust, noninvasive, and scalable label-free solution that simplifies image analysis workflows, improves reproducibility, and increases confidence in data driven decisions for cell-based fundamental research and translational applications.

Introduction

Traditional cell segmentation often relies on fluorescent labeling, which requires genetic manipulation or chemical processing that can disrupt the natural physiology of cells. In real-time live-cell experiments, labels can introduce artifacts such as increased cell death,[1] reactive oxygen species accumulation,[2] mitotic arrest, or impaired signaling pathways.[3] This is particularly critical for physiologically relevant primary cell types, such as patient-derived induced pluripotent stem cells,[4] where labeling may not be feasible. As a result, there has been growing interest in robust label-free image analysis that supports longitudinal, nonperturbing measurements.

Accurate segmentation is fundamental for quantitative microscopy-based assays. It enables measurement of total cell number in proliferation, apoptosis, and cytotoxicity assays, where small segmentation errors can distort growth and potency curves over time. When combined with fluorescence imaging, segmentation also supports analysis of protein expression and subpopulation kinetics at the single-cell level. In drug discovery, these readouts are routinely collected across many wells and time points, so analysis must be robust, consistent, and scalable. However, traditional segmentation methods (such as thresholding, region growing, and watershed) are sensitive to image contrast, background variation, and cell density.[5-7] They often require manual parameter tuning, which can introduce user-to-user variability and reduce reproducibility.

Open-source and commercial tools can address some of these challenges, but many still require careful setup and assay-by-assay optimization. Convolutional neural networks (CNNs) offer an alternative approach by learning cell features directly from representative images and generalizing across morphology and imaging conditions.[8]

The xCELLigence RTCA eSight software AI-powered cell segmentation feature leverages trained CNNs to deliver automated, label-free brightfield segmentation at the single-cell level. In practice, this replaces iterative parameter tuning with a preview-and-run workflow designed for high-content, time-lapse datasets. This application note verifies AI-powered cell segmentation across diverse adherent and nonadherent cell types and demonstrate how AI-based brightfield cell counts support high-throughput, physiologically relevant insights into cell health and treatment response.

Together, these features reduce hands-on analysis time, minimize user-dependent variability, and improve cross-experiment reproducibility. For scientists running longitudinal live-cell studies, this translates to faster time-to-answer and greater confidence in kinetic trends.

Assay principle

The Agilent xCELLigence RTCA eSight software AI-powered cell segmentation feature is trained on various cell types with multiple morphologies, including dispersed, aggregated, epithelial-like, and fibroblast-like cells. Over 500 million cells and more than 1.2 million images were used for software validation, supporting accuracy and broad applicability. After the initial model set up, fine tuning was conducted to improve identification of neuron like cells and enhance robustness to scratches and background shadows. The resulting software achieved both reliability and versatility (Figure 1).

To apply AI-powered cell segmentation, select the **Use AI Algorithm box**, preview representative images, and then run image analysis on the full dataset. Segmentation and downstream per-cell measurements are performed within the same analysis run, requiring minimal user input. Compared to threshold-based methods, this workflow reduces analysis time by 80% and delivers more accurate segmentation, resulting in reliable cell count data, even under high confluence conditions (> 90%) or low contrast. By delineating single-cell boundaries, the software also improves fluorescence-based classification and enables dynamic subpopulation analysis (Figure 2). Figures 4 and 5 show examples of phagocytic activity and live/dead classification based on fluorescence signals.

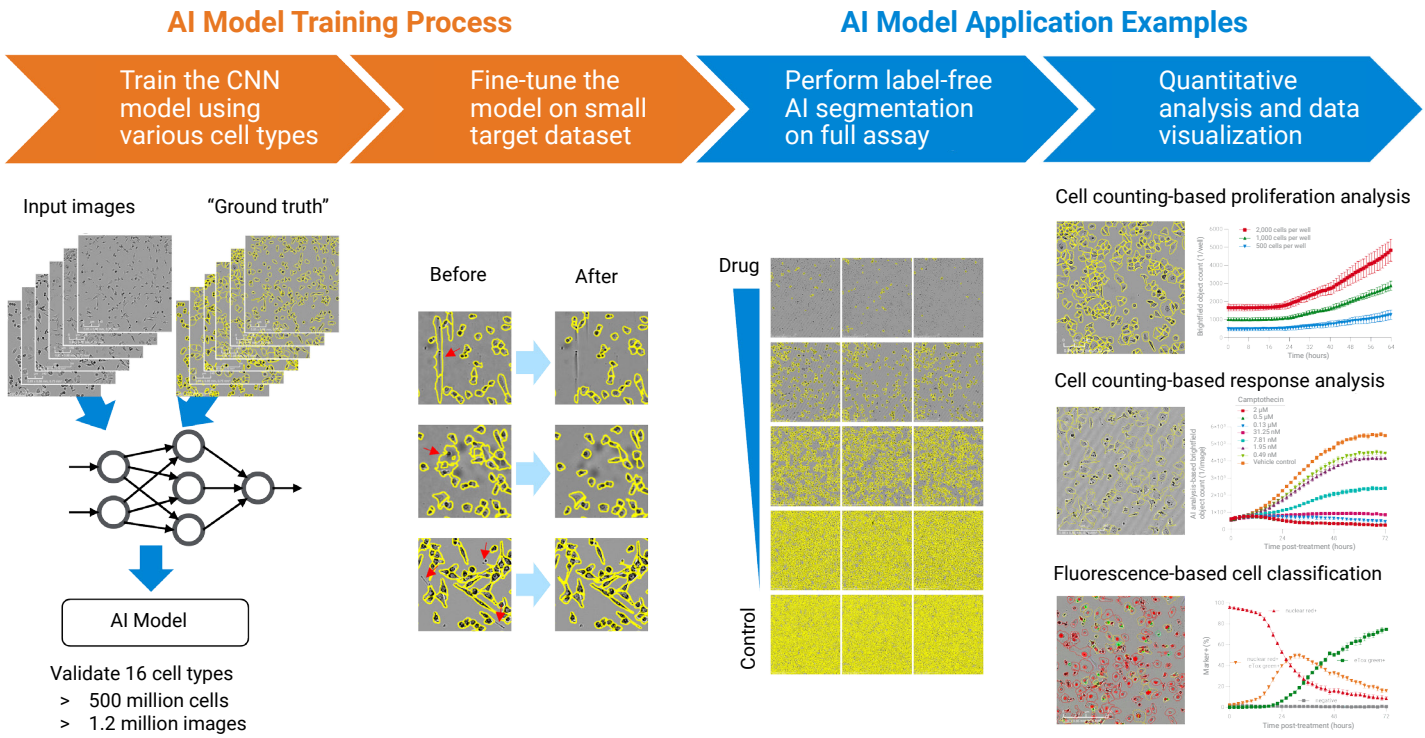

Figure 1. The Agilent xCELLigence RTCA eSight software AI-powered cell segmentation feature undergoes a large-scale training and validation process. The resulting software enables accurate cell segmentation for robust cell counting and fluorescence-based cell classification.

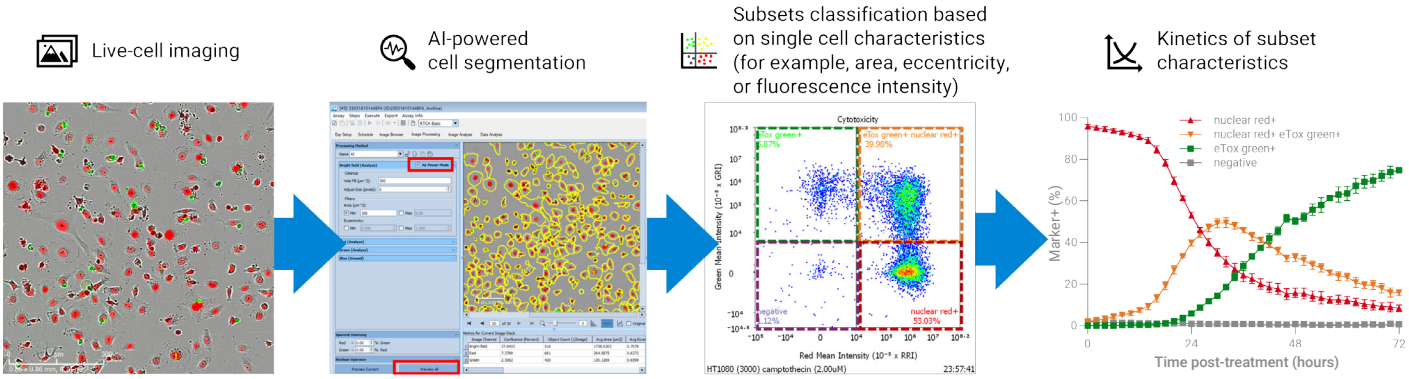

Figure 2. The Agilent xCELLigence RTCA eSight software AI-powered cell segmentation feature enables accurate cell segmentation for robust fluorescence-based cell classification.

Experimental

Cell lines

Cell maintenance and assays were conducted in a 37 °C incubator with 5% CO_2. Cell lines and their growth media are shown in Table 1. Fetal bovine serum (FBS) (Gibco, part number 10099-141) and penicillin/streptomycin (pen/strep) (HyClone, part number SV30010) were used as the media supplements.

Table 1. Cell lines and their culture media.

Cell Lines	Basal Media (Vendor, Part Number)	Media Supplements
BxPC3, SKBR3, Hcc1954	RPMI 1640 (Gibco, 11875-093)	10% FBS + 1% pen/strep
HELA	EMEM (ATCC, 30-2003)	10% FBS + 1% pen/strep
SKOV3	McCoy's 5A (Gibco, 16600-082)	10% FBS + 1% pen/strep
A549	Ham's F-12K (Gibco, 21127-022)	10% FBS + 1% pen/strep
NIH-3T3, Raw264.7	DMEM (Gibco, 12430-054)	10% FBS + 1% pen/strep
A172	DMEM/F12(Gibco, 12634010)	10% FBS + 1% pen/strep
U87	50% F12 (Gibco, 11765-054) + 50% MEM (Gibco, 11095-080)	10% FBS + 1% pen/strep

A549-Red (a stable cell population) was generated from the parental A549 cells by transduction with lentiviral particles harboring the gene encoding red fluorescent protein (Agilent eLenti Red reagent, part number 8711011) and subsequent a short puromycin selection step.

Cell seeding and treatment

Collect cells in the logarithmic growth phase, resuspend, and seed 2,000 to 6,000 cells per well (100 µL) into 96-well plates. Incubate overnight, then add 50 µL of fresh medium containing the treatment at a 3x final assay concentration for the cytotoxicity and phagocytosis assay. For 384-well plates, moisten wells with 20 µL of complete medium, centrifuge at 1,500 rpm for 5 minutes to remove bubbles, and add 30 µL of medium containing 1,000 to 2,000 cells. Incubate overnight, then add 25 µL of medium with treatment and eTox green (Agilent, part number 8711008) at a 3x final assay concentration. Table 2 lists the treatments used in this application note.

Table 2. Reagents used in this application note.

Reagents	Vendor and Part Number	Dilutions
Staurosporine	Sigma-Aldrich, 569397	From 2 µM to 0.49 nM, at 1:4 dilutions
Camptothecin	Sigma-Aldrich, 390238	From 2 µM to 0.49 nM, at 1:4 dilutions From 3 µM to 0.46 nM, at 1:3 dilutions
Taxol	Sigma-Aldrich, 5.08227	From 200 nM to 0.27 nM, at 1:3 dilutions
Vinorelbine	MedChemExpress, HY-12053	From 2 µM to 0.49 nM, at 1:4 dilutions
Doxorubicin	MedChemExpress, HY-15142	From 10 µM to 2.44 nM, at 1:4 dilutions
MG-132	Sigma-Aldrich, 474790	From 10 µM to 2.44 nM, at 1:4 dilutions
pHrodo Red E. coli Bioparticles Conjugate	Thermo Fisher, 354234	From 10 µg/mL to 0.63 µg/mL, at 1:2 dilutions

All assays were run on cradles one to five of the RTCA eSight system, capturing four images per well every one to two hours with a 10x objective. Brightfield exposure was auto-adjusted, while red and green channel exposures were manually set to 100 to 400 ms based on fluorescence brightness. Instrument and software bundles are listed in Table 3.

Table 3. AI software and instrument bundle.

Description	Part Number
Agilent xCELLigence RTCA eSight instrument AI bundle (GPU workstation and software licenses included)	S2836AA
AI software license (individual)	S2807-90102

Results and discussion

AI-powered cell segmentation enables label-free, time-lapse cell counting for proliferation analysis

First, we evaluated segmentation performance across a panel of cell lines with diverse morphologies to reflect common real-time live-cell workflows. In Figure 3A, representative brightfield images show accurate single-cell boundaries (yellow outlines) for aggregated cells (BxPC3, SKBR3, Hcc1954), epithelial-like cells (HELA, SKOV3, A549), and cells with prominent protrusions (NIH3T3, A172, U87). These results demonstrate consistent recognition and segmentation across cell types. When applied across time, the resulting counts generate proliferation curves suitable for comparing growth kinetics between conditions (Figure 3B).

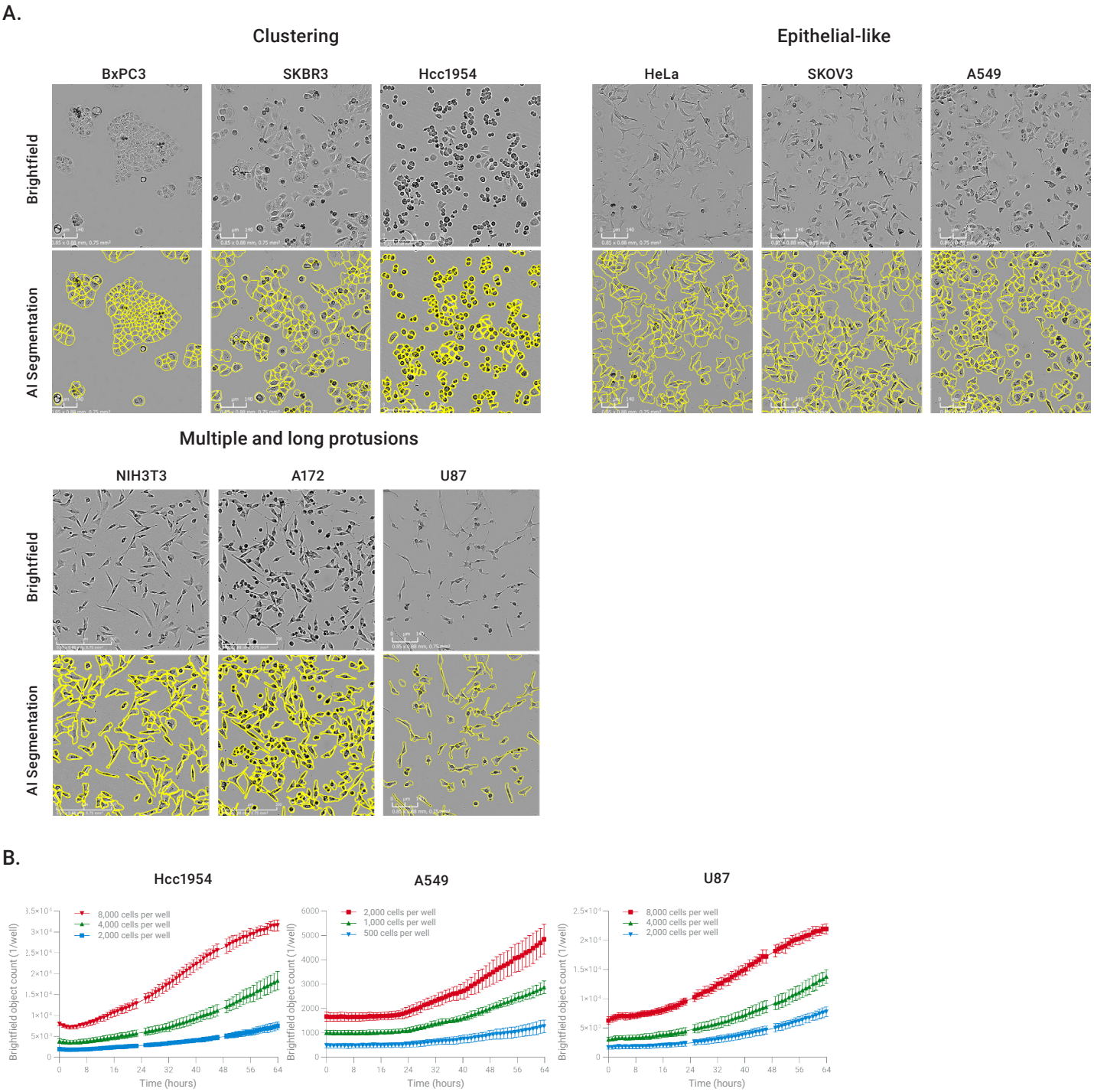

Figure 3. AI-powered cell segmentation enables label-free cell counting for proliferation analysis. (A) Representative images show that the software accurately segments a wide range of cell types with diverse morphologies. (B) Cell counting over time plot.

AI-powered cell segmentation maintains accurate cell boundaries in the presence of bioparticles (phagocytosis assay)

The software was optimized to maintain accurate segmentation despite impurities, fibers, scratches, and biological particles. Figure 4 shows Raw264.7 macrophages exposed to 5 µg/mL pHrodo Red E. coli Bioparticles. These bioparticles are nonfluorescent at neutral pH but emit red fluorescence after internalization. Using cell-by-cell analysis, cells were classified by fluorescence: phagocytic cells are outlined in magenta, whereas nonphagocytic cells are outlined in blue (Figure 4A). Quantitative analysis showed a positive correlation between red fluorescence intensity and bioparticle concentration (Figure 4B). The percentage of phagocytic cells increased within one to three hours and then plateaued. At 5 µg/mL, ~ 95% of cells were phagocytic, and this fraction decreased with lower bioparticle concentrations (Figure 4C). Because segmentation remains stable in the presence of particles, fluorescence intensity can be quantified per cell across time without manual mask retuning.

AI-powered cell segmentation enables label-free cell counting and fluorescence-based cell classification in cytotoxicity assays

In Figure 5A, the yellow boundary outlines show that the software segments both normally proliferating A549 cells and rounded cells consistent with camptothecin-induced cell death. Although the current software version does not yet provide direct label-free live/dead calls (such as with two separate label-free masks), it supports fluorescence-based live/dead classification and subpopulation quantification using the cell-by-cell analysis feature (Figure 5B).

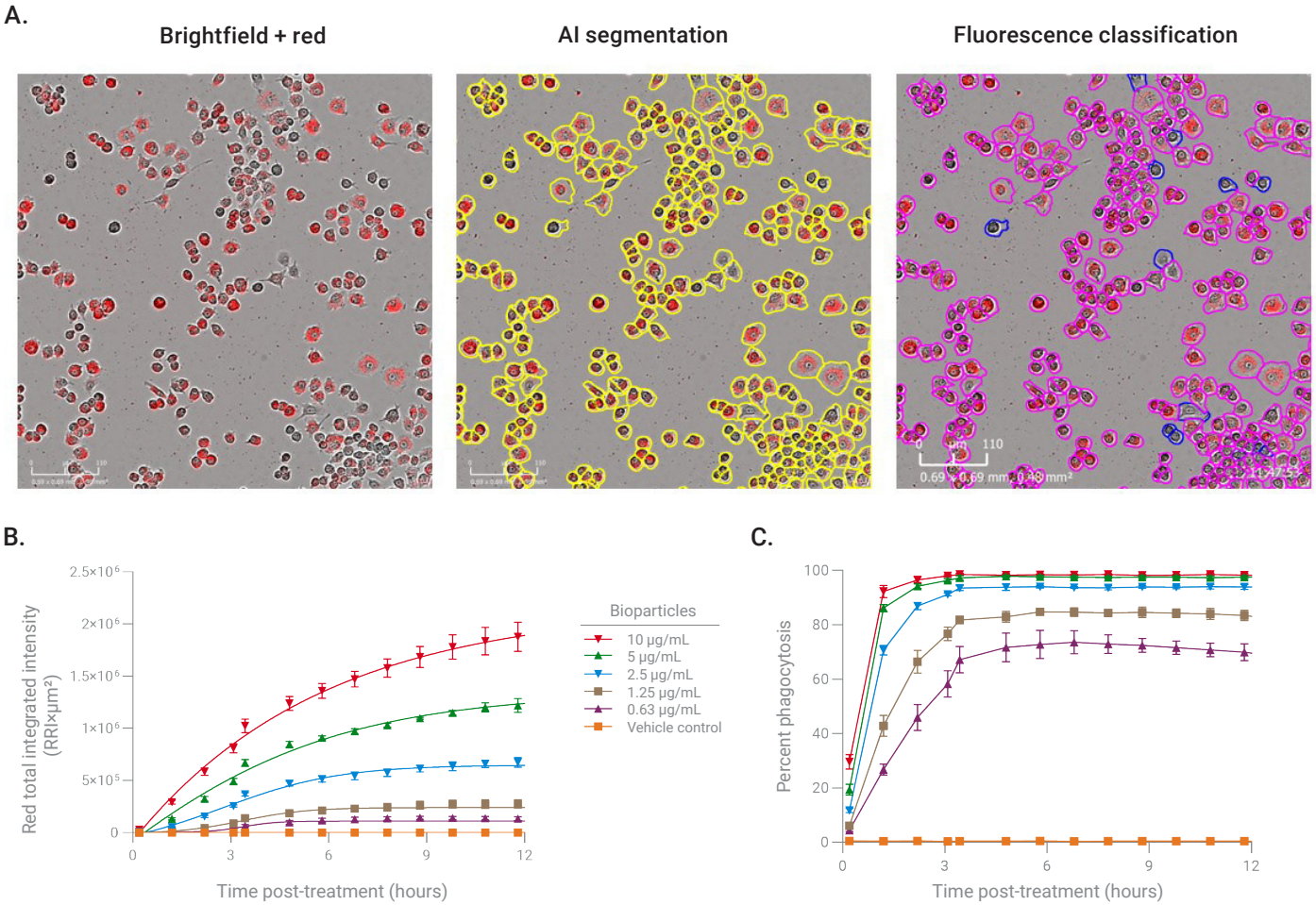

Figure 4. Robust AI-powered cell segmentation enables accurate quantification of phagocytic cells. Raw264.7 cells are seeded at a density of 10,000 cells/well into a 96-well plate and incubated overnight. Then, pHrodo™ Red E. coli BioParticles were added into the wells at various concentrations (10 µg/mL to 0.63 µg/mL, 1:2 dilution). (A) Representative images show RAW264.7 cells treated with 5 µg/mL bioparticles after 12 hours. The software accurately segments cells (yellow brightfield outline), while fluorescence classification distinguishes phagocytic cells (magenta outline, red fluorescence) from non-phagocytic cells (blue outline, no fluorescence). (B) Red total integrated intensity plot. (C) The percentage of phagocytic cells plot.

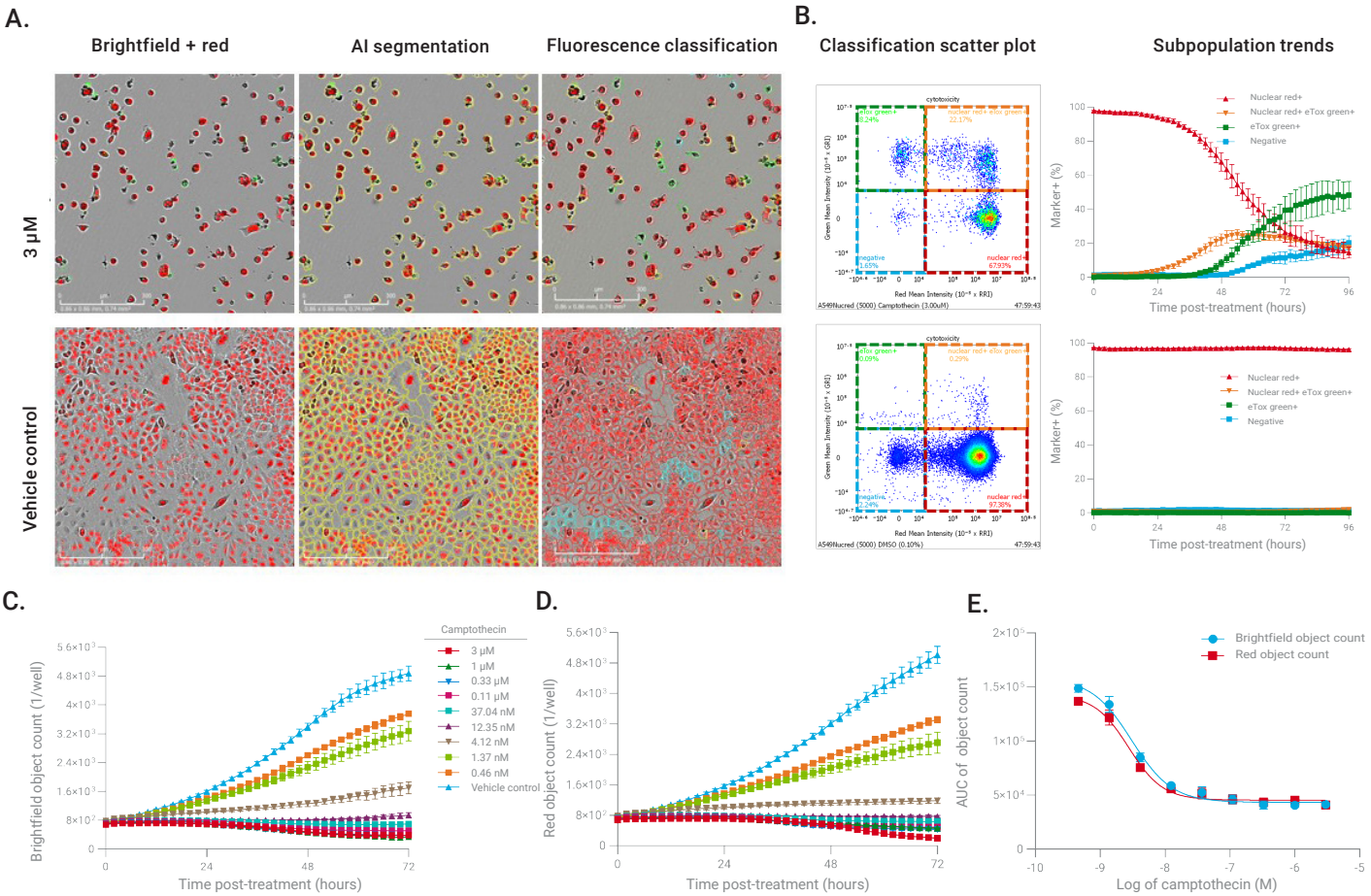

Figure 5. AI-powered cell segmentation enables quantifying the A549 cell response to camptothecin through cell counting and fluorescence-based live/dead cell classification. A549 red cells were seeded and treated with camptothecin (3 µM to 0.46 nM, 1:3 dilutions) after overnight incubation, and eTox green was used to selectively stain the nuclei of dead cells. Representative cell images 24 hours after treatment with a yellow brightfield segmentation mask and fluorescence classification masks (red = RFP+eTox-, orange = RFP+eTox+, green = RFP-eTox+, cyan = double negative) are shown (A). Cell subsets were classified based on red and green fluorescence using the eSight Cell-by-Cell Analysis function, and the percentage of subpopulations change over time (B). (C) Brightfield object count as a function of time. (D) Red object count as a function of time. (E) Dose-response curves generated from (C) and (D).

This allows users to run a label-free segmentation workflow while optionally anchoring viability readouts to fluorescence markers, depending on assay needs.

We further evaluated whether AI-based brightfield object counts can quantify drug response. In Figures 5C–E, brightfield object counts decrease with increasing camptothecin concentration, matching the trend in viable cell numbers measured by Agilent eLenti Red labeled nuclei. The RTCA eSight software calculated IC_{50} values of 3.05 nM (brightfield counts) and 2.74 nM (red counts) (Figure 5E). The close agreement supports the use of label free counts for quantitative drug effect assessment.

AI-powered cell segmentation enables one-click analysis of diverse drug-induced cellular morphological changes

With traditional threshold-based workflows, drug-induced morphological changes often require extensive image-processing parameter tuning and sometimes separate parameter sets for each treatment condition. The AI-powered segmentation workflow avoids this reoptimization: it runs with a single click, reduces user-introduced bias, and returns objective, accurate results. This is particularly useful in time-lapse studies, where maintaining consistent segmentation across time points is critical for interpreting kinetic response profiles.

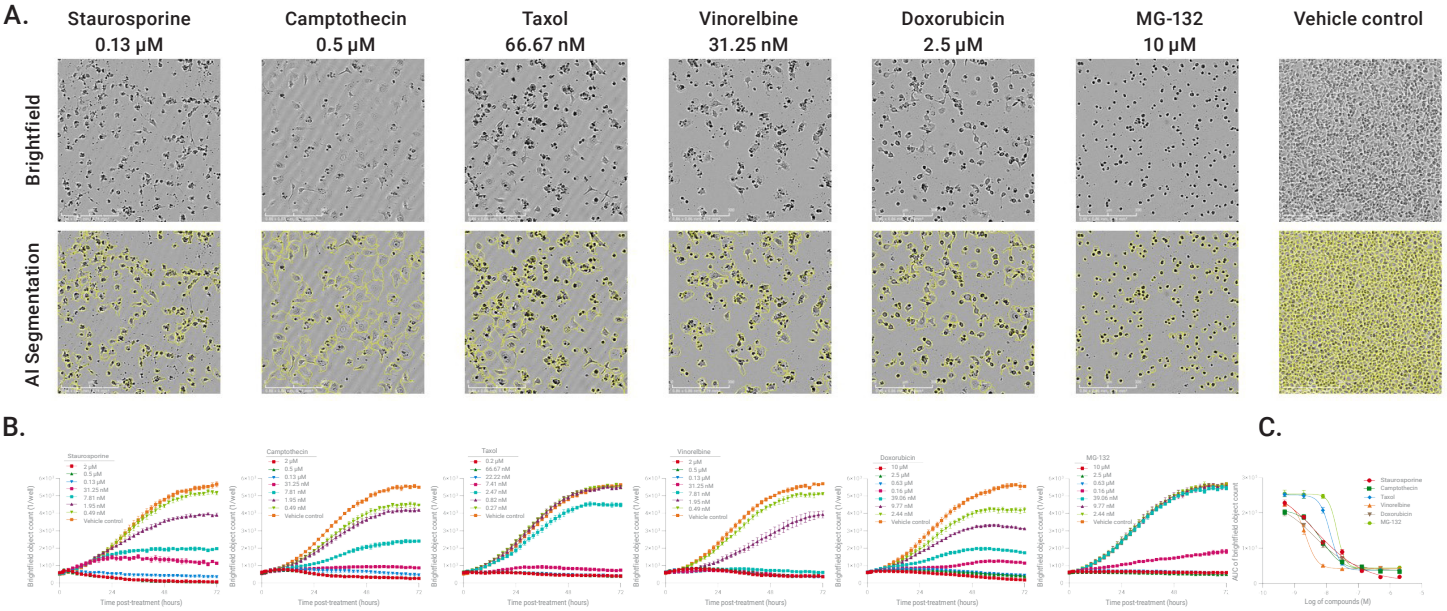

Figure 6. AI-powered cell segmentation simplifies compound evaluation in high-throughput 384-well microplates. HT1080 cells are seeded at a density of 10,000 cells per well into a 384-well plate and incubated overnight. Then compounds were added to the wells at multiple concentrations. (A) Representative images were captured 64 hours post-treatment, featuring a yellow brightfield segmentation mask outline. (B) Time courses of brightfield object count display the concentration-dependent decrease in cell counts. (C) Dose-response curves generated from (B).

In Figure 6A, the software accurately segments HT1080 cells treated with six compounds (staurosporine, camptothecin, taxol, vinorelbine, doxorubicin, and MG 132) in a 384-well plate. Label-free cell counting reveals dose-dependent cytotoxicity for each compound (Figure 6B). Based on cell counts, the RTCA eSight software calculated IC_{50} values of 4.92, 7.06, 13.09, 2.25, 4.18, and 21.42 nM, respectively (Figure 6C).

Conclusion

The AI-powered cell segmentation feature integrates deep-learning-based segmentation into the Agilent xCELLigence RTCA eSight system workflow to support reproducible, label-free analysis of time-lapse imaging datasets. It enables robust recognition and precise segmentation across heterogeneous cell morphologies, provides reliable cell counts, and supports quantitative analysis of proliferation and treatment response. In addition, AI-based segmentation can be combined with fluorescence classification to quantify live/dead subpopulations in toxicity assays and phagocytic cells in phagocytosis assays. By reducing manual parameter tuning and maintaining consistent segmentation across wells and time points, the workflow improves throughput and strengthens confidence in kinetic trends. The streamlined AI analysis workflow reduces the time required for traditional threshold-based analysis by 80%, minimizing user input while delivering more reproducible and unbiased results.

References

1. Liu, H. S.; Jan, M. S.; Chou, C. K.; Chen, P. H.; Ke, N. J. Is green fluorescent protein toxic to the living cells?. Biochem. *Biophys. Res. Commun.* **1999**, *260* (3), 712–717. DOI: 10.1006/bbrc.1999.0954
2. Dixit, R;, Cyr, R. Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy. *Plant J*. **2003**, *36*(2), 280–90. DOI: 10.1046/j.1365-313x.2003.01868.x
3. Agbulut, O.; Coirault, C.; Niederländer, N.; Huet, A.; Vicart, P.; Hagège, A.; Puceat, M., Menasché, P. GFP expression in muscle cells impairs actin-myosin interactions: implications for cell therapy. *Nat. Methods*. **2006**, *3*(5), 331. DOI: 10.1038/nmeth0506-331
4. Saito-Diaz, K.; Zeltner, N. Induced pluripotent stem cells for disease modeling, cell therapy and drug discovery in genetic autonomic disorders: a review. *Clin. Auton. Res.* **2019**, *29*(4), 367–384. DOI: 10.1007/s10286-018-00587-4
5. Edlund, C.; Jackson, T. R.; Khalid, N.; Bevan, N.; Dale, T.; Dengel, A.; Ahmed, S.; Trygg, J.; Sjögren, R. LIVECell-A large-scale dataset for label-free live cell segmentation. *Nat. Methods.* **2021**, *18*(9), 1038–1045. DOI: 10.1038/s41592-021-01249-6
6. Otsu, N. A Threshold Selection Method from Gray-Level Histograms. *IEEE. Trans. Syst. Man. Cybern.* **1979**, *9*(1), 62–66. DOI: 10.1109/TSMC.1979.4310076
7. Wu, Y.; Li, Q. The Algorithm of Watershed Color Image Segmentation Based on Morphological Gradient. *Sensors (Basel)*. **2022**, *22*(21), 8202. DOI: 10.3390/s22218202
8. Convolutional neural network. *Wikipedia*. https://en.wikipedia.org/wiki/Convolutional_neural_network (accessed 2026-05-06)
9. F-score. *Wikipedia*. https://en.wikipedia.org/wiki/F-score (accessed 2026-05-06)
10. Dice-Sørensen coefficient. *Wikipedia*. https://en.wikipedia.org/wiki/Dice-S%C3%B8rensen_coefficient (accessed 2026-05-06)

Products used in this application

Agilent products

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