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Application Note

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Quantifying Chemotherapeutic Cytotoxicity in Glial Cells using AI-Driven Label-Free Cell Analysis

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Introduction

Glioblastoma multiform (GBM) is an aggressive glioma that originates from astrocytes and is associated with poor prognosis.[1] Several barriers exist in the treatment of GBM, including the localization of the tumor within the brain, a high rate of malignant invasion, tumor heterogeneity, and an intrinsic resistance to conventional therapies. Despite concerted efforts to overcome these, a lack of translational *in vitro* models and robust analytical tools makes deciphering the complex molecular interactions challenging.[2,3]

Technological progression has facilitated the adoption of advanced cellular models, such as primary cells or induced pluripotent stem cells (iPSCs), that more precisely represent human phenotypes. This has simultaneously driven the requirement for label-free, non-perturbing analytical methods to accurately capture biologically relevant data in complex models and provide critical insights into disease processes.[4,5] By eliminating fluorescent labels, this ensures that experimental observations are not attributed to the label, or the labeling process, and provides a method that is amenable to cell types where labeling is not feasible, such as sensitive or rare cells.[6,7]

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Live-cell imaging enables the acquisition of phase contrast images in a non-perturbing manner. Alongside the incorporation of Artificial Intelligence (AI) into image analysis workflows, this has empowered accurate quantification of a broad spectrum of cellular models, making it a powerful approach to make data-driven decisions and further understanding of cancer biology. These innovative technologies, based on neural network algorithms, are more complex than traditional image analysis and facilitate more accurate segmentation of heterogenous cell morphologies whilst minimizing user-introduced bias.[4,8]

In this application note, we describe a robust solution for label-free cell segmentation and live/dead classification of individual cells using integrated AI-based software. We exemplify how this approach can provide high-throughput insights into glial cell health in response to clinically relevant chemotherapeutic treatments.

Assay Principle

The Incucyte® AI Cell Health Analysis Software Module enables label-free quantification of cell viability. The analysis module uses trained convolutional neural networks (CNNs), which automatically analyzes phase contrast images to segment individual cells and classify them as live or dead, all in one step. This streamlined workflow (Figure 1) requires minimal user input, providing unbiased results which can be directly compared across assays.

Phase contrast images are acquired using AI Scan acquisition with 10X or 20X objectives in microplates up to 384-well throughput. These images can be analyzed using the Incucyte® AI Cell Health Analysis Software Module which provides metrics such as Total Cell Count (all objects), as well as the number and percentage of live and dead cells. In cases where optional fluorescence images are acquired, the mean and total integrated intensity within all cells, as well as within the live or dead subpopulations, will be provided. Fluorescence classification can be performed as an additional analysis, again providing metrics describing the count and percentage of high vs. low fluorescence within total cells, and within live or dead subpopulations.

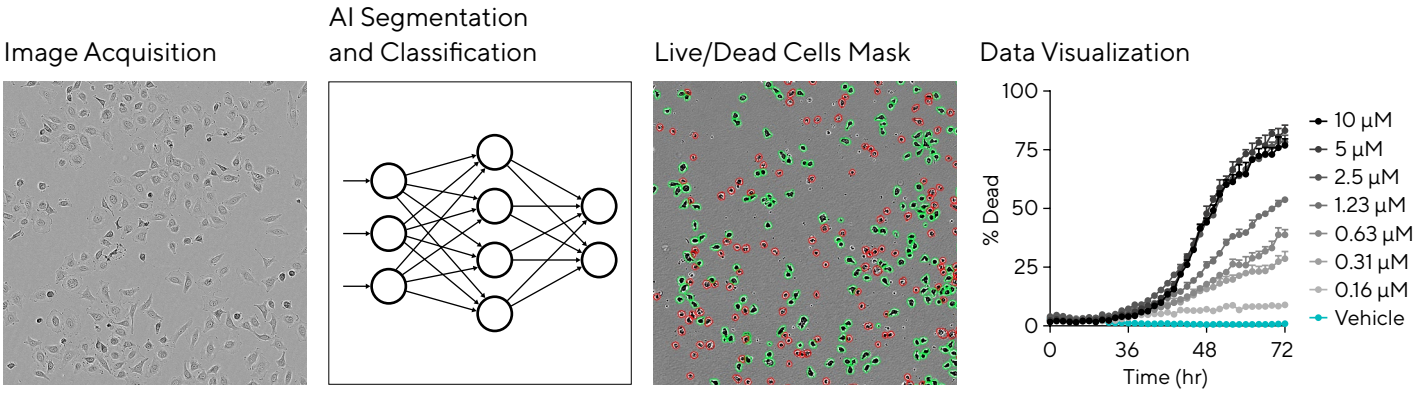


Figure 1: *Incucyte® AI Cell Health Analysis Workflow.*

Phase contrast images are acquired and processed using neural networks (CNN), to automatically segment and classify cells as live or dead.

Precise segmentation provides accurate cell count data even at high cell confluence and yields reliable proliferation data. Label-free classification of cells as live or dead enables quantification of cell viability within a physiologically relevant and non-perturbing environment. The combination of label-free analysis with optional fluorescence readouts from the live or dead subpopulations provides additional insight into mechanisms of cell death.

AI-Driven Cell Segmentation

The AI Cell Health segmentation model was trained using phase contrast images which were manually annotated to identify the boundary of individual cells as described previously.[9,10] The resulting segmentation is highly accurate and adaptable to numerous cell morphologies. Figure 2 shows the AI segmentation applied to different glial cell types including an adherent glioblastoma cell line (A172), a semi-adherent microglial cell line (BV2), flat and transparent primary rat cortical astrocytes (Incucyte® rAstrocytes), and dead cells are accurately outlined (T98G cells treated with Taxol (paclitaxel, 500 nM)).

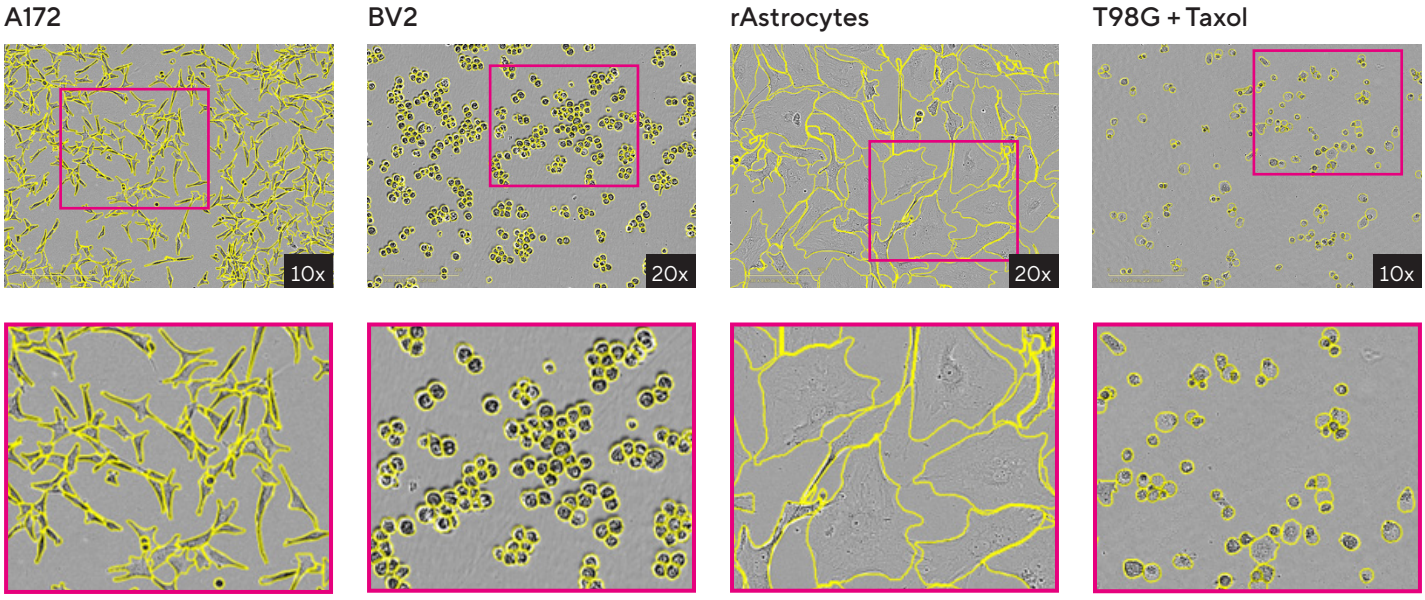


Figure 2: *Incucyte® AI Cell Health Analysis Accurately Segments a Wide Range of Live and Dead Cell Types With Diverse Morphologies.*

HD phase contrast images were acquired using the Incucyte® Live-Cell Analysis System at 10X or 20X magnification. AI Cell segmentation (yellow outline) shown for glial cell types.

AI-Driven Live/Dead Classification

AI Cell Health Analysis can identify live and dead cells without the requirement for a fluorescent reagent. For the validation of AI-driven classification, a wide range of cell types were treated with cytotoxic compounds in the presence of Incucyte® Cytotox Dye which enters non-viable cells, increasing their fluorescence intensity.[10] Here, we demonstrate this validation process applied to glial cell lines of interest (Figure 3).

T98G glioblastoma cells, A172 glioblastoma cells, and BV2 microglia cells were treated with concentration ranges of compounds to induce cell death in the presence of Incucyte® Cytotox Green Dye (Figure 3). Quantification of cell death was performed using both Incucyte® AI Cell Health Live/Dead classification (AI-driven, label-free analysis) and fluorescence classification of Cytotox positive cells. Phase and fluorescence blended images show live and dead cells for each cell type (Figure 3A). Time courses show percentage of dead cells in response to compound treatment and display comparable time- and concentration-dependent responses between fluorescence (Cytotox) and Incucyte® AI Cell Health classification (label-free) across all conditions tested (Figure 3B). Concentration response curves for T98G and A172 (72h) or BV2 cells (24h) confirm analogous compound efficacies using label-free and fluorescence analysis (Figure 3C). This confirms that the label-free Incucyte® AI Cell Health Analysis accurately identifies cell death induced by compounds with different mechanisms of action across a heterogenous group of glial cell types.

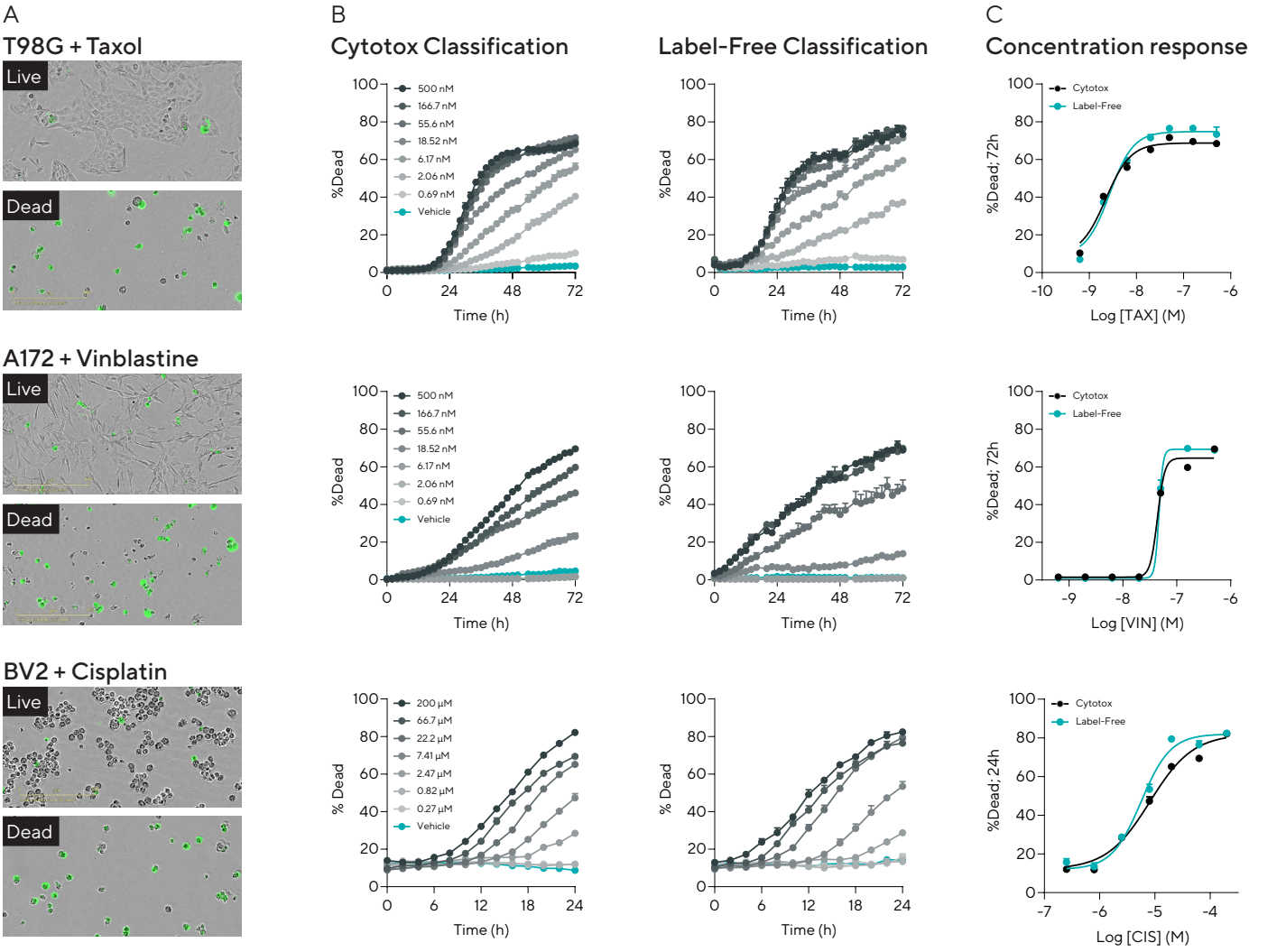


Figure 3: *Incucyte® AI Cell Health Analysis Produces Cytotoxicity Data Comparable to Standard Fluorescence Methods.*

A172, T98G, or BV2 cells were treated with concentration ranges of 3 different cytotoxic compounds in the presence of Incucyte® Cytotox Dye. Phase and fluorescence images are shown for live and dead cells for each cell type (A). Time courses show the percentage of dead cells over time using fluorescence classification (cytotox) or Incucyte® AI Cell Health Analysis (label-free). Concentration response curves plot cell death at 72 (T98G and A172) or 24 hours (BV2) (C). Data shown as mean ± SEM, n= 3 replicates.

Chemotherapeutic Response of Glioblastoma Cells in Microplate Throughput

Genetic heterogeneity of GBM is well recognized and is considered a contributing factor in the lack of effective chemotherapy for new and recurrent disease.[11] The Akt-PI3K signaling pathway, which controls cell growth and survival, is negatively regulated by the tumor suppressor phosphatase and tensin homolog (PTEN).[12] Loss of PTEN function has been associated with uncontrolled proliferation and tumor resistance and PTEN is commonly deleted or mutated in up to 80% of GBMs.[3] Information on genetic alterations and the mechanisms associated with tumor resistance has the potential to provide the fundamental basis for more precise or novel therapeutic strategies.

To examine drug sensitivity, we compared 3 glioblastoma cell lines with different PTEN expression status in their response to chemotherapeutic compounds with varied mechanisms of action. Initially, A172 (PTEN-negative[3]), T98G (PTEN expressing[13]), and U87-MG cells (PTEN-deficient[14]) were treated with 13 clinically relevant chemotherapeutics consisting of high and low concentrations in a 96-well plate (Figure 4). Microplate views of percentage of dead cells over time (Figure 4A) and heat maps at 72 hours (Figure 4B) were utilized to identify compound hits that suggested differential sensitivity across the three cell lines. Phase contrast images were used to confirm the detected levels of cell death (Figure 4C). For most compounds we observed that U87-MG had lower percentages of cell death compared to A172 and T98G cells, suggesting reduced compound sensitivity.

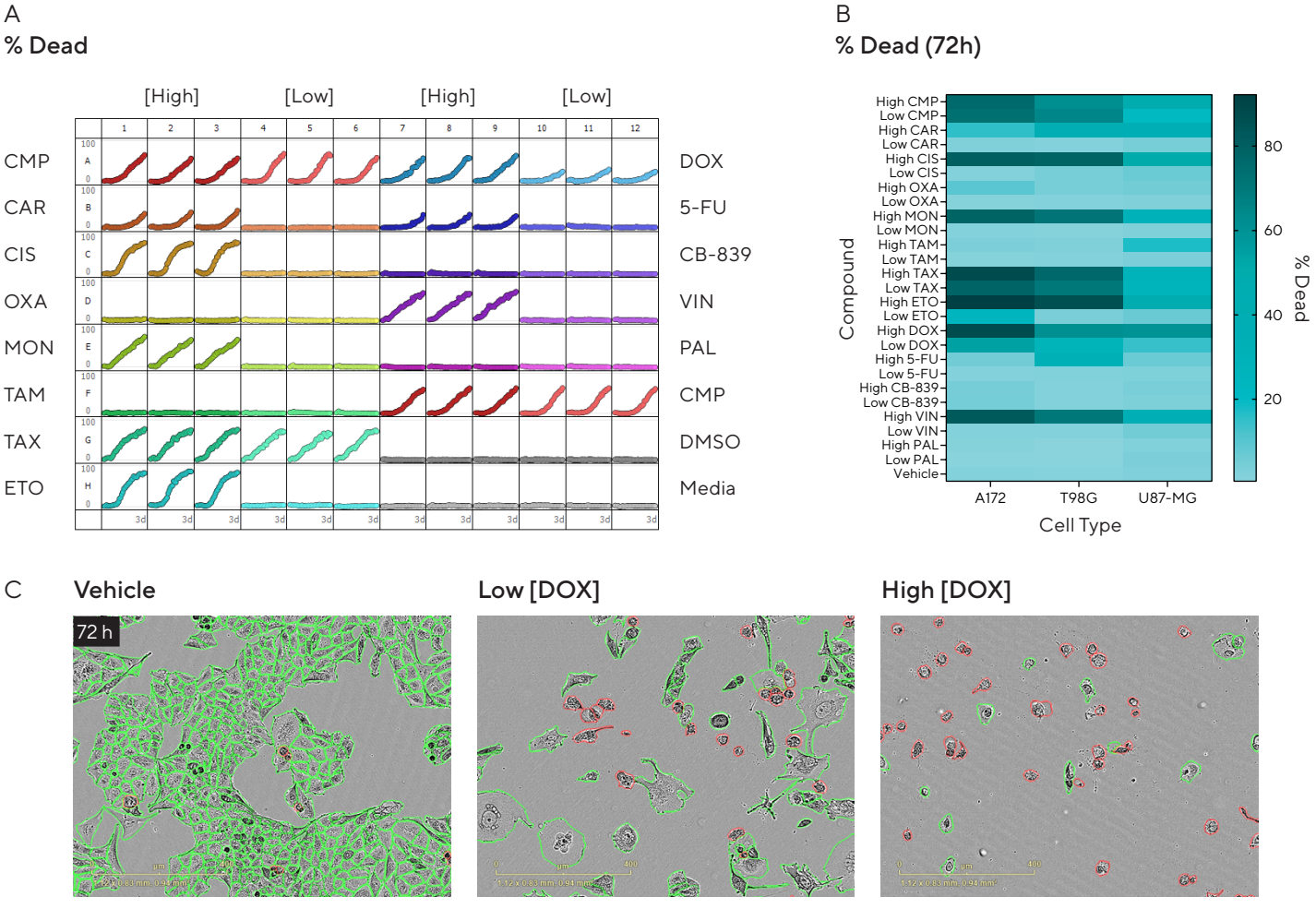


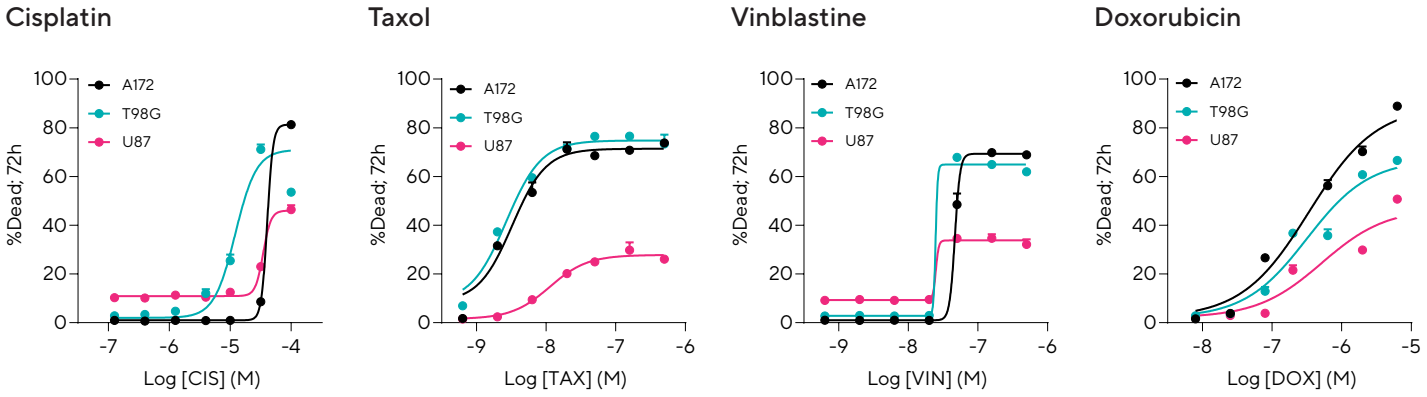
Figure 4: *Label-Free Screening of Glioblastoma Responses to Chemotherapeutic Compounds in Microplate Format.*

A172 (PTEN-negative), T98G (PTEN-expressing), and U87-MG (PTEN-deficient) glioblastoma cell lines were seeded into 96-well plates (2,000 cells/well) and once adhered, treated with high or low concentrations of 13 chemotherapeutic compounds. Microplate view shows the percentage of dead cells for T98G cells over 3 days (A). Heat map of the percentage of dead cells at 72 hours allows for comparison of compound toxicity across glioblastoma cell lines (B). Representative phase images show Incucyte® AI Cell Health Live (green) and Dead (red) classification masks for Doxorubicin (DOX) treated or vehicle T98G cells at 72 hours. Data shown as mean ± SEM, n= 3 replicates.

Four compounds were selected to investigate further based on observed differences in cell death. The glioblastoma cell lines were seeded into 96-well plates, treated with a 3-fold serial dilution of cisplatin, Taxol (paclitaxel), vinblastine, and doxorubicin, and cell death was monitored over 3 days (Figure 5). Concentration response curves (Figure 5A) for each compound are shown at 72 hours, with reported EC_{50} and maximal percentage cell death values (Figure 5B). Data revealed differences in compound cytotoxic effects across cell lines and across compounds with different mechanisms of action, with U87-MG exhibiting reduced sensitivity compared to T98G and A172 cells for all compounds. For example, Taxol, a highly potent anti-cancer agent commonly used against solid tumors, induced comparable levels of efficacy in A172 and T98G cells with maximal cell death values of 73.8 % and 76.6 %, respectively, and identical EC_{50} values of 0.003 µM. However, efficacy was reduced in U87-MG cells inducing maximal cell death of 27.1 % and an EC_{50} value of 0.014 µM.

This suggests that the PTEN status could be one of multiple factors, as part of complex regulatory signaling pathways, that modify the efficacy of these of compounds. Overall, the data highlights how label-free analysis can robustly be used to examine multiple conditions in high-throughput, with the potential to gain deeper insights into the genetic influences on drug resistance in an unbiased manner.

A



B

Compound	Mechanism of Action	A172		T98G		U87-MG	
		EC_{50} (µM)	Max (%)	EC_{50} (µM)	Max (%)	EC_{50} (µM)	Max (%)
Cisplatin	DNA alkylating agent	40.6	80.5	12.7	70.8	36.5	43.8
Taxol	Inhibitor of microtubule dissociation	0.003	73.8	0.003	76.6	0.014	27.1
Vinblastine	Disrupts microtubule assembly	0.054	69.8	0.028	67.8	0.026	31.4
Doxorubicin	DNA intercalator; topoisomerase II inhibitor	0.33	88.9	0.52	66.6	0.69	46.5

Figure 5: *Profiling Cell Death Responses to Concentration Ranges of Chemotherapeutic Compounds in Glioblastoma Cell Lines.*

A172 (PTEN-negative), T98G (PTEN-expressing), and U87-MG (PTEN-deficient) cells were seeded into 96-well plates (2,000 cells/well) and once adhered treated with concentration range of Cisplatin, Taxol, Vinblastine, and Doxorubicin. Transformed data at 72 hours compares compound efficacies (A). Table reports EC_{50} and maximal the percentage of dead cells values observed at 72 hours across all conditions studied (B).
Data shown as mean ± SEM, n= 3 replicates.

Label-Free Analysis Enables Examination of Compound Mechanisms of Action in Primary Astrocytes

Traditionally the field of neuro-oncology has heavily relied on tumor cell lines such as neuroblastomas or glioblastomas. Whilst these approaches remain useful, they have limited translational value and are unable to fully recapitulate the complexity and heterogeneity observed in brain tumors. Consequently, there is an increase in the use of more sensitive or rare cell types, such as iPSCs and primary cells. To support drug discovery and accelerate these advanced models, the continued development of non-perturbing *in vitro* assays and analytical approaches are essential.

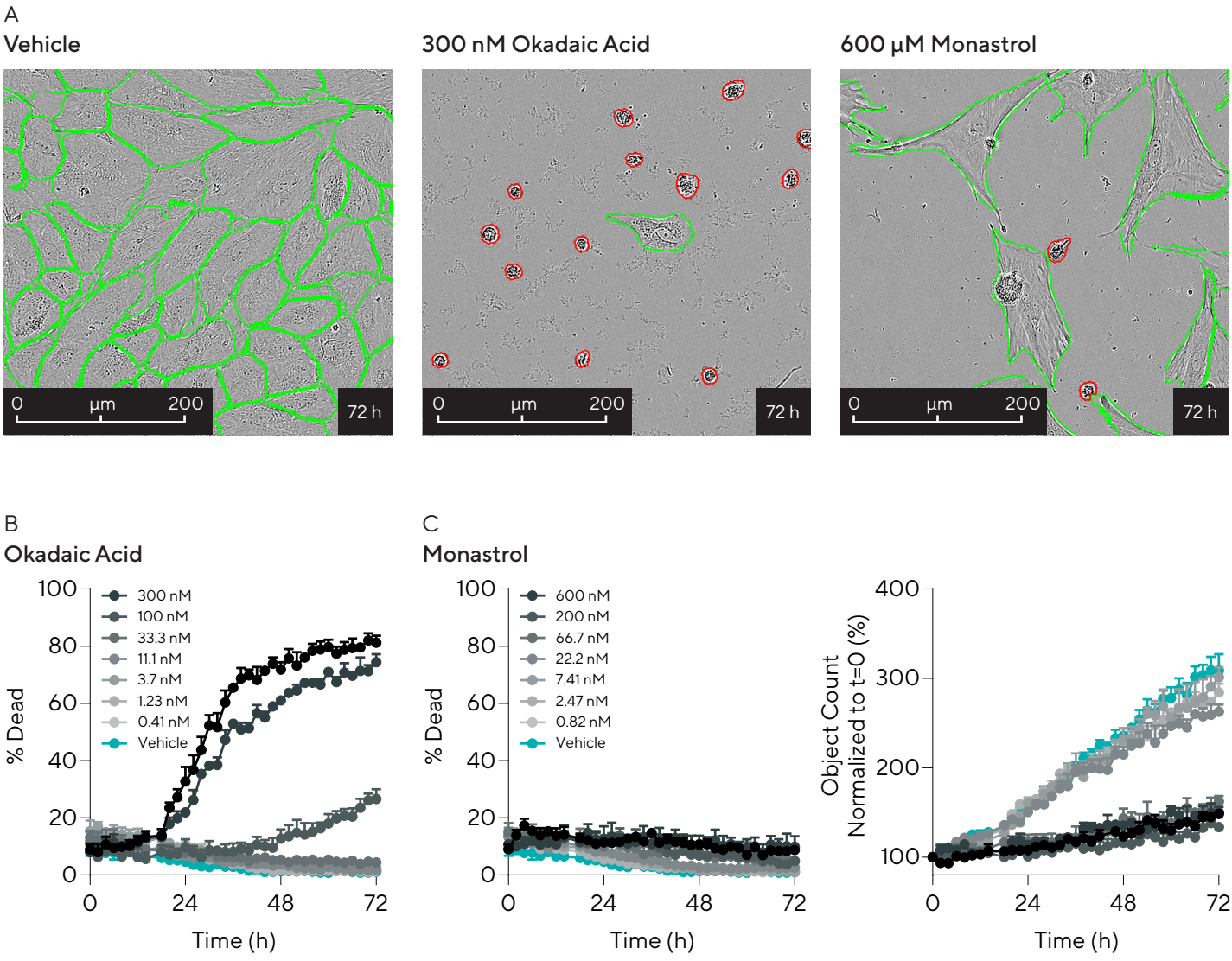


Figure 6: *Label-Free Analysis of Compound Mechanisms of Action.*

Primary rat cortical astrocytes (Incucyte® rAstrocytes) were seeded onto Poly-L-Lysine (PLL) coated 96-well TPP plates (2,500 cells/well) and treated with a concentration range of okadaic acid or Monastrol. Cell death was quantified using Incucyte® AI Cell Health Analysis Software Module. Images of vehicle and drug-treated conditions show AI Live (green) and Dead (red) classification masks and enable visualization of morphological changes (A). Time course shown for okadaic acid quantifying the percentage of dead cells (B). Time courses shown for % dead and total cell count for Monastrol (C). Data shown as mean ± SEM, n= 3 replicates.

To demonstrate the utility of this label-free analysis we investigated mechanisms of drug action in primary astrocytes. Rat cortical astrocytes were treated with a concentration range of two compounds, okadaic acid (dual protein phosphatase inhibitor) and Monastrol (small molecule inhibitor of kinesin-5) and were monitored over 3 days using the Incucyte® Live-Cell Analysis System (Figure 6). Images at 72 hours post-treatment show AI Live and Dead classification and revealed okadaic acid (300 nM) induced cell death whilst Monastrol (600 µM) was not toxic but induced morphological changes compared to vehicle (Figure 6A).

Quantification of cell death showed okadaic acid had a concentration-dependent cytotoxic effect on the primary astrocytes (Figure 6B). In contrast, Monastrol exerted an all-or-nothing cytostatic effect as revealed by little-to-no cell death but a reduction in cell proliferation (object count) with increasing compound concentrations (Figure 6C). This is consistent with known mechanisms of action and exemplifies how this assay is amenable to examination of compound effects in non-perturbing physiologically relevant conditions.

Combined Live-Cell Analysis and Flow Cytometry Approach Yields Additional Insights into Cell Death

In the central nervous system, microglia primarily function as phagocytes in responding to infection or damage. Under pathological conditions, glioma associated microglia play an important role in the tumor microenvironment by responding to oncogenic signaling via the secretion of chemokines and cytokines that further promote tumor progression.[15] Research is focusing on expanding our understanding of these biological roles and interactions, with therapies targeting microglia showing potential complements to current treatments.[16]

We used a combined approach of live-cell analysis and advanced flow cytometry to examine cell death in a microglial cell line. Incucyte® AI Cell Health Analysis Software Module enables label-free real-time monitoring of cell death and is non-exhaustive, which facilitates end-point selection and allows for downstream experiments to be performed to probe deeper into mechanisms of cell death. The iQue® Human 4-Plex Apoptosis Kit is a flow-cytometry based multi-parameter assay that enables quantification of four different apoptosis measurements per well including caspase 3/7 activity, annexin V binding, cell viability, and mitochondrial membrane potential (MMP) (Figure 7).

BV2 cells were treated with 3 chemotherapeutics, camptothecin (CMP), cisplatin (CIS), and carboplatin (CAR), all of which are known to cause DNA damage and activate apoptotic pathways.[17,18] Images were acquired every 2 hours and cell death monitored using the Incucyte® Live-Cell Analysis System. At 24 hours post-treatment, cells were harvested and labeled with the iQue® Apoptosis Kit using a no-wash protocol. The samples were assessed on the iQue® Advanced Flow Cytometry Platform and analyzed using integrated Forecyt® Software. The data in Figure 7 highlights the gating strategy used for each of the apoptosis readouts (Figure 7A). The Forecyt® heat map shows concentration dependent increases in the percentage of caspase positive cells in response to camptothecin and cisplatin, with a partial increase observed for carboplatin (Figure 7B). We observed similar responses across all four apoptosis readouts (data not shown). Comparison of the concentration response curves between Incucyte® label-free and iQue® caspase positive readouts revealed analogous results in compound efficacy (Figure 7C). Taking camptothecin as an example, we observed EC_{50} values of 0.35 and 0.34 µM, respectively. This approach could also be used to examine each apoptosis pathway in more detail for example through assessing caspase-dependent or independent mechanisms.

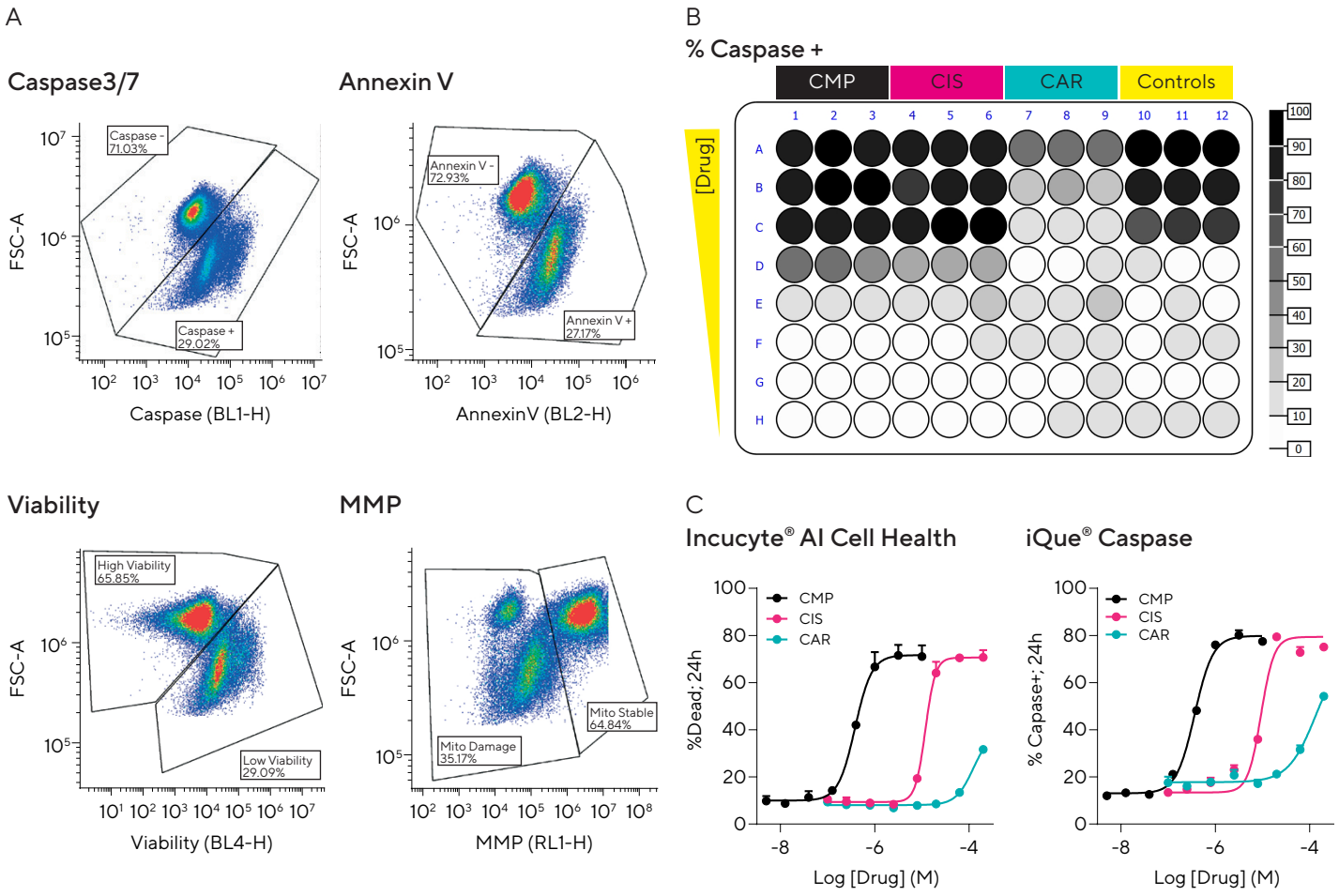


Figure 7: *Combined Approach to Quantify Cell Death in Microglia Using Live-Cell Analysis and Flow Cytometry.*

BV2 microglia cells were seeded onto a poly-L-ornithine (PLO) coated 96-well plate (8,000 cells/well) and once adhered treated with concentration ranges of camptothecin (CMP), cisplatin (CIS) and carboplatin (CAR). Images were acquired every 2 hours in the Incucyte® Live-Cell Analysis System over 24 hours and cell death quantified using Incucyte® AI Cell Health Analysis Software Module. At 24 hours, cells were harvested, transferred to a 96-well v-bottomed plate, labeled using the iQue® 4-Plex Apoptosis Kit (Cat. No. 90053) and run on the iQue® Advanced Flow Cytometry Platform. Dot plots show gating strategy used for each apoptosis readout (A). Heat map of 96-well plate shows caspase positive cells expressed as a percentage of single cells (B). Transformed data shows EC_{50} curves for Incucyte® quantifying the percentage of dead cells and iQue® showing the percentage of caspase positive cells at 24 hours (C). Data shown as mean ± SEM, n= 3 replicates.

Summary & Conclusion

The response of tumor cells, such as glioblastomas, to cytotoxic chemotherapeutic compounds is a complex and dynamic process that is crucial to understand in neuro-oncology research. The Incucyte® AI Cell Health Analysis Software Module uses pre-trained neural networks to accurately segment individual cells and classify them as live or dead. The data shown demonstrates that this is a powerful, robust approach for assessing cytotoxicity in glial cell types and is amenable to screening of therapeutic candidates. The label-free analysis enables non-perturbing quantification of cytotoxicity, which is especially important when using sensitive cell types, and enables downstream experiments, such as flow cytometry, to be performed for additional insights into the mechanisms of apoptosis.

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