

Investigating Novel Therapeutic Targets for Triple Negative Breast Cancer using an Automated Kinetic Proliferation Assay



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Introduction

Measuring cell proliferation is a vital aspect of cancer research and therapeutic drug development. Most current methods for evaluating cell proliferation rely on end point assays, often using indirect measurements to approximate the size of a cell population based on a single predetermined time point. Imaging-based kinetic proliferation assays offer considerable advantages over these conventional approaches by capturing a detailed profile of cell population growth over time using direct cell counts or percent confluence values.

Although fluorescent label-based methods are available to determine either cell counts or percent confluence, they present substantial constraints concerning kinetic applications. The use of intercalating nuclear stains limits the maximum duration of experiments due to cytotoxic effects; while creating stable cell lines expressing a fluorescent marker is a costly and time-consuming task.

Given these limitations, label-free techniques for measuring cell population size and determining proliferation rates are preferable over methods that require fluorescent labels. BioTek's kinetic label-free proliferation assay simultaneously determines both direct cell counts and percent confluence values using high contrast brightfield imaging. The fully automated imaging system provides complete environmental control for up to eight microplates, enabling long-term proliferation experiments for diverse applications.

Here we describe a study in which this novel technique was used to evaluate a promising class of drug targets for specifically limiting the growth of triple negative breast cancer (TNBC) cells. Five different kinesin motor proteins involved in regulating mitotic spindle integrity were inhibited in three TNBC subtypes (basal-like 1, basal-like 2, and mesenchymal). The versatility of this assay enabled accurate evaluation of cell proliferation rates for each cell type across a range of cell densities and conditions. Our findings indicate that inhibition of the kinesin Kif18A significantly slows TNBC proliferation but not normal cells, supporting the development of Kif18A-targeted therapeutics for TNBC.

There Are No Targeted Therapies for TNBC

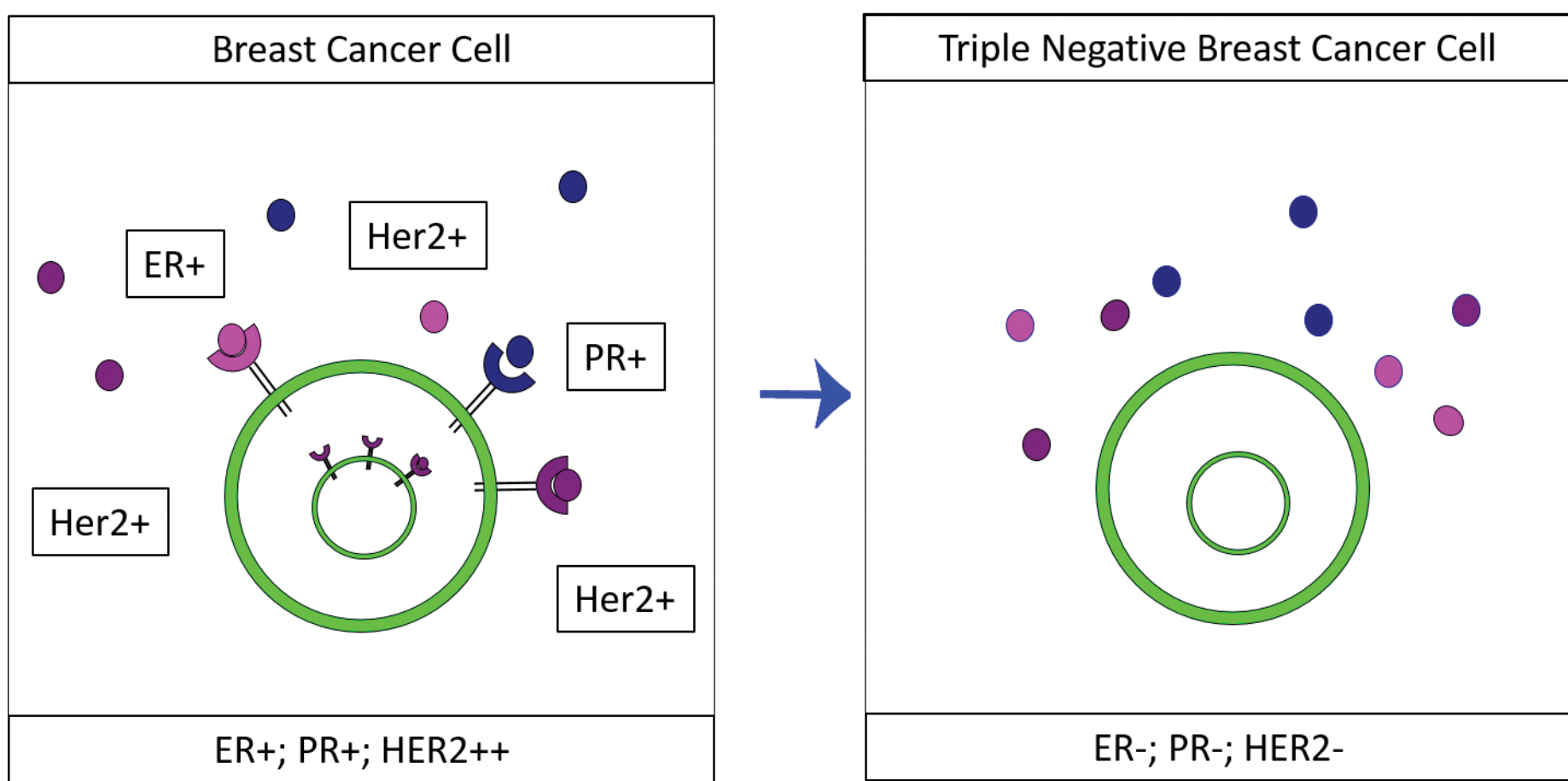


Figure 1. TNBC is usually treated with combinations of chemotherapy, radiation therapy, and surgery.

BioTek Instrumentation



Figure 2. BioSpa 8 Automated Incubator and Cytation 5 Cell Imaging Multi-Mode Reader.

BioSpa 8™ Automated Incubator

- Fully automated incubator with temperature and gas control, humidity monitoring
- Routinely delivers microplates to coupled Cytation
- Accommodates up to eight microplates and other labware
- Option to integrate with washer or dispenser for complete workflow automation

Cytation 5™ Cell Imaging Multi-Mode Reader

- Versatile automated imaging system with wide range of LED filter cube sets available and objectives spanning 1.25x to 60x
- Brightfield, color brightfield, high contrast brightfield, phase contrast, and fluorescence channels
- Optimized for live cell imaging with temperature and CO₂/O₂ control, shaking, and dual reagent injectors
- Hybrid optical design offers filter-based fluorescence and monochromator optics

Materials and Methods

Kinesin motors are efficiently depleted from normal breast epithelial cells and TNBC cell lines using specific siRNAs

Cell types included in this study	TNBC subtype
MCF10A	Normal breast epithelial
HCC1806	Mesenchymal
MDA-MB-468	Basal-like 1
MDA-MB-231	Basal-like 2

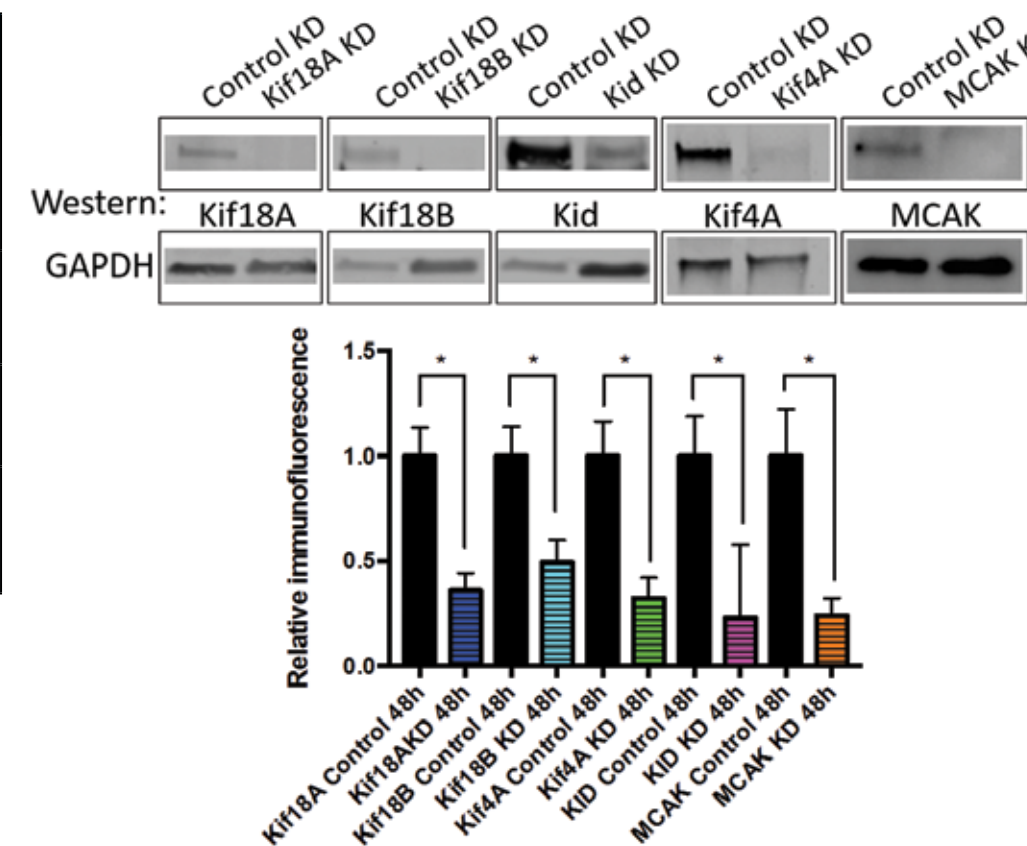


Figure 3. Included cell types and quantification of mitotic kinesin depletion. This study focused on the three TNBC subtypes (mesenchymal, basal-like 1, and basal-like 2) with the greatest level of disruption in cell cycle gene expression, and one normal breast epithelial cell line (MCF10A). Five mitotic kinesins (Kif18A, Kif18B, Kif4A, Kid, and MCAK) were inhibited in each cell type to measure their effect on proliferation rates. siRNA knockdown of kinesin motors was optimized and quantified by Western blot analysis using antibodies against each of the five kinesins tested.

Label-Free Kinetic Proliferation Assay

Assay setup

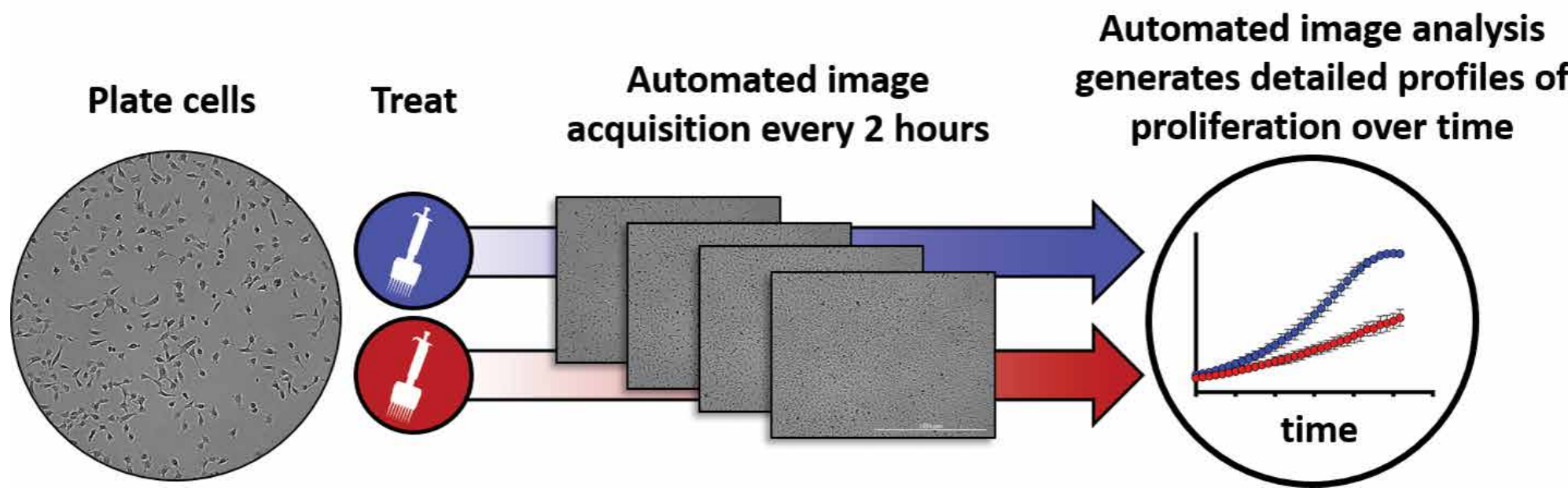


Figure 4. The kinetic proliferation assay provides a sensitive and robust evaluation of cell population growth. Treated cells were plated in 96-well microplates at 1000 cells per well. Up to eight plates were loaded into BioSpa Automated Incubator before starting automated imaging routine on linked Cytation. High contrast brightfield images were captured every two hours over a 96-hour time-course, creating a continuous record of the entire experiment. Automated image processing and analysis is conducted in real time, generating detailed proliferation profiles over time using direct cell counts or percent confluence.

High contrast (HC) cell counting technique

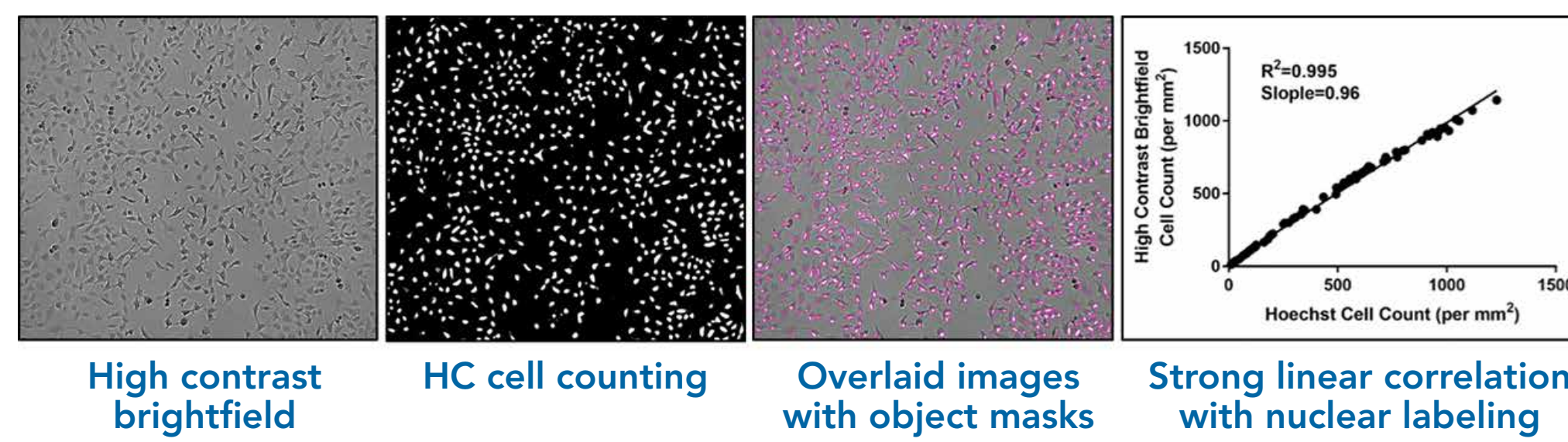


Figure 5. HC cell counting uses a modified form of high contrast brightfield to determine accurate label-free cell counts for normalization of experimental data and kinetic proliferation assays. In HC cell counting mode, refraction of white light by each cell produces a distinct bright spot against a dark background. Gen5™ Microplate Reader and Imager Software identifies each object to generate HC cell counts that are comparable to counts achieved using nuclear labels, across a wide range of cell densities.

Direct cell counts are a more sensitive and accurate metric for quantifying cell population size compared to % confluence

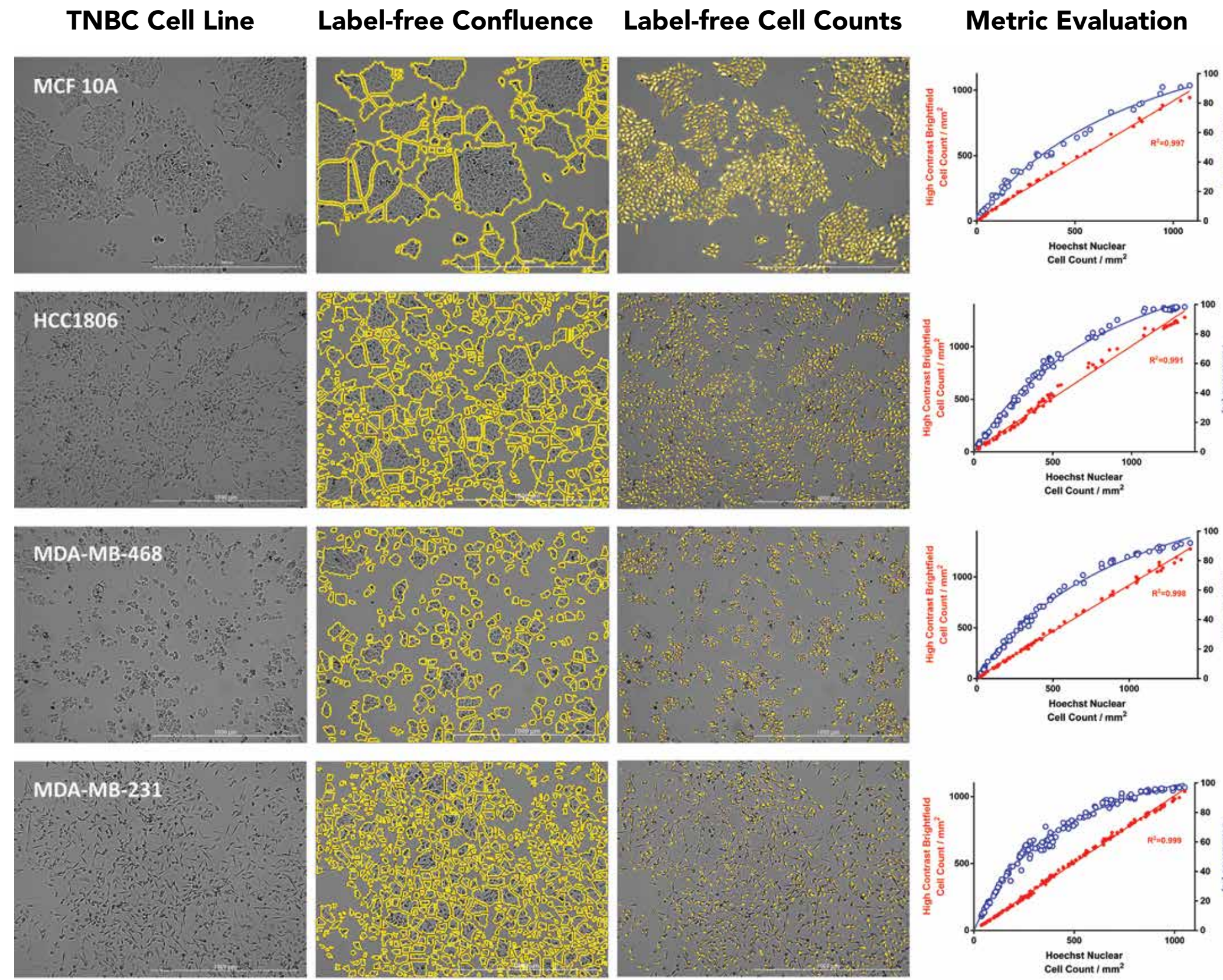


Figure 6. The automated kinetic proliferation assay simultaneously determines both direct HC cell counts and percent confluence values using high contrast brightfield imaging. Representative high contrast brightfield images with either confluence or HC cell count masks applied for analysis. The availability of both metrics enables the user to determine the most appropriate analysis for each condition. Although percent confluence provides insight into the relative size of a population, the relationship between cell confluence and number of cells present is not linear at many culture densities (graphs on the right). HC cell counts are therefore often preferable over confluence for measuring the size of a cell population. However, some cell types and conditions are not amenable to the HC cell counting technique due to irregular cell morphology or a tendency to form cell clusters. For this study, the accuracy of HC cell counts across a full range of cell densities was determined for each cell type by comparing them to counts of the same population using Hoechst 33342 stained nuclei. For each cell type, HC cell counts had a strong linear correlation with nuclear cell counts ($R^2 > 0.99$; slope = 0.92 to 1).

Shaker step improves accuracy of cell counts by dispersing cellular debris from surface

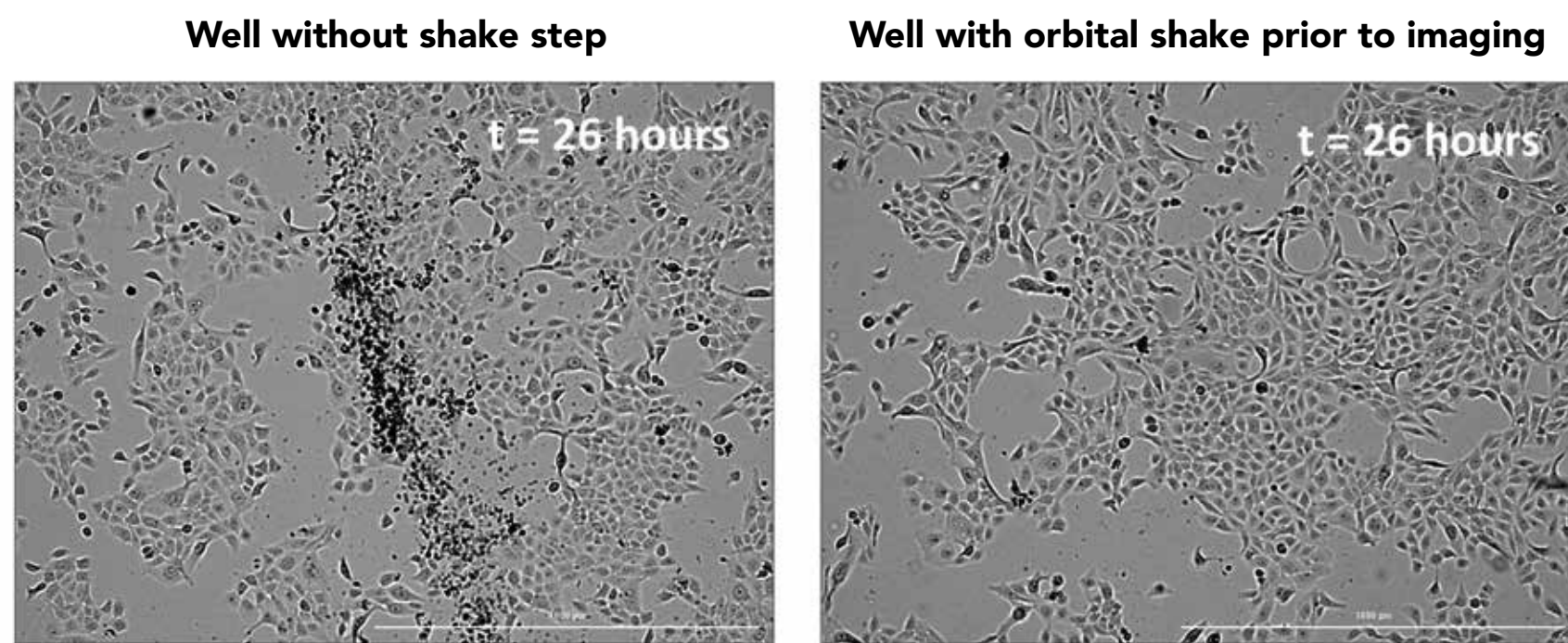


Figure 7. Treatment effects can lead to accumulation of cellular debris and cause inaccurate evaluation of cell population size. MCF10A cells after 26 hour incubation. An optional orbital shake step within the Cytation prior to imaging gently disperses debris for optimized confluence measurements and cell counts.

Results

Depletion of mitotic kinesins inhibits the proliferation of TNBC cells but not normal breast epithelial cells

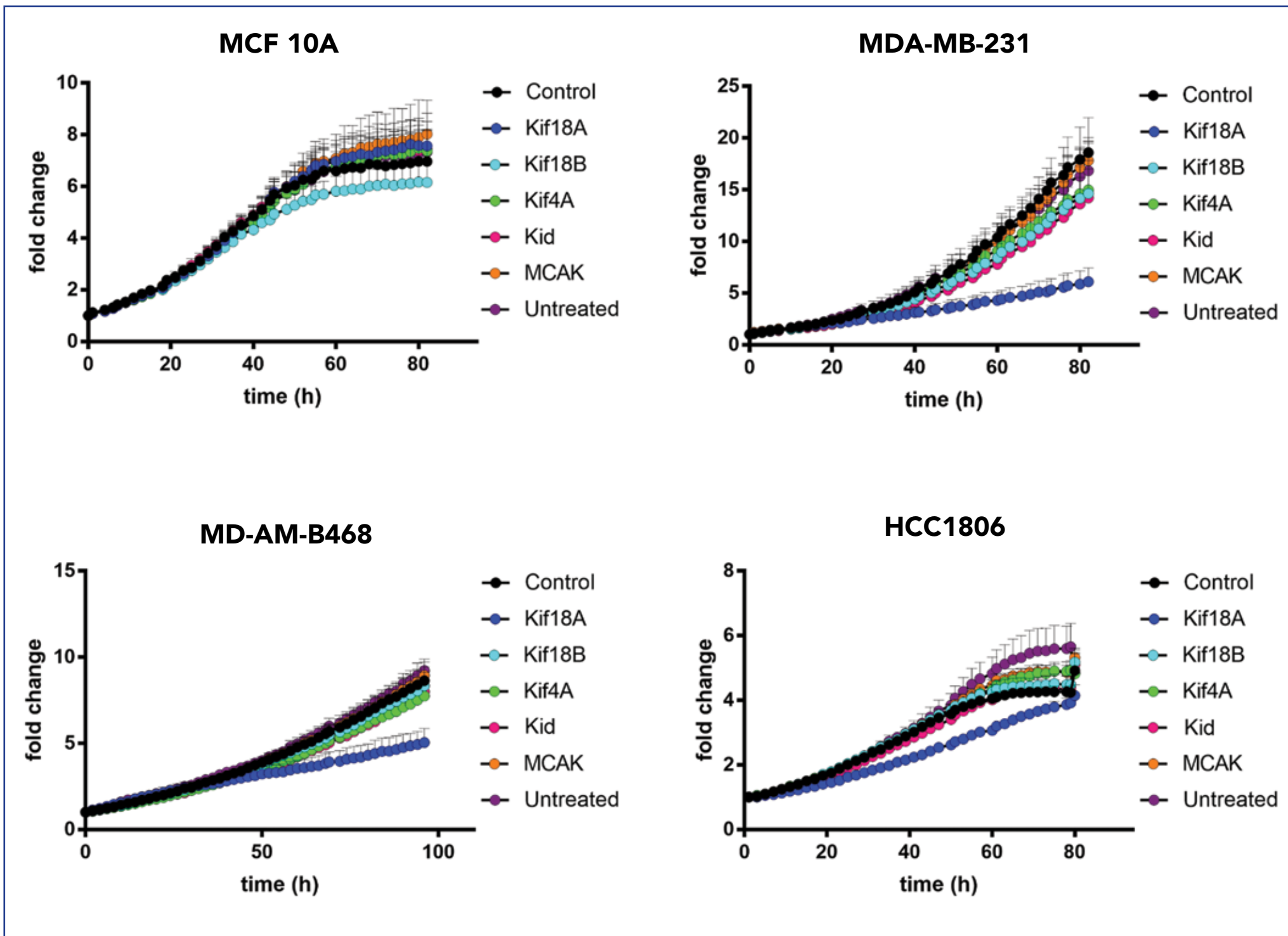


Figure 8. Kinetic cell proliferation profiles generated from HC cell counts over time enable sensitive detection of changes in TNBC proliferation rates. Cell count fold change (cell count / cell count t_0) were calculated every two hours over a four day period or until cell counts plateaued due to overcrowding.

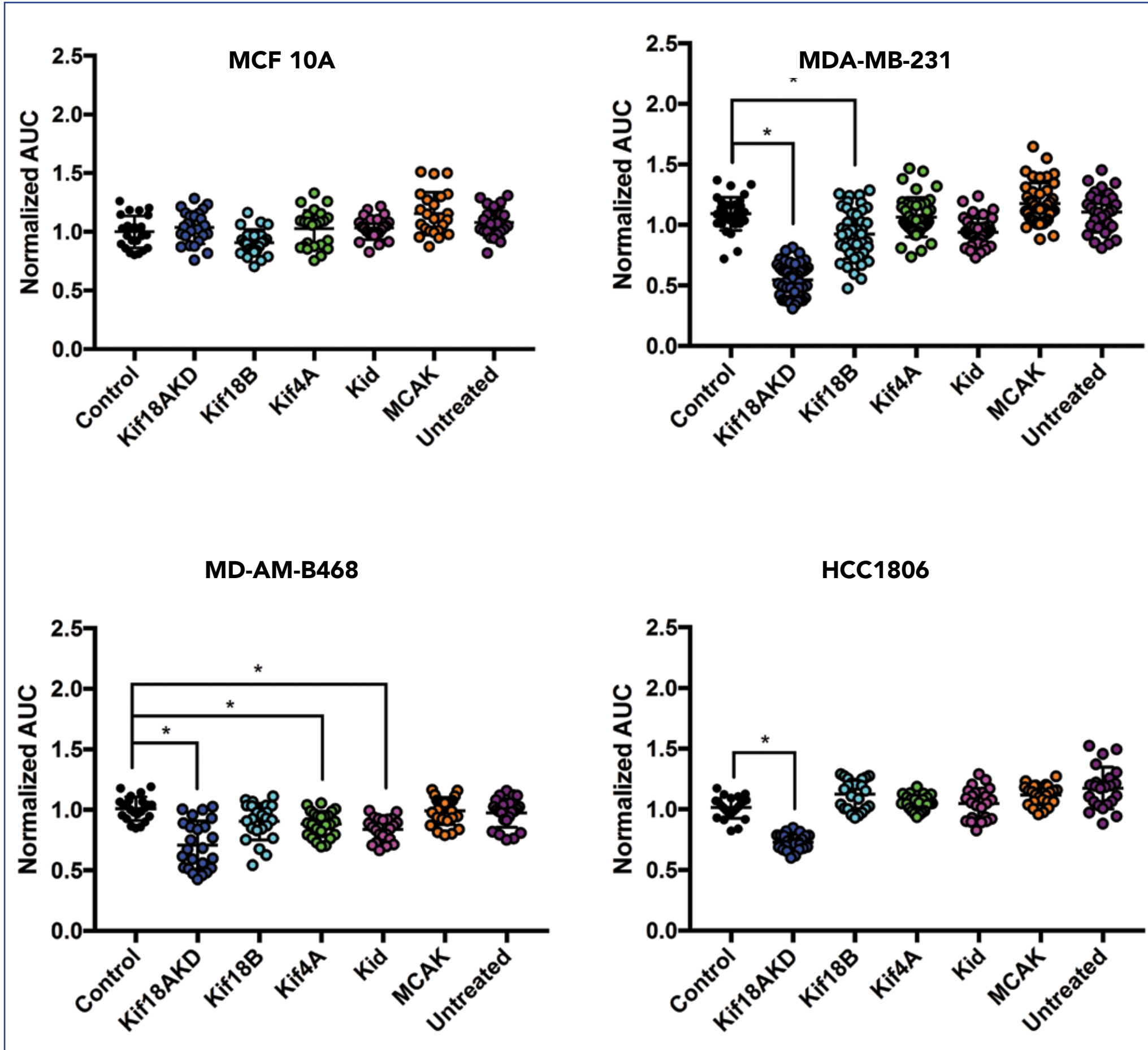


Figure 9. Area under the curve (AUC) calculations were used to compare proliferation rates across treatment conditions for each cell type. Results from three independent experiments were combined, with $n \geq 8$ replicates per experiment. Normalized AUC (AUC/AUC₀) values were derived from kinetic profiles, extending out to the time point in which untreated cells reached full confluence or 96 hours, whichever came first.

Conclusions

- Coupling the fully automated cell-handling abilities of the BioSpa 8 with the imaging and image analysis capabilities of the Cytation 5 provides a powerful system to conduct long-term proliferation studies.
- HC cell counting is an accurate label-free method to quantify cell proliferation with considerable advantages over % confluence measurements.
- Inhibition of the mitotic kinesin Kif18A significantly slows the growth of BL1, BL2, and mesenchymal TNBC subtypes.
- In contrast, the growth and mitotic progression of normal breast epithelial cells were not affected by depletion of mitotic kinesins, including Kif18A, suggesting that the effects of inhibiting these motors are specific to tumor cells.
- Taken together, our data support a model in which TNBC cells contain "fragile" spindles that are sensitive to loss of normally non-essential kinesin motors.

Fragile Spindle Hypothesis

