ESSEN BioScience

Application Note

CellPlayer™ Kinetic Proliferation Assay

Katherine Artymovich, Kalpana Patel, Clare Szybut, Patricia M. Garay,
Tim O'Callaghan, Tim Dale, Del Trezise, and Daniel M. Appledorn
Essen BioScience – Ann Arbor, Michigan—Welwyn Garden City, U.K.

Introduction

Cell proliferation assays are a cornerstone of cancer therapeutic, developmental biology, and drug safety research. Analysis of the sustained signalling pathways that underlie the progression of tumours, for example, accounts for >10,000 manuscripts in PubMed, the majority of which contain some measurement or other of cell proliferation. Despite this, there has not been a direct, straightforward, industrialized method for quantifying cell proliferation as a continuous event. Rather, the majority of measures are end points or at best a series of concatenated endpoints to measure the time-course. Many biochemical detection measures (e.g. LDH, ATP detection) are indirect and subject to artifacts that cannot be readily verified by morphology changes. Antibody- and chemical probe-based high content imaging methods address this limitation but are not amenable to long term temporal assays. The introduction of IncuCyte live cell imaging enabled non-invasive fully kinetic measures of cell growth based on area (confluence) metrics, which remains a high value and valid approach for many applications (Figure 1).

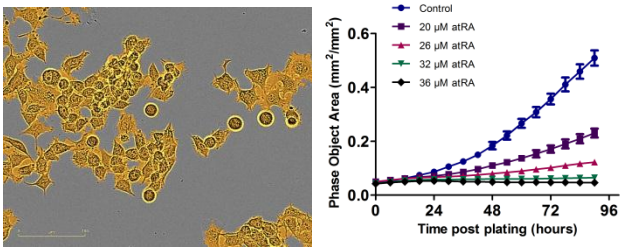

Figure 1. Proliferation is successfully measured using an IncuCyte phase-only processing module. An IncuCyte mask (goldenrod) analyzes a phase-contrast image of proliferating neuro-2a cells. The proliferation of neuro-2a cells decreases under increasing concentrations of all-*trans* retinoic acid.

However, cells with very low contrast can prove difficult to quantify, and the relationship between confluence and true cell number becomes non-linear both when cell morphology changes (e.g. cells shrink or expand) and when full confluence is achieved. Furthermore, analysis of multiple cells types in co-culture—a model often demonstrated to be more representative of *in vivo* biology—is impossible using confluence measurement alone. To resolve these issues, we introduce a method to measure kinetic cell proliferation based on direct cell (nuclear) counting using the IncuCyte platform

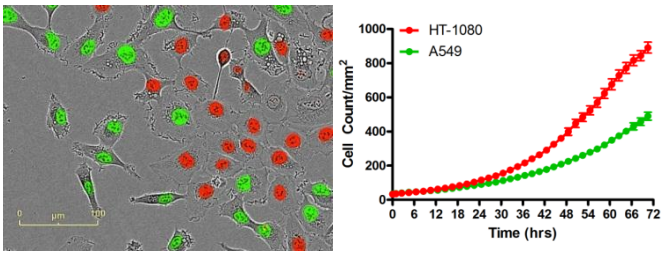

Figure 2. A wide range of co-culture-based assays are possible by fluorescently labeling the nuclei of one or more cell types (HT-1080 red; A549 green) with a non-perturbing nuclear-targeted fluorescent protein. IncuCyte fluorescent object counting software successfully quantifies real-cell proliferation.

and novel nuclear-restricted green or red fluorescent proteins termed NucLight Green and NucLight Red (Figure 2). This integrated approach enables a medium-throughput, content-rich, fully kinetic solution. In this note we will demonstrate the validity and flexibility of the NucLight reagents in combination with the IncuCyte platform. We explore the proliferation of cancer cells in co-culture with fibroblasts. Furthermore, we establish the applicability of the IncuCyte platform to screening studies of cellular proliferation.

Approach and Methods

Cancer cell co-culture: growth conditions and experimental set-up

SK-BR-3 (ATCC, Cat. #HTB-30), CCD-1068Sk (ATCC, Cat. #CRL-2086), and HMF (ScienCell, Cat. #7630) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Cat. #10313) with 10% FBS, 2% Glutamax (Invitrogen, Cat. #35050), and 1xPenStrep-Glutamine (Invitrogen, Cat. #15140). SK-BR-3 cells were infected with NucLight Red (Cat. #4476, Lenti, EF1α, puromycin) at an MOI of 3 transducing units/mL in the presence of 8 µg/mL Polybrene® (Sigma-Aldrich, Cat. #H9268). (See Supplemental Data for an analysis of the NucLight reagent.) Two days after infection, complete media containing 1 µg/mL puromycin was added to cells to select for SK-BR-3 cells expressing NucLight Red. Cells were maintained in complete media containing 0.5 µg/mL puromycin (Invitrogen, Cat. #A11138). Co-culture

experiments were performed in complete media without puromycin.

The co-culture experiment was set up by seeding CCD-1068Sk cells or HMF cells into 32 wells each in a 96-well plate at a density of 9,000 cells/well. Cells were incubated overnight at 37°C, 5% CO_2. The following day (DIV 1), media was removed and 9,000 cells/well of SK-BR-3 cells expressing NucLight Red were seeded in all 96 wells, resulting in two co-cultures and one monoculture. ~6 hours later, lapatinib (LC Laboratories, Cat. #L-4804), serially diluted 3-fold in 100% DMSO, was added to cells in medium at a final concentration range of 6.86 nM to 5000 nM. The DMSO concentration was consistent at 0.5% in all wells. Cells were imaged in IncuCyte ZOOM for 8 days, and 67% of the media was changed on DIV 4.

Cancer cell co-culture: IncuCyte™ ZOOM data quantification and analysis

Phase-contrast and red fluorescent images were collected on IncuCyte ZOOM for 8 days. A specific processing definition was applied to count red nuclear objects for the duration of the assay. Nuclear objects per mm^2 from each well at each time point was exported into GraphPad Prism Software. The area under the curve (AUC) was calculated for each concentration (n=4) and used to calculate IC_{50} values of Lapatinib for SK-BR-3 grown in monoculture, SK-BR-3 grown with HMF, and SK-BR-3 grown with CCD-1068Sk.

Screening assay

Stable populations of NucLight Green HT-1080 cells were made by transducing parent cell lines with Essen's CellPlayer™ NucLight Green reagent (Cat. #4475, Lenti, Ef1α, puromycin) at an MOI of 3 TU/mL in the presence of 8 µg/mL polybrene (see Supplemental Data for an analysis of the NucLight reagent). Populations expressing green fluorescent protein restricted to the nucleus were selected for in complete medium containing 1 µg/mL puromycin for 3-5 days, and then maintained in complete medium containing 0.5 µg/mL puromycin.

HT-1080 cells were plated in full media/serum at 2000 cells/well for 6-12 h on 384-well microtiter plates (Corning, CLS3985) using a Multidrop automated cell dispenser. Compound plates comprising 16 different compounds each at 11 different test concentrations in duplicate wells, were prepared in 100% DMSO. Low volume (1 µL) plate copies were created using a 384-well PlateMate Plus liquid handler, and diluted to 100 µL in media/serum. Media was then removed from the cell plate using an aspirator manifold and replaced with 50 µL of the compound containing fresh media. Cell plates were returned to the IncuCyte and scanned every 3h for 72h.

Screening experiment: IncuCyte™ FLR data quantification and analysis

Phase-contrast and fluorescent images were collected to detect increase in cell number (via NucLight Green nuclear object counting). The integrated object counting algorithm was used to isolate the fluorescent nuclear signal from background. Nuclei were reported on a per-area (mm^2) basis for each time point. Each cell proliferation time-course was analyzed for peak cell number via an export of the temporal object count data into Microsoft Excel. Concentration-response curves for inhibition of proliferation were constructed and fitted to a four parameter logistic equation using XLfit to yield IC_{50} values. Quantification was verified with representative images and time-lapse videos of the cells.

Results and Discussion

Quantitative measurement of cancer cell proliferation using NucLight Red reagent in a co-culture model

Certain cancers are resistant to chemotherapy due to the biological activity of their neighboring cells, or within the context of the tumor microenvironment. For example, stromal cells have been observed to rescue tumors from drug-induced toxicity by secreting growth factors that impede apoptotic pathways[1]. Such cell interactions may be illuminated by juxtaposing monocultures and co-cultures through in vitro assays. In this study, we examined the culture-dependence of the SK-BR-3 cell line, a type of breast adenocarcinoma cell that overexpresses HER-2. A previous study demonstrated that monocultures of SK-BR-3 cells are sensitive to the drug lapatinib, which induces cell death by inhibiting tyrosine kinase activity of the HER-2 and EGFR pathways[2]. However, another study showed that when SK-BR-3 cells were co-cultured with normal skin fibroblasts (CCD-1068Sk), these stromal cells were able to rescue the inhibitory effect of lapatinib. Interestingly, no such rescue effect was observed when SK-BR-3 cells grown in co-culture with normal human mammary fibroblasts (HMF)[1]. In a similar study, using the combination of IncuCyte ZOOM and CellPlayer NucLight Red reagents, we continuously and kinetically monitored the proliferation of SK-BR-3 cells in monoculture and co-culture in the presence of increasing concentrations of lapatinib. SK-BR-3 cells were quantified using IncuCyte's nucleus counting algorithm. This method allows real-time cell counting based on nuclear restricted fluorescent protein expression. Kinetic graphs of nuclear counts per mm^2 over time show SK-BR-3 cells grown with CCD-1068Sk fibroblasts grow at a

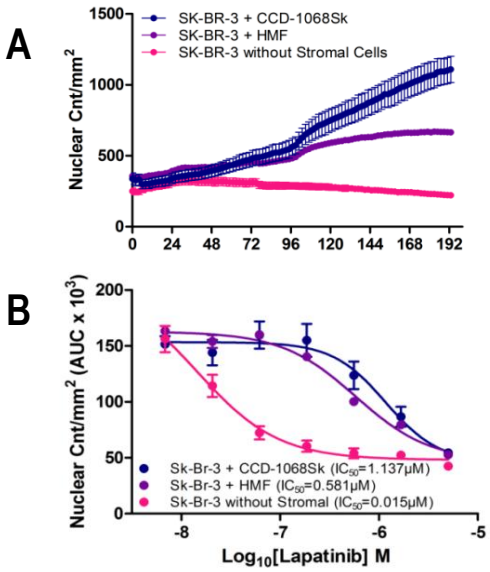

Figure 3. Proliferation of SK-BR-3 cells in co-culture and monoculture under lapatinib treatment. SK-BR-3 cells expressing a nuclear restricted red protein were grown with normal skin fibroblasts (CCD-1068Sk), human mammary fibroblasts (HMF), or in monoculture, and treated with varying concentrations of lapatinib for 8 days. (A) Nuclear counts per mm^2 of SK-BR-3 cells grown with or without stromal cells in the presence of 556 nM lapatinib illustrate the rescue effect of CCD-1068Sk fibroblasts compared to HMFs and mono-culture. (B) Area under the curve of nuclear counts per mm^2 over time for each concentration (n=4) was used to calculate and compare IC_{50} values of SK-BR-3 cells grown with or without stromal cells.

significantly higher rate than those grown alone or with HMF's in the presence of ≈500 nM lapatinib (Figure 3A).

In addition, IC_{50} values (calculated using the area under the curve (AUC) of nuclear counts per mm^2 over time) provide quantitative evidence for the differences in drug response between monoculture and co-cultures in the presence of lapatinib. Specficially, SK-BR-3 cells grown with CCD-1068Sk fibroblasts are the least sensitive to lapatinib with an IC_{50} value of 1.162 µM, followed by SK-BR-3 cells grown with HMF's with an IC_{50} value of 0.581 µM (Figure B). Interestingly, SK-BR-3 cells grown in mono-culture remain effectively sensitive to lapatinib with an IC_{50} value of 0.015 µM (Figure B) which is comparable to the published IC_{50} value, 0.037±0.031 µM (Konecny, Cancer Res 2006). This data was repeated in 2 independent experiments. These striking data very clearly illustrate the difference in SK-BR-3 cell proliferation in the presence of stromal cells, thus highlighting the importance of considering the effect that the tumor microenvironment can have on drug resistance.

High-throughput compound testing using NucLight Green labeled HT-1080s

High-throughput compound testing is essential for efficiently siphoning potential drugs through the drug discovery pipeline. To examine the ability of the IncuCyte system to meet this need, we measured cell proliferation over time in a higher-throughput format. (See Supplemental Data for 96-well experiments on seeding density, FBS concentration, and cycloheximide treatments.) To assess many pharmacological agents simultaneously, sixteen literature-standard compounds (Table 1) were applied to HT-1080 tumor-derived fibrosarcoma in a 384-well format (Figure 5). An 11-point concentration response curve was constructed for each compound (Figure 6). Of the 16 compounds tested, the rank order of potency for inhibition of cell proliferation was: doxorubicin = staurosporine = camptothecin > mitomycin C > cycloheximide = RITA > PD-98059 > FAK inhibitor 14 = cisplatin > 10-DEBC = Chrysin = Compound 401. TAME, PAC1, KU0063794 and FPA-124 had little or no effect on cell proliferation under the conditions of the experiment.

Table 1. Drugs identified in literature as relevant to cell proliferation

Drug	Description
Doxorubicin	chemotherapy drug, intercalates DNA[3]
Camptothesin	alkaloid inhibits topoisomerase, causing DNA damage[3]
Staurosporine	potent alkaloid inhibitor of protein kinase[4]
Mitomycin C	chemotherapy drug, alkylates DNA[5]
Cycloheximide	protein synthesis inhibitor[6]
RITA	(reactivation of p53 and *i*nduction of *t*umor cell *a*poptosis) a small molecule, binds p53[7]
PD-98059	MAPK1/2 inhibitor[8]
Cisplatin	chemotherapy drug acts through crosslinking DNA[9]
FAK-inhibitor 14	selective inhibitor of focal adhension kinase[10]
10-DEBC	selective inhibitor of Akt[11]
Chrysin	a flavonoid observed to inhibit growth in cancer cells[12]
TAME	tert-Amyl methyl ether; a gasoline additive with suspected toxic effects upon inhalation[13]
PAC1	(pro-caspase activating compound-1), a small-molecule activator of procaspase-3 to caspase-3[14]
KU0063794	specific inhibitor of mTORC1/2 [15]
FPA-124	Akt inhibitor[16]
Compound 401	inhibitor of DNA-dependent kinase and mTOR[17]

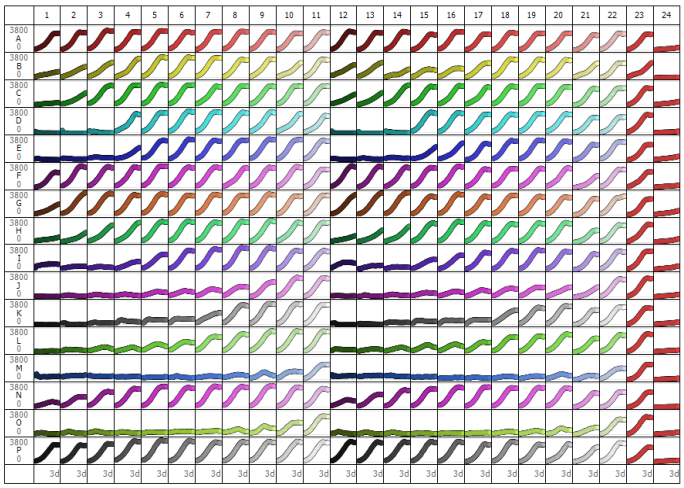

Figure 5. 384-well microplate view of HT-1080 NucLight Green cell proliferation with 16 different compounds, 11-point concentration-response curves in duplicate (different colors, high to low concentrations left to right). Columns 15 and 16 are vehicle (0.5% DMSO) and CHX (3 µM) controls, respectively. Note the potent concentration-dependent inhibition of cell proliferation for certain compounds (e.g. row J, row M, row O), and weaker effects/inactivity of others (e.g. row A, row P). Abscissa: time (0-72h), ordinate: fluorescent object count per well (0-3800).

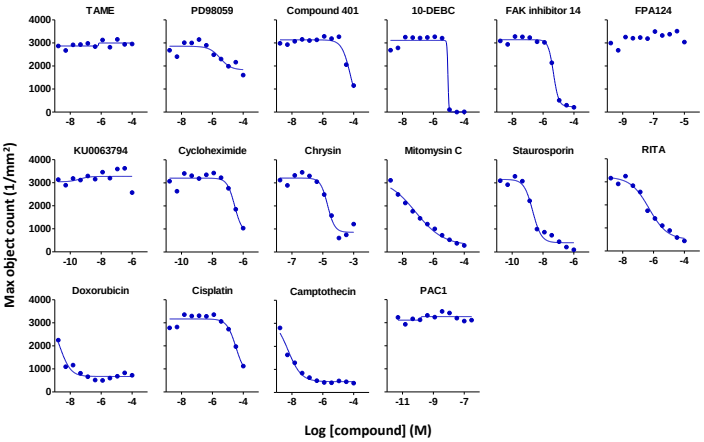

Figure 6. Concentration-response curves for a selected set of literature standard inhibitors of cell proliferation in HT-1080 NucLight Green cells. Abscissa: -log M [compound], ordinate: maximum nuclear count (per mm²). Each point represents the average data from 2 wells from a single 384-well compound plate. The lines of best fit are a 4-parameter logistic equation calculated in GraphPad Prism.

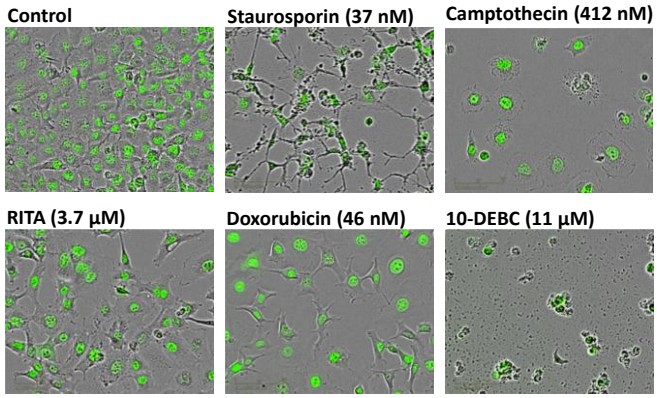

Figure 7. Representative 'blended' phase contrast/fluorescence images of HT-1080 NucLight Green cells treated with different test compounds as labeled. Images shown were taken 24 h post compound treatment (IncuCyte FLR 10x). Note the reduction in total cell number for each treatment compared to the vehicle control, and the profound differences in cell morphology.

To verify and extend these findings, representative images and time-lapse videos of the cells exposed to test compounds at selected concentrations were inspected (Figure 7). Following exposure to an IC_{80} concentration for 24h, staurosporine produced profound changes in cell morphology, with extensive branching and condensation of the nucleus and cell body. The cells lost motility and there was clear evidence of cytotoxicity. In contrast, the inhibition of cell proliferation by RITA (also IC_{80}, 24h) was not accompanied by any obvious morphological changes. At anti-proliferative concentrations, both camptothecin and doxorubicin treated cells appeared healthy with no evidence of cell death, suggesting that senescence had occurred. 10-DEBC (11 mM, 24h) produced overt cytotoxicity and complete cell lysis. These data show the potential of this kinetic and morphological approach to the screening, prioritization, and classification of compounds in drug discovery.

Conclusions

The IncuCyte™ Live-Cell Imaging Systems in conjunction with NucLight reagents provides a live cell, kinetic assay for the measurement of proliferation. This strategy has demonstrated quantitative and reproducible analysis of cells in monoculture and importantly, co-culture. It also gives the user the ability to monitor morphological changes in parallel with quantification, the combination of which is a powerful and unique tool for detecting pharmacological or genetic manipulations that alter cell viability.

The experiments described in this application note demonstrate:

1. Using stably-expressing NucLight Green and Red cell lines, kinetic proliferation assays based on direct, true cell (nuclear) count can be performed in both monoculture and co-culture models. Full time courses (>7 days) of changes in cell number can be observed and quantified, which can furthermore aid in future experiment planning.

2. Rate constant (k) and doubling time values can be used to compare the effects of test conditions both within and across cell types. Statistical analyses, such as area under the curve, can also be determined. Arbitrarily defined end points are not required.

3. Proliferation assays can be run in microplates (96-well and 384-well) with high precision and reproducibility. In 384-well plates, a mix and read assay is exemplified whereby full concentration-response curves of 16 standard anti-proliferative agents were compared. In a single IncuCyte instrument 6 x 384-well plates can be monitored providing >2000 wells of parallel data acquisition.

4. All data and time points can be verified by inspecting individual images and/or time-lapse movies. Cell morphology observations provide additional validation and insight into mechanistic differences between treatments or conditions.

We conclude that our combination of NucLight reagents and cell lines expressing nuclear restricted red or green fluorescent proteins, IncuCyte phase and fluorescence live cell imaging technology, and the accompanying easy to use software tools provides a powerful solution for kinetic cell proliferation measurements and pharmacology assays.

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