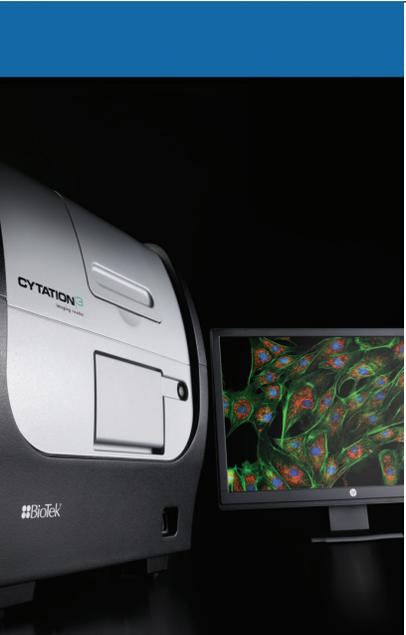


Multiplexed Assay for IL-6 Secretion and Cell Viability Using an Epithelial Ovarian Cancer Cell Line

Using the Cytation™3 Cell Imaging Multi-Mode Reader to Monitor IL-6 Secretion Using HTRF Detection and Cell Viability Using Digital Widefield Fluorescence Microscopy



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Epidermal growth factor receptor (EGFR) is expressed in up to 70% of epithelial ovarian cancers (EOCs). High levels of IL-6 have also been found in the ascites of EOC patients. It has been shown that ligand stimulated EGFR activated NFkB dependent transcription and induced secretion of the pro-inflammatory IL-6 cytokine. In this work we will demonstrate a multiplexed assay for inhibitors at the level of EGFR and NFkB to monitor IL-6 secretion and cell viability. The work flow of the multiplexed assay uses an assay plate and HTRF detection plate. Following treatment of the cells, supernatant is transferred to the HTRF detection plate where IL-6 concentrations are determined; then cell viability is assessed in the assay plate using fluorescent probes and imaging. All assays were conducted on the Cytation™3 Cell Imaging Multi-Mode Reader.

Introduction

According to the Centers of Disease Control and Prevention (CDC), each year about 20,000 women in the United States get ovarian cancer [1]. 90% of these cancers are classified as "epithelial" and are believed to arise from the surface (epithelium) of the ovary and are termed Epithelial Ovarian Cancer (EOC). While prognosis is good for early diagnosis in disease stages I/II, symptoms are non-specific and difficult to trace. The majority of ovarian cancers are diagnosed at late stage III/IV, where symptoms become more evident. Unfortunately at this point, prognosis is poor. A common manifestation of EOC at this late stage of its progression is a build-up of fluid in the abdominal cavity (ascites). It has been shown that high levels of IL-6 are present in these ascites [2].

The origin of the high expression of IL-6 is linked to the receptor Epidermal Growth Factor Receptor (EGFR). EGFR is expressed in up to 70% of EOCs, and its altered expression is associated with late stage disease and poor prognosis [3]. EGFR, a member of ErbB family of receptor tyrosine kinases, activates multiple signaling cascades including the activation of NFkB, which is known to activate the transcription of inflammation-related proteins such as IL-6 [4]. In a recent publication, it was shown that EGFR ligand binding induces the expression of IL-6 via the NFkB pathway in advanced-stage epithelial ovarian cancer [5].

In this application note we demonstrate an in vitro microplate assay that can monitor IL-6 secretion from plated SKOV-3 ovarian carcinoma cells induced through EGFR ligand binding and NFkB activation. The assay workflow involved a 2 plate protocol where cells are plated and EGFR ligand-activated. IL-6 measurements are made in a separate microplate by transferring a portion of the cell supernatant. We also showed that IL-6 secretion can be inhibited at the level of either EGFR or NFkB using known inhibitors. Inhibitors that are potentially toxic to the plated cells can be assessed through digital widefield fluorescence microscopy using fluorescent probes. This provides a quantitative determination of whether IL-6 suppression is caused by the inhibition of receptor/transcription factor activation or through cell toxicity. All microplate measurements were made on the Cytation™3 Cell Imaging Multi-Mode Reader.

Key Words:

Microscopy

Cell Viability

HTRF

TR-FRET

IL-6

Cytokine

EGFR

Biotherapeutics

Biomarkers

Materials and Methods

Materials

Cells

Ovarian carcinoma SKOV-3 cells (Catalog No. AKR-253) were obtained from Cell Biolabs, Inc. (San Diego, CA). The cells were propagated in RPMI 1640 Medium (Catalog No. 11875) plus Fetal Bovine Serum, 10% (Catalog No. 10437) and Pen-Strep-Glutamine, 1x (Catalog No. 10378) from Life Technologies (Carlsbad, CA). The cells were plated at a density of 1.0×10^5 cells/mL in serum-free medium for 24 hours prior to performing the assay.

EGFR Signaling Cascade Inducer

Epidermal Growth Factor (EGF) (Catalog No. CYT-217) from ProSpec (Rehovot, Israel) was used to stimulate the EGFR signaling cascade, leading to eventual secretion of the pro-inflammatory cytokine, IL-6.

Inhibitors

AG 1478 (Catalog No. 1276), Cardamonin (Catalog No. 2509), U0126 (Catalog No. 1144), and LY 294002 (Catalog No. 1130) were purchased from R&D Systems (Minneapolis, MN). Cetuximab was provided by Cisbio Bioassays (Codolet, France). Anti-EGFR antibodies 225 (Catalog No. LS-C88001) and 111.6 (Catalog No. LS-C88141) were purchased from LifeSpan BioSciences (Seattle, WA).

Cell Plates

96-Well Flat Clear Bottom, Black PS, TC-Treated Microplates (Catalog No. 3904), and 384-Well Low Volume White Round Bottom PS NBS Coated Microplates (Catalog No. 3673) were purchased from Corning Life Sciences (Corning, NY).

Instrumentation

CytationTM3 Cell Imaging Multi-Mode Reader

Cytation3 combines automated digital widefield microscopy and conventional microplate detection. This patent pending design provides rich phenotypic cellular information with well-based quantitative data.

Equipped with BioTek's patented Hybrid TechnologyTM for microplate detection, Cytation3 includes both high sensitivity filter-based detection and a flexible monochromator based system for unmatched versatility and performance. The upgradable automated digital fluorescence microscopy module provides researchers rich cellular visualization analysis without the complexity and expense of standard microplate-based imagers.

Fluorescence microscopy is a powerful technique for visualizing cellular responses to understand cell proliferation, protein expression, cytotoxicity and other cellular processes. The ability to perform both conventional quantitative fluorescence measurements and cell imaging provides unique capabilities such as screening microplate wells for a fluorescence intensity threshold that triggers the reader to follow-up the screen with imaging of those wells that passed the intensity threshold. This serves to reduce analysis time and data storage requirements by imaging only those wells of interest which pass the intensity threshold.

Cytation3's design places special emphasis on live-cell assays: features include temperature control to 45 °C, CO₂/O₂ gas control, orbital shaking and full support for kinetic studies with BioTek's Gen5TM Data Analysis Software, specifically designed to make plate reading and image capture easy. Other technology advances are found throughout Cytation3's design including high-intensity LED light sources, matched filter cubes, hard coated optical filters, Olympus objectives, and superior autofocus for totally software controlled digital microscopy.

The filter-based system was used to detect the 665 nm and 620 nm fluorescent emissions from the HTRF[®] IL-6 assay chemistry with the following settings: Delay after plate movement: 0 msec; Delay after excitation: 150 µsec; Integration time: 500 µsec; Read height: 10.5 mm. Imaging was then performed with the Nuclear-IDTM Blue/Red Cell Viability assay using the microscopy capabilities. Gen5 software was used for initial data analysis.

Assay Chemistries

HTRF® Human IL-6 Assay

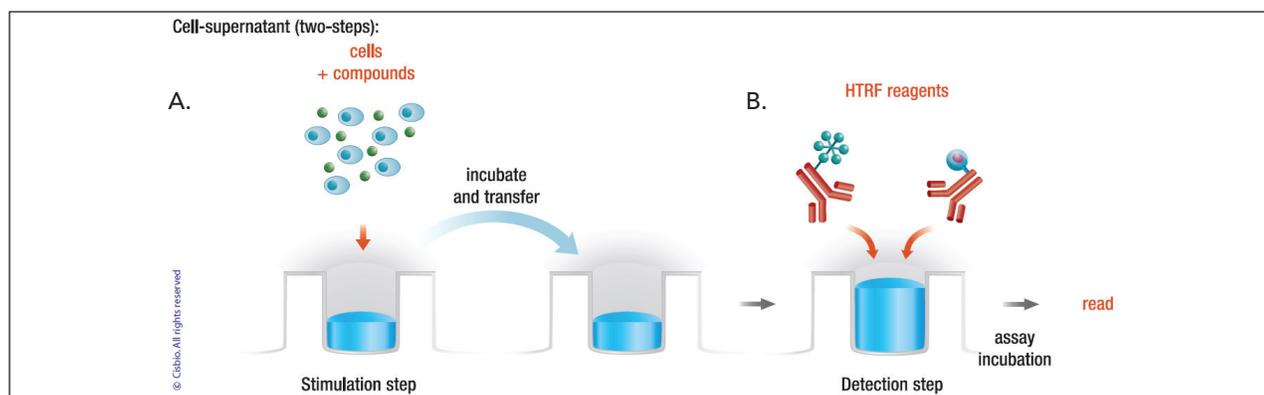


Figure 1. 2-Plate HTRF® Human IL-6 Assay.

In the assay, IL-6 is measured using a sandwich immunoassay involving two monoclonal antibodies: anti-IL-6 (MAb1) labeled with Eu-Cryptate and anti-IL-6 (MAb2) labeled with XL665. These antibodies may be pre-mixed and added in a single dispensing step, to further streamline the protocol. The assay is run in two steps (Figure 1). (A) In the stimulation step cells are incubated with activators and inhibitors. (B) In the detection step supernatant containing the secreted IL-6 is then transferred to a second plate, followed by antibody addition.

Nuclear-ID™ Blue/Red Cell Viability Assay

The Nuclear-ID™ Blue/Red cell viability reagent (Catalog No. ENZ-53005) from Enzo Life Sciences (Farmingdale, NY) is a mixture of a blue fluorescent cell-permeable nucleic acid dye and a red fluorescent cell-impermeable nucleic acid dye that is suited for staining dead nuclei. Nuclei from viable cells will stain blue. As cell viability decreases, their membranes lose integrity and the red fluorescent dye is then able to stain the nucleus. The staining pattern arising from the simultaneous combination of these two dyes permits determination of live and dead cell populations by fluorescence microscopy.

Methods

2-Plate Assay Protocol

SKOV-3 cells, in a volume of 100 μ L, were added to the 96-well cell plates and incubated for 24 hours in serum-free medium. 50 μ L of 3x EGF or 25 μ L of 6x EGF and inhibitor was then added to the well and incubated for the appropriate time. Following incubation, 16 μ L of supernatant was transferred to a separate low-volume 384-well plate. 4 μ L of HTRF antibody mix was then added and incubated for 4 hours before reading.

The remaining medium was removed from the cell plate, and the plate was washed once with 1X PBS. 50 μ L of PBS containing the Nuclear-ID reagent was then added to the wells and incubated at 37 °C/5% CO₂ for 30 minutes. Upon completion the plate was washed twice with PBS, and a final volume of 50 μ L PBS was added to the wells before imaging.

Delta F(%) Calculation

$$\left(\frac{\text{HTRF Value}_{(\text{Test Well})} - \text{HTRF Value}_{(\text{Neg Ctl})}}{\text{HTRF Value}_{(\text{Neg Ctl})}} \right) * 100.$$

EGFR Pathway Stimulation Optimization

An initial experiment was performed to assess the level of IL-6 secretion upon stimulation of the EGFR signaling pathway. An 11-point titration of human EGF was created using serial 1:4 dilutions starting at a 1x concentration of 2000 ng/mL. The growth factor was added to the SKOV-3 cells and incubated for 24, 48, 72, or 96 hours. The remaining portion of the HTRF IL-6 assay was then performed as previously explained.

EGFR Pathway Inhibitor Confirmation

Small molecule and anti-EGFR antibody inhibitors were then tested for their ability to attenuate IL-6 secretion as well as cytotoxic properties. Compounds included the EGFR inhibitor AG 1478, the anti-inflammatory Cardamonin, known to inhibit NF κ B activation, the MAP kinase inhibitor U0126, and the PI3 kinase inhibitor LY 294002. Three anti-EGFR antibodies with known human reactivity, 225, 111.6, and Cetuximab, were also included. Inhibitors and EGF were added to the SKOV-3 cells and co-incubated for 48 hours prior to performing the IL-6 and cytotoxicity assays.

Gen5™ 2-Plate Microplate Reader/Imager HTRF IL-6 and Cytotoxicity Protocol

A single 2-plate protocol was created with the Gen5 software to allow efficient processing of the HTRF assay plate and cell plate, as well as eliminating the need to image the entire cell plate, therefore obviating unnecessary data generation and storage. The plate layout created in Gen5 for the HTRF assay plate identifies the location of control and test wells (Figure 2A). Data analysis steps also convert the raw fluorescence data into Delta F(%) and identify "hit" wells where inhibition of IL-6 secretion is $\geq 50\%$ (Figure 2B).

A.

B.

Figure 2. (A) Gen5 plate layout for HTRF IL-6 384-well assay. (B) Data reduction steps for conversion of raw fluorescence values and determination of positive inhibition wells.

Using the data reduction and *Cutoff Analysis* performed following the HTRF assay plate read, wells are identified which demonstrate $\geq 50\%$ inhibition of IL-6 secretion (Figure 3).

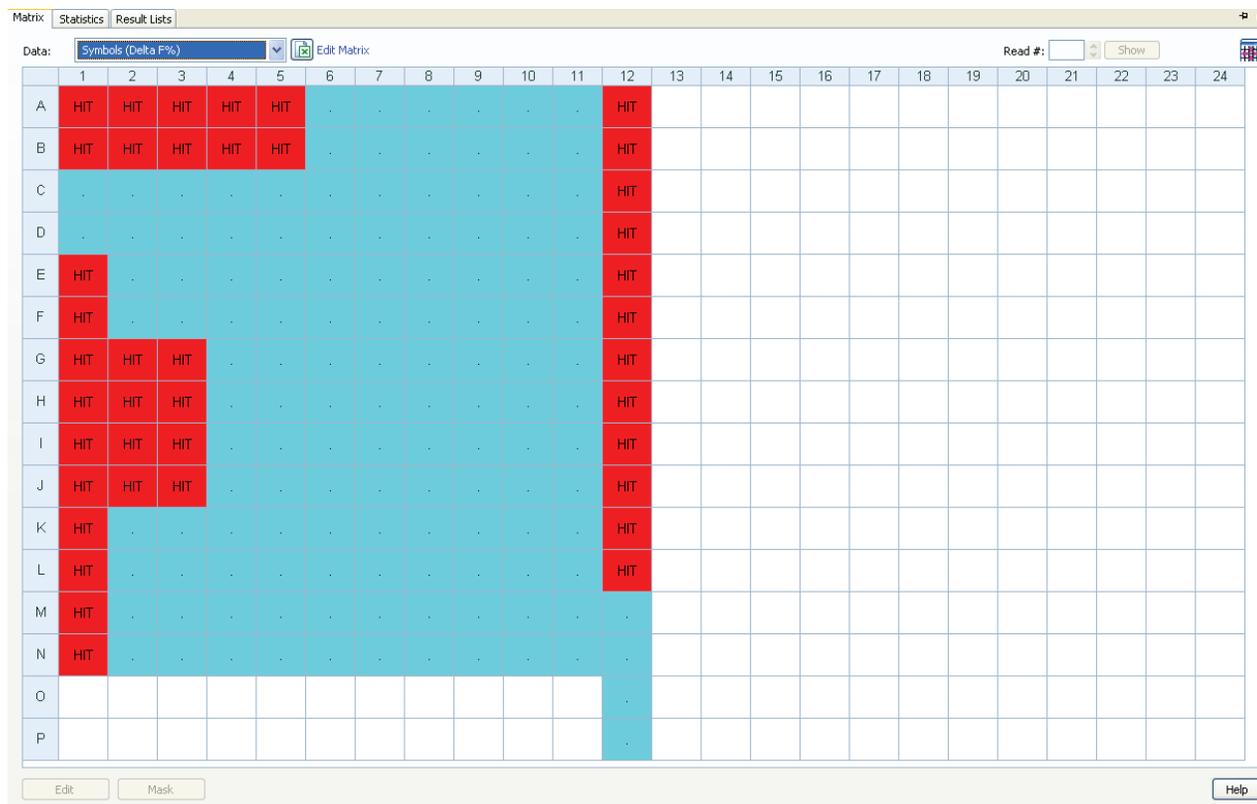


Figure 3. Results from *Cutoff Analysis* of inhibition data. Wells showing Delta F(%) values $\leq 50\%$ of maximum stimulated wells labeled in red. All other wells labeled in green.

In the same *Experiment File* as that used to generate the HTRF results, wells are chosen within the original cell plate to assess potential cytotoxic effects from the inhibitor concentrations of interest (Figure 4).

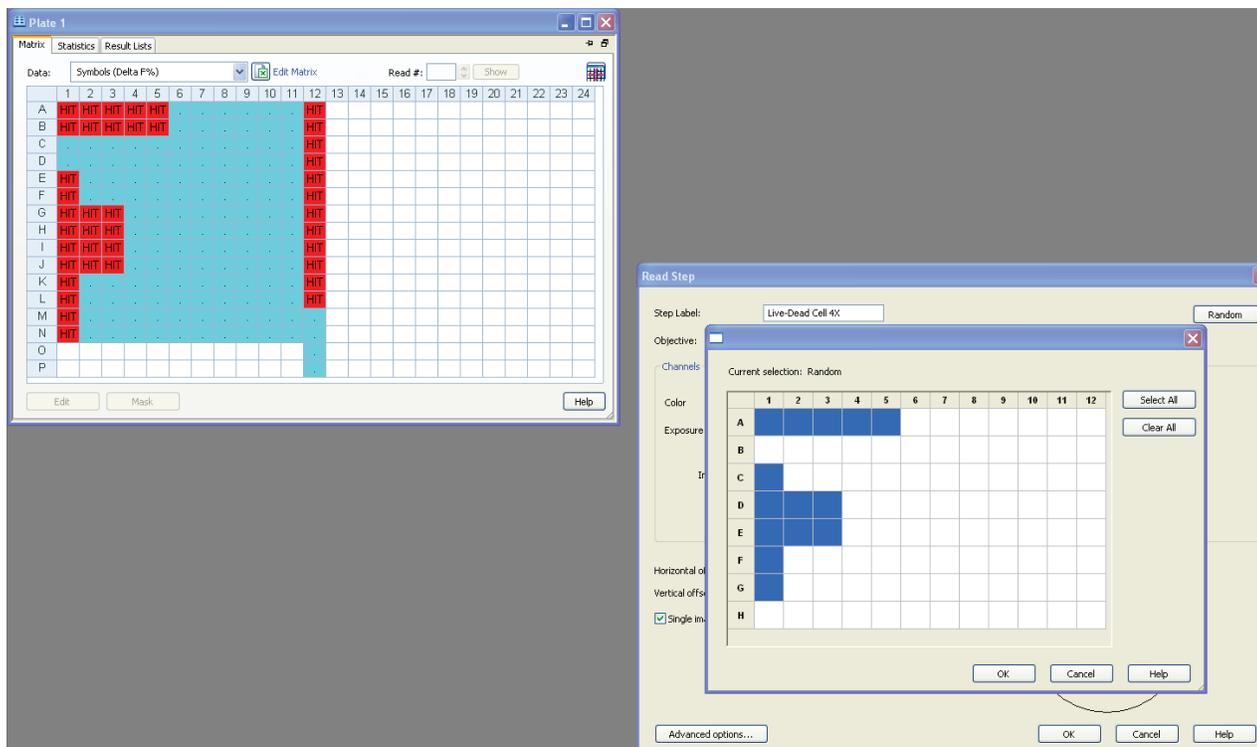


Figure 4. Individual plate layout selected for imaging of cell plate (Plate 2).

4x and 20x images of live and dead cell nuclei, stained with the Nuclear-ID™ Cell Viability Reagent, are then captured (Figure 5).

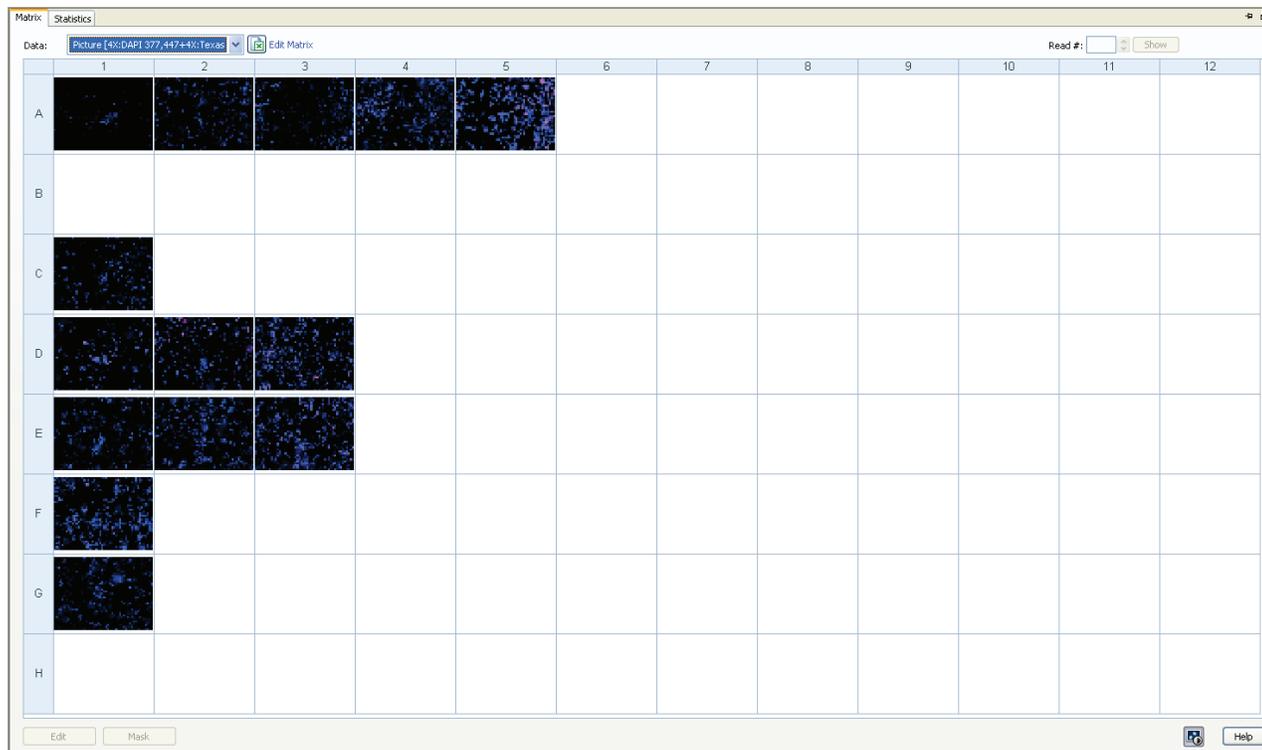


Figure 5. Thumbnail view of 4x blue and red images for selected wells.

The Cellular Analysis tool is then used to determine the number of live and dead cells captured in each 4x image. Object size and Threshold fluorescence value criteria are used to guarantee that the appropriate cells are selected for each count (Figure 6).

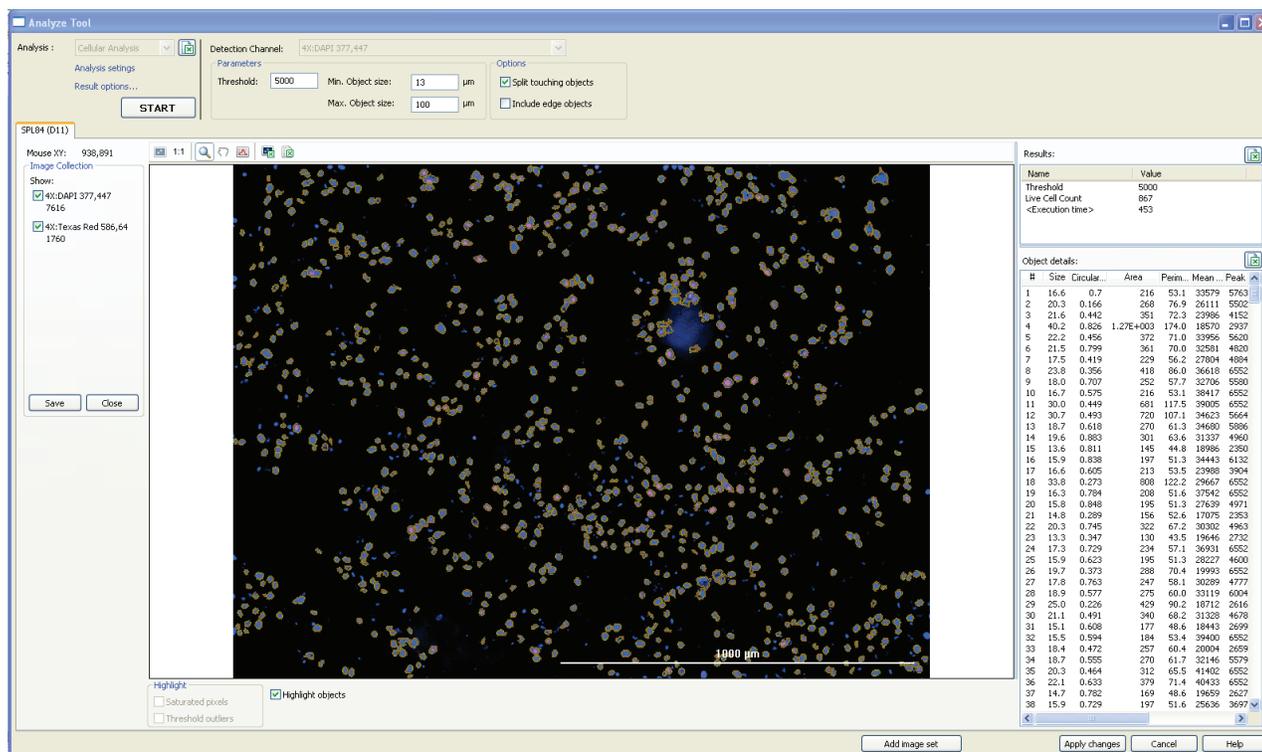


Figure 6. Gen5™ Cellular Analysis imaging tool.

Results and Discussion

EGFR Pathway Stimulation

The results generated in the multi-day pathway stimulation analysis (Table 1) demonstrate that a 48 hour EGF incubation with the serum starved SKOV-3 cells provides the largest change in signal between wells containing stimulated and unstimulated cells, or assay window.

Ratio (Delta F(%)) _{Maximum IL-6 Stimulation} /Delta F(%)) _{Unstimulated}			
24 Hours	48 Hours	72 Hours	96 Hours
2.8	4.3	2.8	2.8

Table 1. Multiple Incubation Time EGF Stimulation Assay Window.

The 24 hour incubation time may not be long enough to see peak stimulation of IL-6 secretion, while the cytokine may begin to be degraded by the SKOV-3 cells with extended incubations such as 72 and 96 hours. Therefore, the 48 hour incubation time was to be used for inhibitor testing.

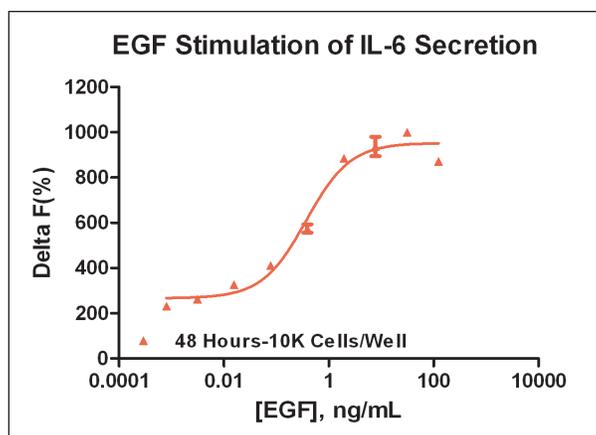


Figure 7. EGF Stimulation of IL-6 Secretion. IL-6 secretion stimulation curve for 48 hour EGF incubation with SKOV-3 cells.

From the 48 hour stimulation curve (Figure 7) it can be seen that maximum stimulation occurs between 1 and 100 ng/mL EGF. A concentration of 35 ng/mL was used for subsequent inhibition analyses.

Confirmation of EGFR Pathway Inhibitors

Inhibition curves for all compounds and anti-EGFR antibodies tested were plotted from the Delta F(%) values calculated by the Gen5™ software using the original 620 nm and 665 nm emission signals (Figure 8).

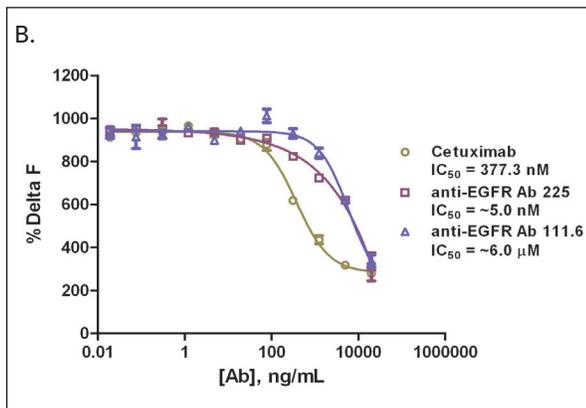
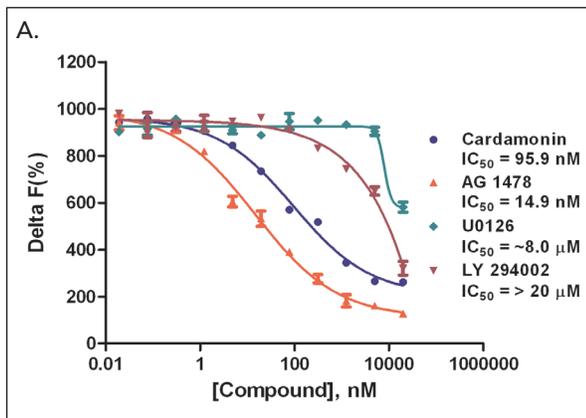


Figure 8. Inhibition of IL-6 secretion for (A) Small Molecule Inhibitors and (B) anti-EGFR antibodies.

While a decrease in IL-6 secretion was seen with all inhibitors tested, a more significant decrease was seen from AG 1478, Cardamomin, and Cetuximab. This is consistent with previous findings which demonstrated that inhibition at the level of receptor and NFκB pathway activation led to a subsequent decrease in EGF-stimulated IL-6 secretion [5]. The lack of appreciable inhibition by U0126 and LY 294002 indicates that ligand-dependent EGFR/MEK/ERK and EGFR/PI3K/ AKT activation plays a diminished role leading to IL-6 secretion in SKOV-3 cells. Finally the increased potency of Cetuximab compared to the other anti-EGFR antibodies tested also agrees with results published from previous comparisons [6].

Cytotoxic effects from inhibitors was assessed by capturing 4x and 20x images of live and dead cells from the predetermined wells of the original cell plate identified in the *Cutoff Analysis* (Figure 9). Additional wells were also imaged in order to determine potential cytotoxicity at the identified IC₅₀ value, as well for the no compound, negative control wells.

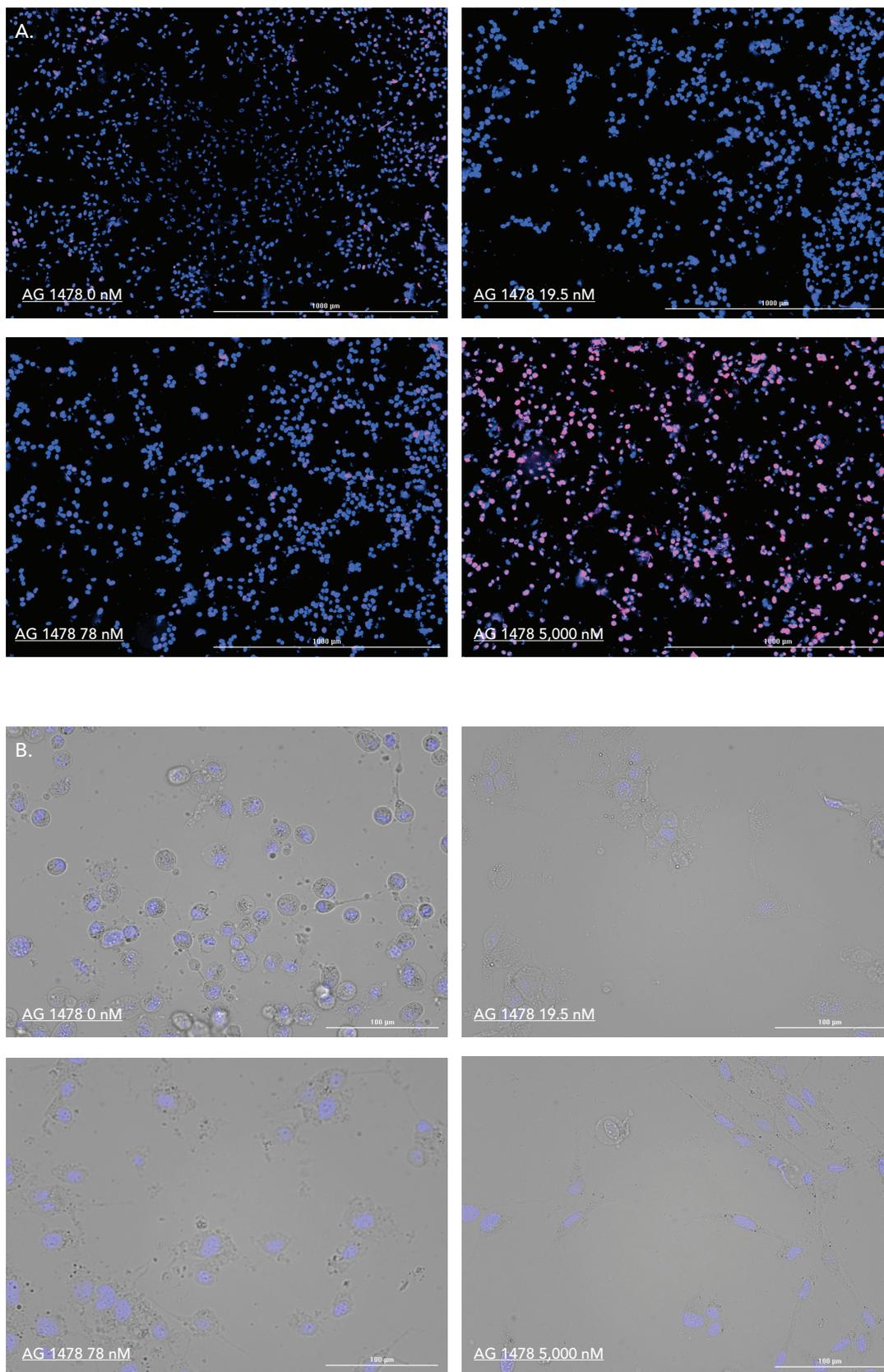


Figure 9. (A) 4X live/dead cell images for AG 1478. Images shown for two pre-selected wells in addition to IC_{50} concentration and no inhibitor control. (B) 20X blue/brightfield images showing cell morphology and stained nuclei.

From the *Cellular Analysis* performed on the 4X images (Figure 6), cytotoxicity curves were generated.

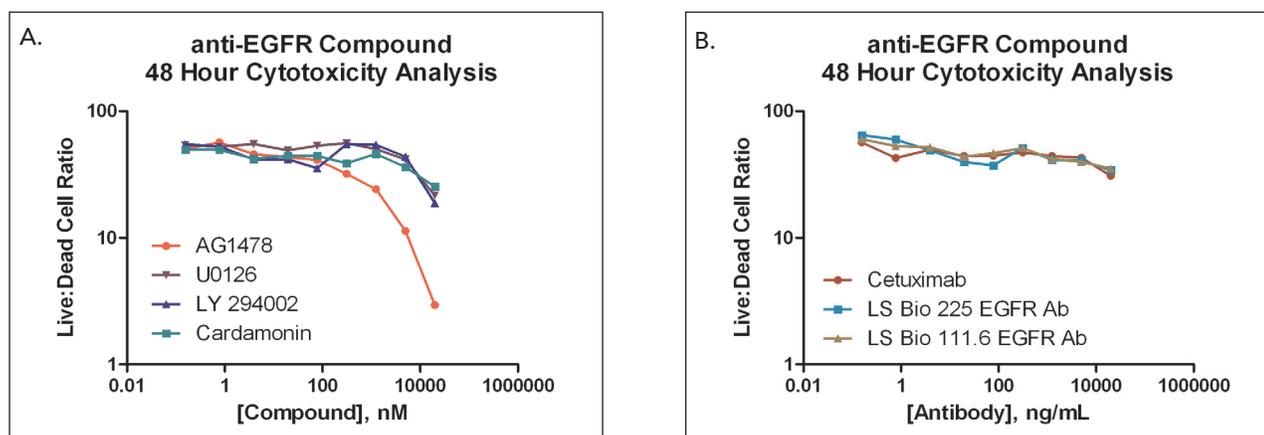


Figure 10. Live/dead cell ratios calculated for all concentrations tested with (A) small molecule and (B) anti-EGFR antibody inhibitors.

The results from the live/dead cell imaging demonstrate that cytotoxic effects are minimal at the IC_{50} concentrations determined when using a 48 hour incubation period (Figure 10). Only at the highest concentrations of small molecule inhibitors tested is there a noticeable decrease in cell viability. A final experiment was performed to confirm receptor binding of the anti-EGFR antibodies. Each primary antibody was added to the SKOV-3 cells, followed by the addition of XL665 labeled goat anti-human Fc antibody. A red fluorescent image is expected where 1° antibody:receptor and 2° anti-human Fc antibody binding takes place (Figure 11).

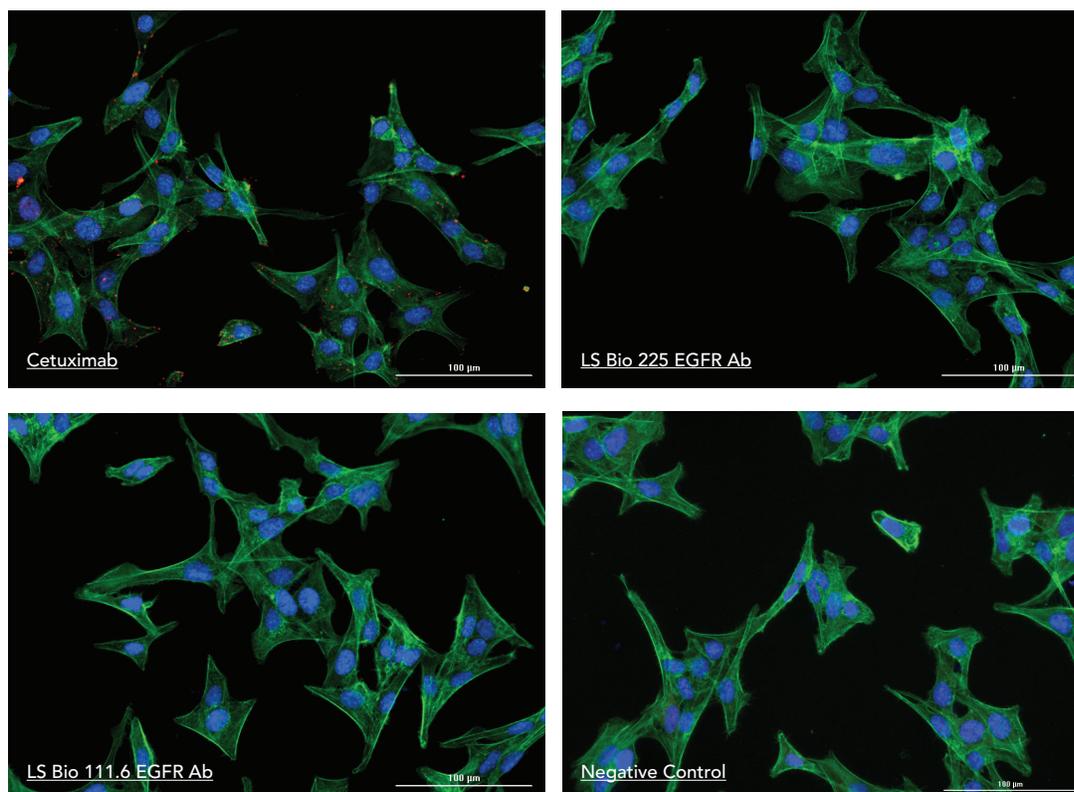


Figure 11. Anti-EGF receptor antibody binding 20x images. Blue indicated Dapi stained nucleus. Green indicates Phalloidin actin staining.

EGF receptor and anti-human Fc antibody binding was seen with Cetuximab only. This was due to the fact that the antibody contains humanized Fab and Fc regions. The 225 and 111.6 antibodies are mouse origin, and therefore demonstrate only human reactivity at the Fab region. Binding was also not seen with the negative control (no 1^o antibody) confirming that non-specific receptor binding of the 2^o antibody does not take place.

Conclusions

The HTRF[®] IL-6 assay provides an easy-to-use, sensitive method for the assessment of cytokine secretion from cancer cell models. When run using the 2-plate protocol, the assay can be multiplexed with the Nuclear-ID™ Blue/Red cell viability assay for rapid evaluation of live and dead cell populations. The combined capabilities of the Cytation™3 afford the ability to easily perform each assay with one instrument. The advanced optics and Gen5™ Data Analysis Software features ensure accurate detection of both reader-based and microscopy assays, and provide efficient performance of the entire experimental workflow.

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