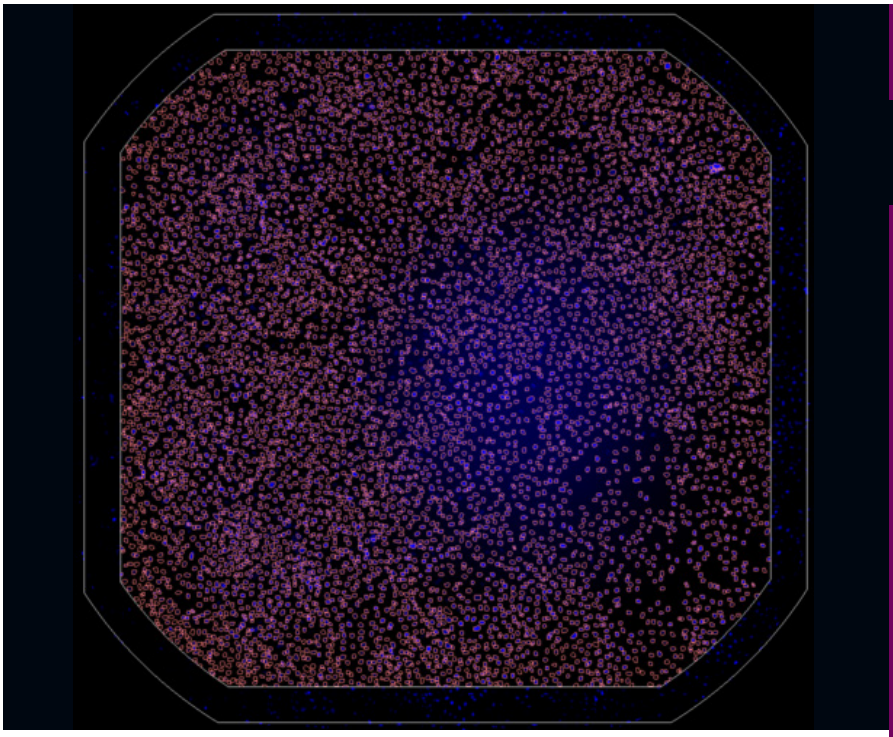

APPLICATION NOTE

AlphaLISA Technology and EnSight Multimode Plate Reader

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Biomarker Detection and Cellular Imaging in the Same Well: A Normalization Approach

Introduction

One concern when assessing the effect of multi-day drug treatment on protein expression in a cellular context is the collateral impact on cell number by either affecting proliferation or inducing cell death pathways. One solution is to count the total number of cells, something that can easily be done for a population of cells in suspension. This is more difficult for adherent cells since the cells must be detached from the plate in a process which can damage extracellular membrane-associated proteins. We present here a simple, high-throughput assay workflow that includes labeling cell nuclei, automated well-imaging and image analysis to count the cells in each well, followed by cell lysis and rapid quantitation of protein biomarkers using the AlphaLISA® bead-based technology. Biomarker concentrations are then normalized to the number of cells per well which provides multiparametric data from the same well. The data we present shows the differential effects of a two-day treatment with Epidermal Growth Factor (EGF) on cellular proliferation and the expression of the EGF receptor (EGFR) and programmed death ligand-1 (PD-L1) in two human cancer-derived cell lines. Both cellular imaging and biomarker detection were performed using the EnSight® multimode plate reader, an integrated imaging and multi-label detection platform in combination with optimized AlphaLISA reagent kits and high-quality microplates as a total solution approach.

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AlphaLISA® technology allows for the detection of molecules of interest in a homogeneous, no-wash format. In addition, AlphaLISA assays require low sample volumes (5 µL or less) and produce highly sensitive results. In the AlphaLISA assay illustrated in Figure 1A, a biotinylated anti-EGFR antibody binds to streptavidin-coated Alpha Donor beads while another anti-EGFR antibody is conjugated directly to AlphaLISA Acceptor beads. Both antibodies bind to human EGFR and bring the Donor and Acceptor beads into proximity. Excitation of the Donor beads with light at 680 nm initiates the release of singlet oxygen molecules that activate the Acceptor beads resulting in an emission of light at 615 nm. The intensity of emission is proportional to the concentration of EGFR protein in the sample.

Methods

Cell Culture and Treatment

The human skin cancer-derived A431 cell line (A-431; ATCC®, CRL-1555™) and human lung cancer derived A549 cell line (A549; ATCC®, CRL-185™) were grown in DMEM (ATCC, #30-2002) and F-12K Medium (ATCC, #30-2004) respectively, supplemented with 10% FBS (ThermoFisher, #11875-093). For all-in-one well assays, A549 cells were seeded at 1,250 cells in 25 µL per well in black ViewPlate™-384 (PerkinElmer, #6007460) microplates. For assays using 96-well format, A431 cells were seeded at 10,000 cells in 100 µL per well into ViewPlate-96 (PerkinElmer, #6005225) microplates. Cells were given time to attach overnight and then treated for two days with varying concentrations of recombinant human EGF (rh EGF; R&D Systems, #236-EG-200).

Cell Staining and Well Cytometry using the EnSight

Two days after treatment, cellular nuclei were labeled by the addition of Hoechst 33342 (Life Technologies, #H3570) diluted in culture media at 5 µg/mL final concentration. After 15 minutes, microplates were imaged using the EnSight multimode plate reader (Figure 1B). Automated cellular imaging and fluorescence measurement of Hoechst-labeled cells were done using the well-imaging module and Kaleido™ software on the EnSight using UV filters. Cellular nuclei identified via Hoechst staining were automatically counted from the specified field and total cell number were extrapolated per well based on well area (Figure 1D).

AlphaLISA Detection Assays

Immediately after imaging (less than 30 minutes from Hoechst addition), culture media were aspirated and cells lysed by the addition of AlphaLISA Lysis Buffer (PerkinElmer, #AL003) for 30 minutes at room temperature on a rotational shaker (DELFIA® PlateShake set at 600-700 RPM). For direct assays in 384-well plates, each well was lysed with 5 µL of lysis buffer. These wells were then assayed using the AlphaLISA detection assays for EGFR and PD-L1 (PerkinElmer, #AL340 and #AL355, respectively). White BackSeals (PerkinElmer, #6005189) were applied to the black ViewPlates in order to boost signal detection. For large-volume assays in 96-well plates, 100 µL of lysis buffer was used, lysates were transferred to polypropylene StorPlates™ (PerkinElmer, #6008290) and frozen at -20 °C. These samples were thawed later and 5 µL samples were taken from the same wells for measuring multiple biomarkers using various AlphaLISA assay kits. AlphaLISA assays were performed in gray assay plates (AlphaPlate™-384, PerkinElmer, #6005350) and carried out as indicated in the kit manuals with a total incubation time of two hours.

AlphaLISA assays were measured using the EnSight multimode plate reader using the default values for standard Alpha detection (Figure 1B). Standard curves were prepared in AlphaLISA Lysis Buffer using recombinant human protein provided in each kit. Data were plotted in GraphPad Prism© and standard curves were fit with nonlinear regression analysis using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting (Figure 1C). The lower detection limit (LDL) of the assay is calculated by taking three times the standard deviation of the average background values and interpolating from the standard curve. Interpolated concentrations represent the amount of protein in a 5 µL sample. All data shown is the average of a minimum of triplicate measurements unless otherwise specified.

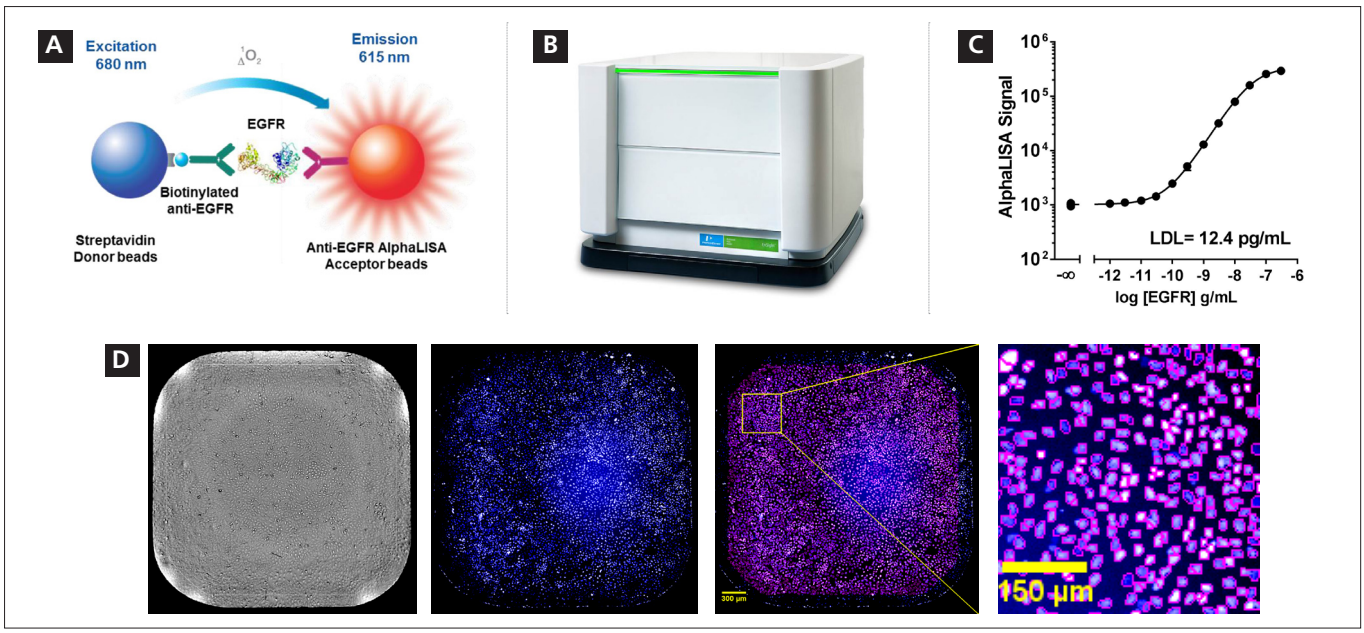

Figure 1. A) Schematic of the AlphaLISA EGFR assay; B) EnSight multimode plate reader with well-imaging capabilities; C) Standard curve of recombinant human EGFR illustrates the AlphaLISA assay sensitivity and dynamic range. D) Representative well-images of A549 cells cultured for two days in a 384-well plate stained with Hoechst and taken using brightfield (gray-scale) and UV (blue) optics using the EnSight. Individual nuclei were automatically identified with the EnSight's Kaleido software and are illustrated by the purple-pink outlines in the images on right.

Results

Hoechst Stain Does Not Interfere With AlphaLISA in Assays

When assessing the effects of an agonist or inhibitor on a cellular process in a specific cell line, it is important to determine if the treatment affects cellular proliferation independent of the effects observed on protein expression. In order to do this, one simple way is to measure cell number with and without treatment. Hoechst 33342 is a commonly used cell-permeable DNA stain that binds preferentially to adenine-thymine regions of DNA of both fixed and live cells. Hoechst dye that is bound to DNA is excited by ultraviolet light (maximally at 361 nm) and emits blue fluorescence at 460 – 490 nm, whereas unbound dye emits rather weakly in the 510 - 540 nm range. AlphaLISA assays require excitation at 680 nm and maximally emit at 615 nm which suggests that residual Hoechst present in cellular lysates should not interfere with the AlphaLISA assay signal. In order to show that residual Hoechst dye in labeled cells do not interfere with the AlphaLISA signal, A549 cells were cultured in 384-well black ViewPlates and treated for two days with varying concentrations of EGF to stimulate EGFR. The A549 cell line, derived from human adenocarcinoma of the lung, expresses EGFR and is used as a model for non-small cell lung carcinoma (NSCLC).[1] After two days, half the wells were labeled with Hoechst and all wells were imaged using brightfield and UV optics on the EnSight, a procedure that takes only 6.5 minutes to image and analyze an entire 384-well plate. Immediately following imaging, culture media were aspirated, cells were lysed, and the AlphaLISA detection assays were run directly in the plate wells (with and without Hoechst). Data show that both raw signal and interpolated concentrations of EGFR and PD-L1 detected using AlphaLISA technology were not influenced by Hoechst labeling (Figure 2). The data also illustrate how the treatment with EGF differentially regulates the expression of each of these biomarkers. EGFR concentrations decrease with increasing concentrations of EGF (Figure 2D), whereas PD-L1 concentrations increase (Figure 2E). The wells that were labeled with Hoechst were imaged and the total cell number (nuclei) quantified per well using the EnSight system. Representative images from a control well (no EGF) are shown in Figure 1D. The resulting data (Figure 2F) suggest no apparent effect of EGF treatment on A549 cell proliferation as shown by cell counting, indicating that biomarker expression changes are independent of cellular proliferation in A549 cells.

Effects of EGF on A431 Cell Number and Morphology

The experiments in 384-well plates illustrate how EGF treatment can influence common cancer biomarker expression levels in A549 cells without an effect on cellular proliferation (cell counts). In order to illustrate the advantage of normalization, we assessed the effects of EGF treatment on a cellular model known to demonstrate anti-proliferative effects. Treatment with EGF has been shown to induce changes in cell morphology and attachment and affect the proliferative capacity of A431 cells.[2] To examine this, A431 cells were seeded into 96-well plates, allowed to adhere overnight, and then treated with varying concentrations of EGF. The 96-well format allows for seeding larger cell numbers and provides the growth area for longer treatment windows in cells that proliferate rapidly, as well as producing larger sample volumes which can be divided and used to quantify multiple biomarkers from the same well. After two days of EGF stimulation, cell nuclei were labeled with Hoechst and the plate imaged on the EnSight.

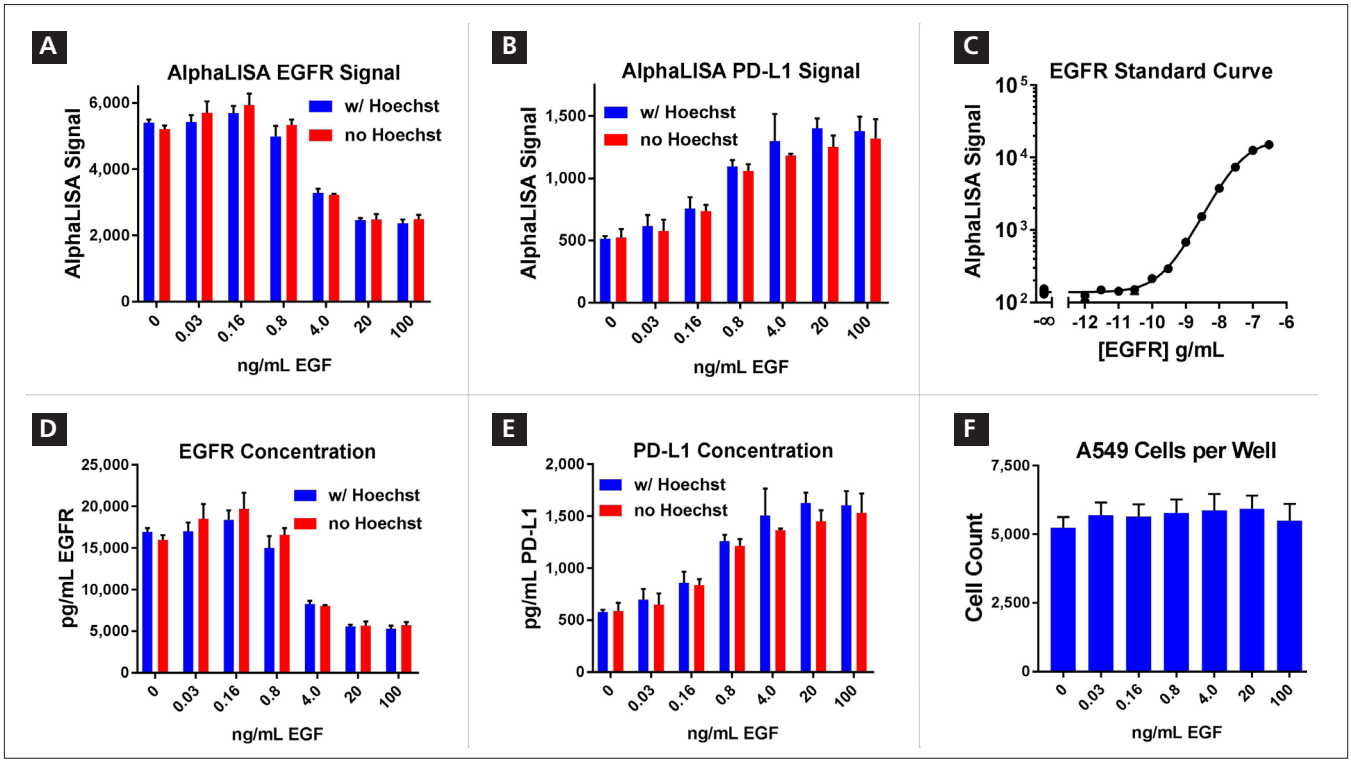

Figure 2. Hoechst stain does not interfere with AlphaLISA assay signal or observed protein concentrations for two AlphaLISA detection assays. After staining half the wells with Hoechst, all wells were imaged and nuclei counted using the EnSight. After imaging, the cells were lysed and AlphaLISA assays for EGFR and PD-L1 detection performed AlphaLISA signal intensities from (A) EGFR and (B) PD-L1 assay kits were not affected by the presence of Hoechst in cell lysate samples. C) A standard curve of recombinant human EGFR run in separate wells of the same plate was used to quantify protein concentrations in cell lysates. D) There is no effect of Hoechst observed on concentrations in pg/mL of EGFR and (E) PD-L1 at all concentrations of EGF tested. F) EGF treatment does not influence the number of A549 cells per well.

The images in Figure 3A of a control well (no EGF) from a 96-well plate show the capability of the Kaleido software to parse and count cells in this format. Magnified images from representative wells receiving different treatment concentrations illustrate the dramatic effects of EGF at higher concentrations on A431 cellular morphology (Figure 3B), producing more sparse cultures of narrow, spindle-shaped cells. To quantify the effects of EGF on total cell number, the EnSight was used to automatically identify and count nuclei, and extrapolate the total number of cells per well. The data in Figure 3C indicates that increasing concentrations of EGF result in a decrease in A431 cell numbers, consistent with a report that high concentrations of EGF can reduce proliferation and induce cell death pathways.[3]

Expression Levels of EGFR and PD-L1 Differentially Regulated by EGF in A431 Cells

We next examined the expression of EGFR and PD-L1 in A431 cultures to determine if they are regulated by treatment with EGF independent of cell numbers. To do this, immediately after imaging and cell counting on the EnSight, media were aspirated, and cultures lysed with AlphaLISA Lysis Buffer. Lysates were examined for EGFR receptor expression and data presented in Figure 4A. From biomarker data alone, it appears that increasing concentrations of EGF result in a decrease in EGFR concentration in A431 cells per well as observed in A549 cells (Figure 2D). However, EGF treatment affected A431 proliferation such that higher concentrations of EGF result in a decrease in the number of A431 cells (Figure 3C). In order to account for these effects of EGF in the interpretation of EGFR detection results, we normalized biomarker data to cell number by dividing the concentration of EGFR (listed here in pg/mL) by the total cell number in each well and multiplied by a factor of 10,000 (for graphing purposes). When this is done, we observe that the EGF effect on EGFR concentration appears to be mainly due to the reduction in cellular proliferation at the higher concentrations (Figure 4B). When PD-L1 concentrations are assessed from the same wells (Figure 4C), it appears that higher concentrations of EGF induce upregulation of PD-L1 up to a point (4.8 ng/mL) but then levels decrease. However, when the effect of EGF on the number of A431 cells per well are accounted for, the normalized data (Figure 4D) indicate that PD-L1 levels continue to increase *per cell* in wells treated with the highest concentrations of EGF. This would not be apparent if biomarker data were not normalized to cell number. Since the lysate sample volume collected from the 96-well plate was 100 µL per well and AlphaLISA detection assays require a maximum of 5 µL, multiple biomarkers can be assessed from the same wells and normalized to cell number. In addition, supernatant samples can be taken prior to Hoechst staining for assessment of secreted factors like cancer-regulated chemokines and growth factors. For further reading about other common EGFR-regulated biomarkers assessed in these cultures, see the associated application note.[4]

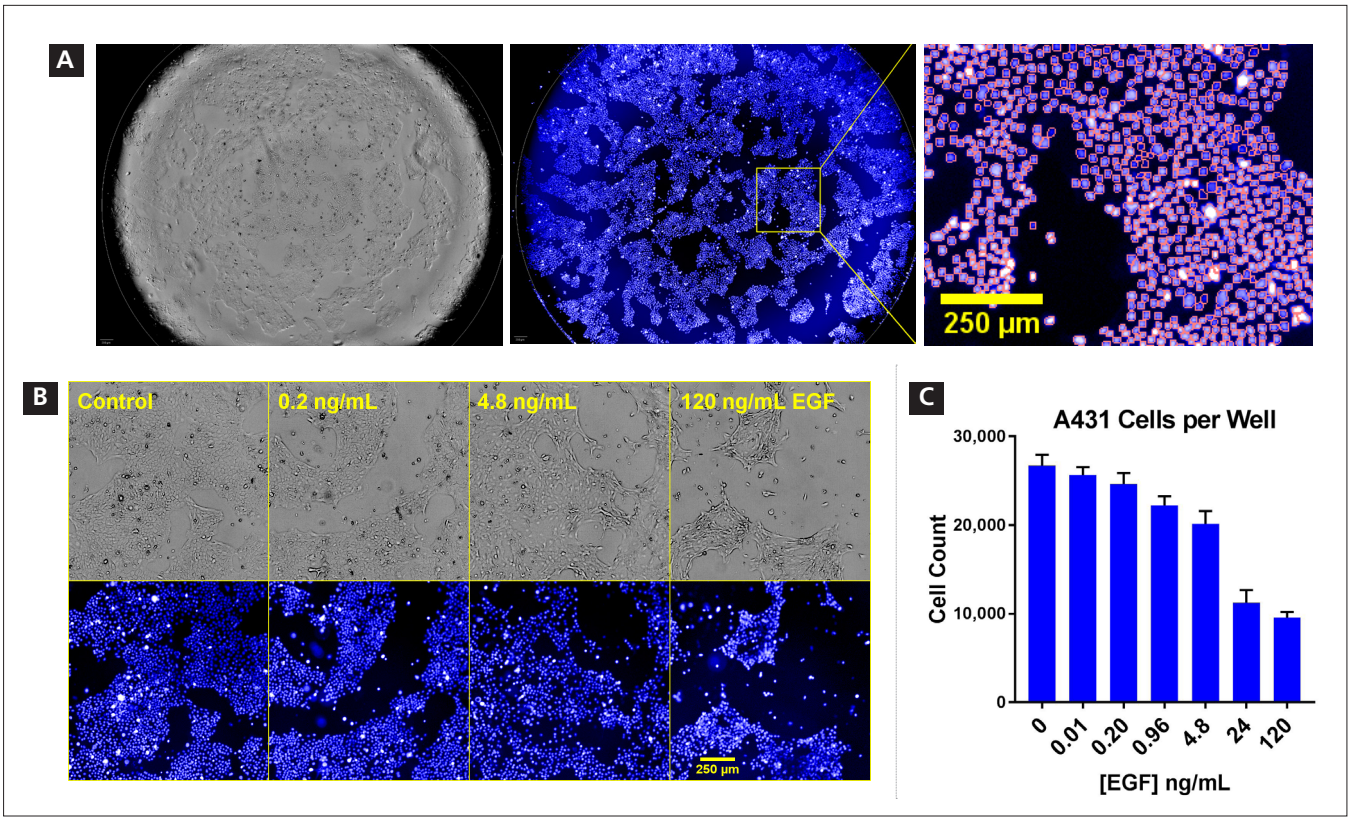

Figure 3. A) Brightfield (grayscale) and Hoechst-stained (blue) images of A431 cells cultured for three days in a 96-well plate. B) Representative images of A431 cells illustrating the effects of treatment for 2 days with 3 concentrations of EGF on cellular proliferation and morphology. C) A431 cells treated for 2 days with varying concentrations of EGF were labeled with Hoechst and counted on the EnSight.

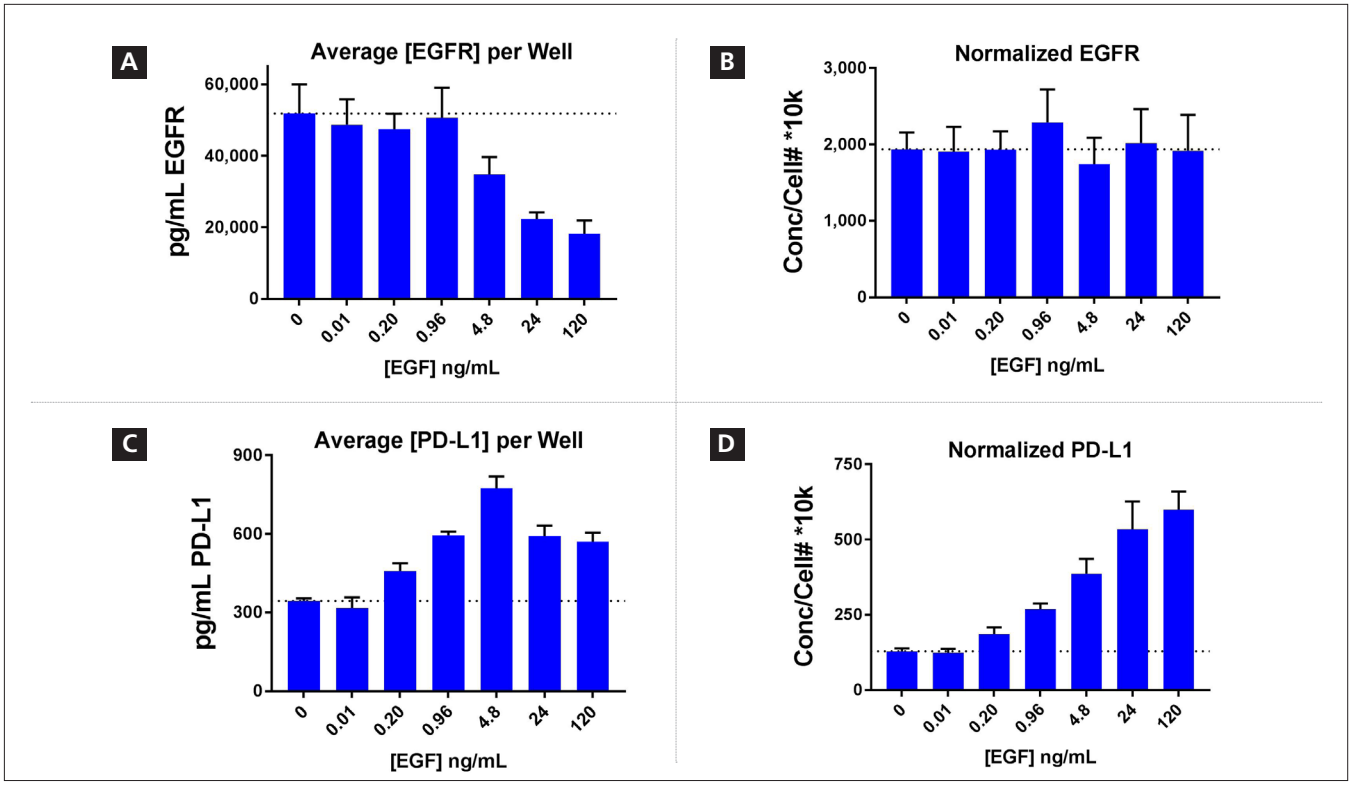

Figure 4. Normalizing biomarker concentrations to the number of cells per well using the EnSight multimode plate reader to both count cells and measure protein concentrations with AlphaLISA assays performed on lysate samples from the same well. (A) EGFR and (B) PD-L1 concentrations were measured in A431 lysate samples from 96-well plates using AlphaLISA detection assays. Protein concentrations in each well were normalized to cell number counted using the EnSight and multiplied by a factor of 10,000 (y-axes labeled Conc/Cell#*10k). Normalized data illustrate that the EGF-induced reduction in EGFR is correlated to a decrease in cell number as seen by the lack of an effect after normalization (B), whereas PD-L1 expression continues to increase with higher EGF concentrations (D).

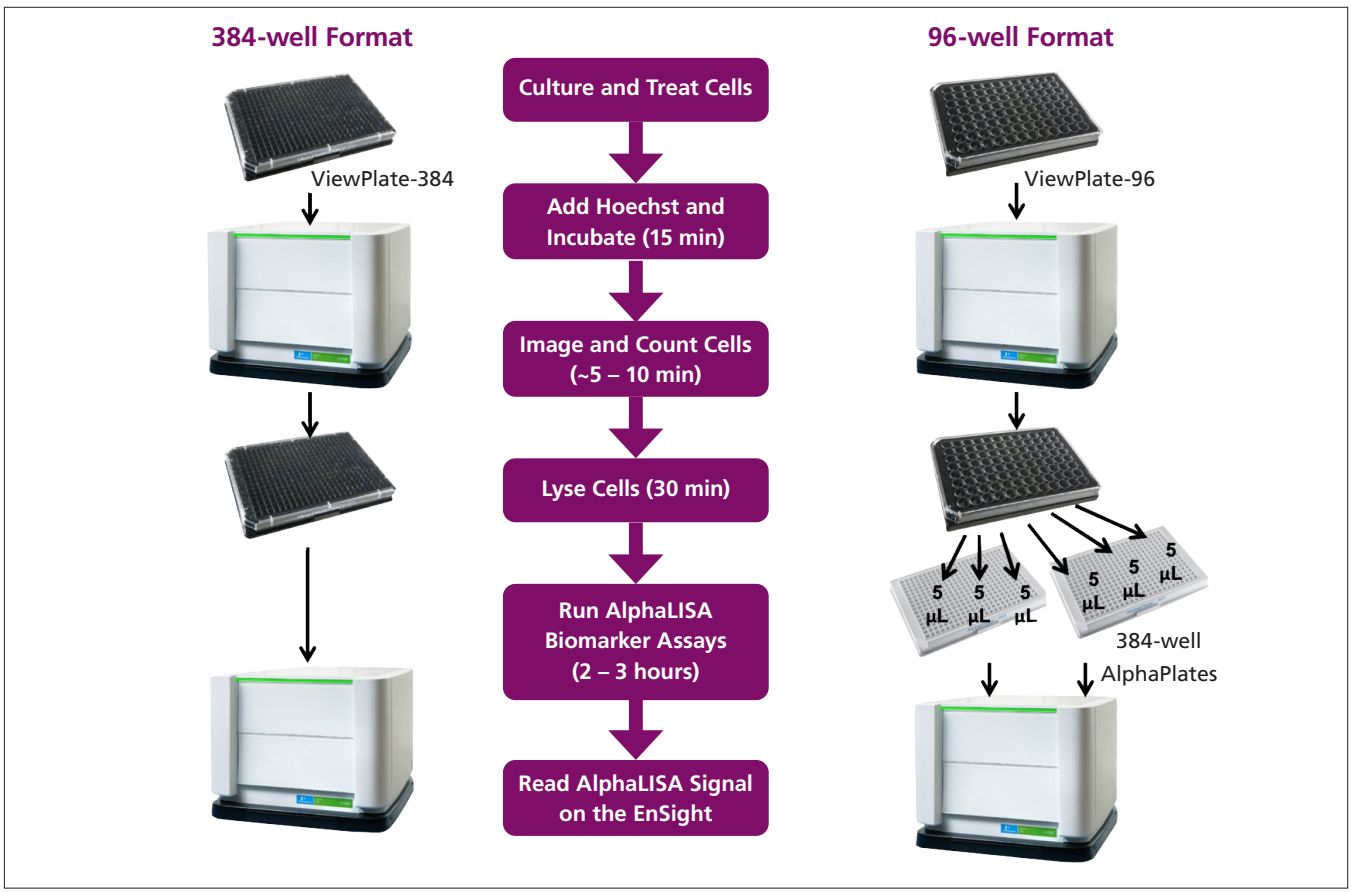

Figure 5. Assay workflows for labeling adherent cell cultures with Hoechst, well imaging, cell counting, and biomarker detection and measurement using AlphaLISA assays and the EnSight multimode plate reader.

Conclusion

The assessment of treatment effects of various agonists and inhibitors on protein expression in a cellular context can be complicated by collateral effects on cellular proliferation. Here, we present a method to rapidly measure the effects of EGF treatment on cellular growth and the expression of key biomarkers regulated by activation of EGFR (Figure 5). We demonstrate the ease at which one can normalize biomarker concentration data to cell number using the EnSight multimode plate reader to both count cells and measure biomarkers with AlphaLISA detection assays in two separate human cancer-derived culture models. There is no interference observed from labeling cells with Hoechst prior to AlphaLISA signal detection or biomarker quantitation. This method works well in both a direct 384-well context and in 96-well cultures where larger sample volumes can be divided up to assay for multiple biomarkers from the same culture well. We illustrate here the importance of normalizing biomarker concentration data to cell number before drawing final conclusions by factoring in effects on proliferation of a given treatment as seen with EGFR and PD-L1 in the A431 cell line (Figure 4). We also demonstrate how different cellular models expressing the same receptor can show differential effects in biomarker expression and proliferation that can be easily measured by combining Alpha technology and the imaging capabilities of the EnSight multimode plate reader.

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