Roche

xCELLigence System

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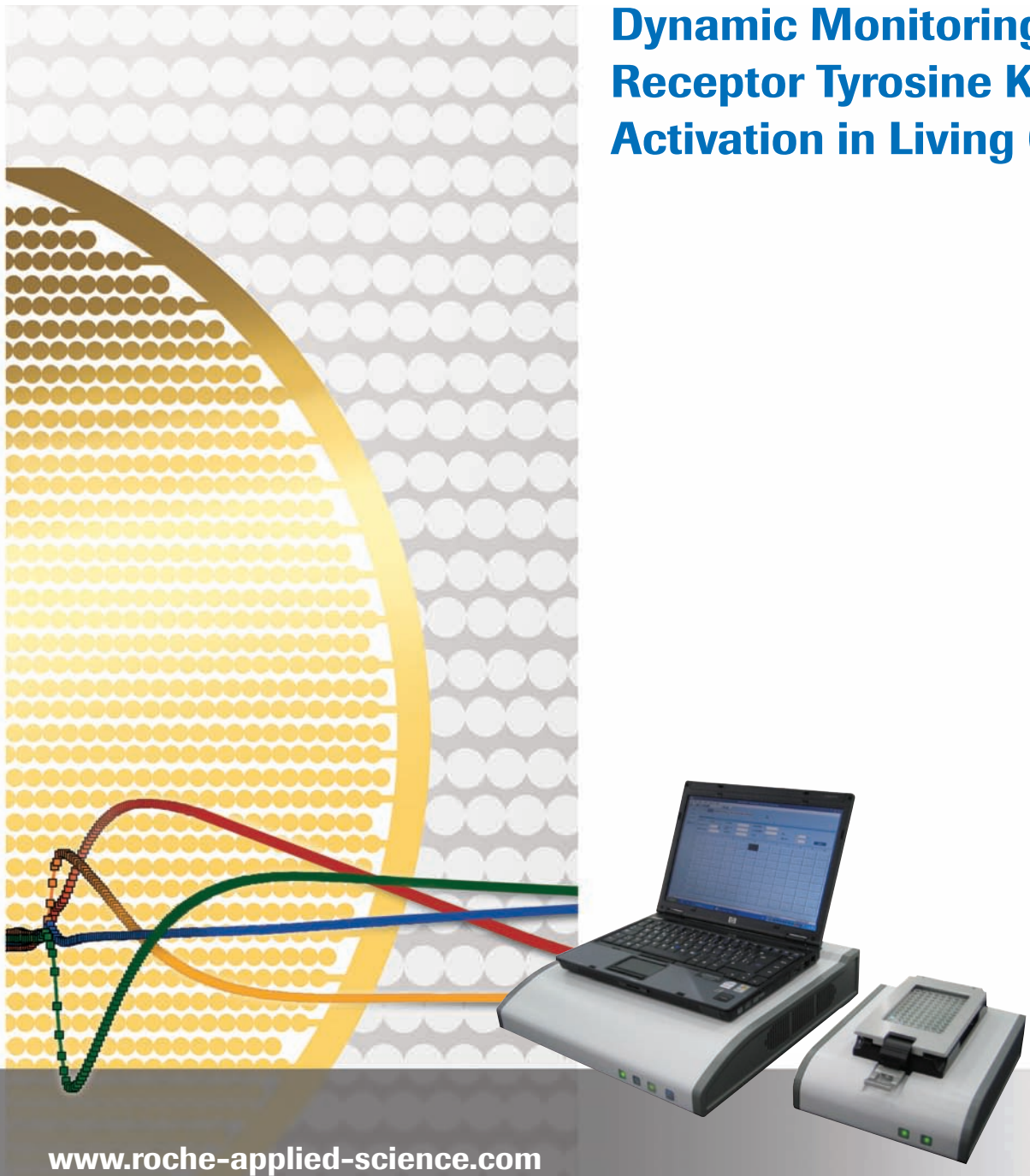
Dynamic Monitoring of Receptor Tyrosine Kinase Activation in Living Cells



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ACEA Biosciences, Inc.

Dynamic Monitoring of Receptor Tyrosine Kinase Activation in Living Cells

Introduction

Over 500 different protein kinases have been identified, constituting ~1.7% of the human genome; of these, 11% are known to be receptor tyrosine kinases (RTKs) (1). RTKs and their growth factor ligands mediate important cellular processes including proliferation, survival, differentiation, metabolism, motility and gene expression. Loss of regulation of RTK expression or activity has been implicated in initiation and progression of cancer, inflammation, diabetes and cardiovascular disease. Their central role in these cellular processes and disease states has made RTKs an attractive and important target for the development of inhibitors that could be therapeutic for these diseases. Several antibody- and small molecule-based inhibitors specific for various RTKs have been approved by the FDA for the treatment of different cancers.

RTKs are membrane receptors that contain an intracellular kinase domain, which transfers a phosphate group from an ATP molecule to the hydroxyl group on tyrosine residues. Upon ligand binding, RTKs dimerize or oligomerize, resulting in autophosphorylation and increased activation of their intrinsic kinase activity. This leads to phosphorylation of several downstream effector proteins, resulting in activation of multiple signaling pathways. These pathways include activation of Ras/MAPK, phosphoinositide-3 kinase and PLC pathways. Another pathway activated as a result of growth factors binding to their cognate RTKs is the phosphorylation of effector proteins such as Src, Paxillin and FAK. Activation or phosphorylation of these proteins leads to cytoskeletal changes that include membrane ruffling, lamellipodia and filopodia formation (2). These cellular changes are a result of actin remodeling, and are mediated by the activities of small GTPases Rac, Rho and Cdc42 (3).

Numerous screening platforms have been developed for the identification of inhibitors for RTK. They are generally subdivided into:

- Antibody-dependent technologies including AlphaScreen, TR-FRET, FP, TRF, SPA, Luminex and ELISA
- Antibody-independent methods such as incorporation of radioactivity, ATP consumption and technologies based on change of substrate size and charge

Although these technologies offer some advantages, they are limited by one or more of the following factors: complicated and tedious optimization steps, limited substrate capacity, assay component interference, and expensive assay components. All of these factors can affect the signal, throughput, time and utility of the assay.

ACEA Biosciences developed a unique cell sensor system, namely electrodes integrated into the wells of a microtiter plate (E-Plate). These sensors are arrayed in a novel design that covers 80% of the well surface area, allowing for sensitive, quantitative detection of cellular changes. Signals from these sensors are relayed to a real time, impedance-based system (RT-CES®) that can monitor and analyze the kinetic aspects of cellular behavior. This system is the predecessor of the new xCELLigence System jointly developed by Roche Applied Science and ACEA Biosciences.

The signals relayed to the system are impedance changes resulting from an ionic environment that is created by application of an electric field. Disruption of this ionic environment on the sensor surface, due to the presence of cells or changes in cell morphology, can cause changes in measured impedance, which is then converted to a cell index value. The extent of the cell-electrode impedance response depends on the quality of the cell attachment and the sensor area covered by the cell. When cell number or degree of attachment increases, it causes a corresponding increase in measured impedance value, and, therefore, in observed cell index. This system has been successfully used in monitoring cell proliferation and cytotoxicity, cell adhesion, and G-protein coupled receptor function.

This report demonstrates the development and utility of an alternative RTK assay that uses this impedance-based system. This assay addresses several of the limitations mentioned above, and provides a facile, easy platform for identification and further characterization of RTK inhibitors.

Introduction continued

It is known that growth factor binding to RTK results in immediate morphological changes. The impedance-based system makes it possible to quantitatively assay these cellular changes and, hence, measure receptor tyrosine kinase activity and function. Experiments described here show that these cell assays are specific, robust, reproducible and in concurrence with other RTK cell-based assays, such as ELISA. Therefore, the impedance-based system was used to screen a small diverse library of inhibitors and a collection of kinase inhibitors; this screen identified a specific and potent EGFR inhibitor. The assay was also used to generate dose response curves, thereby further characterizing the inhibitor.

Materials and Methods

Cell Culture and Reagents. COS7 cells were acquired from ATCC. They were maintained in DMEM supplemented with 10% fetal bovine serum and incubated at 37°C with 5% CO_2. Cells were plated on E-Plates at 1 x 10^4 cells per well and incubated overnight. On the day of assay, cells were serum-starved in DMEM supplemented with 0.25% BSA for a total of 4 hours. If pretreated with inhibitors, cells were incubated with the inhibitors during the last hour of serum starvation and then stimulated with growth factors. Inhibitors (Calbiochem) and LOPAC enzyme inhibitor ligand set (Sigma) were resuspended and stored according to manufacturers instructions.

RTK Assays Using Impedance Technology. Cells were continuously monitored with the impedance-based RT-CES® system. RTK-induced effects were detected as changes in impedance and expressed in arbitrary cell index units.

ELISA. Cells were plated on E-Plates at 1 x 10^4 cells per well and incubated overnight. On the day of assay, cells were serum-starved in DMEM supplemented with 0.25% BSA for a total of 4 hours. If pretreated with inhibitors, cells were incubated with the inhibitors during the last hour of serum starvation and then stimulated with growth factor for 15 minutes. After growth factor stimulation, cells were washed 2 times with cold PBS and lysed. ELISA (Biosource) assay was performed to detect total EGFR and phospho-EGFR (1068); assays were read at 450 nm.

Statistical and Data Analysis. All dose response curves were generated by plotting the average % control (+/- standard deviation) versus ligand or inhibitor concentrations. The average % control was calculated relative to samples treated with growth factor alone without inhibitor; samples were measured in quadruplicates. The EC_{50} for ligands and IC_{50} for inhibitors were determined from a fitted curve generated by XL*fit* 4.0.

Results and Discussion

Specificity of cellular response to EGF and insulin treatments

Cells plated on the E-Plates were monitored from the time of plating to the end of the experiment. This allows the cells and assay conditions to be monitored constantly before and during the time of experimentation. 1 x 10^4 COS7 cells in E-Plates were serum-starved for a total of 4 hours and stimulated with 25 ng/mL EGF or insulin, then monitored every minute from the time of ligand addition.

Ligand addition results in a rapid and transient increase in cell index for both EGF- and insulin-treated cells (Figure 1A). This increase was immediately followed by a decrease in cell index, with EGF-treated cells showing a faster decrease than insulin-treated cells. The transient increase in cell index is a result of cytoskeletal rearrangements due to growth factor treatment, which is a well-documented (2) effect of RTK activation.

To characterize the specificity of these responses to ligand treatment, cells were pretreated for one hour with 10 µM of the EGFR inhibitor (EGFRI), 4557W, before addition of EGF or insulin. Since the inhibitor is specific to EGFR, application of the EGFRI should only affect cellular changes induced by EGF treatment. Indeed, after ligand addition, insulin-

treated cells showed the transient increase in cell index, but EGF-treated cells did not (Figure 1B). The absence of cell response in EGF-treated cells was a result of the specific inhibition of EGFR and its signaling pathways by the EGFRI. The specificity of this inhibitor and ligand response is demonstrated by the lack of effect on the transient cell index increase in insulin-treated cells.

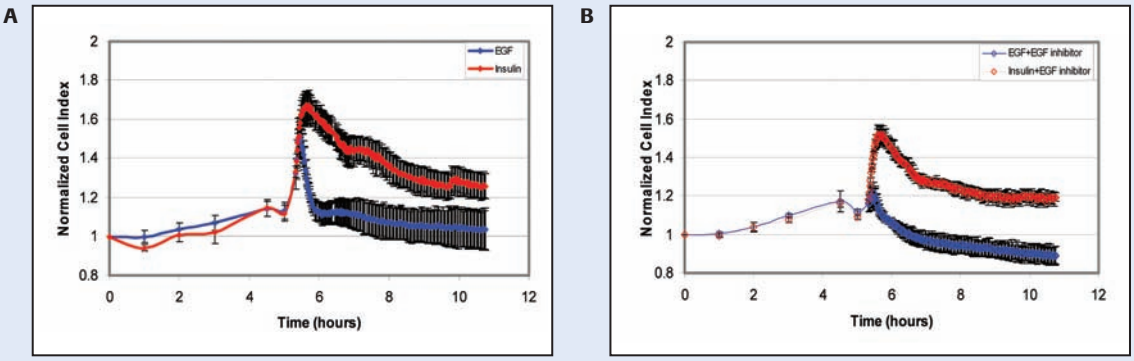


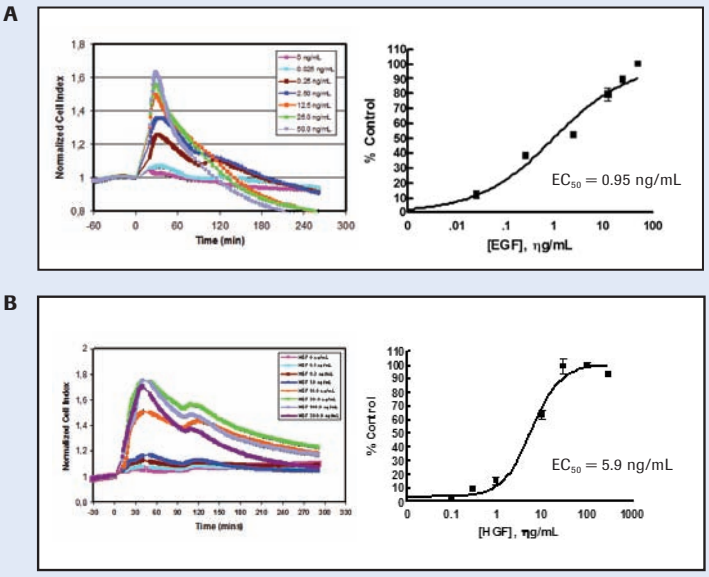
Figure 1: Assessment of specificity of cellular response to EGF and insulin treatments. COS7 cells were pretreated for 1 hour with either a specific EGFR inhibitor or vehicle. Cells were then stimulated with insulin or EGF. (A) Cells treated with insulin or EGF showed a characteristic rise in cell index. (B) When pretreated with 10 µM EGFR inhibitor, 4557W, the EGF response is inhibited while the insulin response remains intact.

Characterization of COS7 cellular response to EGF and HGF treatments

To further characterize this cellular response, a wide range of EGF and HGF concentrations were used to determine the ligand EC_{50} (Figure 2A and 2B). For each concentration, the cell index was measured every minute over several hours. Cells treated with low concentrations of the ligand showed transient small changes in peak cell index, while increasing the concentration of ligand resulted in an increase in the amplitude of the cell index peak. The magnitude of the cell index was directly related to the concentration of ligand used and reached a saturable response.

These cell index traces were used to determine the maximum cell index for each ligand concentration, and to calculate the % control (*i.e.* relative to the response of the sample when it was treated with the maximum concentration of ligand); these % control values were plotted versus ligand concentration. From the fitted curves the EGF and HGF EC_{50} were calculated to be 0.95 ng/mL and 5.9 ng/mL, respectively.

An important consideration for establishing the validity of this new method was to show that these results are consistent with other RTK assays. To compare the EC_{50} values derived from this new system with a well-established assay used to monitor RTK activity, ELISA assays were performed to detect phosphorylated EGFR on COS7 cells that had been treated with varying concentrations of EGF, HGF and EGFR (Figure 2C). From the fitted curve an EC_{50} value of 2.6 ng/mL was calculated. This value was comparable to EC_{50} values determined using impedance technology, thereby demonstrating the validity of using this system as an alternative or complementary assay to existing RTK assays.



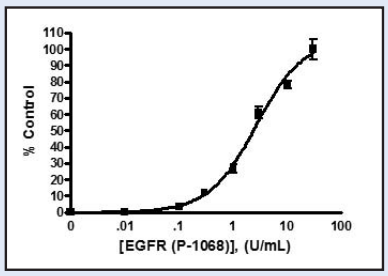
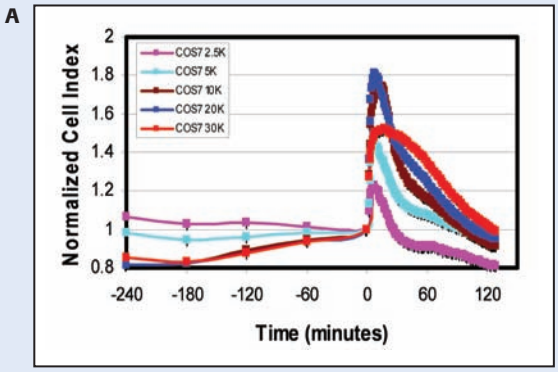
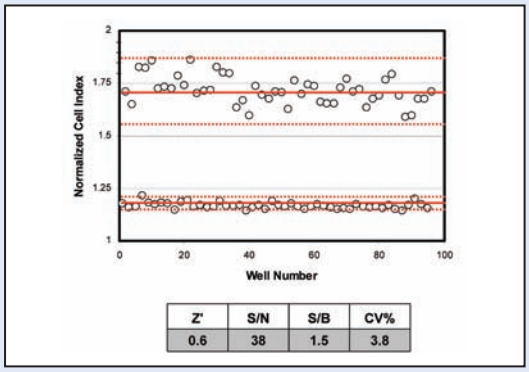


Figure 2: Characterization of COS7 cellular response to EGF and HGF treatments.
Cell index traces of COS7 cells treated with EGF (A) and HGF (B). Maximum cell indices were determined from each trace and dose response curves were generated by plotting % control versus ligand concentration. (C) ELISA assay of phosphorylated EGFR was performed on COS7 cells treated with varying concentrations of EGF. Dose response curves were generated by plotting % control of absorbance readings versus ligand concentration.

Optimization of assay conditions for screening of inhibitors against RTKs

Before the new system could be used to screen for EGFR inhibitors, a few system parameters had to be optimized. First, the optimum concentration of cells needed to achieve the maximum signal-to-noise ratio was determined (Figure 3A). A range of COS7 cells were plated and tested for response to EGF. The peak in cell index due to EGF treatment increased as the number of cells plated increased. However, there was a critical cell density, above which further increase in cell number resulted in a decrease in cell index. This decrease is thought to be due to the absence of available space between the cells, which prevents the lateral expansion of cell membranes over the sensors during ligand-mediated cytoskeletal rearrangement. Second, the ligand concentration was titrated to determine the maximum amount of ligand needed to produce the highest signal, as well as the appropriate ligand concentration for the type of assay used.
After these parameters were optimized, statistical parameters, including the Z' factor of the assay, were calculated (Figure 3B). The Z' factor is a statistical parameter used for evaluating assay quality; the value calculated for this assay, 0.6, is above the acceptable limit for a robust and consistent assay, and the S/N values is 38.





Z'	S/N	S/B	CV%
0.6	38	1.5	3.8

Figure 3: Optimization of assay conditions for screening of inhibitors against RTKs. (A) Increasing number of COS7 cells were plated and treated with EGF. Cell indices were measured every minute over several hours. B) Statistical evaluation of label-free EGFR inhibitor screening assay. Z', S/N, S/B and % CV were determined to assess quality of assay.

Validation of the impedance-based assay system via inhibitor screening

In order to validate this assay, it was used to screen a diverse collection of small molecule inhibitors from Sigma (Figure 4A). This library was supplemented with a specific EGFRI. The library was arrayed in a 96-well plate. The plate contained a single concentration, between 5 and 10 µM, of each component in the inhibitor ligand set from Sigma; it also contained several wells of full activity (positive) and zero activity (negative) reference controls. Maximum cell index due to EGF treatment was determined for each inhibitor-treated sample. In addition, the % control (relative to the positive reference, *i.e.* EGF-treated cell without inhibitor) was calculated. Using 60 % (or 40% inhibition) as the cutoff criteria, the screening study identified only a single potent inhibitor or "hit". This inhibitor was the EGFRI, 4557W, which had been added to the library. The assay was also tested against a collection of kinase inhibitors, and similarly identified the EGFRI as the one that produced the most significant inhibition (Figure 4B). A dose response curve was generated for this inhibitor (Figure 4C). From the fitted curve, an IC_{50} of 161 nM was calculated.

These experiments demonstrate that the system is able to identify a potent and selective inhibitor from a diverse inhibitor library and also from a chemically focused kinase inhibitor library. The system can also be used to further characterize identified hits.

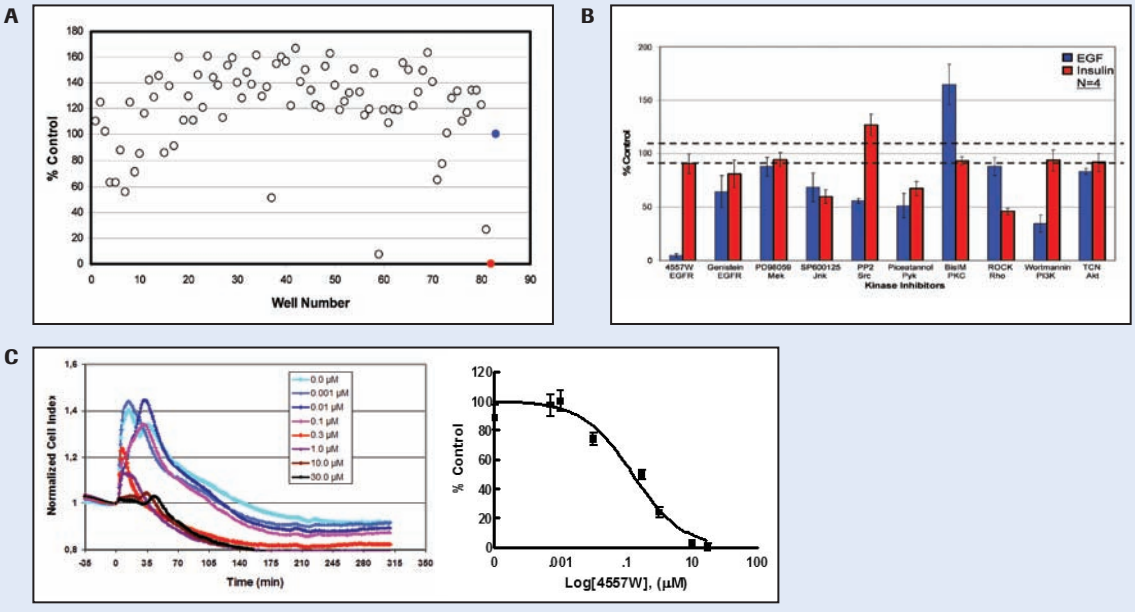


Figure 4: Validation of the impedance-based assay system via inhibitor screening (A) Graphical representation of a screen of 81 compounds, mostly from the enzyme inhibitor Ligand Set of Sigma. Compounds were screened in singlets at 5-10 µM concentrations. The red circle represents the negative control; the blue circle represents the positive control. (B) A collection of kinase inhibitors were screened for inhibition of EGFR activity. (C) EGFRI, 4557W, was identified as potent inhibitor of EGFR signaling from both screens. Cellular response to EGF was measured after the cells were pretreated with varying concentrations of inhibitor. Dose response curves were generated by plotting % control of maximum cell index versus ligand concentration.

Results and Discussion continued

Reaction of selected immortalized cell lines to treatment with various ligands for RTK

In addition to insulin and EGF, other growth factors, including HGF, FGF and PDGF, have produced a cellular response in different mammalian cancer cells. To explore the responses to these growth factors, several human cell lines were plated and treated with different growth factors (Figure 5). Cell lines such as A431 showed a robust response to EGF and HGF, but responded only minimally to other growth factors.

In conclusion, these data demonstrate a facile and novel cell-based assay for RTK activity and function. This assay quantifies morphological changes in response to growth factor treatment and therefore mimics proximal events in kinase activation.

Unlike other RTK assays, this assay is:
- cell-based
- label-free
- capable of monitoring cellular changes in real-time
- non-invasive

Furthermore, the assay provides valuable information about the state of the cell and the signaling pathways being activated. In addition, the RTK assay described here does not require expensive reagents, *e.g.* purified antibody or peptides, nor suffer from assay component interference. Since the readout is non-invasive, multiple treatments can be performed in the same well. The assay can also be used in conjunction with other existing cell-based assays for RTK. Finally, it requires very little optimization and user training, making this assay amenable for use in both primary and secondary screens.

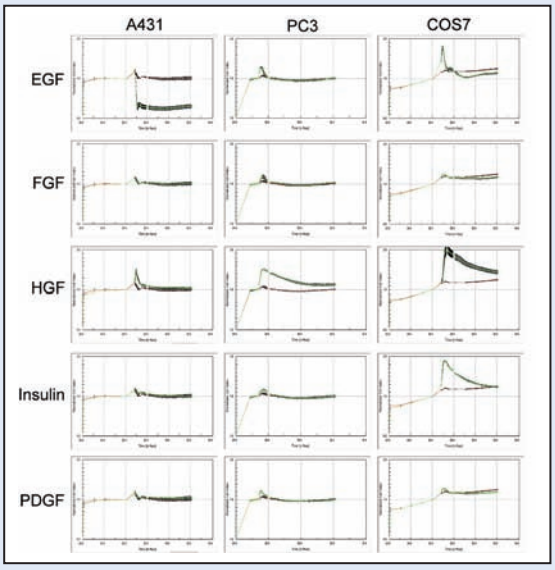


Figure 5: Comparison of unique signaling patterns of selected immortalized cell lines after treatment with various ligands for RTK. Cells were plated, serum-starved and treated with ligands. Green traces represent cells treated with ligand, and red traces represent cells treated with vehicle. Response was measured every minute and data normalized to time of ligand addition. Error bars represent a standard deviation of n=4.

References

1) Manning G et al., (2002) *Science* **298**:1912
2) Hall, A (1998) *Science* **279**:509
3) Etienne-Manneville S and Hall A, (2002) *Nature* **420**:629

The original impedance-based assay system (RT-CES®), which was used to perform the experiments described in this application note, is the predecessor of the new xCELLigence System jointly developed by Roche Applied Science and ACEA Biosciences. While retaining the advantages of impedance-based technology described in this publication, the xCELLigence System will have improved functionality over the original system.

Intended Use:
The xCELLigence System is intended for life science research only. It is not intended for use in diagnostic procedures.

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