Application Note

Monitoring of EGFR (Receptor Tyrosine Kinase) activation in parental and genetically modified cells

Topic:
EGFR (RTK)
Cells:
HEK 293 parental,
HEK 293 ΔG7 knockout
Tools:
AtlaZ

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Summary

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors that play crucial roles in various cellular processes, including cell growth and proliferation, differentiation, migration or metabolism [1]. They function by binding extracellular ligands, which triggers receptor dimerization and activation of their intracellular tyrosine kinase domains. This initiates downstream signaling cascades that regulate key cellular functions (Figure 1). RTKs are highly relevant targets within drug discovery because they are frequently dysregulated in cancer and other diseases. Many successful targeted therapies inhibit RTKs (e.g. imatinib, erlotinib), showing that the receptors provide opportunities for developing selective inhibitors [1,2].

RTK (receptor tyrosine kinase) measurements using AtlaZ
Impedance-based AtlaZ assays have several important advantages for RTK-targeted drug discovery. The system allows real-time, non-invasive measurement of cellular responses without the need for labels or reporters. Furthermore, it captures integrated cellular responses resulting from complex signaling networks downstream of RTKs and it enables tracking of both rapid and prolonged cellular responses to RTK modulation. Subtle changes in cell morphology, adhesion, and behavior induced by RTK inhibitors are monitored, making the assay suitable for studying various RTK-mediated processes like proliferation, migration, and differentiation. The time-resolved kinetic data allow determination of important pharmacological parameters like onset of action and duration of effect. By providing rich, time-resolved data on cellular responses to RTK modulation, impedance-based assays have become a valuable tool in the drug discovery process [3], enabling more efficient screening and characterization of RTK-targeted compounds, potentially accelerating the development of new therapeutics.

Relevance of RTK measurements in genetically modified cells
Genetically modified cells allow researchers to manipulate specific components of signaling pathways, helping to elucidate the roles of individual proteins and their interactions in RTK activation and downstream signaling [1]. For instance, by introducing cancer-associated RTK mutations into cells, researchers can investigate how these alterations affect signaling dynamics, cell behavior, and drug responses. Furthermore, genetically modified cells expressing specific RTK

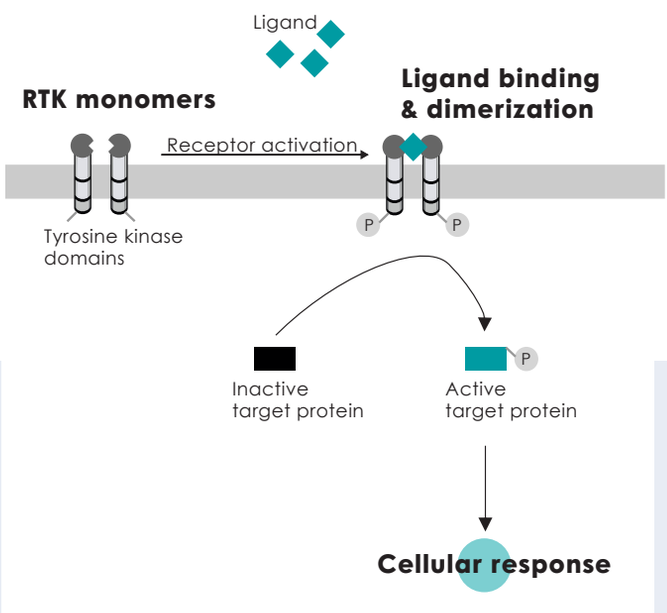


Figure 1: ***Receptor tyrosine kinase activation.***
Ligand binding activates receptors, triggering auto-phosphorylation. This activates target proteins, initiating intracellular signals that regulate genes and induce biological responses.

variants can be used to screen and evaluate the efficacy of targeted therapies, helping to identify potential drug candidates [1]. Engineered cells can also serve as models for specific disease conditions, allowing researchers to study how RTK signaling contributes to pathological processes. Lastly, genetic modifications can help reveal how cells regulate RTK signaling through feedback loops and compensatory mechanisms, and, genetic manipulation of RTK pathways can help confirm the relevance of specific targets for therapeutic intervention.

The strength of the AtlaZ assay approach with regards to RTKs as a target is its (i) time resolution, (ii) throughput, (iii) the fact that it is label-free and (iv) independent of genetic engineering. The technology is applicable to primary cultures or finite cell lines with endogenous receptor density [4]. A crucial advantage over standard assays that rely on labels for optical or radiometric detection, is the continuity of cell monitoring. Endpoint assays using dyes or labels just report on the status of the cells at a single time point but neglect the kinetics of the cell response.

Results

In this study, we used HEK 293 parental cells endogenously expressing the EGFR and a Δ7 knockout HEK 293 cell line where the G protein subunits Gas/olf/q/11/12/13/z were knocked out by CRISPR/Cas9 genome-editing. This cell line is severely impaired in responding with morphology changes to stimulation of G protein-coupled receptors (GPCRs) but should, in principle, remain sensitive to RTK-mediated cell activation.

The aim of the study was to perform proof of concept measurements showing that, using the AtlaZ system, the EGFR can be stimulated and that the resulting morphological changes in HEK 293 cells can be detected. The knockout cell line was used exemplarily to investigate if RTK signaling is maintained.

EGF (Epidermal Growth Factor) is a key ligand that activates the EGFR (Epidermal Growth Factor Receptor), which is an important RTK expressed endogenously in HEK 293 cells. Firstly, HEK 293 parental cells were seeded on AtlaZ sensor plates (NSP-Z) with and without fibronectin coating to see if coating improves cell attachment and proliferation (Figure 2). The adherence of cells was improved by fibronectin, resulting in a faster increase of the Cell Signal (impedance signal) and a larger amplitude. Furthermore, not only over the adherence but also during the proliferation phase, starting approx. at t = 7 h, the Cell Signal was higher at all times. Therefore, in the following experiments, plates were coated with fibronectin.

EGF Stimulation and RTK Activation

EGF binding triggers EGFR dimerization and autophosphorylation. This leads to activation of the receptor's tyrosine kinase domain, and a signaling cascade initiation. Activated EGFR recruits and phosphorylates downstream signaling proteins. HEK 293 parental cells endogenously expressing the EGFR or a ΔG7 knockout HEK 293 cell line were seeded in culture medium at a density of 60.000 cells/well. On day 2, cells were stimulated with 1 µM EGF in HBSS + HEPES + 0.01% BSA buffer. At the beginning of the assay, cells were allowed to adapt to fresh assay buffer within 2 h prior to stimulation. The recording mode used here was "multiple frequencies" i.e. the impedance is recorded at several designated AC frequencies. Here, the impedance was recorded at 10 frequencies between 100 Hz and 100 kHz equally spaced on a logarithmic scale. For the EGF experiments, a recording frequency of 10 kHz was chosen (see also Figure 4).

EGF stimulation can produce either transient or sustained RTK activation, here we found that EGF stimulates a Cell Signal

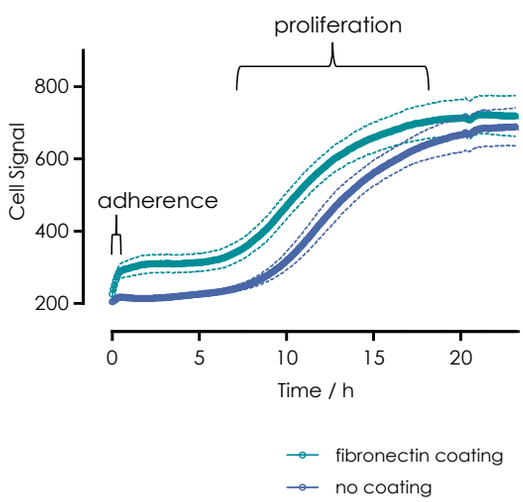
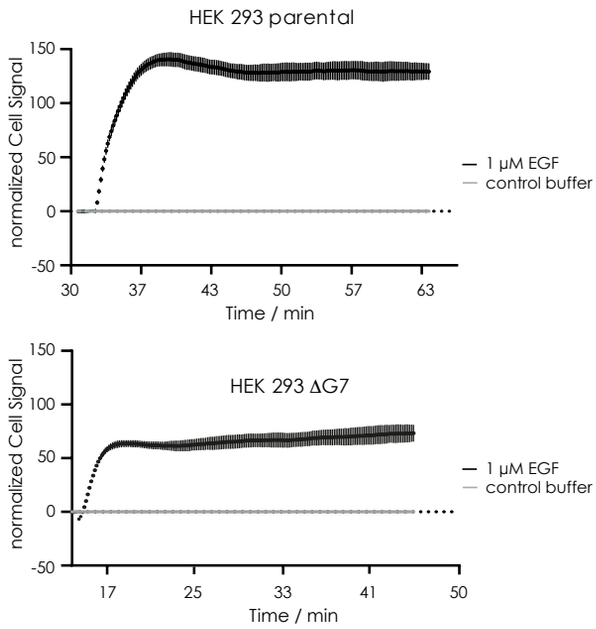


Figure 2: ***HEK 293 cell adhesion and growth****. Typical growth curve of HEK 293 parental cells over a timeframe of 1 day. 60 k cells were seeded per well at t = 0. Note that fibronectin promotes faster adherence.*

Figure 3: ***Time-resolved impedance profiles of endogenous EGFR in HEK 293 parental and ΔG7 cells.*** *Stimulation of HEK 293 parental or ΔG7 cells via 1 µM EGF. Data were normalized to control and are shown +/- SEM, n = 1, triplicate.*



with an amplitude increase in the first 6 minutes in HEK 293 parental cells (Figure 3). The Cell Signal then remained stable over the entire recording time. In HEK ΔG7 cells, 1 µM EGF induced a Cell Signal increase within 4 minutes, resulting in a constantly remaining high amplitude. The overall signal hub was approximately 50 % smaller than the effect in HEK 293 parental cells indicating that the absence of functional G proteins also impacts RTK-mediated morphology changes, this being consistent with the notion that RTKs also use components of GPCR-mediated signaling.

In conclusion, the AtlaZ system offers a highly automated approach capable of studying the pharmacology of RTK signaling pathways using a single platform. In addition, AtlaZ does not require labeled reagents and provides the cell response to receptor stimulation in real-time. The latter has proven its usefulness, for instance, when photoswitchable GPCR agonists [6] or receptor desensitization [5] have been studied. Thus, straightforward assay design, minimal artifacts, and short assay time, contribute to higher and more efficient throughput provided by the AtlaZ platform.

It is also important to emphasize that impedance-based monitoring of RTK stimulation is sensitive enough to work with endogenously expressed receptors, like in primary cultures or stem cells. This approach yields the most physiologically relevant results when all components of the signaling pathway are expressed at their natural levels. Moreover, it avoids the need for genetic engineering prior to receptor characterization and thereby reduces the biological safety requirements when conducting the assay.

Impedance-based cell monitoring is often called a *holistic approach* because it measures the electrical properties of the entire cell without focusing on specific molecular interactions. Unlike many label-based techniques that target particular cellular components, this method offers an integrated view of the cell's overall state and behavior. As a result, it provides a broad, unbiased perspective on cellular responses and dynamics. The lack of molecular specificity may require some extra careful assay procedures and controls to avoid false-positive results, but it unfolds its full strength when signaling pathway-unbiased measures of cellular activity that discriminate wildtype from mutant RTKs are needed. In addition, integrated responses to endogenous and synthetic ligands as well as drugs targeting this therapeutically important receptor family are detected.

Methods

AtlaZ platform

The AtlaZ platform (Nanion) provides quantitative live-cell analysis by measuring the impedance (Ohm, Ω), displayed as Cell Signal, of adherent cells grown on 96-well plates with embedded planar gold-film electrodes (NSP-Z plates, Nanion). The methodology, Electrical Impedance Spectroscopy (EIS), provides a unique richness of information from cells. Depending on the data acquisition frequency, the system detects predominantly the resistive part of cell-cell and cell-matrix contacts at lower frequencies or predominantly capacitive currents across the cell membranes at higher frequencies as an indicator for electrode coverage. The latter is the basis for cell adhesion or migration assays. Thus, real-time impedance data provide insights into various cell phenotypes, such as cell morphology changes as a result of signaling, proliferation, lateral migration or cytotoxicity even over prolonged periods of time.

The readout, displayed as the Cell Signal, represents raw impedance values. For the specific recordings in this study we

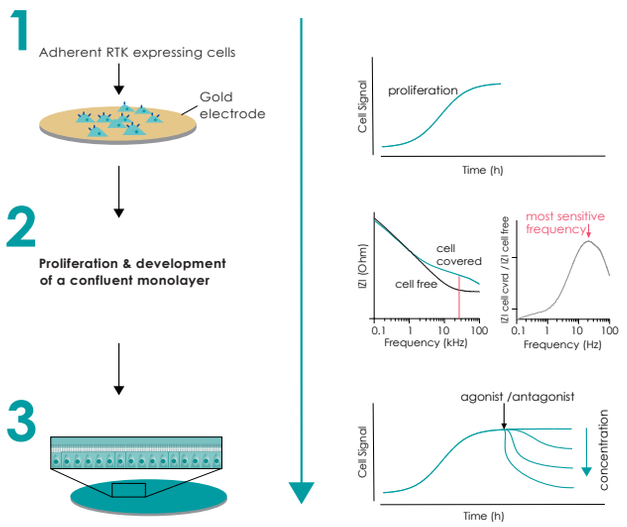


Figure 4: *Workflow of a EGFR assay.* *Target cells adhere and proliferate (<= 48 h) (1). Upon reaching a plateau phase, spectra for cell-free and cell-covered electrodes reveal the most sensitive frequency (2) by plotting the ratio of the impedance magnitude |Z| of electrodes with cells and the impedance |Z| of electrodes without. Next, the treatment can be executed (3). The Cell Signal is monitored continuously over time, revealing the kinetics of stimulus-induced effects.*

have chosen a 10 kHz recording frequency, which is best suited for these type of pathway investigations. The optimal frequency is cell-type specific and has to be determined empirically, but for most electrode types it ranges between 1 kHz and 50 kHz [7]. As a general guideline, the sensitive frequency for detection of changes in cell layer properties can be determined by dividing the impedance magnitude |Z| of a cell-covered electrode by the impedance of a cell-free electrode along the frequency spectrum (Figure 4). When these ratios are plotted for each individual frequency, one obtains a bell-shaped curve from which the maximum ratio reflects the frequency with the broadest range of relative change in impedance (Figure 4), i.e. the most sensitive frequency.

References

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Key findings

- Our results demonstrate the capability of AtlaZ to detect morphological changes of cells upon stimulation of EGFR, a receptor tyrosine kinase.
- AtlaZ allows for cellular research on cell adhesion, proliferation, cytotoxicity and RTK-mediated signal transduction effects, label-free and in real-time.
- Recordings can be performed for up to 6 x 96 = 576 samples either simultaneously or independently.

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