Chapter 8

A High-Content Assay to Screen for Modulators of EGFR Function

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Abstract

Cell-based assays have the potential and advantage to identify cell-permeable modulators of kinase function, and hence provide an alternative to the conventional enzymatic activity-driven discovery approaches that rely on purified recombinant kinase catalytic domains. Here, we describe a domain-based high-content biosensor approach to study endogenous EGFR activity whereby EGF-induced receptor activation, subsequent trafficking, and internalization are imaged and quantified using time-dependent granule formation in cells. This method can readily be used to search for EGFR modulators in both chemical and RNAi screening; with potential applicability to other receptor tyrosine kinases.

Key words RNAi, siRNA, Iressa, Kinase, Inhibitor, High-content assay, EGFR, Cancer, Drug discovery, INCA2000, INCA3000, Microscopy, HTS

1 Introduction

Receptor tyrosine kinases (RTKs) regulate key cellular processes including proliferation, differentiation, survival, migration, and metabolism. Aberrant activities of RTK are invariably associated with carcinogenesis. RTKs, therefore, constitute important targets for cancer therapeutics exemplified by several FDA-approved small-molecule drugs targeting EGFR function, among them, gefitinib, erlotinib, and lapatinib. All three drugs belong to the 4-anilinoquinazoline class of chemicals that share a common mechanism of action by competing for ATP binding. However, the efficacy of such drugs is hampered by primary and acquired resistance as a result of mutations in the kinase domain or in the ATP-binding site of the target EGFR [1]. RTK targeting drug discovery has been focusing primarily on the use of recombinant kinase domain-based in vitro kinase assays, which unfortunately can be associated with high rate of attrition where exceptionally potent inhibitors are found to lack cellular activity. Though there have been several reported efforts for developing cell-based EGFR

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activation quantification [2–6], we could not identify published outcomes as to their use in chemical screening.

We have recently adopted and optimized an image-based live cell biosensor reporter platform for assaying EGFR activation, trafficking, and internalization [7]. The assay relies on stably expressed Src Homology 2 (SH2) of growth receptor-bound protein 2 (GRB2) tagged with GFP in A549, a human non-small-cell lung cancer cell line, termed A549-EGFRB cells [8]. Specifically, upon ligand-stimulated activation by epidermal growth factor (EGF), EGFR undergoes a conformational change and tyrosine phosphorylation, allowing for high-affinity binding of SH2-GRB2 to phosphotyrosine residues on the receptor kinase domain followed by EGFR-bound GFP-GRB2-SH2 intracellular granule formation. Subsequently, these formed granules can be imaged and quantified using automated fluorescent microscopy as a surrogate for measuring endogenous EGFR function. We have further validated the assay in a pilot screen against a library of 6912 chemicals and identified EGFR function modulators: 82 inhibitors and 66 activators. Among the inhibitors were 12 well-known and characterized EGFR inhibitors present in the screened library [9].

In the next sections, we guide the readers through the experimental steps for assay setup, image acquisition, image analysis, and data interpretation.

2 Materials

2.1 Cell Culture

1. The A549-EGFRB cell line was obtained as previously described [8] and can be purchased from Sigma-Aldrich (catalog # CLL1097-1VL).
2. RPMI-1640 media (Sigma-Aldrich).
3. Puromycin (Sigma-Aldrich).
4. EGF (Sigma # E-9644).
5. Dimethylsulfoxide (DMSO).
6. Triton X-100.
7. Penicillin (Thermo Fisher Scientific).
8. Streptomycin (Thermo Fisher Scientific).
9. Fetal bovine serum (FBS) (PAA Laboratories).
10. Cell culture media: RPMI-1640 media supplemented with 10 % FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL puromycin.
11. Trypsin solution: 0.05 % trypsin-EDTA (Thermo Fisher Scientific).
12. Phosphate-buffered saline (PBS) (Thermo Fisher Scientific).

13. Nuclease-free water.
14. OptiMEM media (Thermo Fisher Scientific).
15. Hoechst solution: 10 μM Hoechst 33342 in 0.05 % Triton X-100 (v/v) (Thermo Fisher Scientific).
16. Dharmafect-1 transfection Reagent (Thermo Fisher Scientific).
17. Paraformaldehyde (32 %, w/v) (Electron Microscopy Sciences).
18. PFA Solution: 4 % PFA in PBS (v/v).
19. Iressa (gefitinib) (LC Laboratories # G-4408).
20. T-175 cm^2 tissue culture flask and 384-well microtiter tissue culture-treated black plate (Corning # 3985).
21. siRNA duplex targeting EGFR (siEGFR; catalog # SKI 2027) was designed and custom synthesized at the HTS Core Facility at MSKCC [7].
22. Non-targeting control siRNA duplex (siCtrl; Thermo Fisher Scientific # 4390843).
23. Anti-EGFR mouse mAb conjugated to AF647 (Santa Cruz Biotech).
24. Goat serum.

2.2 Instrumentation (See Note 1)

1. CytoMat (Thermo Fisher Scientific): Automated temperature- and humidity-controlled incubator set at 37 °C, 5 % CO_2–95 % air.
2. PP-384-M Personal Pipettor (Apricot Designs).
3. Multidrop 384 liquid dispenser (Thermo Fisher Scientific).
4. ELx405 automated plate washer (BioTek Instruments).
5. ABgene 300 plate sealer (Thermo Fisher Scientific).
6. Imaging systems: The IN Cell Analyzer 2000 (INCA2000): automated epifluorescence microscope, and the IN Cell Analyzer 3000 (INCA3000): automated laser scanning confocal microscope (GE Healthcare, *see* **Note 2**).

2.3 Image Analysis Software

The IN Cell Developer 1.7 software (GE Healthcare): Image analysis software equipped with a variety of object segmentation analysis modules for size, shape, and intensity.

3 Methods

The basic schematic of the assay is shown in Fig. 1. In response to EGF, the activated EGF receptor internalizes resulting in granule formation. In the absence of EGF, no granule formation is observed as all receptors are localized at the cell surface in these resting cells; this would constitute the baseline of inhibiting EGF function [7].

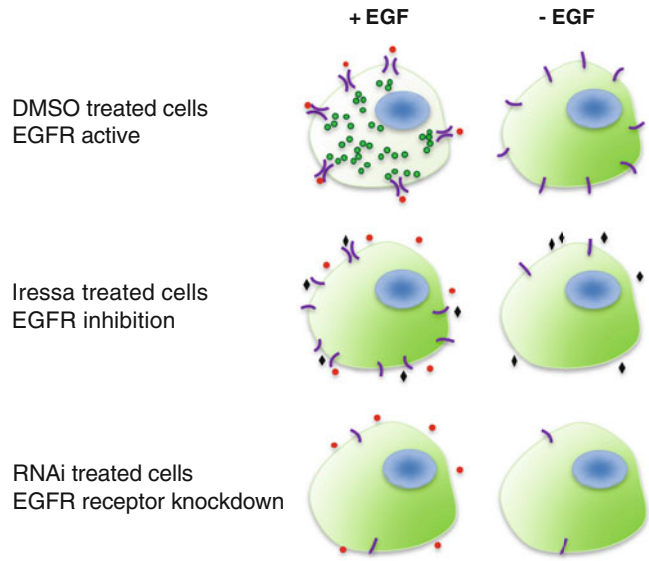

Fig. 1 EGFR biosensor assay principle: Assay schematic demonstrates the principle of the EGFR biosensor in A549-EGFRB cells in absence and in presence of EGF stimulation. GFP expression is diffuse in the cytoplasm in the absence of EGF stimulation and EGF addition leads to the recruitment and clustering of the EGFR biosensor, enabling live imaging and quantification of granule formation as a surrogate for measuring endogenous EGFR activity. EGFR biosensor activation by EGF is prevented by small-molecule EGFR inhibitor and by EGFR receptor knockdown using RNAi

3.1 Tissue Culture and Cell Maintenance

1. Maintain EGFRB cells in a 5 % CO_2-humidified incubator at 37 °C in cell culture media.
2. Passage cells every 3–4 days when reaching ~80 % cell confluency.

3.2 Assay Setup for Compounds

1. Dispense 5 μL per well of Iressa (*see* **Note 3**) from 100 μM stock in 10 % DMSO (v/v), along with 10 % DMSO (v/v) as a positive control to 384-well assay plates.
2. Dispense 2000 cells in 45 μL per well, yielding a final concentration of Iressa of 10 μM in 1 % DMSO (v/v), and incubate for 16 h at 37 °C.
3. Aspirate 35 μL of media from assay plates using the ELx405 automated plate washer.
4. For half the wells, replace with 35 μL per well of antibiotic-free media containing 500 nM EGF and for the other half supplement with 35 μL per well of antibiotic-free media.
5. Incubate for 70 min at 37 °C and perform fixing and staining as per Subheading 3.4.

3.3 Assay Setup for siRNA Duplexes

1. Dispense 2000 cells in 40 μL per well in antibiotic-free media and incubate for 24 h at 37 °C.
2. Prepare RNAi complex by mixing Dharmafect-1 transfection reagent and the siRNA duplex (siCtrl or siEGFR) in optiMEM media at a final siRNA concentration of 100 nM and 0.1 μL Dharmafect-1 reagent per well and incubate for 15 min at room temperature. This incubation time here is highly critical to achieve best knockdown.
3. Add 10 μL of the pre-prepared siRNA:lipid complex per well and incubate the cells for an additional 96 h at 37 °C.
4. Aspirate of media from assay plates using the ELx405 automated plate washer.
5. For half the wells, replace with 35 μL per well of antibiotic-free media containing 500 nM EGF and for the other half supplement with 35 μL per well of antibiotic-free media alone.
6. Incubate for 70 min at 37 °C and perform fixing and staining as per Subheading 3.4.

3.4 Cell Fixing, Nuclei Staining, and EGFR Immunostaining

1. Aspirate media from assay plates and wash cells once with 50 μL per well PBS.
2. Fix cells by dispensing 50 μL per well of PFA solution and incubate for 20 min at room temperature.
3. Wash cells once in PBS with 50 μL per well.
4. Permeabilize cells and stain nuclei by dispensing 50 μL per well of Hoechst solution and incubate for 15 min at room temperature.
5. Wash plate twice with 50 μL per well PBS.
6. Block cells with 10 % goat serum in PBS for 1.5 h at room temperature.
7. Immunostain EGFR using mouse anti-EGFR monoclonal antibody conjugated to Alexa647 and diluted 1:20 in 1 % goat serum (v/v) in PBS.
8. Incubate for 1 h at room temperature.
9. Wash plate twice with 50 μL PBS per well.
10. Dispense 50 μL of PBS per well; seal the assay plates using the ABgene plate sealer and store in the dark at 4 °C until ready for image acquisition.

3.5 Image Acquisition on the INCA2000

Images of cells in 384-well microtiter plates were acquired using the INCA2000 and imaging was performed at 20× magnification using a 20× ASAC objective (0.45NA). Images of nuclei stained with Hoechst in the blue channel were acquired using 350/50 nm excitation and 455/58 nm emission at an exposure time of 100 ms.

Images of GFP in the green channel were acquired using 490/20 nm excitation and 525/36 nm emission and GFP was imaged at an exposure time of 1.2 s [7].

3.6 Image Acquisition on the INCA3000

Images of cells in 384-well microtiter plates were acquired using the INCA3000 and imaging was performed at 40× objective magnification. Images of nuclei stained with Hoechst in the blue channel were acquired using 364 nm excitation and 450/65 nm emission in the blue channel at an exposure time of 1.5 ms. Images of GFP in the green channel were acquired using 488 nm excitation and 535/45 nm emission at an exposure time of 1.5 ms. Images of EGFR immunostaining in the red channel were acquired using 647 nm excitation and 695/55 nm emission at an exposure time of 1.5 ms [7].

3.7 Image Analysis

Images acquired by the INCA2000 were analyzed with the Developer Toolbox 1.7 software (GE Healthcare) using a custom-developed image analysis protocol. GFP granules were identified using object-based segmentation on the green channel. Hoechst-stained nuclei were identified after processing using object-based segmentation on the blue channel. Automated image analysis using a custom-developed protocol allowed us to extract granule and nuclei count, respectively, used for quantification of EGFR function and cytotoxicity [7].

3.8 Data Analysis (See Note 4)

1. Resting cells in the absence of EGF show high cell surface expression of endogenous EGFR (Fig. 2b) and upon activation with EGF, most of the expression disappears as the receptor is being internalized and forming granules (Fig. 2a).
2. Treatment of cells with non-targeting siRNA duplex (siCtrl) reveals similar results as for untreated cells (Fig. 2c, d), endogenous EGFR expression is unaffected and it is responsive to the addition of EGF. Treatment of cells with siRNA duplex targeting EGFR (siEGFR) shows near-complete knockdown of the receptor with a total absence of granules (Fig. 2f) even upon addition of EGF (Fig. 2e). These results are consistent with the granule formation being totally dependent on both the presence and activation of the EGFR.
3. To assess the robustness of this miniaturized assay, the control experiment is used consisting of several wells that contained 1 % DMSO (v/v) measuring maximum granule formation as the high control of the assay and those that contained 10 μM Iressa in 1 % DMSO (v/v) measuring complete inhibition of granule formation as the low control of the assay using the described assay workflow under Subheading 3.2.

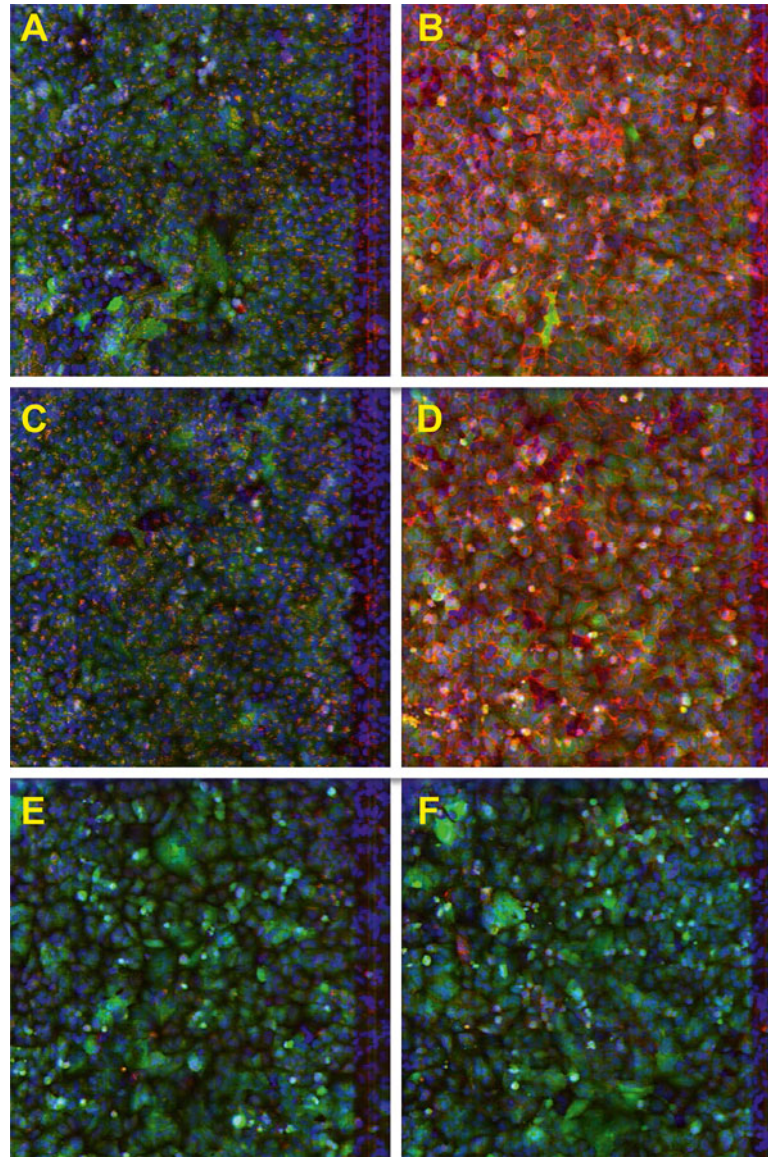

Fig. 2 Granule formation is stimulated in the presence of EGF and inhibited by RNAi. EGFR biosensor activation is induced by addition of EGF (**a**) only and reports EGFR activation; the absence of EGF shows resting cells with no apparent granules (**b**). A549-EGFRB cells treated with non-targeting siRNA duplex (siCtrl) show depletion of cell surface expression of EGFR in the presence of EGF (**c**) and not in its absence (**d**). A549-EGFRB cells treated with EGFR-targeting siRNA duplex (siEGFR) show depletion of protein at cell surface in resting cells; no added EGF (**f**); and in stimulated cells added EGF (**e**), demonstrating that granule formation is both EGF and EGFR dependent. A549-EGFRB cells imaged with the confocal microscope INCA3000 at 40× objective magnification. *Green* channel: EGF biosensor (GFP), *blue* channel: Hoechst staining of nuclei, *red* channel: immunostaining of EGFR; images are shown as an overlay of the three channels

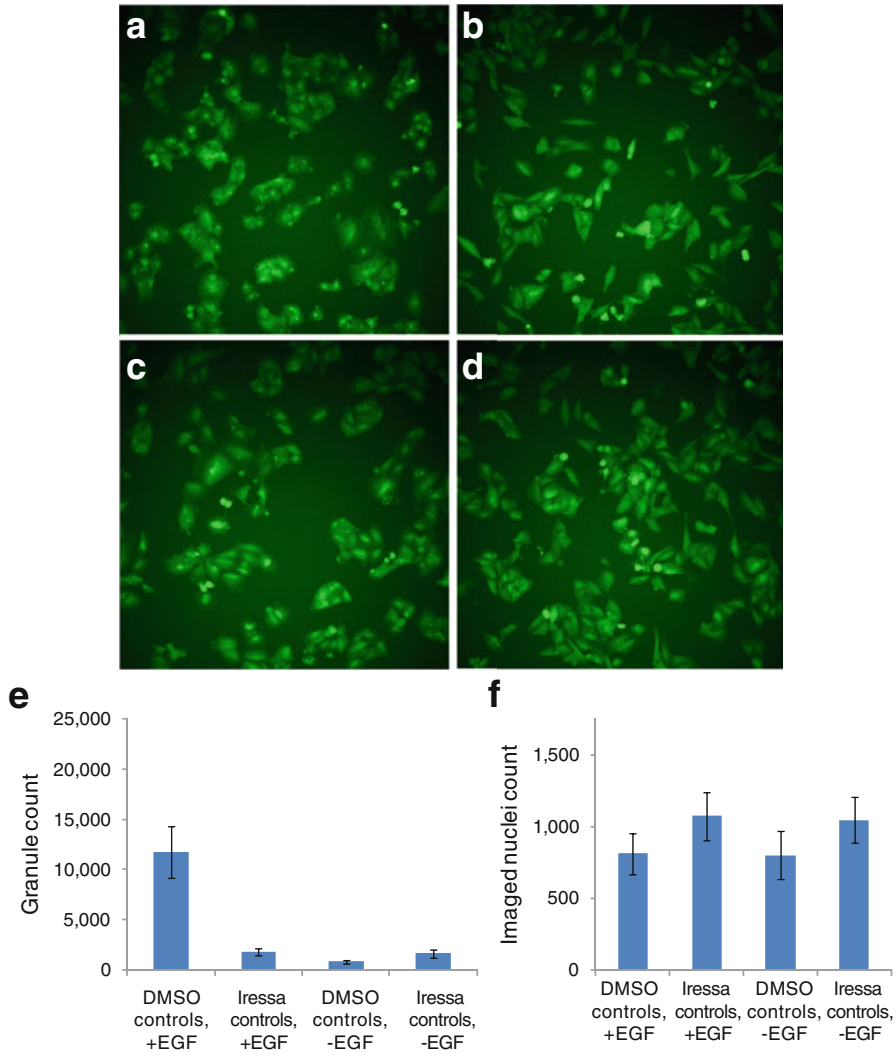

Fig. 3 EGFR granule-forming activity is inhibited by Iressa in A549-EGFRB cells. Granule formation is evident in A549-EGFRB cells, in the presence of EGF, upon treatment with 1 % DMSO (v/v) control (**a**), but totally absent in resting cells with no EGF treatment (**b**). The absence of granules is also found when cells were treated with 10 μM Iressa in 10 % DMSO (v/v) regardless of addition or not of EGF (**c**, **d**). Quantification of granule count can easily be obtained as total count per well (**e**). Quantification of nuclei count is also obtained as means to assess for cytotoxicity (**f**). A549-EGFRB cells imaged with the epifluorescence microscope INCA2000 at 20× objective magnification. *Green* channel: EGF biosensor (GFP)

4. DMSO treatment in the presence of EGF clearly shows visible granules (Fig. 3a) and a range of 10,000 to 15,000 granule count should be obtained (Fig. 3f), whereas treatment with 10 μM Iressa, in the presence of EGF, completely abrogates granule formation (Fig. 3c) with a granule count range from 0 to 1500 counts (Fig. 3f). Untreated EGF wells, regardless of DMSO or Iressa addition, did not show any substantial amount of granule counts (Fig. 3b, d).

5. Hoechst staining of cells provides an additional measure of whether the treatment was cytotoxic to the cells. Measured nuclei counts across the four treatments show little or no toxic effects (Fig. 3f). This additional measure provides a way to bin toxic versus nontoxic compounds obtained from screening chemical libraries as an example.

4 Notes

1. The list of instruments described here can easily be substituted with alternative commercially available ones.
2. Alternative automated imaging systems, such as BD Pathway 435™ (BD Biosciences, San Jose, CA); IN Cell Analyzer 1000, IN Cell Analyzer 2200, and IN Cell Analyzer 6000 (GE Healthcare); Opera and Operetta (Perkin Elmer, Waltham, MA); and BD Pathway 855™ (BD Biosciences), can easily be used for this assay.
3. Additional EGFR modulators [7, 9] can easily be purchased from commercial sources.
4. To better understand the workings of this domain-based biosensor assay, it is important to confirm that granule formation is dependent on both addition of EGF and expression of endogenous EGFP; its expression can easily be knocked down using siEGFR [7] or its activation can easily be inhibited by Iressa [7].

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