Receptor-Mediated MAPK and Akt Signaling are Maintained in Gamma-Irradiated FroZen Cells

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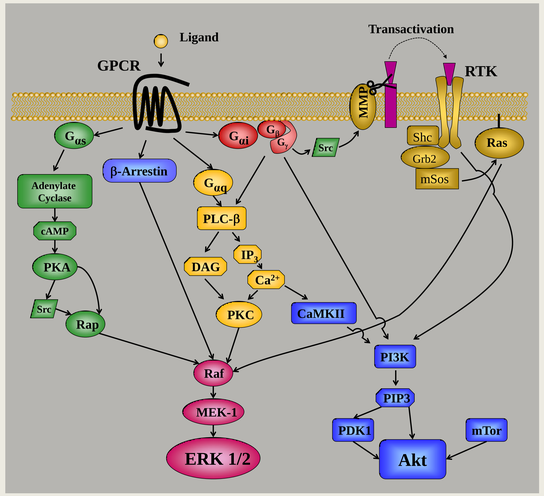
1 Abstract

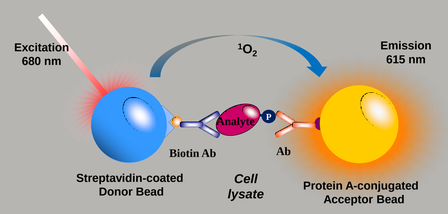
The use of frozen cells for cell-based screening has become widely accepted within the drug discovery community. Separating cell production from the actual screening campaign not only increases your flexibility but also improves the data consistency as the cellular material can be controlled and validated before running the functional assay. One of the methods used to deliver frozen cells as a consumable to the final user is to gamma-irradiate the cells, so that cells cannot resume growth after thawing.
This material is currently available for GPCR-expressing cells: AequoZen® cells, validated for calcium flux assays (AequoScreen® or fluorescence assays) and cAMPZen® cells, validated for the LANCE® cAMP assay.
The increasing amount of evidence for biased agonism (collateral efficacy and varying potencies according to the signal transduction pathway observed), and the search for more physiologically relevant recording of the activity of drugs in development, results in an increasing demand for assays relating to other signaling pathways activated by GPCRs.
Amongst these GPCR-triggered pathways are numerous kinase cascades, including MAP Kinase which leads to ERK activation. The location of this kinase downstream from the activation of many GPCRs makes measuring the phosphorylation ideal for evaluating pathway activation/inhibition in the presence of small molecules. AlphaScreen® *SureFire*® is a homogenous assay format for measuring ERK phosphorylation in cells. In this assay system, activated ERK binds a combination of two antibodies, one of which can only bind when ERK is phosphorylated. Only modified proteins that bind both antibodies are detected, using the Alpha technology (PerkinElmer) containing streptavidin coated Donor beads and Protein A coated Acceptor beads.
We show here that commercially available frozen, gamma-irradiated cAMPZen cells can be used together with ERK, MEK and Akt AlphaScreen® *SureFire*® assays to assay GPCR stimulation of the MAP Kinase pathway.

2 ERK-MAPK and Akt signaling

GPCR activation can lead to the activation of Mitogen-activated protein kinase (MAPK) pathways. Amongst these is the Raf/MEK/ERK pathway, linked to cell proliferation. Various pathways leading to ERK phosphorylation can be activated from the stimulation of $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ proteins. In addition, ERK phosphorylation can result from β-Arrestin activation, or proceed through the transactivation of receptor tyrosine kinases (RTKs) from the local release of their agonists. Depending on the GPCR and on the cellular background, the ERK activation will proceed through one or several of the indicated pathways, or as well through other pathways not represented here. Several pathways also lead to the activation of Akt.



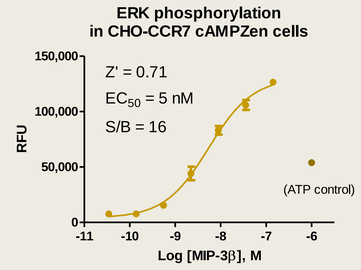
3 AlphaScreen® *SureFire*® Assay Principle

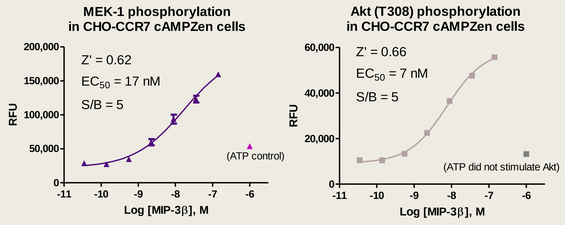


In AlphaScreen® *SureFire*® assays, following treatment of the cells with an agonist for the GPCR, the endogenous phospho-protein is captured in the cell lysate using an antibody against the phospho-protein and another antibody against the total (phospho- and non-phospho-) protein. An AlphaScreen® signal is generated when beads are brought into close proximity via simultaneous capture of the two antibodies respectively by the AlphaScreen® Streptavidin Donor beads and the AlphaScreen® protein A Acceptor beads.

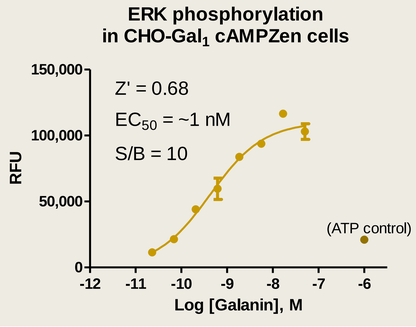
4 MEK, ERK and Akt assays on γ-irradiated CHO-CCR7 FroZen cells

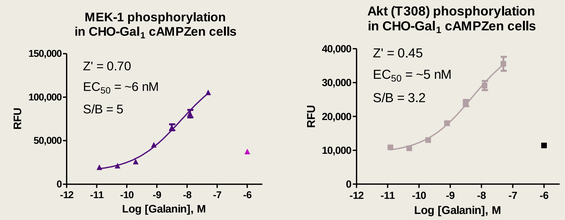
CHO-CCR7 cAMPZen FroZen cells (panel 4) or CHO-GAL1 cAMPZen FroZen cells (panel 5) were thawed, seeded at 40,000 cells/well, and the next day were stimulated with either a dose-response of Mip-3β or galanin, respectively. A single concentration of ATP, acting on an endogenous P2Y2 receptor in CHO cells, was used as a control agonist. The same cell lysates were used to assess MEK, ERK and Akt phosphorylation using the corresponding AlphaScreen® *SureFire*® kits. Both CCR7 ($G_{\alpha i}$-coupled) and Galanin 1 ($G_{\alpha i}$-coupled) stimulated the MEK-ERK pathway, as well as the PI3 kinase, as demonstrated by the Akt response. ATP ($G_{\alpha q}$-coupled P2Y2 receptor) stimulated the MEK-ERK pathway to a lesser extent, and did not elicit a response of the PI3 Kinase pathway.





5 MEK, ERK and Akt assays on γ-irradiated CHO-Gal_1 FroZen cells





6 Materials and methods

cAMPZen, γ-irradiated FroZen cells (PerkinElmer CCR7: Cat no. ES-140-CF; Gal_1: ES-510-CF) were rapidly thawed and seeded at 40,000 cells/well in 96-well plates, in Ham's F12 medium containing 10% FBS. After 10 min stimulation with the indicated concentrations of agonists, cells were lysed for 10 min with 50 µL of 1X-lysis buffer, (350 rpm plate shaking) and 4 µL of this cell lysate was used to detect ERK, MEK and Akt phosphorylation as recommended in the instructions of the AlphaScreen® *SureFire*® Assay Kits (PerkinElmer Cat no. TGRES, TGRMS and TGRA3S). The dose-response curves are representative of 2 to 3 independent experiments. Z′ values were calculated using 8 unstimulated, and 8 stimulated (EC_{100}) values.

7 Conclusions

FroZen, γ-irradiated cells, are a well established product, that can be ordered as a consumable, and readily used to perform Aequorin functional assays (AequoZen) or cAMP assays (cAMPZen). While, for the $G_{\alpha s}$–coupled receptors, an increase of cAMP is detected in such an assay, for $G_{\alpha i}$-coupled receptors an inhibition of the forskolin-stimulated level of cAMP is detected. This negative read-out may not be the preferred setting to develop an assay that is robust and easy to manage.

The data presented here show that these cAMPZen cells can be used as well in AlphaScreen® *SureFire*® assays, where the stimulation of $G_{\alpha i}$-coupled receptors leads to a positive read-out. So γ-irradiation does not prevent the use of such cells for assaying the MEK-ERK or the PI3kinase pathways.

This will provide additional flexibility for the characterization in multiple assays of drugs in development and allow an easier introduction of the detection of protean agonism (collateral efficacy) in drug discovery programs.

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