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New tools for drug discovery: Monitoring intracellular second messengers in primary cells using high-throughput formats

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1. Abstract

Primary cells allow for a higher predictability of drug reactions in humans. These cells endogenously express relevant drug targets at physiological level and genuinely carry the components required for specific signal transduction. They can be derived from the actual tissue of interest. These are significant advantages over immortalized cell lines, which may be derived from irrelevant tissue, be of non-human origin, and often express transfected drug targets at non-physiological levels. For these reasons, there is a growing demand for primary cells in drug screening and hit validation.

Here we show that Clonetics™ primary cells and Poietics™ stem cells can be used in high throughput formats (i.e. 96-well and 384-well plate) to monitor intracellular Ca^{2+} fluxes and changes of intracellular cAMP concentration. The cells transiently expressing either Axxam's luminescent calcium biosensor i-Photina® or Promega's luminescent cAMP biosensor GloSensor™ can be cryopreserved without loss of functionality. Cells expressing i-Photina® can be loaded with coelenterazine, substrate for the calcium-sensitive photoprotein, prior to freezing and are therefore ready to use after recovery.

The functional expression of i-Photina® was demonstrated through pore-forming ionomycin causing Ca^{2+} influx, the functional expression of the GloSensor™ by forskolin directly activating the cAMP producing enzyme adenylyl cyclase. Functionality and specificity of endogenously expressed G-protein coupled receptors triggering intracellular Ca^{2+} release or cAMP production was shown through receptor-binding agonists and antagonists in dose-dependent manner. EC_{50} and IC_{50} values nicely confirmed published data. Z' values above 0.6 facilitate the use in high-throughput screenings. We also provide evidence for a versatile assay system, cells co-expressing calcium and cAMP biosensor. Both pathways can consecutively be monitored in the same sample.

These new technologies are non-toxic and non-destructive to the cells, and unlike with fluorescence-based assays, there is virtually no background signal and no interference from fluorescent compounds. These ready-to-use cell based assay systems are excellent tools to study drug effects on signaling in primary cells and are expected to help open new roads for more predictable compound screening.

2. Materials and methods

Monitoring intracellular calcium levels in human primary cells expressing a calcium biosensor

Lonza primary cells (human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells of the lung (HMVEC-L), human mesenchymal cells isolated from bone marrow (hMSC)) were transiently transfected with an expression plasmid encoding i-Photina® using the Nucleofector™ technology. After Nucleofection™ cells were incubated in a humidified incubator at 37°C, 5% CO_2 for 6 hours. In the last 2 hours of the incubation time the cells were loaded with 10 µM native coelenterazine right before freezing. The cells were frozen in vials in cryoprotective agent. For performing the Ca^{2+}-assay cryopreserved cells were thawed, seeded on a 96-well-plate, and were allowed to recover for around 16 hours in a humidified incubator at 37°C, 5% CO_2. To remove the cryoprotective agent medium was exchanged 4 hours after thawing. Injection of stimulating agonists was carried out with a microplate reader equipped with automatic dispensers. Luminescence signals were recorded at 25°C every second for a total of 35 seconds.

Analysis of intracellular cAMP levels in hMSC using Promega's pGloSensor™-22F

HMSC were transiently transfected with the pGloSensor™-22F cAMP plasmid (Promega) using the Nucleofector™ technology. Cells were frozen in cryoprotective agent after a 6 hour incubaton period in a humidified incubator at 37°C, 5% CO_2. For performing the cAMP assay cryopreserved cells were thawed as described in the previous section. The cells were equilibrated with the GloSensor™ cAMP reagent (Promega) according to manufacturer's protocol 2 hours before performing the assay. Cells were stimulated with different agonists in 96-well or 384-well format and luminescence was analyzed for up to 30 min at 25°C in a microplate reader. For the antagonist experiments cells were incubated with various propranolol concentrations for 15 min and afterwards stimulated with 250 nM isoproterenol in 96-well format.

Monitoring intracellular calcium and cAMP levels in one sample

HMSC were co-transfected with GloSensor™ and i-Photina® expression vectors as described above. Incubation after Nucleofection™, freezing the cells, reactivating frozen cells for performing the Ca^{2+} and cAMP-assay was carried out exactly as described in the previous sections. Stimulation with two agonists was done simultaneously in one sample. The i-Photina® flash luminescence and the GloSensor™ luminescence were consecutively recorded using the Hamamatsu FDSS®/µCell (see below).

Luminescence recording

The luminescence signals were either detected on a dispenser-equipped plate reader with photomultiplier tube (PMT)-based technology or, where indicated, on a CCD camera-based FDSS®/µCell from Hamamatsu. Settings of the FDSS®/µCell for recording i-Photina® flash luminescence in 96-well were: Dispense speed 150 µl/sec, dispense height 2 mm, detection interval 2 sec; in 384-well: dispense speed 50 µl/sec, dispense height 2 mm, exposure 1.7 sec. For recording GloSensor™ luminescence (96-well only): dispense speed 50 µl/sec, dispense height 2 mm, exposure 9.7 sec. Sensitivity setting for all measurements was set to 5.

3. Results

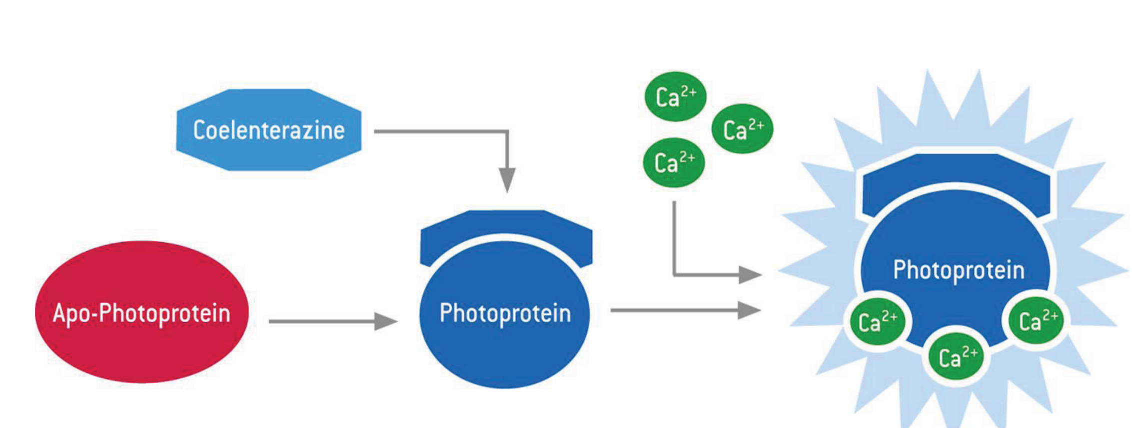

Figure 1. **Mechanism of the i-Photina® reaction.** Incubation of cells, expressing the i-Photina® apo-photoprotein, with coelenterazine in the presence of oxygen leads to formation of a stable complex, the active photoprotein. Calcium released from intracellular stores upon stimulation of the cells with agonists via G-protein coupled receptors binds to the photoprotein. The excited photoprotein converts coelenterazine to coelenteramide and emits a flash of blue luminescence.

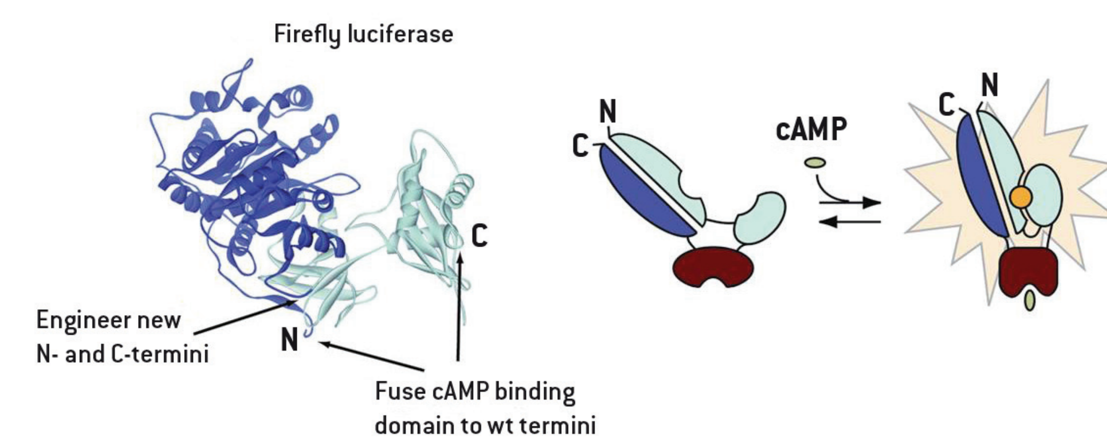

Figure 2. **GloSensor™ technology.** Firefly luciferase has been fused to the cAMP-binding domain of human protein kinase A (red). Upon binding of cAMP the whole protein molecule undergoes a conformational change. This activates the luciferase domain which converts luciferin to oxyluciferin and emits luminescence. See www.promega.com/glosensor for more information on the live-cell biosensor

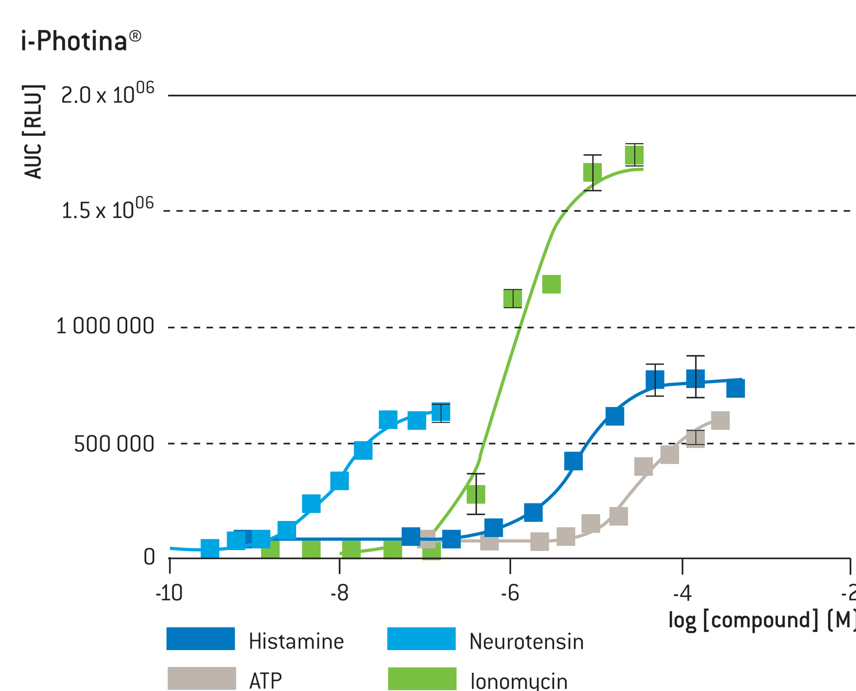

Figure 3. **HUVEC calcium biosensor responds to different GPCR ligands in dose-dependent fashion.** The assay was carried out as described in the materials and methods section. Ionomycin serves as a positive control for functional expression of the biosensor protein. EC50 values: ionomycin 1 µM, histamine 6 µM, neurotensin 8.8 nM, ATP 33 µM.

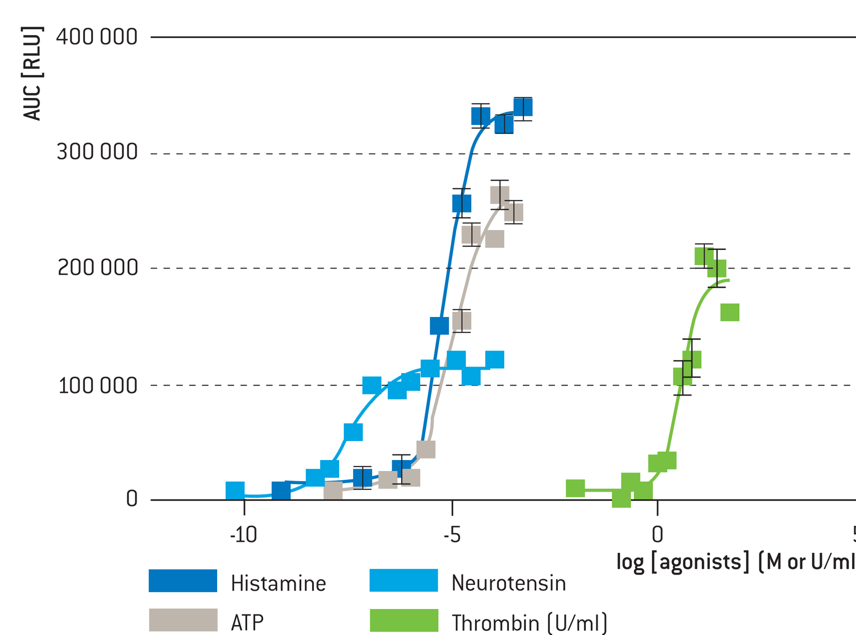

Figure 4. **HMVEC-L calcium biosensor responds to GPCR ligands in dose-dependent fashion.** The assay was carried out as described in the materials and methods section. EC_{50} values: histamine 8.5 µM, neurotensin 39 nM, ATP 11 µM, thrombin 4.3 U/ml.

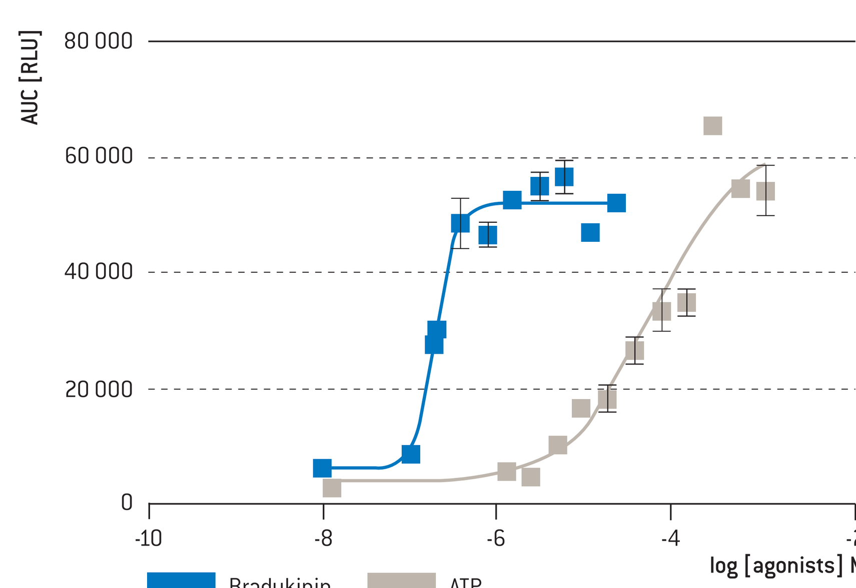

Figure 5. **hMSC calcium biosensor responds to GPCR ligands in dose-dependent fashion.** The assay was carried out as described in the materials and methods section. EC_{50} values: bradykinin 201 nM, ATP 64 µM.

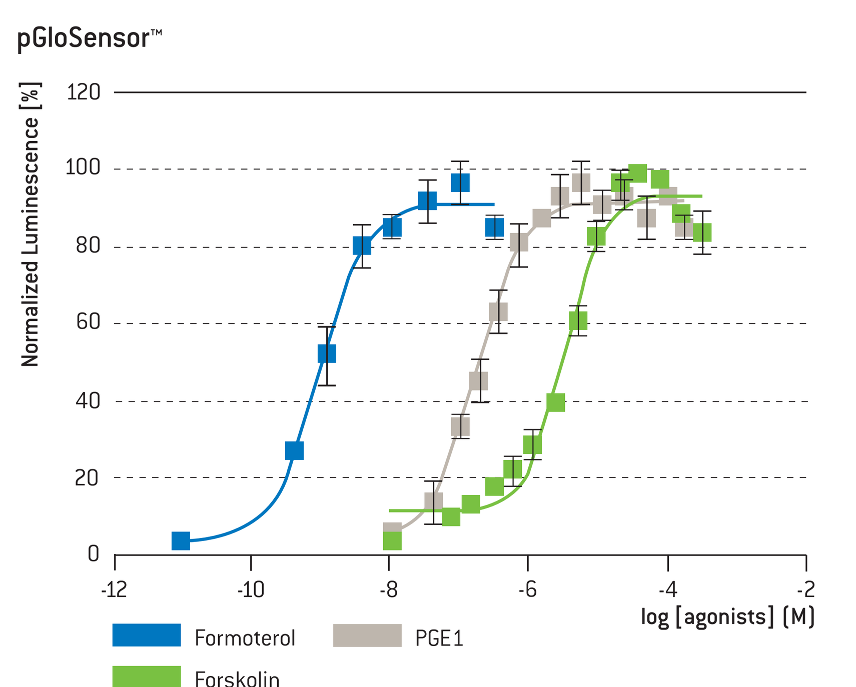

Figure 6. **hMSC cAMP biosensor responds to GPCR ligands in dose-dependent fashion.** The assay was carried out as described in the materials and methods section. PGE1 and forskolin responses were recorded on a PMT-based reader, formoterol on the Hamamatsu FDSS®/µCell in 96-well format. EC_{50} values: prostaglandin E_1 177 nM, formoterol 0.9 nM, forskolin 3.2 µM.

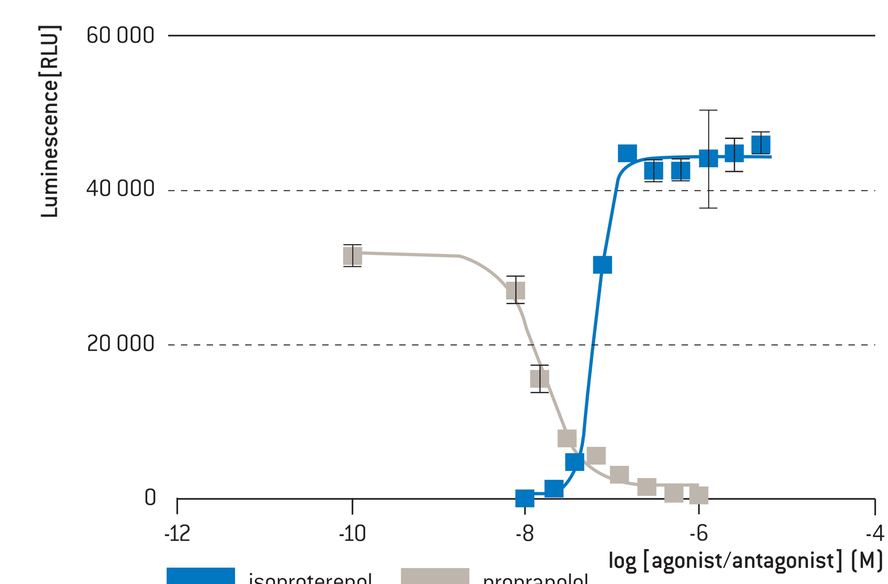

Figure 7. **Specific inhibition of β2-adrenergic receptor signaling in hMSC.** The assay was carried out as described in the materials and methods section. Luminescence was recorded on the FDSS®/µCell. EC_{50} isoproterenol 65 nM, IC_{50} propranolol 16 nM.

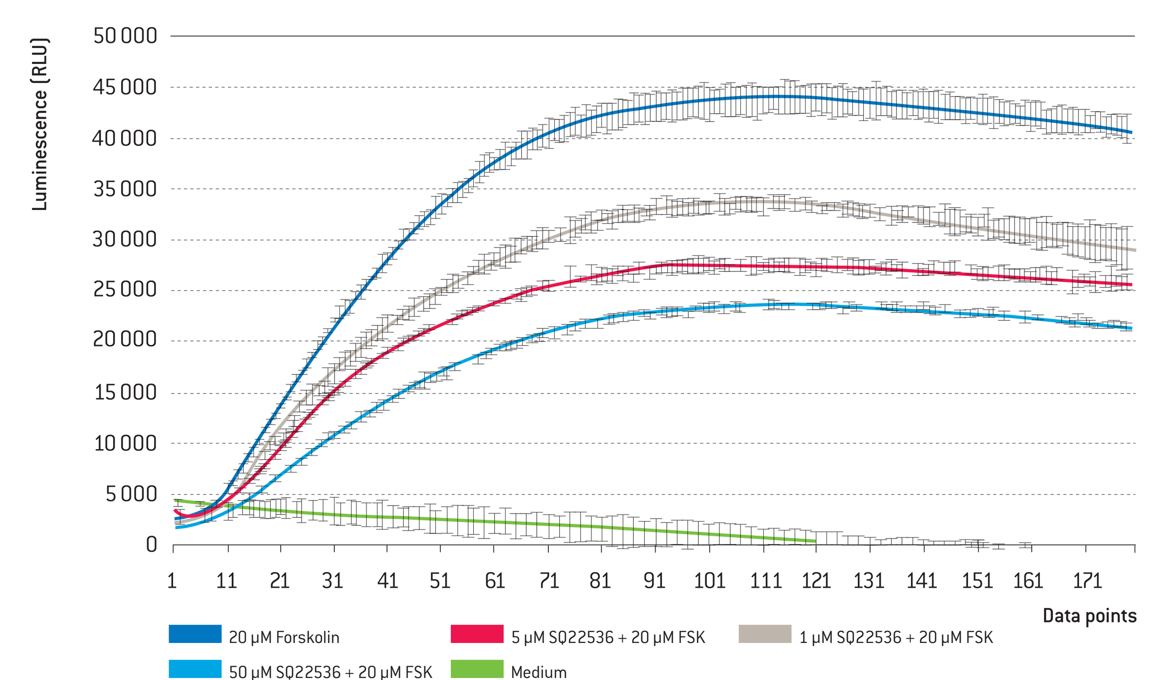

Figure 8. **Monitoring decreasing cAMP levels in hMSC.** The assay was carried out as described in the materials and methods section. Luminescence was recorded on the Hamamatsu FDSS®/µCell. The cells were preincubated with different concentrations of the adenylyl cyclase inhibitor SQ22536 for 15 min, followed by stimulation with 20 µM forskolin.

Co-expression of i-Photina® and pGloSensor™

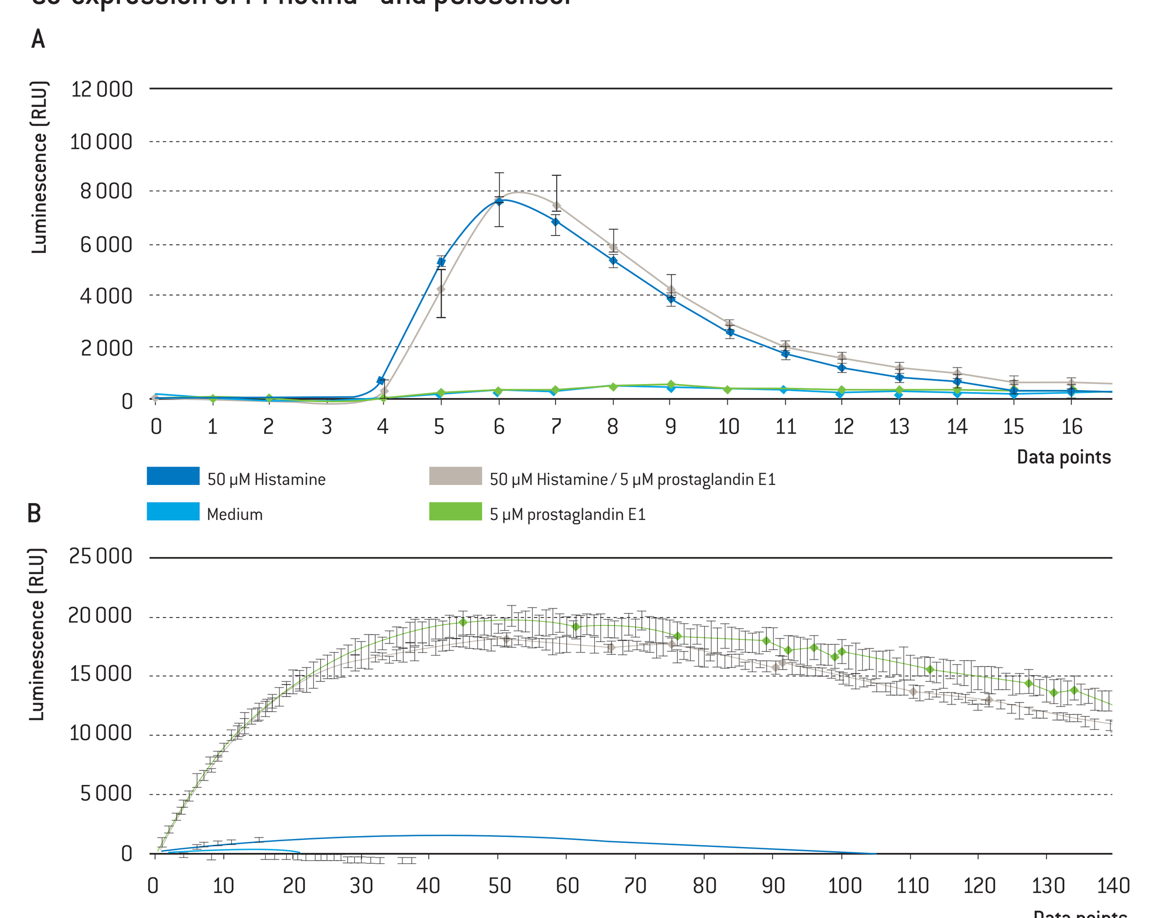

Figure 9. **Multiplexing of calcium and cAMP detection in hMSC.** Calcium and cAMP signaling were triggered simultaneously in the same sample by applying a mixture of two specific agonists. The i-Photina® flash luminescence (A) and the GloSensor™ luminescence (B) were consecutively detected on the FDSS®/µCell.

4. Conclusion

We have shown here that cAMP and Ca^{2+} signaling can be reliably monitored in primary cells after transient transfection of Promega's GloSensor™ and Axxam's i-Photina® using the Amaxa™ Nucleofector™ technology. For cAMP and calcium biosensor we demonstrated pharmacologically relevant dose-dependent responses with agonists of different classes of G-protein coupled receptors. Both assays can simultaneously be performed with cells cryopreserved after transfection. Thus, monitoring intracellular calcium and cAMP levels can be multiplexed in primary cells. This allows for identifying signaling pathways of compounds with fewer cell samples. Given these results, plus Z' values ranging from 0.6 to 0.7 (data not shown), delivered ready-to-use in cryopreserved form with unaltered functionality, we provide a basis for using primary cells in drug development endeavours.

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