Primary Cell Types Provide a Ready-to-Use Platform for Cell-Based Assays

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1. Abstract

In drug discovery research cell-based assays are a widely accepted tool for high throughput screening of potential drug candidates. To date usually immortalized cell lines are employed in cell based assays. They bear some limitations as they are (1) often not originated from the actual tissue or native cell of interest, (2) sometimes non-human (e.g. hamster derived CHO cell line), and (3) often expressing the transfected targets at non-physiological levels.
In contrast primary cell types can be derived from the tissue of interest. They allow for a higher predictability of the drug reaction in humans. The cells express relevant drug targets at physiological level and carry all the components necessary for specific signal transduction.
There is a growing demand for primary cells in secondary and even primary drug screens, although the major draw-back has been the unreliable availability of high quality primary cells.
Here we show that Clonetics® and Poietics® primary cells (human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells of the lung (HMVEC-L), aortic smooth muscle cells (AoSMC), human mesenchymal stem cells (hMSC) and normal human dermal fibroblasts adult (NHDF-ad) can be used in high throughput formats (i.e. 96-well and 384-well plate) to monitor intracellular Ca^{2+} fluxes.
The cells were transiently transfected with the luminescent calcium biosensor i-Photina® using the Amaxa® 96-well Shuttle® Nucleofector® Technology. After subsequent loading with the biosensor's substrate coelenterazine the cells could be used either directly or they could be cryopreserved and reactivated as needed.
Stimulation through agonists of endogenously expressed surface receptors lead to dose dependent Ca^{2+} responses at a very high signal to background ratio.
This ready-to-use cell based assay system is an excellent tool to study agonist effects on calcium signaling in primary cells and will open new roads for more predictable drug screenings.

2. Introduction

The data presented here demonstrate that Clonetics® and Poietics® primary cells transiently expressing a calcium dependent photoprotein can be employed for detecting intracellular Ca^{2+} release upon stimulation of different signaling pathways.
Monitoring changes in intracellular calcium concentrations through calcium-binding bioluminescent proteins offers the advantage of an instant signal after stimulus. The i-Photina® apo-protein used in this study covalently binds coelenterazine as substrate which is converted to coelenteramide upon binding of Ca^{2+} to the protein accompanied by emission of a light flash (Fig. 1).The method is non-toxic and, unlike with fluorescent dye-based Ca^{2+} assays, there is virtually no background signal and no interference from fluorescent compounds.
We were able to monitor Ca^{2+} release not only by stimulation with PMA/Ionomycin but also by triggering endogenously expressed receptors. In addition the system allows the analysis of antagonists like mepyramine. Transferring the assay to 384-well format, a Z'-value of 0.6 and cryopreservation of transiently transfected cells with unaltered signal to noise ratio facilitates the use in high-throughput screening.

3. Material and Methods

Transfection of primary cells

Clonetics® and Poietics® primary cells (human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells of the lung (HMVEC-L), aortic smooth muscle cells (AoSMC), human mesenchymal stem cells (hMSC) and normal human dermal fibroblasts adult (NHDF-ad) were transiently transfected with an expression plasmid encoding i-Photina® using the appropriate Amaxa® 96-well Nucleofector® Kits and the Amaxa® 96-well Shuttle® Nucleofector.

Detection of intracellular calcium release

The transfected cells were loaded with 10 µM native coelenterazine 20 hours after transfection for 4 hours.
Measurement of luminescence was carried out in 96-well or 384-well format with a micro-plate reader equipped with injection devices. The compounds (ATP, Histamine, PMA/Ionomycin (all Sigma Aldrich), 2-pyridylethylamine, Mepyramine (both Tocris)) were injected into the wells. The luminescence signals were recorded at 25°C every second for a total of 30 to 60 seconds.
For freezing experiments cells were incubated after Nucleofection® for 4 hours. The loading with 10 µM native coelenterazine was done for another 2 hours right before freezing (HUVEC and HMVEC-L). The cells were frozen in vials in cryoprotective agent. In order to perform the Ca^{2+}-assay cryopreserved cells were thawed, seeded on a 96-well plate, and were allowed to recover for 20 hours. 4 hours after thawing medium was exchanged for HEPEs buffered medium to remove the cryoprotective agent.
NHDF, hMSC and AoSMC were loaded with coelenterazine after reactivation for 4 hours right before stimulation.
Dose-dependent responses were calculated using area under the curve (AUC) integration.

4. Results

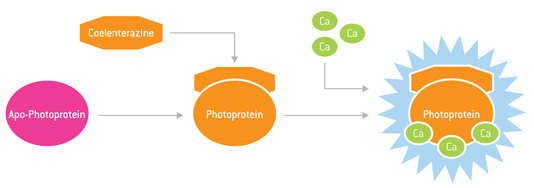


Figure 1. Mechanism of Calcium Biosensor. Primary cells transiently transfected with the Calcium Biosensor express the protein in an inactive state called apo-photoprotein. Upon incubation of the cells with the substrate coelenterazine in the presence of oxygen a stable complex of coelenterazine and the active photoprotein is build up. Stimulation of cells with agonists and antagonists regulating calcium signalling via G-Protein coupled receptor binding induces calcium release from internal stores. Binding of calcium to the complex causes a conformational change to an excited state. The following rapid reaction results in a blue luminescence light flash which can be detected by a photo-multiplier in a plate reader.

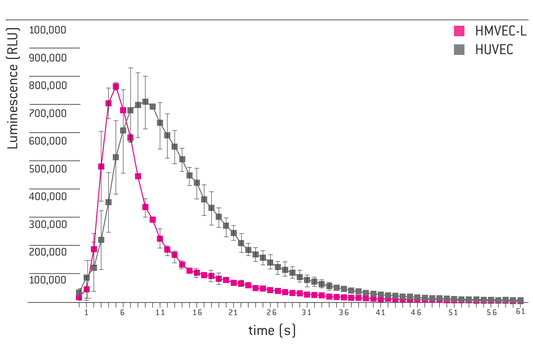


Figure 2. Signal kinetics of HUVEC and HMVEC-L Calcium Biosensors upon stimulation with 100 µM ATP. Cells were stimulated 24h after transfection with the agonist ATP, which is a ligand for the P2Y family of purinergic receptors. These receptors modulate mainly intracellular calcium levels. Luminescence signal was recorded every second for a total of 60 seconds.

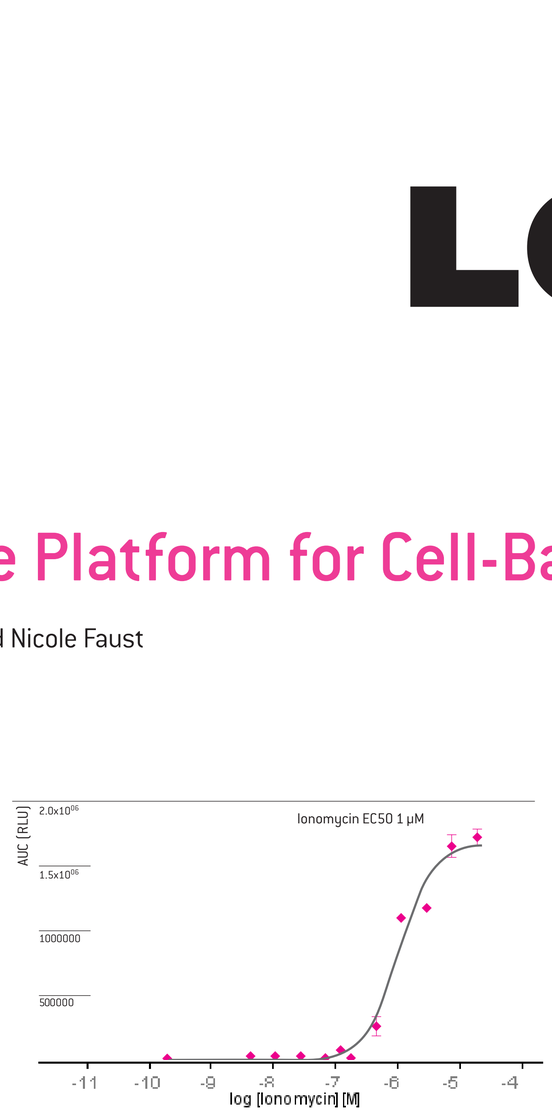


Figure 3. Dose-dependent response to PMA/Ionomycin in HUVEC Calcium Biosensor. After thawing and recovery over night cells were stimulated with various concentrations of Phorbol Ester PMA and Ca^{2+} ionophor Ionomycin.

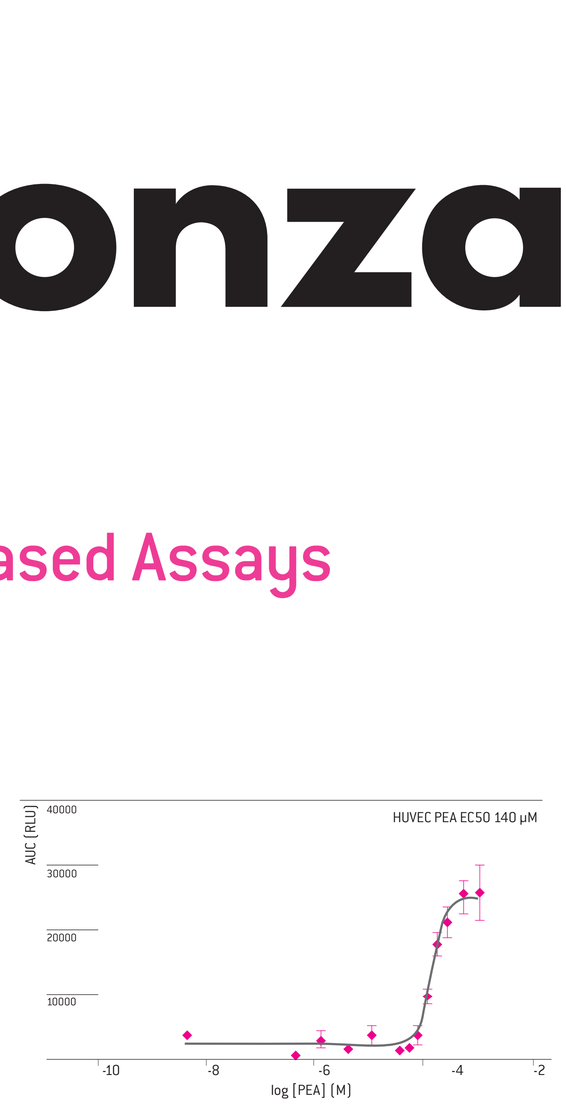
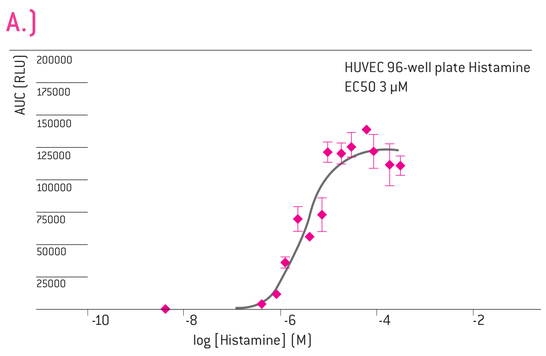


Figure 4. Dose-dependent response to H1 receptor agonist PEA in HUVEC Calcium Biosensor. After thawing and recovery over night cells were stimulated with various concentrations 2-pyridylethylamine (PEA), like histamine a ligand for the H1 receptor.



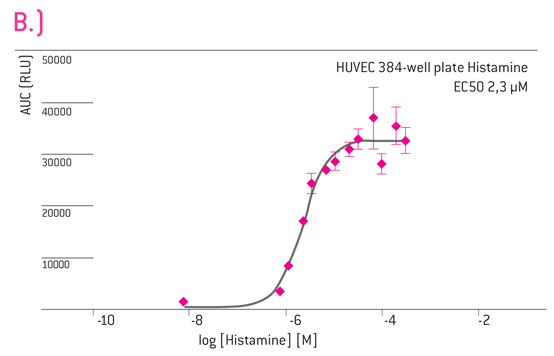


Figure 5. Dose-dependent response to histamine in HUVEC Calcium Biosensor. After thawing on 96-well (A) and 384-well (B) format and recovery over night cells were stimulated with various concentrations of histamine. Histamine binds to the H1 receptor which is expressed throughout the whole body, specifically e.g. on endothelial cells, in the central nervous system.

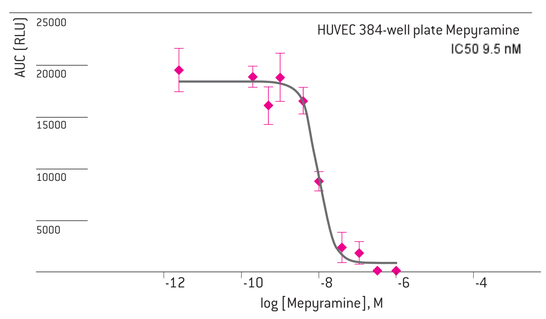


Figure 6. Dose-dependent responses to the H1R antagonist Mepyramine in HUVEC Calcium Biosensor. After thawing on 384-well plate and recovery over night cells were treated with various concentrations of mepyramine (for 5-15 min) and then stimulated with 7.5 µM histamine.

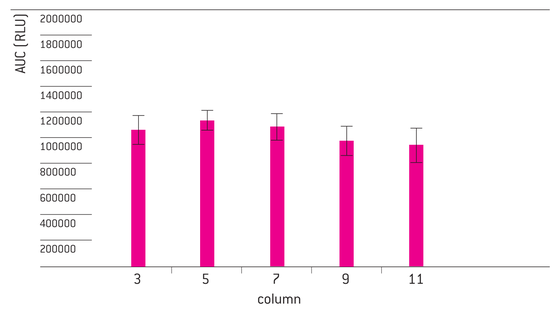


Figure 7. Z' for HUVEC Calcium Biosensor. After thawing and recovery over night cells were seeded on a 96-well plate and were stimulated with 50 µM histamine. Consistent signals with low standard deviations between the columns on the plate result in a Z' value of 0.6.

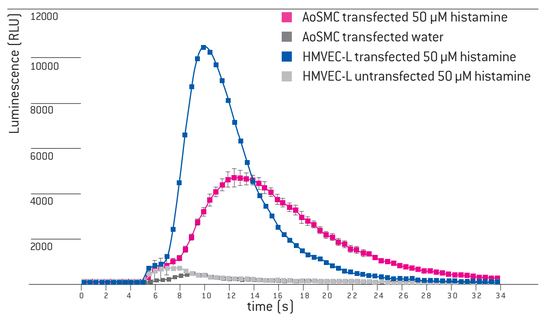


Figure 8. Signal kinetic of i-Photina® in transiently transfected HMVEC-L and AoSMC upon stimulation with histamine. After thawing and recovery over night cells were stimulated with 50 µM histamine. Luminescence signal was recorded every second for a total of 35 seconds starting with a 5 second pre-run for baseline recording.

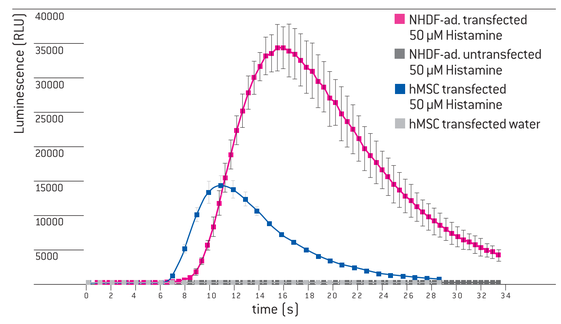


Figure 9. Signal kinetic of i-Photina® in transiently transfected NHDF-adult and hMSC upon stimulation with histamine. After thawing and recovery over night cells were stimulated with 50 µM histamine. Luminescence signal was recorded every second for a total of 35 seconds starting with a 5 second pre-run for baseline recording.

5. Conclusion

Primary cells transiently transfected with a Calcium Biosensor can be employed for monitoring intracellular Ca^{2+} release in high-throughput formats. The cells expressing the biosensor and loaded with the substrate coelenterazine are provided frozen ready-to-use. They can be seeded directly into different plate formats and can be used for cell-based assays. Lonza's Clonetics® Primary Sensors are a groundbreaking system to study agonist triggered calcium signaling in primary cells. This should open new roads for more predictable drug screenings.

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