BioResearch

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New Tools for Drug Discovery: Monitoring Intracellular Ca^{2+} Fluxes in Primary Cell Types with High-Throughput Formats

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1. Abstract

Primary cells allow for a higher predictability of drug reactions in humans. These cells express relevant drug targets at physiological level and genuinely carry all 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, of non-human origin, and often express transfected drug targets at non-physiological levels.

Thus, in spite of the so far unreliable availability of high quality primary cells, there is a growing demand for primary cells in secondary and even primary drug screens.

Here we show that Clonetics™ and Poietics™ primary cells, human endothelial cells of the umbilical vein (HUVEC), of the lung microvasculature (HMVEC-L), aortic smooth muscle cells (AoSMC), human mesenchymal stem cells (hMSC), human mammary epithelial cells (HMEC) 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® and subsequently loaded with the biosensor's substrate coelenterazine. They were used either directly, or cryopreserved and reactivated as needed. The functional expression of i-Photina® was demonstrated through pore forming ionomycin causing Ca^{2+} influx. Functionality of receptors stimulating intracellular Ca^{2+} release was shown through specific ligands. The histamine response of HUVEC is solely elicited through the H1-receptor demonstrated in dose-dependent manner by specific agonists and antagonists. EC50 and IC50 were within the range of published data. In addition with other known ligands such as adenosintriphosphate, thrombin, and neurotensin endogenously expressed receptors on endothelial cells could be stimulated to release intracellular Ca^{2+}. Transferring the assay to 384-well format, Z' values ranging from 0.5 to 0.7 and cryopreservation of transiently transfected cells with unaltered functionality facilitate the use in high-throughput screenings.

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. This ready-to-use cell based assay system is an excellent tool to study drug effects on calcium signaling in primary cells and will help open new roads for more predictable compound screening.

2. Materials 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), human mammary epithelial cells (HMEC) 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.

The cells were incubated after Nucleofection™ for 6 hours. For HUVEC and HMVEC-L the loading with 10 µM native coelenterazine was done for 2 hours during this incubation time right before freezing. The cells were frozen in vials in cryoprotective agent.

Detection of intracellular calcium release

In order to perform the Ca^{2+}-assay cryopreserved cells were thawed, seeded on a 96-well or 384-well plate, and were allowed to recover over night. 4 hours after thawing medium was exchanged for HEPES-buffered medium to remove the cryoprotective agent.

NHDF, hMSC, HMEC and AoSMC were loaded with coelenterazine for 4 hours after over night reactivation right before stimulation. Measurement of luminescence was carried out with a micro-plate reader equipped with an automatic dispenser. Compounds (ionomycin, thrombin, histamine , ATP (all Sigma Aldrich)) were injected into the wells, or manually added to the wells for a 5 – 15 min preincubation (antagonist mepyramine (Tocris)). Luminescence was recorded every second at 25°C for 5 seconds prior to injection (base-line recording) and for a total of 30 seconds after injection of the compound. Dose-dependent responses were calculated using area under the curve (AUC) integration.

3. Results

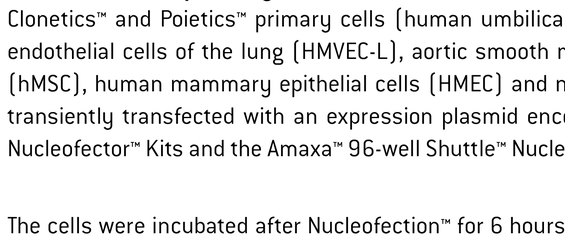

Figure 1. **Primary cells transiently transfected with the Calcium Biosensor express the protein in an inactive state called apo-photoprotein.** Upon incubation of the cells with the substrate coelenterazine in the presence of oxygen a stable complex of coelenterazine and the active photoprotein is build up. Stimulation of cells with agonists 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.

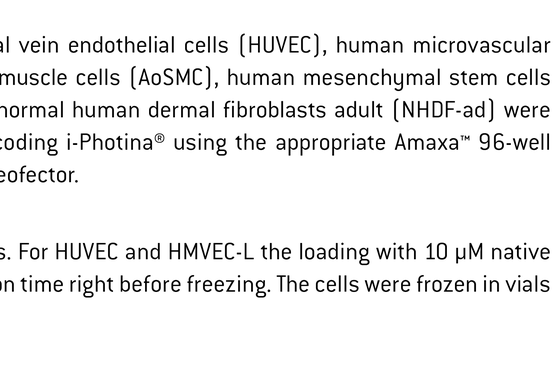

Figure 2. **Transiently transfected HUVEC Calcium Biosensor express functional i-Photina®.** After thawing and recovery over night cells were stimulated with various concentrations of $Ca2^{+}$ ionophor ionomycin (EC50 1.5 µM).

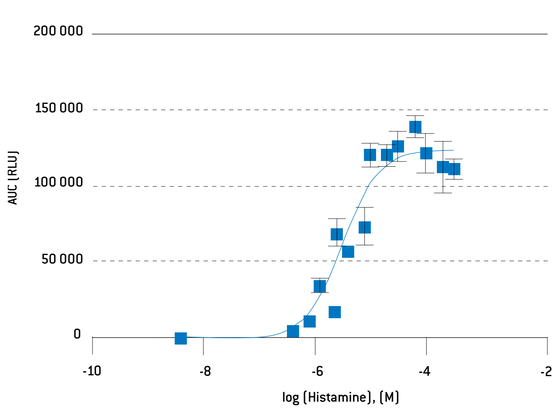

Figure 3. **Histamine elicited Ca^{2+}-response in HUVEC Calcium Biosensor.** Cells were thawed on a 96-well plate and stimulated with different concentrations of the H1 receptor agonist Histamine on the next day. HUVEC Calcium Biosensor shows a dose-dependent response to histamine (EC50 3 µM).

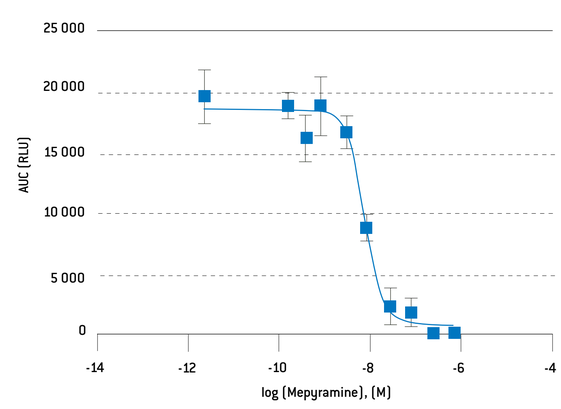

Figure 4. **Histamine response of HUVEC is mediated through H1 receptor.** After thawing on a 384-well plate and recovery over night cells were preincubated with various concentrations of mepyramine, an antagonist specific for histamine receptor 1, and then stimulated with 7.5 µM histamine (IC50 9.5 nM).

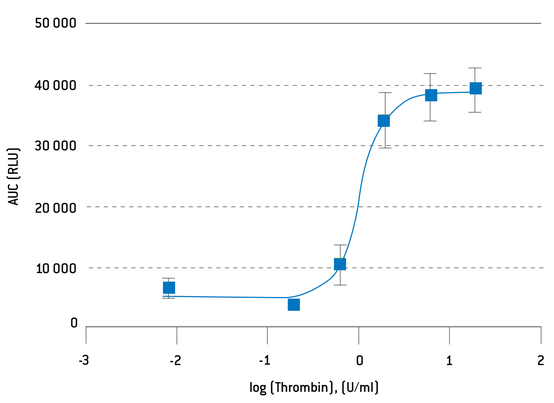

Figure 5 A. **Results with HUVEC Calcium Biosensor are consistent independent of plate format.** After thawing, the cells were seeded on 96-well plate and allowed to recover over night. The cells were stimulated with different concentrations of thrombin (a ligand of the protease-activated receptor type 1) (EC50 1 U/ml).

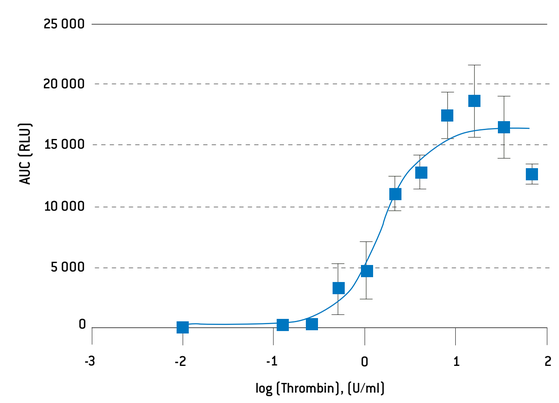

Figure 5 B. **Results with HUVEC Calcium Biosensor are consistent independent of plate format.** After thawing, the cells were seeded on 384-well plate and allowed to recover over night. The cells were stimulated with different concentrations of thrombin (a ligand of the protease-activated receptor type 1) (EC50 1.4 U/ml).

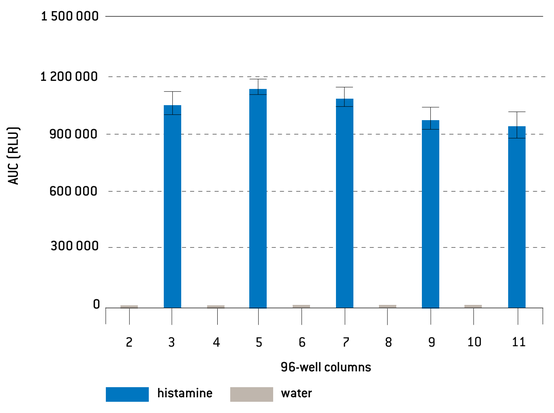

Figure 6. **Plate gradient and Z' value for 96-well format.** After thawing, cells were seeded on a 96-well plate and allowed to recover over night. Cells were stimulated with 50 µM histamine in every other 8-well column. Alternating columns were injected with water as control (Z' value was 0.6).

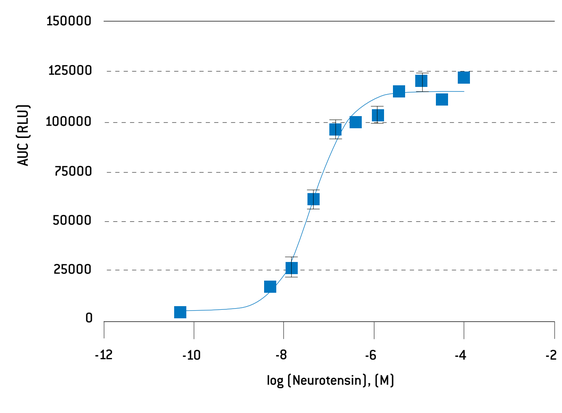

Figure 7. **Neurotensin elicits strong Ca^{2+} response in microvascular endothelial cells (HMVEC-L).** After thawing the cells were seeded on 96-well plate and allowed to recover over night. The cells were stimulated with different concentrations of neurotensin (EC 50 44.6 nM).

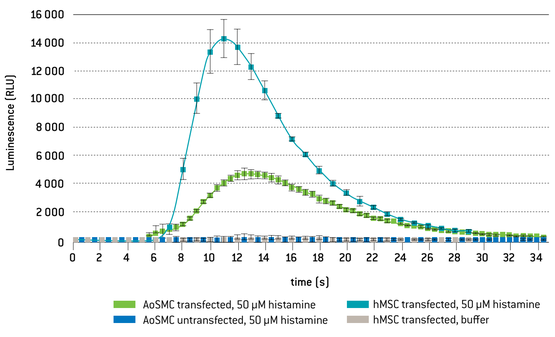

Figure 8 A. **Signal kinetic of i-Photina® in transiently transfected primary smooth muscle and mesenchymal stem cells.** After thawing and recovery over night, cells were stimulated with 50 µM histamine. Luminescence signal was recorded every second for a total of 35 seconds starting with a 5 second pre-run for baseline recording.

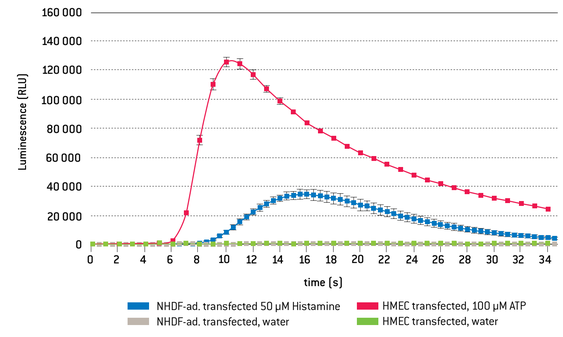

Figure 8 B. **Signal kinetic of i-Photina® in transiently transfected primary epithelial and fibroblast cells.** After thawing and recovery over night, cells were stimulated with either 50 µM histamine or 100 µM ATP. Luminescence signal was recorded every second for a total of 35 seconds starting with a 5 second pre-run for baseline recording.

4. Conclusion

Primary cells transiently transfected with a calcium biosensor can be employed for monitoring intracellular Ca^{2+} release in high-throughput formats. Cells expressing the biosensor and preloaded with the substrate coelenterazine are provided frozen ready-to-use. They can be seeded directly into different plate formats and can be used for monitoring Ca^{2+}-dependent signalling upon stimulation with physiological agonists. Lonza's Clonetics™ Primary Sensors are a groundbreaking system for high-throughput screenings in primary cells.

For further information, questions, comments, please contact Silke.Valentin@lonza.com, Bodo.Ortmann@lonza.com