Berthold Detection Systems GmbH
Bleichstrasse 56–68
D-75173 Pforzheim/Germany

Phone: +49(0)7231/9206-0
Fax: +49(0)7231/9206-50
E-Mail: contact@titertek-berthold.com
Internet: www.titertek-berthold.com

Orion II Microplate Luminometer Simplicity 4

Monitoring Intracellular Ca^{2+} Fluxes with Clonetics™ Primary Sensors using the Orion II Microplate Luminometer

By Silke Valentin, Bodo Ortmann, Kristin Atze, Nadine Breuer, Mathias Kühn, Steffi Franke, Leon de Bruin and Anthony Pitt.
Lonza Cologne GmbH, Nattermannallee 1, 50829 Cologne, Germany.

Introduction

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™ human microvascular endothelial cells of the lung (HMVEC-L) can be used in high throughput formats (i.e. 96-well and 384-well plate) to monitor intracellular Ca^{2+} fluxes on the Orion II microplate luminometer.

The cells were transiently transfected with the luminescent calcium biosensor i-Photina® and subsequently loaded with the biosensor's substrate coelenterazine. After that they were cryopreserved and therefore are ready-to-use. 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 HMVEC-L is solely elicited through the H1-receptor demonstrated in dose-dependent manner by specific agonists and antagonists. EC_{50} and IC_{50} were within the range of published data. In addition with other known ligands like adenosinetriphosphate, thrombin, and neurotensin endogenously expressed receptors on endothelial cells could be stimulated to release intracellular Ca^{2+}. Transferring the assay to 384-well format 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.

Materials and Methods

- Production of HMVEC-L Calcium Biosensor
  Clonetics™ human microvascular endothelial cells of the lung (HMVEC-L) were transiently transfected with an expression plasmid encoding i-Photina® using the appropriate Amaxa™ 96-well Nucleofector™ Kit and the Amaxa™ 96-well Shuttle™ Nucleofector.
  The HMVEC-L were incubated after Nucleofection™ for 6 hours. During this incubation time right before freezing the loading with 10 µM native coelenterazine was done for 2 hours. The cells were frozen in vials in cryoprotective agent.

Orion II Microplate Luminometer Simplicity 4

- Detection of intracellular calcium release using the Orion II microplate reader

  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.

  Measurement of luminescence was carried out with the Orion II microplate reader equipped with automatic dispensers. Compounds (ionomycin, ATP, thrombin, histamine (all Sigma Aldrich), neurotensin (Bachem)) were injected into the wells. For antagonist experiments cells were preincubated for 5 -15 min with mepyramine (Tocris). Afterwards cells were stimulated with histamine. Luminescence was recorded every second for 5 seconds prior to injection (base-line recording) and for a total of 30 seconds after injection of the compound.

  Dose-dependent responses and EC_{50} values were calculated using area under the curve (AUC) integration (GraphPad Prism).

- Measurement parameters on the Orion II microplate luminometer

  Reader: Berthold Detection Systems Orion II microplate luminometer

  Software: Simplicity 4

  Software settings:

  Protocol: Fast Kinetics

  Temperature: room temperature

  Injection: 25µl (384well), 50 µl (96well)

  Kinetic duration: 35 s

  Interval time: 0.7s

  Integration time: 0.7s

Results:

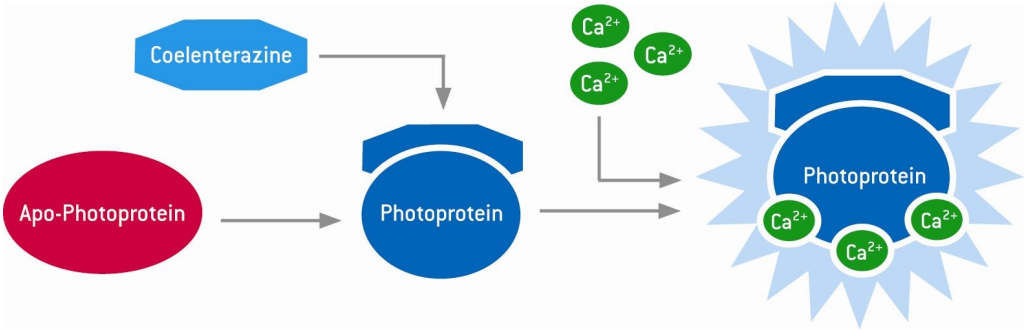


Figure 1. Mechanism of the calcium biosensor i-Photina®.

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.

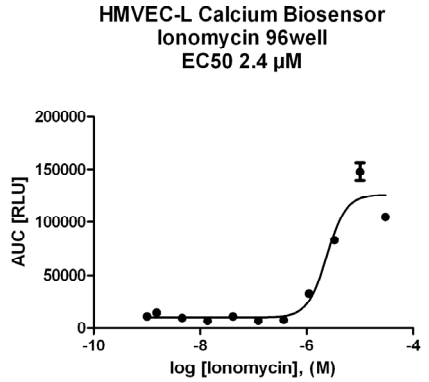


Figure 2. Transiently transfected HMVEC-L Calcium Biosensor express functional i-Photina.

After thawing on a 96-well plate and recovery over night cells were stimulated with various concentrations of Ca^{2+} ionophor ionomycin.

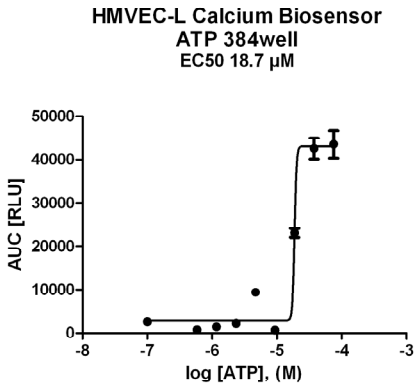
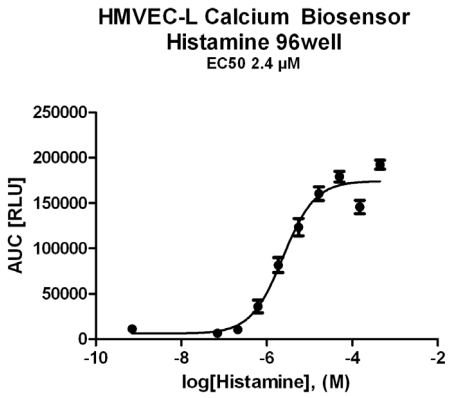


Figure 3. ATP elicits dose-dependent calcium response in HMVEC-L Calcium Biosensor.

Cells were thawed on a 384-well plate and allowed to recover over night. They were stimulated with different concentrations of the purinergic receptor agonist ATP on the next day.



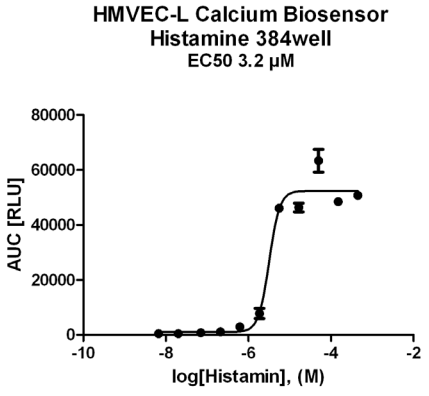


Figure 4. Results with HMVEC-L Calcium Biosensor are consistent independent of plate format.

After thawing the cells were seeded on 96-well and 384-well plate and allowed to recover over night. The cells were stimulated with different concentrations of histamine. Histamine binds to the H1 receptor which is expressed throughout the whole body, specifically e.g. on endothelial cells.

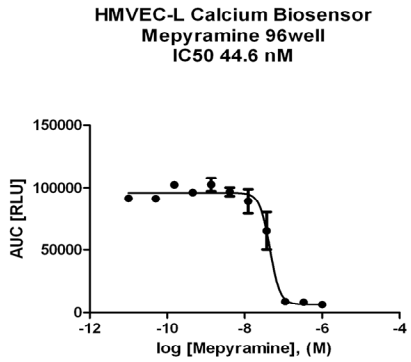


Figure 5. Histamine response of HMVEC-L Calcium Biosensor is mediated through H1 receptor.

After thawing on a 96-well plate and recovery over night, cells were preincubated with various concentrations of mepyramine, an antagonist specific for the H1 receptor. Afterwards cells were stimulated with 7.5 µM histamine.

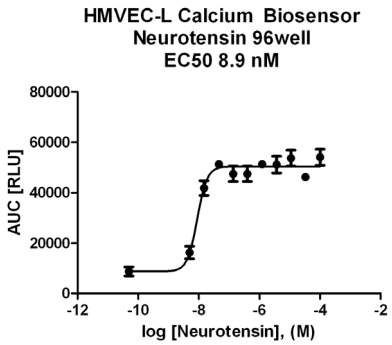


Figure 6. Neurotensin elicits a strong calcium response in HMVEC-L Calcium Biosensor.

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, which is a peptide neurotransmitter involved in different cardiovascular effects, e.g. hypotension or hypertension.

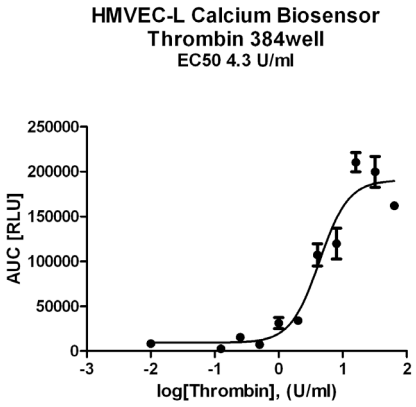


Figure 7. Dose-dependent response to thrombin in HMVEC-L Calcium Biosensor.

After thawing the cells were seeded on a 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.

Conclusion:

Primary cells transiently transfected with the calcium biosensor i-Photina® and available as ready-to-use tool are a groundbreaking new system for monitoring Ca^{2+}-dependent signalling upon stimulation with physiological agonists in high-throughput formats. Here we show that the Orion II microplate reader in combination with the HMVEC-L Calcium Biosensor is very well suited to easily detect dose-dependent Ca^{2+} responses with EC_{50} values consistent with published data.

Acknowledgement:
Data provided by Lonza Cologne GmbH, Germany
Figures and text by courtesy of Lonza Cologne GmbH, Germany

For further information on this product and other related products, please visit our homepage:
www.lonza.com/drugdiscovery