NOMAD® Genetically encoded biosensors for multiplexing PAR2 protease-activated receptor functional assay

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Abstract

Innoprot has developed a technology for multiplexing GPCR functional assays using fluorescent biosensors. This approach allows the measurement of the different signaling pathways involved in drug target activation in one single assay. It is the simplest way to identify GPCR biased ligands. Here we show a multiplexed assay using a stable cell line expressing green Ca^{2+} and red beta-arrestin Nomad biosensors and PAR2 protease-activated receptor to screen a library of 480 compounds. Both signals, calcium concentration and β-arrestin recruitment, were measured simultaneously by fluorescence intensity changes in living cells. After the screening campaign, positive compounds were chosen for further testing, based on the strength of the initial response and the lack of cytotoxicity. Our results indicate that Nomad Biosensors are effective and efficient tools for discovering differentiated GPCR biased ligands.

Results

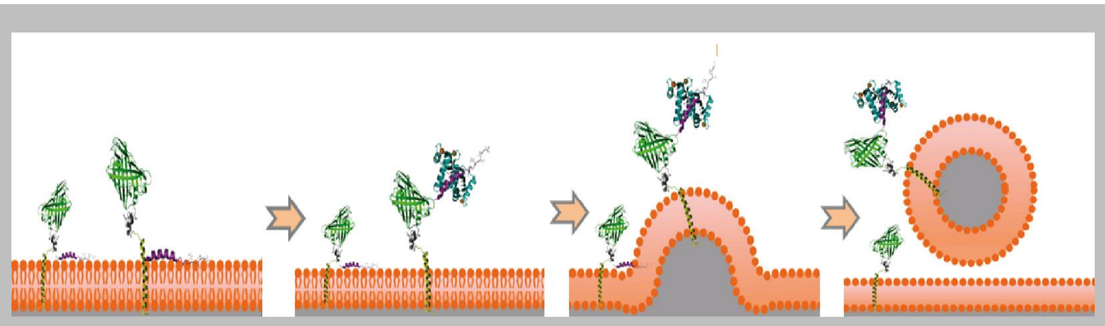

Figure 1. Schematic representation of the Nomad biosensor functioning in living cells. Nomad biosensor is a fluorescent fusion polypeptide capable of changing its localization within the cell from the cell cytoplasmic membrane to retention vesicles, upon an increase in the concentration of second messengers within the cell cytoplasm. A second messenger concentration increase leads to a change in the Nomad Biosensor´s folding promoting a cellular localization change. High-content screening (HCS) in Nomad systems using living cells is a very useful tool in biological research to discover and optimize new drug candidates.

Multiplex $_{Arres\text{-}Ca2+}$Nomad Biosensor-PAR2

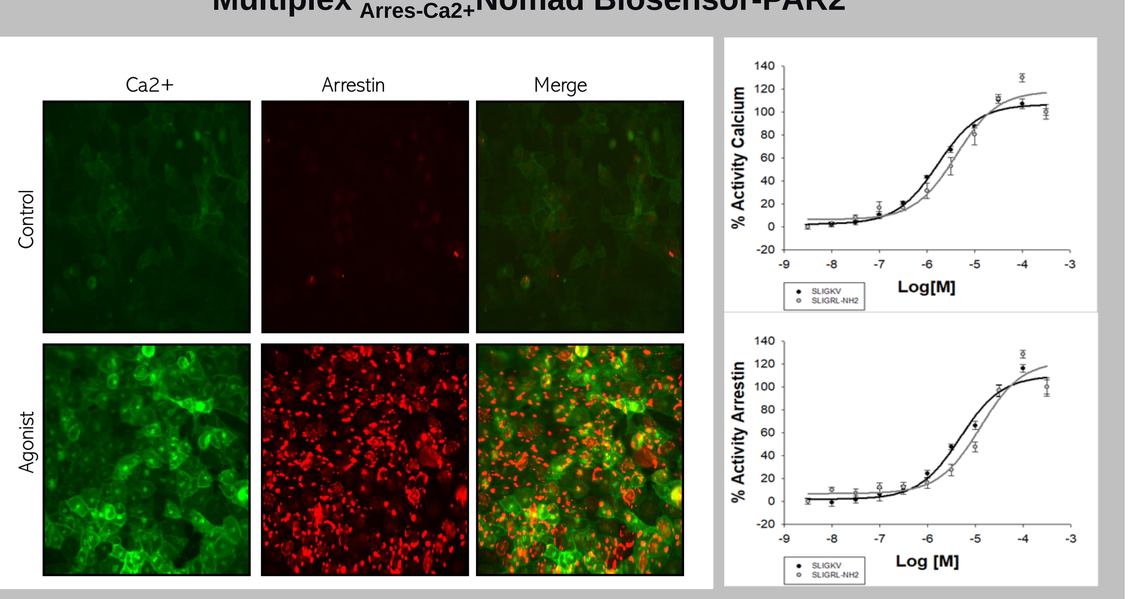

Figure 2. MPX $_{Arres\text{-}Ca2+}$Nomad Biosensor fluorescence intensity images and dose-response curves for SLIGKV and SLIGRL-NH2 using PAR2-$_{Arres\text{-}Ca2+}$Nomad U2OS cell line. Cells were treated with 11 log dilution series (n=5). % Activity was calculated relative to positive (300µM). The increase in the Nomad biosensors' fluorescences were detected and analyzed using "Synergy 2" microplate reader from Biotek after a treatment of 24 h with the agonists. The EC50 for the calcium signal of SLIGKV was $1.67x10^{-6}$ M and EC50 for the SLIGRL-NH2 was $4.06\ x10^{-6}$ M. The EC50 for the arrestin signal of SLIGKV and SLIGRL-NH2 were 4.83x10-6M and $1.27\ x10^{-5}$ M, respectively.

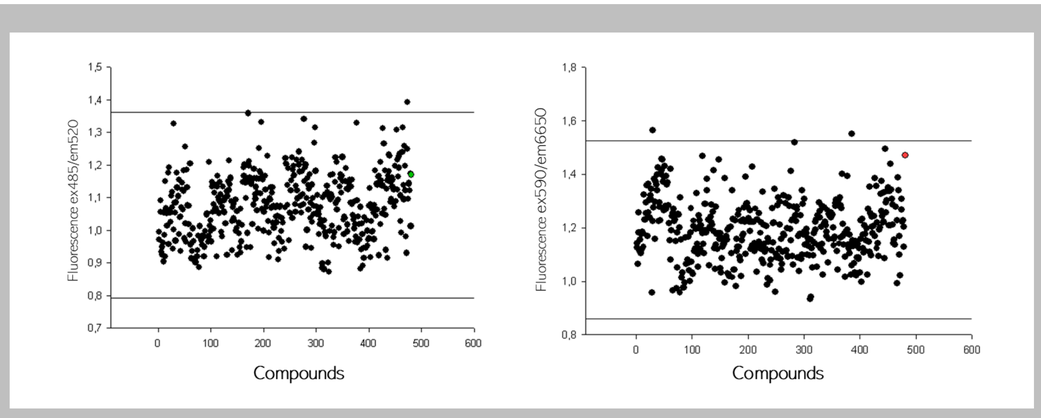

Figure 3. Screening of 480 compounds library using $_{ArresCa2+}$Nomad PAR2 cell line. A chemical library consisting of 480 compounds, sourced from the Prestwick Chemical Library® was used with the objective to identify putative biased compounds implicated in the PAR2 activity regulation. A scatter plot of the calcium response (left) and Arrestin response (right) in the presence of the library compounds. Positive control (SLYGKV) is represented in green for calcium response and in red for Arrestin response. As threshold of hit detection, 3 standard deviation (SD) over the mean was used, resulting in 3 postive hits. The Z' factors calculated from the positive and negative control wells on each plate produced an average Z' of 0.75 ± 0.07 for calcium activity and a Z' of 0.66 ± 0.06 for Arrestin activity.

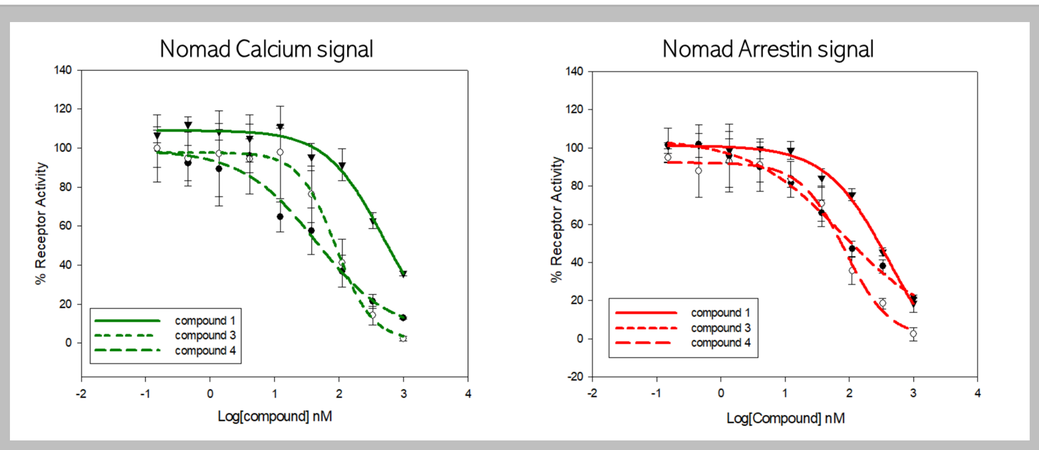

Figure 4. Comparative of antagonism behaviour of different compounds in $_{ArresCa2+}$Nomad PAR2 cell line. Compounds 1, 3 and 4 were incubated in the presence of 10 µM SLIGKV during 24h. Left panel shows the antagonist effect of different compounds in the calcium pathway. Right panel shows the antagonist effect of different compounds in the arrestin pathway. Data points represent the mean ± SD at each condition for a single experiment performed in triplicate. The results were normalized according to 10 µM SLIGKV and vehicle, considering 10 µM SLIGKV and vehicle as 100 % and 0 %, respectively. The EC50 for calcium for compounds 1, 3 and 4 were 467.73, 0.012 and 0.025 nM, respectively. The EC50 for arrestin for compounds 1, 3 and 4 were 416.86, 0.021 and 0.013 nM, respectively.

Materials and methods

Cell culture: U2OS human bone osteosarcoma cell line (DSMZ, Braunschweig Germany), from ATCC (Catalog No. HTB-96), was grown in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich, St. Louis, MO), MEM non-essential amino acids (Sigma-Aldrich, St. Louis, MO) and gentamicin (Sigma-Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere supplemented with 5% CO2. For the fluorescence analysis, cell were seeded in 96-well Imaging Plates (BD, Franklin Lakes, NJ) at a density of 20.000 cells/well.
Liquid Handling : For dispensing of the liquid media containing cells and compounds, the Hamilton's (Reno, NV) Microlab Star automated liquid handling workstation was used. Cells were treated with the agonists for 24 hours in Opti-MEM medium (Thermo Fisher Scientific, Waltham, MA) before image acquisition.
Image acquisition and analysis: Fluorescent images were acquired in the BD (Franklin Lakes, NJ) Pathway 855 High-Content automated image platform with a x20 dry objective and dose-response curves were obtained using "Synergy 2" fluorescence microplate reader from Biotek.

Conclusions

NOMAD® is a fluorescent biosensor platform that comprises the main GPCR signaling pathways, works in living cells and provides accurate quantitative results.

Nomad biosensor provides a robust and homogeneous assays that are amenable to HTS/HCS with high Z' values.

Nomad technology can be multiplexed for the simultaneous measurement of different signaling pathways with label-free receptors.