GPCR regulation through arrestin pathway using Nomad technology

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Abstract

Here we show a screening of compounds for Neurokinin 1 receptor regulation using Nomad technology through arrestin pathway. The Nomad biosensor localization is in the Plasma Membrane but the GPCR activation leads to a modification in its structural folding that promotes its vesicularization. In this work we have screened a library of 600 compounds. Substance P and L733-060 compounds were used as agonist and inhibitor control for this model. 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 indicated that this model is a valid strategy for drug screening.

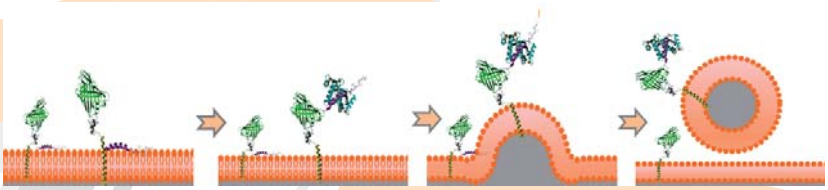

Figure 1. Schematic representation of the Nomad biosensor functioning in living cells. Nomad biosensor is a fluorescent fusion polypeptide capable of changing its localization within the cell from the cell cytoplasmic membrane to retention vesicles. In the case of Arrestin Nomad biosensor, after activation and phosphorylation of GPCR, our biosensor binds to phosphorylated GPCR that leads into a change in the spatial folding of Nomad Biosensor and promoting a cellular localization change. High-content screening (HCS) in Nomad Biosensors using living cells is a very useful tool in biological research to discover and optimize new drug candidates.

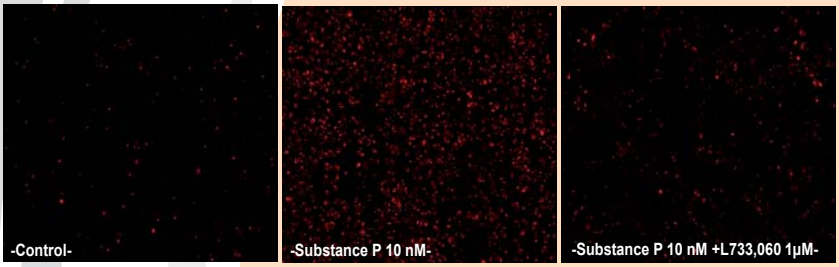

Fig. 2. Nomad biosensor arrestin pathway subcellular localization. Representative images of the Arrestin Nomad NKR1 cell line show an increment in the vesicle number after substance P treatment. This vesicular pattern is inhibited after cellular co-treatment with subtance P (10nM) and L733,060 inhibitor (1uM).

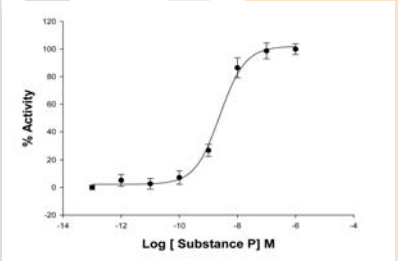

Fig.3 Nomad Biosensor Arrestin pathway dose-response curve of the agonist Substance P using NKR1-Arrestin Nomad cell line. To determine the EC_{50} value for Substance P, cells were treated with a range of concentrations from 1 µM to 0.1 fM during 24h. Data from measurements were fitted to a sigmoid 4-parameter fit logistic model (sigmoidal) with Sigma Plot 11.0 software. Error bars represent the standard deviation among 5 replicate wells. EC_{50} for Substance-P was $2,73.10^{-9}$ M and Z' factor for this experiment was 0.82 +/-0.01.

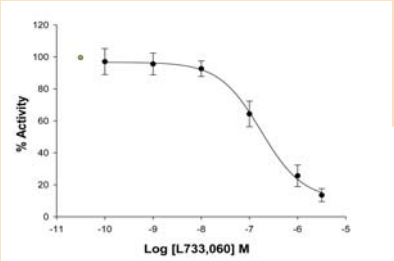

Fig.4 Arrestin pathway Nomad biosensor dose -response curve of the inhibitor L733,060 using NKR1-Arrestin Nomad cell line. To determine the EC_{50} value for L733,060, cells were co-treated with a constant concentration of 10nM of Substance P and a range of concentrations from 10 µM to 0.1 nM **of** L733,060 during 24h. Data from measurements were fitted to a sigmoid 4-parameter fit logistic model (sigmoidal) with Sigma Plot 11.0 software. Error bars represent the standard deviation among 5 replicate wells. IC_{50} for L733,060 was $3,79.10^{-7}$ M and Z' factor for this experiment was 0.70 +/-0.01.

Methods and Materials

Cultured cells:
U2OS based model cells were cultured into 96 wells Imaging Plates BD at 15.10^3 cells/well in 200 µl of DMEM F12 10% FBS and incubated at 37ºC and 5% CO2. After 16 hours, the cells were treated in OptiMEM and incubated with the compounds during 24 hours.
Image acquisition:
The cells were fixed with formaldehyde solution (3.7 wt. %, 20 minutes). Nuclei were stained using DAPI (2 ng/ml) and the fluorescence was measured using a BD Pathway 855 High-Content Bioimager from Becton Dickinson. To detect DAPI, the filters used were 380/10 and 460/10 nm for excitation and emission respectively and to detect arrestin Nomad, the filters were 590/20 nm and 665/8 nm. The images where obtained with an objective of 20X, taking 9 pictures of each well. Cell quantification was performed delimitating the region of interest of the nuclei (stained with DAPI) using Attovision software (Becton Dickinson) and after quantification, the average of each of the 5 replicates was performed. Vesicles quantification was also performed using AttoVision Software. This software application quantified the granularity per cell and the average of granularity per cell of each well was calculated. Approximately 500 cells per field were analyzed.

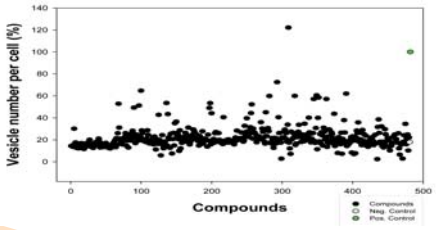

Fig. 5. Screening of 600 compounds library using the NK1R-Arrestin Nomad cell line. Representative data of vesicle number per cell normalized to control. Negative control (DMSO) is represented in white and positive control (Substance P 10 µM) in green.

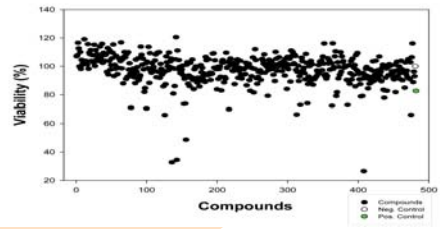

Fig.6. Cellular viability assessment. Toxicity levels were determined as a % of cell dead with respect to the negative control. The positive control (Substance P) is represented in green and the negative control is represented in white.

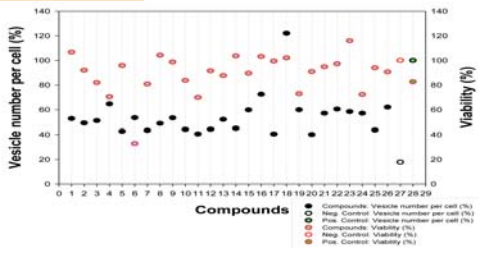

Fig.7. Positive compounds and viability relationship . The compounds that show an increment in the arrestin pathway activation are represented in black and the cell viability percentage is represented in grey with red edge. The positive control (Substance P) is represented in green spots and the negative control (DMSO) is represented in white.

Conclusions

***Nomad is a fluorescent biosensor platform that covers the main GPCR signaling pathways, works in living cells and provides accurate quantitative results.**
***Nomad technology can be used for second messenger and arrestin pathway studies.**
***Nomad biosensor provides a robust and homogeneous assay that is amenable to High Contect Screening with high Z' values.**
***Nomad cell-based assays require no additional reagents. The fluorescence signal can be detected on any standard imaging system and the results can be obtained easily using images analysis algorithms.**
***Nomad technology can be multiplexed for simultaneous measurement of second messengers of different signaling pathways and tagged receptors.**