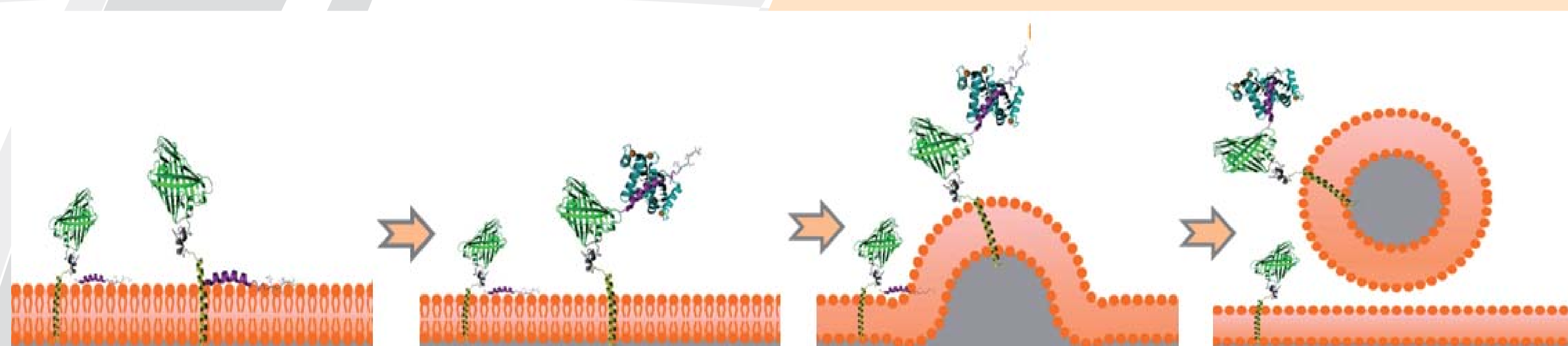


# Arrestin-Ca<sup>++</sup> Nomad: A High Throughput Screening technology for multiplexing GPCR functional assays

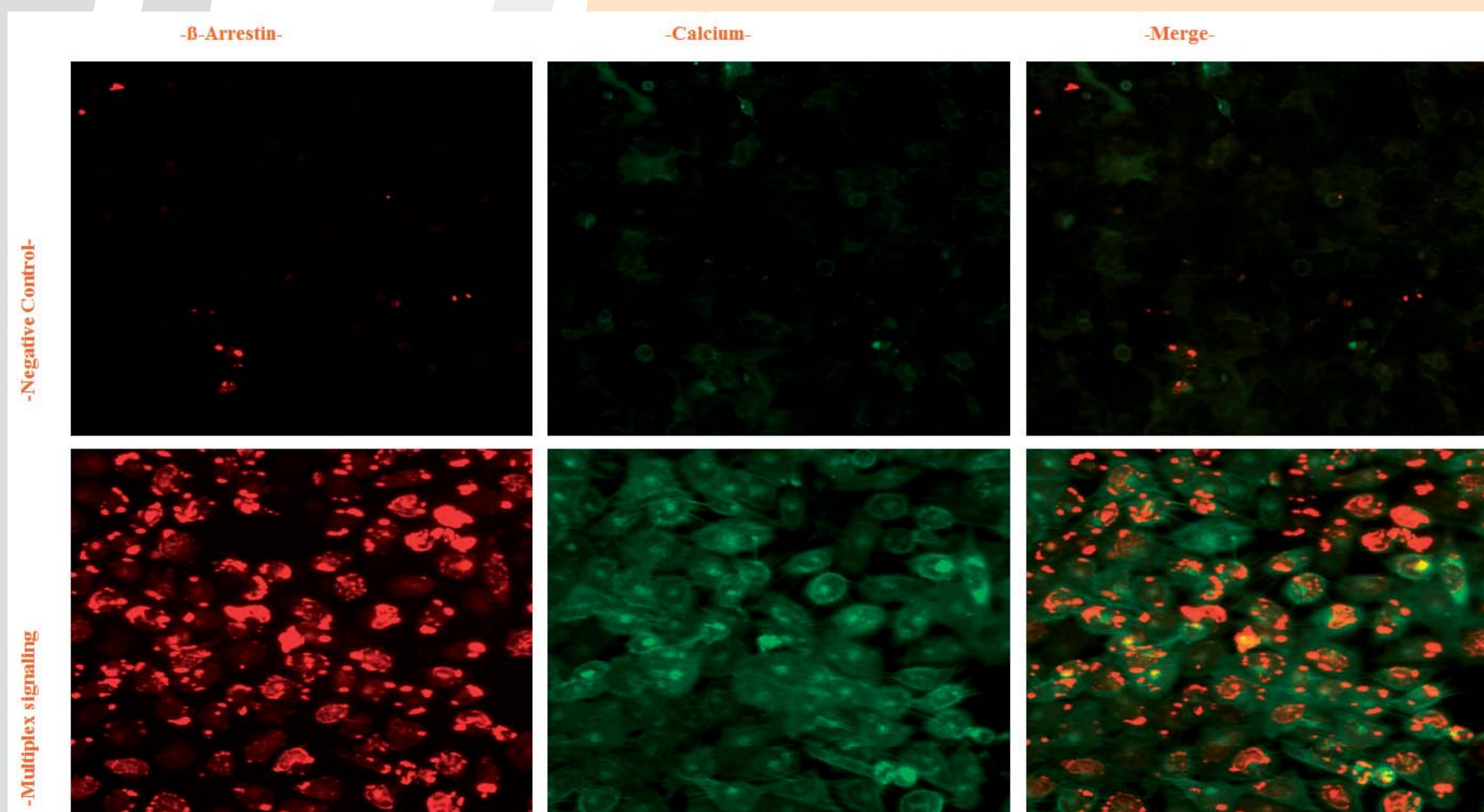
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## Abstract

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<sup>++</sup> & red beta-arrestin Nomad biosensors and Neurotensin receptor 1 to screen a library of 640 compounds. Both signals, calcium concentration and b-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.



**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 the retention vesicles, upon an increase in the concentration of second messengers within the cell cytoplasm. A second messenger concentration increase leads to the Nomad biosensor spatial folding conversion that promotes the cellular localization change. High-content screening (HCS) in Nomad systems uses living cells as a tool in biological research to discover and optimize new drug candidates.



**Figure 2. Cellular fluorescence redistribution in Arrestin-Ca<sup>++</sup> Nomad U2OS cell line.** In this work we describe a novel tool for GPCR screening multiplexing Ca<sup>++</sup> and Arrestin signals with a family of novel biosensors called Nomad. The possibility of tracing both pathways (calcium and arrestin) simultaneously in living cells opens the door to a screening step looking for biased agonists or antagonists. Here, we show red Arrestin Nomad biosensor and green Ca<sup>++</sup> Nomad biosensor expressed together inside the U2OS cells line. The pictures show that an increment in the Ca<sup>++</sup> concentration or in the arrestin activation generates biosensor localization change and an increment in the fluorescence intensity. Both signals can be analyzed using a image analysis equipment, a microplate reader or any other fluorescence device.

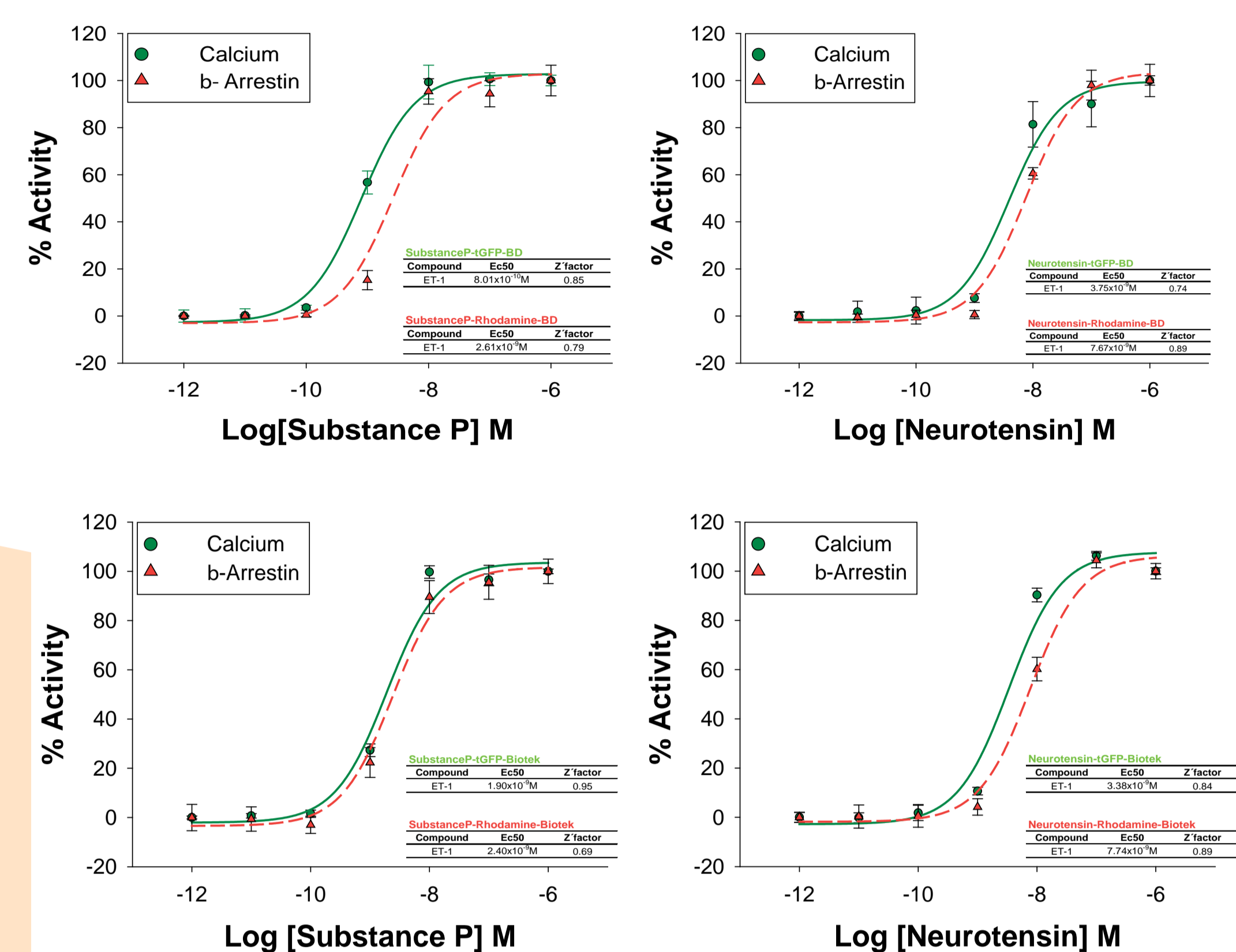
## Methods and Materials

**Cell cultures:** U2OS human bone osteosarcoma cell line 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% CO<sub>2</sub>. For the screening image analysis, cell lines were cultured into 96 wells Imaging Plates (BD, Franklin Lakes, NJ) at a density of 10,000 cells/well.

**Generation of stable cell line:** Nomad Arrestin-FP602 fusion protein was created by subsequent cloning of the FP602 red fluorescent protein (Evrogen, Moscow, Russia) cDNAs into the puromycin resistant pPURO vector designed in our lab, under the control of the CMV (cytomegalovirus) promoter. On the other hand, Nomad-calcium tGFP fusion protein was also created by the cloning of the turboGFP (tGFP) green fluorescent protein cDNA (Evrogen, Moscow, Russia) into the geneticin resistant expressing vector (Evrogen, Moscow, Russia), under the CMV promoter as well.

**Liquid Handling and phenotype-based screening assay:** For dispensing of the liquid media containing cells and compounds, the Hamilton's (Reno, NV) Microlab Star automated liquid handling workstation was used. Arrestin-Ca<sup>++</sup> Nomad stably expressing cells were treated with positive control NST-1 at 1 μM for 24 hours and library compounds at 10 μM for 24 hours in Optimem medium (Thermo Fisher Scientific, Waltham, MA) before image acquisition. Redistribution of Parkin and its localization to mitochondria was quantified after treatment. Cellular viability was determined by counting nuclei.

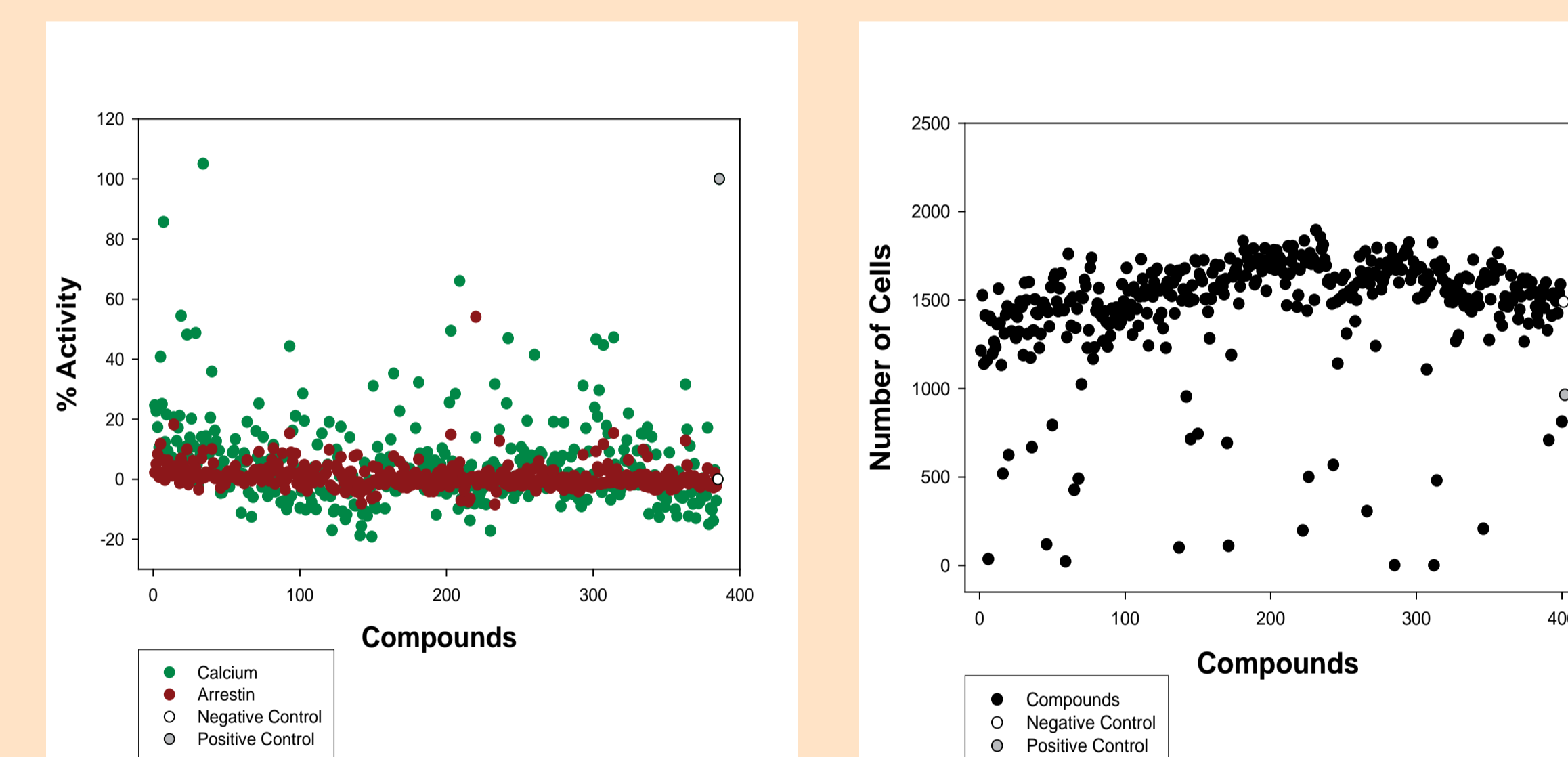
**Image acquisition and analysis:** Plates were fixed with phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) containing 3.7% formaldehyde (FA; Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature (RT), permeabilized with 0.3% Triton-X100 (Sigma-Aldrich, St. Louis, MO) in PBS for 3 min at RT and cell's nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, MO) for 5 min at RT. Fluorescent images were acquired in the BD (Franklin Lakes, NJ) Pathway 855 High-Content automated image platform with a x20 dry objective.



**Figure 3. Multiplex cell-based assays in two different Arrestin-Ca<sup>++</sup> Nomad cell lines stably expressing Neurotensin-1 Receptor and Neurokinin 1 Receptor.** Arrestin-Ca<sup>++</sup> Nomad NTSR1 and NK1R cell lines were treated with 7 log dilution series of corresponding agonist (n=6) during 24h. Cells were fixed and the nuclei were stained with DAPI.

**Upper panels** show results analysis using a "BD Pathway 855" High-Content Bioimager equipment.

**Lower panels** show results analysis using a Synergy 2 Multi-Mode Microplate reader. % Activity was calculated relative to positive (1 μM). Multiplexed assay was validated with an average of Z'<sup>2</sup>=0.7 +/- 0.01 for High Content Screening and it was validated with an average of Z'<sup>2</sup>=0.6 +/- 0.01 for high throughput Screening.



**Figure 4. Screening of 640 compounds library using Arrestin-Ca<sup>++</sup> Nomad NSTR1 cell line.** A chemical library consisting of 640 compounds, sourced from the Prestwick Chemical Library<sup>®</sup> was used with the objective to identify putative biased compounds implicated in the NTSR1 activity regulation.

- **(Left)** Compounds activity measure by the green calcium Nomad biosensor and Red Arrestin Nomad biosensor. Vehicle control (DMSO) is represented in white and positive control NST1) in black. The results of the compounds were normalized according to NST-1 and vehicle.

- **(Right)** Cellular viability expressed as number of cells after compounds treatment.

Compound	Ca <sup>++</sup>	Arrestin
Neurotensin	100.00	100.00
Flurandrenolide	40.77	11.78
Levonorefrin	44.31	15.28
Nylidrin	49.42	14.84
Aminocaproic acid	44.69	11.67
Gestrinone	47.20	15.35
Nilvadipine	85.70	6.42
Pranlukast	54.40	6.86
Tetraethylenepentamine pentahydrochloride	48.14	9.96
Dydrogesterone	48.69	3.75
Beta-Escin	105.04	9.55
Merbromin	244.38	8.10
Doxazosin mesylate	66.02	5.58
Flubendazol	46.94	4.62
(+)-Isoproterenol (+)-bitartrate salt	41.41	3.50
Florfenicol	46.57	9.29
Halcinonide	20.67	18.20
Secnidazole	35.84	10.06
Sulfadimethoxine	11.37	10.27
Luteolin	13.89	54.05
Cloquinol	16.50	12.78
Etilerine hydrochloride	31.63	12.88

**Figure 5. Screening analysis results using Arrestin-Ca<sup>++</sup> Nomad NSTR1 cell line.** Positive compounds were considered those compounds with a relative activity over 40% in the case of Ca<sup>++</sup> Nomad and over 10% in the case of ArrestinNomad.

**YELLOW PANEL:** Hit compounds that activate both Arrestin and calcium signalling pathway.

**GREEN PANEL:** Hit compounds that induce calcium signalling pathway activation.

**RED PANEL:** Hit compounds that induce arrestin signalling pathway activation.

## Conclusions

**\* Nomad is a fluorescent biosensor platform that covers the main GPCR signaling pathways, works in living cells and provides accurate quantitative results.**

**\*Nomad biosensors can be combined inside the same cells obtaining different second messenger signals and b-arrestin activation pathway simultaneously. So, Nomad biosensor permit to analyze easily the putative biased compounds.**

**\*Nomad biosensor provides a robust and homogeneous assay that is amenable to High Throughput Screening with high Z' values.**

**\*Nomad cell-based assays require no additional reagents. The fluorescence signal can be detected on any standard fluorescence 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 without any tagged receptor.**