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Multiplexed Assays for Co-expressed GPCRs Involved in Appetite Control

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Chi S. Ho, Colleen Harvey, and M. Ines Morano

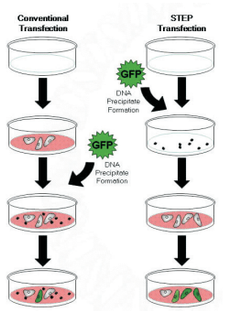
Originus Inc., 3985 Research Park Dr., Ann Arbor, Michigan 48108

Introduction

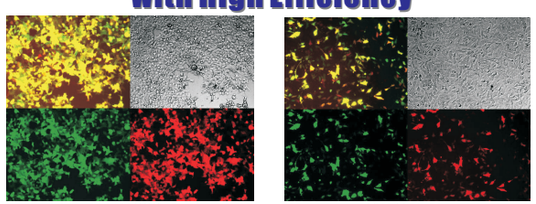
The biology of appetite, eating and weight control involves the interplay of a number of physiological systems including the brain, pituitary and gastrointestinal tract. These systems require in turn the appropriate functioning of an ever-growing list of molecular effectors. The development of drugs for obesity and eating disorders represents a major target in the pharmaceutical industry. Novel targets for such drug development arise from the recent explosion of information on molecules and neural circuits involved in appetite regulation. This study focuses on two families of G-Protein-Coupled Receptors (GPCRs) associated with appetite control and eating behavior in the central nervous system--Melanocortin (MCRs) and Orexin Receptors (OXRs). Co-existence of receptors from these two families is found in the ventromedial hypothalamus and paraventricular nucleus, where they may work as an ensemble to control neural signaling. Using a patented transfection technology developed at the University of Michigan termed Surface Transfection and Expression Protocol (STEP) to facilitate co-transfections of multiple expression constructs for receptors, G-proteins, and reporters simultaneously into cultured cells, we established a reproducible system for GPCR assay without generating stable cell lines. Multiple cell based assays for various GPCRs have been developed in Hek293T and neuronal cell lines. In this study, OXR and MCR were co-introduced into the same cell to mimic the co-localization of these two receptors in hypothalamus, nevertheless, their activations could be detected distinctively. This system has been examined with known agonists, antagonists, and chemical libraries. This multiplexed assay system not only can enhance the efficiency and reduce cost of screening new drug candidates, it also allows the detection of receptor interactions including heterodimer formation. The use of this platform is being extended to other potentially interacting GPCRs such as D2 dopamine receptor and CB1 cannabinoid receptor. (Supported by NIH SBIR Mh070995.)

STEP Technology

Conventional transfection methods can be divided into two systems: transient and stable cell transfection. Time delays of 3-9 months are necessary to produce a stable cell line; they are not always successful and involve high tissue culture costs and FTEs for long term maintenance; and these immortalized cells can lose their potency over time. Transient (solution-based) cell transfections have their advantages, but are limited to relatively few cell lines and it is difficult to co-transfect multiple DNAs into a single cell. STEP (Surface Transfection and Expression Protocol) solid phase transfection overcomes many of the disadvantages of conventional transient transfection and involves the formation of transfection complexes that are applied directly to the growth surface for the cells of interest. This enhances both the Transfection efficiency and co-transfection efficiency for multiple plasmids. The STEP complex coated microplates can be used for HTS experiments just by adding adherent cells.



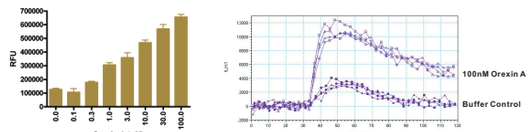
STEP Delivers Multiple Plasmids with High Efficiency



HEK-293T SH-SY5Y

STEP complexes were formed with equal amounts of expression vectors for green and red fluorescent proteins (5.0 % of total DNA each). Twenty four hours after application of cells, expression of the two fluorescent proteins was determined. Transfection efficiency on HEK-293T cells was over 90% and co-transfection efficiency was over 95%(Left panel). While in SH-SY5Y cells, the transfection efficiency using the same complex was over 60% and co-transfection efficiency was over 90% (Right panel). The co-transfection efficiency in HEK-293T cells remained over 90% even with a STEP complexing containing 0.5% of each fluorescence protein DNA. Such high transfection and co-transfection efficieny by STEP allows fine tuning of the stoichiometry of heterologously expressed proteins and facilitates development of robust cell based assays for high throughput screening.

Robust HTS with STEP

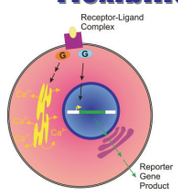


The data show an example of a calcium mobilization assay using STEP technology. Each well of a 384-well plate was coated with the same STEP complex containing human OX1R Orexin receptor and the plate was subjected to assay at 48 hours after plating HEK-293T cells. A serial dilution of Orexin A was applied to individual wells in quadriplicates at 30 seconds of the 2 minutes observation in a FlexStation-II 384. The accumulated changes in fluorescence signal are plotted on the left panel. The raw data from the buffer control and the 100nM positive control were shown on the right. The 4 replicates of each group virtually overlapped with each other indicating the reproducibility of the assay.

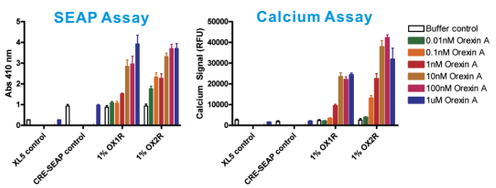
No. of Plates	10
No. of Compounds	3520
% Activity (mean ± SD)	103.0 ± 20.1
Threshold for Positives (% Activity)	42.7
Number of Positives	112
Number of Positives (% of Compounds)	2.6
SD of High Controls (% Activity)	6.4
SD of Low Controls (% Activity)	3.7
Z'-Factor (All Controls)	0.70
Z'-Factor (Mean of Intraplate Z'-Factors)	0.70 ± 0.05

High Throughput Screening parameters for another GPCR screen using STEP transfection were found to be superior when compared to a stable cell line expressing the same receptor. A Z'- factor of 0.7 was obtained indicating the robustness of the assay.

Multiplexed Assays for Flexibility and Hit Validation

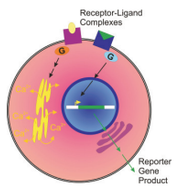


By providing the appropriate G-proteins, the ligand-activated GPCR can be coupled to different assay read outs, such as Calcium mobilization and a reporter gene assay. With the high co-transfection efficiency of STEP, the GPCR, G protein(s), and reporter gene(s) can be co-introduced into cells simultaneously. Such system allows the end user to choose an assay depending on equipment availability. In addition, the second read-out also provides a mean for hit validation without repeating the whole experiment.



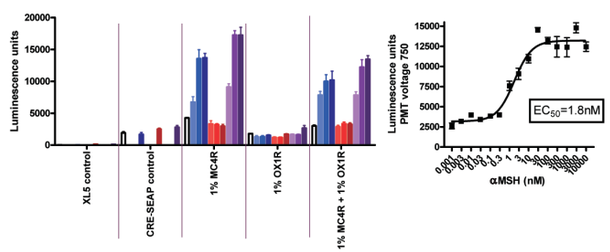
Four different STEP complexes were coated on two identical plates. The first 2 complexes were negative controls containing the filler DNA (pCMV6-XL5) and 1% Gq/Gs chimera, or with the addition of CRE-SEAP reporter. The third and fourth STEP complexes contained further addition of 1% Orexin 1 or Orexin 2 receptor expression construct, respectively. At 48 hours after plating cells, various doses of Orexin A were added to the plates and subjected to Calcium assay instantaneously, or SEAP assay after 8 hours of incubation. The activation of Orexin receptors was coupled to internal Calcium release through the endogenous Gq pathway, while activation of the PKA pathway was mediated by the supplementary Gq/Gs and was measured as up-regulation of CRE-SEAP expression. HEK293T cells transfected with either OXR containing complexes showed strong dose-dependent responses to Orexin A in both assays. Moreover, the SEAP assay can also be performed from the media of the same well after Calcium assay for the purpose of hit validation.

Multiplexed GPCRs in Assays with Distinctive Read-outs

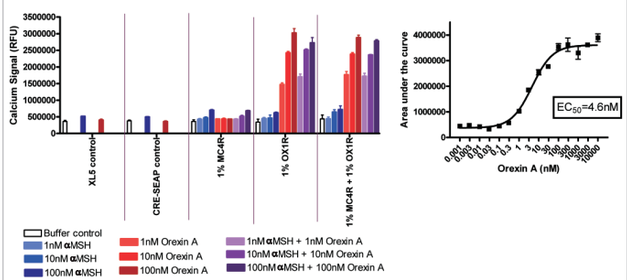


Co-expression of GPCRs *in vivo* may play a role in the modulation of the triggered response. In most HTS, a single target was assessed, which is in part due to the difficulty in generating stable cell lines with multiple heterologous DNA and control of the expression levels of each construct. The STEP technology allows co-transfection of multiple GPCR expression constructs with fine tuning of individual receptors. With careful selection of cell lines and supplement of chimeric G-proteins, different read-outs can be coupled to each introduced receptor individually. Such a system not only facilitates the study of GPCR pathway interactions, it also permits the formation of potentially heterodimeric receptors.

SEAP Assay for MC4R Activity



Calcium Assay for OX1R Activity

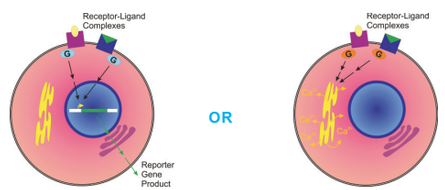


In this experiment, the Melanocortin receptor MC4R and the Orexin receptor OX1R were tranfected individually or co-introduced into HEK-293T cells using STEP technology. The activation of OX1R by Orexin A was coupled to intracellular Calcium release through endogenous Gq, while the activation of MC4R by α-MSH was coupled to the PKA pathway through endogenous Gs and was detected by up-regulation of CRE-SEAP expression. The response of singly expressed receptor to its corresponding agonist was indifferent from the one with both receptor expressed. Moreover, the double receptor transfected cells showed response to either agonist. The agonist for OX1R did not cross-react with MC4R and vice versa. Dose reponse curves of Orexin A on OX1R and α-MSH on MC4R were performed on the same plate using corresponding assays. This experiment demonstrated the potential of STEP technology in cell-based assays for multiple targets with independent read-outs.

Summary

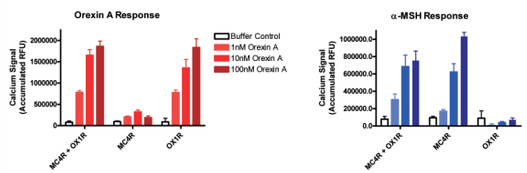
1. STEP technology offers high transfection and high co-transfection efficiencies.
2. Robust GPCR assays can be achieved using STEP technology without developing stable cell lines.
3. Multiple read-outs, such as Calcium mobilization, reporter genes, and membrane potential assays, have been implemented on the STEP platform.
4. Multiple read-outs from the same target are made possible by STEP. It provides flexibility in the choice of assay, and it also provides a means for rapid hit validation without repeating the whole experiment.
5. Multiplexed GPCRs in a single well provide a system for studying Receptor and pathway interactions. Different read-outs can be used to monitor the activities of individual receptors distinctively while under the modulation of each other.
6. Multiple targets can be combined in a single assay using STEP to increase the throughput and to reduce cost in drug screening.

Multiplexed GPCRs in Assays to Increase HTS Efficiency



In a typical HTS, not more 2-5% compounds are identified as positives. By combining 2 targets within the same well in the primary screening, the overall throughput can be nearly doubled. With the STEP technology, each receptor construct is typically less than 2% of the total DNA in the complex. Therefore, a second, third, or even more receptor DNAs may be added to the complex. Although technically 10 or more receptors can be multiplexed into a single well using STEP, 2-3 targets seems to be optimal as the increased number of total hits would also multiply the amount of work in hit validation. Different chimeric G protein(s) may be required to direct different GPCR signal into the same monitoring pathway and the expression level of each receptor needs to be adjusted to produce similar activity upon full activation.

Multiplexed MC4R and OX1R in Calcium Assay



In this experiment, three STEP complexes containing 2% OX1R, 2% MC4R, or 2% of each were used to transfect HEK-293T cells. Since MC4R requires Gα16 to transduce its activation to Calcium signal, 1% Gα16 was added in all complexes for a fair comparison. The cells expressing individual receptors were compared to the multiplexed ones in responses to Orexin A and α-MSH. Basically, the Orexin 1 receptor responsed to Orexin A equally well in both STEP transfected cells expressing OX1R only or with both OX1R and MC4R. Similarly, the MC4R responsed to α-MSH disregarding the addition of OX1R. Similar experiment using CRE-SEAP reporter has also been conducted with similar finding. This experiment demonstrated the feasibility of combining multiple GPCRs in HTS.