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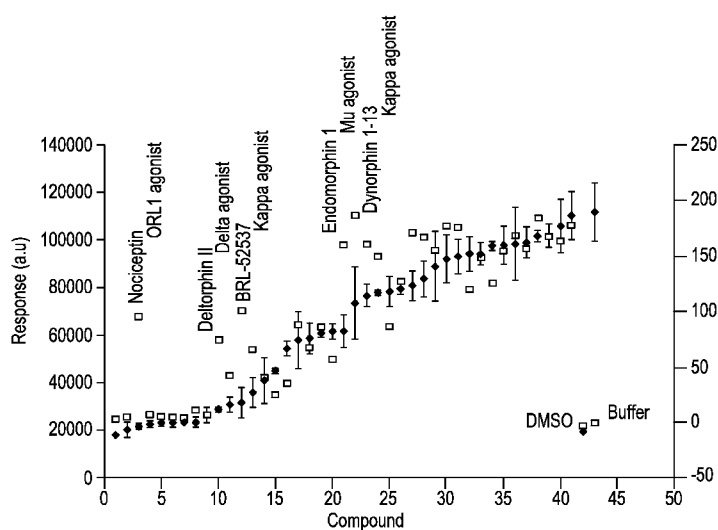
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FIG. 4



(57) Abstract: Disclosed are methods and machines for assessing opioid receptor and materials related thereto.

ASSESSMENT OF OPIOID RECEPTOR LIGANDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. § 119 of U.S. Provisional Application Serial No. 61/447,990 filed on March 1, 2011 the content of which is relied upon and incorporated herein by reference in its entirety.

BACKGROUND

[0002] GPCR ligands are often classified as full agonists, partial agonists, neutral antagonists and inverse agonists, based on single read-out measurements. However, single read-out measurements generally lead to non-correlation between *in vitro* testing results and *in vivo* actions. Label-free biosensor cellular assays can overcome the poor correlation between *in vitro* testing results and *in vivo* actions.

[0003] The methods disclosed herein are related to methods to identify molecular pharmacology using label-free integrative pharmacology. The methods disclose label-free methods to characterize opioid receptor ligands using label-free biosensor cellular assays. Specifically, the methods disclose the use of a pair of cell lines (an engineered cell line and its parental cell line) for characterizing the on-target pharmacology of ligands acting on a specific opioid receptor ligands using a battery of assays wherein cellular background is manipulated using a selected set of chemicals and biochemicals. The methods disclosed herein can be applied to three opioid receptors, mu, delta and kappa receptors. The methods disclosed herein can also be applicable to endogenous opioid receptors wherein a native cell line expresses at least one opioid receptor. The methods also disclose bucket opioid receptor ligands into different clusters, based on their selectivity and mode of action including biased agonism, at the receptor family level. The methods also disclose methods that can filter data such that such an on-target pharmacology assessment is validated and effective.

[0004] The disclosed compositions and methods can also be used for assessing Gi-coupled receptors in general. The methods can also include clustering analysis to determine the similarity of an unknown molecule with a known reference molecule whose pharmacology is at least partially known, thus to determine the pharmacology of the unknown molecule.

SUMMARY

[0005] Disclosed herein are methods to determine the on-target pharmacology of GPCR ligands. In some forms the GPCR ligands can be opioid receptor ligands. The disclosed methods are related to label-free cellular assays and label-free cellular integrative pharmacology. The disclosed methods can use a battery assay to determine distinct aspects of molecular pharmacology acting through an opioid receptor. The disclosed methods can also use a numerical matrix to describe the label-free integrative pharmacology of molecules, and can use clustering algorithms to classify the pharmacology of molecules. In some forms the opioid receptors can be, for example, mu-opioid receptors, delta-opioid receptors or kappa-opioid receptors. Also disclosed are engineered cells expressing opioid receptors.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] Figure 1 shows a representative example of the disclosed methods using the label-free on-target pharmacology approach to determine the on-target pharmacology of the opioid receptor agonist DAMGO acting on mu opioid receptor (MOR). A heat map is also shown for the representative compounds. (A) The DAMGO DMR in HEK293 having no mu opioid receptor; (B) The DAMGO DMR in HEK-MOR stably expressing MOR; (C) The DAMGO DMR in the CTOP pretreated HEK-MOR; (D) The CTOP DMR in the DAMGO-pretreated HEK-MOR; (E)-(L) The DAMGO DMR in the DAMGO-, PTX-, CTX-, forskolin-, U0126-, SB202190-, SP100625- and LY294002-pretreated HEK-MOR cells, respectively. 10 μ M of DAMGO was used in these experiments, also 10 μ M of CTOP, U-0126, SB202190, SP100625, LY294002 was used in these experiments. All pretreatments were carried out 1hr before the stimulation, except that PTX of 100ng/ml was used to pretreat cells overnight and CTX of 400ng/ml were used to pretreat cells 4hrs before stimulation.

[0007] Figure 2 shows a heat map showing the clusters of known opioid receptor ligands against five different cell lines (HEK293, HEK-MOR, HEK-DOR, HEK-KOR, and SH-SH-5Y). The heat map was generated using a one-dimension similarity analysis, wherein a compound's DMR signal in a cell line was described using 3-time domain responses. The 3 time domain responses were the real values of a DMR signal at 3min, 9min, and 30min post stimulation. This clustering method is useful to identify the selectivity of opioid ligands

across the family members, as well as a moderate resolution classification of pharmacology (agonist vs antagonist).

[0008] Figure 3 shows a heat map to classify the on-target pharmacology of mu receptor-active ligands using a battery of assays, as described in Figure 1. This heat map was generated using a one-dimension similarity analysis, wherein a compound's DMR signal in a cell line was described using 3-time domain responses. The 3 time domain responses were the real values of a DMR signal at 3min, 9min, and 30min post stimulation.

[0009] Figure 4 shows the correlation between cAMP and DMR signals of opioid ligands acting on HEK-MOR cells. For cAMP measurements the cells were co-stimulated with forskolin (4micromolar) for each ligand (10 micromolar). After 30min the cells were lysated and the phosphodiesterase inhibitors were added to prevent cAMP hydrolysis, the the total cAMP concentration was measured using Promega cAMP reagent kits. The DMR signal of each ligand was calculated based on its amplitude at 3min post stimulation.

DETAILED DESCRIPTION

A. G-Protein Coupled Receptors (GPCR)

[0010] G-Protein Coupled Receptors OPKR1 is a seven transmembrane G protein coupled receptor (GPCR) [Yasuda et al. (1994), Kozak et al. (1994), Jordan and Devi (1999), Statnick et al. (2003)]. Many medically significant biological processes are mediated by signal transduction pathways that involve G-proteins [Lefkowitz, (1991)]. The family of G-protein coupled receptors (GPCRs) includes receptors for hormones, neurotransmitters, growth factors, and viruses. Specific examples of GPCRs include receptors for such diverse agents as dopamine, calcitonine, adrenergic hormones, endotheline, cAMP, adenosine, acetylcholine, serotonin, histamine, thrombin, kinine, follicle stimulating hormone, opsins, endothelial differentiation gene-1, rhodopsins, odorants, cytomegalovirus, G-proteins themselves, effector proteins such as phospholipase C, adenyl cyclase, and phosphodiesterase, and actuator proteins such as protein kinase A and protein kinase C.

[0011] GPCRs possess seven conserved membrane-spanning domains connecting at least eight divergent hydrophilic loops. GPCRs, also known as seven transmembrane, 7TM, receptors, have been characterized as including these seven conserved hydrophobic stretches of about 20 to 30 amino acids, connecting at least eight divergent hydrophilic loops. Most GPCRs have single conserved cysteine residues in each of the first two extracellular loops, which form disulfide bonds that are believed to stabilize functional protein structure. The

seven transmembrane regions are designated as TM1, TM2, TM3, TM4, TM5, TM6, and TM7. TM3 is being implicated with signal transduction. Phosphorylation and lipidation (palmitoylation or farnesylation) of cysteine residues can influence signal transduction of some GPCRs.

[0012] Most GPCRs contain potential phosphorylation sites within the third cytoplasmic loop and/or the carboxy terminus. For several GPCRs, such as the beta-adrenergic receptor, phosphorylation by protein kinase A and/or specific receptor kinases mediates receptor desensitization.

[0013] For some receptors, the ligand binding sites of GPCRs are believed to comprise hydrophilic sockets formed by several GPCR transmembrane domains. The hydrophilic sockets are surrounded by hydrophobic residues of the GPCRs. The hydrophilic side of each GPCR transmembrane helix is postulated to face inward and form a polar ligand binding site. TM3 is being implicated with several GPCRs as having a ligand binding site, such as the TM3 aspartate residue. TM5 serines, a TM6 asparagine, and TM6 or TM7 phenylalanines or tyrosines also are implicated in ligand binding.

[0014] GPCRs are coupled inside the cell by heterotrimeric G-proteins to various intracellular enzymes, ion channels, and transporters. Different G-protein alpha- subunits preferentially stimulate particular effectors to modulate various biological functions in a cell. Phosphorylation of cytoplasmic residues of GPCRs is an important mechanism for the regulation of some GPCRs. For example, in one form of signal transduction, the effect of hormone binding is the activation of the enzyme, adenylate cyclase, inside the cell. Enzyme activation by hormones is dependent on the presence of the nucleotide GTP. GTP also influences hormone binding. A G- protein connects the hormone receptor to adenylate cyclase. G-protein exchanges GTP for bound GDP when activated by a hormone receptor. The GTP-carrying form then binds to activated adenylate cyclase. Hydrolysis of GTP to GDP, catalyzed by the G-protein itself, returns the G-protein to its basal, inactive form. Thus, the G- protein serves a dual role, as an intermediate that relays the signal from receptor to effector, and as a clock that controls the duration of the signal.

[0015] Over the past 15 years, nearly 350 therapeutic agents targeting 7TM receptors have been successfully introduced into the market. This indicates that these receptors have an established, proven history as therapeutic targets.

1. Opioid Receptors

[0016] Opioid receptors make up a family of G protein-coupled receptors (GPCRs). GPCRs are the largest group of cell-surface receptors that are involved in signaling. Opioid receptors have been reported to interact with a multitude of cell surface receptors, which modulate numerous physiological responses such as nociception, hormone secretion, neurotransmitter release, respiratory depression, and opiate addiction. There are three known classes of opioid receptors: Mu, Kappa and Delta. Through successive genetic knockouts of each of these opioid receptors, it has been shown that the Mu opioid receptor (MOR) is primarily responsible for producing addictive behaviors, and mediating morphine responses such as analgesia and dependence. Recently, more emphasis has been put on the importance of Kappa and Delta opioid receptors (KOR and DOR, respectively). KOR and DOR agonists trigger decreased excitability of neurons, similar to the activation of MOR; also recent evidence shows that KOR and DOR agonists can produce analgesic effects.

[0017] Although opioid receptors are well-known to act individually, they also interact with each other to elicit important cellular responses. Heteromeric interactions, for example, significantly affect ligand efficacy and downstream signaling; it has been shown that interconversion between the dimeric and monomeric forms plays a role in opioid receptor internalization.

[0018] Opioid receptors are Gi/Go-coupled GPCRs; activation of MOR leads to inhibition of adenylyl cyclase, activation of potassium conductance, inhibition of calcium channels, and inhibition of neurotransmitter release. The modulation in potassium and calcium conductance (increase and decrease, respectively) serves to reduce the membrane excitability, which leads to the decrease in secretion of neurotransmitters. These effects are produced by both exogenous and endogenous ligands, and they function to modulate nociception in the central nervous system. Opioid receptors, like most GPCRs, are regulated by multiple mechanisms, including receptor desensitization and internalization; understanding how these mechanisms affect signaling is important for elucidating the physiological basis of opioid dependence. This understanding will ultimately lead to a better understanding of opioid receptor pharmacology.

[0019] There are four known coupling classes of GPCR (Gi/Go, Gs, Gq and G12/G13). The literature shows that all of the opioid evoked responses are blocked by pertussis toxin, and therefore is fully dictated by Gi/Go receptor coupling. However, there

are indications that a Gs component is involved in some opioid receptor activation. There are also indications that this Gs-coupled component of opioid signaling is related to the determinants of tolerance and the phenomenon of addiction, but the specific mechanisms are still mostly unknown.

[0020] Many opioid compounds bind to the opioid receptor family. These compounds can be endogenous or exogenous, natural or derived. Different opioid ligands have different effects on internalization, desensitization, ligand efficacy and ultimately addiction. Receptor ligands can be classified as full agonists, partial agonists, neutral antagonists or inverse agonists, based on efficacy. Opioid receptors have “pathway-selective signaling” where one group of agonists will activate one pathway, while another will only activate a separate different signaling pathway. This functional selectivity is crucial for drug development, as this phenomenon raises the possibility of selecting and/or designing novel ligands that differentially activate only a subset of functions of a single receptor, thereby optimizing the therapeutic effect. While the existence of functional selectivity has been demonstrated in the literature the correlation between agonist-selective signaling and the functionality of the opioid receptors still need to be investigated to fully utilize this biased agonism in order to use the knowledge of signaling to design signaling pathway specific therapeutics. Opioids are incredibly valuable in medicinal settings, and manipulating agonist selectivity would greatly enhance their therapeutic functionality. For example, opioids are powerful analgesics used to treat unremitting pain. However, they have many negative side effects including tolerance, dependence and ultimately addiction. Label free methods described herein can uncover the full pharmacology of the opioid receptors. The information gained from the pharmacology characterization will allow for growth in future therapeutic uses of opioid receptor signaling. The label free methods can be carried out using the Epic system.

i. Mu-opioid receptors

[0021] The mu(μ)-type opioid receptor (MOR) is a member of the G-protein- coupled receptor (GPCR) family. It has an extracellular N-terminus and intracellular C-terminus, with seven membrane-spanning domains that comprise the binding pocket for exogenous drugs. Upon activation, these seven transmembrane (7TM) domain GPCRs initiate molecular changes resulting in inhibition of nerve, immune, and glial cells that play a role in the onset and maintenance of pain. MOR induces analgesia via pertussis toxin (PTX)-sensitive

inhibitory G protein ($G_{ai/o}$), which inhibits cAMP formation and Ca^{2+} conductance and activates K^+ conductance, leading to hyper-polarization of cells thereby, exerting an inhibitory effect. See Crain and Shen, Pain, 84, 121-131 (2000). Nevertheless, the opposite, stimulatory effects of opiates also have been demonstrated, depending on the experimental concentration of the drug and the duration of incubation. See Crain and Shen, Pain, 84, 121-131 (2000); and Rubovitch et al., Brain Res. Mol. Brain Res., 110, 261-266 (2003).

ii. Delta-opioid receptors

[0022] Delta Opioid Receptors (DOR) were first described in 1977 (Lord et al, 1977) and subsequently, several classes of peptide and non-peptide based molecules have been synthesized that selectively stimulate this receptor. Selective DOR molecules include the modified enkephalin analog, DPDPE (Mosberg et al., 1983), deltorphin-based peptides (Kreil et al, 1989) and analogs of BW373U86 (e.g., SNC80; Bilsky et al, 1994; Calderon et al, 1994; 2004). Preclinical efficacy studies of these compounds, along with DOR- selective antagonists, provide a convincing rationale for pursuing DOR agonists as analgesic agents. In addition, DOR knockout mice exhibit increased pain behaviors following an inflammatory or neuropathic-based insult (Gaveriaux-Ruff et al, 2008; Nadal et al, 2006). Interestingly, upregulation and altered trafficking of DOR occurs following induction of various pain states in rodents (Cahill et al, 2003; 2007; Walwyn et al, 2005). DOR selective compounds also have significantly reduced the tumor burden in multiple animal models.

iii. Kappa-opioid receptors

[0023] Kappa opioid receptors (KORs) are present in the brain, spinal cord, and on the central and peripheral terminals and cell bodies of the primary sensory afferents (somatic and visceral), as well as on immune cells.

B. Methods

[0024] Disclosed herein are methods relating to label-free on-target pharmacology for assessing GPCR ligands. In some forms the GPCR ligands can be opioid receptor ligands. In some forms the methods can be specifically acting on stably expressed mu, delta and/or kappa receptors, as well as endogenously mu, delta and opioid like receptor subtype 1 (ORL1) in a native cell. The disclosed methods are related to label-free cellular assays and label-free cellular integrative pharmacology. The disclosed methods can use a battery of assays to determine aspects of molecular pharmacology acting through an opioid receptor. The disclosed methods can also use a numerical matrix to describe the label-free integrative

pharmacology of molecules, and can use clustering algorithms to classify the pharmacology of molecules. In some forms the opioid receptors can be, for example, mu-opioid receptors, delta-opioid receptors or kappa-opioid receptors.

[0025] The disclosed methods can use a battery of assays specifically tailored for on-target pharmacology assessment of opioid receptors. The choice of assays is linked with the signaling pathways and possible biased agonism activity of opioid receptor ligands. These assays for a ligand include: (1) agonism response of the ligand in the parental cell having no opioid receptor (e.g., HEK-293), wherein this assay defines the possible activity of the ligand acting on an endogenous target; (2) agonism response of the ligand in an engineered cell stably expressing a specific opioid receptor (e.g., HEK-MOR), wherein this assay can determine the possible agonism of the ligand acting on the opioid receptor; (3) the ability of the ligand to cause the engineered cell responding to succeeding stimulation with the known receptor antagonist, wherein this assay can determine the sustainability of the test molecule-induced cellular response and the ability of the antagonist to reverse its signal; (4) the agonism activity of the ligand in the engineered cells preconditioned via pretreatment with distinct chemicals or biochemicals, including (a) the known receptor antagonist which can determine the specificity of the ligand agonism acting on the receptor, (b) the known receptor agonist which can determine the desensitization-resensitization pattern of the ligand acting via the receptor, (c) the Gi protein killer pertussis toxin (PTX) which can determine the possible Gi-independent/dependent component of the ligand mediated biased agonism, (d) the Gs protein killer cholera toxin (CTX) which can determine the possible Gs-dependent component of the ligand mediated biased agonism, (e) the adenylyl cyclase activator forskolin which can determine the possible Gi-dependent component of the ligand mediated biased agonism, (f) the MEK1/2 inhibitor U0126 which can determine the possible biased agonism of the ligand acting via MAPK pathway. (g) the p38 MAPK inhibitor SB210290 which can determine the possible biased agonism of the ligand acting via p38 MAPK pathway, (h) the JNK inhibitor SP100625 which can determine the possible biased agonism of the ligand acting via JNK pathway, and (i) the PI3K inhibitor LY294002 which can determine the possible biased agonism of the ligand acting via PI3K pathway.

[0026] The disclosed methods can filter compounds for determination of their on-target pharmacology. First, any compounds that show agonism activity in the parental cell line are not included in on-target pharmacology assessment. Second, any compounds that have low

potency (with an EC_{50} or K_i or K_d higher than 5 μM) acting on the target receptor are also not included in on-target pharmacology assessment. The reason being that for on-target pharmacology assessment, a fixed dose of 10 μM is used for all compounds. Alternatively, for low potency compounds, a higher concentration can be used, or dose-dependent responses can be assayed in order to carry out effective on-target pharmacology assessment.

[0027] The disclosed methods can also use a simplified matrix to describe the ligand-induced DMR signals acting via one or more opioid receptors, which only uses a 3 time domain matrix, preferably 3min, 9min and 30min post stimulation. Such a matrix allows for effective clustering of opioid ligands using the described on-target pharmacology methods.

[0028] The disclosed methods can also use a similarity analysis to classify on-target pharmacology of opioid ligands. The appropriate clustering algorithms include, but not limited to, Hierarchical, K-means and MCL clustering. The Hierarchical clustering is a cluster analysis method which seeks to build a hierarchy of clusters based on linkages. The K-Means clustering is a partitioning algorithm that divides the data into k non-overlapping clusters, where k is an input parameter, and also the Number of clusters. One of the challenges in K-Means clustering is that the number of clusters must be chosen in advance, and in general are close to the square root of $1/2$ of the number of nodes. Markov Clustering Algorithm (MCL) is a fast divisive clustering algorithm for graphs based on simulation of the flow in the graph. For label-free integrative pharmacology approach, Hierarchical clustering can be used, and is used throughout in the disclosed experimental examples.

[0029] Clustering is a widely established technique for exploratory data analysis with applications in statistics, computer science, biology, social sciences, or psychology. It is applied to empirical data in basically any scientific field to gain an initial impression of structural similarities. For this purpose, it is of great advantage to have an efficient and easy-to-use tool that can be applied ubiquitously to a large scope of data types. However, the applications of clustering analysis in label-free cellular assays have not been explored.

[0030] The clustering analysis is generally carried out using conventional pairwise similarity functions to determine similarity (or distance) for each unordered pair in the dataset, leading to a similarity matrix. The conventional pairwise similarity functions include, but not limited to, Hierarchical, and k-Means. Both Hierarchical and K-means have been applied to cluster expression or genetic data. Hierarchical and k-Means clusters may be displayed as hierarchical groups of nodes or as heat maps. Other known methods, such as

MCL and FORCE, can also be used. Both MCL and FORCE create collapsible "meta nodes" to allow interactive exploration of the putative family associations, and thus are often used for clustering similarity networks to look for protein families (and putative functional similarities).

[0031] Hierarchical clustering is a method of cluster analysis which seeks to build a hierarchy of clusters. Strategies for hierarchical clustering generally fall into two types: agglomerative and divisive. The agglomerative clustering is a "bottom up" approach – each observation starts in its own cluster, and pairs of clusters are merged as one moves up the hierarchy. The divisive clustering is a "top down" approach – all observations start in one cluster, and splits are performed recursively as one move down the hierarchy. In order to decide which clusters should be combined (for agglomerative), or where a cluster should be split (for divisive), a measure of dissimilarity between sets of observations is required. In most methods of hierarchical clustering, this is achieved by use of an appropriate distance metric (a measure of distance between pairs of observations), and a linkage criteria which specifies the dissimilarity of sets as a function of the pairwise distances of observations in the sets. The choice of an appropriate metric will influence the shape of the clusters, as some elements may be close to one another according to one distance and farther away according to another. Common distance metrics include Euclidean distance, squared Euclidean distance, Manhattan distance, maximum distance, Mahalanobis distance, and cosine similarity. The Euclidean distance is found to be the most preferred metric for label-free integrative pharmacology applications, and is used throughout in the disclosed experimental examples.

[0032] Hierarchical clustering builds a dendrogram (binary tree) such that more similar nodes are likely to connect more closely into the tree. Hierarchical clustering is useful for organizing the data to get a sense of the pairwise relationships between data values and between clusters. The dendrogram is generated by using linkage criteria. The linkage is referred to a measure of "closeness" between the two groups. The linkage criteria determine the distance between sets of observations as a function of the pairwise distances between observations. There are four different types of linkage. In agglomerative clustering techniques such as hierarchical clustering, at each step in the algorithm, the two closest groups are chosen to be merged. The linkage methods include: (1) pairwise average-linkage (i.e., the mean distance between all pairs of elements in the two groups), (2) pairwise single-linkage (i.e., the smallest distance between all pairs of elements in the two groups), (3)

pairwise maximum-linkage (i.e., the largest distance between all pairs of elements in the two groups) and (4) pairwise centroid-linkage (i.e., the distance between the centroids of all pairs of elements in the two groups). The pairwise maximum-linkage is found to be the most preferred for label-free integrative pharmacology applications.

[0033] For Hierarchical clustering, there are several ways to calculate the distance matrix that is used to build the cluster. Typically, the distances represent the distances between two rows (usually representing nodes) in the matrix. The distance metrics used includes, but not limited to, (1) Euclidean distance which is the simple two-dimensional Euclidean distance between two rows calculated as the square root of the sum of the squares of the differences between the values; (2) City-block distance which is the sum of the absolute value of the differences between the values in the two rows; (3) Pearson correlation which is the Pearson product-moment coefficient of the values in the two rows being compared. This value is calculated by dividing the covariance of the two rows by the product of their standard deviations; (4) Pearson correlation, absolute value which is similar to the value indicated in (3), but using the absolute value of the covariance of the two rows; (5) Uncentered correlation which is the standard Pearson correlation includes terms to center the sum of squares around zero. This metric makes no attempt to center the sum of squares. (6) Centered correlation, absolute value which is similar to the value indicated in (5), but using the absolute value of the covariance of the two rows; (7) Spearman's rank correlation which is Spearman's rank correlation (ρ) is a non-parametric measure of the correlation between the two rows; (8) Kendall's tau which ranks correlation coefficient (τ) between the two rows. The choice of distance metric for label-free integrative pharmacology is found to be dependent on the types of data. For on-target pharmacology classification, uncentered absolute correlation is preferable.

[0034] The similarity analysis can further use a predefined clustering threshold (a density parameter, also termed as similarity threshold) to compute a similarity matrix. Such a threshold gives the boundary between similar and dissimilar objects, and thus is used to control the density of the clustering analysis. High (restrictive) values make it more expensive to add most of the edges, resulting in many small clusters. On the other hand, lower values make it cheap to add edges but expensive to remove them, resulting in few big clusters (meaning lower resolution). For label-free integrative pharmacology, the clustering threshold can be variable, and often depending on the desired resolution of clustering.

[0035] For label-free integrative pharmacology, the data contain the list of all numeric node and edge attributes that can be used for hierarchical clustering. The node is often the molecule. The edge attribute represents the response of the molecules either alone (i.e., a given response at a specific time *i* for the molecule primary profile in a cell), or represents the modulation percentage of the molecule against a marker (i.e., the modulation percentage of the marker biosensor response, such as P-DMR, or N-DMR, by the molecule at a specific concentration). At least one edge attribute or one or more node attributes must be selected to perform the clustering. If an edge attribute is selected, the resulting matrix will be symmetric across the diagonal with nodes on both columns and rows. If multiple node attributes are selected, the attributes will define columns and the nodes will be the rows. Under certain circumstances, it may be desirable to cluster only a subset of the nodes in the network. For example, to identify molecules sharing a specific mode of action, only a subset of the nodes displaying such mode of action is examined.

[0036] For label-free integrative pharmacology approach, certain normalization or data pretreatments may be necessary for effectively clustering. For example, data filtering may be necessary. For similarity analysis based on molecule biosensor primary indices, an effective data filtering mean is to use the max-min difference (e.g., only molecules whose DMR signal having a max-min difference between different time points greater than 40picometer within one hour post-stimulation are subject to similarity analysis).

[0037] For label-free on-target pharmacology studies, both one-dimensional and two-dimensional clustering analysis can be used. The one-dimensional clustering primarily is focused on the similarity among molecules (nodes). The two dimensional clustering includes clustering both attributes and nodes. In such method, the clustering algorithm will be run twice, first with the rows in the matrix representing the nodes and the columns representing the attributes. The resulting dendrogram provides a hierarchical clustering of the nodes given the values of the attributes. In the second pass, the matrix is transposed and the rows represent the attribute values. This provides a dendrogram clustering the attributes. Both the node-based and the attribute-base dendrograms can be viewed. As shown in disclosed examples, the first clustering allows one to cluster molecules in term of their similarity and dissimilarity. The second clustering can serve different purposes, depending on the types of label-free integrative pharmacology analysis.

[0038] The similarity analysis typically leads to dendrogram which consists of interconnected or independent clusters of molecules, each cluster of molecules share similar mode(s) of action (i.e., pharmacology). The clusters can also be viewed as heat map. Similarity analysis for gene expression analysis and protein network analysis has resulted in three types of heat map display, including HeatMapView (unclustered), Eisen TreeView, and Eisen KnnView. These heat map display approaches can be directly used to view the clusters and relations of molecules in terms of their label-free integrative pharmacology. Gene expression analysis often shows the results of hierarchically clustering the nodes (i.e. genes) and a number of node attributes (typically expression data under different experimental conditions). Clustering based on label-free integrative pharmacology also displays the results of hierarchically clustering the nodes (i.e., the molecules) and a number of node attributes. The node attributes used are dependent on the types of analysis. For on-target pharmacology classification, the node attributes can be the absolute responses at a number of time points of a biosensor signal induced by the molecule under different assay conditions. The node attributes can also be the modulation percentages of the molecule against each marker in the marker panel. The modulation percentage is often calculated by normalizing the marker biosensor response in the presence of a molecule to the marker biosensor response in the absence of the molecule. Such normalization is often based on signal amplitudes of a particular biosensor event (e.g., P-DMR, N-DMR or RP-DMR) but not the kinetics of the respective event, since it is the signal amplitude, but not the kinetics, that is associated with molecule efficacy (when the molecule is an agonist or activator for a pathway or a cellular process) or potency (when the molecule is an antagonist or inhibitor for a pathway or a cellular process).

[0039] Among the heat map display approaches developed to date, the Eisen TreeView is the most common approach. Here Hierarchical clustering results are usually displayed with a color-coded "Heat Map" of the data values and the dendrogram from clustering. Alternatively, when k-means clustering is used, the results can be shown with the Eisen KnnView.

1. Specific embodiments

[0040] Disclosed herein is a method of assessment of opioid receptors comprising, obtaining data on an opioid receptor parameter for a test molecule, wherein the parameters included endogenous cell activity, opioid agonism activity, antagonism reversal activity, and

preconditioned activity, and wherein obtaining the endogenous cell activity parameter comprises analyzing the agonism response of the test molecule in a parental cell line having no opioid receptor, wherein obtaining the opioid agonism activity parameter comprises analyzing the agonism response of the test molecule in an engineered cell line stably expressing the opioid receptor (e.g., HEK-MOR), wherein obtaining the antagonism reversal activity parameter comprises analyzing the ability of the test molecule to cause the engineered cell of responding to successive stimulation with a known receptor antagonist, wherein the antagonism reversal activity cause successive stimulation determines the sustainability of the test molecule-induced cellular response and the ability of the antagonist to reverse its signal, and wherein obtaining the preconditioned activity parameter comprises analyzing the agonism activity of the test molecule in the engineered cells, wherein the engineered cells have been preconditioned via pretreatment with a preconditioning molecule. Preferably the engineered cell line is originated from the same parental cell line, although the engineered cell line from different cellular background can be used. Alternatively, a native cell line that expresses an opioid receptor can be used, wherein an engineered cell line can be made from this native cell line by conventional gene deletion or RNA interfering methods to delete or suppress the expression of the opioid receptor.

[0041] In some forms of the methods analyzing can comprise using a marker. The marker can be any kind of marker. In some forms, the marker can be an antagonist or an agonist.

[0042] In some forms of the methods, the parental cell line can comprise a HEK-293 cell line. In some forms of the methods the HEK-293 cell line does not comprise an opioid receptor. In some forms, the parental cell line comprises Chinese ovary hamster (CHO-K1), Cos-7, or HeLa cell lines.

[0043] In some forms of the methods, the opioid receptor can comprise a mu opioid receptor, a delta opioid receptor, a kappa opioid receptor, or opioid-like receptor 1. In some forms of the methods the opioid receptor can comprise subtype1 receptor. In some forms of the methods, the opioid receptor can comprise a mu opioid receptor. In some forms of the methods, the opioid receptor can comprise a delta opioid receptor. In some forms of the methods, the opioid receptor can comprise a kappa opioid receptor. In some forms of the method the opioid receptors can be stably expressed.

[0044] In some forms of the methods the preconditioning molecule can comprises the known receptor antagonist, the known receptor agonist, a Gi protein killer, a Gs protein killer, a adenylyl cyclase activator, a MEK1/2 inhibitor, a p38 MAPK inhibitor, a JNK inhibitor, or a PI3K inhibitor. In some forms of the methods the preconditioning molecule antagonizes or agonize the target. In some forms of the methods the target is an opioid receptor. In some forms of the methods the known receptor agonist can comprise DAMGO. In some forms of

the methods the Gi protein killer can comprise pertussis toxin (PTX). In some forms of the methods the Gs protein killer can comprise cholera toxin (CTX). In some forms of the methods adenylyl cyclase activator can comprise forskolin. In some forms of the method the MEK1/2 inhibitor can comprise U0126. In some forms of the methods the p38 MAPK inhibitor can comprise SB210290. In some forms of the methods, the JNK inhibitor can comprise SP100625. In some forms of the methods, PI3K inhibitor can comprise LY294002.

[0045] In some forms of the methods, the methods can further comprise the step of prefiltering the test molecule. In some forms prefiltering the test molecule can comprise assaying the molecule against a cell line. In some forms the cell line has no opioid receptors. In some forms a biosensor response disqualifies the test molecule from further assays. In some forms of the method the test molecule has been prefiltered.

[0046] In some forms of the methods the molecule does not show agonism activity in the parental cell line. In some forms the test molecule has an EC₅₀, K_i, or K_d less than 0.01, 0.1, 1, 5, 10, or 50 micromolar.

[0047] In some forms of the methods the concentration of the test molecule and preconditioning molecule can be about 10 micromolar or greater than its corresponding K_d value. In some forms of the methods the preconditioning molecule can be 10 micromolar.

[0048] In some forms of the methods each step of analyzing can be performed using label free methods. In some forms the label free methods comprise label free biosensor methods. In some forms the label free methods can comprise a Resonant Waveguide Grating biosensor. In some form the methods can further comprise the step of analyzing the test molecule induced DMR signals. In some forms the test molecule can be a known molecule. In some forms the test molecules can be an unknown molecule. In some forms the DMR signals can be analyzed using a three time domain matrix. In some forms the three time domain matrix uses three specific periods post stimulation. In some forms the three domain matrix can use 3 minutes, 9 minutes, and 30 minutes post stimulation. In some forms the three domain matrix can be selected from three time periods, 1-5min, 5-15min, 15-60min post stimulation. Examples are (2min, 10min, 45min), or (5min, 15min, 60min).

[0049] In some forms the methods can further comprise the step of performing a similarity analysis on the data for at least one parameter. In some forms the similarity analysis on the data is performed for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 parameters. In some forms the similarity analysis can comprise a clustering analysis. In some forms the clustering can comprise performing Hierarchical, K-means, FORCE, or MCL clustering.

[0050] In some forms the methods can further comprise the step of pretreating the data. In some forms the pretreating can comprise normalization or filtering. In some forms the filtering can comprise performing a max-min difference analysis. In some forms the clustering analysis comprises a one-dimensional analysis. In some forms the clustering analysis can comprise a two-dimensional analysis. In some forms the clustering analysis can comprise utilizing a heat map. In some forms the display of the heat map can comprise a HeatMapView (unclustered), Eisen TreeView, or Eisen KnnView. In some forms the clustering can comprise nodes. In some forms the nodes can comprise a node attribute.

[0051] In some forms of the methods the node attributes can comprise the absolute responses at a number of time points of a biosensor signal induced by the test molecule under different assay conditions, the modulation percentages of the molecule against each marker in the marker panel.

[0052] Also disclosed are methods, wherein the cluster analysis comprises performing a Hierarchical clustering method, wherein the Hierarchical clustering method comprises an agglomerative method, wherein the Hierarchical clustering method comprises a divisive method, comprising a measure of dissimilarity between sets of observations, wherein the measure of dissimilarity comprises a distance metric and a linkage criteria, or alone or in any combination with any step, machine, or article herein.

[0053] Disclosed are methods, wherein the distance metric comprise a Euclidean distance method, squared Euclidean distance method, City-block distance method, Manhattan distance method, Pearson correlation method, Pearson correlation absolute value method, Uncentered correlation method, Centered correlation method, Spearman's rank correlation method, Kendall's tau method, maximum distance method, Mahalanobis distance method, or a cosine similarity method.

[0054] Also disclosed are methods, wherein when the data set comprises data from a primary indice the distance metric comprises the uncentered correlation with absolute value, wherein when the data set comprises data from a molecule modulation indice the distance metric comprises either the uncentered correlation with absolute value method or the centered correlation with absolute value method, wherein the distance metric comprises a Euclidean distance method, wherein the linkage criteria comprises a pairwise average-linkage, a pairwise single-linkage, a pairwise maximum-linkage, or a pairwise centroid-linkage, wherein the linkage criteria comprises a pairwise maximum-linkage, comprising a distance

matrix, wherein the distance matrix is made up of distances between two rows in the matrix, wherein the rows represent nodes in the distance matrix, or alone or in any combination with any step, machine, or article herein.

[0055] Disclosed are methods, further comprising a predefined clustering threshold, such as density parameter or similarity threshold, or alone or in any combination with any step, machine, or article herein.

[0056] Disclosed are methods, wherein the predefined clustering threshold is a biosensor parameter, or alone or in any combination with any step, machine, or article herein.

[0057] Also disclosed are methods, wherein performing the clustering analysis produces a similarity matrix, wherein the node comprises the molecule used in the biosensor assay, wherein the edge attribute comprises the a parameter of the cell response to the molecule or a parameter of a modulation indice (i.e. modulation percentage of the molecule against a marker), wherein an edge attribute is selected, wherein multiple node attributes are selected, wherein only a subset of the nodes are selected, or alone or in any combination with any step, machine, or article herein.

[0058] Disclosed are methods, further comprising a normalization or data pretreatment step, or alone or in any combination with any step, machine, or article herein.

[0059] Also disclosed are methods, wherein the data pretreatment step comprises data filtering, wherein when the data set comprises data from a primary indice and the data filtering comprises a max-min difference computation, wherein the max-min difference computation selects data points that have at least a 40 picometer max-min difference within one hour post stimulation, wherein when the data set comprises data from a modulation indice and the data filtering step comprises removing molecules whose biosensor modulation indice contain less than or equal to 15% modulation against all the markers or a specific set of markers, wherein the clustering analysis comprises a two-dimensional clustering analysis, wherein the clustering algorithm is first run with the nodes of the matrix producing a hierarchical clustering of the nodes given the values of the attributes and then with the attributes of the matrix, producing a hierarchical clustering of the attributes for a given node, wherein the clustering algorithm is first run with the attributes of the matrix and then with the nodes of the matrix, or alone or in any combination with any step, machine, or article herein.

[0060] Disclosed are methods, further comprising the step of producing a heat map, or alone or in any combination with any step, machine, or article herein.

[0061] Also disclosed are methods, wherein the heat map comprises an unclustered map, wherein the unclustered map comprises a HeatMapView, wherein the heat map comprises an Eisen TreeView or an Eisen KnnView, wherein the edge attribute comprises an absolute response of a biosensor response, predetermined kinetic parameter, or modulation percentage, wherein the method is a computer implemented method, further comprising the step of outputting results from the cluster analysis, or alone or in any combination with any step, machine, or article herein.

[0062] Disclosed are methods of analyzing a label free biosensor data set comprising; receiving a label free biosensor data set record and performing a cluster analysis, wherein the record contains biosensor data measuring a biosensor response and outputting results from the cluster analysis, or alone or in any combination with any step, machine, or article herein.

[0063] Also disclosed are methods, wherein the method is a computer implemented method, wherein receiving the label free biosensor data set record comprises receiving the label free biosensor data set record from a storage medium, wherein receiving the label free biosensor data set record comprises receiving the record from a computer system, wherein receiving the label free biosensor data set record comprises receiving the record from a biosensor system, wherein receiving the label free biosensor data set record comprises receiving the label free biosensor data set record via a computer network, or alone or in any combination with any step, machine, or article herein.

[0064] Disclosed are one or more computer readable media storing program code that, upon execution by one or more computer systems, causes the computer systems to perform the any of the methods herein, or alone or in any combination with any step, machine, or article herein.

[0065] Disclosed are computer program products comprising a computer usable memory adapted to be executed to implement any of the methods herein, or alone or in any combination with any step, machine, or article herein.

[0066] Also disclosed are computer programs, comprising a logic processing module, a configuration file processing module, a data organization module, and data display organization module, that are embodied upon a computer readable medium, or alone or in any combination with any step, machine, or article herein.

[0067] Disclosed are computer program products, comprising a computer usable medium having a computer readable program code embodied therein, said computer readable program

code adapted to be executed to implement a method for generating the cluster analysis of any method disclosed herein, said method further comprising: providing a system, wherein the system comprises distinct software modules, and wherein the distinct software modules comprise a logic processing module, a configuration file processing module, a data organization module, and a data display organization module, or alone or in any combination with any step, machine, or article herein.

[0068] Also disclosed are methods, further comprising a computerized system configured for performing the method, further comprising the outputting of the results from the cluster analysis, or alone or in any combination with any step, machine, or article herein.

[0069] Disclosed are computer-readable media having stored thereon instructions that, when executed on a programmed processor perform any of the methods, or alone or in any combination with any step, machine, or article herein.

[0070] Disclosed are cluster analysis systems, the systems comprising: a data store capable of storing label free biosensor data set; a system processor comprising one or more processing elements, the one or more processing elements programmed or adapted to: receive the label free biosensor data set; store the label free biosensor data set in the data store; perform a cluster analysis on the label free biosensor data set; and output a result from the cluster analysis, or alone or in any combination with any step, machine, or article herein.

[0071] Also disclosed are systems, wherein the system receives the label free biosensor data from a biosensor system, wherein the system receives the label free biosensor data via a computer network, further comprising a biosensor system, or alone or in any combination with any step, machine, or article herein.

C. Biosensors and Biosensor Cellular Assays

[0072] Label-free cell-based assays generally employ a biosensor to monitor molecule-induced responses in living cells. The molecule can be naturally occurring or synthetic, and can be a purified or unpurified mixture. A biosensor typically utilizes a transducer such as an optical, electrical, calorimetric, acoustic, magnetic, or like transducer, to convert a molecular recognition event or a molecule-induced change in cells contacted with the biosensor into a quantifiable signal. These label-free biosensors can be used for molecular interaction analysis, which involves characterizing how molecular complexes form and disassociate over time, or for cellular response, which involves characterizing how cells respond to stimulation. The biosensors that are applicable to the present methods can include, for example, optical

biosensor systems such as surface plasmon resonance (SPR) and resonant waveguide grating (RWG) biosensors, resonant mirrors, ellipsometers, and electric biosensor systems such as bioimpedance systems.

1. Acoustic biosensors

[0073] Acoustic biosensors such as quartz crystal resonators utilize acoustic waves to characterize cellular responses. The acoustic waves are generally generated and received using piezoelectric. An acoustic biosensor is often designed to operate in a resonant type sensor configuration. In a typical setup, thin quartz discs are sandwiched between two gold electrodes. Application of an AC signal across electrodes leads to the excitation and oscillation of the crystal, which acts as a sensitive oscillator circuit. The output sensor signals are the resonance frequency and motional resistance. The resonance frequency is largely a linear function of total mass of adsorbed materials when the biosensor surface is rigid. Under liquid environments the acoustic sensor response is sensitive not only to the mass of bound molecules, but also to changes in viscoelastic properties and charge of the molecular complexes formed or live cells. By measuring the resonance frequency and the motion resistance of cells associated with the crystals, cellular processes including cell adhesion and cytotoxicity can be studied in real time.

2. Electrical biosensors

[0074] Electrical biosensors employ impedance to characterize cellular responses including cell adhesion. In a typical setup, live cells are brought in contact with a biosensor surface wherein an integrated electrode array is embedded. A small AC pulse at a constant voltage and high frequency is used to generate an electric field between the electrodes, which are impeded by the presence of cells. The electric pulses are generated onsite using the integrated electric circuit; and the electrical current through the circuit is followed with time. The resultant impedance is a measure of changes in the electrical conductivity of the cell layer. The cellular plasma membrane acts as an insulating agent forcing the current to flow between or beneath the cells, leading to quite robust changes in impedance. Impedance-based measurements have been applied to study a wide range of cellular events, including cell adhesion and spreading, cell micromotion, cell morphological changes, and cell death, and cell signaling.

3. Optical biosensors

[0075] Optical biosensors primarily employ a surface-bound electromagnetic wave to characterize cellular responses. The surface-bound waves can be achieved either on gold substrates using either light excited surface plasmons (surface plasmon resonance, SPR) or on dielectric substrate using diffraction grating coupled waveguide mode resonances (resonance waveguide grating, RWG). For SPR including mid-IR SPR, the readout is the resonance angle at which a minimal in intensity of reflected light occurs. Similarly, for RWG biosensor including photonic crystal biosensors, the readout is the resonance angle or wavelength at which a maximum incoupling efficiency is achieved. The resonance angle or wavelength is a function of the local refractive index at or near the sensor surface. Unlike SPR which is limited to a few of flow channels for assaying, RWG biosensors are amenable for high throughput screening (HTS) and cellular assays, due to recent advancements in instrumentation and assays. In a typical RWG, the cells are directly placed into a well of a microtiter plate in which a biosensor consisting of a material with high refractive index is embedded. Local changes in the refractive index lead to a dynamic mass redistribution (DMR) signal of live cells upon stimulation. These biosensors have been used to study diverse cellular processes including receptor biology, ligand pharmacology, and cell adhesion.

[0076] The present invention preferably uses resonant waveguide grating biosensors, such as Corning Epic® systems. Epic® system includes the commercially available wavelength integration system, or angular interrogation system or swept wavelength imaging system (Corning Inc., Corning, NY). The commercial system consists of a temperature-control unit, an optical detection unit, with an on-board liquid handling unit with robotics, or an external liquid accessory system with robotics. The detection unit is centered on integrated fiber optics, and enables kinetic measures of cellular responses with a time interval of ~7 or 15sec. The compound solutions were introduced by using either the on-board liquid handling unit, or the external liquid accessory system; both of which use conventional liquid handling systems. Different RWG biosensor systems including high resolution imaging systems as well as high acquisition optical biosensor systems can also be used.

4. SPR Biosensors and Systems

[0077] SPR relies on a prism to direct a wedge of polarized light, covering a range of incident angles, into a planar glass substrate bearing an electrically conducting metallic film

(e.g., gold) to excite surface plasmons. The resultant evanescent wave interacts with, and is absorbed by, free electron clouds in the gold layer, generating electron charge density waves (i.e., surface plasmons) and causing a reduction in the intensity of the reflected light. The resonance angle at which this intensity minimum occurs is a function of the refractive index of the solution close to the gold layer on the opposing face of the sensor surface

5. RWG Biosensors and Systems

[0078] An RWG biosensor can include, for example, a substrate (e.g., glass), a waveguide thin film with an embedded grating or periodic structure, and a cell layer. The RWG biosensor utilizes the resonant coupling of light into a waveguide by means of a diffraction grating, leading to total internal reflection at the solution-surface interface, which in turn creates an electromagnetic field at the interface. This electromagnetic field is evanescent in nature, meaning that it decays exponentially from the sensor surface; the distance at which it decays to $1/e$ of its initial value is known as the penetration depth and is a function of the design of a particular RWG biosensor, but is typically on the order of about 200 nm. This type of biosensor exploits such evanescent wave to characterize ligand-induced alterations of a cell layer at or near the sensor surface.

[0079] RWG instruments can be subdivided into systems based on angle-shift or wavelength-shift measurements. In a wavelength-shift measurement, polarized light covering a range of incident wavelengths with a constant angle is used to illuminate the waveguide; light at specific wavelengths is coupled into and propagates along the waveguide. Alternatively, in angle-shift instruments, the sensor is illuminated with monochromatic light and the angle at which the light is resonantly coupled is measured.

[0080] The resonance conditions are influenced by the cell layer (e.g., cell confluency, adhesion and status), which is in direct contact with the surface of the biosensor. When a ligand or an analyte interacts with a cellular target (e.g., a GPCR, an ion channel, a kinase) in living cells, any change in local refractive index within the cell layer can be detected as a shift in resonant angle (or wavelength).

[0081] The Corning® Epic® system uses RWG biosensors for label-free biochemical or cell-based assays (Corning Inc., Corning, NY). The Epic® System consists of an RWG plate reader and SBS (Society for Biomolecular Screening) standard microtiter plates. The detector system in the plate reader exploits integrated fiber optics to measure the shift in wavelength of the incident light, as a result of ligand-induced changes in the cells. A series

of illumination-detection heads are arranged in a linear fashion, so that reflection spectra are collected simultaneously from each well within a column of a 384-well microplate. The whole plate is scanned so that each sensor can be addressed multiple times, and each column is addressed in sequence. The wavelengths of the incident light are collected and used for analysis. A temperature-controlling unit can be included in the instrument to minimize spurious shifts in the incident wavelength due to the temperature fluctuations. The measured response represents an averaged response of a population of cells. Varying features of the systems can be automated, such as sample loading, and can be multiplexed, such as with a 96 or 386 well microtiter plate. Liquid handling is carried out by either on-board liquid handler, or an external liquid handling accessory. Specifically, molecule solutions are directly added or pipetted into the wells of a cell assay plate having cells cultured in the bottom of each well. The cell assay plate contains certain volume of assay buffer solution covering the cells. A simple mixing step by pipetting up and down certain times can also be incorporated into the molecule addition step.

6. Electrical Biosensors and Systems

[0082] Electrical biosensors consist of a substrate (e.g., plastic), an electrode, and a cell layer. In this electrical detection method, cells are cultured on small gold electrodes arrayed onto a substrate, and the system's electrical impedance is followed with time. The impedance is a measure of changes in the electrical conductivity of the cell layer. Typically, a small constant voltage at a fixed frequency or varied frequencies is applied to the electrode or electrode array, and the electrical current through the circuit is monitored over time. The ligand-induced change in electrical current provides a measure of cell response. Impedance measurement for whole cell sensing was first realized in 1984. Since then, impedance-based measurements have been applied to study a wide range of cellular events, including cell adhesion and spreading, cell micromotion, cell morphological changes, and cell death. Classical impedance systems suffer from high assay variability due to use of a small detection electrode and a large reference electrode. To overcome this variability, the latest generation of systems, such as the CellKey system (MDS Sciex, South San Francisco, CA) and RT-CES (ACEA Biosciences Inc., San Diego, CA), utilize an integrated circuit having a microelectrode array.

7. High Spatial Resolution Biosensor Imaging Systems

[0083] Optical biosensor imaging systems, including SPR imaging systems, ellipsometry imaging systems, and RWG imaging systems, offer high spatial resolution, and can be used in embodiments of the disclosure. For example, SPR imager®II (GWC Technologies Inc) uses prism-coupled SPR, and takes SPR measurements at a fixed angle of incidence, and collects the reflected light with a CCD camera. Changes on the surface are recorded as reflectivity changes. Thus, SPR imaging collects measurements for all elements of an array simultaneously.

[0084] A swept wavelength optical interrogation system based on RWG biosensor for imaging-based application can be employed. In this system, a fast tunable laser source is used to illuminate a sensor or an array of RWG biosensors in a microplate format. The sensor spectrum can be constructed by detecting the optical power reflected from the sensor as a function of time as the laser wavelength scans, and analysis of the measured data with computerized resonant wavelength interrogation modeling results in the construction of spatially resolved images of biosensors having immobilized receptors or a cell layer. The use of an image sensor naturally leads to an imaging based interrogation scheme. 2 dimensional label-free images can be obtained without moving parts.

[0085] Alternatively, angular interrogation system with transverse magnetic or *p*-polarized TM_0 mode can also be used. This system consists of a launch system for generating an array of light beams such that each illuminates a RWG sensor with a dimension of approximately $200\ \mu\text{m} \times 3000\ \mu\text{m}$ or $200\ \mu\text{m} \times 2000\ \mu\text{m}$, and a CCD camera-based receive system for recording changes in the angles of the light beams reflected from these sensors. The arrayed light beams are obtained by means of a beam splitter in combination with diffractive optical lenses. This system allows up to 49 sensors (in a 7x7 well sensor array) to be simultaneously sampled at every 3 seconds, or up to the whole 384well microplate to be simultaneously sampled at every 10 seconds.

[0086] Alternatively, a scanning wavelength interrogation system can also be used. In this system, a polarized light covering a range of incident wavelengths with a constant angle is used to illuminate and scan across a waveguide grating biosensor, and the reflected light at each location can be recorded simultaneously. Through scanning, a high resolution image across a biosensor can also be achieved

8. Dynamic Mass Redistribution (DMR) Signals in Living Cells

[0087] The cellular response to stimulation through a cellular target can be encoded by the spatial and temporal dynamics of downstream signaling networks. For this reason, monitoring the integration of cell signaling in real time can provide physiologically relevant information that is useful in understanding cell biology and physiology.

[0088] Optical biosensors including resonant waveguide grating (RWG) biosensors, can detect an integrated cellular response related to dynamic redistribution of cellular matters, thus providing a non-invasive means for studying cell signaling. All optical biosensors are common in that they can measure changes in local refractive index at or very near the sensor surface. In principle, almost all optical biosensors are applicable for cell sensing, as they can employ an evanescent wave to characterize ligand-induced change in cells. The evanescent-wave is an electromagnetic field, created by the total internal reflection of light at a solution-surface interface, which typically extends a short distance (~hundreds of nanometers) into the solution at a characteristic depth known as the penetration depth or sensing volume.

[0089] Recently, theoretical and mathematical models have been developed that describe the parameters and nature of optical signals measured in living cells in response to stimulation with ligands. These models, based on a 3-layer waveguide system in combination with known cellular biophysics, link the ligand-induced optical signals to specific cellular processes mediated through a receptor.

[0090] Because biosensors measure the average response of the cells located at the area illuminated by the incident light, a highly confluent layer of cells can be used to achieve optimal assay results. Due to the large dimension of the cells as compared to the short penetration depth of a biosensor, the sensor configuration is considered as a non-conventional three-layer system: a substrate, a waveguide film with a grating structure, and a cell layer. Thus, a ligand-induced change in effective refractive index (i.e., the detected signal) can be, to first order, directly proportional to the change in refractive index of the bottom portion of the cell layer:

$$\Delta N = S(C)\Delta n_c$$

[0091] where $S(C)$ is the sensitivity to the cell layer, and Δn_c the ligand-induced change in local refractive index of the cell layer sensed by the biosensor. Because the refractive index of a given volume within a cell is largely determined by the concentrations of bio-

molecules such as proteins, Δn_c can be assumed to be directly proportional to ligand-induced change in local concentrations of cellular targets or molecular assemblies within the sensing volume. Considering the exponentially decaying nature of the evanescent wave extending away from the sensor surface, the ligand-induced optical signal is governed by:

$$\Delta N = S(C)\alpha d \sum_i \Delta C_i \left[e^{\frac{-z_i}{\Delta Z_c}} - e^{\frac{-z_{i+1}}{\Delta Z_c}} \right]$$

[0092] where ΔZ_c is the penetration depth into the cell layer, α the specific refraction increment (about 0.18/mL/g for proteins), z_i the distance where the mass redistribution occurs, and d an imaginary thickness of a slice within the cell layer. Here the cell layer is divided into an equal-spaced slice in the vertical direction. The equation above indicates that the ligand-induced optical signal is a sum of mass redistribution occurring at distinct distances away from the sensor surface, each with an unequal contribution to the overall response. Furthermore, the detected signal, in terms of wavelength or angular shifts, is primarily sensitive to mass redistribution occurring perpendicular to the sensor surface. Because of its dynamic nature, it also is referred to as dynamic mass redistribution (DMR) signal.

9. Cells and biosensors

[0093] Cells rely on multiple cellular pathways or machineries to process, encode and integrate the information they receive. Unlike the affinity analysis with optical biosensors that specifically measures the binding of analytes to a protein target, living cells are much more complex and dynamic.

[0094] To study cell signaling, cells can be brought in contact with the surface of a biosensor, which can be achieved through cell culture. These cultured cells can be attached onto the biosensor surface through three types of contacts: focal contacts, close contacts and extracellular matrix contacts, each with its own characteristic separation distance from the surface. As a result, the basal cell membranes are generally located away from the surface by ~10-100nm. For suspension cells, the cells can be brought in contact with the biosensor surface through either covalent coupling of cell surface receptors, or specific binding of cell surface receptors, or simply settlement by gravity force. For this reason, biosensors are able to sense the bottom portion of cells.

[0095] Cells, in many cases, exhibit surface-dependent adhesion and proliferation. In order to achieve robust cell assays, the biosensor surface can require a coating to enhance cell

adhesion and proliferation. However, the surface properties can have a direct impact on cell biology. For example, surface-bound ligands can influence the response of cells, as can the mechanical compliance of a substrate material, which dictates how it will deform under forces applied by the cell. Due to differing culture conditions (time, serum concentration, confluency, *etc.*), the cellular status obtained can be distinct from one surface to another, and from one condition to another. Thus, special efforts to control cellular status can be necessary in order to develop biosensor-based cell assays.

[0096] Cells are dynamic objects with relatively large dimensions – typically in the range of tens of microns. Even without stimulation, cells constantly undergo micromotion – a dynamic movement and remodeling of cellular structure, as observed in tissue culture by time lapse microscopy at the sub-cellular resolution, as well as by bio-impedance measurements at the nanometer level.

[0097] Under un-stimulated conditions cells generally produce an almost net-zero DMR response as examined with a RWG biosensor. This is partly because of the low spatial resolution of optical biosensors, as determined by the large size of the laser spot and the long propagation length of the coupled light. The size of the laser spot determines the size of the area studied – and usually only one analysis point can be tracked at a time. Thus, the biosensor typically measures an averaged response of a large population of cells located at the light incident area. Although cells undergo micromotion at the single cell level, the large populations of cells give rise to an average net-zero DMR response. Furthermore, intracellular macromolecules are highly organized and spatially restricted to appropriate sites in mammalian cells. The tightly controlled localization of proteins on and within cells determines specific cell functions and responses because the localization allows cells to regulate the specificity and efficiency of proteins interacting with their proper partners and to spatially separate protein activation and deactivation mechanisms. Because of this control, under un-stimulated conditions, the local mass density of cells within the sensing volume can reach an equilibrium state, thus leading to a net-zero optical response. In order to achieve a consistent optical response, the cells examined can be cultured under conventional culture conditions for a period of time such that most of the cells have just completed a single cycle of division.

[0098] Living cells have exquisite abilities to sense and respond to exogenous signals. Cell signaling was previously thought to function via linear routes where an environmental

cue would trigger a linear chain of reactions resulting in a single well-defined response. However, research has shown that cellular responses to external stimuli are much more complicated. It has become apparent that the information that cells receive can be processed and encoded into complex temporal and spatial patterns of phosphorylation and topological relocation of signaling proteins. The spatial and temporal targeting of proteins to appropriate sites can be crucial to regulating the specificity and efficiency of protein-protein interactions, thus dictating the timing and intensity of cell signaling and responses. Pivotal cellular decisions, such as cytoskeletal reorganization, cell cycle checkpoints and apoptosis, depend on the precise temporal control and relative spatial distribution of activated signal-transducers. Thus, cell signaling mediated through a cellular target such as G protein-coupled receptor (GPCR) typically proceeds in an orderly and regulated manner, and consists of a series of spatial and temporal events, many of which lead to changes in local mass density or redistribution in local cellular matters of cells. These changes or redistribution, when occurring within the sensing volume, can be followed directly in real time using optical biosensors

10. DMR Signal is a Physiological Response of Living Cells

[0099] Through comparison with conventional pharmacological approaches for studying receptor biology, it has been shown that when a ligand is specific to a receptor expressed in a cell system, the ligand-induced DMR signal is receptor-specific, dose-dependent and saturate-able. For a great number of G protein-coupled receptor (GPCR) ligands, the efficacies (measured by EC_{50} values) are found to be almost identical to those measured using conventional methods. In addition, the DMR signals exhibit expected desensitization patterns, as desensitization and re-sensitization is common to all GPCRs. Furthermore, the DMR signal also maintains the fidelity of GPCR ligands, similar to those obtained using conventional technologies. In addition, the biosensor can distinguish full agonists, partial agonists, inverse agonists, antagonists, and allosteric modulators. Taken together, these findings indicate that the DMR is capable of monitoring physiological responses of living cells.

11. Biosensor parameters

[00100] A label-free biosensor such as RWG biosensor or bioimpedance biosensor is able to follow in real time ligand-induced cellular response. The non-invasive and manipulation-free biosensor cellular assays do not require prior knowledge of cell signaling. The resultant

biosensor signal contains high information relating to receptor signaling and ligand pharmacology. Multi-parameters can be extracted from the kinetic biosensor response of cells upon stimulation. These parameters include, but not limited to, the overall dynamics, phases, signal amplitudes, as well as kinetic parameters including the transition time from one phase to another, and the kinetics of each phase (see Fang, Y., and Ferrie, A.M. (2008) “label-free optical biosensor for ligand-directed functional selectivity acting on β 2 adrenoceptor in living cells”. FEBS Lett. 582, 558–564; Fang, Y., et al., (2005) “Characteristics of dynamic mass redistribution of EGF receptor signaling in living cells measured with label free optical biosensors”. Anal. Chem., 77, 5720-5725; Fang, Y., et al., (2006) “Resonant waveguide grating biosensor for living cell sensing”. Biophys. J., 91, 1925-1940).

D. Definitions

[00101] Various embodiments of the disclosure will be described in detail with reference to drawings, if any. Reference to various embodiments does not limit the scope of the disclosure, which is limited only by the scope of the claims attached hereto. Additionally, any examples set forth in this specification are not intended to be limiting and merely set forth some of the many possible embodiments for the claimed invention.

1. A

[00102] As used in the specification and the appended claims, the singular forms "a," "an" and "the" or like terms include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a pharmaceutical carrier” includes mixtures of two or more such carriers, and the like.

2. Abbreviations

[00103] Abbreviations, which are well known to one of ordinary skill in the art, may be used (e.g., “h” or “hr” for hour or hours, “g” or “gm” for gram(s), “mL” for milliliters, and “rt” for room temperature, “nm” for nanometers, “M” for molar, and like abbreviations).

3. About

[00104] About modifying, for example, the quantity of an ingredient in a composition, concentrations, volumes, process temperature, process time, yields, flow rates, pressures, and like values, and ranges thereof, employed in describing the embodiments of the disclosure, refers to variation in the numerical quantity that can occur, for example, through typical measuring and handling procedures used for making compounds, compositions, concentrates

or use formulations; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of starting materials or ingredients used to carry out the methods; and like considerations. The term “about” also encompasses amounts that differ due to aging of a composition or formulation with a particular initial concentration or mixture, and amounts that differ due to mixing or processing a composition or formulation with a particular initial concentration or mixture. Whether modified by the term “about” the claims appended hereto include equivalents to these quantities.

4. Adenylyl cyclase activator

[00105] Adenylyl cyclase activator or the like terms refer to a molecule or ligand that can determine the Gi-dependent component of the ligand mediated biased agonism. An adenylyl cyclase activator can, for example, be forskolin.

5. Analyzing

[00106] Analyzing or the like terms refer to evaluating an event. For example, analyzing can be done by performing or using biosensors or label free methods, such as label free biosensor methods.

6. Antagonism reversal activity

[00107] An antagonism reversal activity or the like terms refer to a molecular event that reverses the direction of an agonist-induced cellular response at a specific time after the cells are first stimulated with the agonist.

7. Assaying

[00108] Assaying, assay, or like terms refers to an analysis to determine a characteristic of a substance, such as a molecule or a cell, such as for example, the presence, absence, quantity, extent, kinetics, dynamics, or type of an a cell's optical or bioimpedance response upon stimulation with one or more exogenous stimuli, such as a ligand or marker. Producing a biosensor signal of a cell's response to a stimulus can be an assay.

8. Assaying the response

[00109] “Assaying the response” or like terms means using a means to characterize the response. For example, if a molecule is brought into contact with a cell, a biosensor can be used to assay the response of the cell upon exposure to the molecule.

9. Agonism and antagonism mode

[00110] The agonism mode or like terms is the assay wherein the cells are exposed to a molecule to determine the ability of the molecule to trigger biosensor signals such as DMR

signals, while the antagonism mode is the assay wherein the cells are exposed to a marker in the presence of a molecule to determine the ability of the molecule to modulate the biosensor signal of cells responding to the marker.

10. Biosensor

[00111] Biosensor or like terms refer to a device for the detection of an analyte that combines a biological component with a physicochemical detector component. The biosensor typically consists of three parts: a biological component or element (such as tissue, microorganism, pathogen, cells, or combinations thereof), a detector element (works in a physicochemical way such as optical, piezoelectric, electrochemical, thermometric, or magnetic), and a transducer associated with both components. The biological component or element can be, for example, a living cell, a pathogen, or combinations thereof. In embodiments, an optical biosensor can comprise an optical transducer for converting a molecular recognition or molecular stimulation event in a living cell, a pathogen, or combinations thereof into a quantifiable signal.

11. Biosensor Response

[00112] A “biosensor response”, “biosensor output signal”, “biosensor signal” or like terms is any reaction of a sensor system having a cell to a cellular response. A biosensor converts a cellular response to a quantifiable sensor response. A biosensor response is an optical response upon stimulation as measured by an optical biosensor such as RWG or SPR or it is a bioimpedance response of the cells upon stimulation as measured by an electric biosensor. Since a biosensor response is directly associated with the cellular response upon stimulation, the biosensor response and the cellular response can be used interchangeably, in embodiments of disclosure.

12. Biosensor Signal

[00113] A “biosensor signal” or like terms refers to the signal of cells measured with a biosensor that is produced by the response of a cell upon stimulation.

13. Cell

[00114] Cell or like term refers to a small usually microscopic mass of protoplasm bounded externally by a semipermeable membrane, optionally including one or more nuclei and various other organelles, capable alone or interacting with other like masses of performing all the fundamental functions of life, and forming the smallest structural unit of

living matter capable of functioning independently including synthetic cell constructs, cell model systems, and like artificial cellular systems.

[00115] A cell can include different cell types, such as a cell associated with a specific disease, a type of cell from a specific origin, a type of cell associated with a specific target, or a type of cell associated with a specific physiological function. A cell can also be a native cell, an engineered cell, a transformed cell, an immortalized cell, a primary cell, an embryonic stem cell, an adult stem cell, a cancer stem cell, or a stem cell derived cell.

[00116] Human consists of about 210 known distinct cell types. The numbers of types of cells can almost unlimited, considering how the cells are prepared (e.g., engineered, transformed, immortalized, or freshly isolated from a human body) and where the cells are obtained (e.g., human bodies of different ages or different disease stages, etc).

14. Cell culture

[00117] “Cell culture” or “cell culturing” refers to the process by which either prokaryotic or eukaryotic cells are grown under controlled conditions. “Cell culture” not only refers to the culturing of cells derived from multicellular eukaryotes, especially animal cells, but also the culturing of complex tissues and organs.

15. Cellular Response

[00118] A “cellular response” or like terms is any reaction by the cell to a stimulation.

16. Cellular process

[00119] A cellular process or like terms is a process that takes place in or by a cell. Examples of cellular process include, but not limited to, proliferation, apoptosis, necrosis, differentiation, cell signal transduction, polarity change, migration, or transformation.

17. Cellular target

[00120] A “cellular target” or like terms is a biopolymer such as a protein or nucleic acid whose activity can be modified by an external stimulus. Cellular targets commonly are proteins such as enzymes, kinases, ion channels, and receptors.

18. Cluster

[00121] A cluster as used herein is a means of using variables to divide cases or test molecules into groups or sets which are related.

19. Characterizing

[00122] Characterizing or like terms refers to gathering information about any property of a substance, such as a ligand, molecule, marker, or cell, such as obtaining a profile for the ligand, molecule, marker, or cell.

20. Comprise

[00123] Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises," means "including but not limited to," and is not intended to exclude, for example, other additives, components, integers or steps.

21. Consisting essentially of

[00124] "Consisting essentially of" in embodiments refers, for example, to a surface composition, a method of making or using a surface composition, formulation, or composition on the surface of the biosensor, and articles, devices, or apparatus of the disclosure, and can include the components or steps listed in the claim, plus other components or steps that do not materially affect the basic and novel properties of the compositions, articles, apparatus, and methods of making and use of the disclosure, such as particular reactants, particular additives or ingredients, a particular agents, a particular cell or cell line, a particular surface modifier or condition, a particular ligand candidate, or like structure, material, or process variable selected. Items that may materially affect the basic properties of the components or steps of the disclosure or may impart undesirable characteristics to the present disclosure include, for example, decreased affinity of the cell for the biosensor surface, aberrant affinity of a stimulus for a cell surface receptor or for an intracellular receptor, anomalous or contrary cell activity in response to a ligand candidate or like stimulus, and like characteristics.

22. Components

[00125] Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these molecules may not be explicitly disclosed, each is specifically contemplated and described herein. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules

D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the subgroup of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

23. Contacting

[00126] Contacting or like terms means bringing into proximity such that a molecular interaction can take place, if a molecular interaction is possible between at least two things, such as molecules, cells, markers, at least a compound or composition, or at least two compositions, or any of these with an article(s) or with a machine. For example, contacting refers to bringing at least two compositions, molecules, articles, or things into contact, i.e. such that they are in proximity to mix or touch. For example, having a solution of composition A and cultured cell B and pouring solution of composition A over cultured cell B would be bringing solution of composition A in contact with cell culture B. Contacting a cell with a ligand would be bringing a ligand to the cell to ensure the cell have access to the ligand.

[00127] It is understood that anything disclosed herein can be brought into contact with anything else. For example, a cell can be brought into contact with a marker or a molecule, a biosensor, and so forth.

24. Compounds and compositions

[00128] Compounds and compositions have their standard meaning in the art. It is understood that wherever, a particular designation, such as a molecule, substance, marker, cell, or reagent compositions comprising, consisting of, and consisting essentially of these designations are disclosed. Thus, where the particular designation marker is used, it is understood that also disclosed would be compositions comprising that marker, consisting of that marker, or consisting essentially of that marker. Where appropriate wherever a particular designation is made, it is understood that the compound of that designation is also disclosed.

For example, if particular biological material, such as EGF, is disclosed EGF in its compound form is also disclosed.

25. Control

[00129] The terms control or "control levels" or "control cells" or like terms are defined as the standard by which a change is measured, for example, the controls are not subjected to the experiment, but are instead subjected to a defined set of parameters, or the controls are based on pre- or post-treatment levels. They can either be run in parallel with or before or after a test run, or they can be a pre-determined standard. For example, a control can refer to the results from an experiment in which the subjects or objects or reagents etc are treated as in a parallel experiment except for omission of the procedure or agent or variable etc under test and which is used as a standard of comparison in judging experimental effects. Thus, the control can be used to determine the effects related to the procedure or agent or variable etc. For example, if the effect of a test molecule on a cell was in question, one could a) simply record the characteristics of the cell in the presence of the molecule, b) perform a and then also record the effects of adding a control molecule with a known activity or lack of activity, or a control composition (e.g., the assay buffer solution (the vehicle)) and then compare effects of the test molecule to the control. In certain circumstances once a control is performed the control can be used as a standard, in which the control experiment does not have to be performed again and in other circumstances the control experiment should be run in parallel each time a comparison will be made.

26. Detect

[00130] Detect or like terms refer to an ability of the apparatus and methods of the disclosure to discover or sense a molecule- or a marker-induced cellular response and to distinguish the sensed responses for distinct molecules.

27. Direct action (of a test molecule)

[00131] A "direct action" or like terms is a result (of a test molecule) acting independently on a cell.

28. DMR signal

[00132] A "DMR signal" or like terms refers to the signal of cells measured with an optical biosensor that is produced by the response of a cell upon stimulation.

29. DMR response

[00133] A “DMR response” or like terms is a biosensor response using an optical biosensor. The DMR refers to dynamic mass redistribution or dynamic cellular matter redistribution. A P-DMR is a positive DMR response, a N-DMR is a negative DMR response, and a RP-DMR is a recovery P-DMR response.

30. Endogenous cell activity

[00134] Endogenous cell activity or the like terms refer to cellular activity that originates from within the cell, and is activity which has not been engineered into the cell through recombinant biotechnology.

31. Engineered parental cell line

[00135] An engineered parental cell line or the like terms refer to a parental cell line that has been manipulated through recombinant biotechnology to express a protein or gene not expressed in the non-engineered cognate cell. For example, a HEK-293 cell line stably expressing Mu-opioid receptors, Delta-opioid receptors or Kappa-opioid receptors can be an engineered parental cell line.

32. Efficacy

[00136] Efficacy or like terms is the capacity to produce a desired size of an effect under ideal or optimal conditions. It is these conditions that distinguish efficacy from the related concept of effectiveness, which relates to change under real-life conditions. Efficacy is the relationship between receptor occupancy and the ability to initiate a response at the molecular, cellular, tissue or system level.

33. Higher and inhibit and like words

[00137] The terms higher, increases, elevates, or elevation or like terms or variants of these terms, refer to increases above basal levels, e.g., as compared a control. The terms low, lower, reduces, decreases or reduction or like terms or variation of these terms, refer to decreases below basal levels, e.g., as compared to a control. For example, basal levels are normal in vivo levels prior to, or in the absence of, or addition of a molecule such as an agonist or antagonist to a cell. Inhibit or forms of inhibit or like terms refers to reducing or suppressing.

34. Hierarchical clustering

[00138] The Hierarchical clustering method is a method of cluster analysis which seeks to build a hierarchy of clusters based on linkages.

[00139] Hierarchical clustering is a method of cluster analysis which seeks to build a hierarchy of clusters. Hierarchical clustering does not require a preset number of clusters. Hierarchical clustering builds a “tree” in which each leaf represents an individual data item and each interior node, or branch point represents a cluster of data items. Strategies for hierarchical clustering generally fall into two types: agglomerative and divisive. Agglomerative clustering is a “bottom up” approach – each observation starts in its own cluster, and pairs of clusters are merged as one moves up the hierarchy. Divisive clustering is a “top down” approach – all observations start in one cluster, and splits are performed recursively as one moves down the hierarchy. In order to decide which clusters should be combined (for agglomerative), or where a cluster should be split (for divisive), a measure of dissimilarity between sets of observations is required. In most methods of hierarchical clustering, this is achieved by use of an appropriate distance metric (a measure of distance between pairs of observations), and a linkage criteria which specifies the dissimilarity of sets as a function of the pairwise distances of observations in the sets. The choice of an appropriate metric will influence the shape of the clusters, as some elements may be close to one another according to one distance and farther away according to another. Common distance metrics include Euclidean distance, squared Euclidean distance, Manhattan distance, maximum distance, Mahalanobis distance, and cosine similarity. The Euclidean distance is found to be the most preferred metric for label-free integrative pharmacology applications, and is used throughout in the disclosed experimental examples.

[00140] Hierarchical clustering builds a dendrogram (binary tree) such that more similar nodes are likely to connect more closely into the tree. Hierarchical clustering is useful for organizing the data to get a sense of the pairwise relationships between data values and between clusters. The dendrogram is generated by using linkage criteria. The linkage is referred to as a measure of “closeness” between the two groups. The linkage criteria determine the distance between sets of observations as a function of the pairwise distances between observations. There are four different types of linkage. In agglomerative clustering techniques such as hierarchical clustering, at each step in the algorithm, the two closest groups are chosen to be merged. The linkage methods include: (1) pairwise average-linkage (i.e., the mean distance between all pairs of elements in the two groups), (2) pairwise single-linkage (i.e., the smallest distance between all pairs of elements in the two groups), (3) pairwise maximum-linkage (i.e., the largest distance between all pairs of elements in the two

groups) and (4) pairwise centroid-linkage (i.e., the distance between the centroids of all pairs of elements in the two groups). The pairwise maximum-linkage is found to be the most preferred for label-free integrative pharmacology applications.

[00141] For Hierarchical clustering, there are several ways to calculate the distance matrix that is used to build the cluster. Typically, the distances represent the distances between two rows (usually representing nodes) in the matrix. The distance metrics used includes, but not limited to, (1) Euclidean distance which is the simple two-dimensional Euclidean distance between two rows calculated as the square root of the sum of the squares of the differences between the values; (2) City-block distance which is the sum of the absolute value of the differences between the values in the two rows; (3) Pearson correlation which is the Pearson product-moment coefficient of the values in the two rows being compared. This value is calculated by dividing the covariance of the two rows by the product of their standard deviations; (4) Pearson correlation, absolute value which is similar to the value indicated in (3), but using the absolute value of the covariance of the two rows; (5) Uncentered correlation which is the standard Pearson correlation includes terms to center the sum of squares around zero. This metric makes no attempt to center the sum of squares. (6) Centered correlation, absolute value which is similar to the value indicated in (5), but using the absolute value of the covariance of the two rows; (7) Spearman's rank correlation which is Spearman's rank correlation (ρ) is a non-parametric measure of the correlation between the two rows; (8) Kendall's tau which ranks correlation coefficient (τ) between the two rows. The choice of distance metric for label-free integrative pharmacology is found to be dependent on the types of data. For similarity analysis based on the molecule biosensor primary indices, the uncentered correlation with absolute value is preferable. However, for similarity analysis based on the molecule modulation indices, both the uncentered correlation with absolute value and the centered correlation with absolute value can be used.

[00142] The similarity analysis can further use a predefined clustering threshold (a density parameter, also termed as similarity threshold) to compute a similarity matrix. Such a threshold gives the boundary between similar and dissimilar objects, and thus is used to control the density of the clustering analysis. High (restrictive) values make it more expensive to add most of the edges, resulting in many small clusters. On the other hand, lower values make it cheap to add edges but expensive to remove them, resulting in few big clusters (meaning lower resolution). For label-free integrative pharmacology, the clustering

threshold can be variable, and often depending on the desired resolution of clustering (e.g., at the cell type level, or at the specific pathway level, or at the specific target level).

[00143] For label-free integrative pharmacology, the data contain the list of all numeric node and edge attributes that can be used for hierarchical clustering. The node is often the molecule. The edge attribute represents the response of the molecules either alone (i.e., a given response at a specific time i for the molecule primary profile in a cell), or represents the modulation percentage of the molecule against a marker (i.e., the modulation percentage of the marker biosensor response, such as P-DMR, or N-DMR, by the molecule at a specific concentration). At least one edge attribute or one or more node attributes must be selected to perform the clustering. If an edge attribute is selected, the resulting matrix will be symmetric across the diagonal with nodes on both columns and rows. If multiple node attributes are selected, the attributes will define columns and the nodes will be the rows. Under certain circumstances, it may be desirable to cluster only a subset of the nodes in the network. For example, to identify molecules sharing a specific mode of action, only a subset of the nodes displaying such mode of action is examined (see example in Figure 6).

[00144] For label-free integrative pharmacology approach, certain normalization or data pretreatments may be necessary for effectively clustering. For example, data filtering may be necessary. For similarity analysis based on molecule biosensor primary indices, an effective data filtering mean is to use the max-min difference (e.g., only molecules whose DMR signal having a max-min difference between different time points greater than 40picometer within one hour post-stimulation are subject to similarity analysis). On the other hand, for similarity analysis based on molecule biosensor modulation indices, an effective data filtering mean is to ignore molecules whose biosensor modulation indices contain less than 15% modulation against all the markers, or a specific set of markers.

[00145] For label-free integrative pharmacology approach, a two-dimensional clustering analysis is preferred. Such analysis includes clustering both attributes and nodes. In such a method, the clustering algorithm will be run twice, first with the rows in the matrix representing the nodes and the columns representing the attributes. The resulting dendrogram provides a hierarchical clustering of the nodes given the values of the attributes. In the second pass, the matrix is transposed and the rows represent the attribute values. This provides a dendrogram clustering the attributes. Both the node-based and the attribute-base dendrograms can be viewed. As shown in disclosed examples, the first clustering allows one to cluster

molecules in term of their similarity and dissimilarity. The second clustering will serve different purposes, depending on the types of label-free integrative pharmacology analysis. For analysis based on the molecule biosensor primary indices, this clustering allows one to identify the minimal numbers of kinetic parameters needed for effective clustering molecules, and also to investigate the regulation mechanisms of the kinetic responses (i.e., pathways involved in the early response, versus pathways involved in the late response of a molecule acting on the cell(s)). For analysis based on the molecule biosensor modulation indices, this clustering not only allows one to identify the polypharmacology and phenotypic pharmacology of a molecule, but also to investigate the pathway interactions among different markers acting in a specific cell or a panel of cells.

[00146] The similarity analysis typically leads to dendrogram which consists of interconnected or independent clusters of molecules, each cluster of molecules share similar mode(s) of action (i.e., pharmacology). The clusters can also be viewed as a heat map. Similarity analysis for gene expression analysis and protein network analysis has resulted in three types of heat map displays, including HeatMapView (unclustered), Eisen TreeView, and Eisen KnnView. These heat map display approaches can be directly used to view the clusters and relations of molecules in terms of their label-free integrative pharmacology. Gene expression analysis often shows the results of hierarchically clustering of the nodes (i.e., genes) and a number of node attributes (typically expression data under different experimental conditions). Clustering based on label-free integrative pharmacology also displays the results of hierarchically clustering of the nodes (i.e., the molecules) and a number of node attributes. The node attributes used are dependent on the types of analysis. For the molecule biosensor primary indices-based similarity analysis, the node attributes are the absolute responses at a number of time points of a molecule acting on a cell or a panel of cells. Alternatively, for the molecule biosensor primary indices-based functional selectivity analysis, the node attributes can also be the predetermined kinetic parameters (e.g., amplitude, kinetics and duration of a P-DMR and/or a N-DMR event). On the other hand, for the molecule biosensor modulation indices based similarity analysis, the node attributes can be the modulation percentages of the molecules against each marker in a cell. The modulation percentage is often calculated by normalizing the marker biosensor response in the presence of a molecule to the marker biosensor response in the absence of the molecule. Such normalization is often based on signal amplitudes of a particular biosensor event (e.g., P-

DMR, N-DMR or RP-DMR) but not the kinetics of the respective event, since it is the signal amplitude, but not the kinetics, that is associated with molecule efficacy (when the molecule is an agonist or activator for a pathway or a cellular process) or potency (when the molecule is an antagonist or inhibitor for a pathway or a cellular process).

[00147] Among the heat map display approaches developed to date, the Eisen TreeView is the most common approach. Here Hierarchical clustering results are usually displayed with a color-coded "Heat Map" of the data values and the dendrogram from clustering.

Alternatively, when k-means clustering is used, the results can be shown with the Eisen KnnView.

35. Gi protein killer

[00148] Gi protein killer or the like terms refer to a molecule or biochemical that can inhibit the activity of the Gi protein. The Gi protein killer can also be used to determine the Gi-independent/dependent component of the ligand mediated biased agonism. A Gi protein killer can, for example, be pertussis toxin (PTX).

36. Gs protein killer

[00149] Gs protein killer or the like terms refer to a molecule or ligand that can decouple the interaction between a receptor and the Gs protein. The Gs protein killer can inhibit the Gs protein (e.g, a Gs protein inhibitor) or cause the Gs protein constitutive (e.g, cholera toxin). The Gs protein killer can also be used to determine the Gs-dependent component of the ligand mediated biased agonism. A Gs protein killer can, for example, be cholera toxin (CTX).

37. In the presence of the molecule

[00150] "in the presence of the molecule" or like terms refers to the contact or exposure of the cultured cell with the molecule. The contact or exposure can be taken place before, or at the time, the stimulus is brought to contact with the cell.

38. Index

[00151] An index or like terms is a collection of data. For example, an index can be a list, table, file, or catalog that contains one or more modulation profiles. It is understood that an index can be produced from any combination of data. For example, a DMR profile can have a P-DMR, a N-DMR, and a RP-DMR. An index can be produced using the completed date of the profile, the P-DMR data, the N-DMR data, the RP-DMR data, or any point within these, or in combination of these or other data. The index is the collection of any such

information. Typically, when comparing indexes, the indexes are of like data, i.e. P-DMR to P-DMR data.

i. Biosensor Index

[00152] A “biosensor index” or like terms is an index made up of a collection of biosensor data. A biosensor index can be a collection of biosensor profiles, such as primary profiles, or secondary profiles. The index can be comprised of any type of data. For example, an index of profiles could be comprised of just an N-DMR data point, it could be a P-DMR data point, or both or it could be an impedance data point. It could be all of the data points associated with the profile curve.

ii. DMR index

[00153] A “DMR index” or like terms is a biosensor index made up of a collection of DMR data.

39. JNK inhibitor

[00154] A JNK inhibitor or the like terms refer to a molecule or ligand that can inhibit the activity of JNK. The JNK inhibitor can also be used to determine the biased agonism of the ligand acting via JNK pathway. A JNK inhibitor can, for example, be SP100625.

40. K-Means

[00155] The K-Means clustering is a partitioning algorithm that divides the data into k non-overlapping clusters, where k is an input parameter, and also the Number of clusters. One of the challenges in k-Means clustering is that the number of clusters must be chosen in advance, and in general are close to the square root of $\frac{1}{2}$ of the number of nodes.

41. Known molecule

[00156] A known molecule or like terms is a molecule with known pharmacological/biological/physiological/pathophysiological activity whose precise mode of action(s) may be known or unknown.

42. Known modulator

[00157] A known modulator or like terms is a modulator where at least one of the targets is known with a known affinity. For example, a known modulator could be a PI3K inhibitor, a PKA inhibitor, a GPCR antagonist, a GPCR agonist, a RTK inhibitor, an epidermal growth factor receptor neutralizing antibody, or a phosphodiesterase inhibition, a PKC inhibitor or activator, etc.

43. Known modulator biosensor index

[00158] A “known modulator biosensor index” or like terms is a modulator biosensor index produced by data collected for a known modulator. For example, a known modulator biosensor index can be made up of a profile of the known modulator acting on the panel of cells, and the modulation profile of the known modulator against the panels of markers, each panel of markers for a cell in the panel of cells.

44. Known modulator DMR index

[00159] A “known modulator DMR index” or like terms is a modulator DMR index produced by data collected for a known modulator. For example, a known modulator DMR index can be made up of a profile of the known modulator acting on the panel of cells, and the modulation profile of the known modulator against the panels of markers, each panel of markers for a cell in the panel of cells.

45. Known receptor agonist

[00160] A known receptor agonist or like terms refer to molecule with known agonistic pharmacological/biological/physiological/pathophysiological activity towards a receptor and whose precise mode of action(s) may be known or unknown. A known receptor agonist is, for example, DAMGO.

46. Known receptor antagonist

[00161] A known receptor antagonist or like terms refer to molecule with known antagonistic pharmacological/biological/physiological/pathophysiological activity towards a receptor and whose precise mode of action(s) may be known or unknown.

47. Label free methods

[00162] Label free methods or the like terms refer to techniques and machines that can detect molecular events in real or lapsed time without the need of labeling biomolecules for detection of the molecular event. For example, label free methods can use biosensors or label free biosensor such as the Epic® system.

48. Ligand

[00163] A ligand or like terms is a substance or a composition or a molecule that is able to bind to and form a complex with a biomolecule to serve a biological purpose. Actual irreversible covalent binding between a ligand and its target molecule is rare in biological systems. Ligand binding to receptors alters the chemical conformation, i.e., the three dimensional shape of the receptor protein. The conformational state of a receptor protein

determines the functional state of the receptor. The tendency or strength of binding is called affinity. Ligands include substrates, blockers, inhibitors, activators, and neurotransmitters. Radioligands are radioisotope labeled ligands, while fluorescent ligands are fluorescently tagged ligands; both can be considered as ligands are often used as tracers for receptor biology and biochemistry studies. Ligand and modulator are used interchangeably.

49. Library

[00164] A library or like terms is a collection. The library can be a collection of anything disclosed herein. For example, it can be a collection, of indexes, an index library; it can be a collection of profiles, a profile library; or it can be a collection of DMR indexes, a DMR index library; Also, it can be a collection of molecule, a molecule library; it can be a collection of cells, a cell library; it can be a collection of markers, a marker library; a library can be for example, random or non-random, determined or undetermined. For example, disclosed are libraries of DMR indexes or biosensor indexes of known modulators.

50. Marker

[00165] A marker or like terms is a ligand which produces a signal in a biosensor cellular assay. The signal is, must also be, characteristic of at least one specific cell signaling pathway(s) and/or at least one specific cellular process(es) mediated through at least one specific target(s). The signal can be positive, or negative, or any combinations (e.g., oscillation).

51. Marker panel

[00166] A “marker panel” or like terms is a panel which comprises at least two markers. The markers can be for different pathways, the same pathway, different targets, or even the same targets.

52. Marker biosensor index

[00167] A “marker biosensor index” or like terms is a biosensor index produced by data collected for a marker. For example, a marker biosensor index can be made up of a profile of the marker acting on the panel of cells, and the modulation profile of the marker against the panels of markers, each panel of markers for a cell in the panel of cells.

53. Marker DMR index

[00168] A “marker biosensor index” or like terms is a biosensor DMR index produced by data collected for a marker. For example, a marker DMR index can be made up of a profile

of the marker acting on the panel of cells, and the modulation profile of the marker against the panels of markers, each panel of markers for a cell in the panel of cells.

54. Markov Clustering Algorithm

[00169] Markov Clustering Algorithm (MCL) is a fast divisive clustering algorithm for graphs based on simulation of the flow in the graph.

55. MCL and FORCE

[00170] Both MCL and FORCE create collapsible "meta nodes" to allow interactive exploration of the putative family associations, and thus are often used for clustering similarity networks to look for protein families (and putative functional similarities).

56. Material

[00171] Material is the tangible part of something (chemical, biochemical, biological, or mixed) that goes into the makeup of a physical object.

57. MEK ½ inhibitor

[00172] MEK1/2 inhibitor or the like terms refer a molecule or ligand that can inhibit the activity of MEK1/2. A MEK1/2 inhibitor can also be used to determine the biased agonism of the ligand acting via MAPK pathway. A MEK ½ inhibitor can, for example, be U0126.

58. Mimic

[00173] As used herein, "mimic" or like terms refers to performing one or more of the functions of a reference object. For example, a molecule mimic performs one or more of the functions of a molecule.

59. Modulate

[00174] To modulate, or forms thereof, means either increasing, decreasing, or maintaining a cellular activity mediated through a cellular target. It is understood that wherever one of these words is used it is also disclosed that it could be 1%, 5%, 10%, 20%, 50%, 100%, 500%, or 1000% increased from a control, or it could be 1%, 5%, 10%, 20%, 50%, or 100% decreased from a control.

60. Modulator

[00175] A modulator or like terms is a ligand that controls the activity of a cellular target. It is a signal modulating molecule binding to a cellular target, such as a target protein. A modulator can be, for example, a opioid receptor modulator.

61. Modulation comparison

[00176] A “modulation comparison” or like terms is a result of normalizing a primary profile and a secondary profile.

62. Modulator biosensor index

[00177] A “modulator biosensor index” or like terms is a biosensor index produced by data collected for a modulator. For example, a modulator biosensor index can be made up of a profile of the modulator acting on the panel of cells, and the modulation profile of the modulator against the panels of markers, each panel of markers for a cell in the panel of cells.

63. Modulator DMR index

[00178] A “modulator DMR index” or like terms is a DMR index produced by data collected for a modulator. For example, a modulator DMR index can be made up of a profile of the modulator acting on the panel of cells, and the modulation profile of the modulator against the panels of markers, each panel of markers for a cell in the panel of cells.

64. Modulate the biosensor signal of a marker

[00179] “Modulate the biosensor signal or like terms is to cause changes of the biosensor signal or profile of a cell in response to stimulation with a marker.

65. Modulate the DMR signal

[00180] “Modulate the DMR signal or like terms is to cause changes of the DMR signal or profile of a cell in response to stimulation with a marker.

66. Molecule

[00181] As used herein, the terms “molecule” or like terms refers to a biological or biochemical or chemical entity that exists in the form of a chemical molecule or molecule with a definite molecular weight. A molecule or like terms is a chemical, biochemical or biological molecule, regardless of its size.

[00182] Many molecules are of the type referred to as organic molecules (molecules containing carbon atoms, among others, connected by covalent bonds), although some molecules do not contain carbon (including simple molecular gases such as molecular oxygen and more complex molecules such as some sulfur-based polymers). The general term “molecule” includes numerous descriptive classes or groups of molecules, such as proteins, nucleic acids, carbohydrates, steroids, organic pharmaceuticals, small molecule, receptors, antibodies, and lipids. When appropriate, one or more of these more descriptive terms (many of which, such as “protein,” themselves describe overlapping groups of molecules) will be

used herein because of application of the method to a subgroup of molecules, without detracting from the intent to have such molecules be representative of both the general class "molecules" and the named subclass, such as proteins. Unless specifically indicated, the word "molecule" would include the specific molecule and salts thereof, such as pharmaceutically acceptable salts.

67. Molecule mixture

[00183] A molecule mixture or like terms is a mixture containing at least two molecules. The two molecules can be, but not limited to, structurally different (i.e., enantiomers), or compositionally different (e.g., protein isoforms, glycoform, or an antibody with different poly(ethylene glycol) (PEG) modifications), or structurally and compositionally different (e.g., unpurified natural extracts, or unpurified synthetic compounds).

68. Molecule biosensor index

[00184] A "molecule biosensor index" or like terms is a biosensor index produced by data collected for a molecule. For example, a molecule biosensor index can be made up of a profile of the molecule acting on the panel of cells, and the modulation profile of the molecule against the panels of markers, each panel of markers for a cell in the panel of cells.

69. Molecule DMR index

[00185] A "molecule DMR index" or like terms is a DMR index produced by data collected for a molecule. For example, a molecule biosensor index can be made up of a profile of the molecule acting on the panel of cells, and the modulation profile of the molecule against the panels of markers, each panel of markers for a cell in the panel of cells.

70. Molecule index

[00186] A "molecule index" or like terms is an index related to the molecule.

71. Molecule-treated cell

[00187] A molecule-treated cell or like terms is a cell that has been exposed to a molecule.

72. Molecule modulation index

[00188] A "molecule modulation index" or like terms is an index to display the ability of the molecule to modulate the biosensor output signals of the panels of markers acting on the panel of cells. The modulation index is generated by normalizing a specific biosensor output signal parameter of a response of a cell upon stimulation with a marker in the presence of a molecule against that in the absence of any molecule.

73. Molecule pharmacology

[00189] Molecule pharmacology or the like terms refers to the systems cell biology or systems cell pharmacology or mode(s) of action of a molecule acting on a cell. The molecule pharmacology is often characterized by, but not limited, toxicity, ability to influence specific cellular process(es) (e.g., proliferation, differentiation, reactive oxygen species signaling), or ability to modulate a specific cellular target (e.g, PI3K, GPCR, opioid receptors, MAPK or MEK2).

74. Normalizing

[00190] Normalizing or like terms means, adjusting data, or a profile, or a response, for example, to remove at least one common variable. For example, if two responses are generated, one for a marker acting a cell and one for a marker and molecule acting on the cell, normalizing would refer to the action of comparing the marker-induced response in the absence of the molecule and the response in the presence of the molecule, and removing the response due to the marker only, such that the normalized response would represent the response due to the modulation of the molecule against the marker. A modulation comparison is produced by normalizing a primary profile of the marker and a secondary profile of the marker in the presence of a molecule (modulation profile).

75. Opioid

[00191] The term "opioid" or "opioids" as used herein refers to a natural or synthetic substance that have opiate -like activities such as have effects on perception of pain, consciousness, motor control, mood, and autonomic function, and can induce physical dependence.. Opioids or opiates include, but are not limited to alfentanil, allylprodine, alphaprodine, anileridine, benzylmorphine, bezitramide, buprenorphine, butorphanol, clonitazene, codeine, cyclazocine, desomorphine, dextromoramide, dezocine, diampromide, diamorphine, dihydrocodeine, dihydromorphine, dimenoxadol, dimepheptanol, dimethylthiambutene, dioxaphetylbutyrate, dipipanone, eptazocine, ethoheptazine, ethylmethylthiambutene, ethylmorphine, etonitazene fentanyl, heroin, hydrocodone, hydromorphone, hydroxypethidine, isomethadone, ketobemidone, levallorphan, levorphanol, levophenacylmorphine, lofentanil, meperidine, meptazinol, metazocine, methadone, metopon, morphine, myrophine, nalbuphine, narceine, nicomorphine, norlevorphanol, normethadone, nalorphine, normorphine, norpipanone, opium, oxycodone, oxymorphone, papaveretum, pentazocine, phenadoxone, phenomorphan, phenazocine, phenoperidine, piminodine,

piritramide, propheptazine, promedol, properidine, propiram, propoxyphene, sufentanil, tilidine, and tramadol.

76. Opioid agonism activity

[00192] An opioid agonism activity or the like terms refer to a cellular activity triggered when a molecule or ligand binds to an opioid receptor.

77. Opioid receptor

[00193] Opioid receptor or the like terms refer to a receptor that can bind to an opioid. An opioid receptor can for example be a Mu-opioid receptor, Delta-opioid receptor or Kappa-opioid receptor.

78. Opioid receptor parameter

[00194] An opioid receptor parameter or the like terms refer to a measureable activity in a cell.

79. Optional

[00195] “Optional” or “optionally” or like terms means that the subsequently described event or circumstance can or cannot occur, and that the description includes instances where the event or circumstance occurs and instances where it does not. For example, the phrase “optionally the composition can comprise a combination” means that the composition may comprise a combination of different molecules or may not include a combination such that the description includes both the combination and the absence of the combination (i.e., individual members of the combination).

80. Or

[00196] The word "or" or like terms as used herein means any one member of a particular list and also includes any combination of members of that list.

81. p38 MAPK inhibitor

[00197] A p38 MAPK inhibitor or the like terms refer to a molecule or ligand that can inhibit a p38 MAPK activity. A p38 MAPK inhibitor can be used to determine the biased agonism of the ligand acting via p38 MAPK pathway. A p38 MAPK inhibitor can, for example, be SB210290

82. Parental cell line

[00198] A parental cell line refers to a cell line that is the source of other cell lines. For example, a parental cell line can be a cell line that is used to develop engineered cell lines. For example, HEK-293 can be a parental cell line. Alternatively, any other native cell lines

not expressing opioid receptor can be used as a parental cell line, given that it is possible to engineer such a cell line to express the opioid receptor.

83. PI3K inhibitor

[00199] A PI3K inhibitor or the like terms refer to a molecule or ligand that can inhibit a PI3K inhibitor. A PI3K inhibitor can be used to determine the possible biased agonism of the ligand acting via PI3K pathway. A PI3K inhibitor can, for example, be LY2940002.

84. Preconditioned activity

[00200] A preconditioned activity or the like term refers to pretreatment of cells with distinct molecules, ligands, chemicals or biochemicals. The molecules, ligands, chemicals or biochemicals can for example be a known receptor antagonist or a known receptor agonist.

85. Preconditioning molecule

[00201] A preconditioning molecule is a molecule that is used for the pretreatment of cells.

86. Profile

[00202] A profile or like terms refers to the data which is collected for a composition, such as a cell. A profile can be collected from a label free biosensor as described herein.

i. Primary profile

[00203] A “primary profile” or like terms refers to a biosensor response or biosensor output signal or profile which is produced when a molecule contacts a cell. Typically, the primary profile is obtained after normalization of initial cellular response to the net-zero biosensor signal (i.e., baseline)

ii. Secondary profile

[00204] A “secondary profile” or like terms is a biosensor response or biosensor output signal of cells in response to a marker in the presence of a molecule. A secondary profile can be used as an indicator of the ability of the molecule to modulate the marker-induced cellular response or biosensor response.

iii. Modulation profile

[00205] A “modulation profile” or like terms is the comparison between a secondary profile of the marker in the presence of a molecule and the primary profile of the marker in the absence of any molecule. The comparison can be by, for example, subtracting the primary profile from secondary profile or subtracting the secondary profile from the primary profile or normalizing the secondary profile against the primary profile.

87. Panel

[00206] A panel or like terms is a predetermined set of specimens (e.g., markers, or cells, or pathways). A panel can be produced from picking specimens from a library.

88. Positive control

[00207] A "positive control" or like terms is a control that shows that the conditions for data collection can lead to data collection.

89. Potentiate

[00208] Potentiate, potentiated or like terms refers to an increase of a specific parameter of a biosensor response of a marker in a cell caused by a molecule. By comparing the primary profile of a marker with the secondary profile of the same marker in the same cell in the presence of a molecule, one can calculate the modulation of the marker-induced biosensor response of the cells by the molecule. A positive modulation means the molecule to cause increase in the biosensor signal induced by the marker.

90. Potency

[00209] Potency or like terms is a measure of molecule activity expressed in terms of the amount required to produce an effect of given intensity. For example, a highly potent drug evokes a larger response at low concentrations. The potency is proportional to affinity and efficacy. Affinity is the ability of the drug molecule to bind to a receptor.

91. Prefiltering

[00210] Prefiltering or the like terms refer to assaying a test molecule to determine if the test molecule triggers unwanted cellular responses.

92. Publications

[00211] Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

93. Receptor

[00212] A receptor or like terms is a protein molecule embedded in either the plasma membrane or cytoplasm of a cell, to which a mobile signaling (or "signal") molecule may attach. A molecule which binds to a receptor is called a "ligand," and may be a peptide (such

as a neurotransmitter), a hormone, a pharmaceutical drug, or a toxin, and when such binding occurs, the receptor goes into a conformational change which ordinarily initiates a cellular response. However, some ligands merely block receptors without inducing any response (e.g. antagonists). Ligand-induced changes in receptors result in physiological changes which constitute the biological activity of the ligands.

94. “Robust”

[00213] A “robust” when used in conjunction with an assay or parameter or condition is a one in whose amplitude(s) is significantly (such as 3x, 10x, 20x, 100x, or 1000x) above either the noise level, or the negative control response. The negative control response is often the biosensor response of cells after addition of the assay buffer solution (i.e., the vehicle). The noise level is the biosensor signal of cells without further addition of any solution. It is worthy of noting that the cells are always covered with a solution before addition of any solution.

95. “Robust DMR signal”

[00214] A “robust DMR signal” or like terms is a DMR form of a “robust biosensor signal.”

96. Ranges

[00215] Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that when a value is disclosed that “less than or equal to” the value, “greater than or equal to the value” and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value “10” is disclosed the “less than or equal to 10” as well as “greater than or equal to 10” is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and

ranges for any combination of the data points. For example, if a particular data point “10” and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

97. Response

[00216] A response or like terms is any reaction to any stimulation.

98. Sample

[00217] By sample or like terms is meant an animal, a plant, a fungus, etc.; a natural product, a natural product extract, etc.; a tissue or organ from an animal; a cell (either within a subject, taken directly from a subject, or a cell maintained in culture or from a cultured cell line); a cell lysate (or lysate fraction) or cell extract; or a solution containing one or more molecules derived from a cell or cellular material (e.g. a polypeptide or nucleic acid), which is assayed as described herein. A sample may also be any body fluid or excretion (for example, but not limited to, blood, urine, stool, saliva, tears, bile) that contains cells or cell components.

99. Substance

[00218] A substance or like terms is any physical object. A material is a substance. Molecules, ligands, markers, cells, proteins, and DNA can be considered substances. A machine or an article would be considered to be made of substances, rather than considered a substance themselves.

100. Subject

[00219] As used throughout, by a subject or like terms is meant an individual. Thus, the “subject” can include, for example, domesticated animals, such as cats, dogs, etc., livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), laboratory animals (e.g., mouse, rabbit, rat, guinea pig, etc.) and mammals, non-human mammals, primates, non-human primates, rodents, birds, reptiles, amphibians, fish, and any other animal. In one aspect, the subject is a mammal such as a primate or a human. The subject can be a non-human.

101. Test molecule

[00220] A test molecule or like terms is a molecule which is used in a method to gain some information about the test molecule. A test molecule can be an unknown or a known molecule.

102. Test molecule-induced cellular response

[00221] A test molecule induced cellular response is a cellular response initiated, caused or as a result of a test molecule.

103. Three time domain matrix

[00222] Three (3) time domain matrix or the like terms refer to system that allows for effective clustering analysis of ligand on-target pharmacology by measuring the on-target pharmacology at three specific time intervals. The time intervals can, for example, be 3min, 9min and 30min post stimulation.

104. Treating

[00223] Treating or treatment or like terms can be used in at least two ways. First, treating or treatment or like terms can refer to administration or action taken towards a subject. Second, treating or treatment or like terms can refer to mixing any two things together, such as any two or more substances together, such as a molecule and a cell. This mixing will bring the at least two substances together such that a contact between them can take place.

[00224] When treating or treatment or like terms is used in the context of a subject with a disease, it does not imply a cure or even a reduction of a symptom for example. When the term therapeutic or like terms is used in conjunction with treating or treatment or like terms, it means that the symptoms of the underlying disease are reduced, and/or that one or more of the underlying cellular, physiological, or biochemical causes or mechanisms causing the symptoms are reduced. It is understood that reduced, as used in this context, means relative to the state of the disease, including the molecular state of the disease, not just the physiological state of the disease.

105. Trigger

[00225] A trigger or like terms refers to the act of setting off or initiating an event, such as a response.

106. Values

[00226] Specific and preferred values disclosed for components, ingredients, additives, cell types, markers, and like aspects, and ranges thereof, are for illustration only; they do not

exclude other defined values or other values within defined ranges. The compositions, apparatus, and methods of the disclosure include those having any value or any combination of the values, specific values, more specific values, and preferred values described herein.

[00227] Thus, the disclosed methods, compositions, articles, and machines, can be combined in a manner to comprise, consist of, or consist essentially of, the various components, steps, molecules, and composition, and the like, discussed herein. They can be used, for example, in methods for characterizing a molecule including a ligand as defined herein; a method of producing an index as defined herein; or a method of drug discovery as defined herein.

107. Unknown molecule

[00228] An unknown molecule or like terms is a molecule with unknown biological/pharmacological/physiological/pathophysiological activity.

108. Data output

[00229] A data output refers to the collected result occurring after performing an assay using an analytical machine, such as a label free biosensor. For example, the data output of a label free biosensor could be a DMR signal. It is understood that data output can be manipulated, for example, into an Index. It is also understood that there can be any kind of data output that the assay is performed with, such as a molecule, Marker, inhibitor, marker-molecule, etc. It is also understood that any two outputs can be compared, such as a molecule data output and a data output forming a comparison. Typically, such a comparison will be performed with analogous data outputs, such as a DMR data output to a DMR data output.

E. Examples

1. Experimental procedures

i. Reagents

[00230] Pertussis toxin, cholera toxin, forskolin and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). DAMGO, DPDPE, BRL-53527, CTOP, naltrindole hydrochloride, norbinaltorphimine, U0126, SB202190, SP600125, and LY294002 were purchased from Tocris Biosciences. The Opioid Compound Library (consisting 64 compounds of pan-specific and receptor subtype-specific agonists and antagonists, each at 10mM in DMSO) was obtained from Enzo Life Sciences.

ii. Cell culture

[00231] The HEK293 and SH-SY5Y cell lines were obtained from American Type Cell Culture (Manassas, VA). The culture medium for these cell types was Dulbecco's modified Eagle's medium (DMEM GlutaMAX-I, Gibco) supplemented with 10% non-heated inactivated fetal bovine serum and 1% penicillin-streptomycin.

[00232] The HEK293 cells with stably-transfected FLAG-tagged Mu Opioid Receptors, HEK293 cells with stably-transfected FLAG-tagged Delta Opioid Receptors and HEK293 cells with stably-transfected FLAG-tagged Kappa Opioid Receptors (provided by the Levenson Lab) were cultured in DMEM GlutaMAX I with 10% non-heat inactivated fetal bovine serum, 1% penicillin-streptomycin and 400ug/mL of Geneticin.

[00233] All cells were maintained at 37°C and 5% CO₂ in a humidified incubator. All overexpressed cells including the HEK293 cell line doubled roughly every 24 hours. The SH-SY5Y cells had a doubling time of about 48h.

iii. Optical biosensor cellular assays

[00234] The Epic® wavelength interrogation system (Corning Inc, Corning, NY) was used for whole cell sensing. This system consists of a temperature-controlled unit, an optical detection unit, and an on-board liquid handling unit with robotics. The detection unit is centered on integrated fiber optics, and enables kinetic measurements of cellular responses with a time interval of ~15sec.

[00235] The RWG biosensor is capable of detecting minute changes in local index of refraction near the sensor surface. Since the local index of refraction within a cell is a function of density and its distribution of biomass (e.g. proteins, molecular complexes), the biosensor exploits its evanescent wave to non-invasively detect ligand-induced dynamic mass redistribution (DMR) in native cells. The evanescent wave extends into the cells and exponentially decays over distance, leading to a characteristic sensing volume of ~150nm, implying that any optical response mediated through the receptor activation only represents an average over the portion of the cell that the evanescent wave is sampling. The aggregation of many cellular events downstream of the receptor activation determines the kinetics and amplitude of a ligand-induced DMR.

iv. Cell Assay Methods

[00236] Cells were seeded at the following densities: MOR and KOR at 20K, DOR at 18K per 40uL of complete media with Geneticin (DMEM GlutaMAX I with 10% non-heat

inactivated fetal bovine serum, 1% penicillin-streptomycin and 400ug/mL of Geneticin) per well. The cells were seeded on fibronectin coated 384-well Epic® plates (available commercially). HEK 293 cells were seeded at 16K per 40uL of complete media (DMEM GlutaMAX I with 10% non-heat inactivated fetal bovine serum, 1% penicillin-streptomycin) per well, also on fibronectin coated plates. SH-SY5Y cells were seeded at 15K per 40uL of complete media per well.

[00237] After seeding, the Epic® plates were left in hood for 30 minutes to allow the cells to settle to the well bottom and to minimize edge effects in assay results. The plates were then transferred to an incubator for 24 hours, for all HEK based cell lines. SH-SY5Y cells were incubated for 48 hours, to cover their slower doubling time.

[00238] One hour prior to starting the Epic® assay, cells were washed twice (50uL each) with 1x HBSS containing 20mM HEPES (pH 7.1), and then maintained in 30uL of the HBSS in the commercial instrument for 1 hour. While incubating, compound plates were prepared by diluting stock solutions (typically 10-100mM in DMSO) of each compound in the HBSS buffer, and then transferring to 384well polypropylene plates (Corning). For 1-step or step 1 assays, compound plates were prepared at 4X the final concentration. For 2-step assays, step 2 compound plates were prepared at 5X the final concentration. The compound plates were typically loaded inside the Epic® instrument minutes before the start of the assay.

[00239] All cell assays were run using the CIP_CBA_nomix method. This method consisted of a 2-min baseline read, followed by a 10uL compound addition step (which was carried out by an on-board liquid handler), then a final 50-min assay read.

[00240] For 2-step assays to study the effect of one compound (e.g. an antagonist or pathway modulator) on another compound-induced response, a second stimulation with the second compound a fixed dose (typically EC100) was applied. The resonant wavelengths of all biosensors in the microplate were normalized again to establish a second baseline, right before the second stimulation. The stimulations were usually separated by ~1h.

[00241] All assays were carried out at a controlled temperature of 26.0°C. Each data point represents an average of at least two replicates performed in the same assay. The assay coefficient of variation was found to be <10%.

2. Example 1: Receptor specificity assays

[00242] The first part of this assay was carried out to establish the potency and efficacy of the opioid receptors. It is well known that opioid receptor trafficking and opioid

signaling is highly agonist dependent. The parental line for the over-expressed opioid receptors is HEK293 (in which the native cells do not contain opioid receptors). Thus, the first set of background studies, focusing on the activation in HEK293 cells, assessed the opioid compound library for off-target signaling. Baseline studies were also performed on each of the opioid cell lines (HEK-MOR, HEK-DOR, HEK-KOR and SH-SY5Y) to visualize the efficacies of the compounds when acting on each receptor subtype. Each ligand was and should be looked at specifically in this platform in the cells lines, as classifications of efficacies can be affected by many factors, including the level of receptor expression and strength of stimulus-response coupling.

[00243] In order to further characterize the compounds (to determine full agonism, partial agonism, neutral antagonism or inverse antagonism), receptor specificity assays were also completed. Known agonists and antagonists (i.e. DAMGO and CTOP as the agonist and antagonist for MOR, respectively) were utilized in sequence with the opioid compound library. High concentrations of the agonists and antagonists were used, in order to establish that the receptors were fully saturated. Cells were either treated with a high concentration of the CTOP in step one and the opioid compound library in step two, or alternatively by introducing the cells to the opioid receptor library in step one and treating with DAMGO or CTOP in assay two.

[00244] For the initial studies of HEK293 cells, the cells were seeded according to standard procedure (seeded at optimized concentration, left in hood for 30 minutes then stored in the incubator for 24 hours). Cells were then washed one hour prior to the experiment and then incubate in the commercial Epic platform. During the one hour incubation, the compound plate of the opioid compound library was prepared. 1 uL of each solution having each compound at 10mM was diluted in 200 mL of 1x HBSS containing 20mM HEPES, and then transferred into a 384-well polypropylene plate (Corning). The assay was carried out as a one-step assay, in which the cultured cells were introduced to a compound in the opioid receptor library (the compound may be pan-specific or receptor subtype specific, and may be either an agonist or an antagonist). 10uL of the solution containing the compound was added to each well, by an on-board liquid handling system. The assay was run for 50 minutes, during which time cellular responses were monitored, to determine if there was off-site activation from any of the compounds in the library. It was determined that two of the compounds in the opioid receptor library including BNTX and

etonitazenyl isothiocyanate led to non-opioid specific activation of the HEK293 cells, thus, these compounds were excluded in the follow up studies.

[00245] Target specificity was further strengthened using antagonism assays as well as receptor desensitization and resensitization assays. For the MOR, the cells were prepared according to standard procedures (seeded at an optimized concentration of 20K per well, left in hood for 30 minutes then stored in the incubator for 24 hours). Cells were then washed one hour prior to the experiment and then incubate in commercial Epic platform. The cultured cells were treated with HBSS with 20mM HEPES buffer and then standard 50-minute assay was completed, to monitor the cellular responses. Second, the buffer-pretreated cells were introduced to a compound in the opioid receptor library (compound may be pan-specific or receptor subtype specific, and either an agonist or an antagonist). 10uL of the solution containing the compound was added to each well, by an on-board liquid handling system. The assay was run for 50 minutes, during which time the efficacy of each ligand was monitored. This was performed in a two-step assay so that it would also serve as a control for the other assays, which were all carried out in a two-step format. At the conclusion of this study, activity of all of the compounds in the opioid library was characterized, in order to use as controls for later studies.

[00246] Receptor specificity was completed in three studies for each of the opioid cell lines. For the MOR cells, cells were prepared according to standard procedure (seeded at an optimized concentration, left in hood for 30 minutes then stored in the incubator for 24 hours). Cells were then washed one hour prior to the experiment and then incubate in commercial Epic platform. All three assays were carried out using a two-step protocol. The first two assays determined the effects of the opioid receptor compound library on known agonists and antagonists. This was carried out as a two-step assay in which the cultured cells were introduced to a compound in the opioid receptor library (compound may be pan-specific or receptor subtype-specific agonist or antagonist) for step one. A 50-minute assay was completed, to monitor the cellular responses. Second, the compound-stimulated cells were introduced to a known agonist or antagonist (1uM concentration of DAMGO as the agonist for the MOR cells, and 5uM of CTOP as the antagonist). These assays were run for 50 minutes, and at the conclusion, results were used to compare the differences between the opioid agonist/antagonist-induced cellular responses in the absence & presence of the compound pretreatment. The third assay used to characterize receptor specificity was done

using the same methodology, except the cells were treated with compounds in the opposite order. Step one introduced the cultured cells to CTOP (the receptor subtype specific antagonist) and step two introduced the antagonist-stimulated cells to an opioid compound library. This allowed for comparison in the different antagonists, to determine compounds were acting as neutral antagonists or inverse antagonists.

[00247] The results from the assays allowed for all compounds in the opioid receptor library to be classified by their mode of activity on all three opioid receptors.

3. Experimental example 2: Receptor-level signaling pathway deconvolution assays

[00248] Opioid receptors are GPCRs, which activate signaling at the receptor level via G-protein signaling pathways, as well as the PKA pathway due to changes in cAMP levels. It is known that opioid receptors are coupled to the Gi signaling pathway. The literature shows that activation of opioid receptors leads to an inhibition of adenylyl cyclase, an increase in potassium conductance, an inhibition of calcium channels, and an inhibition of neurotransmitter release. All of these responses are indicative of Gi coupling, and are blocked by pretreatment of pertussis toxin (PTX). Yet, while it has been shown that Gi-coupling is predominant in opioid signaling, more recent reports indicate that there is potentially an aspect of Gs coupled signaling upon opioid receptor activation. In order to examine the effects of the Gs pathways in opioid signaling, cholera toxin (CTX) is utilized. CTX inhibits GTPase activity (thus inhibiting the Gs pathway) along with enhancing the GTP-dependent adenylyl cyclase activity. Also playing an important role at the receptor level is adenylyl cyclase. There are indications that opioid inhibition of adenylyl cyclase is a mechanism by which opioids inhibit primary afferent excitability and relieve pain. One of the most commonly used modulators of adenylyl cyclase activity is forskolin; pretreatment with forskolin stimulates adenylyl cyclase activity.

[00249] In order to determine which pathways are playing a key role in opioid signaling, and hence to deconvolute receptor-level signaling, assays were completed using pretreatments with PTX, CTX and forskolin. These assays were carried in order to visualize the effects of Gi, Gs and cAMP on opioid signaling. DMRs which were completely abolished by PTX, but unaffected by CTX were identified to be due to Gi-coupled receptors. Those which were affected by CTX indicate that a Gs component is involved in their signaling events.

[00250] For all three assays, cells were seeded as described above. Specifically, HEK-MOR cells were seeded at 20K per well on fibronectin coated Epic plates. To break down the Gi and Gs signaling components, 2 hours after seeding the cells, Gi and Gs pathway inhibitors, PTX and CTX respectively, were added to the 384-well plates. PTX was added at concentration of 50ng/mL in columns 2-12 of the plate, while CTX treatment was 10ng/mL on columns 13-23 of the MOR cells. Columns 1 & 24 were left at just complete media overnight, to serve as controls. After PTX and CTX addition, the cells were put back into the incubator for the remained of the 24 hours.

[00251] Day 2, the cells were washed by hand (due to the toxic nature of the pathway inhibitors) 50uL each with 1x HBSS containing 20mM HEPES), and then maintained in 30uL of the HBSS in the commercial instrument for 1 hour. During the one hour, the compound plate of the opioid compound library was prepared. 1 uL of each solution containing each compound at 10mM was diluted in 200 mL of 1x HBSS containing 20mM HEPES, then transferred into a 384-well polypropylene plate (Corning). Next, the assay was run by introducing the pretreated cells to a compound in the opioid receptor library (the compound may be pan-specific or receptor subtype specific, and may be either an agonist or an antagonist). 10uL of the solution containing the compound was added to each well, by an on-board liquid handling system. The assay was run for 50 minutes, and at the conclusion the differences between the opioid-compound induced cellular responses in the absence or presence of pretreatment with a modulator (PTX/CTX) were studied. It was observed that while most of the opioid signaling was Gi-dependent (and therefore blocked by PTX), there did appear to be a Gs component to some of the receptor-specific agonist responses. This was manifested by partial inhibition of the DMR signal on pretreatment with CTX. In each case, the response was ligand-specific.

[00252] Along with looking at the roles of Gi and Gs pathways in this study of receptor-level signaling deconvolution, the role of the PKA pathway was also examined. To achieve this, the cells were treated with forskolin, a cell-permeable activator of adenylyl cyclase. The PKA pathway is a GPCR-coupled receptor triggered signaling pathway, in which adenylyl cyclase binds directly to a G-protein subunit.

[00253] For this assay, the cells were seeded using standard procedures (seeded at an optimized concentration, left in hood for 30 minutes then stored in the incubator for 24 hours). Cells were then washed one hour prior to the experiment and then incubated in the

commercial Epic platform. The forskolin assay was carried out as a two-step assay, in which the cultured cells were first introduced to 10uM of forskolin and cellular responses were monitored for 50min. Second, the forskolin-pretreated cells were introduced to a compound in the opioid receptor library (the compound may be pan-specific or receptor subtype specific, and may be either an agonist or an antagonist). 10uL of the solution containing the compound was added to each well, by an on-board liquid handling system. The assay was run for 50 minutes, and at the conclusion the differences between the opioid-compound induced cellular responses in the absence or presence of pretreatment with the cAMP modulator were studied. It was observed that the presence of forskolin cAMP signaling was attenuated in Gi-coupled responses.

4. Example 3: Downstream-level signaling pathway deconvolution assays

[00254] There are multiple ways to study pathway deconvolution of GPCR signaling. As described elsewhere herein, studies evaluated functional selectivity at the receptor level. Downstream signaling is also vital in order to understand the mechanisms and implications of agonist-selective signaling. The different pathways activated can have profound effects on the physiological effects of the ligands.

[00255] Kinases, and specifically the family of mitogen-activated protein kinase (MAPKs), have been shown to be activated by downstream signaling of GPCRs. These kinases (specifically ERK, JNK, p38, and PI3K for this study) have been defined as having such roles as proliferation, plasticity, long-term potentiation and survival and differentiation. These functions of MAP kinases provide a possible connection for neuronal adaptations to decrease plasticity with accompanying opioid abuse. It is known that protein kinases modulate internalization and desensitization in cellular signaling-pathways; these processes are important in opioid receptor activation as they appear to play a critical role in opioid tolerance and addiction. Adding another layer of complexity to the deconvolution of the kinase phosphorylation cascade stems from the perception that not only does the type of kinase signaling activated have a role in functional selectivity, but also the duration of activation. There are indications that different agonist will induce either transient or chronic activation of ERKs.

[00256] For these studies, assays were developed to individually and specifically inhibit parts of the kinase signaling pathway in order to more fully determine the downstream effects of opioid receptor activation and agonist-selective signaling.

[00257] The downstream receptor signaling deconvolution studies were carried out for each of the opioid cell lines. The full set of experiments comprised four assays, each using a specific pathway modulator: U0126 (ERK inhibitor), SP600125 (JNK inhibitor), SB202190 (p38 MAPK inhibitor) and LY294002 (PI3K inhibitor). For the MOR cells, cells were prepared using standard procedures (seeded at an optimized concentration of 20K per well, left in hood for 30 minutes then stored in the incubator for 24 hours). Cells were then washed one hour prior to the experiment and then incubated in a commercial Epic platform. All four assays were carried out using the same two-step protocol. The assays were performed to determine the effects known kinase inhibitors on the downstream signaling of known ligands. For step one, the cultured cells were introduced to a kinase specific inhibitor. U0126 was used at final concentration of 5uM, while SP600125, SB202190 and LY294002 were used at a final concentration of 10uM. A 50-minute assay was completed, to monitor the cellular responses. Second, the inhibitor-pretreated cells were introduced to a compound in the opioid compound library, which may be pan-specific or receptor sub-type specific agonist or antagonist. Step two, assays were run for 50 minutes, and at the conclusion, differences were compared between the opioid compound-induced cellular responses in the absence and presence of pretreatment with the kinase inhibitor. It was seen that these various ligands did have downstream agonist-specific effects on signaling, which could be shown utilizing these kinase inhibitors.

5. Example 4: On-target pharmacology of mu opioid ligands

[00258] For assessing the on-target pharmacology of mu opioid receptor ligands, a library of 61 opioid receptor ligands were assembled to form a library. Both DAMGO and BLR52537 were assayed as duplicate for control purpose. Using the parental HEK293 as a control, two ligands that displayed agonism activity, BNTX and etonitazenyl isothiocyanate, were excluded in follow up analysis. Using the binding affinity (K_i , IC_{50} and K_d) as a matrix, 20 additional ligands that displayed no-activity or weak activity acting on mu receptor were also excluded from the follow up analysis. Therefore, a total of 41 ligands were included in the on-target pharmacology assessment. Figure 1 shows an example of the known mu opioid receptor agonist DAMGO, wherein the corresponding DMR signals were recorded in real time and used as a basis for on-target pharmacology assessment. Results showed: (A) DAMGO led to an insignificant DMR signal in HEK293; (B) DAMGO led to a robust DMR in HEK-MOR cell; (C) DAMGO led to a small DMR in the MOR antagonist CTOP

pretreated cell; (D) DAMGO caused the HEK-MOR cells responding to succeeding stimulation with CTOP with a small N-DMR; (E) DAMGO caused the HEK-MOR cells desensitized to succeeding stimulation with DAMGO; (F) PTX almost completely attenuated the DAMGO DMR; (G) CTX slightly potentiated the DAMGO DMR; (H) forskolin significantly potentiated the DAMGO DMR; (I) U0126 slightly attenuated the DAMGO DMR; (J) SB202190 also slightly attenuated the DAMGO DMR; (K and L) both SP100625 and LY294002 had little impact on the DAMGO DMR signal. Taken together, these results suggest that DAMGO is an agonist to mu receptor, and mediates signaling mostly Gi protein, and propagates primarily via MAPK pathway.

[00259] Figure 2 shows the selectivity and mode of action of all opioid receptor ligands acting against the family of opioid receptors in engineered and native cells. The heat map indicates that these ligands can be classified in different categories.

[00260] Figure 3A shows the functional selectivity of a group of mu-active opioid agonists, as well as the antagonism of a group of mu-active ligands. The results showed that mu agonists are diverse in their behaviors in distinct assays. Cluster analysis identified full agonists including DAMGO, partial agonists such as tramadol, and many biased agonism in different pathways (e.g., JNK pathway, PI3K pathway, or MAPK pathway). Mu antagonists can also be clustered in two categories, based on their label-free profiles, exemplified by naloxone methiodide that does not show any biased agonism, and Nalbuphine that displays biased agonism when certain pathways are blocked (e.g., MAPK pathway is attenuated by the MEK inhibitor U0126). These results indicate that the label-free on-target pharmacology offers high resolution characterization of opioid receptor ligands.

F. References

Dynamic Mass Redistribution as a Means to Measure and Differentiate Signaling via Opioid and Cannabinoid Receptors” ASSAY and Drug Development Technologies 2011 Feb 16 (ahead of print)

"Activation profiles of opioid ligands in HEK cells expressing delta opioid receptors“ BMC Neuroscience 2002, 3:19

“Pharmacological profiles of opioid ligands at Kappa opioid receptors” BMC Pharmacology 2006, 6:3

“Activity of opioid ligands in cells expressing cloned μ opioid receptors” BMC Pharmacology 2003, 3:1

M. B. Eisen, P. T. Spellman, P. O. Brown, and David Botstein: Cluster analysis and display of genome-wide expression patterns. PNAS, 95(25):14863-8 (1998)

SP10-091P. Fang, Y. and Ferrie, A.M. Label-free on-target pharmacology methods. [####PLEASE PROVIDE PUBLICATION NUMBER OR APPLICATION NUMBER IF AVAILABLE####]

Claims

We claim:

1. A method of assessment of opioid receptors comprising, obtaining data on an opioid receptor parameter for a test molecule, wherein the parameters included endogenous cell activity, opioid agonism activity, antagonism reversal activity, and preconditioned activity,

and wherein obtaining the endogenous cell activity parameter comprises analyzing the agonism response of the test molecule in a parental cell line having no opioid receptor,

wherein obtaining the opioid agonism activity parameter comprises analyzing the agonism response of the test molecule in an engineered parental cell line stably expressing the opioid receptor ,

wherein obtaining the antagonism reversal activity parameter comprises analyzing the ability of the test molecule to cause the engineered cell responding to successive stimulation with a known receptor antagonist, wherein the antagonism reversal activity determines the sustainability of the test molecule-induced cellular response and the ability of the antagonist to reverse its signal,

and wherein obtaining the preconditioned activity parameter comprises analyzing the agonism activity of the test molecule in the engineered cells, wherein the engineered cells have been preconditioned via pretreatment with a preconditioning molecule.

2. The method of claim 1, wherein the at least one step of analyzing comprises using a marker.

3. The method of claim 1, wherein the parental cell line comprises a HEK-293 cell line, or Chinese ovary hamster (CHO-K1), Cos-7, or HeLa cell line.

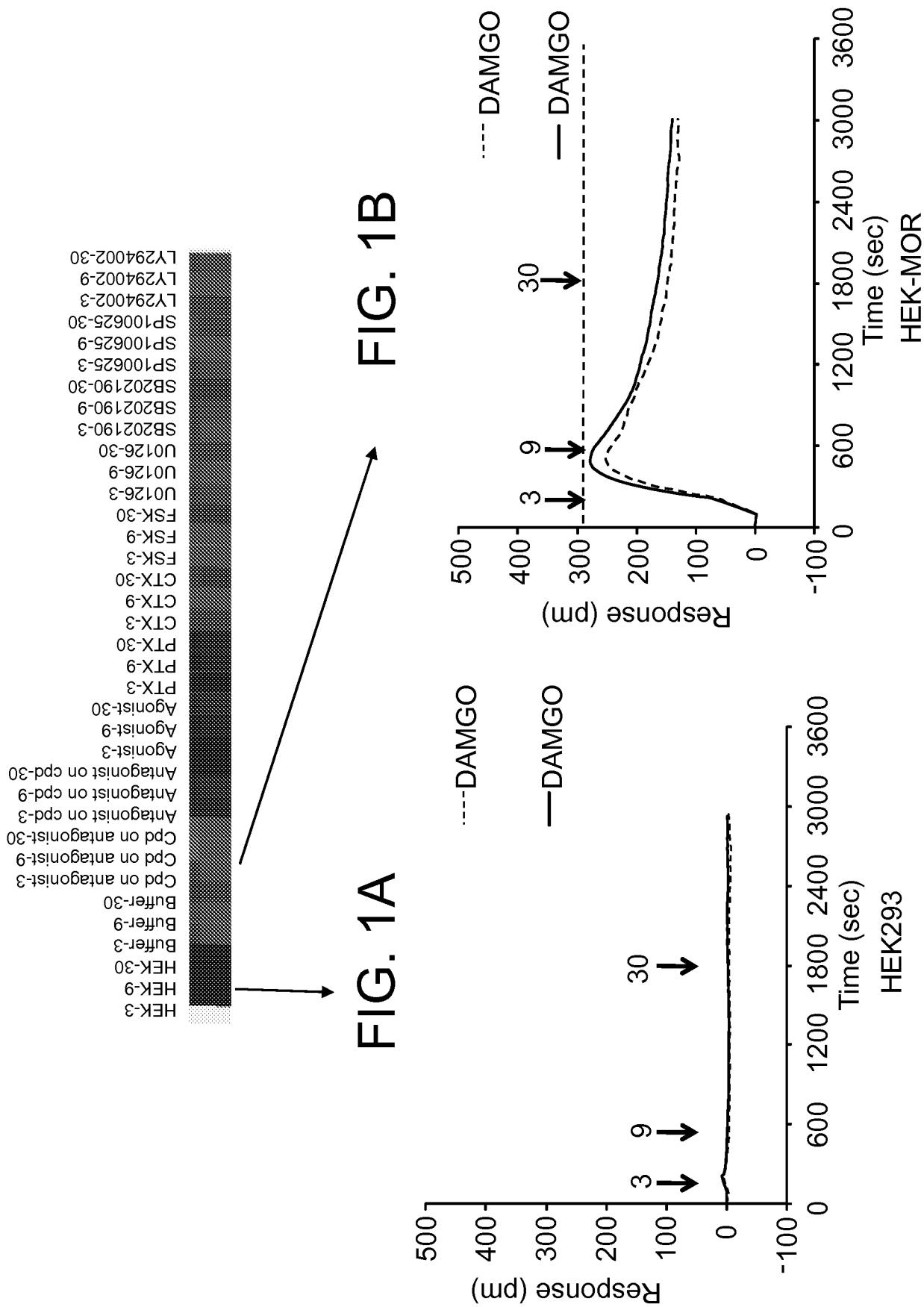
4. The method of claim 1, wherein the opioid receptor comprises a mu opioid receptor, a delta opioid receptor, a kappa opioid receptor, or opioid-like receptor 1.

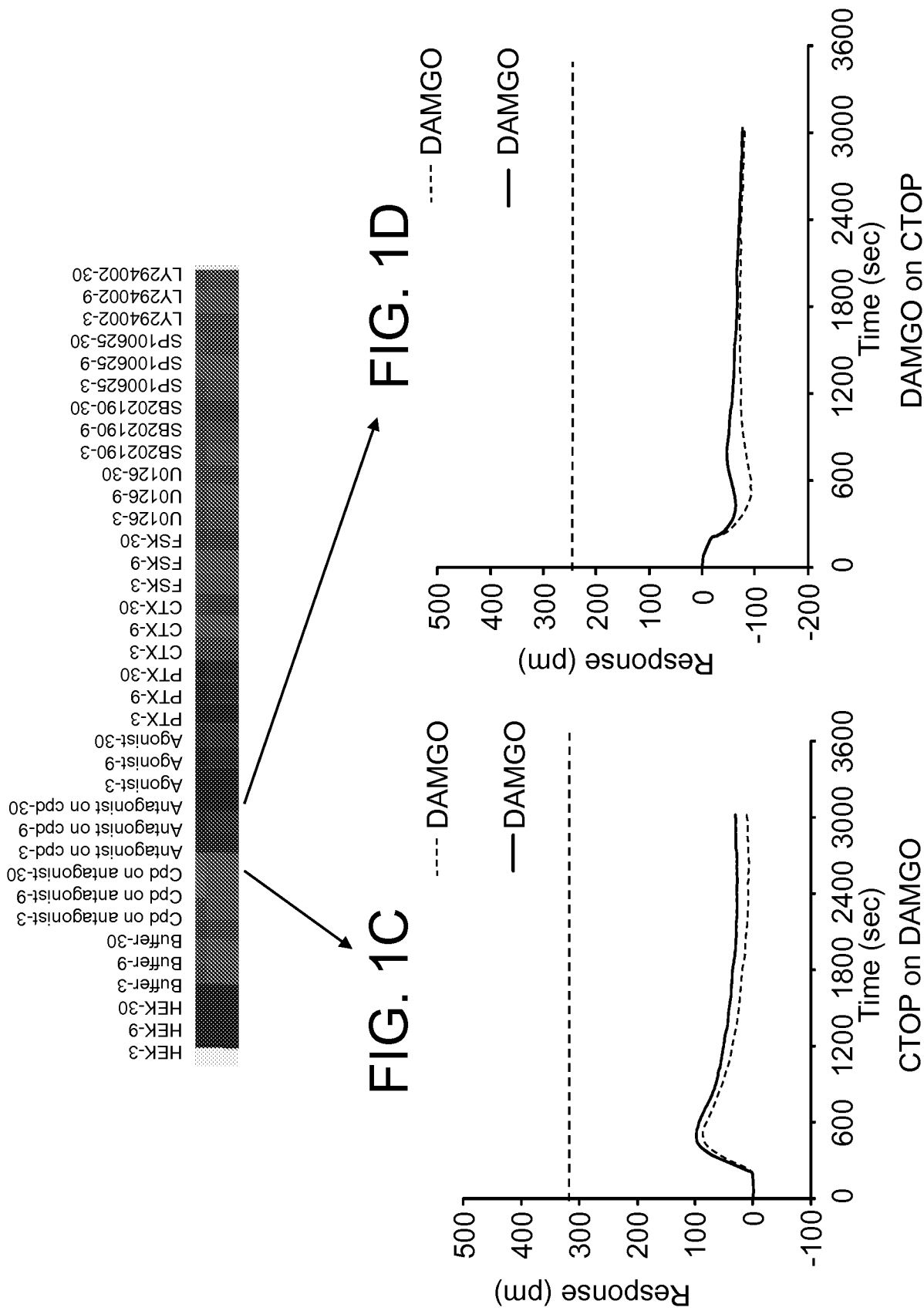
5. The method of claim 1, wherein the preconditioning molecule comprises the known receptor antagonist, the known receptor agonist, a Gi protein killer, a Gs protein killer, a adenylyl cyclase activator, a MEK1/2 inhibitor, a p38 MAPK inhibitor, a JNK inhibitor, or a PI3K inhibitor.

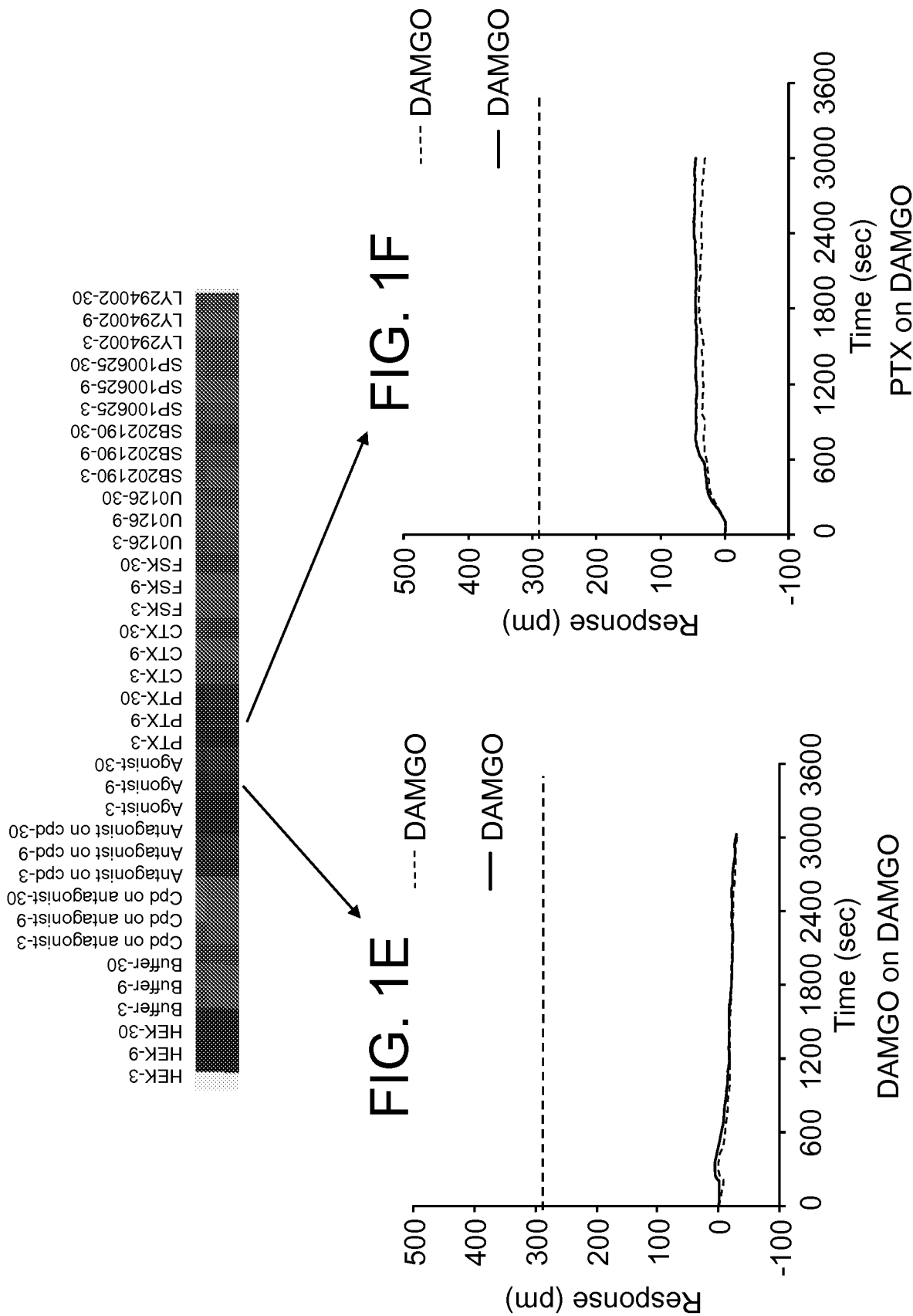
6. The method of claim 5, wherein the known receptor agonist comprises DAMGO.

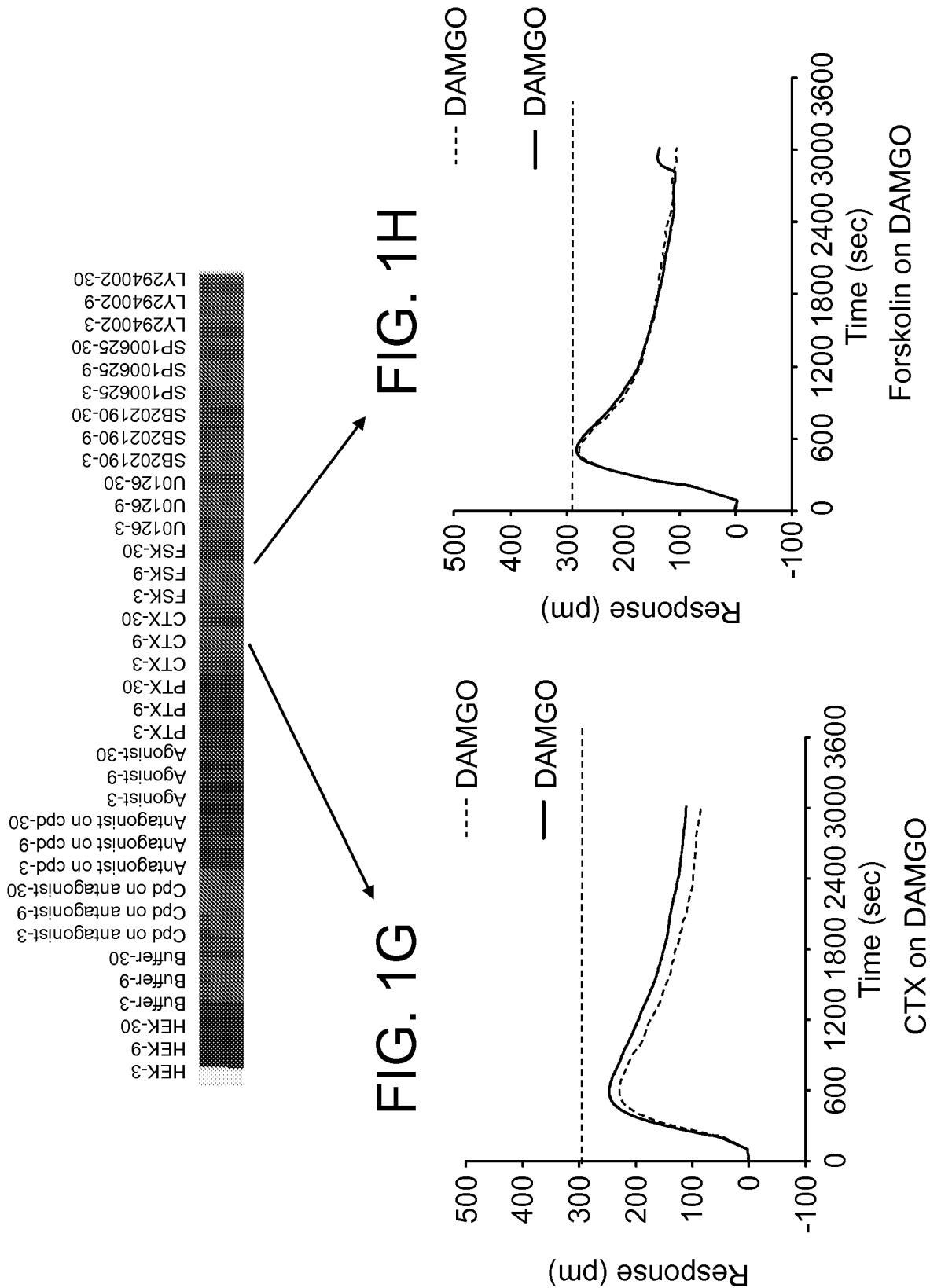
7. The method of claim 5, wherein the Gi protein killer comprises pertussis toxin (PTX).
8. The method of claim 5, wherein the Gs protein killer comprises cholera toxin (CTX).
9. The method of claim 5, wherein the adenylyl cyclase activator comprises forskolin.
10. The method of claim 5, wherein the MEK1/2 inhibitor comprises U0126.
11. The method of claim 5, wherein the p38 MAPK inhibitor comprises SB210290.
12. The method of claim 5, wherein the JNK inhibitor comprises SP100625.
13. The method of claim 5, wherein the PI3K inhibitor comprises LY294002.
14. The method of claim 1, further comprising the step of prefiltering the test molecule.
15. The method of claim 1, wherein the test molecule has been prefiltered.
16. The method of claim 1, wherein the test molecule does not show agonism activity in the parental cell line, wherein the test molecule has an EC₅₀, K_i, or K_d less than 5 micromolar.
17. The method of claim 1, wherein the concentration of the test molecule and preconditioning molecule is about 10 micromolar or greater than its corresponding K_d value.
18. The method of claim 1, wherein each step of analyzing is performed using label free methods.
19. The method of claim 18, wherein the label free methods comprise a Resonant Waveguide Grating biosensor.
20. The method of claim 19, further comprising the step of analyzing the test molecule induced DMR signals.
21. The method of claim 20, wherein the DMR signals are analyzed using a three time domain matrix.
22. The method of claim 21, wherein the three time domain matrix uses 3 minutes, 9 minutes, and 30 minutes post stimulation.
23. The method of claim 22, further comprising the step of performing a similarity analysis on the data for at least one parameter.

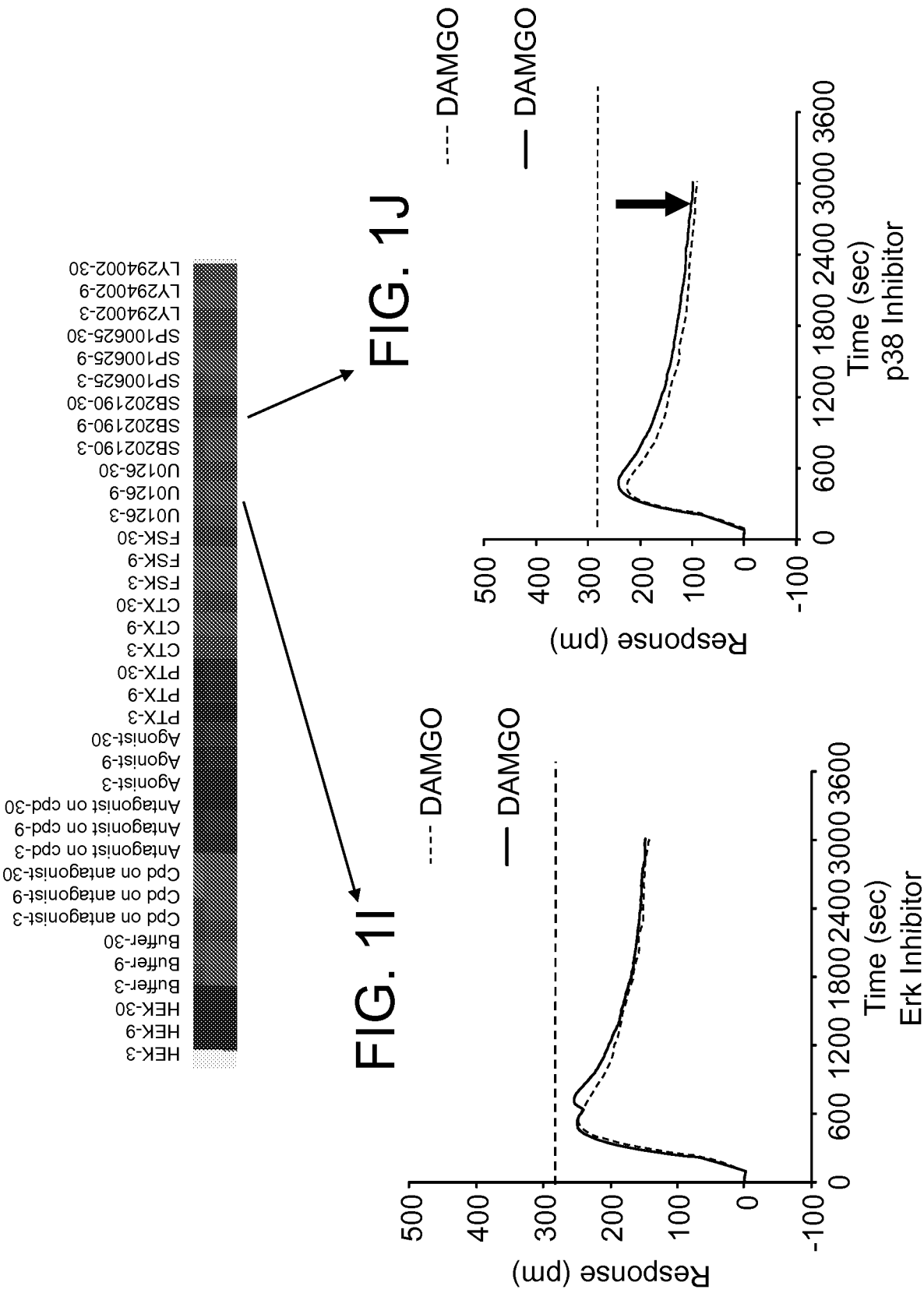
24. The method of claim 23, wherein the similarity analysis comprises a clustering analysis.
25. The method of claim 24, wherein the step of clustering comprises performing Hierarchical, K-means, FORCE, or MCL clustering.
26. The method of claim 25, further comprising the step of pretreating the data.
27. The method of claim 26, wherein the step of pretreating comprises normalization or filtering.
28. The method of claim 27, wherein the filtering comprises performing a max-min difference analysis.
29. The method of claim 24, wherein the clustering analysis comprises a one-dimensional analysis.
30. The method of claim 24, wherein the clustering analysis comprises a two-dimensional analysis.
31. The method of claim 24, wherein the clustering analysis comprises utilizing a heat map.
32. The method of claim 31, wherein the display of the heat map comprises a HeatMapView (unclustered), Eisen TreeView, or Eisen KnnView.
33. The method of claim 24, wherein the clustering comprises nodes.
34. The method of claim 33, wherein the nodes comprise a node attribute.
35. The method of claim 34, wherein the node attributes comprise the absolute responses at a number of time points of a biosensor signal induced by the test molecule under different assay conditions, the modulation percentages of the molecule against each marker in the marker panel.











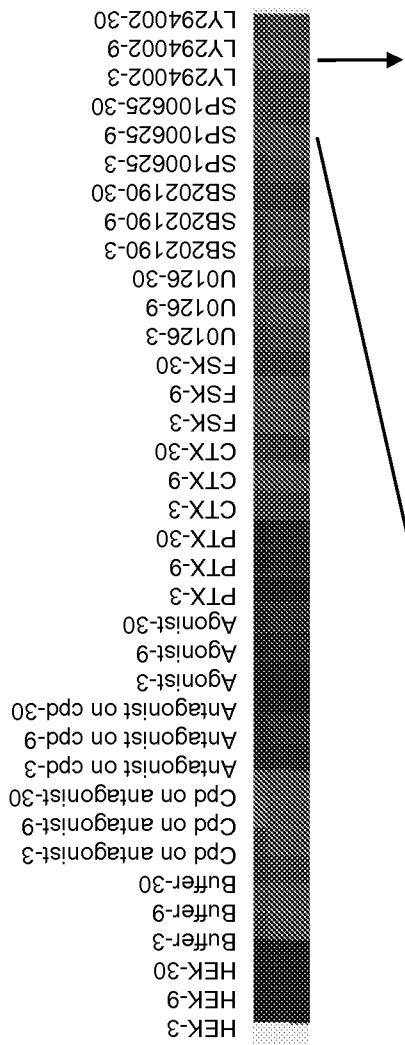


FIG. 1K

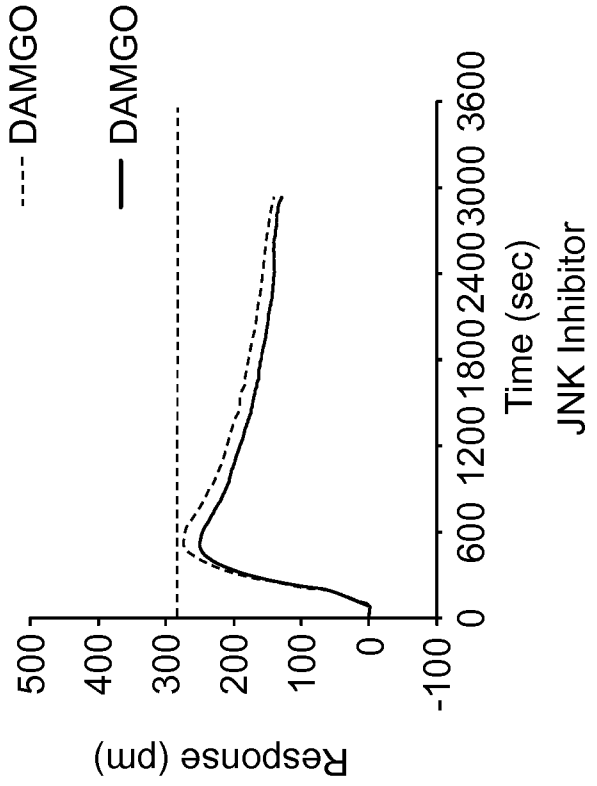


FIG. 1L

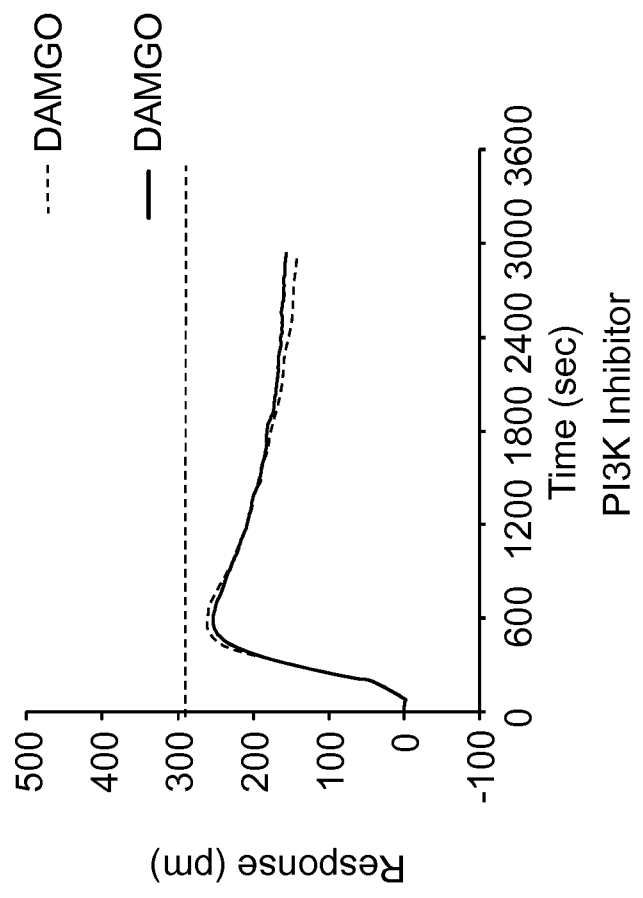
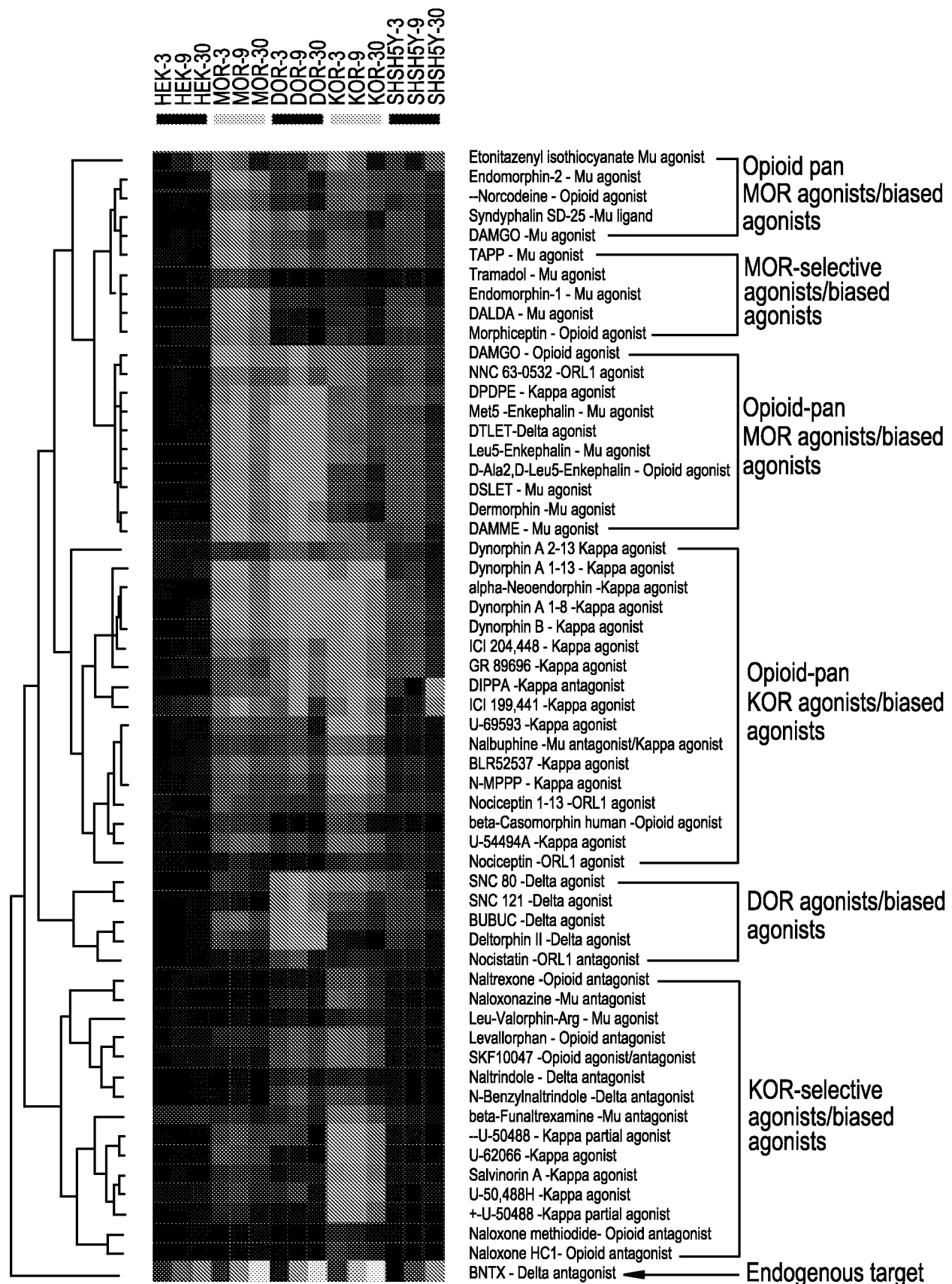


FIG. 2



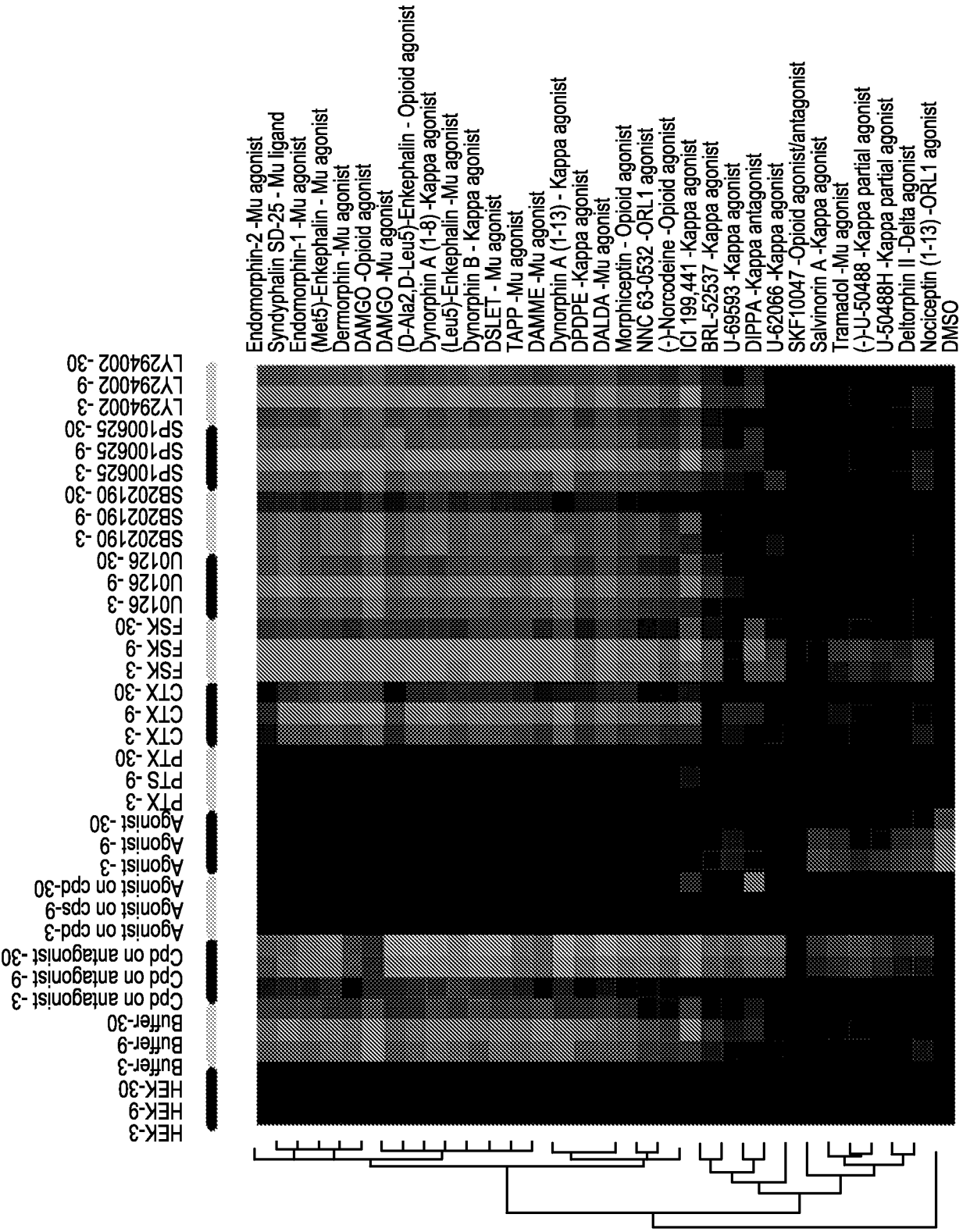
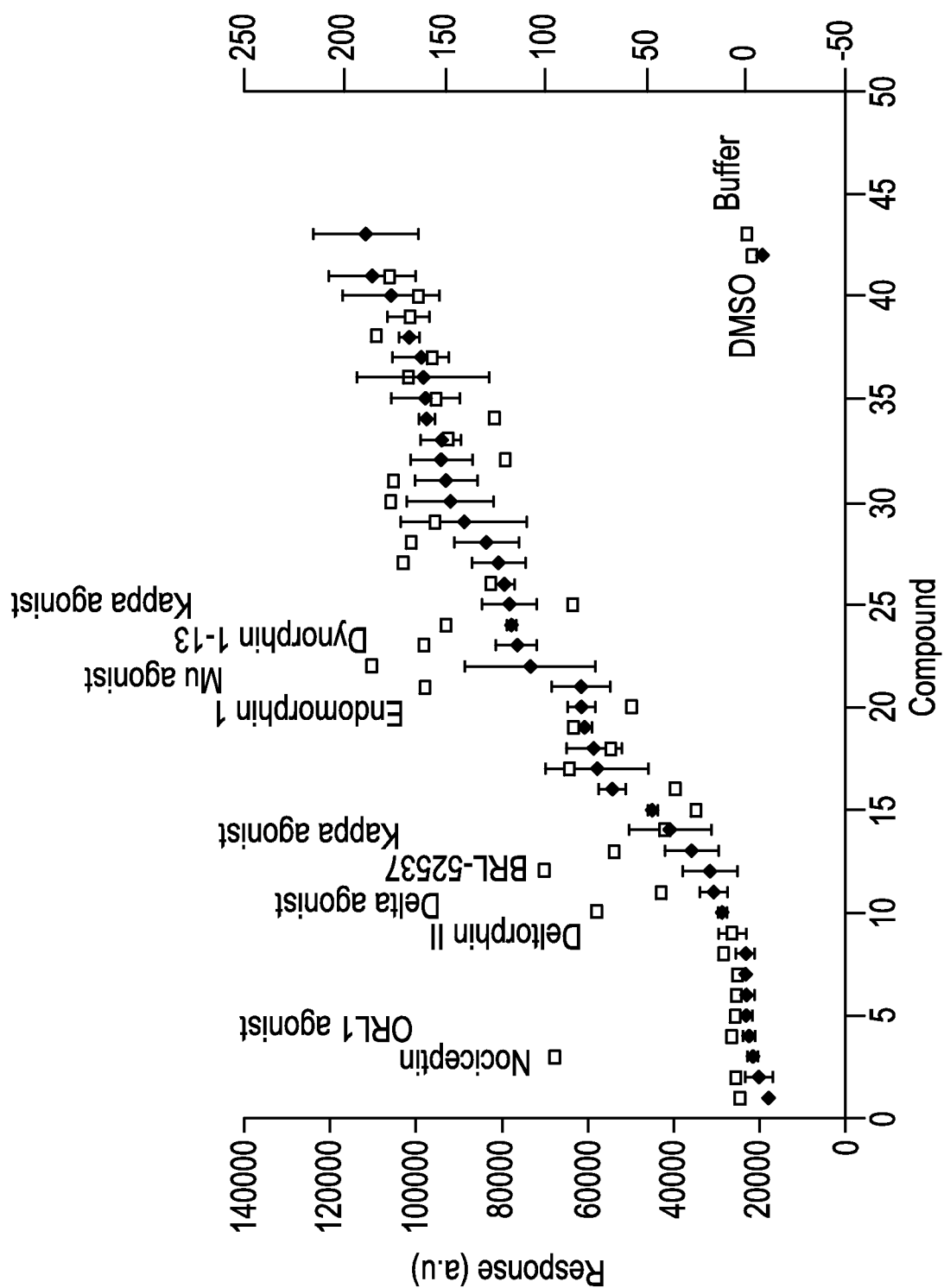


FIG. 4



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/027164

A. CLASSIFICATION OF SUBJECT MATTER
 INV. G01N33/50
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CODD ELLEN E ET AL: "Dynamic mass redistribution as a means to measure and differentiate signaling via opioid and cannabinoid receptors.", ASSAY AND DRUG DEVELOPMENT TECHNOLOGIES, vol. 9, no. 4, 16 February 2011 (2011-02-16), pages 362-372, XP002676696, ISSN: 1557-8127, DOI: 10.1089/ADT.2010.0347 PUBMED:21323580 figures 1-2, 7; tables 1-3 page 363, column 2, line 29 page 364, column 2, line 9 - page 366, column 1, line 40 page 366, column 2, line 35 - page 367, column 2, line 11 page 369, column 1, lines 3-7 ----- -/--	1-6, 17-20



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Date of the actual completion of the international search

29 May 2012

Date of mailing of the international search report

14/06/2012

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Landré, Julien

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/027164

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SALAMON Z ET AL: "Plasmon-waveguide resonance studies of ligand binding to integral proteins in membrane fragments derived from bacterial and mammalian cells", ANALYTICAL BIOCHEMISTRY, vol. 387, no. 1, 1 April 2009 (2009-04-01) , pages 95-101, XP025988678, ACADEMIC PRESS INC, NEW YORK ISSN: 0003-2697, DOI: 10.1016/J.AB.2009.01.019 [retrieved on 2009-01-19] abstract; figure 3 page 95, column 2, lines 22-26 page 96, column 1, lines 6-9 page 96, column 2, lines 19-49 page 99, column 2, lines 8-14 -----	7-16, 21-35
Y	LEE P H ET AL: "Evaluation of dynamic mass redistribution technology for pharmacological studies of recombinant and endogenously expressed G protein-coupled receptors", ASSAY AND DRUG DEVELOPMENT TECHNOLOGIES, vol. 6, no. 1, 1 February 2008 (2008-02-01), pages 83-94, XP002568980, MARY ANN LIEBERT, INC. PUBLISHERS, US ISSN: 1540-658X, DOI: 10.1089/ADT.2007.126 [retrieved on 2008-03-12] abstract; figures 2-6 page 84, column 1, lines 41-55 page 86, column 2, lines 6-39 page 88, column 2, line 4 - page 91, column 1, line 5 page 92, column 1, line 28 - page 92, column 2, line 47 page 93, column 1, lines 1-10 page 93, column 1, lines 35-48 page 93, column 2, lines 15-18 ----- -/--	7-16, 21-35

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2012/027164

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TRAN ELIZABETH ET AL: "Duplexed label-free G protein--coupled receptor assays for high-throughput screening.", JOURNAL OF BIOMOLECULAR SCREENING, vol. 13, no. 10, December 2008 (2008-12), pages 975-985, XP002676697, ISSN: 1087-0571 abstract; figures 4-6 page 976, column 1, lines 47-52 page 976, column 2, line 25 - page 977, column 2, line 8 page 977, column 2, lines 47-51 page 978, column 1, line 48 - page 978, column 2, line 6 page 980, column 1, lines 12-15 page 983, column 2, lines 37-53 -----	7-16, 21-35
Y,P	US 2011/230359 A1 (FANG YE [US] ET AL) 22 September 2011 (2011-09-22) the whole document -----	1-35
X,P	MORSE MEGAN ET AL: "Ligand-directed functional selectivity at the mu opioid receptor revealed by label-free integrative pharmacology on-target.", PLOS ONE, vol. 6, no. 10, E25643, 2011, pages 1-13, XP002676698, ISSN: 1932-6203 the whole document -----	1-35
Y,P	FERRIE ANN M ET AL: "Label-free integrative pharmacology on-target of drugs at the [beta](2)-adrenergic receptor.", SCIENTIFIC REPORTS, vol. 1, no. 33, 2011, pages 1-8, XP002676699, ISSN: 2045-2322 the whole document -----	1-35

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2012/027164

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011230359 A1	22-09-2011	US 2011230359 A1	22-09-2011
		WO 2011116260 A1	22-09-2011
