it is important to also identify off-target interactions (e.g., lower affinity). Most methods used today for identifying the targets of bioactive agents rely on the enrichment of these targets from complex cellular lysate using "bi-functionalized" compounds that contain a selective moiety (e.g., the bioactive agent or related compound) and a sorting moiety (e.g., affinity tag or solid support). As the enrichment is based on the binding properties of the compounds, where the inherent affinity of these compounds for their target is insufficient, compound analogs are designed to covalently bind to the target (e.g., photo-crosslink). By either approach, the efficacy and specificity of target isolation is essential, and the failure rate of these methods is high. Failure of these approaches can be due to insufficient capture of the target or high background due to non-specific capture. Contributing factors to this failures include: compounds binding multiple targets with low to moderate affinity with these relative weak interactions difficult to detect; lack of robust, straightforward, unbiased technologies to characterize the detected interactions; inability to perform target isolation within the native cellular environment upon which interactions may depend; limited information provided about the binding potency of targets in the cell; and high background of false positive interactions due to non-specific binding to the solid support or functionalized small molecule.

SUMMARY

In some embodiments, the present ~~invention~~ disclosure provides BRET assay systems comprising: (a) a bioactive agent tethered to a chromophore (e.g., fluorophore); (b) a cellular target fused to a bioluminescent reporter; and (c) a substrate for the bioluminescent reporter. In an aspect, the present disclosure provides an assay system comprising: (a) a bioactive agent tethered to a fluorophore; (b) a cellular target fused to a bioluminescent reporter; and (c) a substrate for the bioluminescent reporter. In some embodiments, the bioactive agent is a small molecule. In some embodiments, the bioactive agent is an inhibitor of protein function, e.g., an enzyme inhibitor or a receptor inhibitor. In some embodiments, the chromophore is a fluorophore. In some embodiments, the fluorophore is a carboxy rhodamine analog. In some embodiments, the bioluminescent reporter comprises a polypeptide with at least 70% sequence identity (e.g., 75% identity... 80% identity... 85% identity... 90% identity, 95% identity... 98% identity... 99% identity) with SEQ ID NO: 1. In some embodiments, (b) is a cellular target fused to a portion, or subunit, or component of a bioluminescent reporter. In some embodiments, (b) is a cellular target fused to a polypeptide that requires interaction with another polypeptide to produce luminescence. In some embodiments, (a) and (b) are within a cell. In some embodiments, (b) is expressed intracellularly as a fusion protein with a protein of interest, e.g., cellular target. In some embodiments, the cellular target is composed of more than one component, subunit or polypeptide, e.g., the cellular target is a protein complex. In some embodiments, the bioluminescent reporter is composed of more than one component, subunit or polypeptide, e.g., the bioluminescent reporter is a protein complex. In

some embodiments, (a) is added extracellularly and enters the cell. In some embodiments, (a) is present both within a cell and in the medium surrounding the cell. In some embodiments, (a) is present both bound to a cell and in the medium surrounding the cell. In some embodiments, the amount of (a) present in the surrounding medium is significantly greater than the amount in the cell or bound to the cell, e.g., greater by at least 2 fold, at least 5 fold, 10 fold, 30 fold, or 100 fold. In some embodiments, the cellular target is a binding partner of the bioactive agent. In some embodiments, the emission spectrum of the bioluminescent reporter overlaps with the absorption spectrum of a fluorophore. In some embodiments, upon binding of the bioactive agent to the cellular target, conversion of the substrate to a reaction product by the bioluminescent reporter results in excitation of a fluorophore by BRET and light emission from a fluorophore. In some embodiments, (a) is one of a library of agents or compounds tethered to a chromophore. In some embodiments, (a) is one of a library of agents or compounds tethered to fluorophores. In some embodiments, (b) is one of a plurality of potential cellular targets fused to a bioluminescent reporter. In some embodiments, (b) is a library of cells expressing one of a plurality of potential cellular targets fused to a bioluminescent reporter.

In some embodiments, the present ~~invention~~ disclosure provides methods of detecting, analyzing, characterizing, etc. the binding of a bioactive agent to a cellular target. In an aspect, the present disclosure provides a method of detecting the binding of a bioactive agent to a cellular target comprising the system as disclosed herein. In some embodiments, the cellular target may be the primary drug target. In some embodiments, the cellular target is an off-target liability that may cause undesirable side effects *in vivo*. In some embodiments, binding by the bioactive agent to the cellular target may have no discernible biological effect. In some embodiments, the present ~~invention~~ disclosure provides cells comprising one or more of (e.g., each of) (a) a bioactive agent tethered to a chromophore (e.g., fluorophore); (b) a cellular target fused to a bioluminescent reporter; and (c) a substrate for the bioluminescent reporter. In an aspect, the present disclosure provides a cell comprising the system as disclosed herein. In some embodiments, the binding of a bioactive agent to a cellular target is non-covalent. In some embodiments, the chromophore is a fluorophore. In ~~some embodiments~~an aspect, the present ~~invention~~ disclosure provides methods for detection of an interaction between a bioactive agent and a cellular target comprising: (a) expressing in a cell a fusion of said cellular target and a bioluminescent reporter that emits energy at a first wavelength (e.g., range of wavelengths, spectral distribution, etc.); (b) contacting said cell with said bioactive agent tethered to a fluorophore, wherein said fluorophore accepts energy at said first wavelength and emits energy at a second wavelength (e.g., range of wavelengths, spectral distribution, etc.); (c) contacting said cell with a substrate for said bioluminescent reporter; (d) detecting energy at said second wavelength, wherein the presence of said energy at said second wavelength indicates the interaction of said bioactive agent with said cellular target.

In some embodiments, the present ~~invention~~ disclosure provides methods for detection of an interaction between a bioactive agent and a cellular target comprising: (a) providing a fusion of said cellular target and a bioluminescent reporter; (b) contacting said fusion with said bioactive agent tethered to a chromophore (e.g., fluorophore); (c) contacting said fusion with a substrate for said reporter; (d) detecting a change in the spectral distribution of emitted light relative to said fusion contacted with said substrate in the absence of said chromophore. In some embodiments, the present ~~invention~~ disclosure provides a method for detection of an interaction of a second bioactive agent and a cellular target comprising: (a) providing a fusion of said cellular target and a bioluminescent reporter; (b) contacting said fusion with both a first bioactive agent tethered to a chromophore (e.g., fluorophore) and with said second bioactive agent; (c) contacting said fusion with a substrate for said reporter; (d) detecting a change in the spectral distribution of emitted light relative to said fusion contacted with said first bioactive agent and said substrate in the absence of said second bioactive agent. In some embodiments, the change in spectral distribution of emitted light is caused by displacement of said first bioactive agent by said second bioactive agent. In some embodiments, said displacement is competitive displacement. In some embodiments, the change in spectral distribution of emitted light is used to estimate binding affinity of the bioactive agent to cellular target. In some embodiments, said second bioactive agent is one of a plurality of bioactive agents. In some embodiments, the change in spectral distribution of emitted light is used to estimate the relative binding affinity of a plurality of bioactive agents to a cellular target. In some embodiments, said first and said second bioactive agents are synthetic molecules. In some embodiments, said cellular targets are in living cells, permeabilized cells or cell lysates. In some embodiments, the change in the spectral distribution of emitted light is determined by measuring the ratio of light intensity at two different wavelengths, or two different ranges of wavelengths. In some embodiments, the change in spectral distribution over time is detected. In some embodiments, the chromophore is a fluorophore.

In some embodiments, the present ~~invention~~ disclosure provides a BRET assay system comprising: (a) a bioactive agent tethered to a fluorophore; (b) a first interaction partner fused to a structurally complementary peptide of a bioluminescent reporter; (c) a second interaction partner fused to a structurally complementary polypeptide of a bioluminescent reporter; and (d) a substrate for the bioluminescent reporter, wherein the first and second interaction partners interact to form an interaction complex, and wherein the first interaction partner, second interaction partner, and/or interaction complex are binding partners for the bioactive agent. In some embodiments, the first interaction partner and the second interaction partner are proteins or polypeptides that interact to form a protein complex. In some embodiments, the first interaction partner and the second

interaction partner are brought together by the interaction of the structurally complementary polypeptides. In some embodiments, the structurally complementary polypeptides are brought together by the interaction of first interaction partner and the second interaction partner. In some embodiments, the interaction of the first interaction partner and the second interaction partner is determined by an increase in luminescence. In some embodiments, the first and second interaction partners form the interaction complex in the presence or absence of the bioactive agent. In some embodiments, the interaction complex is a binding partner for the bioactive agent, but neither the first or second interaction partners alone are binding partners for the bioactive agent. In some embodiments, one of the interaction partners, but not the other is a binding partner for the bioactive agent. In some embodiments, interaction complex formation requires binding of the bioactive agent to an interaction partner. In some embodiments, interaction complex formation is independent of binding of the bioactive agent. In some embodiments, the present ~~invention~~ disclosure provides BRET assay systems comprising: (a) a bioactive agent tethered to a fluorophore; (b) a cellular target fused to a structurally complementary peptide of a bioluminescent reporter; (c) a structurally complementary polypeptide of a bioluminescent reporter; and (d) a substrate for the bioluminescent reporter. In some embodiments, the complementary peptide and polypeptide of the bioluminescent reporter associate to form active bioluminescent reporter enzyme. In some embodiments, upon binding of the bioactive agent to the cellular target and association of the complementary peptide and polypeptide, conversion of the substrate to a reaction product by the bioluminescent reporter enzyme results in excitation of the fluorophore by BRET and fluorescence emission from the fluorophore.

In some embodiments, the present ~~invention~~ disclosure provides an assay system comprising: (a) a bioactive agent tethered to a fluorophore; (b) a first binding partner fused to a complementary peptide of a bioluminescent reporter; (c) a second binding partner fused to a complementary polypeptide of a bioluminescent reporter; and (c) a substrate for the bioluminescent reporter. In some embodiments, the complementary peptide and polypeptide of the bioluminescent reporter associate to form active bioluminescent reporter enzyme. In some embodiments, when the first and second binding partners interact, the bioactive agent binds to the interacting partners and association of the complementary peptide and polypeptide occurs, conversion of the substrate to a reaction product by the bioluminescent reporter enzyme results in excitation of the fluorophore by BRET and fluorescence emission from the fluorophore.

In some embodiments, the bioactive agent is tethered to a fluorescence or luminescence quencher (e.g., Dabcyl). In such embodiments, titration of the untethered bioactive agent produces a gain in signal.

In some embodiments, the present ~~invention~~ disclosure provides imaging (e.g., BRET imaging, via charge couple device camera, etc.) of any of the systems described herein to confirm and/or identify the location (e.g., intracellular, extracellular, etc.) of fluorescence and/or BRET signals resulting from the presence and/or interactions of system components (e.g., bioactive agent, cellular target, etc.).

In some embodiments systems and methods described herein (See, e.g., Example 23 for an exemplary embodiment) are useful for the measurement of intracellular selectivity and affinity of a bioactive agent against a panel of putative cellular targets. In some embodiments, the effect on BRET is monitored as the bioactive agent/cellular target complex is competitively disrupted. In some embodiments, affinities are inferred by the IC50 value generated via competitive disruption. In some embodiments, the affinities of inhibitors to individual domains of a cellular target are determined via genetic fusion of a bioluminescent reporter to a segregated domain of the cellular target.

DRAWINGS

Figure 1 shows a schematic representation of an embodiment of the present invention: (A) fluorophore-tethered bioactive agent and bioluminescent-reporter-tethered cellular target; (B) binding of bioactive agent to cellular target; (C) addition of reporter substrate results in BRET; and (D) displacement by excess untethered bioactive agent results in loss of BRET and fluorescence.

Figure 2 shows graphs comparing the BRET profiles of two different fluorophore-SAHA derivatives as BRET acceptors for NANOLUC-HDAC6 fusions in live cells.

Figure 3 shows graphs depicting the effect of dilution of expression on assay performance. The first graph demonstrates the effect of lowered expression on the BRET assay window using competitive displacement of fluorophore-tethered SAHA from NANOLUC-fused HDAC6. The second graph shows the effect of lowered expression on both BRET assay window, as well as observed compound potency (EC50) for a drug tracer binding to NanoLuc-Histamine H1 receptor.

Figure 4 shows graphs demonstrating the requirement of the cellular milieu for pro-drug processing.

Figure 5 shows graphs demonstrating the ability to use permeabilization agents to potentiate the entry of impermeable drug tracers.