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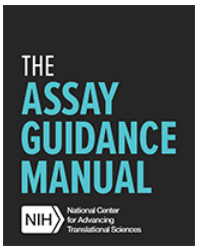

Inhibition of Protein-Protein Interactions: Cell-Based Assays

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Abstract

There is a strong interest in discovering compounds that inhibit protein-protein interactions. High-throughput screening (HTS) approaches include formats using purified proteins (see AGM chapter Inhibition of Protein-Protein Interactions: Non-Cellular Assay Formats) (1) and those using whole cells. This chapter describes two types of cell-based HTS assays, energy transfer (Förster resonance energy transfer and bioluminescence resonance energy transfer) and protein complementation (fluorescence or enzymatic, e.g. luciferase).

Introduction

Protein-protein interactions (PPI) are a diverse and challenging group of targets for small-molecule discovery efforts (2). PPI interfaces come in a wide variety of sizes (from 4 amino acids to thousands of $Å^2$) and vary greatly in their binding affinities, dynamics, and complexity (from two proteins to tens of proteins in a complex). In order to capture the complexity of the PPI environment and maintain the integrity of the PPI complex, several groups have developed assays to monitor protein complexes in cells.

Cellular PPI assays offer different advantages and disadvantages when compared to biochemical approaches using purified proteins *in vitro*. Biochemical formats offer the advantage that the targets are studied in isolation, and hits from such screens are likely to interact directly with one of the proteins (see AGM chapter Inhibition of Protein-Protein Interactions: Non-Cellular Assay Formats) (1). However, the cellular context is lost in a protein-based assay. In cells, proteins are in their native environment, including discrete subcellular locations and formation of multi-protein complexes. This complexity provides additional mechanisms of interaction for a small molecule, and ensures that active compounds are cell permeable and able to reach the PPI target.

This chapter describes the development of energy transfer and protein complementation screening assays for identifying small molecules that modulate PPI in cells. General introductions to assay development for HTS, cell culture for HTS, and high content imaging can be found in dedicated chapters in this *Assay Guidance Manual*.

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Background

Energy transfer assays include Förster resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET). In a FRET assay, the fluorescence energy from the donor protein is transferred to the acceptor protein, which then emits light; in BRET, luciferase-induced chemiluminescence provides the donor energy that is then transferred to a fluorescent protein or dye. For energy transfer assays, donor and acceptor proteins are fused to each partner of a PPI. Formation of the PPI complex brings the two proteins close enough to undergo energy transfer.

Bimolecular protein complementation assays utilize a reporter protein that has been genetically split into two chains that do not fold into active protein on their own. Each chain is fused to one of the target protein partners; when the protein partners come into contact, the split reporter protein is reconstituted and its fluorescence or enzymatic activity is restored. Protein fragment complementation assays fall into 'indirect' and 'direct' reporter classes. For direct reporters, complementation of the two fragments leads to reconstitution of reporter function – such as fluorescence (for example, GFP) or enzymatic activity (such as luciferase). Indirect reporters trigger additional events that lead to the assay readout, such as transcription via the 2-hybrid assay (3-6). The direct reporter system provides a more proximal measurement of a PPI, and is therefore generally preferred for the discovery of PPI inhibitors.

Table 1 contrasts the energy-transfer and protein-complementation formats. All methods rely on generating fusion proteins with the reporter pairs. The signals that derive from energy transfer and protein complementation require that the two proteins of interest be in molecular contact. These assay formats are distinct from co-localization assays that are used to demonstrate that two proteins are in the same subcellular region.

The selection of a cell-based energy transfer versus protein complementation assay depends on several factors. Due to its relatively low signal and narrow dynamic range, FRET is generally read through an imaging-based assay or flow cytometry; by contrast, enzyme complementation and BRET assays utilize a standard multi-modal plate reader. An important distinction among formats is whether they are reversible. Upon reading out a dynamic process, it is highly advantageous for the reporter signal to also be reversible. FRET, BRET and bimolecular luminescence protein complementation (BiLC) are reversible (7-12), whereas bimolecular fluorescent protein complementation (BiFC), β-galactosidase complementation, and β-lactamase complementation are generally not reversible (6, 13-23).

Table 1: Cell-based PPI assays described in this chapter

Format	Notable features
Förster Resonance Energy Transfer (FRET)	**Read out: high content imaging or flow cytometry** **Benefits: reversibility, suited to live-cell assay** **Limitations: low signal/dynamic range, photobleaching, need to correct spectral overlap**
Bioluminescence resonance energy transfer (BRET, NanoBRET)	**Read out: plate reader with luminescence detection** **Benefits: reversibility, high signal/low background, suited to live-cell assay, cell-permeable chemiluminescent substrates and fluorescent tags available** **Limitations: Older configurations of BRET have too much spectral overlap resulting in narrow dynamic range**
Bimolecular fluorescent protein complementation (BiFC)	**Generally not reversible**

Table 1 continued from previous page.

Format	Notable features
Bimolecular luminescence protein complementation (BiLC)	**Read out: plate reader with luminescence detection** **Benefits: reversibility, high signal/low background, suited to live-cell assay, cell-permeable chemiluminescent substrates available** **Limitations: signals vary depending on fusion constructs and selection of luciferase species/ substrate; compounds can inhibit luciferase directly**
Other enzyme protein complementation (β-galactosidase, β -lactamase)	**Read out: plate reader with fluorescence detection** **Benefits: high signal/low background, suited to live-cell assay, cell-permeable substrates available** **Limitations: generally not reversible**

General Considerations for Developing Cell-Based PPI Assays

Yeast versus mammalian cells: Methods to measure PPI in mammalian cell-based assays are robust and in most cases provide sufficient throughput to serve as primary screening formats. We therefore recommend using cells of the same species as the target PPI. *Saccharomyces cerevisiae* (yeast) has been extensively used to discover protein-protein interactions and has also been used to discover the mechanisms of action and off-target effects of bioactive compounds (24). Several of the assays described below can be used in yeast cells. However, there are some drawbacks to yeast expression of mammalian proteins. First, yeast lacks some proteins that might be required to form multi-protein complexes and might not produce the posttranslational modifications required for some mammalian PPIs. Second, accumulation of some test compounds in yeast is prevented by the expression of multidrug transporters, though yeast strains with increased drug sensitivity are available (25).

Cell type considerations: Any transfectable cell line can be used, but there is an increasing trend towards using cell lines and primary cells that best recapitulate the native biology. For instance, primary astrocytes have been stably transfected and used in a high-throughput screen to detect neuronal cells (26). An important factor for run-to-run reproducibility is to maintain a consistent passage number for cells. We highly recommend that a screen-sized batch of cells are grown and frozen in single-use aliquots prior to initiating the screen.

Transient versus stable transfection: If at all possible, stable transfection is highly preferable to transient transfection. First, stable transfection increases run-to-run reproducibility, which is critical for high-throughput screening (HTS). Second, expression levels can be selected based on resistance markers or FACS-based selection. Higher expression levels are often obtained through transient transfection, which can increase signal strength but might decrease physiological relevance.

Orientation of the protein fusions: In protein-complementation (BiFC and BiLC) assays, steric hindrance can sometimes prevent reconstitution/proper folding of the split protein. For example, GFP N- and C-terminal regions might not be oriented correctly upon protein-protein interaction, and thus fluorescence will not be observed. FRET and BRET efficiencies depend on distance and angle (see **Equations 2 and 3** in the next section), so orientation can also affect energy-transfer efficiency. Therefore, fusions to the PPI partners should be tested in all orientations and combinations to determine the optimal pair. In some cases, the biology of the PPI under study will limit the configuration options. For example, fusion of polypeptides to the C-terminus of the *r*eally *i*nteresting *n*ew *g*ene (RING) domain can inhibit biological function. Additionally, placing a linker (such as a serine/glycine sequence) between the proteins of interest and each half of the split protein is recommended to reduce steric hindrance. Including an epitope tag in this linker region can also be useful for quantifying the relative amount of each fusion partner present in the cell.

Full-length versus fragments of the proteins of interest: There are biological and practical considerations to consider. The use of full-length proteins is recommended when possible, because additional domains remote

from the primary site of interaction can sometimes contribute to binding. However, steric effects or alterations in the stability, expression level, or localization of the proteins are sometimes prohibitive. In this case, fragments rather than full-length proteins might be required for interrogating some PPIs.

Making the system inducible: Consistent expression of the PPI is preferable if the protein pair is constitutively expressed in the native case. On the other hand, there are potential advantages for regulated expression of the fusion proteins. First, if the system is under a doxycycline-regulated promoter (Dox-on), graded expression of the protein can be achieved by titrating in the amount of inducer. This aids assay development, as the minimal amount of fusion protein required to generate sufficient signal can be determined. Second, an inducible system allows cells to be pre-incubated with compounds before expression of the interacting partners. This might increase the number of compounds identified in the primary screen if the PPI is essentially irreversible.

To lyse or not to lyse: Sometimes the term 'cell-based' PPI assay is used when the cells are lysed before the complex is measured. *An important issue with this assay format is that protein-protein and protein-small molecule interactions will re-equilibrate during lysis and dilution.* These assays might therefore be more precisely defined as 'lysate-based' PPI assays. There are situations in which a lysate-based assay is beneficial. For instance, when the PPI is difficult to express in a native form, one could express the interacting protein partners in the cell and prepare lysates before performing the screen. In this way, one can also perform large-scale preparations from cells grown in bulk and generate assay-ready lysate that can be stored. It is also noteworthy that compounds that are not cell-permeable can still be identified through a lysate-based assay. However, if the goal is to screen for PPI inhibitors in whole cells, a non-lytic format should be used.

Demonstrate that the PPI under study drives the complementation and not vice-versa: For fragment complementation assays, it is important to demonstrate that the affinity of the PPI of interest is not significantly altered by the addition of the protein fragments. Control constructs containing point mutation(s) that destroy the PPI can be used for this purpose. If available, a known small molecule inhibitor should be used as a positive control. As with any type of screen, the controls for specificity and selectivity of 'hits' identified in protein fragment complementation screens are critical.

Statistical Considerations: The Z′-factor should be used to evaluate the quality or performance of the optimized assay prior to implementing HTS (27). The Z′-factor takes into account the measurement variability for the high-signal (c^+) and low-signal controls (c^-) in addition to the overall dynamic range of the assay signal as shown below where σ_{c+} μ_{c+} and σ_{c-} μ_{c-} are the standard deviation and mean values for the high-signal and low-signal controls, respectively (**Equation 1**):

$$Z'\ factor = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

An assay with a Z′-factor value between 0.5–1 is considered to be robust with low assay variability and suitable for HTS. Variability within the plate, between plates, and between days needs to be established. Hits should be defined by a threshold of three standard deviations from the baseline and the percent coefficient of variation (in the absence of a positive control) should be less than 10%. For additional information on assay validation see the AGM Chapter HTS Assay Validation.

Assay artifacts and interferences: Prior to selecting an assay for optimization, it is important to carefully consider all potential sources of artifacts and interferences. In comparing multiple assay approaches for high-throughput screening, the primary assay should be selected to minimize susceptibility to interferences, provided that the assay is robust, feasible and of suitable quality for screening. Counter screens can be developed to identify compounds that interfere with the detection method or have intractable mechanisms of action. Additionally, orthogonal assays can be optimized that are susceptible to different types of artifacts compared with the primary assay, and also increase the evidence for on-target activities of hit compounds (see AGM chapters Interferences with Luciferase Reporter Enzymes and Interference with Fluorescence and Absorbance)

(28, 29). Depending on the design and readout, assays have different susceptibilities to artifacts and interferences. The methodologies described in this chapter rely on the measurement of fluorescence and luminescence (luciferase) light as a readout. Test compounds in chemical libraries can interfere with the detection measurement due to autofluorescence properties, scattering of light, or by absorbing light at the excitation or emission wavelengths (inner filter effect) (28, 29). Fluorescence spectroscopic profiling studies of large compound libraries indicated that interference is most prominent in the UV and blue range, whereas it is substantially reduced for red-shifted readouts (30, 31). Therefore one can reduce library compound interferences by simply selecting the most red-shifted detection reagents that are available and applicable. Luciferase assays are susceptible to interference by colored compounds that can quench luminescence as well as enzymatic inhibitors (see AGM chapters Interferences with Luciferase Reporter Enzymes and Interference with Fluorescence and Absorbance) (28-30, 32). It is critical to ensure that the signals being measured are in the linear range of the detection instrumentation and that the appropriate hardware (such as filters and mirrors) are in place. While some interference mechanisms are assay or readout dependent, other mechanisms such as chemical reactivity (see AGM chapter Assay Interference by Chemical Reactivity) (33) and aggregation (see AGM chapter Assay Interference by Aggregation) (34) can affect a wide range of methodologies and should be tested for by appropriate assays during the hit triage process.

Förster Resonance Energy Transfer (FRET)

Introduction

Förster Resonance Energy Transfer (FRET) occurs when energy from an excited fluorophore (donor) is absorbed by another molecule (acceptor). FRET is a powerful method for measuring distances between two fluorophores and is used in unimolecular systems (e.g., the donor and acceptor on the same protein) and biomolecular systems (e.g., the donor and acceptor on two interacting proteins; **Figure 1**).

The underlying principles of FRET were introduced over fifty years ago (reviewed in (35)). Cell-based FRET was first used in flow cytometry (11), but it is also an established format for high content assays and plate-reader formats.

For a FRET assay, the donor and acceptor molecules must be in close proximity to one another (typically 10-100 Å). In addition, there must be overlap between the fluorescence emission spectrum of the donor and the excitation spectrum (usually the same as the absorption spectrum) of the acceptor (**Figure 2**). The extent of overlap is referred to as the spectral overlap integral (*J*). Förster demonstrated that the efficiency (*E*) of the energy transfer depends on the inverse sixth-distance between donor and acceptor (36) (**Equation 2**):

$$E = \frac{Ro^6}{\left(Ro^6 + r^6\right)}$$

Where *Ro* is the Förster distance at which half the energy is transferred to the acceptor and *r* is the distance between donor and acceptor. Förster distance (*Ro*) is dependent on a number of factors, including the fluorescence quantum yield of the donor in the absence of acceptor ($_d$), the refractive index of the solution (η), the dipole angular orientation of each molecule (k^2), and the spectral overlap integral of the donor and acceptor (*J*) (**Equation 3**):

$$Ro = 9.78 \times 10^3 \times \left(\eta^{-4} \times f_d \times k^2 \times J\right)^{\frac{1}{6}} \times \mathring{A}$$

The *Ro* distance is between 30-50 Å for the FRET pairs in Table 2. While this range could be important for large complexes, FRET pairs are generally chosen based on spectral characteristics rather than Ro. Energy transfer leads to a reduction in donor fluorescence and an increase in acceptor fluorescence, and is usually reported as the ratio donor emission/acceptor emission. Cell-based FRET generally uses fluorescent proteins, which have

broad excitation and emission peaks and small Stokes shift (the distance between the emission and excitation wavelengths). It is therefore important to carefully select the donor and acceptor fluorescent proteins to maximize the distance between fluorescence wavelengths of donor and acceptor, and use narrow bandpass filters to avoid counting the overlap between the donor and acceptor (called cross-talk). Crosstalk should be measured and corrected (see below).

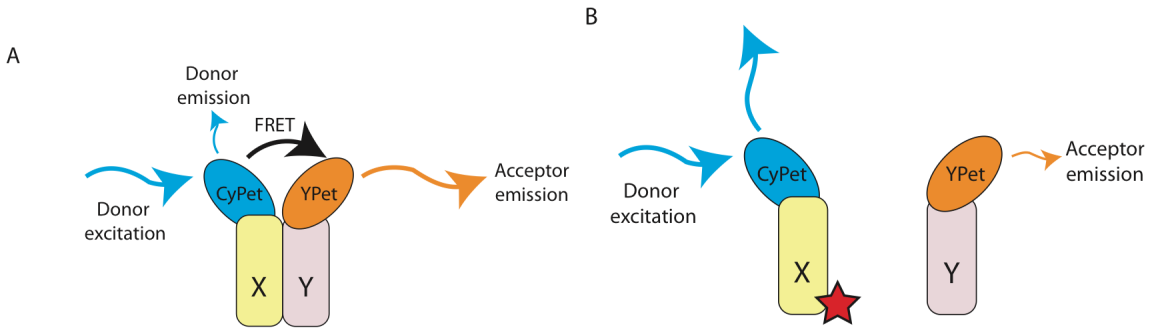

Figure 1. Schematic representation of FRET between two proteins (X and Y) fused to donor- and acceptor fluorescent proteins (CyPet and YPet, respectively). When excited, CyPet emits light, unless that energy is transferred to the YPet acceptor molecule. The YPet then emits light at its characteristic wavelength. If the PPI is inhibited (e.g., by a small molecule, red star) the CyPet donor and YPet acceptor are no longer in proximity, and FRET is reduced.

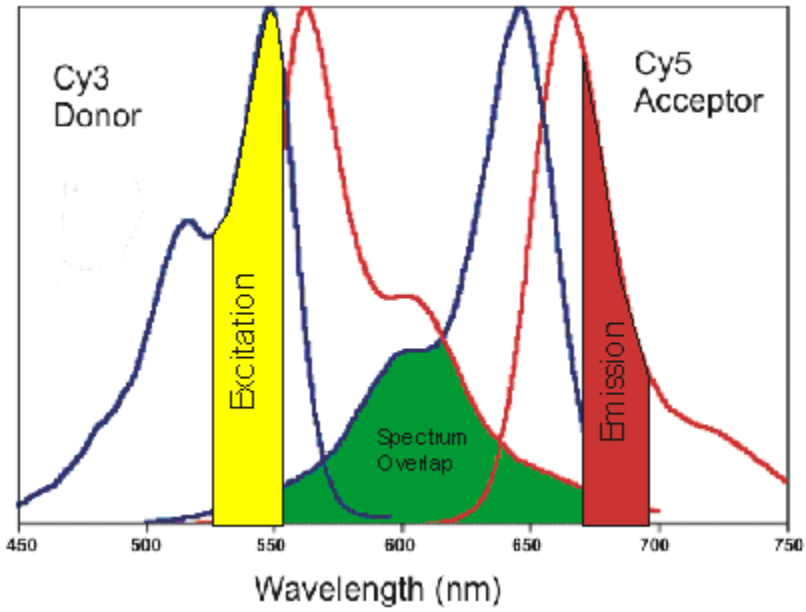

Figure 2. Spectral overlap and FRET. The donor (Cy3) is excited at a maximal wavelength of 550 nm, and emits with light at 650 nm. The acceptor (Cy5) absorbs light at 570 nm and emits at 670 nm. Energy transfer occurs due to the spectral overlap between the emission of the donor and excitation spectrum of the acceptor (shown in green). Crosstalk occurs when donor emission overlaps with acceptor emission, such that the donor emission is counted as FRET; similarly, the acceptor can be directly excited during donor excitation if their absorbance spectra overlap (as they do here).

Table 2. Common Donor/Acceptor pairs for intracellular FRET

Donor (ex/em)	Acceptor (ex/em)	Reference
Sirius (355/424)	mseCFP (434/474)	(62)
mTagBFP (402/457)	sfGFP (485/510)	(63)
CFP (433/475)	eYFP (514/527)	
CFP (433/475)	Venus (515/528)	
mTurquoise2 (434/474)	Venus (515/528)	(64)
CFP (433/475)	mCitrine (516/529)	

Table 2. continued from previous page.

Donor (ex/em)	Acceptor (ex/em)	Reference
mTFP1 (462/492)	mCitrine (516/529)	(65)
eCFP (439/476)	YPet (517/530)	(66)
CyPet (435/477)	YPet (517/530)	
Venus (515/528)	mKOκ (551/563)	(67)
mAmetrine (406/526)	tdTomato (554/581)	(65)
Sapphire (399/511)	DsRed (558/583)	(62)
CFP (433/475)	mRFP (584/607)	(68)
CFP (433/475)	mCherry (587/610)	(64)
eYFP (514/527)	mCherry (587/610)	(64)
Venus (515/528)	mCherry (587/610)	
mOrange (548/562)	mCherry (587/610)	(66)
mKOκ (551/563)	mLumin (587/621)	(67)

Assay Development

Instrumentation**:** For imaging, there are a number of instruments well suited for high-throughput imaging assays (e.g. Molecular Devices' Image Express, GE Healthcare InCell Analyzer, PerkinElmer Operetta). Many commercial plate readers can be used to detect the signal from FRET, and are appropriate for lysate-based assays. These include TECAN's Safire (now TECAN M1000), BMG's PherastarFS, BioTek's Synergy2 and -4, PerkinElmer's Envision.

Plates: Black solid or clear bottom plates are compatible with FRET. For imaging assays, there are plates optimized for microscopy with flat bottom, uniform, and thin plastic wells to minimize distortion (e.g., from Aurora Biotechnologies, Costar, and Greiner).

Cell types**:** FRET has been performed in many cell types including U2OS, HEK293, HeLa, COS-7, CHO, HCT-116. Selecting the most relevant cell type generally leads to identification of more relevant hits.

Buffers**:** For cell-based HTS in intact cells, phenol red-free medium should be used. The medium should be buffered against changes in pH by the addition of HEPES, for example.

Choice of FRET pair: Select the appropriate fluorescent protein pair to use in FRET (see Table 2). Ideal pairs have maximal overlap of donor emission and acceptor excitation, but minimal direct excitation of the acceptor at the excitation maximum of the donor. Historically, CFP/YFP has been the choice for FRET. However, with the continual expansion of the fluorescent protein palette, a large number of additional pairs are now available. Commonly used pairs include modified GFP/Venus (37, 38) and CyPet/YPet (12). Many of the newer pairs contain red-shifted acceptors, as this provides the opportunity for multiplexing with green-shifted acceptors in order to study more than one interaction simultaneously. Red-shifted variants are also advantageous as they can reduce the noise generated by cellular autofluorescence, which is generally in the green range. However, caution should be taken when choosing earlier versions of red-shifted proteins (such as DsRed or mRFP), as they generally have lower quantum yields than green fluorescent proteins.

For measuring PPI on the cell membrane, one can use donor and acceptor labeled antibodies raised against the proteins of interest. This method has been successfully used to detect changes in the interaction between the ERBB1/ERBB2 heterodimer, for example. (39)

Assay Conditions**:** The ratio of the donor and acceptor is critical for accurate FRET. This can be tricky for bimolecular FRET, where two fusion proteins are expressed at different levels or have different stabilities. This might lead to an excess of emission donors that do participate in the PPI, which will in turn mask the true FRET signal. An excess of acceptor can also complicate interpretation of the FRET signal, particularly if the acceptor is susceptible to direct excitation. In addition to expression level, other factors such as pH and temperature (in the case of live cell FRET) can interfere with the assay readout because they alter the rate of acceptor or donor maturation, and thus affect their concentrations.

Data Analysis

The type of analysis performed is linked to the precise FRET methodology used in the screen. In the simplest version, the ratio between acceptor and donor emission following donor excitation is calculated before and after compound addition. In order to overcome the issue of spectral cross-talk (in which the acceptor is excited directly by the donor excitation wavelength, or in which donor emission is present in the acceptor channel), filter cubes with bandpasses very specific for each FRET pair should be used. Ideally, several additional control cell lines should also be used in order to obtain a more accurate measurement of FRET efficiency. These include untransfected cells, and cells that express either the donor or the acceptor FRET partner. Measuring the fluorescence emission in these cells following excitation at donor and acceptor wavelengths provides information that can be used to calibrate the system. FRET is a powerful method but it does require careful analysis of images including measurement of bleed-through from each signal and normalization if the expression levels are different.

Alternatively, where ratio is not 1:1, the acceptor photobleaching (donor dequenching) FRET method might be considered, as the FRET efficiency calculated using this approach is independent of the ratio (see (39) for an example of FRET calculated using this method). In terms of HTS, however, this technique is relatively low throughput, as it generally requires laser scanning microscopy and high magnification, and bleaching times can be in the order of minutes for some photostable proteins.

Fluorescence lifetime microscopy (FLIM) is a third alternative to the measurement of FRET. This technique is independent of the ratio between donor and acceptor, as it only measures changes in donor fluorescence lifetime following illumination. Specifically, participation of the donor in FRET reactions with the acceptor alters the donor fluorescence lifetime, which can then be used to estimate changes in FRET efficiency in the presence and absence of compounds. However, conventional FLIM is unsuitable for HTS as its measurement is time-consuming and requires sophisticated instrumentation. Recently, FLIM microplate readers compatible with SBS standard labware have been introduced on the market (for example, see http://www.fluorescenceinnovations.com/cells.html). Furthermore, the throughput of FLIM has been improved, to give 96 well plate reads of tens of minutes, rather than hours (40). Because spectral bleed-through is not an issue with this technique, additional control cell lines expressing individual FRET proteins are not required. However, quantification of FLIM does require a series of calculations following image segmentation (for examples see (40) and (41)).

Assay Validation Steps

1. Test which orientation of the donor and acceptor fluorescent protein fusions produces the most robust FRET signal (depending on the protein of interest, validation of correct subcellular localization or biochemical activity might also be prudent). Also determine whether full-length proteins or only the interaction domains should be used for the FRET constructs. Inclusion of an epitope tag in the linker between the donor and acceptor fluorescent proteins and the proteins of interest can help determine relative ratio. If one member of the pair is more highly expressed, this protein should be selected as the acceptor because it reduces the potential of free donors contributing to spectral cross talk.

2. In the absence of small molecule positive controls, create point mutants or deletion constructs that disrupt the interaction under investigation.
3. Generate cells in which neither, or only one of the FRET pair is expressed in order to establish the degree of spectral cross-talk and bleed-through from each fluorophore.
4. Select specific filters based on the FRET pair chosen (i.e., it is not sufficient to use a general 'YFP' filter for all of the new yellow acceptor fluorescent protein variants). Use of dual band excitation/emission filters to allow simultaneous read of donor and acceptor emission can reduce variability during plate reads.
5. Optimize the illumination conditions for FRET: low illumination might result in poor signal to noise, whereas strong illumination can lead to photobleaching, both of which will complicate ratiometric analysis.
6. For automated image acquisition, minimize the exposure time for auto-focusing in order to avoid photobleaching effects.

Statistical Considerations for FRET

Enough cells should be prepared in bulk to run the full screen to avoid passage-number variation. Batch-to-batch (day-to-day) variations can also be significant with FRET. Inclusion of the same control on all plates within and between batches is recommended. The same statistics that have been described for other HTS methods apply to FRET-based assays, See the AGM chapter (see the AGM Chapter HTS Assay Validation) (42). See the overall statistical considerations section at the beginning of this chapter.

Sample Validation Data

Example 1. Development of FRET Assay into Quantitative and High-throughput Screening Technology Platforms for Protein–Protein Interactions by Song and colleagues (12). This manuscript describes development of a FRET-based assay for measuring the signaling cascade of SUMOylation using the FRET pair CyPet and YPet. Assay development and a pilot high-throughput screen in 384-well plates is described. Compounds are incubated with the stably transfected HEK293 cells for two hours, before reading the signal in a Molecular Devices FlexStation reader.

Example 2. Förster resonance energy transfer (FRET)-based subcellular visualization of pathogen-induced host receptor signaling by Buntru and colleagues (43). This study measures a protein-protein interaction in intact live cells using flow cytometry measurements. The authors further validated their assay with confocal microscopy and acceptor photobleaching to localize the sites of bacteria-host cell contact. This assay could be adopted for high-throughput screening.

Troubleshooting Guide

No or low FRET: The absence of energy transfer might be related to the actual FRET donor and acceptor constructs. Steric issues might prevent optimal FRET, while some combinations of fluorescent proteins and interacting proteins might be inherently unstable. Check expression of all orientations of N- and C-terminal fusions of fluorescent proteins for both donor and acceptor during assay development. An excess of free donor can reduce apparent FRET efficiency, and in this case one should titrate the FRET pairs during transfection optimization to favor an excess of acceptor. Suboptimal filter sets can significantly compromise FRET; thus the bandpass of the filters should be tailored to precisely match the absorption/emission spectra for the fluorescent proteins. If FRET is being estimated ratiometrically, care must be taken to avoid photobleaching. Reduce illumination intensity using neutral density filters, and illuminate only donor when scanning plates to identify cells to analyze. In order to maximize FRET efficiency, choose partners with minimal excitation of acceptor at maximal excitation of donor, and maximal overlap of donor emission and acceptor excitation.

Low signal-to-noise: Low signal-to-noise can be due to a low FRET signal (see above) or due to fluctuations in the light source. It is critical that the light source is stable for this assay. Another trivial explanation for low signal-to-noise is the mislabeling of donor and acceptor images during analysis.

Ratio is very high or low: In microscopy-based FRET, outliers with very high or low FRET ratios can be due to analysis of out-of-focus images or differences in expression in transient tranfections or pooled transfectants. In this case, different ROIs or cells must be selected and re-analyzed.

High FRET with negative controls: The fluorescent proteins should incorporate mutations to prevent oligomerization (44). If the genetic controls using binding-deficient mutants give a high FRET signal, this might indicate that the fluorescent proteins are oligomerizing and driving a non-specific interaction. Alternatively the expression level of the FRET pair might be too high and in this case re-optimization of expression is required. Also one should examine the signal overlap between donors and acceptors. Another possibility is that the interaction under investigation is more complex than originally thought. The investigator should validate the sequences of plasmid DNAs to confirm the presence of disruptive mutations.

Bioluminescence Resonance Energy Transfer (BRET)

Introduction

Bioluminescence Resonance Energy Transfer (BRET) is also a proximity-based assay that measures the energy transfer from a donor to an acceptor, in this instance luciferase is used as the donor, and a fluorescent protein as the acceptor (10). The NanoBRET method employs a NanoLuc fusion protein as the bioluminescent donor and a fluorescently labeled HaloTag fusion protein as the acceptor (**Figure 3**). The optimized blue-shifted NanoLuc donor paired with the red-shifted HaloTag acceptor minimizes spectral overlap, resulting in low bleed-through and improved signal-to-background and dynamic range. The extremely bright and stable NanoLuc® also allows for expression of the donor at or near physiological levels further reducing background and improving sensitivity.

Assay Development

Instrumentation: To measure BRET assays, an instrument capable of sequentially measuring dual-filtered luminescence values equipped with appropriate filters is required. For the NanoBRET configuration the ideal filter setup will include a band pass (BP) filter centered around 460nm to measure the donor signal (Ex. Emission 450nm/BP 80nm) and a long pass (LP) filter starting at around 600–610nm to measure the acceptor signal (Ex. Emission 610nm/LP). Filters outside of these ranges will miss critical measurements and compromise data quality. Commercial plate readers pre-equipped with the proper filter set up include Promega's GloMax Discover and BMG's CLARIOstar. Instruments that can be equipped with optional filters include Thermo's Varioskan (Edmunds Optics filters: donor 450nm CWL, 25mm diameter, 80nm FWHM, Interference Filter and acceptor 1 inch diameter, RG-610 Long Pass Filter) and PerkinElmer's EnVision (Chroma filters: Emission Filter (for EmSlot4) Cat. # AT600LP and Second Emission Filter (for EmSlot1) Cat. #AT460/50m).

For instruments using mirrors, select the luminescence mirror. An integration time of 0.2–1 second is typically sufficient. Ensure that the gain on the photomultiplier tube is optimized to capture the highest donor signal without reaching instrument saturation.

Plates: White tissue culture grade plates are preferred. Black plates can quench luminescence. Formats include 96 and 384 wells. 1,536 well formats might require optimization and a specialized instrument.

Cell types: NanoBRET can be performed in any cell line that can be transfected including HEK293, HeLa, HCT-116, NIH3T3, CHO, and Jurkats. For difficult to transfect cells, optimization might be required.

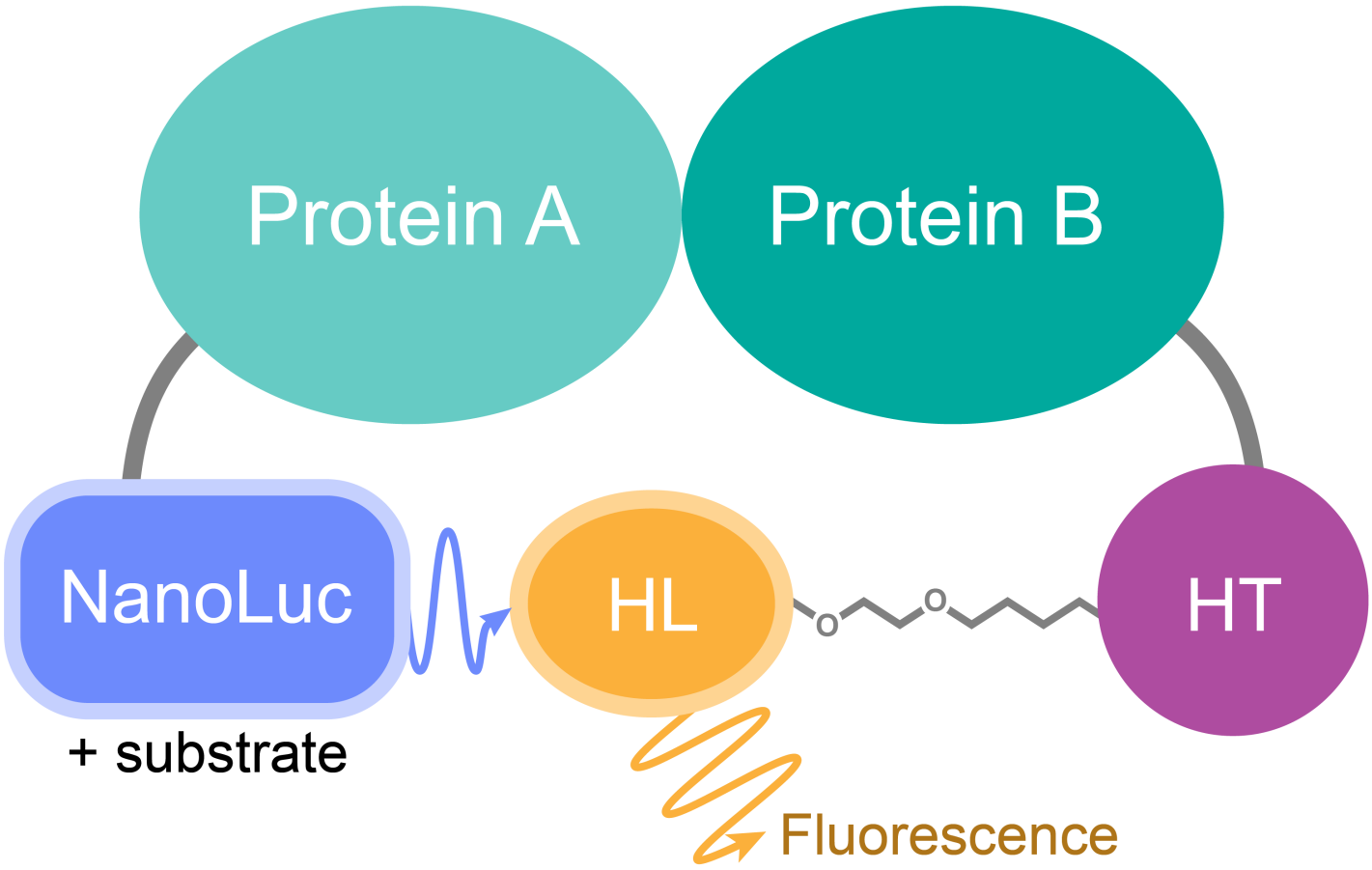

Figure 3. Overview of the NanoBRET assay principle. Energy is transfered from a NanoLuc-Protein A fusion (energy donor) to a fluorescently labeled HaloTag-Protein B fusion (energy acceptor) upon interaction of Protein A and Protein B.

Generation of stable cell lines expressing one or both protein partners is highly preferred, for the reasons described above.

Media: To avoid interference with the acceptor signal, phenol red-free medium should be used. Reduced serum media such as Opti-MEM + 4% FBS is recommended. The use of higher amounts of FBS can increase variability in 384 and 1,536 well formats.

Assay conditions: Find the optimal ratio of donor and acceptor expression levels to minimize unbound donor, which reduces background and maximizes dynamic range (see Assay Validation Steps below). Cells are transfected and grown under typical tissue culture conditions. Because the HT protein is not intrinsically fluorescent, a set of samples without ligand can be plated and used as a background control. The non-lytic assay is completed by the addition of the NanoLuc substrate furimazine and donor and acceptor signal measurements.

Kits and reagents: Kits for generating NanoLuc and HaloTag fusion clones and kits containing HaloTag ligand and NanoLuc substrate reagents are available from Promega.

Data Analysis

It is critical to use the appropriate filters to capture the specific donor and acceptor signals for NanoBRET, generic filters for other BRET configurations might not be applicable.

The donor emission occurs at 460nm, a band pass (BP) filter that covers close to 460nm with a band pass range of 8–80nm is preferred. A short pass (SP) filter that covers the 460nm area also can be used but it might result in an artificially large value for the donor signal measuring the bleed-through into the acceptor peak. This artificially large value could compress the ratio calculation and reduce the assay window. The acceptor emission occurs at 618nm. To measure the acceptor signal, a long pass filter starting at 600–610nm is preferred.

To calculate the BRET ratio the acceptor signal is divided by the donor signal (**Equation 4**):

$$\frac{618\ nm_{Em}}{460\ nm_{Em}} = Raw\ NanoBRET\ ratio\ = BU$$

To convert raw BRET units (typically decimal values) to milliBRET units (mBU; whole numbers), multiply each raw BRET value by 1,000 (**Equation 5**):

$$\frac{618\ nm_{Em}}{460\ nm_{Em}} = BU \times 1000 = mBU$$

To account for any background contribution, the mean ratio from the no ligand control samples is subtracted from the ligand containing samples to yield a corrected ratio (**Equation 6**):

$$Mean\ mBU(experimental) - Mean\ mBU(no\ ligand\ control) = Mean\ corrected\ mBU$$

Assay Validation Steps

1. Determine the donor and acceptor configuration with the optimal distance and geometry for efficient energy transfer between the protein partners. There might be instances when there is prior knowledge of the protein biology that would prevent a certain terminus from being tagged. If no such constrains exist, both proteins of interest can be tagged with either NanoLuc or HaloTag on either the N- or C-terminus resulting in up to 8 possible constructs and 8 possible combinations. Full-length proteins or protein domains might be used. Test the various combinations in the assay to see which one(s) give the most robust ratio(s).
2. Optimize transfection conditions for the top pair configuration(s). This is done by finding the ratio of donor to acceptor DNA that minimizes unbound donor, reducing the background and maximizing dynamic range. Generally, the amount of HaloTag DNA is kept at a higher concentration while the NanoLuc DNA is reduced to $1/10^{th}$ to $1/1{,}000^{th}$ relative to the amount of HaloTag DNA.
3. If a known modulator of the interaction is available, either an inhibitor or an activator, confirm the proper biological response. In some cases the highest possible ratio pair might not always equate the most robust response to the modulator. It is advisable to test more than one combination to find the most responsive to the expected biology. In addition, some small molecules might have been developed against a particular isolated domain or using *in vitro* assays and might not elicit the same response when using full-length proteins or inside the cell.
4. If no known modulator is available, donor saturations assays (DSA) might be performed to show assay specificity (8). In a DSA, the amount of NanoLuc donor DNA is kept constant while steadily increasing the amount of acceptor DNA. As the acceptor-to-donor (A/D) ratio increases, a specific BRET assay will show ratios that increase in a hyperbolic manner and reach a plateau representing complete saturation of all donors with acceptor molecules. Non-specific interaction, as it can be tested with a negative control protein, will generate much weaker ratios that plot in a linear manner known as bystander BRET (**Figure 4**).

Statistical Considerations for BRET

The ratiometric nature of the BRET assay intrinsically results in low variability. Use the corrected mBU and standard deviation values to calculate the Z'-factor (see **Equation 1**).

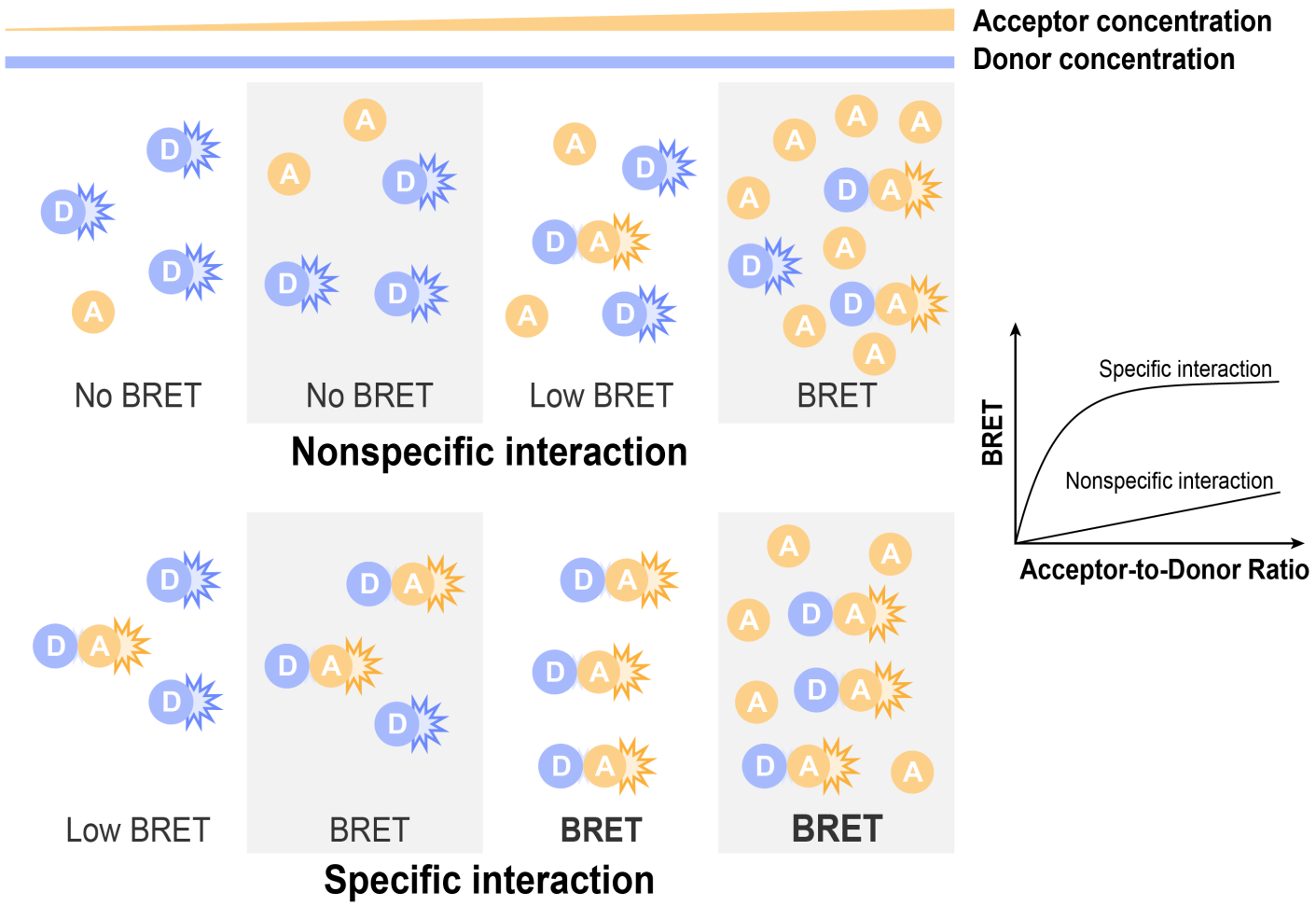

Figure 4. Principle of the Donor Saturation Assay (DSA) to validate BRET assay specificity.

Sample Validation Data

Example 1. NanoBRET—A Novel BRET Platform for the Analysis of Protein–Protein Interactions by Machleidt and colleagues (9). In this example, the authors describe the development of the NanoBRET configuration of BRET and its application to a novel assay developed for analyzing the interactions of bromodomain proteins with chromatin in living cells.

Example 2. Generation of a Selective Small Molecule Inhibitor of the CBP/p300 Bromodomain for Leukemia Therapy by Picaud and colleagues (45). The authors report the development and preclinical evaluation of a novel, potent inhibitor targeting CBP/p300 bromodomains that impairs aberrant self-renewal of leukemic cells. This is a good example of a small molecule that when studied in live cells, exhibits differential inhibitory profiles in full-length proteins vs. individual protein domains

Troubleshooting Guide

No or low BRET:

a. Ensure filters are specific for NanoBRET: 460nm (with 8–80nm band pass) for donor signal and 600–610nm long pass for acceptor signal. Make sure the gain on the photomultiplier tube is set to detect donor

signal without instrument saturation. Confirm ratio calculation is acceptor divided by donor values (618nm/460nm).

b. Test all possible donor/acceptor combinations to find best pair.
c. Ensure protein expression by checking luminescence for NanoLuc constructs. Expression for HaloTag constructs can be checked by fluorescently labeling with a HaloTag ligand such as TMR which can then be run on SDS-PAGE and analyzed using a fluorescence scanner.
d. Optimize transfection conditions and relative amounts of donor and acceptor DNA. Excess donor will compress assay window.
e. Some PPI might be dependent on specific biological events or stimuli to activate specific pathways. Ensure proper pathway activators are added. If possible, check proper phenotypical responses by other means.
f. Confirm cell health by a viability assay (see AGM chapter Cell Viability Assays).
g. Absolute raw values and ratios will vary among PPI systems. Absolute BRET values depend on the proximity of the protein partners, the affinity of the interaction, the relative occupancy with other interacting proteins and the instrument setup. When possible, check specificity with a known modulator such as an inhibitor or by the Donor Saturation Assay (DSA).

High variability: Optimize all parameters of the assay aiming for Z'-factors between 0.5 and 1. If the assay parameters have been optimized, consider automated dispensing to reduce variability.

Unable to detect proper expected biology in the presence of small molecule: Increase the concentration of small molecule, treat overnight or both to see maximum effect. If the compound was developed *in vitro* against the domain or region alone, it might not disrupt the interaction of the full-length proteins. Test region or domain alone versus full-length protein. Always consider the possibility that the compound might affect the luciferase activity without actually disrupting the PPI (see next section).

Fluorescent Protein Fragment Complementation

Introduction

Direct reporter complementation assays have split reporter proteins fused to the protein partners of interest; formation of the PPI causes reconstitution of the reporter protein and induction of fluorescence or enzymatic activity (**Figure 5**).

The use of split GFP as a PPI reporter was first described by Regan and colleagues (46). In this method, a GFP variant was separated between residues 157 and 158 into two polypeptides, each of which were fused to interacting leucine zippers. Reconstitution of fluorescence was not observed when the GFP fragments were co-expressed without the leucine zippers, indicating that PPI could be reliably detected using this method. GFP and additional fluorescent proteins were rapidly adopted for the study of PPIs. Hu and Kerppola extended the use of split GFP to measure protein-protein interactions in mammalian cells (16). This so-called '*Bi*molecular *F*luorescence *C*omplementation' (BiFC) has been extended to many other fluorescent proteins, greatly expanding the potential combinations that could be applied *in vivo*. BiFC offers some advantages over other fluorescence-based methods for PPI detection. For example, FRET requires careful control of the ratio between interacting partners, and is also less sensitive than BiFC due to the presence of background fluorescence emitted by the acceptor fluorophore (**Section 2**) (47). Many BiFC pairs have been demonstrated (**Table 3**).

Because the primary amino acid sequence of different fluorescent proteins varies at only a few residues, it is possible to 'multiplex' BiFC. For example, the C-terminal amino acid sequences of GFP, YFP, and BFP are identical after amino acid 155, whereas their N-terminal sequences differ at several residues. In this way, the interaction of one protein with 2 potential partners can be interrogated simultaneously. For example, interaction of the bJun transcription factor with its binding partners bFos and bATF2 has been demonstrated (16).

Despite its versatility, BiFC does suffer from some limitations. First, refolding of the fluorescent chromophore fragments is essentially irreversible (48, 49). While this might be advantageous for detecting weak or transient PPIs, the irreversible nature of BiFC in its present form greatly complicates its application as a discovery platform for inhibitors of PPIs. To get around this issue, one can engineer an inducible BiFC reporter (using a doxycycline-responsive promoter, for example); the reporter cell lines can then be incubated with compounds prior to expression of the BiFC constructs, providing a time window in which their activity can be detected.

A second issue with BiFC (and other complementation assays in general) is that the signal intensity, compared to that achieved with the full-length protein, is often orders of magnitude lower. Although the background of BiFC is low, the reduced signal can sometimes reduce the robustness of the HTS assay. Variants of Venus (a YFP derivative) have been reported to significantly improve the signal/background for complementation (13).

Finally, a fluorescence-maturation step follows re-association of the split chromophore. In some cases, this maturation is temperature-dependent; the BiFC signal from some YFP fragments and from mCherry is greatly enhanced if the temperature is lowered to 25-30 °C prior to readout (13, 17). This change in temperature presents an additional logistical consideration and potential source of variability for BiFC-based HTS campaigns.

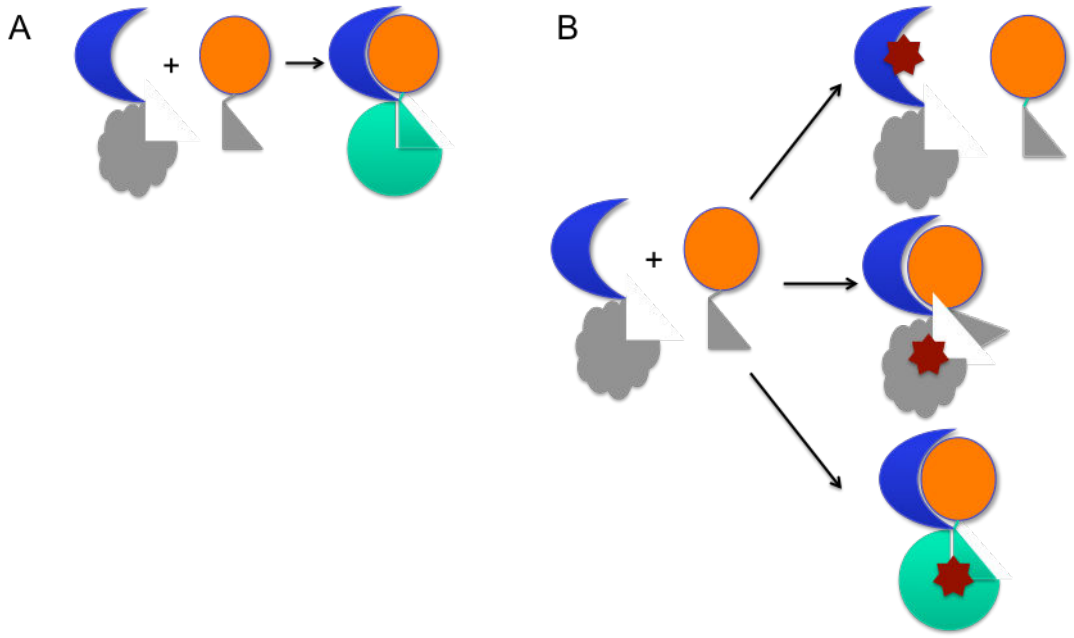

Figure 5: Principle of protein fragment complementation. (A) Two interacting proteins (blue) are fused to N- and C-terminal fragments of a reporter protein such as GFP or luciferase (gray). Following interaction, the reporter is reconstituted (green). (B) A small molecule (red star) can affect the reporter signal in several ways. The molecule might bind to and specifically inhibit the PPI under interrogation, a 'true positive' (top). Alternatively, false positive compounds could interfere with reconstitution of the reporter protein (middle) or block the catalytic activity of the reporter (bottom). Secondary screens are required to identify such false positive events.

Table 3. Examples of BiFC partners

Fluorescent Protein	N-terminus	C-terminus	Comments	Reference
GFP	1-157	158-238	Utilized a brighter variant of wtGFP	(69)
Venus	1-154	155-238	A brighter variant of YFP	(13)
Venus	1-210 (V150A)	210-238	Additional mutations added to Venus to improve S/N ratio	(14)
YFP	1-154	155-238	Increasingly replaced by other 'yellow' variants (Citrine and Venus) due to sensitivity to high temperatures	(15)
CFP	1-154	155-238		(16)
mCitrine	1-172	173-238		(70)
mCherry	1-159	160-237	Sensitive to high temperatures, requires longer maturation times	(17)
mLumin	1-151	152-233	Brighter mKate variant, less sensitive to high temperature compared to other red variants	(71)

Assay Development

Chromophore selection**: Table 3** lists common fluorescent proteins used in split constructions. Each reference contains additional variations in terms of the location of the split, and point mutations to improve fluorescence emission or stability in the BiFC application. Splitting chromoproteins can also slightly alter the maximum excitation and emission spectra compared to the full-length proteins, and wavelength scanning to identify the optimal excitation and emission is therefore recommended when using new BiFC pairs.

Instrumentation**:** BiFC can be measured on high content fluorescence microscopes (e.g., Molecular Devices Image Express and Image Express Ultra, GE Healthcare INCell Analysts 2200 or 6000, or the PerkinElmer

Operetta or Opera) or flow cytometry (e.g., BD Biosciences, Intellicyte). If the fluorescence intensity is sufficiently high, the assay might be readable on a fluorescent plate reader (e.g., TECAN Safire^2 or M1000, BMG Pherastar, BioTek Synergy series, Molecular Devices M5 or Flexstation, PerkinElmer Envision). Reading fluorescence from the bottom of the well is less sensitive to the effects of fluorescence interference from the medium, but top reading plates with a z-plane optimization function can also be used. If a new BiFC reporter is being evaluated, monochromator-based readers can be useful to determine the peak emission of the reporter during assay development.

Plates: Due to the fluorescent readout, black-walled plates are recommended. Clear bottom plates, such as µClear plates from Greiner, are suitable for imaging or for detecting fluorescence from the bottom of the plate.

Cell types: a variety of cells have been used for BiFC assays, including U2OS osteosarcoma, HEK293, COS-1, HeLa and NIH3T3. A primary consideration for high content imaging applications is that the cells should adhere well to the plate. For plate-reader based assays, it might be necessary to perform multiple reads per well (several plate readers allow reading at different spots within a well) to compensate for unequal cell distribution.

Buffers: Cells can be grown in the medium recommended by ATCC. However, for maximal signal (especially when using GFP) it is preferable to use phenol red-free and low-glucose medium in live cells, or switch to PBS if fixed cells are used. These solutions can overcome some of the issues associated with autofluorescence coming from medium components. Fixation should be optimized to reduce quenching of fluorescent proteins.

Assay Conditions**:** In general, lower temperatures (between 4 °C and RT) favor fluorescence complementation. However, fluorescence is reconstituted in many BiFC pairs (for example, mCherry) following incubation at higher temperatures (RT to 30 °C). With newer versions such as Venus, the temperature dependence might be less critical. The temperature and time of incubation for each BiFC pair must be determined empirically, and binding-deficient mutants should be included to control for non-specificity of the BiFC reporter. This is particularly important at lower temperatures, where complementation can occur independently of the proteins to which the fragments are fused. Following completion of the screen, cells might be visualized live, or can be washed in PBS and fixed in 4% paraformaldehyde and stored protected from light until examination. Include additional negative control wells where possible (medium in the absence of cells, for example) to determine the background auto-fluorescence.

Data Analysis

General considerations: Stably transfected cells are highly preferable due to the improved run-to-run stability and ability to subsequently separate cells with the desired expression profiles. If the BiFC screen is performed by transient transfection, however, it is critical to co-transfect a full-length fluorescent protein that is spectrally distinct from the BiFC pair as an internal control for transfection efficiency. In this way, the BiFC signal can be normalized to the full-length fluorescent protein signal on a per cell basis, and the results plotted as a histogram. Ideally, the control protein is expressed from the same plasmid as the BiFC pair; otherwise, not all cells will receive both the BiFC and the full-length fluorescent protein with the same ratio. During assay development, plates should include cells with genetic controls for non-interacting mutants (e.g. N- and C-terminal fragments of the fluorescent protein fused to point or deletion mutants of one of the interacting pair). These constructs can be used to validate that PPI formation drives BiFC and set background readings for the assay.

Imaging-based assays: a nuclear stain (e.g., Hoechst 33342) is added to live or fixed cells. Plates are imaged at the appropriate resolution (typically 10x) with the appropriate fluorescence filters for the nuclear stain and fluorescent protein. Cells are identified by nuclear stain and by the intensity and localization of the fluorescent protein. Fluorescence can be monitored in several ways, such as intensity/cell, number of cells above an appropriate threshold, or number or intensity of puncta/cell. The most reproducible and robust measure should be used to calculate the signal for high-signal and low-signal controls and to determine Z'-factor values (see

Equation 1). Normalization of fluorescence to cell number is important to account for inconsistencies in cell plating or cell-growth and to identify toxic compounds. Dead or dying cells can be excluded by morphological criteria (e.g., cell rounding, nuclear condensation) during image segmentation.

Plate-reader based assays: If a plate reader is used for the BiFC measurement, readings are best performed in cells that have been washed to remove dead and floating cells that can give confounding bright fluorescent signals. Normalization of the BiFC to cell number is important to exclude false positives that are toxic. With a genetically engineered system, this normalization can be achieved using an IRES driving expression of a second fluorescent protein or luciferase. Alternatively, a fluorescent indicator of cellular viability (such as resazurin or CellTiter-Fluor) can be used as an indirect measure of cell number. Care should be taken to ensure the spectral overlap between this marker and the BiFC pair is minimal.

Assay Validation

Note: many of the assay validation considerations are similar to those described for BRET.

Optimal protein pair configuration: Determine the optimal orientation of the fusion proteins for both partners testing both N- and C-terminal orientations (total of 8 combinations). Linker design is also a critical factor at this stage; Gly-Ser rich sequences of varying lengths, as well as RPACKIPNDLKQKVMNH and AAANSSIDLISVPVDSR have been used successfully. In order to validate expression by western blot, an epitope tag such as HA or FLAG can also form part of the linker.

Assay validation controls: Known small-molecule inhibitors are ideal, but if no such modulator is available, design a negative control construct where one of the interacting proteins is mutated to disrupt binding. Alternatively, donor saturation can be used (see Assay Validation Steps for NanoBRET).

Optimize expression: Optimize transfection conditions by varying the ratio of the donor and acceptor plasmids. Determine the optimal temperature and time following induction to yield maximal fluorescence. Ideally, fast maturing fluorescent proteins should be chosen, although due to the split, the maturation times for the BiFC pairs might differ from the full-length fluorescent proteins. If the assay is to be performed transiently, titrate the BiFC pair in order to give the most stable signal with the lowest expression of protein. Also establish the shortest time at which the signal is stable, to avoid issues associated with overexpression. Perform both microscopy and plate reader based analyses at this step. Note that the level of fluorescence from split fluorescent proteins is far less than the full-length counterparts, so adjust gain accordingly. Maximal emission might be slightly offset compared to the full-length protein, which must be taken into account when selecting filters. Determine whether the subcellular localization (and if possible biochemical activity) of the BiFC pair is consistent with the biology. For a multiwell format and especially if the reading will be high content, optimize cell seeding density. If individual cells need to be segmented, then aim for a final confluence at measurement of around 50-60%.

Determine the timing of compound addition: Because BiFC for most (if not all) fluorescent proteins is irreversible, it is recommended that compounds are added either before or concomitantly with introduction of the BiFC pair. Set high-signal (vehicle only) low-signal (known inhibitor, mutated PPI pair, etc.) controls.

Statistical Considerations for Fluorescent Protein Fragment Complementation

For an assessment of assay robustness, the Z'-factor can be used (see **Equation 1**). Ideally the low-signal control should be a known small molecule inhibitor of the PPI under investigation. Otherwise, the non-interacting genetic control can be substituted, although this might give an artificially low BiFC signal compared to actual small molecule inhibitors. For initial hit determination, each sample can be compared to the plate-based mean BiFC signal, with a cutoff defined as between 1 and 3 x σ from the mean, depending on the assay robustness. Secondary screens should be in place in order to test for selectivity (for instance, a different PPI using the same

BiFC fluorescent reporter) and for assay interference (for example, test the putative hits against the original PPI, but using split luciferase instead of BiFC).

Sample Validation Data

Example 1. Drug screening for autophagy inhibitors based on the dissociation of Beclin 1-Bcl2 complex using BiFC technique and mechanism of eugenol on anti-influena A virus activity by Dai and colleagues (50). The goal of this study was to identify compounds that disrupt the interaction between Beclin and Bcl2, thereby increasing autophagy. BiFC used mCherry (N-terminal mCherry aa1-169-Bcl2 and C-terminal mCherry aa160-262-Beclin1). For 96-well plate assays, A549 cells were transfected with BiFC constructs before compound addition for 8 h. The cell-based assay using a microplate reader gave a Z'-factor of 0.5. Known autophagy inhibitors (ERK inhibitor, MAPK inhibitor) increased the BiFC signal, whereas activators (anisomycin, H_2O_2) reduced the signal.

Troubleshooting Guide

No or low BiFC signal: Check all orientations of the fusion proteins and linker lengths to find maximal signal and check for expression by western analysis. Ensure that the filter is correct; this can also be optimized by performing a wavelength scan on monochromatic instruments. Determine the optimal temperature for the maturation of the fluorescent BiFC construct (a range is from 4 °C for 1 h through 37 °C). Due to the lower fluorescence intensity of split fluorescent proteins, reads of multiwell plates should be performed in optically clear medium, and might require significant optimization of the photomultiplier tube gain.

BiFC signal not affected by positive controls: The level of the BiFC constructs might be too high. In that case, switch to weaker promoters, shorter incubation times, or use a lower dose of the inducing agent if using an inducible system. Pre-dose cells with compound prior to expression of the BiFC constructs. Also, older fluorescent proteins used for BiFC can be more prone to self-assembly. Use newer variants such as Venus (V150A mutant, split at 1-210 and 210-238) (14).

Luciferase Enzyme Fragment Complementation

Introduction

*Bi*molecular *l*uciferase *c*omplementation (BiLC) is proving to be a powerful tool in many discovery projects (51). For example, PPIs involved in pathways associated with cancer (52, 53), Alzheimer's disease (54) and plant signaling (55) have all been interrogated with this method. Compared with BiFC, luciferase complementation has three distinct advantages. First, the interaction between the two fragments does not require covalent bond formation, and is therefore completely reversible (49). Second, background luminescence is extremely low because the overlapping luciferase fragments, have no activity on their own, and can reassemble into an active enzyme when the proteins they are fused to are brought into close proximity by the interacting proteins of interest. In addition, the affinity of the split luciferase fragments for each other is much weaker than that of the interacting proteins of interest to which they are fused. Finally, the enzymatic activity of luciferase combined with a very low background leads to robust assays when evaluated by common screening metrics such as the Z'-factor (27). Several groups have also reported the use of BiLC in xenograft studies in mice (7, 53, 56), opening the possibility of further characterization of lead compounds during preclinical studies. **Table 4** highlights the many flavors of BiLC. Some of these enzymes require ATP (e.g., firefly), while others do not (e.g. Renilla).

As discussed above for fluorescent protein fragment complementation assays, BiLC can also be multiplexed. Multiplexing is possible because the N-terminus within a particular luciferase family determines it spectral characteristics, whereas the C-terminus contributes the enzymatic function (57, 58). Ozawa and colleagues (52) have identified mutations in the C-terminus of click beetle red (CBR) that allow complementation with N-

terminal fragments of firefly, CBR and click beetle green (CBG) luciferases. Thus, the interaction of a 'bait' protein (fused to the C-terminus of CBR) with two competing proteins (each of which is fused to a different N-terminal luciferase fragment) can be interrogated. In a second study, complementing CBR and CBG fragments were rationally designed using a 'bait' fused to the C-terminus of CBG and prey fused to either the N-terminus of CBG or CBR (59).

The multiplexed BiLC setup allows the detection of multiple PPIs using one common substrate (D-Luciferin or commercial reagents), but also requires careful selection of imaging medium, luminescence emission filters, and software to perform spectral deconvolution of the data. In terms of HTS, the time taken to image each microwell plate will depend on the total photon output, which in turn will be determined by the expression level of the constructs and the strength of their interaction.

BiLC also has some limitations, such as sensitivity to false positives (inhibitors of luciferase enzymatic activity, for example) and false negatives (such as autofluorescent test compounds). Additionally, as with all protein fragment complementation assays, the choice and orientation of fusion partners might be limited to some extent by steric effects. Controls for normalization of cell number or transfection efficiency are also critical in this assay. Suitable controls include co-transfection or IRES-driven expression of a reporter such as Renilla luciferase or fluorescent protein. Finally, because the chemiluminescent reaction requires molecular oxygen, this assay might be affected in HTS screens performed at low oxygen concentrations or those performed in strictly anaerobic organisms.

Assay Development

Instrumentation**:** Many commercial multimodal plate readers can be used to detect the signal from BiLC. These include Promega's GloMax, TECAN's Safire^2 (now TECAN M1000), BMG's Pherastar, BioTek's Synergy series, PerkinElmer's Envision.

Choice of luciferase species: There are multiple choices for luciferase species. Click beetle luciferases show little color sensitivity to pH and temperature changes as opposed to firefly luciferase, in which the maximal emission color can vary by as much as 40 nm under different reaction conditions (59).

Plates: Solid white plates (for example Corning 3570) are recommended. If the assay is performed with live cells and is to be combined with a fluorescence readout, then black walled Greiner μClear plates can be used.

Cell types**:** BiLC has been performed in many cell types including U2OS, SaOS-2, HEK293, H4 neuroglioma, HeLa, COS-7, CHO.

Buffers**:** For kinetic BiLC studies in intact cells, phenol red-free medium containing 100 μM D-luciferin (made from a concentrated stock solution) should be used. The medium should be buffered by the addition of HEPES, for example. For endpoint assays in which the cells are lysed, phenol red-free medium is still recommended.

Kits: For large numbers of plates in HTS format, commercially available luciferase detection reagents can be beneficial due to the batch-to-batch consistency. Although these reagents are frequently added at a 1:1 (reagent:medium) ratio, lower ratios still produce robust signals and can be optimized during assay development.

Titration of reagents: In a 384-well format, a cell density between 2000 and 15000 per well is recommended, depending on the cell type. This is based on a stable cell line system; if the assay will be performed using transient transfection, the optimal cell density might differ.

Assay Conditions**:** If stable cell lines with constitutive expression are used, it is important to screen several clones and pick those that exhibit a robust signal with the minimum amount of overexpression**.** An alternative (but more labor-intensive) option is to develop a bidirectional, doxycycline-inducible reporter system, where each

pair of the fragment complementation module is expressed following addition of doxycycline to the medium. This can aid in control of the level of expression, permit compounds to be added before protein complexes are formed, and restrict the expression of potentially toxic fragment pairs.

Timing: the optimal time for detection of luminescence will vary according to the experimental setup. For example, if a doxycycline inducible system is used, robust signal can be detected 6-10 h following the addition of doxycycline. One advantage of a short incubation time is that secondary compound effects, such as cytotoxicity, are likely to be avoided.

As with conventional luciferase assays, it is important to equilibrate the plates to room temperature for up to 30 min before reading. This improves plate Z'-factors. In comparison with full-length luciferase, the window for detection of the BiLC signal might be significantly shorter when using reagents such as SteadyGlo. Therefore during assay development the time post-lysis at which optimal luciferase activity is detected must be determined. This will dictate the number of plates that can be processed per unit time while maintaining acceptable plate metrics.

Data Analysis

Using real-time analysis of click beetle green and click beetle red luciferase heteroprotein fragment complementation, the hybrid proteins rapidly and directly reconstitute an enzymatic reporter activity within the cell, offering the potential benefit of real-time analysis and enhanced sensitivity for detecting weakly interacting proteins.

Assay Validation Steps

To validate the assay, well-characterized binding partners would be useful. For example, the rapamycin-binding domain of human mTOR (residues 2024–2113) and human FK506-binding protein-12 can provide a well-characterized macromolecular interaction platform to validate protein fragment complementation pairs. In the case of Villalobos et al., the N-terminal fragments of all luciferase PCA constructs were each fused to a flexible G-S linker and the C-terminus of the rapamycin-binding domain of human mTor, while the C-terminal fragments of all constructs were each fused to a flexible G-S linker and the N-terminus of FK506 binding protein-12 (59). Subsequently, HEK293T cells were used for the validation and the protein interactions were analyzed by live cell bioluminescence imaging.

It is important to establish that the half-life of the protein of interest was unaffected by the addition of the fusion protein. To rule out that any change is related to a change in the half-life of the protein, monitor the stability of the protein complex in real time using a HeLa TetOn stable line expressing the two binding partners. Ilagan et al. used the luciferase complementation activity in live NΔE-NLuc/CLuc-RBPjκ-expressing cells that was monitored continuously for 6 h in the presence or absence of the protein translation inhibitor cycloheximide (60). The decay in bioluminescence indicated a Notch intracellular domain half-life of ~180 minutes, consistent with the half-life obtained by pulse-chase experiments in HeLa cells.

Another way to validate PPI assays is to use known inhibitors of the interaction if they are available. This allows the demonstration that the complementarity can be inhibited. Without the availability of a known inhibitor, a peptide can be selected that will block the two complementary fragments.

Statistical Considerations for Luciferase Enzyme Fragment Complementation

The investigator should calculate the Z´-factor (see **Equation 1**) to gauge assay quality and suitability for HTS (27). For the low-signal control, omission of the substrate, an inhibitor (small molecule or peptide), or the cell line expressing only one of the complementary fusion proteins can be used.

Sample Validation Data

Example 1: Dual-color click beetle luciferase heteroprotein fragment complementation assays by Villalobos and colleagues (59). This paper describes a set of reversible, multicolored heteroprotein complementation fragments based on the click beetle luciferases that utilize the same substrate as firefly, D-luciferin. Using luciferase fragment complementation a dual-color quantification was performed with two discreet pairs of interacting proteins simultaneously or two distinct proteins interacting with a third shared protein in live cells with real-time analysis. The proteins examined were β-TrCP, an E3-ligase that regulates both β-catenin and IκBα, and GSK3β as the kinase that regulates IκBα processing.

Example 2: Real time imaging of notch activation with a luciferase complementation reporter by Ilagan and colleagues (60). This paper measures ligand binding of Notch with a luciferase complementation assay. Notch receptors undergo ectodomain shedding followed by γ-secretase-mediated release of the Notch intracellular domain. The Notch intracellular domain translocates to the nucleus and associates with the DNA-binding protein CBF1/RBPjκ/Su(H)/Lag and then subsequently activates gene expression. The activation of specific Notch paralogs was monitored in live cells and in real time using luciferase complementation imaging. In order to validate the system, kinetic analyses of Notch signaling with agonist- and ligand-dependent activation were conducted in live cells.

Alternative Enzyme Complementation Assays

Two other enzyme complementation formats – β-lactamase and β-galactosidase – are also used for cell-based assays. The luciferase approach offers reversibility and the ability to multiplex PPI readouts, which are clear advantages. Because the assay development and troubleshooting will be analogous to the luciferase complementation described above, an extensive assay description is not included here. Nevertheless, a couple of important points are worth noting for each system.

Beta-galactosidase is a natural example of protein fragment complementation, because two polypeptides (lacα and Lacω) derived from the LacZ gene assemble to form the active enzyme. Mutagenesis studies led to the identification of weakly complementing Δα and Δω polypeptides, whose interaction is driven by the protein partners to which they are fused (6). One advantage of this system is that there are diverse methods for the detection of β-galactosidase activity, including immunohistochemical, fluorescent and chemiluminescent detection reagents. Thus, PPIs measured using split β-galactosidase can be detected on plate readers, flow cytometers and conventional microscopic systems. Furthermore, a choice of β-galactosidase reagents with different spectral characteristics facilitates multiplexing of this assay with other readouts. As with the BiLC approach, the enzymatic nature of split β-galactosidase assays can also lead to high signal-to-noise ratios, and a robust format for HTS (for examples, see (23)). Measuring PPI by β-galactosidase fragment complementation can also be used as a surrogate measure of other cellular processes, such as GPCR activation (DiscoveRx PathHunter), proteolysis, and cytoplasmic-nuclear translocation (61).

Beta-lactamase is also amenable to protein fragment complementation (21). Because β-lactamase is a smaller protein, its usage might be advantageous in situations where β-galactosidase complementation is perturbed by steric hindrance. Beta-lactamase has been used to monitor the interaction between components of the Bcl2 pathway (18), GPCRs and arrestin (22) and proteins involved in Toll-like receptor signaling, and it provides a robust assay for HTS (19, 20).

Suggested Websites

Engineering split GFP

http://www.stanford.edu/group/boxer/gfp.html

http://en.wikipedia.org/wiki/Green_fluorescent_protein

Protein folding from fragments

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2211703/?tool=pubmed

FRET considerations

http://www.olympusmicro.com/primer/techniques/fluorescence/fret/fretintro.html

https://www.biotek.com/resources/white-papers/fluorescent-proteins-filters-mirrors-and-wavelengths/

Luciferase

Harvard luciferase blackboard green and red (firefly) luciferase

http://2009.igem.org/Team:Harvard/Split

Spectra for different luciferases

http://www.targetingsystems.net/drug-discovery.php

Click Beetle differences

http://www.promega.com/products/reporter-assays-and-transfection/reporter-vectors-and-cell-lines/chroma_luc-vectors/

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