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In Vivo Imaging of Protein-Protein Interactions

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1. Introduction

Protein-protein interaction (PPI) plays a pivotal role in a wide variety of cellular events and physiological functions, such as enzymatic activity, signal transduction, immunological recognition, DNA repair/replication, among others (Valdar and Thornton, 2001). In addition, biological events that regulate proliferation, differentiation, and inflammation are also commonly mediated through PPI (Villalobos et al., 2007). Various techniques in molecular biology have been developed to understand the mechanism of these ubiquitous interactions, including qualitative methods such as yeast-two-hybrid screen (Fields and Song, 1989), immunoprecipitation (Williams, 2000), gel-filtration chromatography (Phizicky and Fields, 1995), etc. Meanwhile, quantitative biophysical methods have also been designed which include analytical ultracentrifugation (Hansen et al., 1994), calorimetry (Doyle, 1997), optical spectroscopy (Lakey and Raggett, 1998), etc. A decade ago, an assay for PPI based on β-galactosidase (gal) complementation was designed and successfully applied in cells (Wehrman et al., 2002).

Despite the success achieved by these techniques, none of them can be employed for interrogating PPI in living subjects due to several major limitations. First, traditional assays for measuring protein interactions require cell lysis, where the experimental conditions are inconsistent with the natural intracellular milieu. Second, these techniques may not be able to detect transient interactions that may have potent effects on cell signalling and intracellular processes. Lastly, the degree of false positives and false negatives vary from method to method, which significantly compromises the reproducibility and reliability of the data. With the tremendous expansion and evolution of the interdisciplinary field of molecular imaging over the last decade, many of these disadvantages have been or can be overcome.

Molecular imaging, "the visualization, characterization and measurement of biological processes at the molecular and cellular levels in humans and other living systems" (Mankoff, 2007), is an extremely powerful tool for imaging of PPI. The major molecular imaging modalities that have been applied for investigating PPI include bioluminescence, fluorescence, and positron emission tomography (PET) imaging. Quantitative and real-time molecular imaging of PPI can not only complement the already existing methodologies,

which are mostly used in vitro or in cell culture, but also provide invaluable insights on PPI that were unavailable or impossible to investigate previously. For example, non-invasive imaging of PPI can dramatically accelerate the evaluation of new drugs in living subjects that promote or inhibit homodimeric/heterodimeric protein assembly (Massoud et al., 2007; Villalobos et al., 2007).

In this chapter, we will summarize the current status of in vivo imaging of PPI with various techniques, including fluorescence, bioluminescence, and PET imaging. A schematic summary of the most commonly used strategies for imaging of PPI are shown in **Figure 1**. To the best of our knowledge, there is no literature available on fluorescence imaging of PPI in animal models. However, since this is an indispensible component of imaging PPI in cell culture, herein we will give a few representative examples on fluorescence imaging of PPI to provide a complete overview of this dynamic research area.

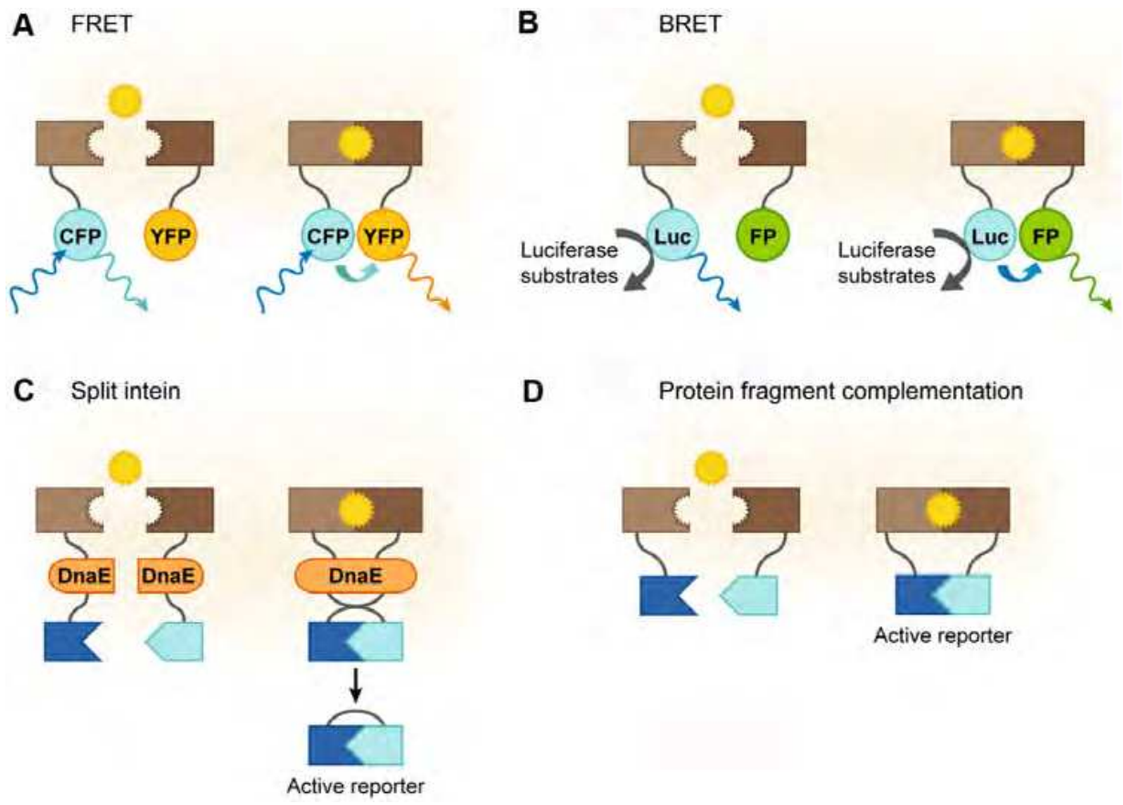

Fig. 1. Commonly used strategies for imaging of PPI. **A.** Fluorescence resonance energy transfer (FRET). **B.** Bioluminescence resonance energy transfer (BRET). **C.** Self-splicing split inteins (DnaE) can splice the two fragments of a reporter protein together into an intact and active reporter protein when they are brought within close proximity of each other. **D.** Protein fragment complementation. Brown fragments are proteins of interest and the yellow star represents an inducer of PPI. Adapted from (Villalobos et al., 2007).

2. Fluorescence imaging of PPI

The (imaging) techniques used to detect or quantify PPI need to be sensitive within the concentration ranges at which proteins are present in cells or tissues, where sometimes fewer than 10^4 protein molecules may be present. Furthermore, these techniques should be capable of identifying interactions of specific proteins against a background of more than 30,000 other proteins within a living cell. As a technology that has had an impact on almost all areas of biology, fluorescent imaging can meet these criteria under certain scenarios and has been widely used for imaging of PPI in vitro.

Fluorescence spectroscopy and fluorescence imaging have been demonstrated to be versatile tools for imaging of PPI. Fluorescent proteins (FPs), specifically variants of the green FP (GFP), are among the most frequently used for imaging of PPI (Giepmans et al., 2006; van Roessel and Brand, 2002). In a typical fluorescence process, an electron in the fluorophore within the FP absorbs photons from suitable excitation light (in the UV or visible range), which raises the energy level of the electron to an excited state. During this short excitation period, some of the energy is dissipated through molecular collisions or transferred to a proximal molecule, and the remaining energy is emitted as a photon to relax the electron back to the ground state (van Roessel and Brand, 2002). Since the energy is lower for the emission photon than the excitation photon, the emission wavelength is longer than the excitation wavelength which can be readily separated by applying a filter of specific wavelength range.

Fluorescence imaging of PPI in cell culture has the potential to provide information on the cellular and sub-cellular distribution of FPs with sub-second time resolution. Fluorescence microscopy techniques, primarily including fluorescence resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCS), are commonly used to quantify the activity, interaction, and dynamics of protein molecules within living cells (Yan and Marriott, 2003). Many protein interactions are transient, or energetically weak, thereby precluding their identification and analysis through traditional biochemical methods such as co-immunoprecipitation. In this regard, the genetically encodable FPs (GFP, yellow FP [YFP], cyan FP [CFP], red PP [RFP], etc.) and their associated overlapping fluorescence spectra have granted researchers the ability to monitor weak interactions in live cells using FRET.

2.1 Imaging of PPI with FRET

FRET requires the measurement of the relative intensity of the emission signal from a pair of fluorophores (Tsien, 2009). The underlying physics is attributed to a quantum mechanical effect between a given pair of fluorophores (i.e. a fluorescent donor and an acceptor) where, upon excitation of the donor, energy is transferred from the donor to the acceptor in a non-radiative manner by means of dipole-dipole coupling (Jares-Erijman and Jovin, 2003). Upon energy transfer, donor fluorescence is quenched and acceptor fluorescence is increased (sensitized), resulting in a decrease in donor excitation lifetime. The FRET efficiency is the quantum yield of the energy transfer transition, i.e. the fraction of energy transfer event occurring per donor excitation event, which is dependent upon several factors including the distance between the donor and the acceptor, the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum, as well as the relative orientation of the donor emission dipole moment and the acceptor absorption dipole moment.

Since FRET is critically dependent upon molecular proximity, it has been described as a molecular ruler. FRET typically operates in a range of 1-10 nm, a distance that is relevant for most molecules engaged in complex formation or conformational changes. FRET from CFP to YFP is a commonly used strategy for monitoring protein interactions or conformational changes of individual proteins. For example, FRET-based assays involving CFP and YFP were designed and employed to monitor receptor interactions on endothelial cells in one report (Seegar and Barton, 2010). However, one disadvantage of FP-based FRET is that protein functions may be perturbed by fusion of FPs since they are quite large in size. In one study, G protein-coupled receptor (GPCR) activation in living cells was used as a model system to compare YFP with a small fluorescent agent (FlAsH), which was targeted to a short tetracysteine sequence (Hoffmann et al., 2005). It was found that FRET from CFP to FlAsH reports GPCR activation in living cells without disturbing receptor function, which is more advantageous than the use of YFP as the FRET acceptor.

FRET has also been employed to visualize the interaction between two FPs, enhanced GFP (EGFP) and mCherry (Albertazzi et al., 2009). One- and two-photon fluorescence lifetime imaging microscopy (FLIM) were used to determine the FRET efficiency values. It was found that this FP pair can be used for effective and quantitative FRET imaging of PPI. Since FLIM can produce images based on the differences in the exponential decay rate of the fluorescence signal from different fluorophores, advances in FRET and FLIM have enabled studies of PPI at the microscopic level. FLIM provides a promising and robust method of detecting molecular interactions via FRET by monitoring the variation of donor fluorescence lifetime, which is insensitive to many factors that can influence the conventional intensity-based measurements, such as fluorophore concentration, photobleaching, spectral bleed-through, donor-acceptor stoichiometry, light path length etc. (Pelet et al., 2006; Zhong et al., 2007). The fact that FRET can deplete the excited state population of the donor and cause a reduction in both its fluorescence intensity and lifetime makes this technique well suited for studies in intact cells.

Interrogating PPI deep inside living tissues requires precise fluorescence lifetime measurements to derive the FRET between two tagged fluorescent markers. In a recent study, FLIM was used in combination with a clinically licensed remote endoscopic cellular resolution imaging modality to map PPI in live cells embedded in a 3D matrix, which served as a model of a diseased organ structure in a patient (Fruhwirth et al., 2010). This strategy allowed accurate measurement of fluorescence lifetime changes on the order of 100 ps, which not only demonstrated the feasibility of studying PPI by FRET in cultured living cells within 3D matrices, but also provided potential instrumentation for other FRET-based assays.

The FRET/FLIM technique can also provide invaluable information for the mechanistic study of PPI in different types of diseases. In one study which investigated the mechanism of metastasis induction by the S100A4 protein, interactions of S100A4 with C-terminal recombinant fragment of non-muscle myosin heavy chain in living HeLa cells were mapped using confocal microscopy, FLIM, and time-correlated single-photon counting (Zhang et al., 2005). The findings indicated that not only there is direct interaction between S100A4 and its target in live mammalian cells, but also that such an interaction contributes to metastasis induction, thus shedding new light onto the mechanism of cancer metastasis. In another

report, FRET/FLIM enabled the study of the interaction between hypoxia-inducible factor-1α (HIF-1α) and HIF-2α with the aryl hydrocarbon receptor nuclear translocator in a hypoxia model, which provided new information about specific gene expression controlled by PPI in hypoxia (Konietzny et al., 2009). FRET/FLIM has also been employed to image dynamic PPI in neurons (**Figure 2**), which enhanced the understanding of nervous system development and function (Timm et al., 2011). Protein kinases of the microtubule affinity regulating kinase (MARK)/Par-1 family play important roles in the establishment of cellular polarity, cell cycle control, and intracellular signal transduction. Disturbance of their function is linked to cancer and various brain diseases. In this recent study, transfected Teal FP (TFP) and YFP were used as FRET donor and acceptor pairs in neurons and imaged by FLIM, which revealed that MARK was particularly active in the axons and growth cones of differentiating neurons (Timm et al., 2011).

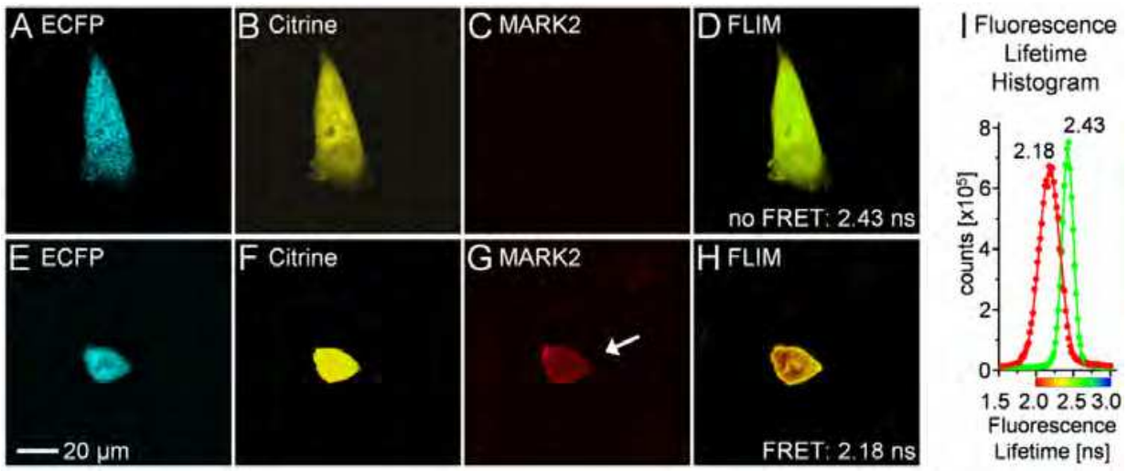

Fig. 2. The upper panel shows both channels of the fluorescence intensity image (**A, B**) of a cell transfected with a construct composed of ECFP (i.e. enhanced CFP) linked to Citrine (i.e. a stable variant of YFP), which does not exhibit FRET in the absence of fluorescently labeled MARK2 (i.e. the inducer of FRET) as indicated by a lack of fluorescence signal in **C**. The pseudo-colored FLIM image is shown in **D**, which has a long fluorescence lifetime of 2.43 ns. FRET between the two FPs (**E, F**) occurs when MARK2 is present, as indicated by the fluorescence signal in **G**. The short fluorescence lifetime of 2.18 ns is shown as red in **H** (high FRET). The graph **I** displays the averaged histograms of cells showing FRET (red dots) or no FRET (green dots) and gaussian fits of the data. Reprinted with permission from (Timm et al., 2011).

Not limited to the imaging of PPI, FRET can also be employed for imaging protein-DNA interactions, such as through the use of near-infrared fluorescent oligodeoxyribonucleotide reporters that can sense transcription factor NF-κB p50 protein binding (Zhang et al., 2008). Recently, a similar approach using hairpin-based FRET probes for the detection of human recombinant NF-κB p50/p65 heterodimer binding to DNA was reported (Metelev et al., 2011). Both of these studies demonstrated that FRET-based technique can give signal changes that are simple to interpret and stoichiometrically correct for detecting transcription factor-DNA interactions.

2.2 Imaging of PPI with FCS

Different from FRET, FCS detects the diffusion rate of single molecules which can give insights regarding whether a protein is part of a larger complex or not (Elson, 2004; Haustein and Schwille, 2007). Based on the analysis of intensity fluctuation of one or a few labeled protein conjugates at nanomolar concentration in a femtoliter volume, which depends on several factors such as the number of fluorescent species in the excitation volume, the diffusion constant of the conjugate, etc., FCS has been used to study PPI, protein-lipid/ligand-receptor interactions, dimerization of membrane receptors and proteins involved in the downstream signalling, DNA dynamics, among others (Elson, 2004; Haustein and Schwille, 2007). The high sensitivity and the possibility to monitor these dynamic interactions makes FCS a powerful tool to study signal transduction in cellular or even tissue environment at physiologically relevant conditions (Hink et al., 2002).

FCS is relatively insensitive to molecular mass. Therefore, species with similar molecular weight cannot be differentiated. Dual color fluorescence cross-correlation spectroscopy (FCCS) measures interactions by cross-correlating two or more fluorescent channels (one channel for each molecule/protein of interest), which can distinguish interactions and dynamics of biomolecules more sensitively than FCS, particularly when the mass change in the reaction/interaction is small. However, the inherent drawback of FCCS is that it suffers from non-ideal confocal volume overlap and spectral cross-talk which severely limits its applications. Fluorescence lifetime correlation/cross-correlation spectroscopy has the potential to resolve this issue, as demonstrated in a recent study (Chen and Irudayaraj, 2010). Interaction of a fluorescently-labeled antagonist antibody with the epidermal growth factor receptor (EGFR)-GFP construct in live HEK293 cells were monitored by both fluorescence lifetime cross-correlation measurements and FLIM, which not only opens up new opportunities in studying PPI in solutions and in live cells but also provides new biological insights in understanding how an antagonist influences EGFR through live cell imaging and quantification.

The field of plant sciences has also benefited from these techniques mentioned above. For example, FRET/FLIM was used to investigate CDC48A, a member of the AAA ATPases (i.e. ATPases associated with diverse cellular activities) family which has various functions in cell division, membrane fusion, as well as proteasome- and ER-associated degradation of proteins (Aker et al., 2007). With the use of FCS, it was shown that CDC48A hexamers are part of even larger complexes.

2.3 Imaging of PPI with other fluorescence techniques

Besides FRET/FLIM and FCS, enzyme complementation was also adopted for fluorescence imaging of PPI a decade ago (Spotts et al., 2002). A reporter technology based on the differential induction of β-lactamase (Bla) enzymatic activity was developed to function as a sensor for the interaction state of two target proteins within single neurons. Bla was split into two separate, complementary protein fragments which can be brought together by phosphorylation-dependent association of the kinase inducible domain of the cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein and the KIX domain of the CREB binding protein (Spotts et al., 2002). Using an intracellular substrate whose fluorescence spectrum changes upon hydrolysis by Bla, time-lapse ratiometric

imaging measurements were achieved after association of CREB and CREB binding protein, which permits direct imaging of PPI in single cells with high signal discrimination.

To investigate the conformational changes of proteins in living cells when external force is applied, a genetically encoded fluorescent sensor was constructed and tested in a myosin-actin model system using the proximity imaging (PRIM) technique, which detects spectral changes of two GFP molecules that are in direct contact (Iwai and Uyeda, 2008). The developed PRIM-based strain sensor module (PriSSM), consisted of the tandem fusion of a normal and circularly permuted GFP, was inserted between two motor domains of dictyostelium myosin II to study the effect of strain. It was suggested that this technology may provide a general approach for studying force-induced protein conformational changes in cells.

2.4 A brief summary of fluorescence imaging of PPI

The FRET/FLIM technique can be used as a versatile tool to characterize the spatial distribution of various proteins and detect/quantify PPI in a living cell, which can measure intermolecular FRET through quite sophisticated mathematical algorithms. However, no in vivo fluorescence imaging of PPI has been reported so far since these techniques (in particular FP-based) cannot be readily used for in vivo imaging applications due to several major limitations.

First, FRET-based techniques require the use of incident light to activate the donor protein. Given that the excitation wavelength is typically in the green range, little excitation light will travel through tissue since most tissues have strong light absorption/attenuation below a wavelength of 600 nm (Frangioni, 2003). Therefore these techniques are intrinsically not suitable for non-invasive imaging studies in live animals. Second, there is strong auto-fluorescence signal from animal tissue which confounds the interpretation of the imaging data. Third, the sensitivity of fluorescence imaging is not very high. Fourth, the relative molar ratios of the FRET donor/acceptor pair are not always 1:1, which can cause significant problems in calibration, detection, and quantification, especially when the situation is exacerbated in vivo when compared to cell-based studies. Lastly, there is significant photobleaching when the FPs are exposed to excitation light for a prolonged period.

3. Bioluminescence imaging (BLI) of PPI

Because of very low background signal and high sensitivity, BLI can be a more suitable technique for in vivo imaging of PPI than fluorescence imaging. The fact that no additional excitation light will be needed in BLI is highly advantageous for reducing the background signal. Two major strategies have been adopted for BLI of PPI: bioluminescence resonance energy transfer (BRET) and enzyme complementation.

3.1 Imaging of PPI with BRET

BRET displays similar characteristics as FRET except the donor is a bioluminescent protein, typically a luciferase, which requires the presence of small molecule substrates but not incident light. Similar to FRET, BRET is also a quantum process in which energy is transferred over a distance, usually < 10 nm, from the donor (e.g. luciferase) to a FP

(Villalobos et al., 2007). However, BRET offers many distinct advantages over FRET because of its higher quantum yield and better detection sensitivity.

As a popular technique for studying PPI in live cells, BRET is particularly suitable for real-time monitoring of such interactions. For example, many cellular signal transduction can be visualized by this technique, such as agonist-induced GPCR/β-arrestin interaction (Pfleger et al., 2006), calcium sensing receptor homodimer formation (Jensen et al., 2002), β2-adrenergic receptor dimerization (Angers et al., 2000), interaction of circadian clock proteins (Xu et al., 1999), etc. Since the potential for studying the modulation of such interactions by agonists, antagonists, inhibitors, dominant negative mutants, and co-expressed accessory proteins is tremendous, high-throughput BRET-based screening system is an ever-expanding area of interest for the pharmaceutical industry. However, imaging PPI with BRET in animal models is very challenging and only a few successful examples are available in the literature (Massoud et al., 2007; Villalobos et al., 2007).

In one early study, a cooled charge-coupled device (CCD) camera-based spectral imaging strategy enabled simultaneous visualization and quantitation of BRET signal from live cells and cells implanted in living mice, where renilla luciferase (RLuc) and its substrate were used as an energy donor and a mutant GFP was used as the acceptor (De and Gambhir, 2005). As a proof-of-principle, the donor and acceptor proteins were fused to FKBP12 and FRB respectively, which are known to interact only in the presence of the small molecule mediator rapamycin (Banaszynski et al., 2005; Choi et al., 1996). Mammalian cells expressing these fusion constructs were imaged using a cooled-CCD camera either directly from culture dishes or after implanting them into mice, where the specific BRET signal was determined by comparing the emission photon yields in the presence and absence of rapamycin. Such CCD camera-based imaging of BRET signal is very appealing since it can seamlessly bridge the gap between in vitro and in vivo studies, thus validating BRET as a powerful tool for interrogating and detecting PPI directly at limited depths in living mice.

Subsequently, a highly photon-efficient and self-illuminating fusion protein, which combines a mutant RFP (mOrange) and a mutant RLuc (RLuc8), was constructed to improve the BRET efficiency/signal (De et al., 2009). This new BRET fusion protein, termed as "BRET3", exhibited several-fold improvement in light intensity when compared with the previous BRET fusion proteins. In addition, BRET3 also exhibits red-shifted light output, which can allow for deeper tissue imaging in small animals. At single cell level, the BRET3 construct (which contains FKBP12 and FRB) was demonstrated to only exhibit BRET signal in the presence of rapamycin. With increased photon intensity, red-shifted light output and good spectral resolution (approximately 85 nm), it was suggested that BRET3-based assays will allow imaging of PPI using a single assay that is directly scalable from living cells to small animals.

Recently, further improvement on the BRET3 construct was reported, which was termed "BRET6" (Dragulescu-Andrasi et al., 2011). Red light-emitting BRET-based reporter systems were developed to allow for assaying PPI both in cell culture and in deep tissues of small animals (**Figure 3**). These BRET systems consist of the newly developed RLuc variants (RLuc8 and RLuc8.6, which serve as BRET donors) and two RFPs (TagRFP and TurboFP635, which serve as BRET acceptors). In addition to the native coelenterazine substrate for RLuc, a synthetic derivative (coelenterazine-v) was also used which further red-shifted the

emission maxima of RLuc by 35 nm. Ratiometric imaging of PPI in the presence of rapamycin-induced FKBP12-FRB association was demonstrated in both cultured cells and small animal tumor models.

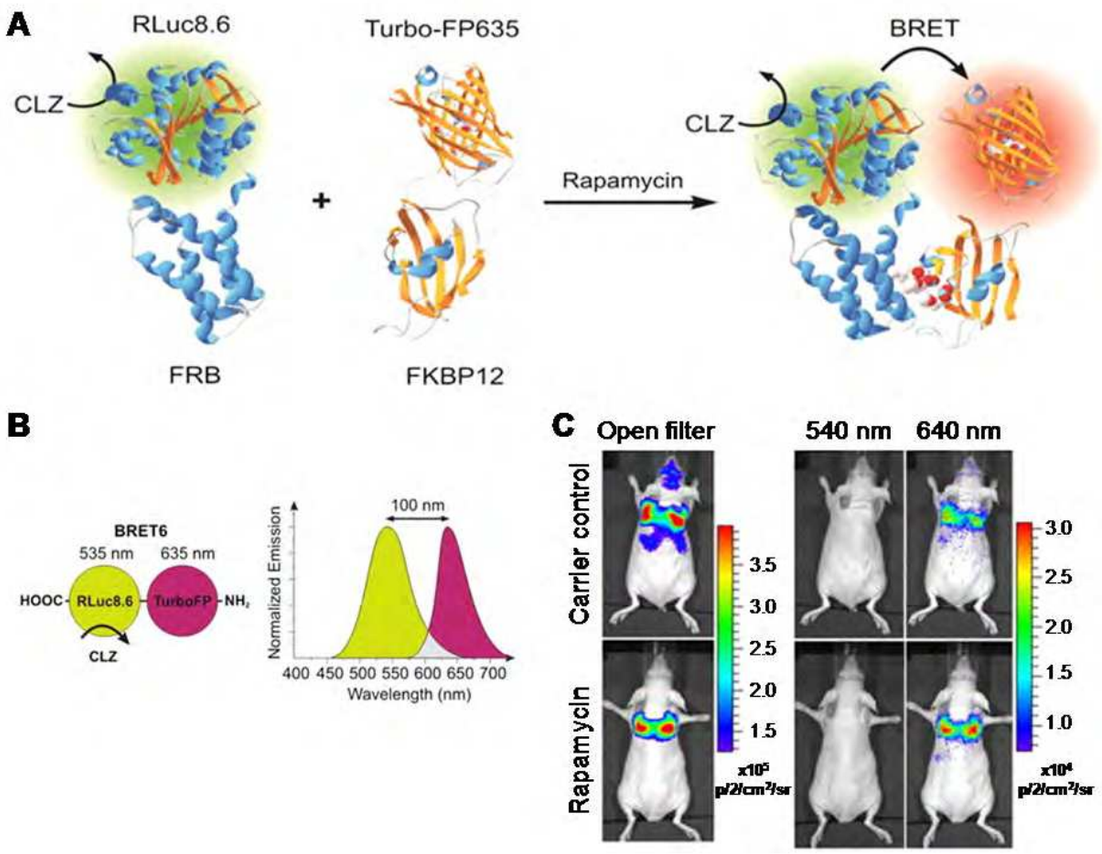

Fig. 3. Imaging of PPI with BRET6. **A.** Schematic illustration of the BRET6 construct for monitoring rapamycin-induced FRB-FKBP12 association. **B.** Schematic representation of the BRET6 fusion construct, the emission spectrum of the RLuc mutant, and the absorption spectrum of the acceptor protein. CLZ denotes coelenterazine (a substrate for RLuc). **C.** Bioluminescence images of cells stably expressing the BRET6 construct, accumulated in the lungs of nude mice after intravenous injection. Mice were also injected with both rapamycin (or control carrier which does not contain rapamycin) and CLZ before imaging. Adapted from (Dragulescu-Andrasi et al., 2011).

Currently, the number of BRET probes reported for the imaging of PPI is significantly lower when compared to FRET-based approaches. Much future work needs to be devoted to BRET-based imaging of PPI. The strategy of combining a fluorescent and a bioluminescent reporter to generate self-illuminated reporter proteins is advantageous to overcome the common problems associated with in vivo fluorescent imaging of PPI. As a genetically encodable approach for ratiometric imaging of PPI in cells and living subjects, light attenuation by tissue is the major challenge for ratiometric analysis of PPI with a BRET system. Since light attenuation varies with the wavelength of the emitted photons

and the tissue depth, red-shifted luciferases and FPs are clearly preferred choices. Meanwhile, consistency of the BRET ratio in different mice should also be monitored carefully to ensure sufficient spatial control to retain the ratiometric characteristics of a BRET sensor.

3.2 Imaging of PPI with complementation of split enzyme

Enzyme complementation assay depends on the division of a reporter enzyme (e.g. luciferase) into two separate inactive components that can regain function upon association (Massoud et al., 2007). When the two enzyme fragments are each fused to two interacting proteins, the enzyme can be reactivated upon PPI. For in vivo BLI applications, the split firefly luciferase (fLuc) with small overlapping sequences is a suitable choice because it consistently yields strong signal and excellent inducible complementation by a variety of PPIs. The reaction kinetics and ease of delivery of the substrate, D-luciferin, also allows for facile application of this technique in BLI assays. Besides fLuc, RLuc has also been investigated for BLI of PPI. However, although the split RLuc system functions quite efficiently, one major limitation of RLuc-based assay is its substrate, coelenterazine, which exhibits poor reaction profile for long-term kinetic experiments. In addition, the hydrophobicity of the molecule also makes it difficult to use for in vivo applications.

The first report on non-invasive BLI of PPI in living subjects based on a split luciferase was achieved a decade ago (Paulmurugan et al., 2002). In this study, split fLuc was designed and constructed for both intein-mediated reconstitution and complementation, where the two fLuc fragments could be brought together by the strong interaction between two proteins, MyoD and Id, both of which are members of the helix-loop-helix family of nuclear proteins. As a demonstration of the proof-of-principle, cells transiently transfected with the split reporter gene construct were used for imaging MyoD-Id interactions, both in cell culture and in cells implanted into living mice.

In a subsequent study, the split fLuc strategy was employed for imaging of PPI in hypoxia (Choi et al., 2008). HIF-1α is well known to regulate the activation of genes that promote malignant progression (Koh et al., 2010). HIF-1α is hydroxylated on prolines 402 and 564 under normoxia, which is targeted for ubiquitin-mediated degradation by interacting with the von Hippel-Lindau protein complex (pVHL). To study the interaction between HIF-1α and pVHL, the split fLuc-based system was used where HIF-1α and pVHL were fused to the amino-terminal and carboxy-terminal fragments of fLuc, respectively. Hydroxylation-dependent interaction between HIF-1α and pVHL led to complementation of the two fLuc fragments, resulting in bioluminescence in vitro and in vivo. Complementation-based bioluminescence was diminished when mutant pVHL with decreased binding affinity for HIF-1α was used. This strategy represents a useful approach for studying PPI involved in the regulation of protein degradation. In another study, split fLuc was also used for investigating epidermal growth factor (EGF)-induced Ras/Raf-1 interaction in mammalian cells (Kanno et al., 2006).

Similar strategy has been adopted to develop an inducible split RLuc-based bioluminescence assay for quantitative measurement of real time PPI in mammalian cells (Paulmurugan and Gambhir, 2003). In a follow-up study, the split RLuc construct was used to evaluate drug-modulated PPI in a cancer model in living mice (**Figure 4**)

(Paulmurugan et al., 2004). The heterodimerization of FRB and FKBP12, mediated by rapamycin, was also utilized in this study. The concentration of rapamycin needed for efficient dimerization, as well as the amount of ascomycin (a competitive binder of rapamycin) required for dimerization inhibition, were investigated. These studies demonstrated that such split reporter-based strategies can be used to efficiently screen small molecule drugs that modulate PPI, and further evaluate the effect of the drugs in living animals.

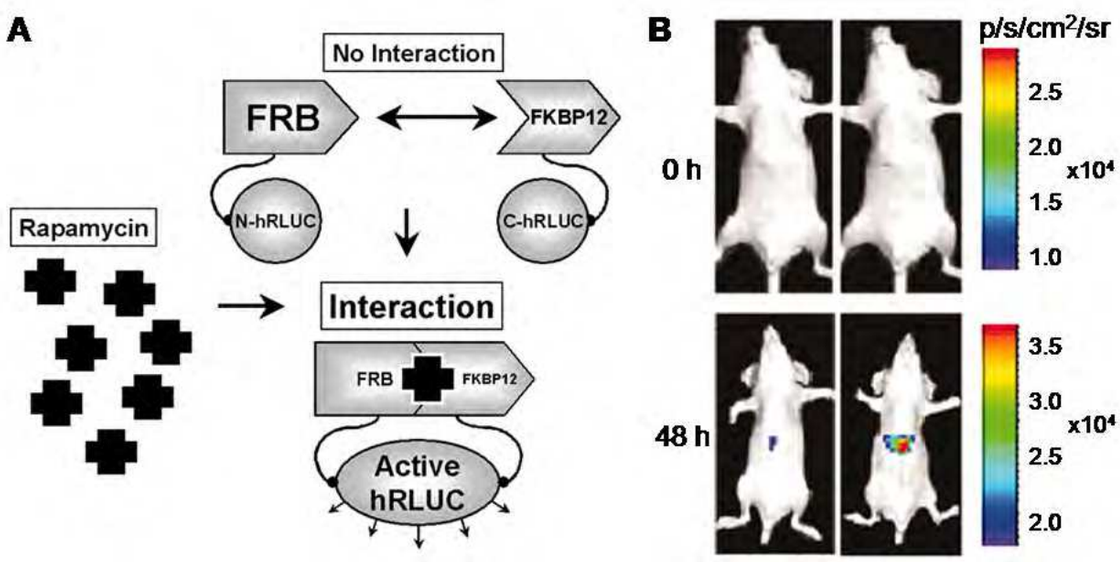

Fig. 4. In vivo imaging of drug-modulated PPI. **A.** Schematic diagram of rapamycin-mediated complementation of the two fragments of synthetic renilla luciferase (hRLUC). **B.** Non-invasive imaging of PPI in living mice, intravenously injected with human 293T embryonic kidney cancer cells that were transiently co-transfected with both split constructs. Mice not receiving rapamycin (left) showed only background signal, whereas the animals receiving repeated injections of rapamycin emitted higher signals originating from the 293T cells in the liver (right). Adapted from (Paulmurugan et al., 2004).

Homodimeric PPI, potent regulators of cellular functions and particularly challenging to study in vivo, can also be visualized by the split RLuc strategy. Split RLuc complementation-based bioluminescence assay was used to study the homodimerization of herpes simplex virus type 1 thymidine kinase (HSV1-TK) in mammalian cells and in living mice (Massoud et al., 2004). Homodimerization of HSV1-TK chimeras containing the N-terminal or C-terminal fragments of RLuc in the upstream and downstream positions, respectively, was visualized and quantified. A mutant of HSV1-TK was used to confirm the specificity of the RLuc complementation signal from HSV1-TK homodimerization. This generalizable assay to screen for molecules that promote or disrupt ubiquitous homodimeric PPI can not only serve as an invaluable tool to understand the biological signaling networks,

but will also be useful in drug discovery/validation in live animal disease models. In a cell-based study, the split RLuc strategy was shown to be useful beyond the visualization and confirmation of the existence of PPI. It also helped in identifying the critical amino acid residues involved in a specific PPI (Jiang et al., 2010).

Besides fLuc and RLuc complementation, split click beetle luciferase has been used to study the interaction between GPCR and β-arrestin (Misawa et al., 2010), whereas split Gaussia luciferase has been employed to image the interaction between calmodulin and other proteins (Kim et al., 2009). However, neither of these split luciferases has been demonstrated for in vivo visualization of PPI. Other split enzymes have also been explored for the imaging of PPI, such as the use of split β-gal for BLI of GPCR interactions in vivo (von Degenfeld et al., 2007). Currently, there is a paucity of sensitive and specific methods for quantitative comparison of the pharmacological properties of GPCRs in physiological and/or pathological settings in live animals. In this study, low affinity and reversible β-gal complementation was developed to quantify GPCR activation via interaction with β-arrestin, which enabled real time BLI of GPCR activity in live animals with high sensitivity and specificity (von Degenfeld et al., 2007). Imaging was achieved by using a recently developed luminescent β-gal substrate, which is a caged luciferin molecule that can be recognized by fLuc to generate light only after it has been cleaved by β-gal (Wehrman et al., 2006). Following implantation of the cells into mice, it was possible to monitor pharmacological GPCR activation and inhibition in their physiological context by non-invasive BLI, suggesting that this technology may have unique advantages to enable novel applications in the functional investigation of GPCR modulation in biological research and drug discovery.

4. PET imaging of PPI

Typically, PPI represents a low-level biological event and is therefore very challenging to detect, locate, and image in intact living subjects. When compared with BLI and fluorescence imaging, PET possesses very high sensitivity, while being quantitative and tomographic (Massoud and Gambhir, 2003). In addition, it is one of the few non-invasive imaging techniques that can be applied in humans for non-invasive monitoring of reporter gene expression (Kang and Chung, 2008). Although PET has enormous potential in imaging complex biological events such as PPI, to date only one example of PET imaging of PPI has been reported (Massoud et al., 2010).

The PET reported gene HSV1-TK was molecularly engineered and cleaved between Thr265 and Ala266, where the fragments were used in a protein-fragment complementation assay to quantify as well as to non-invasively image PPI in mammalian cells and living mice (Massoud et al., 2010). It was found that a point mutation (V119C) could be introduced to markedly enhance the HSV1-TK complementation modulated by several different PPIs such as the rapamycin-mediated FKBP12- FRB, HIF-1α-pVHL, etc. In vivo PET imaging of the FKBP12-FRB interaction modulated through rapamycin was successfully achieved (**Figure 5**). Future applications of this unique split HSV1-TK strategy are potentially far reaching, including accurate monitoring of immune and stem cell therapies, as well as allowing for fully quantitative and tomographic PET localization of PPI in preclinical small and large animal models of various diseases.

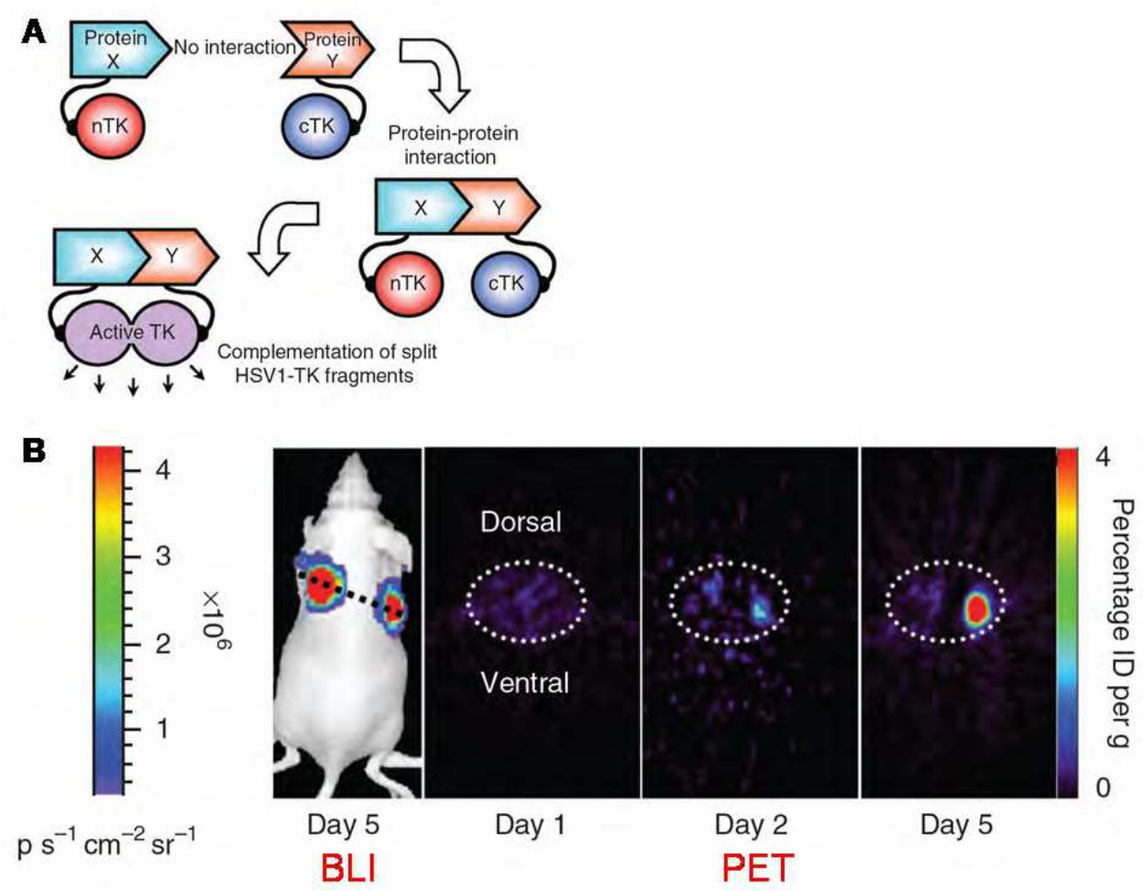

Fig. 5. Non-invasive PET imaging of PPI. **A.** Schematic diagram showing the use of split HSV1-TK to monitor the hypothetical X-Y heterodimeric PPI. **B.** Transaxial PET images of a mouse implanted subcutaneously with mock-transfected 293T cells (left) and 293T cells stably expressing both split constructs of HSV1-TK each fused to FRB and FKBP12 respectively (right). The serial images at different days were acquired after injection of the PET reporter probe for HSV1-TK (i.e. ^{18}F-FHBG). A BLI image of the mouse is also shown to delineate the two tumors. Adapted from (Massoud et al., 2010).

5. Conclusion

The interactions of specific cellular proteins form the basis of a wide variety of biological processes, including many signal transduction and hormone activation pathways involved in maintaining important biological functions. Accurate measurement of PPI can significantly help in deciphering the genetic and proteomic code. The tremendous complexity of cellular events requires assays that can measure different types of PPIs using an array of different methods. Molecular imaging, an extremely powerful tool to study molecular events in living subjects, can provide invaluable information and insight in elucidating the process of various PPIs.

To date, the major molecular imaging modalities used for visualization of PPI include fluorescence imaging (not suitable for in vivo studies), BLI, and PET imaging. All these techniques require extensive efforts in protein engineering due to the complex and challenging nature of imaging PPI in living cells and animals. Particularly for split reporter-based strategies, intensive efforts are needed to obtain better functioning split reporters that exhibit efficient PPI-induced complementation but not self-complementation. At the same time, sufficiently high reporter activity needs to be maintained upon PPI-induced complementation. For in vivo imaging of PPI, PET serves as a better choice over BLI and fluorescence due to its superb sensitivity, excellent tissue penetration, high quantification accuracy, and potential for clinical translation.

Future work on the imaging of PPI may include the design of second-generation complementation reporters with improved signal-to-noise ratios, inducibility, and red-shifted spectral properties for more wide spread use in vivo. The ideal reporter for imaging of PPI should not only serve as an "on/off" signal, but also give a graduated and quantitative response with minimal background signal and excellent induced signal output. Lastly, since no single imaging modality is perfect, combination of different imaging techniques to study the same PPI may provide complementary information.

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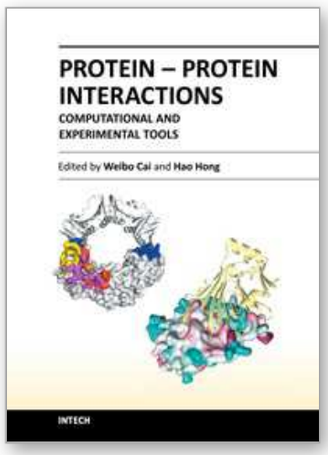

Protein-Protein Interactions - Computational and Experimental Tools

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Proteins are indispensable players in virtually all biological events. The functions of proteins are coordinated through intricate regulatory networks of transient protein-protein interactions (PPIs). To predict and/or study PPIs, a wide variety of techniques have been developed over the last several decades. Many in vitro and in vivo assays have been implemented to explore the mechanism of these ubiquitous interactions. However, despite significant advances in these experimental approaches, many limitations exist such as false-positives/false-negatives, difficulty in obtaining crystal structures of proteins, challenges in the detection of transient PPI, among others. To overcome these limitations, many computational approaches have been developed which are becoming increasingly widely used to facilitate the investigation of PPIs. This book has gathered an ensemble of experts in the field, in 22 chapters, which have been broadly categorized into Computational Approaches, Experimental Approaches, and Others.

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