Time-Gated Luminescence Detection for High-Throughput Screening of Protein Interaction and Inhibitions

BY

ENGIN YAPICI
B.Sc. Middle East Technical University, Ankara, Turkey, 2009
M.Sc. University of Illinois at Chicago, Chicago, USA, 2012

THESIS
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Defense Committee:
Lawrence W. Miller, Chair and Advisor
Wonhwa Cho
Jung-Hyun Min
Justin Lorieau
Terry W. Moore, Medicinal Chemistry and Pharmacognosy
This thesis is dedicated to
my dear wife Esin Soy Yapici,
and my parents, Vildan Yapici and Fuat Zafer Yapici,
without whom it would never have been accomplished.
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>eDHFR</td>
<td>Escherichia coli dihydrofolate reductase</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGS</td>
<td>Ethylene glycol bis[succinimidylsuccinate]</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FKBP</td>
<td>FK506 binding protein 12</td>
</tr>
<tr>
<td>FOXM1</td>
<td>Forkhead box protein M1</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence polarization</td>
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<tr>
<td>FRB</td>
<td>Rapamycin binding domain of mTor</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>ICCD</td>
<td>Intensified charge coupled device</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non essential amino acids</td>
</tr>
<tr>
<td>NLS</td>
<td>Non-linear least squares</td>
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<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
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<tr>
<td>PBS</td>
<td>Dulbecco's phosphate buffered saline</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
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<tr>
<td>POI</td>
<td>Protein of interest</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-protein interaction</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>S:B</td>
<td>Signal-to-background</td>
</tr>
<tr>
<td>S:N</td>
<td>Signal-to-noise</td>
</tr>
<tr>
<td>TR-FRET</td>
<td>Time-resolved fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>TTHA</td>
<td>Triethylenetetraaminehexaacetic acid</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethoprim (5-(3,4,5-Trimethoxybenzyl) pyrimidine-2,4-diamine)</td>
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SUMMARY

High throughput screening (HTS) assays are a key component of the drug discovery processes. They are designed to identify biologically active molecules that can ultimately be used as therapeutic agents. Such assays use the power of automation to test thousands of molecules per day, and they require high reproducibility and robustness to be reliable. Current HTS technologies often employ fluorescence-based detection methods as a means of identifying potentially useful compounds. However, often high levels of non-specific fluorescence background emanating from biological assay components and, especially, library compounds can reduce sensitivity and lead to unacceptable rates of false positive results. The use of time-gated detection in combination with organic complexes of lanthanides, particularly Tb\(^{3+}\) and Eu\(^{3+}\), that have millisecond-scale excited-state lifetimes as luminescent reporters alleviates this problem. By implementing a delay of 10 microseconds or more between sample excitation and detection, nanosecond-scale fluorescence background is eliminated, resulting in HTS assays with very large signal-to-background ratios.

Interactions between proteins are essential for cellular function. Many diseases are caused by mutations that disrupt or otherwise give rise to aberrant protein-protein interactions (PPIs). While enzyme active sites and cell surface receptors have been the most common drug discovery targets, there is an increasing interest in developing small molecule therapeutics that can disrupt PPIs. One of the key challenges in targeting PPIs with small molecules is that the interface between two interacting proteins is a relatively large, flat surface, as opposed to a distinct binding pocket. Moreover, there is often no structural information available about PPI interfaces, and this makes rational design of potential inhibitors difficult or impossible. For this
reason, HTS has proven to be an important tool in the discovery process for PPI inhibitors that have been developed thus far, and it will continue to play an important role in future efforts.

The main objective of the studies described in this dissertation was to design HTS assays for PPI inhibition that can be readily adapted to almost any protein and that can be applied in purified biochemical preparations, cell lysates, or in living mammalian cells. The assay platform is based on the time-gated detection of Förster Resonance Energy Transfer between two overexpressed fusion proteins, one labeled with a luminescent Tb\(^{3+}\) complex and the other attached to a green fluorescent protein. Chapter 1 provides a brief introduction to HTS as well as background on relevant scientific concepts and technologies. In chapter 2, the characterization of a PPI assay that can detect and measure PPIs in impure bacterial lysates is presented. The interaction between two fusion proteins, FRB-eDHFR and GFP-FKBP, was used as a model system to show that the assay is sufficiently sensitive and reproducible for HTS application, and that it can be used to measure dissociation or inhibition constants as well. In chapter 3, the results of assay development efforts using the same model system are presented, but in this case the assay can be performed on proteins expressed in mammalian cells without prior purification. The ability to detect PPIs or their inhibition in living cells in multi-well plate format represents a substantial advance because many proteins either cannot be purified, or require appropriate post-translational modifications or the presence of additional cellular components.

The final chapter of this dissertation describes efforts to develop the HTS assay presented in Chapter 2 to screen for inhibitors of an interaction between the proteins FoxM1b & nucleophosmin (NPM). Evidence in the literature suggested that FoxM1b and NPM bind to one
another and that their interaction may be related to tumorigenesis. The assay development efforts presented in Chapter 4 were unsuccessful, but the results provide insight into the challenges that arise when developing a HTS for PPIs.
CHAPTER 1

THEORETICAL AND PRACTICAL BACKGROUND
1.1 High-throughput screening

1.1.1 Introduction to HTS

High throughput screening (HTS) assays have been a central component of the drug discovery process since the mid 1990s. Such assays are used to identify inhibitors, antagonists or other biologically active small molecules from large chemical libraries, and “high throughput” is defined as the testing of more than 10,000 compounds per day. They leverage the power of automation for quick and accurate content dispensing in miniaturized formats and subsequent signal measurement with several different detection methods. High-throughput, automated sample handling is achieved by robotic instruments in 96-, 384- and 1,536-well plates. The ultimate goal of a HTS is to generate small molecule leads which will either be further analyzed as potential therapeutic agents in the drug discovery campaign or used as a biological tool for research purposes.

HTS emerged during the mid-1990s after improvements in combinatorial chemistry made the rapid generation of chemical libraries possible. Around the same time, several drug targets were discovered using new genomic tools, and HTS became an attractive method for identifying small molecule scaffolds that could serve as a basis for further medicinal chemistry efforts. Chemical libraries are dispensed from and to microtiter plates with either a single compound or multiple compounds pooled in each well. Plates with 96 wells were a standard for the early HTS experiments. However, increases in library size necessitated further miniaturization of the sample volumes and design of new plates with higher well densities. The second generation of microtiter plates had 384 wells with working volumes
ranging between 25-100 µL, and currently some screens are performed in 1536-well plates with working volumes 2.5-10 µL (Figure 1).\(^9,^{10}\)

![Microtiter Plates](image)

**Figure 1**: The different types of microtiter plates used for HTS applications.

HTS assays often incorporate fluorescence-based detection methods including variations such as Förster resonance energy transfer (FRET)\(^{11,12}\), time-resolved energy transfer (TR-FRET)\(^{13-15}\), fluorescence polarization (FP)\(^{16-18}\), fluorescence correlation spectroscopy (FCS)\(^{19,20}\), and fluorescence recovery after photobleaching (FRAP)\(^{21}\). Some other less commonly used HTS detection strategies include chemiluminescence-based technologies\(^{22-24}\), scintillation proximity assay (SPA)\(^{25-27}\), and surface plasmon resonance (SPR)\(^{28,29}\). The biggest advantage of fluorescence-based methods is their sensitivity, as the signal emitted from even very small numbers of fluorescent molecules can be detected with high reliability. Apart from detection sensitivity, numerous fluorescent probes are commercially available for
specifically labeling virtually any biomolecule of interest. Moreover, fluorescent probes can be engineered to respond to changes in protein conformation, post-translational protein modifications, enzyme product production, and many other biochemical events.

HTS campaigns generate large data sets, and HTS data must be sufficiently robust so that “hits” or leads can be identified with high confidence. Various measures are used to assess HTS such as the dynamic range, signal-to-background (S:B), signal-to-noise (S:N), and $Z'$-factor. The dynamic range is the difference between the mean signals of the negative and positive controls. S:B is another measure of the assay signal range, and a value $<2$ is desirable. S:N is closely related to the dynamic signal range and is calculated as,

$$S : N = \frac{\mu_{signal} - \mu_{background}}{\sigma_{background}}$$

where $\mu_{signal}$ and $\mu_{background}$ are the means of signal and background, respectively, and $\sigma_{background}$ is the standard deviation of the background. S:N indicates the confidence with which a given signal can be discriminated from background noise. However, dynamic range, S:B or S:N alone must be combined with measures of data variation in order to judge assay quality$^1$. The $Z'$ factor is a dimensionless statistical measure of data variation associated with the signal measurements in HTS assays$^{30}$. $Z'$ factor is calculated with the following formula:
\[ Z' = 1 - \frac{3\sigma_{\text{max}} + 3\sigma_{\text{min}}}{|\mu_{\text{max}} - \mu_{\text{min}}|} \]

where, \( \sigma_{\text{max}} \) and \( \sigma_{\text{min}} \) are the standard deviations of sample and negative control wells, and \( \mu_{\text{max}} \) and \( \mu_{\text{min}} \) are the means of sample and negative control wells, respectively. \( Z' \) factor takes into account the assay signal dynamic range and the data variation in the control measurements. \( Z' \)-factor values >0.5 for an assay is considered to be excellent while values <0.5 may be acceptable but suggest a need for further optimization.\(^{30}\) A value larger than 0.5 is an indication of large dynamic signal range and/or small data variability.

An HTS assay design requires several considerations. The first one is to determine the detection method depending on the biological system’s response to the library molecules. For example, for one system, the response may be the disruption of a protein-protein interaction\(^{31-34}\), or the inactivation of a cell-surface receptor leading to subsequent signal decreases\(^{35-37}\); while in some other assays it may be transcription of a reporter gene caused by receptor activation and subsequent signal transduction\(^{37,38}\). The second consideration is the response caused by the compound and whether it is affected by other factors in the assay matrix or in the cell. For instance, if the screen is designed to find an antagonist, it may be necessary to test different antagonist amounts in order to simulate different temporal concentrations in the cell.\(^{39,40}\) Third, the kinetic and thermodynamic equilibrium of the small molecule interaction and the duration of the signal response must be considered. Some interactions may have a very quick response time and require very rapid readings (e.g. calcium dependent responses) while others may take minutes or hours to reach to the equilibrium and require endpoint measurements.
1.1.2 Biochemical versus cell-based HTS assays

HTS assays are performed to find inhibitors, agonists or antagonists of biomolecules for therapeutic agent discovery. Depending on the availability of resources and the system in focus, the assay can be designed to run in a cell free, homogenous biochemical environment or in a cell-based format. That said, all HTS campaigns eventually require a live-cell testing stage in order to evaluate the pharmacokinetic performance of any lead compounds. The most significant advantage of using a homogenous biochemical assay is the lack of interference from other components that are found in a complex cell environment. This can yield assays with very large dynamic signal ranges and Z’ factors. On the other hand, these relatively ‘simple’ systems may identify leads that are unsuitable as therapeutic agents due to excessive cytotoxicity, membrane impermeability or other undesirable interactions with cellular components. By incorporating cell-based assays earlier in the HTS process, some of these problems can be circumvented, and a number of other advantages can be gained: 1) more novel systems can be studied in their natural environment, where they may need co-activators, co-repressors, post-translational modifications or some other cellular factors that may not be provided in a solution based assay; 2) proteins that cannot be purified or are very costly to isolate (e.g. membrane proteins) can be studied; and 3) screening of multiple interactions or pathways is possible via multiplexing. Despite these advantages, it is often difficult to design cell-based detection strategies that meet the statistical requirements for HTS. Moreover, cellular assays are often lower in throughput and more costly than screens that are performed with purified biological components.
1.1.3 **Limitations of fluorescence-based HTS.**

As noted, fluorescence is the most commonly used detection method in HTS, but it suffers from some limitations. Principally, high levels of non-specific background caused by the autofluorescence of biological components or fluorescent library compounds reduces assay quality and often results in false positive results. Chemical libraries contain many heterocyclic compounds with conjugated bonds that may emit within the same wavelength range as the reporter fluorophore used in the assay (e.g. green fluorescent protein). Moreover, flavins, NADPH and other biomolecules contribute background fluorescence, especially when near-UV excitation (340-400 nm) is used.\textsuperscript{1,41–43} Biological autofluorescence is particularly problematic in cell-based assays. Various strategies are employed to minimize the effects of non-specific fluorescent background including the use of red-shifted reporter dyes and filter optimization. The use of reporters with long emission lifetimes in combination with time-gated detection methods is a particularly useful way to eliminate background fluorescence, as detailed below.

1.2 **Protein-Protein Interactions (PPIs) as Therapeutic Targets.**

1.2.1 **Importance of protein interactions in disease.**

Protein-protein interactions (PPIs) are involved at some level in most biological processes such as signal transduction, formation of large protein complexes for DNA replication and RNA translation, the immune response and many others. Many diseases are caused by mutations that disrupt or otherwise give rise to aberrant PPIs. One example is the
series of diseases caused by the aberrant regulation of a protein tyrosine phosphatase superfamily member, encoded by the gene \textit{PTPN22}\textsuperscript{44}. A single nucleotide mutation that causes a change of arginine at position 620 to tryptophan was determined to be a common risk factor for several diseases, including type I diabetes\textsuperscript{45}, rheumatoid arthritis\textsuperscript{46,47}, Graves’ disease\textsuperscript{48}, and systemic lupus erythematosus\textsuperscript{49}. Another example is the tumorigenesis caused by the mutations in \textit{TP53} gene, which encodes the p53 protein. p53 is a multifunctional DNA binding protein and a proto-oncogene, meaning a loss-of-function mutation results in cancer\textsuperscript{50}. While enzyme active sites and cell surface receptors have been the most common drug discovery targets, there is increasing interest in developing small molecule therapeutics that can disrupt PPIs\textsuperscript{51,52}.

\subsection*{1.2.2 Small molecule-protein interactions}

Most small molecule drugs that target proteins modulate their function via direct binding and either competitive inhibition or allosteric regulation. The binding site is a well-defined pocket, and often structural data is available that can guide rational drug design. One of the key challenges in targeting PPIs via small molecules is the nature of the interaction itself. The interface between two interacting proteins is usually a relatively large, flat surface, as opposed to a distinct binding pocket. Indeed, for some time, the idea of inhibiting or disrupting PPIs with small molecules was regarded with considerable skepticism in the drug discovery field. However, biophysical studies revealed that often a few key residues in the binding interface that form so-called ‘hot-spots’ (e.g. ion pairs) are responsible for most of the binding energy\textsuperscript{53,54}. Design of a small molecule that can disrupt an interaction over a large surface area or dig into the buried hot-spot is not always, unfortunately, an easy task.
The best approach is to use computational methods to devise a structure that can potentially bind to the hot-spot of one of the proteins but this is possible only if the three dimensional structures of the interacting proteins are known. One example for this structure-based approach is the discovery of inhibitors for the BCL2 and BCL-X\textsubscript{L} interaction. Discovery methods included virtual screening\textsuperscript{55,56}, ligand-based design\textsuperscript{57} and mechanistic analysis of a known compound\textsuperscript{58} by using the three dimensional structures of the binding interfaces of the proteins. Another example is the B7-1 and B7-2 interaction where the protein-protein interface hot-spot was targeted for an inhibitor identification by using the proteins’ three dimensional structures.\textsuperscript{59,60} However, if no structure information is available, the only way to identify an inhibitor is through HTS assays.\textsuperscript{61}

### 1.3 Lanthanide Complexes as Luminescent Probes for Time-Resolved Förster Resonance

**Energy Transfer (TR-FRET)**

Lanthanides (Ln) are the f-block rare earth elements with atomic numbers ranging between 57 and 71 in the periodic table.\textsuperscript{14,62–67} Sensitized complexes of Tb\textsuperscript{3+} and Eu\textsuperscript{3+} have unique features that make them particularly desirable for luminescence-based biological analyses.\textsuperscript{63} Most notably, their unusually long emission lifetimes (0.1-2 ms) make them particularly useful as donor fluorophores in TR-FRET assays that can sensitively detect molecular interactions. In this section, the basics of FRET, lanthanide complexes and TR-FRET as they apply to HTS are described.
1.3.1 Introduction to FRET

Förster resonance energy transfer (FRET) is the mechanism of non-radiative energy transfer between two fluorophores; one being the donor and the other acceptor. The term fluorescence is also commonly used instead of Förster when both chromophores are fluorescent. The donor has to be in an electronic excited state in order to transfer its energy to the acceptor (Figure 2a). The efficiency of this transfer is inversely proportional to the sixth power of the distance between the donor and the acceptor over a range of 1-10 nm: which means, only the chromophores which are in very close proximity can go under this energy transfer. This makes FRET a very powerful technique for biological and medical experiments to detect interactions between molecules of interest, as only the ones who interact will be reflecting the energy transfer. For FRET to occur, the emission band of the donor has to overlap with the excitation band of the acceptor. Moreover, the excitation band of the donor should be reasonably away from that of the acceptor to prevent any direct excitation of the acceptor (Figure 2b). FRET is one of the most commonly used methods to detect interactions both in-vivo and in-vitro and in building biosensors. Without a doubt, it is a very powerful method; however, it has its own limitations including: 1) interference from autofluorescence of the natural fluorophores (in especially live cells) by direct excitation resulting with very low S:B ratios; 2) very low FRET signal dynamic range for fluorescent proteins in live-cell imaging (<5-fold), making it very hard to differentiate between a negative control and the samples, especially in HTS applications; 3) common organic fluorophores’ susceptibility to photobleaching upon a UV or near-UV excitation; and 4) crosstalk and bleedthrough due to wide excitation and emission bands of the donor and acceptors.
Figure 2: Fluorescence resonance energy transfer principles. a) FRET involves energy transfer from electronically excited donor to an acceptor in its ground state. Upon energy transfer, the donor returns to its ground state while acceptor becomes excited. This process happens via nonradiative processes instead of a direct photon emission & absorbance. If the acceptor is fluorescent, it emits photons as it returns to its ground state. b) Representation of the spectral overlap of the donor emission and the acceptor absorption. Note that the donor absorption band does not have any overlap with the acceptor absorption band. This is important to prevent any direct excitation of the acceptor.
1.3.2 Lanthanide chemistry and photophysics

The intricate photophysical properties of lanthanides arise from their electronic [Xe]4f<sup>n</sup> configurations (n=0-14). These configurations generate a variety of electronic levels, energies of which are well defined due to shielding of the 4f orbitals by the xenon core (54 electrons). This shielding makes the valence 4f orbitals ‘inner orbitals’ causing the ion to be minimally sensitive to its environment. This results in special spectroscopic properties with parity-forbidden 4f–4f absorptions having very low molar absorption coefficients (< 3 M<sup>-1</sup>s<sup>-1</sup>) and characteristic narrow-line emission bands (Figure 3a). Another feature of f-f transitions being parity forbidden is the lifetimes of the excited states become very long (microseconds to milliseconds time scale). Long lifetimes in turn make time-resolved measurements possible, where a brief delay (microseconds) is inserted between pulsed excitation and detection; this eliminates short-lived (nanosecond) fluorescence background, thus enabling measurements with high S:B. On the other hand, parity-forbidden transitions also mean that lanthanide ions cannot be directly excited. They have to be complexed with organic ‘antenna’ moieties which contain a sensitizing chromophore with a small singlet-triplet energy gap and a triplet energy at least 1500 cm<sup>-1</sup> above the receiving Ln<sup>+</sup> level. Upon light harvest by the antenna, energy is transferred to the lanthanide for excitation. Energy transfer occurs through the chromophore triplet state mostly, even though transfer through singlet and charge-transfer states has been observed (Figure 3b).
Figure 3: Photophysics of sensitized lanthanide complexes. A) Typical emission spectra of luminescent Tb$^{3+}$ (solid) and Eu$^{3+}$ (dotted) complexes. B) Schematic representation of major energy transitions in a lanthanide complex. S = singlet state, T = triplet state, A = absorption, F = fluorescence, P = phosphorescence, NR = nonradiative, ISC = intersystem crossing, ET = energy transfer, and L = metal luminescence.
1.3.3 **Examples of lanthanide based probes and their properties**

To be able to use the lanthanide probes in biological research and medical imaging, they have to have certain properties including structural stability of the chelated ion in aqueous solutions; emissions with high extinction coefficient (>10,000 M$^{-1}$ cm$^{-1}$) and quantum yield (>0.1); absorption wavelengths above the UV range (>350 nm); functional groups that can be utilized for biomolecule conjugation when needed; and good durability against photobleaching.\(^7\) Long-wavelength absorption is particularly critical as the cells are easily damaged and killed by exposure to the lower wavelengths.\(^7\)

Lanthanide probes have four components: lanthanide ion(s), a sensitizing chromophore, a chelate and one or more functional groups that can be used for biomolecule conjugation. Their electronic and photophysical features strongly depend on the coordination sphere of the metal. The coordination sites of the chelating ligand interact with the lanthanide ion to shield it from the environment while the chromophore acts as an antenna for electron capture and energy transfer to the metal. In general the chromophore can be any aromatic or heteroaromatic $\pi$-conjugated system, which has high efficiency of light absorption and energy transfer capabilities. The chelating molecules can range from acyclic ligands to macropolycyclic ones. In order to keep the positively charged lanthanide ion away from the negatively charged moieties in the solution, a ligand should bear negatively charged functional groups to tightly bind to and shield the metal. Many ligands contain carboxylates, phosphonates, phosphinates and $\beta$-diketonates for this purpose. Even though there are hundreds of lanthanide ligands, only a handful of them has the properties to supply the aforementioned features.\(^6\) The three most commonly used ligands are the
diethylenetriaminepentaacetic acid (DTPA), triethylenetetraaminehexaacetic acid (TTHA) which have been shown to have very stable luminescent complexes with Ln$^{+3}$ and Eu$^{+3}$; and the macrocyclic Lumi4 which very stably encapsulates various lanthanide ions.\textsuperscript{63,73–76}

Various DTPA and TTHA ligands which were conjugated with 7-amino-4-methyl-2(1H)-quinolinone (carbostyril 124; or cs124) antenna moiety (Figure 4a) were developed and shown to have high extinction coefficients ($\sim$10,500 M$^{-1}$ cm$^{-1}$) with relatively long wavelength absorption maximum (341 nm), good quantum yields and proven to have good structural stabilities for live cell usage.\textsuperscript{69,73,74,77–81} Lumi4 is another probe that is a registered trademark macrocycle developed by Lumiphore, Inc. (Richmond, CA), and it is based on a tetradeutate, 2-hydroxyisophthalamide chelating agent developed by Raymond and co-workers. It is one of the most stable and the brightest Tb$^{+3}$ complexes developed to date with a very high extinction coefficient (21,000 M$^{-1}$ cm$^{-1}$) at 340 nm and a very good quantum yield (>50%) (Figure 4b).\textsuperscript{76}
Figure 4: Structures of cs124-polyaminocarboxylate variants and Lumi4. a) Some variants of 7-amino-4-methyl-2(1H)-quinolinone (cs124)-polyaminocarboxylates. b) Lumi4 with four hydroxyisophthalamide moieties that serve as both chelators and sensitizers.
1.3.4 **TR-FRET: its advantages and applications in biological systems**

The use of lanthanide complexes for FRET applications alleviates most of the aforementioned FRET problems due to lanthanides’ unusually long emission lifetimes. First, autofluorescence and crosstalk fluorescence (due to direct acceptor excitation) are eliminated by simply delaying the signal collection by a few microseconds during which time short-lived background and non-specific fluorescence signal diminishes. The detector is turned on after this short delay to collect the long-lived lanthanide luminescence signal for up to a few milliseconds. This particular detection method is called time-resolved (or time-gated) detection and usage of it for energy transfer applications is called TR-FRET (Figure 5a). Second, signal dynamic range and the measurement sensitivity increases tremendously, especially for live-cell studies, because of elimination of the background fluorescence with time-gated detection. There are many groups that have reported usage of lanthanide complexes for HTS assays because of this advantage.\(^{13–15,82,83}\) In fact, this is exploited throughout the following chapters to develop HTS screening assays with large signal windows and unprecedented Z’ factor values. Third, commonly used and commercially available lanthanide complexes have very high resistance to photobleaching. This makes the long experiments possible which need to last for hours and sometimes even days. Fourth, bleedthrough is minimized because of lanthanide’s narrow emission bands (Figure 5b). Finally, each of these narrow emission bands can be used to excite a particular fluorophore species in the environment, paving the way for more powerful multiplexing studies in HTS applications. Through multiplexing, many interactions can be studied in a single experiment; removing the burden of running long and costly immunoassays and proteomics campaigns for interactome analyses.
Figure 5: Time-gated signal collection, Lumi4-Tb absorption and emission spectrum.
a) Long-lived (~ms) Tb$^{3+}$ donor or Tb$^{3+}$-sensitized acceptor emission can be detected without interference from scattering, autofluorescence or directly excited acceptor fluorescence background in time-resolved mode, where a 10-100 µs delay is inserted between pulsed excitation and detection. b) Narrow emission bands of Lumi4-Tb complex prevent bleedthrough and each emission peak can be used to excite a different fluorophore for multiplexing studies.
1.4 Selective Labeling of Recombinant Fusion Proteins with Lanthanide Complexes

1.4.1 Examples of current systems and their applications

The high signal-to-background ratio provided by time-gated detection of the FRET signal has enabled the development of highly sensitive and accurate detection of biological interactions in both cell-free and cell-based systems\textsuperscript{14,83–86}. Being able to selectively label the protein or biomolecule of interest in either live cells or in a biochemical solution provides the necessary specificity needed to monitor the interactions. This selective labeling is achieved by conjugation of the functional groups in the chelate with molecules like trimethoprim (for DHFR labeling)\textsuperscript{87}, biotin (for streptavidin labeling)\textsuperscript{13}, benzylguanine (for SNAP-tag labeling)\textsuperscript{83} and benzylcytosine (for CLIP-tag labeling)\textsuperscript{83}. As one of the biomolecules is labeled with the lanthanide complex, the interacting partner can be labeled with a spectrally suitable fluorophore such as a fluorescent protein or an organic dye. The most commonly used fluorophore for interaction detection is the green fluorescent protein (GFP). Labeling one interacting partner with GFP and the other with a lanthanide complex with one of the above mentioned methods gives rise to FRET signal upon binding (Figure 6). This technique is used to monitor protein-protein interactions with TR-FRET.
Figure 6: Schematic representation of FRET PPI assay mediated by trimethoprim (TMP) / E. coli dihydrofolate reductase (eDHFR) interaction. A TMP-Tb$^{3+}$ complex conjugate binds specifically and tightly to eDHFR ($K_D = \sim 2$ nM). Interaction between eDHFR and GFP fusion proteins and excitation of TMP-Tb (the donor) results in FRET-sensitized emission of GFP (the acceptor).
1.4.2 Trimethoprim/E. coli Dihydrofolate Reductase labeling system

The ease of synthesis of 5-(3,4,5-Trimethoxybenzyl) pyrimidine-2,4-diamine (TMP, or trimethoprim) derivatives allows the introduction of fluorophores or other labels optimized for FRET, chromophore assisted light inactivation of proteins, or other in vivo and in vitro bioassays. In the experiments detailed in following chapters, TMP conjugated Lumi4 or TTHA-cs124 chelators were used to label Escherichia coli dihydrofolate reductase (eDHFR) fusion proteins. TMP non-covalently binds to eDHFR with a dissociation constant ~2 nM. eDHFR is relatively small in size (18 kDa) and does not affect the function of the biomolecule it is incorporated into as shown in later chapters. Moreover, TMP does not interact with mammalian forms of DHFR as strongly, making the selective labeling possible. This rapid and reversible interaction also provides flexibility for ‘effective concentration’ manipulations. For example, if substoichiometric amounts of TMP-probe are used with the eDHFR fusion protein, the signal will be proportionately lower, simulating a lower ‘effective concentration’ of the fusion protein when needed. This is particularly useful if a non-specific, diffusion based FRET is observed due to high concentrations of probes.
CHAPTER 2

AN ADAPTABLE LUMINESCENCE RESONANCE ENERGY TRANSFER ASSAY FOR MEASURING AND SCREENING PROTEIN–PROTEIN INTERACTIONS AND THEIR INHIBITION IN IMPURE MATRICES
2.1 Introduction

Most biological processes are regulated by protein-protein interactions (PPIs). Consequently, bioanalytical methods that can identify and quantify PPIs are critical tools for fundamental biochemistry and proteomics research. Furthermore, compromised interactions as well as interactions between host and pathogen proteins can play a role in disease progression, and high throughput screening (HTS) assays that can identify PPI inhibitors are therefore an important drug discovery tool. Common methods for identifying PPIs include yeast two-hybrid, tandem affinity purification/mass spectrometry and co-immunoprecipitation. A variety of medium- and high-throughput methods can be used to screen for inhibitors or antagonists of PPI in purified biochemical preparations or even in cells including protein fragment complementation assays, fluorescent protein translocation, and methods based on Forster resonance energy transfer (FRET) or fluorescence polarization (FP) that interrogate reporter-labeled proteins. However, methods that offer the sensitivity and throughput necessary for HTS do not typically also allow for accurate measurement of equilibrium dissociation or inhibition constants. For example, while conventional FRET or FP methods are excellent for quantitative interaction analyses, their use in HTS can be problematic because they are susceptible to interference from non-specific fluorescence background signals. Therefore, quantitative PPI affinity or inhibition measurements often require alternative analyses such as classical radioligand binding, surface plasmon resonance or isothermal titration calorimetry. These methods require highly pure samples, which of course precludes their use for many proteins.

In this study, an HTS assay was developed where FK506 binding protein 12 (FKBP) fused to green fluorescent protein (GFP) and rapamycin binding domain of mTor (FRB) fused to
Escherichia coli dihydrofolate reductase (eDHFR) were used in highly impure bacterial cell lysate matrices for both interaction detection and inhibitor screening.

2.2 Materials and Methods

2.2.1 Materials

Oligonucleotides were obtained from Integrated DNA Technologies. Restriction enzymes, Vent DNA polymerase and T4 DNA ligase were purchased from New England Biolabs. Reagents were purchased from the indicated vendors: ascomycin, b-NADPH and Tween80 from Sigma-Aldrich; rapamycin from EMD Chemicals; Halt™ protease/phosphatase inhibitor cocktail and dialysis cassettes from Thermo Scientific; BugBuster® Protein Extraction Reagent from Novagen; HisLink™ protein purification resin from Promega; Luria-Bertani (LB) Broth, LB agar, potassium phosphate, o-phosphoric acid, acrylamide, sodium lauryl sulfate, ampicillin from Fisher Scientific; Coomassie Brilliant Blue G, bovine serum albumin (BSA) and imidazole from Acros Organics; isopropylthio-β-galactoside (IPTG) and HEPES from Invitrogen. Black bottom, 96-well plates were purchased from Costar. Preparation of pRSETb-mTSapphire-eDHFR, pRSETb-GFP-eDHFR plasmids was described in a previous study.73

Bacterial expression constructs were sequenced at UIC Research Resources Center (RRC). Sonications were carried out using a Branson Sonifier 150. Protein concentrations were determined by absorption spectroscopy using a Varian Cary 300 Bio UV-Visible Spectrophotometer. Time-resolved luminescence measurements were made using a fluorescence plate reader (Perkin Elmer, Victor 3V) with 340 nm excitation (60 nm bandpass) and either 520 nm emission (10 nm bandpass) or 615 nm emission (10 nm...
bandpass). Intensity values (1400 µs integration) were measured after a time delay of 100 µs. LRET data was used to generate binding isotherms that were fit non-linearly to the equations described in the text using KaleidaGraph (v4.0, Synergy Software).

The TMP-linked, Tb³⁺ complex used in these studies, TMP-TTHA-cs124(Tb³⁺), was prepared as previously described.⁷⁴

### 2.2.2 Plasmid vector construction

The gene encoding FRB was subcloned from plasmid pcDNA-myc-FRB (Addgene, Plasmid 20228) to pRSETb-mTSapphire-eDHFR to generate pRSETb-FRB-eDHFR. A 316 bp fragment encoding FRB was amplified by PCR from pcDNA-myc-FRB using the primers 5’ – CTC GAG GAT CCA ATC CTC TGG CAT GAG ATG TGGC – 3’ (BamHI, coding strand) and 5’ – CCTC TTC TGA GAT GAG TTT GTA CAC CTT TGA GAT TCG TCGG – 3’ (BsrGI, non-coding strand). This fragment was inserted between the BamHI site and the BsrGI site in pRSETb-mTSapphire-eDHFR to give to pRSETb-FRB-eDHFR. Upon transformation into *E. coli*, pRSETb-FRB-eDHFR expressed the protein fusion MRGSHHHHHHHGMASMTGGQQMGRDLYDDDDKDP-[FRB]-GSGGSG-[eDHFR]. The gene encoding FKBP12 was subcloned from plasmid YFP-FKBP (Addgene, Plasmid 20175) to pRSETb-GFP-eDHFR to generate pRSETb-GFP-FKBP. A 374 bp fragment encoding FKBP12 was amplified by PCR from YFP-FKBP using the primers 5’ – GC ATG GTG TAC AAG TCT GGA AGT GCT GGT GG – 3’ (BsrGI, coding strand) and 5’ – ACC AGC ACA AGC TTC AGC ACT TTC CAG – 3’ (HindIII, non-coding strand). This fragment was inserted between the BsrGI site and the HindIII site in pRSETb-GFP-eDHFR to give to
pRSETb-GFP-FKBP. Upon transformation into *E. coli*, pRSETb-GFP-FKBP expressed the protein fusion MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDP-[EGFP]-SGSAGG-[FKBP]. The integrity of all plasmids was verified by direct sequencing.

### 2.2.3 FRB-eDHFR and EGFP-FKBP12 expression and purification

*E. coli* strain BL21 DE3 (pLysS) cells were transformed with pRSETb-GFP-FKBP and pRSETb-FRB-eDHFR and grown on agar plates with ampicillin (100 µg ml⁻¹). LB broths (50 mL) with ampicillin (100 µg ml⁻¹) were inoculated with single colonies from agar plates and grown overnight at 37 °C. LB broths (1 L) with ampicillin (100 µg ml⁻¹) were inoculated with overnight grown cultures and grown at 37 °C at 250 rpm until OD₆₀₀ ~ 0.6-0.7, at which time IPTG was added (to a final concentration of 1 mM). pRSETb-GFP-FKBP cultures were grown 6 hours and pRSETB-FRB-FKBP cultures were grown 4 hours after IPTG addition until cells were harvested by centrifugation. The pellets were lysed in lysis buffer (25 mL; 1X BugBuster™ Protein Extraction Reagent, 2 units ml⁻¹ DNase I, 1mM PMSF, 100mM HEPES, 10mM imidazole, pH 7.5). Samples were placed on an orbital shaker for 30 min, and then centrifuged (18,500 rcf, 20 min, 4 °C). The supernatants were passed through HisLink™ protein purification resin and washed with gradually increasing concentrations of imidazole solutions until proteins were eluted. The purities of the proteins were tested with sodium dodecyl sulfate polyacrylamide gel electrophoresis and staining with Coomassie Blue. Following purification, the proteins were concentrated (to ~100 µM), dialyzed in phosphate buffer (10 mM K₂HPO₄, KH₂PO₄, pH 7.4), and stored at -80 °C. Concentration of His₆-FRB-eDHFR was determined by 280 nm absorption measurement (ε₂₈₀ = 65,650 cm⁻¹M⁻¹) and
concentration of His\textsubscript{6}-GFP-FKBP was determined by absorbance of GFP at 487 nm ($\varepsilon_{487} = 57,500 \text{ cm}^{-1}\text{M}^{-1}$).

### 2.2.4 Binding assay preparation with pure proteins

Fusion proteins were expressed and purified as explained in section 2.2.3 and the assays were launched with the below configurations:

For the analysis of GFP-FKBP/rapamycin/FRB-eDHFR affinity via titration of GFP-FKBP, below steps were followed:

1. GFP-FKBP (6 µM in assay buffer) was serially diluted into 24 wells containing 50 µL assay buffer.
2. Five rapamycin solutions were prepared (14, 8, 6, 2, 0.8 µM in Assay Buffer), and 25 µL of a given solution was added into wells containing 50 µL GFP-FKBP solutions such that [rapamycin] > [GFP-FKBP].
3. A solution containing FRB-eDHFR (40 nM), TMP-TTHA-cs124(Tb\textsuperscript{3+}) (40 nM), and NADPH (4 µM) was prepared, and 25 µL of it was added into each well.
4. The final concentrations of each component in sample wells were as follows:
   i. GFP-FKBP, ranging from 3.0 µM to 0.4 pM
   ii. Rapamycin, ranging from 3.5 µM to 0.2 µM
   iii. FRB-eDHFR, constant in all wells at 10 nM
   iv. TMP-TTHA-cs124(Tb\textsuperscript{3+}), constant in all wells at 10 nM
   v. NADPH, constant in all wells at 1 µM
5. A solution containing TMP-TTHA-cs124(Tb\(^{3+}\)) (20 nM), and NADPH (2 µM) was prepared for control wells, and 50 µL of it was added into each control well containing 50 µL GFP-FKBP dilutions.

6. The final concentrations of each component in control wells were as follows:
   
   i. GFP-FKBP, ranging from 3.0 µM to 0.4 pM
   
   ii. TMP-TTHA-cs124(Tb\(^{3+}\)), constant in all wells at 10 nM
   
   iii. NADPH, constant in all wells at 1 µM

For the analysis of GFP-FKBP/rapamycin/FRB-eDHFR affinity via titration of FRB-eDHFR, below steps were followed:

1. FRB-eDHFR (2 µM in assay buffer) was serially diluted into 16 wells containing 50 µL assay buffer.

2. A solution containing GFP-FKBP (20 nM), TMP-TTHA-cs124 (2 µM), Tb\(^{3+}\) (0.2 µM), rapamycin (60 nM), and NADPH (4 µM) was prepared, and 50 µL of it was added into each sample well containing 50 µL FRB-eDHFR dilutions and into each control well containing 50 µL assay buffer.

3. The final concentrations of each component in sample wells were as follows:
   
   i. FRB-eDHFR, ranging from 1 µM to 30 pM
   
   ii. GFP-FKBP, constant in all wells at 10 nM
   
   iii. Rapamycin, constant in all wells at 30 nM
   
   iv. TMP-TTHA-cs124, constant in all wells at 1 µM
   
   v. Tb\(^{3+}\), constant in all wells at 0.1 µM
vi. NADPH, constant in all wells at 2 µM

4. The final concentrations of each component in control wells were as follows:
   i. GFP-FKBP, constant in all wells at 10 nM
   ii. Rapamycin, constant in all wells at 30 nM
   iii. TMP-TTHA-cs124, constant in all wells at 1 µM
   iv. Tb³⁺, constant in all wells at 0.1 µM
   v. NADPH, constant in all wells at 2 µM

2.2.5 Binding assay preparation with bacterial cell lysates

Following transformation with pRSETb-GFP-FKBP, growth, and expression, BL21 DE3 (pLysS) cell pellets were resuspended in sonication buffer (2x Halt™ Protease/Phosphatase Inhibitor Cocktail, 2 units ml⁻¹ DNase I, 50 mM K₂HPO₄, KH₂PO₄, pH 7.2) (to the final concentration of 0.1 mg µL⁻¹ (w/v)). The cell suspension was lysed by sonication (2 minutes, 20 s on/10 s off cycles), centrifuged (20,817 rcf, 15 min, 4 °C) and the supernatant was retained for subsequent analysis. GFP-FKBP concentration was quantified by GFP absorbance (ε₄₈₇ = 57,500 cm⁻¹M⁻¹), and total protein concentration in the cell lysate was quantified by absorption at 280 nm.¹⁰⁰

For the analysis of GFP-FKBP/rapamycin/FRB-eDHFR affinity by using proteins in cells lysate, below steps were followed for the assay preparation and launch:

1. Cell lysate (28 mg/ml (w/v)) was serially diluted into 24 wells containing 50 µL assay buffer.
2. GFP-FKBP (6 µM) in cell lysate (28 mg/ml) was serially diluted into 24 wells containing 50 µL cell lysates, in reverse direction of cell lysate dilution.

3. Five rapamycin solutions were prepared (14, 8, 6, 2, 0.8 µM in Assay Buffer), and 25 µL of a given solution was added into each sample well such that [rapamycin] > [GFP-FKBP].

4. A solution containing FRB-eDHFR (40 nM), TMP-TTHA-cs124(Tb³⁺) (40 nM), and NADPH (4 µM) was prepared, and 25 µL of it was added into each sample well.

5. The final concentrations of each component in sample wells were as follows:
   i. GFP-FKBP, ranging from 3.0 µM to 0.4 pM
   ii. Rapamycin, ranging from 3.5 µM to 0.2 µM
   iii. FRB-eDHFR, constant in all wells at 10 nM
   iv. TMP-TTHA-cs124(Tb³⁺), constant in all wells at 10 nM
   v. NADPH, constant in all wells at 1 µM
   vi. Cell lysate, constant in all wells at 14 mg/ml

6. A solution containing TMP-TTHA-cs124(Tb³⁺) (20 nM), and NADPH (2 µM) was prepared for control wells, and 50 µL of it was added into each control well containing 50 µL GFP-FKBP dilutions in cell lysate.

7. The final concentrations of each component in control wells were as follows:
   i. GFP-FKBP, ranging from 3.0 µM to 0.4 pM
   ii. TMP-TTHA-cs124(Tb³⁺), constant in all wells at 10 nM
   iii. NADPH, constant in all wells at 1 µM
   iv. Cell lysate, constant in all wells at 14 mg/ml
2.2.6 Inhibition assay preparation with small molecule inhibitor ascomycin

Measurements of equilibrium binding affinity were carried out in 96-well plates (100 µL/well) at 21-25 ºC with analytes diluted into assay buffer (50 mM K$_2$HPO$_4$, KH$_2$PO$_4$, pH 7.2, 0.1 % (w/v) BSA, and 0.1 % (v/v) Tween80). For a given assay, the titrated analyte was serially diluted well-to-well by a factor of 2 to achieve the desired concentration range, and the remaining analytes were then added to achieve the desired final composition, as detailed below. Each sample titration was performed in triplicate. Plates were measured using a Victor 3V luminescence plate reader and data was analyzed as described in the results and discussion section.

For the analysis of competitive inhibition of GFP-FKBP/rapamycin/FRB-eDHFR interaction using ascomycin, below steps were followed for the assay preparation and launch:

1. Ascomycin (0.4 µM in assay buffer) was serially diluted into 12 wells containing 50 µL assay buffer.
2. A solution containing FRB-eDHFR (60 nM), GFP-FKBP (60 nM), TMP-TTHA-cs124(Tb$^{3+}$) (60 nM), and NADPH (6 µM) was prepared, and 25 µL of it was added into each sample well and into each control well containing 75 µL assay buffer.
3. 25 µL rapamycin solution (60 nM in assay buffer) was added into each sample well.
4. The final concentrations of each component in sample wells were as follows:
   i. Ascomycin, ranging from 200 nM to 98 pM
   ii. GFP-FKBP, constant in all wells at 15 nM
   iii. FRB-eDHFR, constant in all wells at 15 nM
iv. Rapamycin, constant in all wells at 15 nM
v. TMP-TTHA-cs124(Tb$^{3+}$), constant in all wells at 15 nM
vi. NADPH, constant in all wells at 1.5 µM

5. The final concentrations of each component in control wells were as follows:

i. GFP-FKBP, constant in all wells at 15 nM
ii. FRB-eDHFR, constant in all wells at 15 nM
iii. Rapamycin, constant in all wells at 15 nM
iv. TMP-TTHA-cs124(Tb$^{3+}$), constant in all wells at 15 nM
v. NADPH, constant in all wells at 1.5 µM

2.3 Results and Discussion

The assay developed in this study measures the interaction between two fusion proteins; one labeled with a luminescent Tb$^{3+}$ complex and the other fused to GFP. (GFP-)FKBP/Rapamycin/FRB(-eDHFR) system was chosen as a model system due to availability of high number of research papers about this complex interaction and its easy manipulation by simply removing the rapamycin from the medium (Figure 7).
Figure 7: Schematic diagram representing the binding events involved in the formation of a GFP-FKBP/rapamycin/FRB-eDHFR/TMP-Tb complex. The relative magnitudes of the dissociation constants are such that it is the interaction between GFP-FKBP/rapamycin and FRB-eDHFR ($K_D = 12$ nM, shaded) that is measured in a saturation binding assay. By maintaining a constant ratio of FRB-eDHFR relative to TMP-Tb, it is possible to titrate either GFP-FKBP or FRB-eDHFR, and the measured Tb$^{3+}$-to-GFP LRET signal reflects formation of the FKBP/rapamycin/FRB complex.
It was previously shown in our lab that conjugates of trimethoprim (TMP) linked to Tb$^{3+}$ complexes bind selectively and with high affinity to eDHFR in purified preparations, cell lysates and in living cells, thereby offering an effective means of selectively labeling fusion proteins with Tb$^{3+}$ luminophores.\textsuperscript{73–75} Thus, in a mixture containing eDHFR and GFP fusions and a TMP-linked Tb$^{3+}$ reporter, we may expect to detect interaction between the two proteins as Tb$^{3+}$-sensitized, GFP emission from a complex that includes all three components (Figure 8c). Illumination with \textasciitilde340 nm light excites the Tb$^{3+}$ reporter, leading to LRET-sensitized emission of GFP at 520 nm. The sensitized GFP emission can be easily filtered from terbium donor emission (Figure 8a), and time-resolved detection eliminates directly excited GFP fluorescence and non-specific fluorescence from samples or library components (Figure 8b). Here, TMP-TTHA-cs124 (Figure 8d) was used as the luminescent Tb$^{3+}$ label due to its high affinity for eDHFR ($K_D = \sim 2 \text{ nM}$), good brightness ($\varepsilon = \sim 10,000 \text{ M}^{-1}\text{cm}^1$ at 343 nm, quantum yield in water = 0.20), and relatively high metal-binding stability compared to other chelates.\textsuperscript{74}
Figure 8: Key aspects of Luminescence Resonance Energy Transfer (LRET) assays to detect and quantify protein-protein interactions (PPI). (a) The emission spectrum of Tb$^{3+}$ (cyan) overlaps with the excitation spectrum (dotted green) of green fluorescent protein (GFP). The narrow, spiked Tb$^{3+}$ emission facilitates separation of Tb$^{3+}$ donor and LRET-sensitized, GFP acceptor (solid green) emission signals. (b) Long-lived (~ms) Tb$^{3+}$ donor or Tb$^{3+}$-sensitized acceptor emission can be detected without interference from scattering, autofluorescence or directly excited acceptor fluorescence background in time-resolved mode, where a 10-100 µs delay is inserted between pulsed excitation and detection. (c) Schematic representation of LRET PPI assay mediated by trimethoprim (TMP)/E. coli dihydrofolate reductase (eDHFR) interaction. A TMP-Tb$^{3+}$ complex conjugate binds specifically and tightly to eDHFR ($K_D$ = ~2 nM). Interaction between eDHFR and GFP fusion proteins and excitation of TMP-Tb (the donor) results in LRET-sensitized emission of GFP (the acceptor). (d) Structure of TMP-TTHA-cs124, the Tb$^{3+}$ complex of which is used in these studies.
In order to demonstrate the sensitivity, accuracy and precision of our assay for detecting and quantifying PPIs and their inhibition, the well-studied, rapamycin-induced interaction between FKBP and FRB was selected as the model system. Rapamycin-induced heterodimerization of FKBP and FRB fusion proteins has been exploited extensively to regulate protein expression,\textsuperscript{101} glycosylation,\textsuperscript{102} and for other biotechnological applications.\textsuperscript{103} Wandless and coworkers thoroughly characterized the equilibrium binding affinities involved in the formation of the FKBP/rapamycin/FRB complex using fluorescence polarization, surface plasmon resonance, and NMR spectroscopy.\textsuperscript{104} Given the relative magnitudes of the dissociation constants, this LRET binding assay was designed to measure the affinity between the FKBP-rapamycin complex and FRB ($K_D = 12 \text{ nm}$, Figure 7). Here, it is shown that formation of a GFP-FKBP/rapamycin/FRB-eDHFR/TMP-TTHA-cs124(Tb$^{3+}$) complex can be sensitively monitored by detecting long-lifetime ($>100 \text{ us}$), Tb$^{3+}$-to-GFP LRET in 96-well plates using a time-resolved luminescence plate reader. By titrating either GFP-FKBP or FRB-eDHFR while holding all other components of the system at constant concentrations, I was able to generate binding isotherms. As it was detailed below, appropriate correction of the measured LRET signal and non-linear least squares (NLS) analysis of the data allowed for accurate and reproducible measurement of the affinity of GFP-FKBP/rapamycin for FRB-eDHFR in both buffer solution and bacterial lysates.

Initially, affinity measurements were performed in a buffer solution containing purified chimeric proteins, GFP-FKBP and FRB-eDHFR. First, GFP-FKBP was titrated in 96-well plates (3 µM to 0.4 pM in 100 µL of assay buffer) while other component concentrations were held constant, including FRB-eDHFR (10 nM), TMP-TTHA-cs124(Tb$^{3+}$) (10 nM) and NADPH (cofactor necessary for tight TMP/eDHFR binding, 1 µM). Sufficient amounts of rapamycin...
were added to all wells to pre-form the GFP-FKBP/rapamycin complex. Sample titrations were prepared in triplicates (3 sets of 24 wells each), and a single set of negative control wells were prepared that contained identical concentration range of GFP-FKBP but lacked FRB-eDHFR and rapamycin (see Materials and Methods section for complete details of sample preparation). The sample plate was maintained at room temperature and analyzed with 10 minutes intervals over 2 hours by measuring the time-resolved luminescence signal ($\lambda_{ex} = 340$ nm, delay = 100 $\mu$s) at 520 nm (LRET-sensitized, acceptor emission) and at 615 nm (Tb$^{3+}$ donor emission). The 520/615 emission ratio was calculated to minimize well-to-well variability resulting from differences in probe amounts or sample absorbance.$^{105}$ Non-specific signal was observed at 520 nm that increased with GFP-FKBP concentration (Figure 9), and this was attributed to diffusion-mediated LRET between TMP-TTHA-cs124(Tb$^{3+}$) and GFP. Therefore, the 520/615 emission ratio observed in the corresponding negative control was subtracted from the ratio observed in the sample well at a given GFP-FKBP concentration to obtain the LRET signal attributable to formation of the GFP-FKBP/rapamycin/FRB-eDHFR/TMP-TTHA-cs124(Tb$^{3+}$) complex. Then, the percent change in the 520 nm/615 nm emission ratio, $\Delta L\%$, was calculated as

$$\Delta L\% = \left( \frac{520}{615} \right)_{S,[P]} - \left( \frac{520}{615} \right)_{C,[P]}$$

(1)

where, $\left( \frac{520}{615} \right)_{S,[P]}$ and $\left( \frac{520}{615} \right)_{C,[P]}$ are the emission ratios for the sample and negative control wells at a given GFP-FKBP concentration, respectively, and $\left( \frac{520}{615} \right)_{C,12-24}$ is the mean emission ratio for the 12 control wells containing the lowest concentrations of GFP-FKBP.
Figure 9: Raw LRET signal (λ_em = 520 nm) observed for representative binding affinity measurements with GFP-FKBP titration. Signal observed in sample and negative control wells when purified GFP-FKBP was titrated against constant levels of FRB-eDHFR (10 nM) and other assay components. The signal observed in negative controls increases with GFP-FKBP concentration because of non-specific, diffusion-mediated LRET between luminescent terbium complex and GFP. The increased background prevents saturation of the uncorrected LRET sample signal.

In order to obtain a binding isotherm, ΔL% was plotted as a function of GFP-FKBP concentration for each time point analyzed. The system appeared to equilibrate after ~80 minutes, as evidenced by stabilization of the observed data plots (Figure 10). Shown in Figure 11 is the binding isotherm obtained at 120 min.
Figure 10: GFP-FKBP/rapamycin/FRB-eDHFR equilibration with GFP-FKBP titration. System reaches equilibrium after ~80 minutes when purified GFP-FKBP is titrated against purified FRB-eDHFR to measure dissociation constant, as revealed by representative data plots.
Figure 11: Homogeneous, TR-LRET assay detects and measures PPI affinity at high signal-to-background ratio and Z'-factor when GFP tagged protein is titrated. The plot shows the outcome of saturation binding experiment in 96-well plate (100 µL sample volume). The y-axis represents the percent change in the 520 nm/615 nm emission ratio and the x-axis represents concentration of the titrated GFP-FKBP. Complete details of sample preparation and data analysis are given in the text. The data shows the equilibrium binding (120 minutes after sample preparation) of purified GFP-FKBP/rapamycin complex (3 µM to 0.4 pM) to purified FRB-eDHFR (10 nM). Line represents non-linear least squares (NLS) fit to equation 2, yielding $K_D = 7.5 \pm 0.4$ nM.
A NLS fit was applied to this data using the following equation to obtain the $K_D$ for binding of the GFP-FKBP/rapamycin complex to FRB-eDHFR:

\[
\Delta L\% = L_{\text{min}} - (L_{\text{min}} - L_{\text{max}}) \times \\
\frac{([P1]_T + K_D + [P2]_T) - \sqrt{([P1]_T + K_D + [P2]_T)^2 - (4[P1]_T[P2]_T)}}{2[P1]_T}
\]

(2)

where, $L_{\text{min}}$ is the $\Delta L\%$ value observed for of the lowest concentration GFP-FKBP sample, $L_{\text{max}}$ is the maximum $\Delta L\%$ signal observed at saturating GFP-FKBP concentration, $[P1]_T$ is the concentration of the fixed protein (FRB-eDHFR, in this case) and $[P2]_T$ is the concentration of titrated protein (GFP-FKBP). This analysis yielded a $K_D$ of 7.5 ± 0.4 nM. The value measured with this assay differs somewhat from that reported by Wandless and coworkers (12 nM). That study used surface plasmon resonance to analyze binding of FRB to immobilized FKBP/rapamycin, and this may account for the differences in observed affinities. Furthermore, in that same paper, the authors reported an estimated, solution-phase $K_D$ of 6.2 nM for FKBP/rapamycin/FRB interaction based on a fluorescence polarization competition assay, close to our measured value. Moreover, we observed >350 S:B for the LRET signal, suggesting this assay platform would be easily adapted for HTS conditions.

Next, it was sought to determine whether the titration could be reversed; holding GFP-FKBP levels constant while varying FRB-eDHFR concentration. This was attempted in order to demonstrate the versatility of the assay and also to eliminate the varying levels of diffusion-mediated Tb$^{3+}$-to-GFP FRET seen when GFP-FKBP acceptor concentration was varied over a broad range. Here, it was necessary to ensure that the FRB-eDHFR/TMP-TTHA-cs124(Tb$^{3+}$)
concentration remained consistent throughout the titration while minimizing the overall Tb$^{3+}$ luminescence signal. This was accomplished by pre-equilibrating TMP-TTHA-cs124 with a sub-stoichiometric amount of Tb$^{3+}$ and maintaining a relatively high concentration (1 µM) of the TMP conjugate in all sample wells. 96-well plates were prepared such that GFP-FKBP concentration remained constant (10 nM), FRB-eDHFR concentration varied (from 1 µM to ~30 pM), and GFP-FKBP/rapamycin and FRB-eDHFR/TMP-TTHA-cs124 binding were saturated in all wells (see Materials and Methods section for complete details of sample preparation). The plate was then analyzed periodically up to 1 h, and the percent change in 520/615 emission ratio at each time point was calculated as:

$$\Delta \% = \frac{\frac{520}{615}_{S[ip]} - \frac{520}{615}_{C}}{\frac{520}{615}_{C}}$$

where $\frac{520}{615}_{S[ip]}$ is the emission ratios for the sample well at a given FRB-eDHFR concentration, and $\frac{520}{615}_{C}$ is the mean emission ratio for multiple control wells (9, in this case) lacking FRB-eDHFR and rapamycin. The signal change was then plotted against FRB-eDHFR concentration to obtain binding isotherms.

In this case, the signal level equilibrated after ~30 minutes (Figure 12), and the data obtained 45 minutes after sample preparation is shown in Figure 13.
Figure 12: GFP-FKBP/rapamycin/FRB-eDHFR equilibration with FRB-eDHFR titration. System reaches equilibrium after ~30 minutes when purified FRB-eDHFR is titrated against purified GFP-FKBP to measure dissociation constant, as revealed by representative data plots.
Figure 13: Homogeneous, TR-LRET assay detects and measures PPI affinity at high signal-to-background ratio and Z’-factor when GFP tagged protein is kept constant. The plot shows the outcome of saturation binding experiment in 96-well plate (100 µL sample volume). The y-axis represents the percent change in the 520 nm/615 nm emission ratio and the x-axis represents concentration of the titrated FRB-eDHFR. Complete details of sample preparation and data analysis are given in the text. The data shows the equilibrium binding after titration of purified FRB-eDHFR (1 µM to 30 pM) against purified GFP-FKBP/rapamycin complex (10 nM) obtained 45 minutes after sample preparation. NLS fit to equation 2 yielded $K_D = 3.3 \pm 0.6$ nM.

Here, a substantially lower signal change (≈30%) was observed with compared to that seen when GFP-FKBP was titrated. Two reasons for that are: 1) the concentration of signal-generating, GFP-FKBP/rapamycin/FRB-eDHFR/TMP-TTHA-cs124(Tb$^{3+}$) complex is very low at saturation (only ~1 nM); and 2) there was a consistently high level of background signal observed at 520 nm reflecting the relatively high concentration (100 nM) of luminescent Tb$^{3+}$ complex present in all wells (Figure 14).
Figure 14: Raw LRET signal ($\lambda_{em} = 520$ nm) observed for representative binding affinity measurements with FRB-eDHFR titration. Signal observed in sample and negative control wells when purified FRB-eDHFR was titrated against constant levels of GFP-FKBP (10 nM) and other assay components. The signal observed in negative controls is persistently high (~5800 counts) across the titration range because there is a high level (100 nM) of luminescent terbium complex present in all wells.

Despite the lower signal, the data could be fit non-linearly (equation 2) to yield an apparent $K_D$ of 3.3 ± 0.6 nM. This value is ~2-fold lower than that calculated from GFP-FKBP titrations and may reflect the relative noisiness of the data. Nevertheless, the results suggest that the assay can be modified to obtain reasonably accurate $K_D$ estimates across a broad range of experimental conditions and binding affinities (Figure 15).
Figure 15: A broad range of affinities between eDHFR and GFP fusion proteins can be measured by varying the amount of TMP conjugate and/or the amount of terbium in the assay. The plot shows that the expected equilibrium concentration of a luminescent, GFP-eDHFR-TMP-Tb complex (y-axis) can be verified over a similar range under different titration conditions. Calculations were made such that for a given assumed $K_D$ value, $[\text{GFP}] = K_D$, $0.01K_D < [\text{eDHFR}] < 100K_D$, and $[\text{Terbium Complex}]$ were as shown in the plot legend.
Because many proteins cannot be purified, it was sought to determine whether the assay could be used to measure binding affinities in cell lysates where only one of the interacting partners was first subject to purification. Here, GFP-FKBP was expressed in *E. coli* and bacterial lysates containing the fusion protein was titrated against a constant concentration of purified FRB-eDHFR. The concentration of GFP-FKBP in the lysates was determined by using the absorbance measurements and calculation using the known extinction coefficient of EGFP ($\varepsilon_{488\text{ nm}} = 57,500 \text{ M}^{-1}\text{cm}^{-1}$).\textsuperscript{106} Sample plates were prepared such that GFP-FKBP concentration varied (between 3 µM and 0.4 pM), and a constant level of lysate was maintained in all wells (14 mg/mL as total protein concentration) so as to maintain a consistent environment (see Materials and Methods section for complete details of sample preparation). Despite the efforts to maintain well-to-well homogeneity, the plate preparation resulted in substantially lower levels of assay buffer in the highest and lowest ends of the titration range. The assay buffer contains bovine serum albumin and Tween-80 to minimize non-specific binding of TMP-TTHA-cs124(Tb$^{3+}$) to the surface of sample wells and resultant sequestration out of the excitation light path. The inhomogeneity of the samples resulted in a substantial reduction of Tb3+ luminescence at the extreme ends of the titration range and this necessarily affected the measured LRET signal (Figure 16). Nevertheless, by excluding the data points at either end of the titration, we were able to non-linearly fit the data (equation 2) to obtain a $K_D$ of 6.5 ± 0.2 nM, very close to the value seen with purified proteins in assay buffer (Figure 17). These results show that interactions between GFP and eDHFR fusion proteins can be sensitively detected (S:B >100) and quantified in complex matrices even when one of the binding partners is not first purified.
Figure 16: Raw Tb$^{3+}$ signal ($\lambda_{em} = 615$ nm) observed for a representative data set obtained when bacterial lysates containing GFP-FKBP were titrated against a fixed concentration of FRB-eDHFR. The Tb$^{3+}$ luminescence is substantially reduced at the extreme ends of the titration range because these sample wells contain less assay buffer than wells in the mid-range of the titration. The assay buffer is formulated to prevent non-specific binding of TMP-TTHA-cs124(Tb$^{3+}$) to sample wells and resultant sequestration out of the excitation light path. Four data point from either end were omitted for non-linear curve fit calculations (see main text for details).
Figure 17: Homogeneous, TR-LRET assay in impure cell lysate matrix. Data showing equilibrium binding (130 minutes) of impure GFP-FKBP/rapamycin complex (181 nM to 6 pM) to purified FRB-eDHFR (10 nM) in E. coli lysates. The y-axes represent the percent change in the 520 nm/615 nm emission ratio and the x-axes represent concentration of the titrated GFP-FKBP. NLS fit to equation 2 yielded $K_D = 6.5 \pm 0.2$ nM.
Since many HTS assays are used to identify inhibitors of a given interaction, a competitive inhibition assay was performed using ascomycin, which competes with rapamycin for binding to FKBP. The FKPB/ascomycin complex does not bind to FRB. We titrated ascomycin against constant concentrations of rapamycin, GFP-FKBP and FRB-eDHFR (see Materials and Methods section for complete details of sample preparation). The concentration of ascomycin against ΔL% (equation 3) was plotted and the data was fitted using NLS regression to the following equation:

$$\Delta L\% = L_{\text{min}} + \frac{(L_{\text{max}} - L_{\text{min}})}{(1 + 10^{((\log IC_{50}-[I]_T) \times \text{HillSlope})})}$$

where, $L_{\text{min}}$ and $L_{\text{max}}$ are the minimum and maximum observed ΔL% values, respectively, $[I]_T$ is the ascomycin concentration, and Hill Slope is the slope of the steep portion of the curve between 10% and 90% signals. From this fit, a value of $IC_{50} = 9.8\pm0.3 \text{ nM}$ was obtained (Figure 18). As was seen for direct measurements of affinity, a high S:B (>80) was observed between maximum and minimum signals. For HTS assays, one common measure of assay performance is $Z'$-factor:

$$Z' = 1 - \frac{(3\sigma_{\text{max}} + 3\sigma_{\text{min}})}{\left| \mu_{\text{max}} - \mu_{\text{min}} \right|}$$

calculated from the standard deviations and means of the maximum and minimum observed signal levels under controlled conditions (i.e., without library compounds present). $Z'$ can vary between -1 and 1, with values >0.5 considered acceptable. $Z'$-factor for ascomycin inhibition was estimated from the highest and lowest observed signals (Figure 18), obtaining an extremely high value of 0.89.
Figure 18: Ascomycin inhibition isotherm and IC$_{50}$ calculation. Data shows inhibition of GFP-FKBP/rapamycin/FRB-eDHFR (10 nM, 30 nM, and 10 nM, respectively) interaction by ascomycin (200 nM to 100 pM). The y-axis represents the percent change in the 520 nm/615 nm emission ratio and the x-axis represents concentration of the titrated Ascomycin. Line represents NLS fit to equation 3, giving an IC$_{50}$ = 9.8 ± 0.3 nM.
In this study, it was shown that the selective, tight, non-covalent binding of a TMP-linked Tb\(^{3+}\) complex to eDHFR enables high-S:B, LRET-based detection of PPIs and their inhibition as well as accurate measurements of equilibrium dissociation and inhibition constants. GFP-FKBP/rapamycin was titrated against constant levels of FRB-eDHFR/TMP-TTHA-cs124(Tb\(^{3+}\)) and LRET-sensitized, Tb\(^{3+}\)-to-GFP emission was measured to generate equilibrium binding isotherms. NLS fitting revealed nearly identical \(K_D\) values for binding of GFP-FKBP/rapamycin to FRB-eDHFR in both buffer solution with purified proteins (~7.5 nM) and in bacterial lysates where GFP-FKBP was not purified (~6.4 nM). A reverse titration yielded a ~2-fold lower value (\(K_D = \sim 3.3\) nM). These values closely matched previously reported \(K_D\)’s for the FKBP/rapamycin/FRB interaction (6.4 nM, solution-phase; 12 nM, surface-tethered).\(^{104}\) While our proof-of-principle experiments were performed in 96-well plates (100 µL/well), it is evident from the large, observed S:B’s (~100-350 for PPI affinity, ~80 for ascomycin-mediated inhibition) that the assay could easily be miniaturized for HTS in 384-well (20-30 µL/well) or possibly even 1536-well (3-10 µL/well) plates. The hybrid chemical/genetic protein labeling strategy described in this study should allow HTS of a wide variety of target interactions across a broad range of affinities. Moreover, the assay platform can be used to interrogate PPIs directly in bacterial, mammalian or yeast cell extracts, enabling analysis of post-translationally modified proteins or other proteins that are not amenable to purification.
CHAPTER 3

LUMINESCENCE RESONANCE ENERGY TRANSFER ASSAY SCREENING

PROTEIN–PROTEIN INTERACTIONS AND THEIR INHIBITION IN LIVE MAMMALIAN CELLS
3.1 Introduction

Protein–protein interactions (PPIs) are central to biological processes and represent an important class of therapeutic targets. \(^{51,52}\) However, identification of drugs or leads that can inhibit PPIs is challenging due to the large area and flatness of the interfaces, a relative lack of small-molecule scaffolds from which to develop libraries, and the difficulty of characterizing interaction stoichiometry and sites of binding. \(^{52,108}\) Nevertheless, successful drugs have been developed that demonstrate the feasibility of targeting PPIs. These include agents targeting HPV E2/E1, \(^{109,110}\) BCL-X\(_L\)/BAD \(^{111}\), and HDM2/p53 \(^{112,113}\) interactions at doses as low as 1 nM. HTS has been an important tool in identifying these agents, but the screening efforts have relied largely on cell-free systems that require protein purification. Apart from the impossibility of obtaining many proteins in pure form, in vitro assays necessarily occur in artificial contexts that do not account for influences such as subcellular localization, competitive interaction with other cellular factors and post-translational modifications. Moreover, cell-free HTS systems do not discriminate against cytotoxic or membrane-impermeable compounds. \(^{5,114,115}\) Thus, there is a need for high-throughput methods to discover PPI inhibitors that are performed directly within living mammalian cells. \(^{116}\)

A limited number of methods for studying PPIs directly within living cells have been developed that can be adapted to at least a medium-throughput rate of analysis. These include methods based on sub-cellular redistribution of fluorescently labeled proteins (suitable for high-content imagers), \(^{32,117}\) reporter fragment complementation assays (e.g., split GFP, luciferase), \(^{94}\) reporter gene hybrid-like systems, and methods based on FRET or bioluminescence resonance energy transfer (BRET). \(^{97,118,119}\) However, all of these available cell-based PPI assays suffer from
one or more limitations, including low signal-to-background ratio (S:B), high rates of false positives/negatives, and protein sequestration at non-physiologic sites. Therefore, we set out to adapt the in vitro TR-FRET assay described in Chapter 2 so that it could detect PPIs and their inhibition directly within cultured mammalian cells grown in 96-well plates using standard plate reader instrumentation.

The assay developed in this study sensitively measures the interaction between two proteins; FKBP12, which was fused to GFP; and FRB, which was fused to eDHFR and labeled with TMP-Lumi4(Tb\(^{3+}\)) in 96-well plates using a time-resolved fluorescence plate reader by detecting the long-lifetime (>100 µs), Tb\(^{3+}\)-to-GFP FRET. The rapamycin-induced interaction between FKBP12 and FRB was again used as a model PPI. A stably transformed NIH/3T3 fibroblast cell line was prepared that constitutively expresses GFP-FKBP12 and that allows expression of FRB-eDHFR under control of a tetracycline-inducible promoter. Lumi4(Tb\(^{3+}\)) was selected because of its extreme stability in aqueous solutions and it being the brightest terbium complex yet developed with an extinction coefficient of 21,000 M\(^{-1}\)cm\(^{-1}\) at 340 nm and a quantum yield >0.5.\(^{70}\) The details of the FKBP/Rapamycin/FRB interaction and the signal detection principles with terbium complexes were discussed in previous chapters. As detailed in the results and discussion section, appropriate correction of the measured FRET signal allowed for accurate and reproducible measurement of the interaction in both permeabilized and cross-linked mammalian cells with a dynamic signal range of 20-150-fold and \(Z'\) factors of 0.7-0.8. Additionally, a strategy whereby PPIs are interrogated in mammalian cells using a single-chain biosensor approach was initiated, and the details of this strategy and preliminary experimental results are provided.
3.2 Materials and Methods

3.2.1 Materials

Oligonucleotides were obtained from Integrated DNA Technologies. Restriction enzymes and Vent DNA polymerase were purchased from New England Biolabs. Reagents were purchased from the indicated vendors: spectinomycin, β-NADPH and Triton X-100 from Sigma-Aldrich; rapamycin from EMD Chemicals; Hygromycin B solution from Mediatech Inc.; Luria-Bertani (LB) Broth, LB agar, ampicillin from Fisher Scientific; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Opti-MEM I Reduced Serum Media, Dulbecco’s Modified Eagle Medium (DMEM), MEM Non Essential Amino Acids (NEAA), Trypsin, Lipofectamine 2000 from Invitrogen, Life Technologies; Fetal Bovine Serum from Atlanta Biologicals; Ethylene glycol bis[succinimidylsuccinate] (EGS) from Thermo Scientific; In-Fusion® HD Cloning Kit from Clontech. Black flat bottom, 96-well Greiner CELLSTAR® plates were purchased from Sigma-Aldrich. Lumi4® was a generous gift of Lumiphore Inc. pEGFP-Claudin, pPBH-TREtight and pSPB-Transposase plasmids were generous gifts of Jerrold R. Turner Lab at University of Chicago. Preparation of pRSETb-FRB-eDHFR, pRSETb-GFP-FKBP plasmids was described in a previous study.81

Bacterial expression constructs were sequenced at UIC Research Resources Center (RRC). Time-resolved luminescence measurements were made using a fluorescence plate reader (Perkin Elmer, Victor 3V) with 340 nm excitation (60 nm bandpass) and either 520 nm
emission (10 nm bandpass) or 615 nm emission (10 nm bandpass). Intensity values (1800 µs integration) were measured after a time delay of 100 µs.

TMP was coupled to the macrocyclic Tb$^{3+}$ complex, Lumi4 using previously reported methods.\textsuperscript{73}

### 3.2.2 Plasmid vector construction

The gene encoding FKBP12 was subcloned from plasmid pRSETb-GFP-FKBP to pEGFP-Claudin to generate pEGFP-FKBP. A 321 bp fragment encoding FRB was amplified by PCR from pRSETb-GFP-FKBP using the primers 5’ – CT GGA AGT GCT GCT CGA GGA GTG CAG GTG G – 3’ (XhoI, coding strand) and 5’ – GCA GCC GGA TCA AGC TCT AGA TTA TTC CAG TTT TAG AAG CTCC – 3’ (XbaI, non-coding strand). This fragment was inserted between the XhoI site and the XbaI site in pEGFP-Claudin with In-Fusion\textsuperscript{®} Cloning Kit to give to pEGFP-FKBP.

The gene encoding FRB-eDHFR was subcloned from plasmid pRSETb-FRB-eDHFR to pPBH-TRE\textsubscript{tight} to generate pPBH-TRE\textsubscript{tight}-FRB-eDHFR. A 783 bp fragment encoding FRB-eDHFR was amplified by PCR from pRSETb-FRB-eDHFR using the primers 5’ – AC TCT GCA GTC GAC GGT ACC ATG ATC CTC TGG CAT GAG ATG TGG C – 3’ (KpnI, coding strand) and 5’ – GA TCC CGG GCC CGC GGT ACC TCA CTA TTA CCG CCG CTC CAG AAT CTC AAA G – 3’ (KpnI, non-coding strand). This fragment was inserted at the KpnI site in pPBH-TRE\textsubscript{tight} with In-Fusion\textsuperscript{®} Cloning Kit to give to pPBH-TRE\textsubscript{tight}-FRB-eDHFR.
The gene encoding (CMV Promoter)-EGFP-FKBP-(bGH Poly(A) Signal Sequence) was subcloned from plasmid pEGFP-FKBP to pPBH-TRE\textsubscript{tight}-FRB-eDHFR to generate pPBH-TRE\textsubscript{tight}-FRB-eDHFR/GFP-FKBP. A 1850 bp fragment encoding (CMV Promoter)-EGFP-FKBP-(bGH Poly(A) Signal Sequence) was amplified by PCR from pEGFP-FKBP using the primers 5’ – GCCCGTCCCACCAGGTAGTTCCCGTTACATAACTTACG – 3’ (SexAI, coding strand) and 5’ – CGCCTGTTGACCTGGTCGCGTTAAGATACATTGATGAG – 3’ (SexAI, non-coding strand). This fragment was inserted at the SexAI site in pPBH-TRE\textsubscript{tight}-FRB-eDHFR with In-Fusion\textsuperscript{®} Cloning Kit to give to pPBH-TRE\textsubscript{tight}-FRB-eDHFR/GFP-FKBP. The integrity of all plasmids was verified by direct sequencing.

The pPBH-TRE\textsubscript{tight}-FRB-eDHFR-ER/K-GFP-FKBP was prepared by GenScript by synthesizing the open reading frame FRB-eDHFR-ER/K-GFP-FKBP with ER/K linker with the sequence of 5’ – GAA GAG GAA GAG AAA AAA AAA CAG CAG GAA GAG GAA GCA GAA AGG CTG AGG CGT ATT CAA GAA GAA ATG GAA AAG GAA AGA AAA AGA CGT GAA GAC GAA AAA CGT CGA AGA AAG GAA GAG GAG GAA AGG CGG ATG AAA CTT GAG ATG GAA GCA AAG AGA AAA CAA GAA GAA GAA GAG AGA AAG AAA AGG GAA GAT GAT GAA AAA CGC AAG AAG AAG. The synthesized fragment was inserted into pPBH-TRE\textsubscript{tight} vector to give to pPBH-TRE\textsubscript{tight}-FRB-eDHFR-ER/K-GFP-FKBP.
3.2.3 Stable mammalian cell line preparation

First, NIH/3T3 mouse fibroblast cells were analyzed for Hygromycin resistance. A 12-well plate was seeded with $2.3 \times 10^5$ cells per well in DMEM with 1x NEAA, 15 mM HEPES and 10% FBS. Hygromycin was added into the wells with a range of 0.025-1 mg/mL. The media in the wells were replenished in day 3 with the same concentration of Hygromycin in corresponding wells. The plate was analyzed after 4 days of additional incubation (total 7 days from the day of seeding) and the optimal Hygromycin concentration was determined to be 100 µg/mL for cell growth and selection. For the stable transfection of the cells, a sterile 100 mm dish was seeded with $3 \times 10^6$ cells 24 hours before the transfection. Lipofectamine 2000 was used to transfect pPBH-TRE\textsubscript{tight}-FRB-eDHFR/GFP-FKBP and pPBH-TRE\textsubscript{tight}-FRB-eDHFR-ER/K-GFP-FKBP along with their recombination helper plasmid pSPB-Transposase with a Lipofectamine:plasmid ratio of 2.5µL:1µg per plasmid. Plasmid and Lipofectamine solutions were first prepared in separate microcentrifuge tubes in OptiMEM I with a total volume of 1.5 mL. After 5 minutes of incubation at room temperature, the solutions were mixed and kept at room temperature for an additional 20 minutes. The media in 100 mm dish was aspirated and the Lipofectamine + plasmid solution was added into it. The cells were incubated with the solution for 4 hours in tissue culture incubator at 37 °C with 5% CO\textsubscript{2} and then the solution was replaced with 10 mL of fresh DMEM supplied with 1x NEAA, 15 mM HEPES, 10% FBS and 100 mg/mL Hygromycin. The transfections were confirmed with microscopy and flow cytometry by using the GFP emissions.
3.2.4 Cell growth and protein expression in stable cell lines

NIH/3T3 cells were maintained in DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% FBS, 15 mM HEPES and 100 mg/mL Hygromycin at 37 °C and 5% CO₂. The cells were subcultured with 0.05% trypsin/0.53 mM EDTA without HBSS without sodium bicarbonate, calcium & magnesium. Stably transfected cells were sorted through flow cytometry and among 3 distinct populations; the one with medium level GFP expression was selected for the experiments. The expression of FRB-eDHFR was tested with 0.1-2 µg/mL doxycycline 24-48 hours prior to testing. Optimal parameters were determined as 0.5 µg/mL doxycycline and 24 hours growth period. Different numbers of cells ranging between 1,920 – 81,600 were tested (in each 96-well plate well) for the best signal and 40,800 cells were used for the assays. To minimize the edge effect, rows A and H of the 96-well plate were filled with the same volume of growth medium as in sample wells and columns 1 and 12 were omitted from the data analysis in some assay runs.

3.2.5 GFP concentration determination in stable cell lines

NIH/3T3 cells stably expressing GFP-FKBP and FRB-eDHFR were grown in 4, 15 cm dishes to 80% confluency and harvested with 0.05% trypsin/0.53 mM EDTA. The cells were washed twice with ice-cold DPBS (-Ca/-Mg). 3.8 x 10⁷ cells were re-suspended in 1.27 mL mammalian lysis buffer (50 mM Tris•HCl, 0.1 mM DTT, 0.1 mM PMSF, 10 µL protease cocktail) to get 3x10⁷ cells/mL. The cells were lysed on ice by sonication (30% amplitude, 5 sec bursts 10 sec pause). This was repeated until no cells were detected under light microscope. Lysate from non-expressing NIH/3T3 cells was similarly prepared. Purified GFP protein was serially diluted into NIH/3T3 cell lysate in 96-well plates, and a standard curve
was generated by measuring the fluorescence using a plate reader. The curve was used to calculate the concentration of GFP in the lysate from cells expressing GFP-FKBP and FRB-eDHFR. The mean cellular concentration of protein was calculated from the total volume of lysate, the number of cells lysed and an assumed cell volume of 2 pL. This experiment was performed by Megha Rajendran in the Miller lab.

3.2.6 Binding assay preparation with cross-linking

GFP-FKBP/FRB-eDHFR stable cells were seeded at a density of 40,800 cells/well in a 96-well plate and incubated (37 °C, 5% CO₂) ca. 24 h in culture medium containing doxycycline (230—250 µL). Reproducible implementation of the assay required careful addition of reagents and buffers in a precise order, and the below plate preparation protocol was used:

1. Growth media in the wells were discarded carefully and the cells were washed once with 200 uL of colorless DMEM.
2. 85 uL of colorless DMEM with and without 0.5 µM rapamycin was added into the sample and negative control wells respectively. The plate was kept at room temperature for 30 minutes.
3. 5 uL of 36-73 mM EGS in DMSO was added into the wells and the plate was kept at room temperature for 15-20 minutes.
4. 40 uL 50 mM Tris•HCl, pH 7.50 was added into the wells and the plate was kept at room temperature for 15-20 minutes.
5. 20 uL of the 175 nM TMP-Lumi4(Tb⁺³), 17.5 µM NADPH, 0.7% Triton X-100 solution was added into the wells, the plate was kept at room temperature for 15 minutes and the first measurement was taken afterwards.

6. Measurements were recorded every 10 minutes up to 16 hours.

3.2.7 Binding assay preparation with permeabilized mammalian cells

GFP-FKBP/FRB-eDHFR stable cells were seeded at a density of 40,800 cells/well in a 96-well plate and incubated (37 °C, 5% CO₂) ca. 24 h in culture medium containing doxycycline (230—250 µL). The below plate preparation protocol was used:

1. Growth media in the wells were discarded carefully and the cells were washed once with 200 uL of PBS. PBS with 0.5 µM rapamycin and without rapamycin was used for sample and negative control wells respectively.

2. 100 uL of the corresponding PBS solution was added into the wells and the plate was kept at room temperature for 30 minutes.

3. 20 uL of the 150 nM TMP-Lumi4(Tb⁺³), 15 µM NADPH, 0.6% Triton X-100 solution was added into the wells, the plate was kept at room temperature for 15 minutes and the first measurement was taken afterwards.

4. Measurements were recorded every 10 minutes up to 16 hours.
3.2.8 **Inhibition assay with permeabilized mammalian cells**

GFP-FKBP/FRB-eDHFR stable cells were seeded at a density of 40,800 cells/well in a 96-well plate and incubated (37 °C, 5% CO₂) ca. 24 h in culture medium containing doxycycline (230—250 µL). The below plate preparation protocol was used:

1. Growth media in the wells were discarded carefully and the cells were washed once with 200 uL of PBS with 0.333 µM rapamycin and ascomycin with 2-fold serial dilutions with the range of 16.7 µM – 16.3 nM.
2. 100 uL of the corresponding PBS solution was added into the wells and the plate was kept at room temperature for 30 minutes.
3. 20 uL of the 150 nM TMP-Lumi4(Tb³⁺), 15 µM NADPH, 0.6% Triton X-100 solution was added into the wells, the plate was kept at room temperature for 15 minutes and the first measurement was taken afterwards.
4. Measurements were recorded every 10 minutes up to 16 hours.

3.2.9 **Microscopy**

Both time-gated luminescence and continuous wave fluorescence images were acquired using an epi-fluorescence microscope (Axiovert 200, Carl Zeiss, Inc.) modified with the following components: 1) a UV LED emitting at 365 nm (UV-LED-365, Prizmatix, Ltd.); 2) a digital delay generator (DG645, Stanford Research Systems, Inc.); 3) a gated image-intensified CCD camera (ICCD), mounted on the side-port of the microscope (Mega-10EX, Stanford Photonics, Inc.); and 4) a computer running Piper Control software (v2.4.05, Stanford Photonics, Inc.). A 100 W mercury arc lamp was available for continuous
wave fluorescence excitation. Fluorescence and bright-field images were captured using either a conventional CCD (Axiocam MRM, Carl Zeiss, Inc.) mounted on the front port of the microscope or the ICCD operating in “Live” mode, with automatic gain level and acquisition time. Filter cubes containing the appropriate excitation and emission filters and dichroics allowed for wavelength selection. All images were obtained with a 63X/1.25 N.A. EC Plan Neofluar oil-immersion objective (Carl Zeiss, Inc.). The UV LED intensity was measured to be 1.2 mW at the objective back aperture, which corresponds to an irradiance of 0.5 W/cm² at the specimen plane according to calculations described in Grunwald, et al.¹²².

For each time-gated image acquisition, the signal from multiple excitation/emission events was accumulated on the ICCD sensor and read out at the end of the camera frame. The UV LED pulse width and pulse period, the intensifier delay time and on-time, the camera frame length (66.67 ms—2 s) and the intensifier gain voltage could be varied independently. The source/camera timing parameters were the same for all of the time-resolved images and data presented here: excitation pulse width, 1500 µs, pulse period, 3000 µs, delay time, 10 µs, intensifier on-time, 1480 µs. Sensitivity was modulated by either varying the frame length (and thus, the number of integrations) or the intensifier gain voltage. The camera control software enabled summation of multiple frames to yield a single composite. TIFF image with a bit depth equal to 1024 multiplied by the number of frames. All images reported here were summations of four frames (bit depth, 4096), and a feature of the camera control software was enabled that removes large variations in signal resulting from ion-feedback noise of the intensifier.
ICCD images (tagged image file format, .TIFF) were captured with Piper control software (v2.4.05, Stanford Photonics, Inc.), and Axiocam images (.ZVI) were captured with Zeiss AxioVision software (v4.6). Raw, 12-bit images were imported into Adobe Photoshop CC software for all processing operations including cropping and contrast adjustment.

### 3.3 Results and Discussion

To provide good repeatability and low signal variability, it was imperative to use a cell line expressing both fusion proteins in similar concentrations. The only way to provide this was to use the same gene copy number for both proteins via stable transfection. For this purpose, a mammalian expression vector with a homologous recombination cassette (pPBH-TRE<sub>tight</sub>) was used to place the open reading frames of each fusion protein within the same cassette. This approach ensured the integration of both genes in the cell chromosomes with the same copy numbers. The open reading frame of FRB-eDHFR was put under tetracycline responsive elements (TREs), which prevented the protein expression until the tetracycline transactivator protein (tTA) was supplied with either antibiotic tetracycline or one of its derivatives: doxycycline.<sup>123,124</sup> tTA-doxycycline complex induced the FRB-eDHFR expression by binding to TREs which were found in upstream of the constitutive Cytomegalovirus (CMV) promoter. GFP-FKBP open reading frame was placed under the CMV promoter for constitutive expression.

After stable NIH/3T3 FRB-eDHFR & GFP-FKBP cell line preparation was complete, the cells were analyzed and sorted with flow cytometry by using the GFP emission signal. Three different populations were selected and further analyzed under microscope for 3 generations and the one with medium-level GFP expression contained the healthiest cells and it was determined
to have the least signal variability (Figure 19). We decided to use this population for our experiments and intentionally avoided using the population with high-levels of GFP expression because of GFP’s toxicity to the cells at high concentrations. The cellular abundance of GFP-FKBP was estimated to be 3.2 µM in the medium-level GFP expressing cells, as detailed in Materials and Methods. Before the assay development, FKBP/rapamycin/FRB interaction was confirmed by microscopically imaging Tb³⁺-sensitized, GFP emission (i.e., the TR-FRET signal) (Figure 20).

**Figure 19:** Representative images of NIH/3T3 cells stably expressing GFP-FKBP following FACS sort into a) high-level; b) medium-level; c) low-level production of protein.
Figure 20: GFP-FKBP/rapamycin/FRB-eDHFR interaction analysis with TR-FRET. GFP-FKBP and FRB-eDHFR expressing cells were incubated with rapamycin and a cell permeable analog of TMP-Lumi4-Tb\(^{3+}\) complex sequentially, washed and imaged. Representative images showing 1a) GFP emission at 520 nm; 1b) Lumi4-Tb emission at 620 nm; 1c) TR-FRET signal between GFP and Lumi4-Tb detected with 520 nm emission filter. Second row of images show the control cells where rapamycin was lacking.
In order to determine the best doxycycline concentration for FRB-eDHFR induction, each 96-well plate was seeded with 40,800 cells per well and doxycycline was added into the wells with varying concentrations (50-200 ng/mL). 24 hours after the plate preparation, cells were processed for the assay, as described in the materials and methods section, and no significant signal difference was observed between different concentrations of doxycycline (Figure 21). Concentrations of 0.20-0.25 µg/mL were used for the assays performed afterwards.

![Graph showing normalized signal vs log[doxycycline] (ng/µL)](image)

**Figure 21: Doxycycline concentration optimization for NIH/3T3 FRB-eDHFR/GFP-FKBP cells.** Cells were incubated in different doxycycline concentrations (50, 100, 200 ng/mL) for 24 hours. The assay buffer contained 40 nM of TMP-Lumi4(Tb³⁺), 0.1% w/v BSA, 0.1% v/v Triton X-100, 32µM NADPH and 4 µM rapamycin. The signal was normalized by dividing the signal at 520 by the signal at 620 (520/615) and multiplying by 100. Each data point had a total sample number of n = 24. The average signal for control wells in the same plate was 6.25 ± 0.33 (n = 24).
For the initial binding assay, a 96-well plate was prepared with three combinations of assay components: 1) sample wells containing 40,800 cells with GFP-FKBP and FRB-eDHFR expression and rapamycin in the assay buffer; 2) negative control wells containing 40,800 cells with GFP-FKBP and FRB-eDHFR expression but lacking rapamycin in the assay buffer; 3) background wells containing all the components of the assay buffer (including rapamycin) but lacking the cells. The plate was kept in tissue culture incubator (37 °C, 5% CO₂) for 24 hours. On the day of the assay, growth media was removed and the cells were washed with and placed in PBS with and without rapamycin for sample and negative control wells respectively. They were incubated at room temperature for 30 minutes to allow the FKBP/rapamycin/FRB complex to reach equilibrium and then the cell membranes were disrupted with nonionic detergent Triton X-100 immediately prior to signal detection.

The plate was maintained at room temperature throughout the measurements and analyzed at 15-minute intervals over 30 minutes by measuring the time-resolved luminescence signal (λ_ex = 340 nm, delay = 100 µs) at 520 nm (FRET-sensitized, acceptor emission) and at 615 nm (Tb³⁺ donor emission). The 520 nm/615 nm emission ratio (normalized signal) was calculated to minimize well-to-well variability resulting from the differences in probe amounts or sample absorbance. The mean, normalized signal observed in the background control wells was subtracted from the normalized signal of each of the sample and negative control wells. Then, the percent increase in the normalized signal, ΔL%, was calculated as

\[ \Delta L\% = \frac{\frac{520}{615}_s - \frac{520}{615}_B}{\frac{520}{615}_B} \times 100 \]  (6)
where, \( \left( \frac{520}{615} \right)_S \) and \( \left( \frac{520}{615} \right)_B \) are the emission ratios for the sample (or control) and background wells, respectively, and \( \bar{\left( \frac{520}{615} \right)}_B \) is the mean emission ratio for the background wells. With this background correction, we observed a >50-fold increase in the signal in the sample wells relative to that observed in negative control wells, control and sample wells (Figure 22a). A \( Z' \)-factor of 0.70 was estimated from the plate data using the following equation:

\[
Z' = 1 - \frac{3\sigma_{\text{max}} + 3\sigma_{\text{min}}}{\mu_{\text{max}} - \mu_{\text{min}}}
\]

(7)

In the first iteration of this assay, we were able to show the sensitivity and reproducibility of the method for high-throughput applications. However, disrupting the cell membrane constituted a problem: cell contents, including the proteins of interests, were diluted to nanomolar concentrations (3.25 – 4.00 nM). At this concentration, it would not be possible to detect a signal from interactions with >0.1 micromolar range dissociation constants because only a small fraction of the proteins would interact. Therefore, we came up with a new plan for a different assay where we increased the effective concentrations of proteins to micromolar ranges by first cross-linking the proteins prior to cell lysis. We used the N-hydroxysuccinimide ester-based crosslinker ethylene glycol bis(succinimidylsuccinate) (EGS) for this purpose.\textsuperscript{126,127} The cells were incubated with rapamycin prior to cross-linking for FRB/rapamycin/FKBP complex formation and then the crosslinker was added to ‘lock’ the proteins together chemically. After background correction, we observed a >25-fold larger FRET signal in the positive control wells...
(+Rap) relative to that seen in the negative controls. $Z'$ factor was calculated as 0.72 by using equation 7 (Figure 22b).

**Figure 22: Cell-based assay with TR-FRET.** a) Data showing the TR-FRET in permeabilized cells. Average ΔL% for the sample was 36.25 ± 2.87 with a sample size of n = 12 wells, and for the control it was 0.69 ± 0.64 with a sample size of n = 4 wells. The calculated $Z'$ factor was 0.70. b) Data showing the TR-FRET in EGS cross-linked cells.
Average $\Delta L\%$ for the sample was $27.88 \pm 0.34$ with a sample size of $n = 36$ wells, and for the control it was $0.97 \pm 0.08$ with a sample size of $n = 12$ wells. The calculated $Z'$ factor was 0.72. The sample wells contained rapamycin while control wells lacked rapamycin. Error bars indicate the standard deviation. The $\Delta L\%$ was calculated by using equation 6.

After ascertaining that we could obtain a large signal change and good reproducibility for detecting the FKBP/FRB interaction, we sought to determine the EC50 values and the dynamic signal range for FKBP/rapamycin/FRB interaction within 96-well plate with both cross-linked and permeabilized cells. The plates were prepared as described above and in the materials and methods section. However, instead of using a constant concentration of rapamycin, a 2-fold serial dilution of it was applied among the wells in a given row; with a range of 3.33 $\mu$M to 3.26 nM for permeabilized cells and 1 $\mu$M to 0.5 nM for cross-linked cells. The data was fit to the following EC50 function:

$$ Y = L_{\text{min}} + \frac{L_{\text{max}} - L_{\text{min}}}{1 + 10^{(\log EC50 - X) \times HillSlope}} $$

where, $Y$ is the $\Delta L\%$ signal, $X$ is the concentration of rapamycin, $L_{\text{min}}$ is the minimum signal observed at the bottom plateau of the curve, $L_{\text{max}}$ is the maximum signal observed at the top plateau of the curve, and $HillSlope$ is the slope of the steep portion of the curve between 10% and 90% signals. EC50 values of $124 \pm 4$ nM and $182 \pm 4$ nM were calculated for permeabilized and cross-linked samples respectively. (Figure 23).
Figure 23: EC50 calculations with permeabilized and EGS cross-linked cell assays. The plots showing the data fit to the equation 8 to calculate EC50 values. The cells were equilibrated with rapamycin concentrations ranging from 3.33 µM to 3.26 nM for permeabilized cells and 1 µM to 0.5 nM for cross-linked cells. a) The plot for permeabilized cells with a sample size of n = 3 wells for each data point. b) The plot for EGS cross-linked cells with a sample size of n = 5 wells for each data point. Error bars indicate the standard deviation.
The aim of the last set of experiments was to actually test whether this assay format could detect inhibition. The assay was performed using ascomycin as the inhibitor of FKPB/rapamycin-FRB interaction. Ascomycin competes with rapamycin for binding to FKBPs and the FKPB/ascomycin complex does not bind to FRB.\(^\text{107}\) Ascomycin was titrated against a constant concentration of rapamycin and the cells were permeabilized before the readings. The concentration of ascomycin was plotted against ΔL% and the data was fit to equation 8. From this fit, an IC50 value of 0.71 ± 0.14 µM was obtained with a rapamycin concentration of 0.333 µM. An approximately 80-fold increase in mean, normalized FRET signal was observed at the lowest ascomycin concentration relative to the highest. There weren’t enough data points at the high and low ends of the isotherm to be able to calculate a Z’ factor accurately, but the size of the error bars suggests that this assay format easily distinguishes between the presence and absence of inhibition. (Figure 24).
Figure 24: **Ascomycin IC50 calculation with permeabilized cells.** The plot showing the data fit to the equation 8 to calculate IC50 value for ascomycin. The cells were equilibrated with 0.333 µM rapamycin along with varying concentrations of ascomycin (16.7 µM – 16.2 nM). Titration was performed in triplicate. IC50 value was calculated as 0.71 ± 0.14 µM.

The above results show that an interaction between two proteins that are expressed in mammalian cells can be detected by TR-FRET in plate reader format without prior purification. The high dynamic signal range and good reproducibility observed, and the relatively few number of reagent addition steps needed suggest that this assay format could be easily automated. In addition, these assays can be used to estimate the EC50 or IC50 values for follow-up studies. While the proof-of-principle experiments were performed in 96-well plates (50-160 µL/well), it
is evident from the large dynamic signal range that the assays could likely be miniaturized for HTS in 384-well (20-30 µL/well) plates. The TMP/eDHFR protein labeling strategy used here can be extended to any targets that tolerate over-expression as fusion proteins including membrane proteins or others not amenable to purification.

While the initial results are promising, the FKBP/FRB interaction used as a model for this study has a fairly high affinity ($K_D = \sim 7$ nM). For that reason, we were unable to determine whether our chemical cross-linking strategy could effectively preserve a lower affinity interaction so that it could be detected following cell lysis and reagent addition. Moreover, we were unable to detect ascomycin-mediated inhibition when we used the crosslinking protocol. Even if a chemical crosslinking or fixation approach proved to be possible, it would require several additional wash and reagent addition steps, thus resulting in a more costly and slower HTS.

What is needed is a means to reliably preserve the state of a given interaction (on or off) that is occurring in cultured cells at the moment those cells are lysed. To achieve this goal, we began studies to investigate the feasibility of using the so-called SPASM (systematic protein affinity strength modulation) sensor approach for TR-FRET-based HTS of PPIs.

Sivaramakrishnan and Spudich reported a means for interrogating PPIs whereby two interacting proteins, each fused to a FRET partner, are expressed as a single chain with a semi-rigid linker separating them. A polypeptide motif consisting of an alternating sequence of approximately four glutamic acid residues followed by approximately four arginine or lysine residues (an ER/K linker) adopts an extended $\alpha$-helical conformation in solution. When inserted between two proteins, the linker serves to hold the proteins apart unless they bind to one another.
For a given PPI, the fraction of protein-linker-protein constructs in the closed conformation is dependent on the strength of the interaction and the length of the ER/K linker. Importantly, the fraction closed is independent of the overall concentration of the protein chain, and spectroscopic FRET measurements at sensor concentrations as low as 5 nM were reported. Moreover, Sivaramakrishnan and Spudich were able to measure FRET in SPASM sensors comprising interacting proteins with ca. micromolar dissociation constants. Thus, we envisioned a simple strategy where a SPASM sensor is constructed with a general sequence of Protein 1 – eDHFR – ER/K – GFP – Protein 2. Cells stably expressing the sensor would be grown in multi-well plates, library components added, and lastly a lysis/detection reagent solution would be added. Inhibition would be detected as a loss of TR-FRET signal in a given well.

We set about to test this strategy by first generating a construct where FRB-eDHFR and GFP-FKBP were separated by a 73-residue ER/K linker (ca. 10 nm), producing FRB-eDHFR-ER/K-EGFP-FKBP. The fusion protein is under control of under control of a tetracycline-inducible promoter, which gave us the control of switching the expression on or off. Following transient transfection, cells were labeled with a cell permeable TMP-Tb$^{3+}$ complex and imaged using a time-gated luminescence microscope. Unfortunately, initial images revealed no difference in baseline Tb$^{3+}$-to-GFP FRET levels between cells with rapamycin added and those without. It is possible that a longer ER/K linker is required to effectively separate the Tb$^{3+}$-labeled eDHFR and GFP ends of the chain. Further efforts will be undertaken to test various ER/K linker lengths in an effort to detect a robust signal change between positive and negative control populations both microscopically and in plate reader studies.
CHAPTER 4

FORKHEAD BOX PROTEIN M1 (FOXM1) AND NUCLEOPHOSMIN (NPM)

RELATION AND IMAGING WITH TR-FRET
4.1 Introduction

Forkhead box (Fox) proteins are a superfamily of transcription factors that have several different roles affecting cell development and cancer progression. Misregulation of this protein family can alter cell fate and promote tumorigenesis. Fox proteins are involved in cell cycle, growth, metabolism, development, proliferation, migration, apoptosis, differentiation and longevity. FoxM1 gene is a member of the Fox family and is a known human proto-oncogene. Upregulation of FoxM1 has been observed in several major human cancers. The FoxM1 gene in humans consists of 10 exons, and alternative splicing yields three variants: FoxM1a, b and c. FoxM1a is a negative regulator of the other two variants and itself is not transcriptionally active. FoxM1b and c, however, are both transcriptionally active and can induce target gene expression as transcriptional factors. FoxM1b has been shown to be overexpressed in several tumors, playing roles in tumorigenesis and metastasis.

Nucleophosmin (NPM) is another protein with links to tumorigenesis. It is a nucleolar protein that functions in ribosome biogenesis, DNA replication, recombination, transcription and repair, chromatin remodeling, centrosome duplication and apoptosis. It mainly resides in the nucleoli but rapidly shuttles between the nucleus and cytoplasm when performing the listed activities. It is also known to regulate the activity of important tumor suppressor proteins such as p53 and ARF. Therefore, loss of control in NPM expression could contribute to tumorigenesis by multiple distinct mechanisms. NPM has been shown to be overexpressed in highly proliferative cancerous cells, and its upregulation leads to increased cell growth and inhibition of programmed cell death through diverse mechanisms.
In 2011, Bhat et. al. reported that NPM interacts with FoxM1b and that this interaction is crucial for regulation and localization of FoxM1b in the cells$^{159}$. Evidence including co-immunoprecipitation with NPM and FoxM1 antibodies and pull-down from cell lysates using purified NPM showed that NPM and FoxM1b interacted in U2OS osteosarcoma cells and MIA PaCa-2 pancreatic cancer cells. Additionally, immunofluorescence microscopy revealed co-localization of the two proteins in the nuclei of immortal and cancer cell lines but not normal cells. Furthermore, knockdown of NPM in immortal and cancer cells led to significant down-regulation of FOXM1 similar to its levels in normal cells, suggesting that NPM might modulate FOXM1 level.

The fact that FOXM1 is upregulated in a majority of human cancers suggests that growth of cancer cells may depend on FOXM1 activity. Conversely, FoxM1 inhibition or downregulation would seem to be a viable anti-cancer therapeutic strategy. This strategy is supported by work showing that treatment of human cancer cells with thiazole antibiotics resulted in downregulation of FOXM1 and apoptosis.$^{160-162}$ Given the Gartel lab’s work showing that NPM interacts with and down-regulates FoxM1b, UICentre (UICollaborative Engagement in Novel Therapeutic Research and Enterprise) became interested in exploring inhibition of the NPM/FoxM1 interaction as a novel anti-tumor strategy. The Miller lab was engaged to develop a HTS assay to identify potential lead compounds that could disrupt the interaction.

We chose to adapt the assay described in Chapter 2 by preparing plasmids that encoded fusions of FoxM1B and NPM to both GFP and eDHFR. Both bacterial expression vectors as well as mammalian expression constructs were prepared. In order to confirm that the putative interaction between the two proteins could be detected by TR-FRET, we first attempted to visualize co-localization and Tb$^{3+}$-to-GFP sensitized emission in U2OS cells that co-expressed
GFP-FoxM1b and NPM-eDHFR and that were labeled with a cell-permeable TMP-Tb$^{3+}$ complex. Unfortunately, we obtained a negative result; not only was FRET not observed, but the fusion proteins were observed to localize to different sub-cellular locations. Below we describe these initial experiments and discuss possible alternative strategies for a FoxM1/NPM screen as well as the pitfalls and difficulties that can arise when developing HTS for PPIs.

4.2 Materials and Methods

4.2.1 Materials

Oligonucleotides were obtained from Integrated DNA Technologies. Restriction enzymes and Vent DNA polymerase were purchased from New England Biolabs. Reagents were purchased from the indicated vendors: Luria-Bertani (LB) Broth, LB agar, kanamycin from Fisher Scientific; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Opti-MEM I Reduced Serum Media, Dulbecco’s Modified Eagle Medium (DMEM), MEM Non Essential Amino Acids (NEAA), Trypsin from Invitrogen, Life Technologies; Fetal Bovine Serum from Atlanta Biologicals; In-Fusion® HD Cloning Kit from Clontech; pLL-1 (eDHFR) plasmid from Active Motif; H2B-mCherry plasmid from AddGene; pCMV6-AC-tGFP-FOXM1 V3 plasmid from Origene; FuGENE® HD transfection reagent from Promega. Lumi4®-Arg9 was a generous gift of Lumiphore Inc. pGEX-2T-NPM1 plasmid and Homo sapiens bone osteosarcoma (U2OS) cells stably expressing FoxM1b-GFP were generous gifts of Andrei L. Gartel Lab at Department of Medicine, University of Illinois at Chicago.

The cell permeable conjugate of TMP-Lumi4 coupled to nonaarginine was described in Mohandessi, et al.\textsuperscript{163} and provided by Lumiphore, Inc. (Richmond, CA).
4.2.2 Plasmid vector construction

The NPM1 gene was subcloned from plasmid pGEX-2T-NPM1 to pLL-1-eDHFR to generate pLL-1-eDHFR-NPM1. A 873 bp fragment encoding NPM1 was amplified by PCR from pGEX-2T-NPM1 using the primers 5’ – TG GAG CGG CGG TCA TCT AGA GGA AGT GGT TCT GGT TCA ATG GAC ATG GAC ATG AGC – 3’ (XbaI, coding strand), containing (GS)$_5$ linker and 5’ – AT GGC TGA TTA TGA TCT AGA TTA AAG AGA CTT CCT CCA CTG – 3’ (XbaI, non-coding strand). This fragment was inserted into the pLL-1-eDHFR, after the linearization of the vector by XbaI digestion, with In-Fusion$^\text{®}$ Cloning Kit to give to pLL-1-eDHFR-NPM1.

The FoxM1b gene was subcloned from plasmid pCMV6-AC-tGFP-FOXM1 V3 to pLL-1-eDHFR to generate pLL-1-eDHFR-FoxM1b. A 2244 bp fragment encoding FOXM1 was amplified by PCR from pCMV6-AC-tGFP-FoxM1 V3 (FoxM1b) using the primers 5’ – TG GAG CGG CGG TCA TCT AGA GGA AGT GGT TCT GGT TCA ATG AAA ACT AGC CCC CGT CGG CCA – 3’ (XbaI, coding strand), containing (GS)$_3$ linker and 5’ – AT GGC TGA TTA TGA TCT AGA TTA CTG TAG CTC AGG AAT AAA CTG GGA CCA G – 3’ (XbaI, non-coding strand). This fragment was inserted into the pLL-1-eDHFR, after the linearization of the vector by XbaI digestion, with In-Fusion$^\text{®}$ Cloning Kit to give to pLL-1-eDHFR- FoxM1b.

The NPM1 gene was subcloned from plasmid pGEX-2T-NPM1 to pEGFP-FKBP to generate pEGFP-NPM1. A 873 bp fragment encoding NPM1 was amplified by PCR from pGEX-2T-NPM1 using the primers 5’ – ATG GAC GAG CTG TAC AAG GGT TCA GGT
TCA GGT TCA CGA TGG ACA TGG ACA TGA GC – 3’ (BsrGI, coding strand), containing (GS)₃ linker and 5’ – T GAT CAG TTA TCT AGA TTA AAG AGA CTT CCT CCA CTG C – 3’ (XbaI, non-coding strand). This fragment was inserted into the pEGFP-FKBP, after the linearization of the vector by BsrGI and XbaI digestion, with In-Fusion® Cloning Kit to give to pEGFP-NPM1.

4.2.3 Cell growth, transfection and probing with TMP-Lumi4

U2OS/FoxM1-EGFP cells were maintained in DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% FBS, 1X MEM non-essential amino acids, 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and 15 mM HEPES at 37 °C and 5% CO₂. The cells were passaged with 0.05% trypsin/0.53 mM EDTA without HBSS without sodium bicarbonate, calcium & magnesium. FoxM1b-GFP expression was induced with µg/mL doxycycline at least 24 hours prior to experiments.

Cells were prepared to have 80% confluency during the eDHFR-NPM transient transfection. They were transfected with 2 µg of pLL-1-eDHFR-NPM1 and 12 µL FuGENE® HD transfection reagent; or with 2 µg of pLL-1-eDHFR-NPM1 + 2 µg of H2B-mCherry and 24 µL FuGENE® HD transfection reagent for 4 hours in 6-well plate. They were maintained in incubator for 24 hours and then transferred into 8-well plate for microscope imaging. After an additional 24-hour incubation, they were prepared for imaging as detailed below:

1. Aspirate the growth media and wash with 400 µL DPBS, with Ca⁺²/Mg⁺², 3 times.
2. Add 100 µL 10 µM TMP-Lumi4(Tb⁺³)-Arg9 in colorless DMEM (w/o FBS) and incubate at room temperature for 20 min.
3. Aspirate the TMP-Lumi4(Tb$^{3+}$)-Arg9 solution and wash 3X with 400 μL PBS (with Ca$^{+2}$/Mg$^{+2}$).

4. Add 150 μL colorless DMEM containing Patent Blue V (1 mM, to quench extracellular luminescence from non-specifically adsorbed probe).

4.2.4 **TR-FRET microscopy and image processing.**

TR-FRET microscopy was performed as described in Chapter 3.

4.3 **Results and Discussion**

In this study, we designed an HTS assay based on purified eDHFR and GFP fusions of FoxM1b and NPM, analogous to that described for FKBP12 and FRB in Chapter 2. Thus we prepared bacterial expression vectors encoding FoxM1B-GFP and eDHFR NPM with the intention of interrogating the interaction using TR-FRET in multiwell plate format. In parallel to developing a biochemical screen, we also sought to obtain further evidence of an interaction between the two target proteins. To do so, we attempted to observe a FRET signal between the two proteins when they were over-expressed in mammalian cells.

A stable U2OS cell line expressing FoxM1b-EGFP was transiently transfected with plasmid DNA encoding eDHFR-NPM1. Ca. 24 h after transfection, the cells were incubated with a cell permeable TMP-Tb$^{3+}$ complex, washed and imaged using a time-gated luminescence microscope. From steady-state images of GFP fluorescence, FoxM1b-EGFP was predominantly localized to the nucleus, as expected (Figure 25). Time-gated images of Tb$^{3+}$ luminescence indicated that many cells were successfully loaded with the TMP-Tb$^{3+}$
complex. However, time-gated images at the GFP emission wavelength showed only a few cells with a potential Tb$^{3+}$-to-GFP FRET signal (Figure 25). Moreover, in this experiment we did not have a direct mechanism to confirm which cells were expressing eDHFR-NPM as it lacked a fluorescent tag.

Figure 25: TR-FRET image of EGFP-FOXM1b/NPM interaction in U2OS cells. 24 hours after transfection, cells were incubated in medium containing 10 µM Lumi4-Tb complex at 37 °C for 20 minutes. GFP, continuous wave fluorescence showing EGFP-FoxM1 signal; Lumi4-Tb, time-resolved fluorescence image of Tb emission signal (620 nm); TR-FRET, time-resolved fluorescence image of Tb-to-EGFP FRET (520 nm). Arrows in GFP and Lumi4-Tb images indicate cells both expressing EGFP-FoxM1b and loaded with Lumi4-Tb. One cell in FRET channel (indicated with arrow) exhibits a corrected FRET signal, indication of an interaction.
For the second set of experiments, we co-transfected the stable EGFP-FoxM1b U2OS cells with plasmids encoding eDHFR-NPM and H2B-mCherry. Here, the mCherry reporter served as a positive control for transfection. Following transfection, the cells for imaging as detailed above and imaged in both steady-state and time-gated fluorescence modes. In cells that expressed H2B-mCherry (and presumably eDHFR-NPM) we observed a time-gated Tb$^{3+}$ signal localized in sub-nuclear puncta which would be consistent with a nucleolar localization of eDHFR-NPM. Furthermore, fluorescence in the GFP channel shows that GFP-FoxM1 appears to be localized in the nucleoplasmic region but not in the nucleoli. Finally, we could not detect a FRET signal significantly above the background level (Figure 26). From this imaging experiment, we concluded that FoxM1 not only does not bind to NPM when both proteins are overexpressed as fusions in U2OS cells, but they also do not co-localize.

This project was somewhat bold in the sense that the goal was to develop HTS for a PPI that was not intensively studied. Thus far, the only evidence of an interaction between FoxM1 and NPM is that published by the Gartel Lab at UIC. That study used co-immunoprecipitation and pull-down assays as the primary means of determining evidence of interaction. While these assays are commonly accepted methods for indicating interaction, they often result in false positive results. For example, Mackay and co-workers used various biophysical techniques to validate ~20 reported PPIs, predominantly among transcriptional regulators, and they could confirm less than half of those studied. Both Co-IP and pulldown assays can result in false positives due to assay condition-dependent non-specific binding. Also, Co-IP may indicate that two proteins are found together in a complex but not
physically interacting. Generally, more robust biophysical evidence, such as NMR or isothermal titration calorimetry is required to establish that two proteins interact directly.

The fact that we cannot confirm interaction between GFP-FoxM1b and eDHFR-NPM when the proteins are over-expressed in mammalian cells does not bode well for our plan to use bacterially expressed and purified versions of these proteins for screening. Nevertheless, our results thus far do not preclude the possibility of a functional interaction. In the paper by Bhat, et al. most of the evidence provided for a FoxM1/NPM interaction was obtained in cancer cell lines other than U2OS and under different expression conditions. Also, it is possible that binding occurs but is phosphorylation-dependent as FoxM1 has multiple phosphorylation sites. At the present time, further studies are underway to confirm and better characterize the putative interaction between FoxM1 and NPM. If it turns out to be the case that FoxM1 and NPM interact in a manner that is dependent on post-translational modifications of either protein or on cell line or cell growth conditions, the mammalian cell-based approach described in Chapter 3 would be a good candidate for developing an HTS inhibition assay.
Figure 26: EGFP-FOX1b and eDHFR-NPM colocalization in U2OS cells. 24 hours after transfection, cells were incubated in medium containing 10 μM Lumi4-Tb complex at 37 °C for 20 minutes. GFP, continuous wave fluorescence showing EGFP-FoxM1 signal; mCherry, continuous wave fluorescence showing H2B-mCherry signal; Lumi4-Tb, time-resolved fluorescence image of Tb emission signal (620 nm); TR-FRET, time-resolved fluorescence image of Tb-to-EGFP FRET (520 nm). Arrows in images indicate cells both expressing EGFP-FoxM1b, H2B-mCherry and the localized labeling of eDHFR with TMP-Lumi4-Tb. No apparent Tb-to-GFP FRET signal was observed.
REFERENCES


VITA
ENGIN YAPICI

Education:

*University of Illinois at Chicago / Chicago, IL USA*
Department of Chemistry, PhD  
October 2014
Department of Chemistry, MSc  
December 2012

*Middle East Technical University / Ankara, Turkey*
Department of Biological Sciences, BS (High Honors)  
January 2009

Professional Experience:

Aug 2009 – Aug 2014  Graduate Research Assistant, *Department of Chemistry, UIC, USA*

Aug 2009 – May 2014  Teaching Assistant, *Department of Chemistry, UIC, USA*
   • General Chemistry (Chem 112, 114)
   • Inorganic Chemistry (Chem 314)
   • Biochemistry (Chem 452, 455)

Jan 2008 – July 2009  Undergraduate Researcher, *METU, Turkey*

Publications:


Presentations:


**Awards:**

- Chancellor's Student Service & Leadership Award, UIC, 2014
- The Finn Wold Travel Award, 2013
- Protein Society Young Investigator Travel Grant, 2013
- Chancellor's Student Service & Leadership Award, UIC, 2013

**Memberships:**

- American Chemical Society, Member 2012-Present
- UIC Graduate Student Council, Vice President 2012-2014
- UIC Faculty Senate, Student Senator 2012-2014
- UIC Chemistry Graduate Student Association, Treasurer 2012-2013
- UIC Chemistry Graduate Student Association, President 2011-2012
- METU Contemporary Dance Club, President 2008-2009