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Optimization of Bacterial Bioluminescence (\textit{lux}) Expression and Development of Autonomous \textit{lux}-Based Reporters in Human Cell Lines

Tingting Xu
txu2@utk.edu

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To the Graduate Council:

I am submitting herewith a dissertation written by Tingting Xu entitled "Optimization of Bacterial Bioluminescence (\(lux\)) Expression and Development of Autonomous \(lux\)-Based Reporters in Human Cell Lines." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Gary S. Sayler, Major Professor

We have read this dissertation and recommend its acceptance:

Alison Buchan, Timothy E. Sparer, Seung J. Baek, John P. Biggerstaff

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
Optimization of Bacterial Bioluminescence (*lux*)
Expression and Development of Autonomous *lux*-Based Reporters in Human Cell Lines

A Dissertation Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Tingting Xu
December 2012
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A friend of mine once told me that the most difficult time of writing her dissertation was the acknowledgements. I cannot agree any more at this moment when I am writing my own. It is quite an emotional process looking back into the last six years of graduate school to realize what I have achieved thus far. Fortunately, I was not alone. None of this would have been possible without the assistance from many people.

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finish my first 5K, half marathon, full marathon, and mud run races, from which I built a stronger mind. Things I absorbed from these experiences will influence me as a scientist as well as a person and last a lifetime.

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ABSTRACT

Despite its extensive use as a prokaryotic bioreporter, only recently has the bacterial bioluminescence (lux) system been adapted to express at a functional level in the mammalian cellular background. While this novel strategy has the potential to contribute to the fields of high throughput screening and non-invasive \textit{in vivo} imaging due to its autonomous substrate production, it is still constrained because its signal intensity is lower than that of other bioluminescent reporters. This work demonstrates the development of strategies that optimize human cell lux-based bioluminescence to overcome this detriment for advancement towards a fully functional lux reporter system. To enhance lux gene expression, a single vector construct has been developed that improves upon the initial two plasmid expression system. This construct separates the lux genes using viral-derived 2A elements, allowing for simultaneous expression of the six genes from a single promoter in eukaryotic cells. This strategy results in increased bioluminescent output compared to the previous two plasmid system. Additionally, it is demonstrated that gene copy number is the primary limiting factor for bioluminescent output following expression in human cell lines. This limitation has been overcome through the development of a two-step transfection strategy that yields significantly higher transfection efficiency and improves both transgene integration and bioluminescence output. An optimized HEK293 cell line with enhanced bioluminescent production has been constructed using the
two-step transfection strategy. The enhanced signal intensity allows bioluminescent detection from a smaller population size in cell culture compared to their un-optimized counterparts, as well as detection using alternative lower cost instruments. A developmental effort towards lux-based biosensing in human cell lines is also reported in this study. It has been shown that bioluminescence emitted constitutively from lux-expressing human cell lines can be utilized for monitoring population dynamics in a non-invasive manner and that, by regulating the expression of the lux genes, the lux system can function as a fully autonomous bioreporter for continuous monitoring of targets of interest.
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CHAPTER I

Introduction
Background and Research Objectives

Mammalian cells expressing reporter proteins that are capable of producing an optical signal are progressively becoming more widely employed by investigators from diverse backgrounds to interrogate a variety of cellular functions in cell culture and living animals (Baker 2010). These applications include, but are not limited to, tumorigenesis and cancer treatment (Rehemtulla, Stegman et al. 2000; Venisnik, Olafsen et al. 2006), gene expression and regulation (Rettig, McAnuff et al. 2006; Korpal, Yan et al. 2009), cell trafficking (Sheikh, Lin et al. 2007; van der Bogt, Sheikh et al. 2008), viral and pathogenic infection (Andreu, Zelmer et al. 2011), protein stability and function (Laxman, Hall et al. 2002; Wang, Fu et al. 2010), and protein-protein interactions (Paulmurugan, Umezawa et al. 2002; Pichler, Prior et al. 2008). Currently, the mainstays of optical reporter proteins used for these applications are fluorescent proteins (GFP and its other color variants) and bioluminescent proteins (luciferase enzymes) isolated from insects and marine organisms. The optical signal generated by these proteins allows for visualization of the cellular events of interest, but each type of these reporters is associated with its own disadvantages. Fluorescent proteins, for instance, require an initial excitation for light emission. It is this excitation that can produce high levels of background fluorescence from endogenous biological structures in cultured cells and whole animal imaging, thus greatly reducing the sensitivity and resolution of this
technique and interfering with data interpretation.

With little to no endogenous bioluminescent activity in mammalian cells and tissues, the use of luciferase proteins has the advantage over their fluorescent counterparts of near background-free detection, especially for \textit{in vivo} whole animal imaging. However, most of the currently available luciferase proteins require addition of an exogenous substrate for bioluminescent emission. In cell culture based applications, the substrate addition is often associated with cell lysis, therefore only providing measurements on a single time point basis. For \textit{in vivo} whole animal imaging, the required substrate is usually injected directly without animal sacrifice. Although this allows for repeated monitoring of a single subject, due to rapid substrate uptake and consumption (Bhaumik and Gambhir 2002; Inoue, Kiryu et al. 2009), detection using these luciferase proteins has relatively short temporal dynamics, and is thus handicapped for long term monitoring applications.

The only substrate-free bioluminescent reporter system that has been developed to date is the bacterial bioluminescence (\textit{lux}) system. Setting it apart from other luciferase systems is that it is capable of generating its own substrate, therefore eliminating the need for cell destruction or substrate addition for signal detection. Due to this unique feature, the \textit{lux} system is highly amenable towards continuous on-line biosensing. Indeed, it is extensively used as a prokaryotic bioreporter for environmental assessment (Shin 2011; Singh, Abhilash et al.)
and pathogenic infection (Contag, Contag et al. 1995; Francis, Joh et al. 2000; Trcek, Berschl et al. 2010). The advantage offered by the lux system over other substrate-requiring luciferase systems has made it an attractive target for development into a mammalian reporter, but it is not until very recently that it has been adapted to express at a functional level for autonomous bioluminescent production in mammalian cells (Close, Patterson et al. 2010). While this initial demonstration opens the door for continuous real-time monitoring of cellular processes in mammalian cells and in living animals, the current mammalian-adapted lux system is not capable of producing levels of bioluminescent signal as high as any of the other substrate-requiring luciferase systems, which is the major limitation for more widespread applications. To address this issue, this work has been focused on the optimization of lux-based bioluminescent expression and its development as an autonomously bioluminescent reporter in human cell lines. The investigations presented in this dissertation have been performed under the following experimental hypotheses:

**Hypothesis 1:** Through the use of viral 2A peptides as linker regions, it will be possible to simultaneously express up to six genes (the luxCDABEfrp gene cassette) under the regulation of a single promoter for autonomous bioluminescent production in human cell lines.

**Hypothesis 2:** The human cellular background is capable of supplying sufficient endogenous substrates for high level lux-based bioluminescent
production. Therefore, it will be possible to improve bioluminescent expression through increased gene integration.

**Hypothesis 3:** Bioluminescent production dynamics in estrogen-responsive human breast carcinoma T-47D cells can be used as an indicator for estrogen-regulated cell proliferation.

**Hypothesis 4:** By simultaneously regulating the expression of the *lux* genes assembled in a polycistronic single vector, it will be possible to construct a bioluminescent reporter capable of responding to target analyte autonomously and in a near real-time manner.

**Literature Review**

**What is bacterial bioluminescence?**

Bioluminescence – the chemical generation of light within a living organism – is widely distributed in nature. Displayed by over 700 genera, bioluminescence can be found in both terrestrial and aquatic environments. On land it is most commonly observed in the glow of fungi growing on decaying wood or from insects displaying their luminescent signal after dusk, while in marine environments bioluminescence is most commonly observed in bacteria that are found either living freely or in symbiosis with larger hosts. It is these bioluminescent bacteria that are the most abundant and widely distributed of the light emitting organisms on Earth. Despite the widespread prevalence of bacterial
bioluminescence, however, the majority of these organisms are classified into just three genera: *Vibrio*, *Photobacterium*, and *Photorhabdus* (*Xenorhabdus*) (Meighen 1991). Although they are viable as free-living bacterium, these organisms are most commonly observed in symbiosis with a larger host. There is still no consensus as to the evolutionary benefit of bioluminescent production, however, in general it is theorized that the production of light can aid in the consumption of free living bacteria by higher trophic organisms, transferring them to a more controlled, nutrient rich habitat inside the host, or that, likewise, symbiotic bacteria can aid their hosts through the production of light that attracts prey, aids in camouflage, or attracts mates, in return for the shelter and nutrients provided by living within the body of the host organism (Nealson and Hastings 1979).

The genetic system employed for the generation of bioluminescence is well conserved across all known bioluminescent bacteria. Five genes, usually organized in an operon as *luxCDABE*, are essential for bioluminescence production. The luciferase protein catalyzing the bioluminescent reaction is a heterodimer formed by the *luxA* and *luxB* gene products. The long-chain fatty aldehyde substrate required for light emission is produced by a protein complex encoded by the *luxC*, *luxD*, and *luxE* genes. Specifically, the *luxD* gene encodes a transferase that converts intracellular myristyl-ACP, an intermediate for membrane synthesis, to myristic acid, which is further converted to myristyl
aldehyde by the reductase/synthase protein complex encoded by the *luxC* and *luxE* genes. In some species, there is an additional gene, *frp*, which functions as a flavin reductase to aid in the regeneration of the required FMNH₂ substrate. Together with molecular oxygen, these components are all that are required to produce a bioluminescent signal (Meighen 1991) (Figure 1). The overall reaction can be summarized as the following:

\[
\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \rightarrow \text{FMN} + \text{H}_2\text{O} + \text{RCOOH} + h\nu_{490\text{nm}}
\]

**The history of using bacterial bioluminescence as a prokaryotic bioreporter**

**First examples of transgenic lux expression**

Even before the mechanisms responsible for bioluminescence were completely explored in the organisms that naturally expressed it, the *lux* system had gained attention in regards to its potential as a reporter because of the facile detection and quantification of visible light produced by the Lux proteins. Work therefore quickly began to introduce the *lux* genes into non-luminescent bacterial species to determine if the modified organisms could acquire the light-emitting phenotype. These initial attempts to isolate and exogenously express the *lux* genes in non-native hosts such as *E. coli* were driven primarily by the desire to understand the organization, regulation, and function of the genetic components responsible for bioluminescence. The first major advancement in these investigations occurred in 1982 when Belas et al. (1982) demonstrated
Figure 1. Bacterial bioluminescence reaction.

The luciferase that catalyzes the bioluminescent reaction is a heterodimer formed from the \textit{luxA} and \textit{luxB} gene products. The \textit{luxC}, \textit{luxD}, and \textit{luxE} gene products work synergistically to synthesize and regenerate the myristoyl aldehyde co-substrate for the reaction. The \textit{frp} gene encodes a flavin reductase that has been found to facilitate FMNH$_2$ recycling in eukaryotic cells. Used with permission from (Close, Ripp et al. 2009).
bioluminescent production from recombinant *Escherichia coli* containing the *Vibrio harveyi* *luxA* and *luxB* genes upon addition of exogenous aldehyde. In less than one year following this successful demonstration, the entire *lux* cassette (*luxCDABE*), together with its associated regulatory genes, was isolated from *V. fischeri* (more recently designated as *Aliivibrio fischeri*) and introduced into *E. coli* (Engebrecht, Nealson et al. 1983), resulting in the first recombinant strain capable of emitting light without aldehyde supplementation. This process was soon repeated using the *Photorhabdus luminescens* genes, suggesting the universal nature of the operon (Frackman, Anhalt et al. 1990; Fernandezpinas and Wolk 1994). Although these efforts focused primarily at interrogating the functions of the individual *lux* genes, investigators began to appreciate the sensitivity, ease of detection, and non-destructive features the *lux* system offered as a bioluminescent reporter. The practical nonexistence of *in vivo* bioluminescence from bacteria not expressing the *lux* genes provides a high signal-to-noise ratio for the recombinant *lux* reporter system and the fast turnover rate of the bacterial luciferase enzyme allows for rapid light production. Taken together, these attributes have allowed the development of bacterial bioreporters that can be continuously monitored in a near real-time fashion. In addition, because the resulting bioluminescence is emitted in an autonomous manner, these reporters can be employed in a high throughput manner with very low cost, making them attractive options for a large number of biomonitoring applications.
Development of lux as a method for visualizing gene expression

The first use of lux as a biomonitoring technology came soon after its transgenic expression in *E. coli*, when Engebrecht et al. (1985) fused the lux cassette to an inducible promoter that could be used to monitor gene expression *in vivo*. Using this experimental design it became possible to monitor autonomous bioluminescence as an indicator for transcriptional activity of a promoter of interest. Using this method, the first major targets of study were the *E. coli* lac and ara promoters and it was discovered that upon IPTG or arabinose induction, light production in hosts expressing lux fusions increased between 600 to 1000-fold. Following these reports, the lux system was used to monitor regulation of the lateral flagella genes in *Vibrio parahaemolyticus* (Engebrecht, Simon et al. 1985; Belas, Simon et al. 1986), providing its first demonstration in a previously uncharacterized system. These applications represented a significant shift in the way gene expression was investigated because, unlike traditional biochemical assays using enzymatic reporters, the bioluminescent signal from the lux genes could be easily detected and measured with high sensitivity without cell perturbation. This allowed the same sample to be continuously monitored, thus revealing the dynamics of gene expression through changes in bioluminescence over time.

lux-based bioluminescence as a tool for cellular population monitoring

While the Lux proteins do not require exogenous substrate addition, their
function does require continued access to their molecular oxygen, FMNH$_2$, and aldehyde co-substrates. For this reason, their bioluminescence can only be detected in actively growing cells. This knowledge, combined with the discovery that lux bioluminescent output is proportionally correlated to the number of cells present, has therefore been used as a simple, sensitive, and non-destructive means for in situ bacterial monitoring. This was first demonstrated by Shaw et al. (Shaw and Kado 1986) in 1986 when constitutively expressed V. fischeri luxCDABE genes were introduced into the phytopathogen Xanthomonas campestris, and their subsequent invasion of a cauliflower leaf was visualized. Similarly, de Weger and colleagues (de Weger, Dunbar et al. 1991) were successfully able to detect luxCDABE-labeled Pseudomonas fluorescens in the rhizosphere of soybean roots using the same technique. Additionally, through the use of a lux-based system rather than an enzymatic reporter, it was possible for these researchers to achieve detection limits three orders of magnitude lower than what was previously possible, leading to improved signal detection. These early examples highlighted the application of lux-based bioluminescence as a rapid, simple and sensitive tool for in situ detection of living bacteria and established the foundation for future research using lux to monitor genetically engineered microorganisms. In perhaps the most notable use of the lux genes for tracking a cellular population, a P. fluorescens strain was transformed with the lux genes and used for the first bioremediation-related environmental field release of a genetically engineered microorganism in 1996 (Ripp, Nivens et al.
By placing the *lux* genes under the control of promoters in the naphthalene degradation pathway, it was possible to monitor their bioluminescent output as a measure of naphthalene contamination in the soil (King, Digrazia et al. 1990). Using a combination of bioluminescent and traditional culture based detection methods, the release area was monitored for two years after the release of bioluminescent *P. fluorescens*. Over this time, regular sampling was performed to track the amount of bacteria present in the soil, as well as the amount of bioluminescence produced, which were indicative of organism presence and naphthalene degradation, respectively. Based on culture detection methods, the bioluminescent *P. fluorescens* persisted in both contaminated and non-contaminated soils, decaying at similar rates and producing similar colony counts (Ripp, Nivens et al. 2000). The long term nature and difficulty in remote monitoring of bacterial populations presented in this study illustrates how the unique properties of the *lux* operon can provide it with an advantage over its substrate requiring bioluminescent or UV stimulation requiring fluorescent counterparts. Because of its autonomous nature, the *lux*-tagged *P. fluorescens* could be continually surveyed for bioluminescent production without the need for repeated stimulation to induce a reporter signal.

**The use of lux for exogenous target detection**

Following the work that demonstrated how the *lux* cassette could be used as a tool for visualizing gene expression, it soon became clear that these genes
could be adapted for use as a traditional bioreporter target through activation under specific, predetermined conditions as well. By expressing the lux cassette under the control of a promoter with a known inducer, the resultant bioluminescent emission could be used as an indicator for the presence of the given stimulus, and fluctuation of the bioluminescent signal could be interpreted as changes in the bioavailable concentration of the inducer compound. Building upon these ideas, the first use of bioluminescence for monitoring metabolic activity was demonstrated in Pseudomonas putida by Burlage et al. in 1990 (Burlage, Sayler et al. 1990). Here, naphthalene degradation was monitored using a transcriptional fusion of the salicylate inducible nah promoter and the luxCDABE genes. Salicylate is an intermediate metabolite of naphthalene, which is eventually degraded to acetaldehyde and pyruvate in Pseudomonas. Therefore, naphthalene degradation could be correlated to light emission upon induction with naphthalene-derived salicylate. The nondestructive nature of the lux system allowed for this analysis to occur in real time in a growing culture, providing continuous monitoring of naphthalene metabolism across various stages of growth. It was later determined by King et al. (King, Digrazia et al. 1990) that the bioluminescent signal was controlled in a dose/response fashion, therefore demonstrating its usefulness in determining contaminant levels in mixed environmental samples. This opened the door for a multitude of environmental bioreporters featuring lux, such as that developed by Applegate and colleagues that was used to monitor for water soluble benzene, toluene,
ethylbenzene, and xylene (BTEX) compounds indicative of petroleum spills. This reporter, constructed by linking expression of the \textit{lux} cassette to the toluene dioxygenase promoter, was capable of detecting as little as of 30 µg of toluene / L in as quickly as 2 hours and maintained its detection ability for over 100 generations without antibiotic selection (Applegate, Kehrmeyer et al. 1998).

Another common target for \textit{lux}-based environmental sensing has been phenol. Notably, Abd-El-Haleem et al. (Abd-El-Haleem, Ripp et al. 2002) constructed one of the first \textit{lux}-based phenol biosensors by inserting a \textit{mopR}-like promoter fused to the \textit{V. fischeri lux} cassette genes into \textit{Acinetobacter} sp DF4. This reporter was capable of demonstrating a lower detection limit of 2.5 ppm in 4 hours when exposed to phenol, and was only responsive to three of the ten phenol derivatives tested, suggesting that is was relatively specific. This is, however, not by any means the only \textit{lux}-based phenol reporter to be developed. Davidov et al. (Davidov, Rozen et al. 2000) made extensive use of \textit{recA} promoters fused to \textit{lux} cassettes, with each of the reporters containing a slight variation in its promoter sequence, that were expressed either in \textit{E. coli} or \textit{Salmonella typhimurium} and using \textit{lux} genes from either \textit{V. fischeri} or \textit{P. luminescens}. The most sensitive of these reporters was that expressing the \textit{V. fischeri lux} genes in \textit{E. coli}, which was capable of detecting 0.008 mg phenol / L in 2 hours. This same construct, when expressed in \textit{S. typhimurium} was also capable of detecting phenol in 2 hours but required a minimum concentration of 16 mg phenol / L, demonstrating the
differences in host phenol bioavailability.

**Concerns of exogenous gene expression in a surrogate host**

Although nucleic acids serve as the universal genetic material and the central dogma applies to all organisms, exogenous expression of foreign genes is not as straightforward as delivering the target sequence into host cells and waiting for it to be expressed. This is because the gene expression machinery in certain species has evolved in such a way as to manipulate its own genetic material more efficiently than genomic material from other species, a fact that is especially true when the exogenous genetic material is from a very distantly related species. For example, the prokaryotic and eukaryotic cells differ in the basic components for processing genetic information (Table 1). Any discrepancies, such as the genomic characteristics of GC content and codon usage patterns between the native and surrogate hosts will play an important role in the efficiency of exogenous gene expression. In addition, some organisms have also evolved to recognize and remove or silence foreign genetic sequences in order to protect themselves from the deleterious effects of foreign DNA expression. It is only through mimicking, circumventing, or deactivating these mechanisms that it becomes possible to efficiently express a foreign gene in a surrogate host. Therefore, by understanding how these mechanisms work, it increases the likelihood that a strategy can be developed for effective exogenous gene expression.
<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Prokaryotes</th>
<th>Eukaryotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Occurs in cytoplasm</td>
<td>Occurs in nucleus</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Single polymerase</td>
<td>Pol I, Pol II, and Pol III</td>
</tr>
<tr>
<td>Transcription initiation</td>
<td>-10, -35, and UP recognition elements</td>
<td>TATA box and TRIIB recognition elements</td>
</tr>
<tr>
<td>Coding region</td>
<td>Single coding region</td>
<td>Multiple coding regions with exons and introns</td>
</tr>
<tr>
<td>mRNA processing</td>
<td>Rho dependent and independent termination</td>
<td>5’ capping, splicing, and 3’ polyadenylation</td>
</tr>
</tbody>
</table>

Modified from (Close, Xu et al. 2012).
**GC content**

The term GC content refers to the percentage of guanine and cytosine bases in a DNA sequence. It can be used to describe a gene, a chromosome, a genome, or even any region of a particular DNA sequence. Different organisms can vary significantly in their genomic GC content. For example, *Plasmodium falciparum* has an extremely GC-poor genome, with a GC content of approximately 20%, while *Streptomyces coelicolor* possess a GC content as high as 72%.

Due to the difference in thermodynamic stability between the GC bonding pairs and the AT bonding pairs, GC content can affect the formation and stability of both DNA and RNA secondary structures, which are important factors in the regulation of gene expression (Kubo and Imanaka 1989; Kudla, Murray et al. 2009). In bacteria, the Shine-Dalgarno ribosome binding site that is located in the 5’ untranslated region of the mRNA is relatively AU-rich. The presence of this high AT abundance and low secondary structure stability at the 5’ end of a coding region has been found to contribute significantly to producing high translation efficiency in bacteria (Desmit and Vanduin 1990; Allert, Cox et al. 2010). Furthermore, Kudla et al. have demonstrated that the addition of these types of AU-rich leader sequences to the 5’ untranslated region of genes can improve the expression levels of otherwise poorly expressed proteins (Kudla, Murray et al. 2009). In a recent systematic study of 340 genomes from various
groups of organisms including bacteria, archaea, fungi, plants, insects, fishes, birds, and mammals, Gu and colleagues discovered a trend of reduced mRNA stability near the start codon in most organisms except birds and mammals and that this reduction results in changes in mRNA stability that are correlated with genomic GC content (Gu, Zhou et al. 2010).

In birds and mammals, however, the genome-wide trend of reduced mRNA stability near the translation initiation site has not been observed, even though the GC content in these organisms is not significantly different from the species where such a trend was originally observed (Gu, Zhou et al. 2010). The authors speculate that this difference is due to the isochore-type structure in the genomes of these organisms. An isochore is the result of a high variation in GC content over large-scale sequences within a genome (Bernardi 1995). Within an isochore structure, however, the GC content is generally homogeneous regardless of the heterogeneous nature of the remainder of the genome (Eyre-Walker and Hurst 2001). It is important to note that, unlike in E. coli, high GC content within the coding region usually increases expression in mammalian cells (Kudla, Lipinski et al. 2006). Kudla and colleagues have found that GC-rich genes in mammalian cells were transcribed more efficiently than alternate, GC-poor versions of the same gene, leading to higher protein production. In fact, the 5' cap and Kozak consensus sequence located on the 5' untranslated region normally have a GC-rich composition in eukaryotic genes (Kozak 1987).
It is widely accepted that genomic GC content has co-evolved with the gene expression machinery to ensure optimal expression for the fitness of the host (Andersson and Kurland 1990; Kudla, Murray et al. 2009). Therefore, with regards to expression of exogenous genes, the difference in the GC contents between the target genes, especially at the 5’ end, and the expression host can also impact the expression level of foreign genes. The difficulty in expressing *Plasmodium falciparum* genes in *E. coli* is hypothesized to be attributed to its extreme low GC content and the possibility of degradation of mRNA by ribonuclease E (McDowall, Linchao et al. 1994; Plotkin and Kudla 2011). Plotkin and Kudla also predicted that more than 40% of human genes would be expressed poorly in *E. coli* without modification due to the relatively high GC content at the 5’ end of mRNA and subsequent low 5’ folding energy (Plotkin and Kudla 2011).

**Codon usage bias**

In addition to determining mRNA stability and secondary structure organization, another feature of every genome that is impacted by GC content is its codon usage profile. The 20 amino acids commonly found in protein sequences are all encoded from a series of 61 different nucleotide triplets. The redundancy of this coding system necessarily allows the same amino acid to be encoded by several different codons. For example, the amino acids alanine and serine can be encoded using either four or six codons, respectively. This innate
degeneracy that is built into the genetic code has evolved to play a role in protecting DNA sequences from otherwise deleterious mutations by preserving their resultant protein sequences despite the inevitable incorporation of mutations at the genetic level, effectively silencing these mutations. However, the available synonymous codons are not used at equal frequencies across all species, nor across different regions within the same genome, and sometimes not even within the same gene (Andersson and Kurland 1990; Kurland 1991). Predictably, the discrepancy of codon usage profiles is greatest between remotely related species, while more closely related species are more likely to share similar codon preferences. Although the mechanistic processes underlying how an organism develops a specific codon bias has not been completely resolved (Chamary, Parmley et al. 2006; Hershberg and Petrov 2008), the GC content of the preferred codon chosen is thought to be the single most important factor determining codon usage biases across genomes (Plotkin and Kudla 2011).

Although it was initially believed that synonymous codon substitutions were simply examples of fortuitous silent mutations, more recent research has revealed that codon usage patterns can directly affect important cellular processes such as the efficiency of transcription and translation, the accuracy of protein translation and even the process of protein folding (Zhang, Hubalewska et al. 2009; Angov 2011). It is therefore conceivable that the specific codon usage pattern of an organism has co-evolved along with other cellular machinery

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in order to provide for optimal gene expression and protein function of the host genes within their natural environment (Grantham, Gautier et al. 1981). In prokaryotes, for example, the frequency of a codon being used correlates positively with the intracellular abundance of its corresponding tRNA (Bulmer 1987; Dong, Nilsson et al. 1996). It therefore follows that the expression of non-native genes is hampered by the existence of variation in their respective codon usage pattern compared to the host organism. This hypothesis has been supported throughout the long history of exogenous gene expression, revealing that the same DNA sequence is often expressed at different efficiencies in different organisms (Gustafsson, Govindarajan et al. 2004). This is due to the foreign DNA sequence containing codons that are rarely used in the host. A situation that leads to low level translational efficiency and low level protein expression (Kane 1995; Kim and Lee 2006; Rosano and Ceccarelli 2009) due to a reduced translation elongation rate caused by the imbalance between the codons used in the target gene sequence and the available pool of charged tRNA in the host. These expression problems are then compounded by any incompatibility between the host translation machinery and the mRNA secondary structure due to changes in GC content from alternate codon usage patterns (Wu, Jornvall et al. 2004; Kim and Lee 2006).

To overcome these problems, a common strategy aimed at enhancing the expression of non-native genes in a surrogate host is that of codon optimization.
This process encompasses the replacement of rare codons within the DNA sequence in order to closely match the host codon usage bias while retaining 100% identity to the original amino acid sequence. This process of codon optimization also allows for the simultaneous modification of predicted mRNA secondary structures that could result from changes in the GC content. This process is especially helpful in eliminating structures at the 5’ end of coding regions, where they have an increased likelihood of interfering with downstream protein expression (Wu, Jornvall et al. 2004). Cis-acting negative regulatory elements within the coding sequence are also eliminated in order to reduce the chance of repression, therefore improving expression (Graf, Bojak et al. 2000).

The codon optimization process can be achieved experimentally either through multiple stages of site-directed mutagenesis on directly cloned DNA, or by resynthesis of the target gene de novo. The former method may be preferred if there are a limited number of codons that must be changed, however, latter method has become more and more practical due to improvements in the gene synthesis process that have both reduced the cost and time required to generate synthetic DNA sequences. In general, the codon optimization process has been shown to increase expression of a typical mammalian gene five- to fifteen-fold when expressed in an *E. coli* host (Gustafsson, Govindarajan et al. 2004; Burgess-Brown, Sharma et al. 2008). Similarly, expression of prokaryotic genes in eukaryotic cells can be improved significantly using this method as well (Zolotukhin, Potter et al. 1996; Zur Megede, Chen et al. 2000; Patterson, Dionisi...
Mechanisms for removal and silencing of exogenous genes

For an exogenous gene to be expressed in a non-native host, the foreign DNA must be physically delivered into the host cell and then properly integrated into the gene expression and regulation network within the host. Decades of research in the fields of molecular and cellular biotechnology have provided many effective techniques for the introduction of genetic material into both prokaryotic and eukaryotic hosts, however, after the gene has been transferred into the host cell, it needs to be recognized and processed by the host cell’s replication, transcription and translation machinery before it can be expressed as a functional protein. However, because expression of a foreign gene is often deleterious to host survival under wild type conditions, many organisms have evolved defense mechanisms that remove or silence foreign DNA in order to protect themselves from this potentially detrimental process. In bacteria, for example, the invading foreign DNA can be cleaved by restriction endonucleases that recognize specific, non-self, nucleotide sequences, in a phenomenon referred to as restriction. In this process the native genetic material is often methylated at certain positions by methylase enzymes, therefore preventing recognition and degradation by the restriction endonucleases, and ensuring the maintenance and expression of native DNA sequences. This restriction modification system was first discovered in the 1960s and since that time has
been demonstrated to be common in many bacterial species (Wilson and Murray 1991). The restriction system, however, is not the only defense mechanism that has been developed to protect the host from expression of foreign genetic material. It has been demonstrated that Gram-negative bacteria are capable of selectively repressing horizontally acquired genes through their interaction with a histone-like nucleoid structuring (H-NS) protein. This phenomenon, termed xenogeneic silencing, was first discovered in 2006 by Navarre, Lucchini, Oshima and colleagues (Lucchini, Rowley et al. 2006; Navarre, Porwollik et al. 2006; Oshima, Ishikawa et al. 2006). The H-NS protein responsible for xenogeneic silencing belongs to a family of nucleoid-associated proteins that bind to AT-rich DNA sequences with low sequence specificity. In the case of xenogeneic silencing, H-NS protein targets the laterally acquired sequence because it exhibits a lower GC content than the host genome, allowing it to selectively repress the expression of exogenous DNA.

Unlike the prokaryotic approaches for silencing of exogenous DNA sequences, no mechanism for the direct removal of foreign genetic material has yet been proposed to function in eukaryotic organisms. Nonetheless, the expression of exogenous DNA in plants and mammalian cells often suffers from low efficiency due to epigenetic modification. These modifications lead to unstable expression and, in extreme cases, silencing of the transgene over time. Silencing can occur at either the transcriptional or post-transcriptional level.
through changes in the methylation status of the sequence, histone modification, or RNA interference (Pikaart, Recillas-Targa et al. 1998; Pal-Bhadra, Bhadra et al. 2002; Riu, Chen et al. 2007). Regardless of the protective measures taken, these mechanisms are all employed by the host to regulate expression of exogenous genes and protect it from deleterious effects. One final concern that cannot yet be controlled for is that, due to the random integration following chromosomal introduction of an exogenous gene into the host chromosome, expression of the transgene can be highly dependent on the site of insertion. Depending on the location of integration, various position effects and epigenetic events often result in high variation of the expression level between individual expression attempts. While there is no way to reliably control for genomic insertion position of exogenous genes in the majority of cases, several elements have been proposed that can help to counteract the resultant position effects and achieve sustained transgene expression.

**Regulatory sequences that must be considered for optimal exogenous gene expression**

By developing a comprehensive understanding of the mechanisms underlying gene expression and appreciating how factors such as GC content and codon usage bias influence protein expression in non-native hosts, investigators can begin to develop theoretical guidelines for the rational design of DNA sequences optimally tuned for heterologous expression in their target organism. This
approach is especially attractive, with the reduced time and cost of gene synthesis allowing for de novo production of complete genes and even entire expression cassettes making it possible to simply design a gene sequence and begin working. However, there are additional concerns that must be addressed prior to successful expression of an exogenous gene sequence. Besides the optimization of the coding region, regulatory sequences that are not transcribed or translated should also be taken into consideration in order to achieve optimal expression. Although not expressed in the final protein product, these elements are involved in the transcription, translation and long-term maintenance of target genes in the surrogate host, making their optimization just as important as optimization of the coding sequence itself.

**Regulatory elements involved in transcription**

The process leading from a gene to a functional protein starts with transcription by RNA polymerase. Therefore transcription initiation is often an important point of control for exogenous protein expression. The driving force behind recruiting and binding the polymerase that will transcribe the DNA to mRNA is the promoter sequence that is required to recruit the host’s transcription machinery. Even though the promoter itself is not transcribed or translated, choosing a promoter that can be efficiently processed by the host’s machinery therefore has a significant impact on the success of the design strategy. Commonly, strong, constitutive promoters that are normally used to drive the
expression of endogenous housekeeping genes in the expression host are chosen for high level expression of exogenous genes. For example, the T7, alcohol dehydrogenase 1 (ADH1) and human elongation factor 1 α promoters (EF1-α) are commonly employed for heterologous protein expression in *E. coli*, *S. cerevisiae* and mammalian cells, respectively. Viral promoters such as the cytomegalovirus immediate early (CMV IE) promoter and the Simian virus 40 (SV40) regulatory sequences are also used to drive transgene expression in mammalian cells as well. It is important to note, however, that while the strength of the promoter used can at least partially determine the level of transgene expression, different promoters can have variable rates of transcription across different cell lines. For this reason, the selection of an appropriate promoter should be determined on a case-by-case basis. Recent studies have systematically compared many of the commonly used promoters in a variety of cell types (Norrman, Fischer et al. 2010; Qin, Zhang et al. 2010). These types of references are an excellent source of information when designing constructs with specific expression needs.

It is also important to remember that promoter sequences can be designed *de novo* similar to gene sequences, and that designing a specific primer upstream of a gene construct may be beneficial if no native alternative promoter sequences are available. Analysis of a large number of prokaryotic and eukaryotic promoters has revealed that many promoters contain a conserved core sequence that is
essential for recognition and binding of RNA polymerase and its cofactors. Through incorporation of these conserved sequences, it may be possible to specifically design a promoter sequence, allowing one to tailor expression of their genetic construct to their specific needs. In prokaryotes, this conserved sequence is known as the Pribnow box, and consists of a consensus sequence of six nucleotides, TATAAT (Pribnow 1975). In addition, there is another conserved element often found 17 bp upstream of the Pribnow box. This upstream region has a consensus TTGACAT sequence that has been shown to be crucial for transcription initiation (Rosenberg and Court 1979). In eukaryotic organisms, the counterpart to the Pribnow box is the TATA box with a consensus sequence of TATAAA. Besides recruiting the associated transcription machinery, these core promoter elements are also crucial in defining where RNA synthesis starts. In prokaryotes, RNA synthesis usually begins 10 bp downstream of the Pribnow box, whereas the first transcribed nucleotide is located approximately 25 bp downstream of the TATA box in eukaryotes. Therefore in addition to the use of an appropriate core promoter sequence, the location of that promoter sequence relative to the coding region should also be carefully considered to ensure complete transcription of the target genes.

It is important to note that although this minimal core promoter is essential for transcription, it alone is often not adequate to drive high level protein expression. In eukaryotes, DNA elements known as enhancers are often employed in tandem
with the core promoter to enhance gene expression through the recruitment of additional transcription factors. These enhancers can be found at various locations, including upstream of the core promoter, within the introns of the gene driven by the core promoter, and downstream of the genes it regulates as well (Levine and Tjian 2003). Although the mechanistic function of most enhancers is still not well understood, some well-studied viral enhancer elements are often included in common expression vectors as a means to increase the transcription efficiency of exogenous sequences. For example, the CMV IE enhancer has been shown to be capable of improving gene expression level by 8- to 67-fold in lung epithelial cells when combined with several weak promoters (Yew, Wysokenski et al. 1997) and Li and colleagues have further demonstrated that adding a SV40 enhancer to the CMV IE enhancer/promoter or 3’ end of the polyadenylation site can increase exogenous gene expression in mouse muscle cells by up to 20-fold (Li, MacLaughlin et al. 2001).

**Regulatory elements involved in translation**

Just as with the requirement of a core promoter sequence for the initiation of transcription, the presence of certain, conserved sequences at the 5’ untranslated region of mRNA sequences are essential for the initiation of translation. In prokaryotic organisms, the Shine-Dalgarno sequence on the transcribed mRNA serves this function by acting as the ribosome binding site (RBS). This consensus sequence is composed of six nucleotides, AGGAGG,
which are complementary to the anti-Shine-Dalgarno sequence located at the 3’ end of the 16S rRNA in the ribosome. During the initiation of translation the ribosome is recruited to the mRNA by this complementary base paring between the RBS and the 16S rRNA. For this reason, the classic RBS pairing is included as a standard element in the Registry of Standard Biological Parts (http://partsregistry.org/). Also included in the registry is a collection of constitutive prokaryotic RBS containing the Shine-Dalgarno sequence as well as flanking sequences that are known to affect translation. These sequences are invaluable when designing promoter and gene sequences, as their incorporation is required for efficient expression of the synthetic construct.

In eukaryotes, the 40S ribosomal subunit helps to serve this purpose by attaching to initiation factors that assist in the process of scanning the mRNA, with the Kozak sequence acting as the main initiator for translation (Kozak 1986; Kozak 1987). This translational process most commonly begins at the AUG codon closest to the 5’ end of the mRNA, however, this is not always the case. Kozak et al. have demonstrated that the distance from the 5’-end, the sequence surrounding the first AUG codon, and its steric relationship with the 40S ribosomal subunit all contribute to determining the actual initiation site location. However, it has been routinely demonstrated that placing the promoter and Kozak sequence upstream of the initiating codon serves to induce increased expression of target gene sequences (Morita, Kojima et al. 2000).
Besides the optimization of the codon usage pattern in the coding region, additional considerations must be taken into account when expressing prokaryotic genes in eukaryotic hosts or vice versa. Genes cloned directly from the genomic library of a eukaryotic organism usually cannot be expressed successfully in a prokaryotic host due to the presence of intervening, non-coding regions within the sequence. Unlike eukaryotes, prokaryotes lack the RNA splicing mechanisms required to remove these intron sequences and produce a mature mRNA. Therefore, any introns present within the expression construct must be eliminated prior to introduction into the prokaryotic host.

*Elements for simultaneous expression of multiple genes in eukaryotes*

Conversely, a significant obstacle towards the expression genomically cloned bacterial genes in a eukaryotic host is the inability of the host to synthesize proteins polycistronically from a single mRNA. Unlike in prokaryotes, where translation of multiple adjacent genes from one promoter is common, translation in eukaryotic cells normally requires the presence of a methyl-7-G(5’)pppN cap at the 5’ end of the mRNA prior to recognition by the translation initiation complex at the start of peptide synthesis (Pestova, Kolupaeva et al. 2001). There are strategies, however, that allow for co-expression of two or more genes in eukaryotic cells. On the most basic level, it is possible to express each gene independently from its own promoter, either through the introduction of multiple vectors, or introduction of a single vector containing multiple promoters. An
alternate approach is the expression of the multiple genes using a polycistronic expression vector that takes advantage of either IRES (Internal Ribosomal Entry Site) or 2A elements. Derived from a viral linker sequence, the IRES element allows for 5'-cap-independent ribosomal binding and translation initiation directly at the start codon of the downstream gene, thus enabling translation of multiple ORFs from a single mRNA (Jackson 1988; Jang, Krausslich et al. 1988). Although known IRES sequences vary in length and sequence, certain secondary structures have been shown to be conserved and important for the function of the elements (Baird, Turcotte et al. 2006). The most widely used IRES sequence for expression in mammalian cells is the one derived from encephalomyocarditis virus (EMCV) (de Felipe 2002). Similar to the IRES elements, 2A elements are viral sequences that can also be used as a short linker region to provide translation of two or more genes driven off of a single promoter. Translation of the 2A element causes an interaction between the newly synthesized sequence and the exit tunnel of the ribosome. This interaction causes a “skipping” of the last peptide bond at the C terminus of the 2A sequence. Despite this missing bond, the ribosome is able to continue translation, creating a second, independent protein product. To ensure continuous translation, the stop codon of the ORF upstream of the 2A element must be mutated to avoid unnecessary termination. By using a combination of various IRES and 2A elements, investigators have demonstrated polycistronic expression of five genes simultaneously from a single promoter in mammalian cells (Szymczak and
Vignali 2005), illustrating how they can be used to simulate the polycistronic expression of some bacterial genes.

**Elements for sustained maintenance and expression**

Integration of exogenous DNA sequences into a host chromosome is usually required for sustained transgene expression in mammalian cells. Because the insertion event preceding expression is largely random, the expression level of the integrated gene can be greatly impacted by the surrounding sequences and chromatin structure. As a consequence, unstable expression and high variability between individual clones are the two major issues associated with transgene expression. In addition, if insertion of the exogenous genes occurs within or in close vicinity to a required host gene, the health or survivability of the host can be negatively impacted. To aid in controlling for this type of negative regulation, several DNA elements capable of preventing these types of position effects and stabilizing transgene expression have been discovered (Table 2). These DNA elements are naturally found in mammalian genomes and are crucial for regulating the proper expression of endogenous genes. The locus control regions (LCRs) can enhance transcription of linked genes and also enable copy number-dependent gene expression (Li, Peterson et al. 2002), however, their large size and tissue-specific nature constrain their application in a variety of mammalian cell types (Kwaks and Otte 2006). Insulators, also known as barriers or enhancer-blocking elements, are DNA sequences that can protect genes from
Table 2. DNA elements used to enhance and stabilize transgene expression in mammalian cells.

<table>
<thead>
<tr>
<th>Element</th>
<th>Size (kb)</th>
<th>Increased expression</th>
<th>Stability of expression</th>
<th>Cell type-specific</th>
<th>Copy number-dependent</th>
<th>Position-independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCR</td>
<td>16</td>
<td>Unknown</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes, if powerful enough</td>
</tr>
<tr>
<td>Insulator</td>
<td>1.2-2.4</td>
<td>Unknown</td>
<td>Yes</td>
<td>Unknown</td>
<td>No</td>
<td>Majority Yes</td>
</tr>
<tr>
<td>UCOE</td>
<td>2.5-8</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Unknown</td>
<td>Yes</td>
</tr>
<tr>
<td>MAR</td>
<td>~3</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Majority Yes</td>
</tr>
<tr>
<td>STAR</td>
<td>0.5-2</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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the transcriptionally inactive heterochromatin or the action of enhancers and repressors (Recillas-Targa, Valadez-Graham et al. 2004). As an example, the best-characterized insulator, cHS4 (chicken β-globin hypersensitive site 4), has been shown to stabilize transgene expression over a long period of time (Pikaart, Recillas-Targa et al. 1998) and facilitate efficient integration of expressed sequences (Recillas-Targa, Valadez-Graham et al. 2004). Similar to insulators, STARs (stabilizing and antirepressor elements) are specifically used to block repression. Another type of DNA sequence, known as the ubiquitous chromatin opening element (UCOE) is derived from promoters of ubiquitously expressed genes. These elements have been shown to improve and stabilize transgene expression in a tissue-nonspecific manner, most likely through the maintenance of an active chromatin structure (Williams, Mustoe et al. 2005). Matrix attachment regions (MAR) are elements that mediate the attachment of chromosome to the nuclear matrix and, as such, are also widely used DNA for sustained transgene expression. These elements have also been shown to counteract position dependent insertion effects and prevent transgene silencing in a variety of cell types and transgenic animals (reviewed by Harraghy and colleagues (2008)).

Expression of bacterial bioluminescence in mammalian cells

The distinct autonomous feature of the bacterial bioluminescence system and its extensive use as a bacterial whole-cell biosensor has intrigued researchers to expand the applications of lux to eukaryotic reporters. Although many groups
have attempted to express the lux system in eukaryotic organisms, it was almost 30 years after the first demonstration of exogenous expression of lux and acquired bioluminescent phenotype in E. coli (Engebrecht, Nealson et al. 1983) that lux-based autonomous bioluminescence had been successfully expressed in mammalian cells (Close, Patterson et al. 2010). One of the hurdles limiting lux expression in the eukaryotic cellular background is the number of genes needed to be simultaneously expressed. Unlike in prokaryotes, where translation of multiple adjacent genes from one promoter is common, gene expression in eukaryotes often requires that each individual gene be regulated by its own promoter. This is because eukaryotic translation normally requires the presence of a methyl-7-G(5')pppN cap at the 5' end of the mRNA prior to recognition by the translation initiation complex at the start of peptide synthesis (Pestova, Kolupaeva et al. 2001). To overcome this limitation, many groups have attempted to express the Vibrio harveyi luciferase heterodimer (encoded by the luxA and luxB genes) either as fusion proteins (Kirchner, Roberts et al. 1989; Pazzagli, Devine et al. 1992) or as individual subunits using a dual promoter expression vector (Koncz, Olsson et al. 1987). However, it was determined that these strategies were not capable of expressing the LuxA and LuxB proteins at a functional level in mammalian cells, possibly due to improper dimerization and thermo-liability of the V. harveyi luciferase at the optimal mammalian growth temperature. Alternatively, Patterson et al. demonstrated that expression of the luxA and luxB genes from the insect pathogen, P. luminescens as a single
bicistronic transcript with the aid of an IRES element was stable and functional at 37°C in human cells (Patterson, Dionisi et al. 2005). This expression format allowed bioluminescence production at a detectable level in whole cell extracts upon the addition of an aldehyde substrate. It was further demonstrated that the difference in codon usage patterns between bacteria and mammalian species was a limiting factor for efficient translation of bacteria-derived gene sequences in the eukaryotic cellular background. By optimizing the lux gene sequences to mimic the human codon usage pattern without altering the amino acid sequences, bioluminescence increased by more than two orders of magnitude.

Despite the successful expression of the bacterial luciferase dimer, it was also determined that functional expression of the remaining genes in the lux cassette is essential for substrate-free bioluminescence production, as the human cellular background does not provide sufficient substrates to be scavenged by the LuxAB proteins to produce light autonomously. By applying similar strategies used for the expression of the luxAB genes, Close et al. demonstrated that co-expression of human optimized P. luminescens luxCDE and V. harveyi flavin reductase frp genes in an IRES-mediated bicistronic bi-directional vector was capable of providing adequate myristyl aldehyde substrate and FMNH$_2$ co-factor for the bioluminescence reaction in mammalian cells (Close, Patterson et al. 2010). Codon optimization was shown to be a significant contributing factor to the overall bioluminescent output, as expressing the human
optimized versions of these genes led to a 9-fold increase in signal intensity as compared to expression from their wild type counterparts. It was further revealed that cytosolically available FMNH$_2$ was a limiting reagent for the bioluminescent reaction in mammalian cells since supplementation with exogenous flavin oxidoreductase resulted in a greater increase (151-fold vs. 58-fold) in *in vitro* bioluminescent production from cell extracts than that of aldehyde supplementation. This result was consistent with a previous finding that addition of the *frp* gene improved autonomous bioluminescence up to 5.5-fold in *S. cerevisiae* expressing the *luxCDABE* genes (Gupta, Patterson et al. 2003). Therefore, it was believed that including the *frp* gene was an effective strategy and contributed significantly to the overall bioluminescent output.

**Common bioluminescent reporter proteins for mammalian applications**

Despite being widely used as a prokaryotic bioreporter, the *lux* system has yet to be widely employed for use in the mammalian cellular background. Currently, the most commonly used bioluminescent reporter proteins in mammalian cells are firefly luciferase and Renilla luciferase. These bioluminescent proteins are gaining preference over their fluorescent counterparts because the lack of endogenous bioluminescent reactions in mammalian tissue allows for near background-free imaging conditions whereas the prevalence of fluorescently active compounds in these tissues can interfere with target resolution upon exposure to the fluorescent excitation wavelengths required for the generation of
Firefly luciferase (FLuc) is the best studied of a large number of luminescent proteins to be discovered in insects. The genes utilized in most studies are those from the common North American firefly, *Photinus pyralis* (Fraga 2008). The FLuc protein catalyzes the oxidation of reduced luciferin in the presence of ATP-Mg\(^{2+}\) and oxygen to generate CO\(_2\), AMP, PPI, oxyluciferin, and yellow-green light at a wavelength of 562 nm. This reaction was originally reported to occur with a quantum yield of almost 90% (Conti, Franks et al. 1996), however, advances in detection technology have revealed that it is likely actually closer to 40% (Ando, Niwa et al. 2008). Nonetheless, the sufficiently high quantum yield of this reaction is well suited to use as a reporter with as few as 10\(^{-19}\) mol of luciferase (2.4 × 10\(^{5}\) molecules) able to produce a light signal capable of being detected (Gould and Subramani 1988).

Renilla luciferase (RLuc) undergoes a similar method of action to produce bioluminescence. The gene encoding for this protein was originally isolated from the soft coral *Renilla reniformis* and displays blue-green light at a wavelength of 480 nm, however, additional red-shifted variants have been created as well that luminesce at higher wavelengths to promote increased tissue penetration of the luminescent signal. Regardless of the emission wavelength, the RLuc proteins all catalyze the oxidative decarboxylation of its substrate coelenterazine in the presence of dissolved oxygen and perform this reaction at a quantum yield of 7%
(Lorenz, McCann et al. 1991). Because of its dissimilar bioluminescent signal and substrate, RLuc is often used simultaneously with FLuc for multiple reporter studies.

The advantages and disadvantages of FLuc, RLuc, and lux are compared in Table 3. It is clear that improved bioluminescent sensing technology that offers substrate-free and non-invasive monitoring will be beneficial to a variety of research fields. The historical review of lux expression in surrogate hosts presented in this chapter demonstrates that developing lux-based human cell bioreporters will fulfill this niche.
Table 3. Comparison of the lux system with other commonly used bioluminescent proteins.

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Firefly and click beetle luciferase | • High sensitivity and low signal-to-noise ratio  
• Quantitative correlation between signal strength and cell numbers  
• Low background in animal tissues  
• Variations of firefly luciferase (stabilized and red-shifted) and click beetle luciferases (red and green) are available  
• Different colors allow multi-component monitoring | • Requires exogenous luciferin addition  
• Fast consumption of luciferin can lead to unstable signal  
• ATP and oxygen dependent  
• Currently not practical for large animal models |
| • D-luciferin substrate         |                                                                             |                                                                                |
| Renilla and Gaussia luciferase  | • High sensitivity  
• Quantitative correlation between signal strength and cell numbers  
• Stabilized and red-shifted Renilla luciferase are available  
• Secretion of Gaussia luciferase allows for subject-independent bioluminescence measurement | • Requires exogenous coelenterazine addition  
• Low anatomic resolution  
• Increased background due to oxidation of coelenterazine by serum  
• Oxygen dependent  
• Fast consumption of coelenterazine can lead to unstable signal  
• Currently not practical for large animal models |
| • Coelenterazine substrate      |                                                                             |                                                                                |
| Bacterial luciferase            | • High sensitivity and low signal-to-noise ratio  
• Quantitative correlation between signal strength and cell numbers  
• Fully autonomous system, no requirement for addition of exogenous substrate  
• Noninvasive  
• Stable signal  
• Rapid detection permitting real-time monitoring | • Bioluminescence at 490 nm prone to absorption in animal tissues  
• Low anatomic resolution  
• NADPH and oxygen dependent  
• Not as bright as other luciferases  
• Currently not practical for large animal models |

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CHAPTER II

Development of a Single Polycistronic Vector System for Single Promoter Driven Expression of the lux Cassette in Human Cell Lines
Introduction

Setting the bacterial bioluminescence (lux) system apart from other luminescent or fluorescent reporter proteins is its capability to produce light autonomously without the need for substrate addition or external optical excitation. Although the lux reporter system has been used extensively in prokaryotic whole-cell biosensors for environmental assessment (Eltzov and Marks 2011; Ripp, Layton et al. 2011; Shin 2011; Singh, Abhilash et al. 2011; Xu, Close et al. 2012) and for non-invasive visualization of pathogen infection in living animals (Francis, Joh et al. 2000; Francis, Yu et al. 2001; Cronin, Sleator et al. 2008), development of an autonomous mammalian bioluminescent system has long been desired. Unlike the single gene-based firefly luciferase (FLuc) and fluorescent protein (GFP and its color variants) systems which are two of the most widely employed reporter systems in mammalian applications, the lux system requires concurrent expression of six genes (luxCDABEfrp) for substrate-free bioluminescence production in human cell lines. The number of genes that need to be expressed simultaneously have been the major hurdle for expanding the lux system into mammalian cell-based applications.

The simultaneous expression of multiple exogenous genes in eukaryotic cells is complicated relative to their prokaryotic counterparts due to fundamental differences in the genetic architecture and gene expression mechanisms between the two domains. Unlike polycistronic expression of multiple genes often
found in bacteria, gene expression in the eukaryotic cellular background normally requires that each gene be regulated by its own promoter (Pestova, Kolupaeva et al. 2001). Several strategies have been developed to overcome this limitation. On the most basic level, it is possible to express each gene independently from its own promoter, either through the introduction of multiple vectors, or introduction of a single vector containing multiple promoters. However, each gene is transcribed separately, which may lead to imbalanced gene expression (Emerman and Temin 1986). Alternatively, two genes can be translationally fused in one open reading frame (ORF) and transcribed from a single promoter. While it ensures coexpression, decreased protein activities have been observed (Thomas and Maule 2000). Nevertheless, none of these techniques is suitable for expressing more than two proteins.

One method to transcriptionally fuse two or more genes and still allow for separate translation of each gene is to use internal ribosome entry site (IRES) elements. First identified in poliovirus and encephalomyocarditis virus RNA (Jang, Krausslich et al. 1988; Pelletier and Sonenberg 1988), IRES elements are RNA sequences or DNA sequences that upon transcription into mRNA, form a secondary structure that attracts and binds a ribosome to internally initiate translation of the following cistron in a cap-independent manner (Baird, Turcotte et al. 2006). Unlike in fusion proteins, expression of each IRES-linked gene is an independent translation event, thus not interfering with post-translational
interactions between the proteins of interest. For instance, while expressing the bacterial luciferase (LuxAB heterodimer) as fusion proteins was not successful, the use of an IRES-mediated bicistronic vector has been demonstrated to be an effective strategy for generating functional luciferase in human cell lines (Patterson, Dionisi et al. 2005). Polycistronic expression of up to three (Li and Zhang 2004) and four (Bouabe, Fassler et al. 2008) genes from a single promoter have also been made possible with tandem IRES elements. However, the IRES elements have been criticized for their relatively large size (in the range of 500 bp) and lower expression of the downstream genes than the upstream genes (Mizuguchi, Xu et al. 2000).

An alternative method for polycistronically expressing multiple genes in mammalian cells is to use viral 2A elements. Originally identified in foot-and-mouth disease virus (FMDV), 2A elements are in-frame linker regions that separate two genes driven off a single promoter. A highly conserved D(V/I)EXNPGP motif at the C-terminus of these peptides is essential for the function of 2A peptides, as the last G-P bond is “cleaved” during translation. Although it was originally hypothesized that the cleavage was mediated by a proteinase either encoded in the viral genome or in the mammalian host cells, it was demonstrated later that ribosome skipping, rather than proteinase cleaving, is the mechanism for how 2A peptides function (Donnelly, Luke et al. 2001). During translation of the 2A peptides, the ribosome skips the synthesis of the last
Gly-Pro peptide bond at the C-terminus. However, translation of the downstream gene is continued regardless of this missing bond. Two advantages of the 2A elements compared to the IRES elements are their short length (averaging 18 – 22 amino acids) and high efficient stoichiometric expression of flanking genes (de Felipe 2002). To date, 2A elements have been identified in Picornaviruses, Type C rotaviruses and insect viruses, with those from FMDV (F2A), equine rhinitis A virus (E2A), porcine teschovirus-1 (P2A), and *Thosea asigna* virus (T2A) being mostly characterized (Szymczak and Vignali 2005). The ability of the 2A elements to express up to four genes from a single transcript in mammalian cells has been previously demonstrated by many groups (Szymczak, Workman et al. 2004; Carey, Markoulaki et al. 2009).

Autonomous bioluminescence production in human cell lines requires concurrent expression of the luciferase (*luxAB*), the enzymes for synthesis of the aldehyde substrate (*luxCDE*), and the flavin oxidoreductase (*frp*) for recycling the FMNH$_2$ co-factor. The number of genes that need to be delivered and expressed at a functional level represents a challenging hurdle. A two plasmid expression system has been recently developed for expressing the *lux* cassette in human embryonic kidney (HEK293) cells (Close, Patterson et al. 2010). In this system, the *luxAB* genes are expressed from an IRES-mediated bicistronic vector, whereas the *luxCDEfrp* genes are assembled into a second multi-bicistronic vector. Specifically, a total number of three promoters are employed to regulate
gene expression. One optimization to the two plasmid system would be the delivery of all six genes within the context of a single polycistronic vector. It was therefore hypothesized that, through the use of viral 2A peptides as linker regions, it will be possible to simultaneously express up to six genes (the \textit{luxCDABEfrp} gene cassette) under the regulation of a single promoter for autonomous bioluminescent production in human cell lines. This method of expression would as well reduce the number of required transfection steps required for production of bioluminescent cell lines from two to one. This would therefore reduce both the time and effort required to develop additional bioluminescent cell lines, and provide a more simplistic means for regulating bioluminescent production. It is demonstrated here that, through the use of viral-derived 2A elements as linkers, the \textit{luxCDABEfrp} gene cassette can be functionally expressed to produce bioluminescence autonomously from a single promoter in human cell lines. The polycistronic single vector system has also been shown to improve bioluminescence yield above the previously validated two plasmid expression system.

**Materials and Methods**

Human cell lines and plasmid constructs used in this study are summarized in Table 4.
Table 4. Cell lines and plasmid constructs used in this study.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type (Medium)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cells (Dulbecco’s Modified Eagle Medium (DMEM))</td>
<td>ATCC</td>
</tr>
<tr>
<td>HEK293 Tet-On</td>
<td>HEK293 cells stably expressing the tetracycline-regulated transactivator</td>
<td>Clontech</td>
</tr>
<tr>
<td>HEK293/pLuxA:pLuxCDEfrp</td>
<td>Bioluminescent HEK293 cells constructed using the two plasmid expression system</td>
<td>Close et al. (2010)</td>
</tr>
<tr>
<td>HEK293/promo LuxAB</td>
<td>HEK293 cells stably expressing pIRES LuxAB</td>
<td>(Patterson, Dionisi et al. 2005)</td>
</tr>
<tr>
<td>HEK293/promo LuxAB/hygro</td>
<td>HEK293 cells stably expressing pIRES LuxAB/hygro</td>
<td>This study</td>
</tr>
<tr>
<td>HEK293 ECO</td>
<td>Bioluminescent HEK293 cells constructed by re-transfecting HEK293/promo LuxAB/hygro with pCMV Lux</td>
<td>This study</td>
</tr>
<tr>
<td>T-47D</td>
<td>Human breast carcinoma cells (RPMI-1640)</td>
<td>ATCC</td>
</tr>
<tr>
<td>T-47D/Lux</td>
<td>Bioluminescent T-47D cells stably expressing pCMV Lux</td>
<td>This study</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast carcinoma cells (DMEM)</td>
<td>ATCC</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Human colorectal carcinoma cells (McCoy’s 5A)</td>
<td>ATCC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1/hygro(+)</td>
<td>Mammalian expression vector containing a CMV IE promoter and a hygromycin resistance gene</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PCR4-TOPO</td>
<td>TOPO TA cloning vector for cloning of PCR products generated with 3’ A overhangs</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pTRE-Tight-BI</td>
<td>Bi-directional mammalian expression vector containing two divergent CMV IE minimal promoters separated by a tetracycline response element (TRE)</td>
<td>Clontech</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>pd2EGFP-1</td>
<td>Promoter-less vector containing a d2EGFP reporter gene</td>
<td>Clontech</td>
</tr>
<tr>
<td>pIRES Lux&lt;sub&gt;AB&lt;/sub&gt;</td>
<td>IRES-mediated bicistronic vector for expression of the bacterial luciferase (&lt;i&gt;luxAB&lt;/i&gt;) from a single cytomegalovirus immediate early (CMV IE) promoter</td>
<td>(Patterson, Dionisi et al. 2005)</td>
</tr>
<tr>
<td>pEF Lux</td>
<td>Polycistronic vector containing 2A-linked &lt;i&gt;luxCDABEfrp&lt;/i&gt; expressed from a human elongation factor 1-α (EF1-α) promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pCMV Lux</td>
<td>Polycistronic vector containing 2A-linked &lt;i&gt;luxCDABEfrp&lt;/i&gt; expressed from a CMV IE promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pLux-1</td>
<td>Promoter-less 2A-linked polycistronic &lt;i&gt;luxCDABEfrp&lt;/i&gt; vector for downstream cloning</td>
<td>This study</td>
</tr>
<tr>
<td>p2A Lux&lt;sub&gt;AB&lt;/sub&gt;</td>
<td>2A-mediated bicistronic vector for expression of &lt;i&gt;luxAB&lt;/i&gt; from a single CMV IE promoter</td>
<td>This study</td>
</tr>
<tr>
<td>p2A LuxCDEfrp</td>
<td>2A-mediated polycistronic vector for expression of &lt;i&gt;luxCDEfrp&lt;/i&gt; from a single EF1-α promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pEF Lux&lt;sub&gt;CDA-IRES-LuxBEfrp&lt;/sub&gt;</td>
<td>Polycistronic vector containing 2A-linked &lt;i&gt;luxCDE&lt;/i&gt; and 2A-linked &lt;i&gt;luxBEfrp&lt;/i&gt; sequences separated with an IRES element</td>
<td>This study</td>
</tr>
<tr>
<td>pCR4-luxAires</td>
<td>pCR4 TOPO vector hosting the &lt;i&gt;luxA-IRES&lt;/i&gt; sequence cloned from pIRES Lux&lt;sub&gt;AB&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCR4-MluI/CMV/Sall</td>
<td>pCR4 TOPO vector hosting the CMV IE promoter sequence</td>
<td>This study</td>
</tr>
<tr>
<td>pCR4-AIB</td>
<td>pCR4 TOPO vector hosting the &lt;i&gt;luxA-IRES-luxB&lt;/i&gt; sequence for construction of pIRES Lux&lt;sub&gt;AB&lt;/sub&gt;/hygro</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>pIRES LuxAB/hygro</td>
<td>pCDNA3.1/hygro(+) harboring the <em>luxA</em>-IRES-<em>luxB</em> sequence</td>
<td>This study</td>
</tr>
<tr>
<td>pCR4LuxAB</td>
<td>pCR4 TOPO vector hosting the <em>luxA</em>-IRES-<em>luxB</em> sequence with an introduced 5' AgeI site for construction of pERE3-TATA Lux&lt;sub&gt;AB&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pERE3-TATA Lux&lt;sub&gt;AB&lt;/sub&gt;</td>
<td>IRIES-mediated bicistronic expression of <em>luxAB</em> from an estrogen responsive promoter consisting of three tandems of estrogen response element (ERE) and an adenovirus E&lt;sub&gt;1&lt;/sub&gt;B minimal TATA promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pERE3-TATA Lux</td>
<td>Polycistronic vector containing 2A-linked <em>luxCDABEfrp</em> expressed from three tandems of ERE and a TATA promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pERE3-CMVm Lux</td>
<td>Polycistronic vector containing 2A-linked <em>luxCDABEfrp</em> expressed from three tandems of ERE and a CMV IE minimal promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pTet Lux</td>
<td>Single polycistronic vector containing 2A-linked <em>luxCDABEfrp</em> expressed from a tetracycline response element and its associated CMV IE minimal promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>
Design and construction of 2A-linked expression vectors

Divergent viral-derived 2A elements were used to link adjacent lux genes in the polycistronic single vector. Several significant changes had to be made to the lux genes prior to their incorporation into the new single vector expression system. To reduce the likelihood of homologous recombination events, each of the viral 2A elements was sourced from a different virus to avoid the incorporation of repetitive DNA sequences (Table 5). These 2A elements were chosen because they are well-characterized and widely used by various researchers. Furthermore, having been previously demonstrated by Szymczak and colleagues (Szymczak, Workman et al. 2004) that incorporation of flexible linker regions upstream of the 2A elements leads to increased separation efficiency, each 2A element was proceeded by a Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly sequence. Finally, because the 2A linker regions permit continuous translation of the mRNA to protein, the stop codons of all lux genes except for the gene most distal from the promoter were removed. These modifications allowed for the synthetic assembly of a single DNA construct consisting of luxC-flexible linker-2A element-luxD-flexible linker-2A element-luxA-flexible linker-2A element-luxB-flexible linker-2A element-luxE-flexible linker-2A element-frp. The lux genes were organized in such a way to replicate their orders within the lux operon as found in their native bacterial host.
Table 5. Viral 2A elements used for polycistronic expression of the *lux* genes.

<table>
<thead>
<tr>
<th>2A element</th>
<th>Virus source</th>
<th>DNA sequence (5' → 3')</th>
<th>Upstream gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2A</td>
<td>Foot and mouth disease virus</td>
<td>CAGCTGTTGAATTTTGACCTTCTCAAGTTGGCGGGAGACGTGGAGTCCAACCCAGGGCCC</td>
<td>luxC</td>
</tr>
<tr>
<td>E2A</td>
<td>Equine rhinitis A virus</td>
<td>CAGTGTACTAACTACGCTTTGTGAAAACTCGCTGGCGATGTTGAAAGTAAACCGCTCTCCT</td>
<td>luxD</td>
</tr>
<tr>
<td>Ta2A</td>
<td>Synthetic version of wild type T2A</td>
<td>GAAGGTAGGGTTCATTATTTGACCTGTGGAATGTGCAAGAAGGTTACCCAGGACCC</td>
<td>luxA</td>
</tr>
<tr>
<td>P2A</td>
<td>Porcine teschovirus</td>
<td>GCGACAAACTTTAGCTTGCTGAAGCAAGCTGGTGACGTTGAGGAGAATGCCGGACCA</td>
<td>luxB</td>
</tr>
<tr>
<td>T2A</td>
<td><em>Thosea asigna</em> virus</td>
<td>GAGGGCAGAGGAAGTCTTTTACATGCCTTACGAGCTTGGGAGGAGAATCCCGGCCCT</td>
<td>luxE</td>
</tr>
</tbody>
</table>
This sequence was assembled *de novo* by GeneArt and cloned into the human elongation factor 1-α (EF1-α) promoter driven pEF/myc/nuc vector (Invitrogen) using compatible Ncol and XbaI restriction sites to create the vector pEF Lux (Figure 2). It is important to note that, during the synthesis process, the removal of intervening restriction and regulatory sequences was performed according to standard GeneArt synthesis protocols, and the codon optimization process was re-performed on each of the *lux* genes to reflect updated codon usage patterns and improved codon optimization software that has been developed since the previous optimization took place in 2004. This resulted in some changes in the DNA sequence of *luxAB* and minor changes in *luxC*, *luxD*, *luxE*, and *frp* gene sequences, but did not in any way alter the resultant protein products (Table 6).

2A-linked vectors p2A Lux<sub>AB</sub> and p2A Lux<sub>CDEfrp</sub> were also constructed for comparison purposes. To construct vector p2A Lux<sub>AB</sub>, the *luxA*-Ta2A-*luxB* sequence was synthesized *de novo* commercially (GeneArt) and cloned into the original pIRES Lux<sub>AB</sub> vector to replace the *luxA*-IRES-*luxB* sequence. The substrate processing vector p2A Lux<sub>CDEfrp</sub> was generated through a series of modifications on pEF Lux. First, the E2A-*luxA*-Ta2A-*luxB* sequence was removed by blunt end restriction enzymes Pmel and EcoRV, followed by a ligation reaction to join both the open ends of the remaining sequence to form a circular vector. This intermediate vector, however, was frame shifted for *luxE* and *frp* genes as a
Figure 2. A schematic representation of the single promoter polycistronic vector for *lux* expression in human cell lines.

Viral 2A elements are used to link adjacent *lux* genes for polycistronic translation. The stop codons of all *lux* genes except for the gene most distal from the promoter (*frp*) are removed to allow for continuous translation. The *lux* genes are organized to replicate their orders within the *lux* operon as found in their native bacterial host.
Table 6. Comparison of previous and updated versions of codon optimized gene sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>DNA identity to previous optimization (Close, Patterson et al. 2010)</th>
</tr>
</thead>
<tbody>
<tr>
<td>luxA</td>
<td>85%</td>
</tr>
<tr>
<td>luxB</td>
<td>85%</td>
</tr>
<tr>
<td>luxC</td>
<td>95%</td>
</tr>
<tr>
<td>luxD</td>
<td>95%</td>
</tr>
<tr>
<td>luxE</td>
<td>93%</td>
</tr>
<tr>
<td>frp</td>
<td>99%</td>
</tr>
</tbody>
</table>
result of removing the \textit{luxAB} sequence. Site-directed mutagenesis was subsequently performed to delete 2 bp between \textit{luxD} and \textit{luxE} using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) to allow for in-frame translation of the LuxE and Frp protein. The final vector was designated p2A Lux\textsubscript{CDEFrp}.

To compare 2A- and IRES- mediated expression, vector pEF Lux was also modified to replace the Ta2A linker between \textit{luxA} and \textit{luxB} with an IRES element. Because the IRES element attracts a separate ribosome to initial translation of the downstream ORF, it requires that the upstream ORF contains a stop codon for proper translation termination. Therefore, instead of simply replacing the Ta2A sequence with an IRES sequence, the \textit{luxA} (with stop codon)-IRES sequence was first PCR amplified using primers 5'-ATGAAGTTCGGCAACTTCCTG-3' and 5'-GTGGCAAGCTTATCATCGTG-3' and cloned into pCR4-TOPO vector (Invitrogen) to create pCR4-luxAIRES. The \textit{luxA} (without stop codon)-Ta2A sequence was then removed from pEF Lux using blunt end restriction enzymes SwaI and Eco47III and replaced by the \textit{luxA} (with stop codon)-IRES sequence cut from pCR4-luxAIRES using blunt end restriction enzymes Pmel and SnaB1, generating vector pEF Lux\textsubscript{CDA-IRES-LuxBEfrp}.

To facilitate future modification of the single \textit{lux} vector, the EF1-\alpha promoter was removed by restriction digest with Acc65I and Ncol and replaced by a 92 bp sequence containing multiple restriction sites, creating a promoter-less vector
pLux-1. To construct human cytomegalovirus immediate early (CMV IE) promoter regulated lux vectors, the promoter sequence was PCR amplified using pIRES Lux<sub>AB</sub> as template with added 5'-MluI and 3'-SalI site and cloned into pCR4 to generate pCR4-MluI/CMV/SalI. The promoter sequence was then removed from pCR4-MluI/CMV/SalI using MluI and SalI and cloned into similarly digested pLux-1 to generate pCMV Lux.

**Human cell culture and transfection**

Human cell lines used in this study are listed in Table 4. All cell lines were originally obtained from American Type Culture Collection (ATCC) and maintained in recommended growth medium supplemented with 10% fetal bovine serum (FBS) (Hyclone), 0.01 mM non-essential amino acids (Invitrogen), 1X antibiotic-antimyotic (Invitrogen) and 0.01 mM sodium pyruvate (Invitrogen). Cells were cultured at 37°C in a 5% CO<sub>2</sub> environment with medium being refreshed every 2 - 3 days. Cells were passaged regularly upon reaching 80% confluence.

*E. coli* TOP10 cells (Invitrogen) were used to host and propagate plasmids used in this study. *E. coli* cells were routinely grown in Luria Bertani (LB) broth at 37°C with 200 rpm continuous shaking. Ampicillin at a final concentration of 100 µg/ml was used for selection and maintenance of successfully transformed cells.

Transfections were carried out in 6-well tissue culture plates (Corning). The day prior to transfection, cells were plated at a density of 5 × 10<sup>5</sup> cells/well (for
HEK293 and HCT-116 cells) or $7.5 \times 10^5$ cells/well (for T-47D and MCF-7 cells) in each well. Vector DNA purified from overnight *E. coli* culture using PureYield Plasmid Purification System (Promega) was introduced into the cells using Lipofectamine 2000 transfection reagent (Invitrogen) following manufacturer’s instructions.

**Bioluminescent detection in transiently transfected cells**

To validate the effectiveness of the 2A-linked polycistronic lux vectors (pEF Lux and pCMV Lux), bioluminescence production was evaluated in transiently transfected HEK293, T-47D, MCF-7 and HCT-116 human cell lines. Twenty-four hours post transfection, cells were washed with sterile phosphate buffered saline (PBS) and harvested in growth medium. Approximately $1 \times 10^6$ transfected cells or untransfected cells (as negative controls) were plated in 1 ml volume in triplicate wells in a black 24-well plate for imaging. Bioluminescent production was measured in the IVIS Lumina imaging system (Perkin-Elmer) using a 10 min integration time every 30 min for at least 20 hours. Photon counts were analyzed with Living Image 3.2 software (Perkin-Elmer) and reported as average radiance in photons(p)/second(s)/cm$^2$/steridian(sr).

Polycistronic lux expression using a single vector was also compared to a similarly constructed two plasmid expression system. Equal numbers of cells were transfected with pEF Lux or co-transfected with 2A-linked vectors p2A Lux$_{AB}$ and p2A Lux$_{CDEfrp}$. Twenty-four hours post transfection, cells were washed
and PBS, harvested in growth medium and tested for bioluminescent production as described above.

**Results**

*Expression of the 2A-linked polycistronic single lux vector in human cell lines*

To test if the bacterial bioluminescence gene cassette (*luxCDABEfrp*) can be delivered using a single vector and expressed functionally under the regulation of a single promoter in human cell lines, a 2A-linked polycistronic expression vector was designed and synthesized. The effectiveness of the 2A-linked vector pEF Lux for the expression of bacterial bioluminescence was tested in HEK293, T-47D, MCF-7, and HCT-116 cells. Cells transfected with the single *lux* vector emitted bioluminescence at a detectable level (Student’s *t* test *p* < 0.05) compared to untransfected control in all tested cell types (Figure 3A). However, the expression efficiency was not consistent across different cell types. In 1 × 10⁶ transfected HEK293 or T-47D cells, maximal bioluminescence was observed at 2.13 (± 0.10) × 10³ p/s/cm²/sr and 2.37 (± 0.36) × 10³ p/s/cm²/sr, respectively. On the other hand, expression of the same vector in the other two cell lines was significantly lower than that in HEK293 and T-47D cells, with maximal flux of 4.08 (± 0.22) × 10² p/s/cm²/sr and 3.96 (± 0.16) × 10² p/s/cm²/sr being produced in equal numbers of transfected MCF-7 and HCT-116, respectively.
Figure 3. Polycistronic expression of the lux cassette in a single vector in human cell lines.

(A) Maximal bioluminescence yield from equal numbers (1 × 10^6) of shown cell lines transfected with the polycistronic vector pEF Lux. (B) 24-hour bioluminescent profiles of the transfected cells displayed as fold change over untransfected control (n=3, mean ± standard error).
The substrate-free nature of the lux system permitted near real-time, continuous monitoring of bioluminescent production over time on the same cell population without sample destruction or substrate addition. As shown in Figure 3B, different cell types displayed varying light production dynamics. In transfected HEK293 cells, a significant level of bioluminescence ($p < 0.05$) was consistently observed for the first 20 hours with intermittently detectable light production between 20 and 24 hours. Peak bioluminescence at 6.1 ($\pm$ 0.3)-fold over control was detected ~2.5 hours post plating, followed by a rapid decrease to near-background level. Transfected T-47D cells, though displaying similar maximal light production to HEK293 cells (Figure 3A), produced peak bioluminescence approximately 8 hours post plating. Unlike the rapid decrease in HEK293 cells, bioluminescent flux remained relatively stable for the remaining imaging period, with a slight reduction to 67% of maximal flux at 24 hours post plating. Despite the significantly lower overall signal intensity, the transfected MCF-7 and HCT-116 cells displayed a comparable bioluminescent profile over the imaging period to that of the HEK293 and T-47D cells, respectively.

**Determination of an optimal promoter for polycistronic lux expression in multiple human cell lines**

In order to choose a promoter for optimal bioluminescent production across different cell types, and to test the sub-hypothesis that insufficient transcription of the lux genes to mRNA is responsible for the low bioluminescent flux observed in
MCF-7 and HCT-116 cells (Figure 3A), the EF1-α promoter was substituted with a CMV IE promoter. The CMV IE promoter-driven expression of the 2A linked lux genes yielded significantly higher bioluminescent output in all four tested cell types, especially in MCF-7 and HCT-116 cells, displaying maximal bioluminescence of 13.0 (± 0.6)-fold and 28.5 (± 5.1)-fold over untransfected background, respectively (Figure 4A). In each cell line, the bioluminescent profile driven by the CMV IE promoter was comparable to that expressed from the EF1-α promoter (Figure 4B), suggesting that the 2A peptide-mediated translation activity was not promoter-dependent.

**Effect on bioluminescent production of replacing the 2A element separating the luxA and luxB sequences with an IRES element**

To test the hypothesis that 2A peptides are more efficient for polycistronic expression of multiple genes than are IRES elements, the Ta2A sequence linking the luxA and luxB genes in pEF Lux was replaced by a previously validated and commonly used encephalomyocarditis virus IRES element to create pEF LuxCDA-IRES-LuxBEfrp. To allow for proper termination of peptide synthesis of the genes upstream of the IRES element, the luxA sequence was also modified to include a stop codon at the C-terminus without altering the amino acid sequence. Bioluminescent production was compared in HEK293 cells transfected with either pEF Lux or pEF LuxCDA-IRES-LuxBEfrp; two otherwise identical vectors that linked the luxCDE and luxBEfrp sequences with either a 2A or an IRES element.
Figure 4. Bioluminescent profiles from different promoter driven lux expression in a variety of human cell lines.

(A) Comparison of maximal bioluminescence yield from equal numbers ($1 \times 10^6$) of shown cell lines transfected with pEF Lux or pCMV Lux. (B) 20-hour bioluminescent profiles of the transfected cells displayed as fold change over untransfected control (n=3, mean ± standard error).
Transfection of the IRES-containing polycistronic lux vector yielded maximal bioluminescence of $7.44 (± 0.69) \times 10^2$ p/s/cm$^2$/sr, which was only approximately 35% of that produced from the 2A-only pEF Lux expression vector (Figure 5).

**Comparison of single and dual vector expression of the lux cassette**

Having demonstrated the effectiveness of the single vector expression system for autonomous bioluminescence, it was necessary to compare the efficiency of this new expression format to that of the previously demonstrated dual vector system. To avoid biased translational efficiencies between 2A and IRES elements as well as varying efficiencies between transient and stable expression, the previously characterized stable bioluminescent HEK293/pLux$_{AB}$:pLux$_{CDEfrp}$ cells (Close, Patterson et al. 2010) were not included for comparison. Alternatively, 2A-linked vectors p2A Lux$_{AB}$ and p2A Lux$_{CDEfrp}$ were constructed and co-transfected for transient expression analysis. As shown in Figure 6, the single vector expression format was capable of producing significantly higher bioluminescent output than the dual vector system in HEK293. Maximal bioluminescent flux (photons/second) emitted from the two plasmid system was approximately 50-60% of that from equal number of cells transfected with the single vector over the same surface area. It is important to note that the lux$_{AB}$ genes in p2A Lux$_{AB}$ were driven by a CMV IE promoter, whereas the EF1-$\alpha$ promoter was used in the single vector and the substrate processing vector p2A Lux$_{CDEfrp}$. Having demonstrated above that the CMV IE promoter had a
Figure 5. Replacing the 2A element linking luxA and luxB with an IRES element significantly reduced bioluminescent production in HEK293 cells.

Comparison of maximal bioluminescence yield from HEK293 cells transfected with pEF Lux or pEF LuxCDA-IRES-LuxBEfrp (n=3, mean ± standard error).
Figure 6. Comparison of single vector (pEF Lux) and dual vector (p2A Lux\textsubscript{AB} and p2A Lux\textsubscript{CDEfrp}) expression of the \textit{lux} cassette in HEK293 cells.

Comparison of maximal bioluminescence yield from equal numbers ($1 \times 10^6$) of HEK293 transfected with pEF Lux or co-transfected with p2A Lux\textsubscript{AB} and p2A Lux\textsubscript{CDEfrp} (n=3, mean ± standard error).
higher transcription activity than the EF1-α promoter in both cell types, it was speculated that bioluminescence expression from the dual vector format would be even lower had the luxAB genes been regulated by the EF1-α promoter.

Discussion

Since the first case of expressing bacterial bioluminescence genes exogenously in a foreign host (Engebrecht, Nealson et al. 1983), the last three decades have witnessed a growing interest in developing the lux cassette into an autonomous reporter system in both prokaryotic and eukaryotic cells. While the lux system has become one of the most extensively used reporter systems in prokaryotic whole-cell bioreporters (Xu, Close et al. 2012), its expression in mammalian cells has only been recently demonstrated (Close, Patterson et al. 2010) and is still emerging. The major obstacles for the lux system to be adapted as an autonomous mammalian reporter system include 1) low translation efficiency of the wild type lux genes by the mammalian protein synthesis machinery, 2) limited FMNH$_2$ availability in mammalian cells, and 3) the number of genes that need to be expressed for autonomous bioluminescent production. While the first two hurdles have been overcome by optimizing the gene sequences to match the host codon usage pattern (Patterson, Dionisi et al. 2005) and co-expressing an additional frp gene for improved FMNH$_2$ recycling (Gupta, Patterson et al. 2003), the requirement of simultaneous expression of six genes remains challenging. The first demonstration of autonomous bioluminescent
expression in a human cell line employed a two plasmid expression strategy, where the \textit{luxAB} genes are expressed bicistronically through an IRES element in one vector and the \textit{luxCDEfrp} genes are assembled in a second vector by separating the four genes into two transcription units, each containing its own promoter and two ORFs separated by an IRES element. This strategy has been demonstrated to express all six genes at a functional level to provide sufficient luciferase, aldehyde and FMNH$_2$ for autonomous light production (Close, Patterson et al. 2010). However, development of stable bioluminescent mammalian cell lines can be complicated by the necessary transfection steps to co-transfect two plasmids. It is evident that assembling all six genes in a single vector would simplify this process.

Two widely used elements for polycistronic expression of multiple proteins in mammalian cells are the IRES elements, employed by the previously characterized bioluminescent human cell line (Close, Patterson et al. 2010), and the viral 2A peptides. The 2A peptides are superior to the IRES elements for polycistronic expression of the \textit{luxCDABEfrp} gene cassette for two reasons. First, the 2A sequences are shorter (averaging 18 - 22 amino acids or 54-66 bp in DNA) compared to the approximately 500 - 600 bp long IRES elements, allowing for easier assembly of large numbers of genes. Second, they have shown high cleavage efficiency and stoichiometric expression \textit{in vivo} (Szymczak, Workman et al. 2004) whereas the IRES elements are criticized for the lower translation
efficiency of the downstream gene than that of the upstream gene translated in a cap-dependent manner from the 5' end of the mRNA (Mizuguchi, Xu et al. 2000). Based on these justifications, it was decided to exploit the 2A elements for lux expression in a variety of human cell lines.

Although the 2A-mediated single vector polycistronic expression of four exogenous proteins has been previously reported (Graf, Bojak et al. 2000; Carey, Markoulaki et al. 2009), their capabilities to express up to six genes remains to be validated. Especially for bioluminescent expression in human cell lines, specific concerns such as if the Lux proteins can be produced at a level sufficient for detectable bioluminescent production still exists. The EF1-α promoter was initially chosen to drive the transcription of the lux genes since it had been demonstrated to be consistently strong across different cell types in a systematic comparison study (Qin, Zhang et al. 2010). In our study, the polycistronic expression of the 2A-linked lux genes driven by an EF1-α promoter was successfully demonstrated in HEK293 and T-47D cells but only achieved modest success in MCF-7 and HCT-116 cells (Figure 3A). Although a significantly differentiable signal could be detected from background (cells not expressing the lux genes), the low signal-to-noise ratio (~ 1.2:1 to 1.4:1) limits its application as a reliable reporter system in these cell lines. It was not clear at this point if the low signal intensity was a result of inefficient activity of the 2A peptides to produce independent Lux proteins in these cell types, or caused by the low
transcriptional activity of the EF1-α promoter or low transfection efficiency. Because the 2A peptides function independently upon host cellular factors (i.e., no proteinase is required for the cleavage), it is less likely that the 2A elements were selectively deficient in these cells. This hypothesis was approved by the demonstration of significantly higher bioluminescent output by expressing the lux genes from the CMV IE promoter (Figure 4A). Although having been demonstrated to display the most variable strength across cell types and lower activity compared to the EF1-α promoter in some cases (Qin, Zhang et al. 2010), the CMV IE promoter displayed a consistently higher activity than the EF1-α promoter for bioluminescent production in all four cell lines tested here. These discrepancies can be explained by the different cell lines and reporter genes employed between the two studies. The only common cells are the HEK293 cells, in which the CMV IE promoter exhibited a higher activity in both studies. Nevertheless, it is demonstrated here that the 2A peptides are capable of mediating polycistronic expression of up to six genes in various human cell lines.

The unique advantage of the lux system is to allow for non-invasive and continuous monitoring of the same target over time. Bioluminescent profiles produced in transfected cells were monitored over a 20-hour imaging period (Figure 4B). Both promoters used to drive gene expression are known for their constitutive activities, allowing one to relate light production dynamics to either change in cell numbers, or cellular health and metabolism. Since the doubling
times of selected human cell lines are greater than 20 hours, the rapid increase in bioluminescence in the first few hours observed in all four cell types are not likely caused by cell proliferation. It is speculated that switching from a carefully controlled 5% CO₂ incubator environment to the atmospheric condition in the imaging chamber causes a rapid increase in the medium pH value, which may affect the intracellular redox potential and NADPH availability for the bioluminescent reaction. The inability to control the CO₂ level, humidity and air temperature in the imaging system in combination with the continuing consumption of nutrients present in the medium, represent a unique situation where cellular metabolism and health is challenged by unspecified factors whose effects on living systems cannot be easily detected using the conventional reporter systems. The substrate-free nature of the lux system eliminates the requirements of cell destruction or substrate addition, thus permitting the cellular metabolic response to be visualized continuously in a cell-type dependent manner. Two of the cancer cell lines (T-47D and HCT-116) responded robustly to the uncontrolled and/or unfavorable environmental conditions in the imaging chamber and maintained relatively stable bioluminescence over the 20 h period (Figure 4B). In contrast, the bioluminescent production in HEK293 and MCF-7 cells gradually decreased to a near-background level after the peak close to the beginning of the imaging period, suggesting compromised cellular health and even cell death. While the bioluminescent output of cells expressing lux genes from the CMV IE promoter was of a greater magnitude than that of the EF1-α
promoter, their bioluminescent profiles relative to background were comparable under the same imaging conditions (Figure 4B), implying that the cellular metabolic response was not affected by the expression of the Lux proteins.

The advantage of using the 2A elements over the IRES elements for polycistronic lux expression was confirmed by the reduction in bioluminescent expression when the 2A sequence linking the luxA and luxB genes was replaced by an IRES element. The decrease in light production is in agreement with the criticism that translation of the genes downstream of the IRES element is lower than that of the upstream genes (Szymczak and Vignali 2005). Specifically, it has been demonstrated previously that FMNH$_2$ is a major limiting reagent in the bioluminescent reaction in eukaryotic cells (Gupta, Patterson et al. 2003; Close, Patterson et al. 2010). Since the frp gene encoding the oxidoreductase for FMNH$_2$ recycling was downstream of the IRES element and most distal to the promoter, it is speculated that reduced Frp protein synthesis was an important contributor to the decreased bioluminescent output.

Expressing the lux genes from a single promoter, along with the use of the 2A elements for highly efficient stoichiometric protein expression, leads to coupled transcription and translation of these genes. The more balanced gene expression provided by the single vector system was able to produce higher bioluminescent flux than the similarly constructed two promoter/two vector system. Additionally, cell-to-cell variability in promoter activity and gene expression can interfere with
the correlation between bioluminescent production and the number of cells present in a population (Bar-Even, Paulsson et al. 2006; Blake, Balazsi et al. 2006). This variability can be amplified if a phenotype is controlled by multiple promoters. Therefore, it is speculated that the single promoter expression system will allow a tighter correlation between bioluminescence and cell numbers compared to the two plasmid expression system. However, this still remains to be experimentally assessed.

In conclusion, it is demonstrated here for the first time that through the use of divergent viral 2A elements, it is possible to express the luxCDABEfp genes for autonomous bioluminescent production in a range of human cell lines. The single vector expression format improves bioluminescent production over the previously characterized IRES-based two plasmid system, and provides a more simplistic means for the lux cassette expression in other cell types for non-invasive continuous monitoring.
CHAPTER III

Development of an Improved Autonomous Bioluminescent Human Cell Line with Enhanced lux Expression
**Introduction**

Bioluminescent sensing is becoming increasingly applied across a variety of basic and applied biological research fields due to its low background, high throughput, and relative ease of operation in detecting cellular processes in cell culture and living animals (Baker 2010). By using genetically modified cells containing a reporter protein capable of producing an optical signal, investigators can visualize a diverse range of biological events, such as tumorigenesis and cancer treatment (Rehemtulla, Stegman et al. 2000; Venisnik, Olafsen et al. 2006), gene expression and regulation (Rettig, McAnuff et al. 2006; Korpal, Yan et al. 2009), cell trafficking (Sheikh, Lin et al. 2007; van der Bogt, Sheikh et al. 2008), viral and pathogenic infection (Andreu, Zelmer et al. 2011), protein stability and function (Laxman, Hall et al. 2002; Wang, Fu et al. 2010), and protein-protein interactions (Paulmurugan, Umezawa et al. 2002; Pichler, Prior et al. 2008) (reviewed in (Close, Xu et al. 2011)). To date, the main reporter proteins used for these applications have been the firefly luciferase (FLuc), the Renilla luciferase (RLuc), and the Gaussia luciferase (GLuc). These bioluminescent proteins, regardless of their native host organisms, function in a similar fashion. They catalyze the oxidation of a substrate (luciferin for FLuc and coelenterazine for RLuc and GLuc) in the presence of oxygen to produce light (Lorenz, McCann et al. 1991; Verhaegen and Christopoulous 2002; Fraga 2008). These substrates, however, cannot be synthesized in host cells, and therefore
must be added exogenously. Due to rapid substrate consumption (Bhaumik and Gambhir 2002; Inoue, Kiryu et al. 2009), these systems have relatively short temporal dynamics. Although substrate can be added repeatedly for long term monitoring, this also introduces unwanted injection errors, especially in whole animal imaging applications. Meanwhile, these substrates are relatively unstable and expensive, adding a financial burden to the utilization of these systems (Close, Hahn et al. 2011).

The newest addition to the mammalian-based bioluminescent sensing technology is the bacterial bioluminescence (lux) reporter system (Close, Patterson et al. 2010). This system is intrinsically different from other bioluminescent systems because of its ability to synthesize/recycle all required substrates endogenously within the host cells to produce light in a fully autonomous fashion. This is made possible because the lux system contains genes (luxCDEfrp) encoding proteins that are capable of providing the substrate required for light production. Specifically, the luxC, luxD, and luxE gene products are used for synthesizing and recycling a long chain fatty aldehyde substrate from endogenous compounds. For efficient light production in mammalian cells, a sixth gene, frp, encoding a flavin oxidoreductase is also included to facilitate efficient FMNH₂ recycling. Co-expression of these genes together with the luciferase genes (luxAB) can produce sufficient substrate for autonomous bioluminescent expression and therefore obviates the step of exogenous
substrate addition that is essential for the conventional Luc systems.

Since its first expression in mammalian cells, the *lux* system has proven useful for cell culture-based *in vitro* imaging and whole animal *in vivo* imaging (Close, Patterson et al. 2010; Close, Hahn et al. 2011). However, as the first generation of *lux*-expressing cells, they produce a relatively weak signal compared to the conventional FLuc systems. In a side-by-side comparison, it has been determined that \( \sim 1 \times 10^6 \) *lux*-expressing cells produces signal that is comparable to that produced by only approximately 100 FLuc-expressing cells (Close, Hahn et al. 2011). The requirement of a large population size to produce detectable signal significantly restricts adoption of the *lux* system across a wide range of bioluminescent imaging applications. It is hypothesized that the human cellular background is capable of supplying sufficient endogenous substrates for high level *lux*-based bioluminescent production and it will be possible to improve bioluminescent expression through increased gene integration. Here is reported the development of an improved autonomously bioluminescent HEK293 human cell line, as well as an optimization strategy that takes advantage of the previously described two expression system (Close, Patterson et al. 2010) and the recently developed single polycistronic expression system (Chapter II) for high level *lux* expression.
Materials and Methods

Strain growth and maintenance

Cell lines used in this study included wild type HEK293 cells, HEK293/pIRES Lux\textsubscript{AB} cells (Patterson, Dionisi et al. 2005; Close, Patterson et al. 2010), previously characterized bioluminescent HEK293/pLux\textsubscript{AB}:pLux\textsubscript{CDEfrp} cells (Close, Patterson et al. 2010), and HEK293/pIRES Lux\textsubscript{AB}/hygro (Table 4). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Hyclone), 0.01 mM non-essential amino acids (Invitrogen), 1X antibiotic-antimyotic (Invitrogen) and 0.01 mM sodium pyruvate (Invitrogen). HEK293/pIRES Lux\textsubscript{AB} and HEK293/pLux\textsubscript{AB}:pLux\textsubscript{CDEfrp} cells maintained in medium supplemented with Neomycin (Calbiochem) at a concentration of 500 \(\mu g/ml\). HEK293/pIRES Lux\textsubscript{AB}/hygro cells were maintained in medium supplemented with Hygromycin B (Invitrogen) at a concentration of 50 \(\mu g/ml\). Antibiotic concentrations were determined by susceptibility analysis.

Identification of constraints of the bioluminescent reaction in previously characterized stable bioluminescent cells

Vectors carrying either the luciferase genes (p2A Lux\textsubscript{AB}), the substrate processing genes (p2A Lux\textsubscript{CDEfrp}), or the full \textit{lux} cassette (pEF Lux) were transfected into HEK293/pLux\textsubscript{AB}:pLux\textsubscript{CDEfrp} cells to determine constraints of bioluminescent production. Transfections were carried out in six-well tissue
culture plates (Corning) using Lipofectamine 2000 (Invitrogen). Twenty-four hours post transfection, cells were washed with PBS and harvested in growth medium. Equal numbers of transfected cells or untransfected cells (control) in 1 ml volume were plated in triplicate in black 24-well plates for bioluminescent measurement in the IVIS Lumina imaging system (Perkin-Elmer). Light production was measured using a 10 min exposure time every 30 min for 16 h and reported as either total flux values in photons(p)/second(s) or average radiance values in p/s/cm²/steridian(sr).

**Re-transfection of non-bioluminescent luxAB-expressing cells**

To determine if an initial chromosomal integration of the luxAB genes was essential and sufficient for the production of enhanced bioluminescence following re-transfection of the single lux vector, wild type HEK293 cells (control) and HEK293 cells stably expressing pIRES LuxAB (HEK293/pIRES LuxAB) were transfected with pEF Lux or pCMV Lux. Bioluminescent measurements were carried out as described above.

**Development of stable HEK293 cells with enhanced bioluminescence**

To test if the enhanced bioluminescent phenotype observed in transient transfection assays could be stably maintained, selection of stable clones following successive transfections were attempted. However, direct re-transfection and selection was not possible due to the fact that the single lux vector shared the same Neomycin resistance with current HEK293/pIRES LuxAB
cells. To overcome this hurdle, a luxAB expression vector harboring an alternate antibiotic selection marker (Hygromycin) was generated and used to develop a stable cell line for re-transfection.

**Construction of pIRES LuxAB/hygro**

The DNA fragment containing the luxA-IRES-luxB sequence was amplified from pIRES LuxAB by PCR and cloned into a pCR4-TOPO cloning vector (Invitrogen) to create pCR4-AIB. The AIB fragment was then cut from pCR4-AIB with EcoRV and NotI and cloned into pcDNA3.1/hygro(+) (Invitrogen) digested with the same restriction enzymes. The final construct was designated pIRES LuxAB/hygro.

**Selection of cell lines with stable integration of pIRES LuxAB/hygro**

**Transfection**

HEK293 cells were transfected with pIRES LuxAB/hygro to obtain stable cell lines with stably integrated luxAB sequences and Hygromycin resistance. Cells were plated in six-well tissue culture plates the day before transfection and reached approximately 90% confluence at the time of transfection. Vector DNA purified from overnight *E. coli* culture was introduced using Lipofectamine 2000 transfection reagent (Invitrogen). Twenty-four hours post-transfection, transfected cells were harvested and diluted into new 6-well plates in complete growth medium without antibiotic. Starting the next day, selection of stably transfected clones was performed by refreshing complete growth medium supplemented with
50 µg Hygromycin B/ml (Invitrogen) every 3-4 days until all untransfected cells had died and stably transfected cells had formed visible colonies. At this time, colonies were isolated by trypsinization and expanded into individual lines for screening.

**Protein extraction and quantification**

To extract protein for bioluminescence assays, cells were harvested and washed twice with PBS. Cell pellets were re-suspended in 1 ml PBS and subjected to three cycles of freezing in liquid nitrogen for 30 s followed by thawing at 37°C in a water bath for 5 min. Cell debris was removed by centrifugation at 14,000 g for 10 min. Supernatant containing the total soluble protein was retained for bioluminescence assays. Protein concentration was determined by the BCA protein assay (Pierce) following manufacturer’s instructions.

**Cell lysate-based bioluminescence assays**

Bioluminescence assays were performed to determine the activity of the luciferase (LuxAB) proteins in each clone. Bioluminescence was measured using an FB14 luminometer (Zylux) with a 1 s integration time and reported as relative light units (RLU)/mg total protein. To carry out the light assays, 400 µl protein extract was mixed with 1 µM FMN, 0.1 mM NADPH, 0.1% (w/v) BSA, 0.5 U of oxidoreductase from *Vibrio fischeri* (Roche) and 0.001% (w/v) n-decanal (Sigma-Aldrich) in a total volume of 1 ml. Light signals were detected immediately.
following mixing and normalized to total protein. The clone with the highest LuxAB protein expression was selected for re-transfection with the single lux vector.

**Re-transfection and selection of stable bioluminescent cell lines**

The clone with the highest expression of LuxAB proteins was re-transfected with pCMV Lux using Lipofectamine 2000 transfection reagent (Invitrogen) following standard transfection procedures. Successfully transfected cells were selected based on resistance to Neomycin (Calbiochem) at a concentration of 500 µg/ml. Twenty-four clones were isolated by trypsinization and expanded into individual lines for bioluminescent measurement using the IVIS Lumina imaging system (Perkin-Elmer). The clone displaying the highest level of bioluminescence was designated as HEK293 ECO and chosen for further characterization.

**Determination of expression and chromosomal integration of the lux genes by qRT-PCR and qPCR**

Expression of the vector-borne luxC and frp genes as well as the chromosomally integrated non-optimized luxB (wtluxB) gene following transfection into wild type or HEK293/pIRES LuxAB cells were analyzed by qRT-PCR. Twenty-four h post-transfection, cells were washed with PBS, followed by RNA extraction using an RNeasy Mini Kit (Qiagen). Reactions were carried out using the Brilliant II SYBR GREEN One-Step QRT-PCR Master Mix (Agilent) in 25 µl total volume on a PTC-200 Peltier Thermal Cycler (MJ Research). For each
reaction, a total of 12.5 ng of RNA template were used for reverse transcription. The following thermal cycles were used for all reactions: 50°C for 30 min, 95°C for 5 min, and 40 cycles of 30 s at 95°C and 45 s at 60°C. To normalize gene expression across different samples, expression of the β-actin gene was included as an internal standard. Primers used for amplifications are listed in Table 7. Relative gene expression in a given sample and comparison between two samples was analyzed using the $\Delta C_T$ and the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001), respectively.

Chromosomal integrations of the lux genes were analyzed in three stable clones displaying varying levels of bioluminescent production. Genomic DNA was extracted using a Wizard Genomic DNA Prep Kit (Promega). Quantitative PCR was performed using the Brilliant SYBR Q-PCR system (Agilent) in 25 μl total volume on a PTC-200 Peltier Thermal Cycler (MJ Research). The following thermal cycles were used for all reactions: 95°C for 5 min, and 40 cycles of 30 s at 95°C and 45 s at 60°C. The same primers used in qRT-PCR analysis were used for amplifications. To determine gene copies, the single-copy ribonuclease P RNA component H1 $RPPH1$ gene was included as an internal standard (Table 7).
Table 7. Primers for qPCR and qRT-PCR analysis of the *lux* genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>luxC</td>
<td>forward: AGCAAGAAAGCCTGTGCAT</td>
</tr>
<tr>
<td></td>
<td>reverse: CTCGTCGAAGTCCTTCTTGG</td>
</tr>
<tr>
<td>frp</td>
<td>forward: CGAGCAGAGACAGACCATCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGCAGAACCAGAAAACCTCG</td>
</tr>
<tr>
<td>wtluxB</td>
<td>forward: TAATGGTGTTTGCCTGCTC</td>
</tr>
<tr>
<td></td>
<td>reverse: CGCTATTCGGACAGGATGAT</td>
</tr>
<tr>
<td>β-actin</td>
<td>forward: GCGGAATCGTGCACGACATT</td>
</tr>
<tr>
<td></td>
<td>reverse: GATGGAGTTGAAGGTAGTTTCGTG</td>
</tr>
<tr>
<td>RPPH1</td>
<td>forward: AGCTGAGTGCCTGTCCTCACT</td>
</tr>
<tr>
<td></td>
<td>reverse: TCTGGCCCTAGTCTCAGACCTT</td>
</tr>
</tbody>
</table>
Characterization of the enhanced bioluminescent cells

Minimum detectable population size

HEK293 ECO cells were harvested from 75 cm$^2$ tissue culture flasks and counted using the Scepter 2.0 handheld automated cell counter (Millipore). Serial dilutions of cells ranging from $\sim 1 \times 10^6$ to $\sim 1 \times 10^3$ cells were plated in triplicate in 1 ml of DMEM growth medium in each well of a black 24-well plate. Three wells were filled with 1 ml of media without cells as negative controls to determine background. Bioluminescence was evaluated in the IVIS Lumina imaging system using a 10 min integration time every 30 min for 8 h. Statistical analyses were performed using the Student's $t$ tests with a significant $p$ value of 0.05. To establish the relationship of population size to bioluminescent signal, the average radiance values in p/s/cm$^2$/sr were correlated to cell numbers using the Pearson's linear regression model.

Bioluminescent measurement using a plate reader

To test if the enhanced bioluminescent cells could be detected using a plate reader, various numbers of cells (ranging from $5 \times 10^2$ to $5 \times 10^5$ cells) were plated in triplicate in 200 μl of DMEM growth medium in each well of a black 96-well plate. Background noise was assessed using a control of 200 μl of medium in triplicate wells. Bioluminescence was determined in a Synergy 2 Microplate Reader (BioTek) using either a 1 s or 10 s integration time every 30 min for 8 hours. To establish the relationship of population size to bioluminescent signal,
relative light units per second (RLU/s) were correlated to cell numbers using the Pearson’s linear regression model.

**Results**

**Optimization of bioluminescent production in previously characterized HEK293/pLux<sub>AB</sub>:pLux<sub>CDEfrp</sub> cells**

A suite of gene supplementation experiments were performed to identify constraints and potential optimization points of bioluminescent production in previously characterized HEK293/pLux<sub>AB</sub>:pLux<sub>CDEfrp</sub> cells. Additional copies of lux<sub>AB</sub> or lux<sub>CDEfrp</sub> were expressed in these cells to determine if the luciferase levels or the quantities of the substrate processing proteins were limiting light production. Both supplementations improved signal output but to different extents (Figure 7A). Increasing the luciferase concentration by expressing extra lux<sub>AB</sub> genes increased bioluminescence by approximately 2-fold, whereas supplementation with the lux<sub>CDEfrp</sub> expression resulted in an approximately 5-fold increase in light production (Figure 7B). In an attempt to determine if other Lux protein-independent endogenous cofactors for aldehyde and FMNH<sub>2</sub> synthesis/recycling were limiting factors, up to an approximately 80-fold increase in light production was observed when the cells were supplemented with additional copies of all six genes assembled in a single vector (pEF Lux) (Figure 7B). Since such a high level of signal was unexpected, bioluminescent detection
Figure 7. Optimization of the previously characterized bioluminescent HEK293/pLuxAB:pLuxCDEfrp cells.

(A) Pseudocolor representations of bioluminescent production from HEK293/pLuxAB:pLuxCDEfrp cells following transfection with pEF Lux, p2A LuxAB, or p2A LuxCDEfrp in the IVIS Lumina CCD camera using a 10 min integration time. (B) Bioluminescent profiles from transfected cells displayed as fold change over untransfected control over a 16-hour imaging period (n=6, mean ± standard error). (C) Pseudocolor representations of bioluminescence detected in HEK293/pLuxAB:pLuxCDEfrp cells transfected with pEF Lux using 1 s to 30 s integration times.
originally employed the well-established 10 min integration time for imaging \textit{lux}-based mammalian cells (Close, Patterson et al. 2010; Close, Hahn et al. 2011). With an estimated population size of $2.5 \times 3 \times 10^6$ cells used in this experiment, 10 min exposure caused signal saturation of the CCD camera. For this reason, decreasing integration times ranging from 10 min to 1 s were applied. Pseudocolor representations of signal intensity could be conveniently obtained using as low as a 1 s integration time, although 5 s or longer exposure generated more consistent images (Figure 7C).

**Bioluminescent measurements of singly and doubly transfected cells**

Based on the results from direct optimization of the bioluminescent HEK293/pLux\textsubscript{AB}:pLux\textsubscript{CDEfrp} cells (Figure 7), it was hypothesized that the initial chromosomal integration of the partial \textit{lux} sequences was responsible for the unexpectedly high increase in light production. This hypothesis was tested by comparing light production following expression of the single \textit{lux} vector in wild type HEK293 cells and HEK293 cells stably expressing the \textit{luxAB} genes (Figure 8). While the singly transfected cells (wild type cells as host cells) produced a maximal average radiance of $2.13 \pm 0.10 \times 10^3$ p/s/cm$^2$/sr, re-transfection of the HEK293/pIRES \textit{Lux}\textsubscript{AB} cells with the same vector lead to a two orders of magnitude higher light production, with a peak average radiance of $3.10 \pm 0.17 \times 10^5$ p/s/cm$^2$/sr. It was further demonstrated that the different bioluminescent expression levels between singly and doubly transfected cells was not promoter
Figure 8. Bioluminescent expression following transfection of the \textit{lux} polycistronic vector into wild type and \textit{luxAB}-expressing HEK293 cells.

Wild type (HEK293) and \textit{luxAB}-expressing (HEK293/plRES Lux_{AB}) cells were transfected with pEF Lux or pCMV Lux. Bioluminescence was determined in equal numbers ($1 \times 10^6$) of transfected cells ($n=3$, mean ± standard error).
dependent, as the expression of CMV IE-driven *lux* genes also increased from 8.84 (± 0.22) × 10⁴ p/s/cm²/sr in wild type cells to 2.74 (± 0.03) × 10⁶ p/s/cm²/sr in *luxAB*-expressing cells (Figure 8).

**Selection of stable cell lines with enhanced bioluminescence**

To determine if the enhanced bioluminescent phenotype consistently observed in transient transfection assays could be stably maintained, antibiotic resistance-based stable clone selections were attempted. Twenty-four clones were isolated following transfection of pCMV Lux into HEK293/Lux_AB cells and examined for autonomous bioluminescent production. Twelve of the 24 clones displayed significantly distinguishable (*p* < 0.05) bioluminescence over background, achieving a 50% rate of successful transfection. When compared side-by-side using approximately 1 × 10⁶ cells, seven of the 12 positive clones produced a peak bioluminescence at least 10-fold higher of the previously demonstrated HEK293/pLux_AB:pLux_CDEfrp cells (Figure 9). Specifically, the clone (HEK293 ECO) with the highest level of bioluminescence displayed a maximal average radiance value of 2.09 (± 0.09) × 10⁵ p/s/cm²/sr, which was the same order of magnitude as that measured in transient expression assays (Figure 8). The stability of HEK293 ECO cells was monitored by bioluminescent detection in the IVIS imaging system after a 2.5-month storage period in liquid nitrogen. The recovered cells emitted light of 1.93 (± 0.06) × 10⁵ p/s/cm²/sr per one million cells,
Bioluminescent production of seven stable cell lines obtained by transfecting HEK293/pIRES Lux\textsubscript{AB}/hygro cells with pCMV Lux was compared with that of the previously characterized, un-optimized HEK293/pLux\textsubscript{AB}:pLux\textsubscript{CDEfrp} cells (~1 × 10\textsuperscript{6} cells, n=3, mean ± standard error). The cell line (clone 8) displayed the highest level of light production was designated as HEK293 ECO.
which was statistically similar to that of the cells before cryopreservation ($p > 0.05$) (Figure 10).

**Expression and chromosomal integration of the lux genes**

Quantitative RT-PCR analysis revealed that expression of the lux genes following transfection was host cell-dependent. Expression of the vector-borne luxC and frp gene in the luxAB-expressing background increased by ~ 8.5-fold and ~ 4.6-fold, respectively, compared to their expression in wild type HEK293 cells (Figure 11B). In contrast, expression of the chromosomally integrated wtluxB gene reduced from ~ 0.7% of $\beta$-actin expression in untransfected cells to ~ 0.3% of $\beta$-actin expression upon introduction of the single lux vector (Figure 11C).

The contribution of integration position effect on gene expression was evaluated in three stable clones displaying a gradient of light production. The copy numbers of the three lux genes tested were determined to be the highest in the most bioluminescent clone. Insertions of two of the three genes, luxB and frp, were closely correlated to bioluminescent output, each with an $R^2$ value greater than 0.90 (Figure 12), suggesting that differences in the inserted copy numbers were responsible for the majority of light production variations in different stable lines.
Figure 10. Comparison of bioluminescent production of HEK293 ECO cells before and after cryopreservation.

Bioluminescent production of $1 \times 10^6$ HEK293 ECO cells were assayed before cryopreservation and after recovery from a 2.5-month storage period in liquid nitrogen (n=3, mean ± standard error).
Figure 11. Differential transfection methods result in disparate gene expression levels.

(A) Schematic representation of differential transfection methods. pEF Lux was transfected into wild type (HEK293) or luxAB-expressing (HEK293/pIRES LuxAB) cells (chr: chromosomal integrated; homologous regions were indicated by dash lines)). (B) Expression of the plasmid-borne luxC and frp genes following pEF Lux transfection into HEK293 or HEK293/pIRES LuxAB cells was determined by qRT-PCR (n=9). (C) Expression of the chromosomally integrated wtluxB gene in HEK293/pIRES LuxAB cells was examined before and after transfection with pEF Lux (n=6).
Figure 12. Correlation between \textit{lux} gene copy numbers and bioluminescent production.

The correlation between \textit{lux} gene copy numbers and bioluminescent production were determined in three stable bioluminescent cell lines obtained by transfecting HEK293/pIRES Lux\textsubscript{AB}/hygro cells with pCMV Lux. Results represent \textit{RPPH1}-normalized gene copy numbers determined by qPCR (n=3) and bioluminescence from $5 \times 10^4$ cells (n=3).
**Bioluminescent profiles of the enhanced HEK293 ECO bioluminescent cells**

Before the newly developed bioluminescent cell line can be used as a functional optical reporter, its bioluminescent profile under different detection conditions must be characterized. The enhanced bioluminescent ECO cells were assayed over a dynamic population range to determine minimum detectable cell number in the IVIS imaging system using a 10 min integration time in a 24 well plate format (Figure 13A). Compared to the previously described bioluminescent HEK293/pLuxAB:pLuxCDEfrp cells that could not be reliably detectable under \( \sim 1.5 \times 10^4 \) cells using the same imaging conditions (Close, Hahn et al. 2011), a minimum of \( \sim 1 \times 10^3 \) ECO cells were significantly distinguishable from background \( (p = 0.0004) \) (Figure 13B).

One limitation of the previously characterized cells was that their detection was limited to the sensitive yet relatively expensive CCD camera-based system due to their low signal intensity. With the improvement of bioluminescent production in ECO cells, it remained to be determined if the newly developed cells were amenable to other conventional luminescent detecting instruments. The bioluminescent production of ECO cells was surveyed in a 96-well plate format using a photomultiplier tube (PMT)-based Synergy 2 microplate reader. Unlike the increase of signal output in the first few hours detected by in the IVIS,
Figure 13. Minimum detectable population size of the enhanced bioluminescent HEK293 ECO cells.

The minimum detectable population size of HEK293 ECO cells were determined by (A) plating a range of cell concentrations (K: thousand, M: million) in equal volumes of media in triplicate in a black 24-well plate. (B) Approximately $1 \times 10^3$ cells could be significantly distinguished from medium background ($p < 0.05$) (n=3, mean ± standard error).
the Synergy detected signal remained relatively constant over 8 hours (Figure 14A). The minimal detectable population size was determined to be approximately $5 \times 10^3$ cells in 96 well plates. Because of the lower sensitivity of the plate reader, the same population size produced a lesser signal to noise ratio compared to the IVIS system (Table 8).

Despite the intrinsic difference in signal detection mechanisms between the VIS Lumina and Synergy 2 instruments and plating format, the bioluminescent output from HEK293 ECO cells remained linearly correlated to the numbers of cells present in the population ($R^2 > 0.99$), regardless of exposure time (Figure 14B and 14C).

**Discussion**

The recent development of the *lux* system into a functional reporter in mammalian cells provides a novel and attractive tool for bioluminescent imaging. It allows for continuous and non-invasive monitoring of the same target over time both *in vitro* from cell culture and *in vivo* using living small animal models without any substrate being added exogenously (Close, Hahn et al. 2011). Despite this appealing feature, bioluminescent output using current *lux*-expressing strategies is much lower than that of the traditional substrate-requiring firefly luciferase system, limiting wider applications of this system.

As a first step of optimization, we assessed the constraints for bioluminescent
Figure 14. Comparison of bioluminescent detection of the HEK293 ECO cells using the IVIS imaging system and the Synergy microplate reader.

The bioluminescent profiles of the HEK293 ECO cells were surveyed in the IVIS Lumina imaging CCD camera and the Synergy 2 microplate reader using a 24- or 96- well plate format, respectively (n=3, mean ± standard error). Although these two detection schemes displayed varying dynamics over time, exemplified by (A) bioluminescent detection from of approximately $5 \times 10^5$ HEK293 ECO cells plated in each well of a 24 well plate in 1 ml medium (IVIS detection) or in each well of a 96 well plate in 200 µl medium (Synergy detection), tight correlation between signal and population size were obtained under both conditions ((B) IVIS measurements and (C) Synergy measurements).
Table 8. Comparison of bioluminescent detection from HEK293 ECO cells using the IVIS Lumina imaging system and the Synergy 2 microplate reader.

<table>
<thead>
<tr>
<th>Population Size (Cells/Well)</th>
<th>IVIS (24-well plate, 10 min exposure) (n=3)</th>
<th>Microplate Reader (96-well plate, 1 s exposure/well) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Flux (p/s) (± standard error)</td>
<td>Fold Change (± standard error)</td>
</tr>
<tr>
<td>5 × 10²</td>
<td>n.d.</td>
<td>7.0 (± 1.0)*</td>
</tr>
<tr>
<td>1 × 10³</td>
<td>1.95 (± 0.06) × 10⁴</td>
<td>1.5 (± 0.1)</td>
</tr>
<tr>
<td>5 × 10³</td>
<td>4.62 (± 0.14) × 10⁴</td>
<td>3.6 (± 0.1)</td>
</tr>
<tr>
<td>1 × 10⁴</td>
<td>7.81 (± 0.16) × 10⁴</td>
<td>6.1 (± 0.2)</td>
</tr>
<tr>
<td>5 × 10⁴</td>
<td>3.21 (± 0.06) × 10⁵</td>
<td>25.0 (± 0.7)</td>
</tr>
<tr>
<td>1 × 10⁵</td>
<td>6.12 (± 0.04) × 10⁵</td>
<td>47.6 (± 1.0)</td>
</tr>
<tr>
<td>5 × 10⁵</td>
<td>2.60 (± 0.05) × 10⁶</td>
<td>201.6 (± 5.4)</td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>4.59 (± 0.02) × 10⁶</td>
<td>356.9 (± 7.2)</td>
</tr>
</tbody>
</table>

n.d. - not determined.
* - not significantly distinguishable from background (p > 0.05).
production in the previously described HEK293/pLuxAB:pLuxCDEfrp cells. The chemical reaction of the lux-based bioluminescence system is a typical enzyme-substrate reaction (Meighen 1991). The rate of this reaction, reflected by bioluminescent flux (photons/second), is dependent on the concentrations of the luciferase enzyme as well as the availabilities of the aldehyde and FMNH$_2$ substrates present in the host cells. While the luciferase concentration is directly related to the expression of the luxAB genes, synthesis and recycling of the aldehyde substrate and FMNH$_2$ are dependent not only upon the concentrations of the LuxCDE and Frp proteins, but also on the availability of other endogenous substrates and co-factors present in the host cell (such as NADPH). In the previously characterized cells, the lux cassette is split into two expression vectors with a total of three promoters for the regulation of gene expression. The uncoupled expression of the luciferase genes and the substrate processing genes may result in an unbalanced enzyme/substrate ratio that is not optimal for the reaction. Previous \textit{in vitro} substrate supplementation studies imply that aldehyde and FMNH$_2$ are both limiting factors for light production (Close, Patterson et al. 2010). In this study, gene supplementations showed consistent results that introducing additional copies of the luxCDEfrp genes increased light production by a larger fold compared to luxAB addition (5- vs. 2- fold). However, the relative fold change by \textit{in vivo} gene addition is not as prominent as that following direct substrate amendment. There are three possible explanations: 1) even with the additional copies of genes introduced in this manner, protein
synthesis is still insufficient to supply the same level of substrate that is supplemented in vitro, 2) substrate synthesis is limited by other host cellular factors, and 3) increased level of substrate becomes cytotoxic to the host cells (Hollis, Lagido et al. 2001). With the subsequent finding that a substantial increase (~ 80 fold) in bioluminescence production is possible if all six genes are added at the same time (Figure 7), it becomes clear that availability of host co-factors and increased in vivo aldehyde synthesis are not major limiting factors for high level light production.

A surprising discovery from the gene supplementation study is that expressing the single lux vector in HEK293/pLuxAB:pLuxCDEfrp cells produces significantly higher bioluminescence (peak average radiance in the order of $10^5$ p/s/cm$^2$/sr) than that obtained by expressing the same vector in wild type cells (peak average radiance in the range of $10^3$ p/s/cm$^2$/sr). The non-additive, synergistic increase in light production leads to the hypothesis that the initial chromosomal insertion of the lux genes is capable of promoting gene expression in the second transfection event, probably through homologous recombination-mediated gene integration. This hypothesis was investigated in a unique type of cells expressing the stably integrated luxA-wtluxB transcriptional fusion (Figure 11A). In these cells, the chromosomal copy of the luxA gene is a formerly optimized sequence and shares 85% homology to the more recently optimized luxA sequence carried on the single lux vector (Table 6, Chapter II). The
integrated luxB is an un-optimized wild type sequence (wtluxB) derived from its bacterial host P. luminescens, and has 75% identity to the codon-optimized luxB sequence present in the lux vector. Despite the homology between wtluxB and codon-optimized luxB sequence, it was possible to design specific primers to only amplify the chromosomally integrated wtluxB but not the vector-borne luxB sequence. Because transcription of wtluxB is coupled to that of the upstream luxA gene, homologous recombination between the lux vector and the chromosomal luxA sequence, if it occurs, would disrupt the transcription of wtluxB from the promoter and reduce its expression. Therefore, cells expressing this construct allow us to study the effect of homology between the chromosomal and vector-borne luxA sequences on the highly enhanced bioluminescent production, as well as to use the wtluxB expression as a marker for homologous recombination.

As expected, expressing the single lux vector pEF Lux in this genetic background produced similarly high levels of light, with a peak average radiance of \( \sim 3 \times 10^5 \text{ p/s/cm}^2/\text{sr} \) (Figure 8), supporting the hypothesis that initial genomic integration of a partial lux cassette is sufficient to promote high level bioluminescent expression. Furthermore, the luxC and frp transcripts were found to be approximately 8.5-fold and 4.6-fold higher, respectively, when expressed in these cells compared to wild type controls (Figure 11B). The higher mRNA level could be the result of increased transgene integration, augmented transcription
per incorporated transgene copy, or a combination of both. While experimental assessments are still required to determine whether transcription is increased on a per copy basis, there is evidence presented in the literature as well as in this study supporting the idea that the pre-integrated *luxA* sequence may improve targeted integration of the secondly introduced *lux* cassette.

Foreign DNA is integrated into the cell genome using two antagonistic DNA double strand break (DSB) repair pathways, non-homologous end joining (NHEJ) or homologous recombination (Grandjean, Girod et al. 2011). Integrations of transfected DNA rely on the capture DSBs on random genomic sites (for NHEJ-mediated insertion) or on chromosomal loci that are homologous to the foreign DNA molecules (for homology-mediated integration). Because DSBs occur at very low frequency, stable transgene integrations are rare events. However, increasing the homology between transfected nucleotides and the recipient genome can improve integration efficiency to a certain extent. It has been reported previously that recombination between the newly introduced DNA and the homologous region in the host genome is able to mediate highly frequent site specific integration in mammalian cells (Thomas, Folger et al. 1986; Deng and Capecchi 1992; Grandjean, Girod et al. 2011). In rice, there has been evidence showing that during direct plasmid DNA transfer, the initial insertion sites attract successively transferred DNA to integrate at the same locus (Kohli, Leech et al. 1998). In this study, the pre-integrated *luxA* sequence establishes a homologous
region in the chromosome, which may act as a hot spot for integration of the latterly introduced lux cassette. Additionally, transcription of the wtluxB gene is found to be reduced by ~ 60% following introduction of the lux vector (Figure 11C), suggesting possible transcriptional interruption from its promoter or gene disruption, either of which could be a result of recombination at or near the upstream luxA sequence.

The two-step transfection strategy developed here is also associated with a relatively high efficiency for transgene expression, with 50% of cell lines selected based on antibiotic resistance displaying successful bioluminescent production. This level of efficiency is superior to that obtained from single transfection of wild type cells (less than 10%). Stable heterologous protein expression requires the full transgene construct, including the promoter, the protein coding region, and other essential mRNA processing signals, to be integrated into the cell genome as an intact expression unit. Isolation of cells with stable transgene expression, however, is usually initiated by selecting antibiotic-resistant phenotypes conferred by the co-transfected resistance marker. This often results in false positive clones that express only the resistance gene but not desired transgenes, probably due to no integration or integration of only partial transgene expression units. Screening for light-emitting phenotypes within the resistant population is further complicated by the fact that bioluminescent production requires proper integration and expression of six genes (luxCDABEfrp), yielding a relatively high
false positive rate in the case of random integration following transfection into wild type cells. Alternatively, in homology-mediated integration, because integration is less random and more targeted, higher integration of an intact transgene can be obtained (Deng and Capecchi 1992; Liu, Chan et al. 2010). Together with the homology between the pre-integrated luxA and the newly introduced lux cassette, the higher efficiency observed in doubly transfected cells also suggests that the synergistic bioluminescent expression might be a homologous recombination-mediated effect.

In random, non-targeted insertion, transgene expression is often varied by integration sites due to highly dynamic local genetic architecture of the inserted locus (Kwaks and Otte 2006). In site-specific targeting, on the other hand, the position effect is obviated and transgene expression is mainly copy-number dependent. In this study, insertions of the luxB and frp genes were closely correlated with bioluminescent production in stable cell lines developed by the two-step transfection strategy (Figure 12), suggesting that differences in the inserted copy number were responsible for the majority of light production variations in different stable lines.

The stable luxAB-expressing cells used as the recipient for the second transfection are those that have undergone selection for optimal luciferase expression. Therefore, it is possible that the luxAB sequence has been integrated into a region that favors high level gene expression, or into multiple sites. If this
stays true, the secondarily introduced lux vector will be targeted into this region(s) through the proposed homologous recombination-mediated integration, which also partially explains the improved bioluminescent production following transfection into these cells.

It is important to note that homologous recombination-mediated gene integration is a rare event, especially in non-embryotic stem cells. Although evidence present in this study and other literature both implies that homology between the pre-established partial lux sequence and the newly introduced full lux cassette may target more frequent insertion of the vector sequence into the cell genome, other possibilities such as increased random integration into the host genome cannot be excluded and the detailed mechanism of the synergistic lux expression and exact locus for integrations will still require further experimental assessment.

From a practical point of view, the newly optimized bioluminescent cells developed here through the two-step transfection strategy have important advantages over their previously validated counterparts. First of all, the improved cells can be detected at a more dynamic population range compared to the un-optimized cells, with an ~ 15-fold lower minimum detectable population size. While the un-optimized cells are limited to applications that use larger cell populations (≥ 1.5 × 10^4 cells), a minimum of ~ 1 × 10^3 of the enhanced bioluminescent cells are detectable (Figure 13), and thus are suitable for studies
that require relatively small cell populations, such as drug susceptibility testing and long term cell growth analysis. The un-optimized cells have been reported to require a minimum of $\sim 2.5 \times 10^4$ cells for whole animal \textit{in vivo} imaging following subcutaneous injection into a mouse model (Close, Hahn et al. 2011). Although the minimum population size of the optimized cells remains to be determined in the mouse model, it is predicted based on \textit{in vitro} cell culture detection that less cells will be detectable. Furthermore, due to photon scattering and absorption by endogenous chromophores (Rice, Cable et al. 2001), the un-optimized cells are not ideal for imaging from deep tissue. With two orders of magnitude improvement of bioluminescent production, the enhanced cells should overcome this limitation and provide an alternative, substrate-free tool for deep tissue imaging applications that traditionally employ the FLuc reporter. However, it is also important to note that, even though the signal intensity has been upgraded significantly in the newly optimized \textit{lux}-expressing cells, it is still not as strong as that produced from the FLuc reporter cells (Kim, Urban et al. 2010; Close, Hahn et al. 2011). While the newly optimized \textit{lux} system will not completely replace the FLuc system, it certainly provides a substrate-free alternative for some applications if the desired population size is covered by the dynamic ranges of the \textit{lux} system.

Another advantage associated with the enhanced bioluminescent cells is that they are suitable for multiple types of detection. The un-optimized cells are
limited to detection by highly sensitive but more expensive instruments such as the CCD camera-based IVIS imaging system, but are not detectable using relatively less sensitive PMT detection, due to its low signal intensity. In contrast, the newly optimized cells are readily detectable using less expensive instruments such as the Synergy microplate reader, thus facilitating higher-throughput applications.
CHAPTER IV

Towards *lux*-Based Autonomous Biosensing in Human Cell Lines
Introduction

Despite its extensive use in bacterial whole-cell bioreporters for detection of a diverse range of compounds and conditions (reviewed in Xu, Close et al. (2012)), the application of bacterial bioluminescence (lux) in eukaryotic organisms is extremely limited, due largely to the obstacles preventing successful expression of the essential lux genes at a functional level for bioluminescence production in the eukaryotic cellular background. It was not until 20 years after the first demonstration of exogenous lux expression and acquired bioluminescent phenotype in *E. coli* (Engebrecht, Nealson et al. 1983) that the lux cassette had finally been expressed efficiently in lower eukaryotic organisms. By expressing the luxCDABE and frp genes through a combination of separation of genes into two vectors, use of bi-directional expression vectors, and insertion of IRES elements to mimic polycistronic translation, Gupta and colleagues successfully created autonomously luminescent *S. cerevisiae* for the first time (Gupta, Patterson et al. 2003). This breakthrough quickly progressed into the generation of autonomously bioluminescent eukaryotic reporters for rapid detection of estrogenic and androgenic compounds (Sanseverino, Gupta et al. 2005; Eldridge, Sanseverino et al. 2007).

Estrogenic and androgenic compounds represent a unique family of chemical targets that cannot be screened exhaustively using analytical chemical methods, due to the fact that they are defined not by their chemical structures but by their
physiological impacts on living organisms. According to the recent Scientific Statement by The Endocrine Society (Diamanti-Kandarakis, Bourguignon et al. 2009), endocrine disrupting chemicals (EDCs) are "substances in our environment, food, and consumer products that interfere with hormone biosynthesis, metabolism, or action resulting in a deviation from normal homeostatic control or reproduction." There has been a rich body of scientific literature providing evidence on the potential effects of EDCs on reproductive, developmental, and metabolic disorders inclusive of breast, ovarian, testicular, and prostate cancers, early onset of puberty, genital tract abnormalities, reduced sperm counts, and various other health related problems in both humans and wildlife (Diamanti-Kandarakis, Bourguignon et al. 2009). Due to these public health concerns, the U.S. Environmental Protection Agency (EPA) has been required by law (the 1996 amendments to the Safe Drinking Water Act and the 1996 Food Quality Protection Act) to develop a program to screen and test chemicals used in a variety of industries such as pesticides and personal care products for their endocrine disrupting activities. With an estimated number of ~87,000 chemicals to be screened, bioassays for rapid and high-throughput detections are highly desired.

Detection of endocrine disrupting compounds is not feasible using traditional bacterial bioreporters due to their lack of essential receptors and signaling components with which these compounds interact. Even with their eukaryotic
genetic and cellular background, the aforementioned bioluminescent yeast estrogen and androgen bioreporters still require recombinant expression of the human estrogen and androgen receptor, respectively, for proper function. Although yeast-based assays offer the advantage of rapid detection, they can also produce false results regarding human effects, due to factors such as the difference in chemical uptake between the yeast cell wall and the mammalian cellular membrane, varying receptor levels and metabolic capabilities, and in some cases, the inability to differentiate agonists and antagonists (Zacharewski 1997).

Human cell lines, on the other hand, can serve as a native platform for bioreporter development because they directly represent human bioavailability and toxicity. Most of the current estrogen bioreporters using human cell lines as hosts utilize firefly luciferase as the reporting element. These reporters are created by transcriptionally fusing reporter gene expression to one or more estrogen response elements (EREs), which are unique cis-acting regulatory DNA elements recognized by the DNA binding domain of the estrogen receptor. Upon binding to estrogen/estrogenic chemicals, the ligand-receptor complex forms a homodimer that interacts with the EREs to recruit transcriptional factors that regulate the expression of the associated genes. Several Luc-based in vitro estrogen bioreporters have been developed in this manner using the human breast cancer cell lines T-47D and MCF-7, as they naturally express the estrogen
receptors required for reporter gene activation (Balaguer, Francois et al. 1999; Legler, van den Brink et al. 1999; Wilson, Bobseine et al. 2004). These bioreporters are advantageous over their yeast counterparts in regard to their uptake of chemicals, receptor concentrations, signaling pathways, and their ability to distinguish between agonists and antagonists (O’Connor, Cook et al. 2002). However, these cells do not metabolize most of the EDCs, and thus are unsuitable for detection of compounds that are not estrogenic until being metabolized in vivo (for instance, by the liver) (Soto, Maffini et al. 2006). Although a transgenic mouse model can be developed for in vivo detection (Ciana, Di Luccio et al. 2001), it cannot be done in a non-invasive manner because of the requirement of exogenous substrate addition for producing a detectable signal.

The limitations posed by the substrate-requiring Luc system could potentially be overcome by using the lux system that has recently been adapted for use in the mammalian cellular background. In addition, the substrate-free nature of the lux system permits continuous real-time monitoring of cellular functions in a non-invasive manner. Therefore, the autonomous bioluminescent expression from a mammalian cell line could be used to directly detect the human bioavailability of targets of interests. Because no cell destruction or substrate amendment is required, it also has the potential to be developed into an in vivo model. To advance its broader applications, this chapter describes the preliminary steps of developing the lux system towards a fully autonomous reporter for use in human
Materials and Methods

Strain growth and maintenance

Human breast carcinoma T-47D cells were regularly maintained in phenol-red free RPMI-1640 medium (Hyclone) supplemented with 10% FBS, 0.01 mM non-essential amino acids, 1X antibiotic-antimyotic and 0.01 mM sodium pyruvate. Cells were cultured at 37°C in a 5% CO₂ environment with medium being refreshed every 2 - 3 days. Cells were passaged regularly upon reaching 80% confluence. For estrogen treatment assays, assay medium was modified from growth medium by substituting 10% charcoal/dextran-treated FBS (CD-FBS) (Hyclone). Neomycin at the concentration of 500 µg/ml was used for selection and maintenance of stably transfected cells.

HEK293 Tet-On cells expressing the tetracycline-regulated transactivator were purchased from Clontech and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 µg Neomycin/ml, 10% FBS, 0.01 mM non-essential amino acids, 1X antibiotic-antimyotic and 0.01 mM sodium pyruvate. For doxycycline induction assays, assay medium was modified from growth medium by 10% Tet System Approved FBS (Clontech).

Escherichia coli TOP10 cells (Invitrogen) were used to host and propagate plasmids used in this study. E. coli cells were routinely grown in Luria Bertani (LB)
broth at 37°C with 200 rpm continuous shaking. Kanamycin or ampicillin was used at a final concentration of 50 or 100 µg/ml, respectively, for selection and maintenance of successfully transformed E. coli cells.

**Vector construction**

To construct a vector in which expression of *luxAB* genes was regulated by an estrogen responsive promoter, a double-stranded DNA fragment containing three tandem copies of the estrogen response element (ERE) and the adenovirus E1B minimal TATA promoter (GGGTATATAAT) was synthesized by annealing two complementary synthetic oligonucleotides (ERE3-TATA fwd and rev, Table 9) that were designed to include a BglII overhang on the 5’ end and an Xmal overhang on the 3’ end to facilitate downstream cloning. The annealing reaction was carried out at 70°C for 10 min with 1X Taq polymerase buffer. The resulting double-stranded DNA was purified and cloned into the BglII and Xmal cloning sites in pd2EGFP-1 (Clontech) to generate pERE3-TATA-d2EGFP.

To construct pERE3-TATA-LuxA<sub>B</sub>, the *luxA*-IRES-*luxB* sequence was PCR amplified from pIRES-LuxA<sub>B</sub> using primers AIB fwd and rev (Table 9) with introduced 5’ end AgeI restriction site and cloned into a pCR4-TOPO vector (Invitrogen) to generate pCR4LuxA<sub>B</sub>. The d2EGFP protein coding region was then removed from pERE3-TATA-d2EGFP by AgeI and NotI double restriction
### Table 9. ERE sequences and primers used for vector construction.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERE3-TATA fwd</td>
<td><code>gatctAGGTCACTGTGACCTCTCGAGGTCACTGTGACCTCTCGAGGTTATATAATc</code></td>
</tr>
<tr>
<td>ERE3-TATA rev</td>
<td><code>ccgggATTATATACCGCGATCGCAGGTCACAGTGACCTCGAGAAGTCACAGTGACCTAGGTCACAGTGACCTA</code></td>
</tr>
<tr>
<td>AIB fwd</td>
<td><code>ACCGGTGCAGAATTCCCTTGA</code></td>
</tr>
<tr>
<td>AIB rev</td>
<td><code>CTGAGCTCTGGCCCAGTC</code></td>
</tr>
</tbody>
</table>

*underscored* - estrogen response element (ERE).
*shaded bold* - adenovirus E1B minimal TATA promoter.
*lower case italic* - partial restriction sites used for cloning.
digest and replaced by the *luxA*-IRES-*luxB* sequence, which was cleaved from pCR4Lux<sub>AB</sub> with the same restriction enzymes and purified from agarose gel using the Qiaquick Gel Extraction Kit (Qiagen). The successfully ligated plasmid was confirmed by sequencing and designated as pERE3-TATA Lux<sub>AB</sub>.

The ERE3-TATA promoter was then removed from pERE3-TATA Lux<sub>AB</sub> using MluI and SalI and cloned into similarly digested pCMV Lux to replace the CMV IE promoter. The final construct was named pERE3-TATA Lux. A modified version of this vector was created by replacing the TATA box promoter with a CMV IE minimal (CMVm) promoter cloned from pIRES Lux<sub>AB</sub> to create pERE3-CMVm Lux.

To create a reporter construct for tetracycline response element (TRE)-regulated polycistronic expression of the *luxCDABEfrp* genes, the TRE and its associated minimal CMV promoter were removed from the pTRE-Tight-BI vector (Clontech) using Acc65I and XbaI and cloned into similarly digested pERE3-TATA Lux to replace the ERE3-TATA promoter (all vectors used in this study are listed in Table 4).

Transfection of mammalian cells

Transfections were carried out in 6-well tissue culture plates (Corning). The day prior to transfection, cells were plated at a density of 5 × 10<sup>5</sup> cells/well (for HEK293 Tet-On cells) or 7.5 × 10<sup>5</sup> cells/well (for T-47D cells). Vector DNA purified from overnight *E. coli* culture using PureYield Plasmid Purification
System (Promega) was introduced into the cells using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s instructions. Transfected cells were either assayed twenty-four hours post transfection, or subjected to stable transfectant selection as described below.

**Screening for stably transfected reporter cell lines**

T-47D cells transfected with pCMV Lux or pERE3-TATA Lux\textsubscript{AB} were subjected to stable transfectant selection. Twenty-four hours post-transfection, transfected cells were harvested and diluted into new six-well plates in growth medium without antibiotic selection. Starting the next day, selection of stably transfected clones was performed by refreshing with selective medium containing 500 µg Neomycin/ml every 2 - 3 days until all untransfected cells had died and stably transfected cells had formed visible colonies. The colonies were then removed by trypsinization and expanded into individual lines in growth medium supplemented with Neomycin at a 500 µg/ml concentration.

To screen for autonomous bioluminescence following transfection with pCMV Lux, each isolated cell line was grown in individual 25 cm\textsuperscript{2} tissue culture flasks and harvested for imaging upon reaching ~80% confluence. Cells were collected in 1 ml of RPMI-1640 growth medium and plated in each well of a black 24-well plate. Bioluminescent measurements were carried out using the IVIS Lumina Imaging System (Perkin-Elmer) with a 10 min integration time every 30 min for 24 h. The cell line displaying the highest bioluminescent production was
designated T-47D/Lux and used in further analysis.

To screen stable transfectants for estrogen-regulated LuxAB expression following transfection with pERE3-TATA Lux$_{AB}$, each isolated cell line was propagated in 25 cm$^{2}$ tissue culture flasks with RPMI-1640 assay medium (containing 10% CD-FBS). Upon reaching 80% confluence, cells were split into multiple 25 cm$^{2}$ tissue culture flasks containing equal number of cells per flask. Upon reaching 80% confluence 17$\beta$-estradiol (E$_2$) (Sigma-Aldrich) or HPLC-grade ethanol was added at a final concentration of 10 nM or 0.1% (v/v), respectively. Twenty-four hours post treatment, total protein was extracted and assayed for bioluminescence as described below. Positive cell lines were further tested for 24-hour induction with various concentrations of E$_2$ (5 pM to 10 nM) using the same assay.

**Determination of LuxAB activity using the cell lysate-based bioluminescence assay**

Expression of LuxAB proteins in transiently transfected cells or stable transfectants was determined using the cell lysate-based bioluminescence assays. For transient expression, T-47D cells transfected with pERE3-TATA Lux$_{AB}$ were harvested 24 hours post transfection and treated with 10 nM E$_2$ for 6, 12, or 24 hours before being lysed for bioluminescence assay. Stable transfectants were assayed after 24 hours of E$_2$ treatment as described above.
Protein extraction and quantification

To extract protein for bioluminescence assays, cells were harvested and washed twice with sterile phosphate buffered saline (PBS). Cell pellets were re-suspended in 1 ml PBS and subjected to three cycles of freezing in liquid nitrogen for 30 s followed by thawing at 37°C in a water bath for 5 min. Cell debris was removed by centrifugation at 14,000 g for 10 min. Supernatant containing the total soluble protein was retained for bioluminescence assays. Protein concentration was determined by the BCA protein assay (Pierce) following the manufacturer’s instructions.

Cell lysate-based bioluminescence assays

Cell lysate-based bioluminescence assays were performed to determine the activity of LuxAB proteins in cell extracts. Bioluminescence was measured using an FB14 luminometer (Zylux) with a 1 s integration time and reported as relative light units (RLU)/mg total protein. To carry out the light assays, 400 μl protein extract was mixed with 1 μM FMN, 0.1 mM NADPH, 0.1% (w/v) BSA, 0.5 U of oxidoreductase from V. fischeri (Roche) and 0.001% (w/v) n-decanal (Sigma-Aldrich) in a total volume of 1 ml. Light signals were detected immediately following mixing and normalized to total protein.

Correlating population size with bioluminescence in T-47D/Lux cells

Actively growing T-47D/Lux cells were trypsinized and harvested from 75 cm²
tissue culture flasks and counted using a hemacytometer. Groups of either $2.5 \times 10^5$, $1 \times 10^6$, $5 \times 10^4$, $2.5 \times 10^4$, $1 \times 10^4$, $5 \times 10^3$, or $2.5 \times 10^3$ cells were plated in triplicate in 1 ml of phenol red-free RPMI-1640 complete growth medium in each well of a clear-bottom, tissue culture-treated, black 24-well plate. A negative control for monitoring background was performed in triplicate wells containing 1 ml of medium without cells. Bioluminescence was determined in the IVIS Lumina imaging system using a 10 min integration time every 30 min for 16 h. Statistical analysis was performed using Student's $t$ tests with a significant $p$ value of 0.05. To establish the relationship between population size and bioluminescent signal, the total flux values in p/s were correlated to cell numbers using the Pearson’s linear regression model.

**Bioluminescent T-47D/Lux estrogen assay**

The bioluminescent T-47D estrogen screening assay was carried out in clear-bottom, tissue culture treated, black 24-well plates. Approximately $1 \times 10^4$ T-47D/Lux cells were seeded into each well in 1 ml growth medium and were allowed to attach for 24 h. Before addition of E2, medium was refreshed with 1 ml CD-FBS containing assay medium. E2 (using HPLC grade ethanol as the solvent) was added at final concentrations of 0 pM (control), 0.1 pM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, or 100 nM in triplicate wells. Solvent concentration remained constant at 0.1% (v/v) across all wells. Bioluminescent measurements were obtained using the IVIS Lumina imaging system with a 10 min integration time.
every 24 h for 6 days. Cells were incubated at 37°C in a 5% CO₂ environment between measurements.

**Bioluminescent measurement of ERE-lux reporter cells in response to estrogen**

Estrogen-induced bioluminescent expression of pERE3-TATA Lux and pERE3-CMVm Lux was examined in T-47D cells in transient expression assays. Transfections were carried out in assay medium (RPMI containing 10% CD-FBS) as described previously. Twenty-four h post transfection, cells were washed with sterile PBS and counted using a hemocytometer. Approximately 1 × 10⁶ cells were plated in each well in 1 ml assay medium in a black 24-well plate. E₂ was dosed at final concentrations of 0 (control), 1 or 10 nM with a 0.1% (v/v) solvent (ethanol) concentration. Bioluminescent measurements were performed immediately after estrogen amendment using the IVIS Lumina imaging system using a 10 min integration time every 30 min for 24 hours.

**Bioluminescent measurement of Tet-lux reporter cells in response to doxycycline**

Doxycycline-induced bioluminescent expression of pTet Lux was examined in HEK293 Tet-On cells in transient expression assays. Transfections were carried out in assay medium (DMEM containing 10% Tet System Approved FBS) as described previously. Twenty-four h post transfection, cells were washed with sterile PBS and counted using the Scepter 2.0 handheld automated cell counter.
(Millipore). Approximately $1 \times 10^6$ cells were plated in each well in 1 ml assay medium in a black 24-well plate. Doxycycline was added at final concentrations of 0 (control) or 100 ng/ml in replicate wells. Bioluminescent measurements were performed immediately after doxycycline induction using the IVIS Lumina imaging system using a 10 min integration time every 30 min for 19 h.

**Quantitative RT-PCR analysis of lux gene expression**

Expression of the *lux* genes was analyzed by qRT-PCR. The expression of the *luxC* gene was evaluated in T-47D cells transiently expressing the pERE3-CMVm Lux reporter construct after 24-hour exposure to 0 or 1 nM E$_2$, as well as in constitutively bioluminescent T-47D cells stably expressing pCMV Lux. The expression of the *luxC*, *luxB*, and *frp* genes was determined in HEK293 Tet-On cells transiently expressing the pTet Lux reporter construct after 19-hour exposure to 0 or 100 ng doxycycline/ml.

Total RNA was extracted using an RNeasy Mini Kit (Qiagen). For RNA extracted from transiently transfected cells, an additional step of DNase I treatment using a DNA-Free RNA kit (ZymoResearch) was performed to remove co-extracted plasmid DNA. qRT-PCR reactions were carried out using the Brilliant II SYBR GREEN One-Step QRT-PCR Master Mix (Agilent) in 25 μl total volumes on a PTC-200 Peltier Thermal Cycler (MJ Research). For each reaction, a total of 12.5 ng of RNA template were used for reverse transcription. The following thermal cycles were used for all reactions: 50°C for 30 min, 95°C for 5
min, and 40 cycles of 30 s at 95°C and 45 s at 60°C. To normalize gene expression across different samples, expression of the β-actin gene was included as an internal standard. Relative gene expression in a given sample and comparison between two samples was analyzed using the $\Delta C_T$ and the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001), respectively.

**Results**

**Development of a constitutively bioluminescent T-47D/Lux cell line and its potential application in an estrogen screening assay**

**Selection of stable autonomously bioluminescent cell lines**

Thirty-five stable cell lines were established following neomycin selection of T-47D cells transfected with the pCMV Lux expression vector. Each of these lines was interrogated for autonomous bioluminescent production using the IVIS Lumina imaging system. Despite growing efficiently under selective media conditions, only five lines displayed significantly distinguishable light output compared to background. Due to possible integration position effects and variations in inserted copy numbers, these cell lines exhibited varying light output (ranging from $10^4$ to $10^6$ p/s for one million cells). The cell line that showed the highest level of bioluminescent production was designated as T-47D/Lux and chosen for further investigation.

The bioluminescent profile of the T-47D/Lux cells was investigated to
determine the optimal time frame for bioluminescence detection. Since it has been shown previously that transiently transfection T-47D cells maintained relatively stable bioluminescence production for 20 h in the imaging system (Chapter II), long term monitoring of approximately $1 \times 10^6$ stable T-47D/Lux cells was attempted for 96 h to determine the maximal time frame for continuous imaging (Figure 15A). As observed in the transient expression assays, bioluminescent output increased in the first 6 h, and then remained relatively stable between 6 h and 12 h with an average hourly total flux increase of $6.62 (\pm 0.10) \times 10^3$ p/s. A rapid increase of light production was observed between 12 h to 26 h with an average increase in total flux of $4.28 (\pm 0.26) \times 10^4$ p/s per hour, followed by another relatively stable phase between 26 h and 40 h. Following this stable phase was a short but relatively rapid rise in bioluminescent expression between 40 h and 48 h, increasing from $8.50 (\pm 0.59) \times 10^5$ p/s to $9.75 (\pm 0.67) \times 10^5$ p/s. After peaking at approximately 48 h post plating, light production rapidly decrease, indicating comprising cellular health and cell death. No significant bioluminescence was observed after ~ 84 h of imaging.

For these cells to be useful as a reporter, the bioluminescent output must be detectable over a dynamic population range. To determine minimum detectable cell numbers, T-47D/Lux cells at concentrations ranging from $2.5 \times 10^3$ to $2.5 \times$
Figure 15. Bioluminescent profile of the constitutively bioluminescent T-47D/Lux cell line.

(A) Ninety-six hour continuous monitoring of approximately $1 \times 10^6$ autonomously bioluminescent T-47D/Lux cells (n=3, mean ± standard error). (B) Correlation between bioluminescent output and overall population size.
10^5 were plated in triplicate in equal volumes of media over a constant surface area for signal detection. Using a 10 min integration time, approximately 2.5 × 10^3 cells could be significantly differentiated from medium background (p < 0.01). It was also revealed that the bioluminescent flux correlated tightly to the number of cells present in a population in a linear fashion (R^2 > 0.99) (Figure 15B), suggesting that the bioluminescent output could be used to as an indicator of population size.

**Bioluminescent dynamics upon estrogen-stimulated cell proliferation**

The T-47D/Lux cells were subsequently examined for their potential application in an estrogen screening assay. To determine if the bioluminescent dynamics could be used to denote estrogen-stimulated cell proliferation, equal numbers of cells were exposed to varying concentrations of E_2. Due to the inability to maintain long term cellular health in the imaging chamber, continuous imaging was not attempted. Instead, cells were incubated at optimal growth condition (5% CO_2 at 37°C) with bioluminescence being measured every 24 h using the IVIS imaging system. Because cells remained intact and attached to the growing surface during measurements, it was possible to track the bioluminescent dynamics of the same population throughout the course of exposure (Figure 16A). While exposure to 0.1 pM E_2 was not capable of increasing bioluminescence significantly compared to unexposed control throughout the 6-day exposure period (Table 10), a significant change in
Constitutively bioluminescent T-47D/Lux cells were grown in RPMI-1640 assay medium containing 10% CD-FBS supplemented with various concentrations of 17β-estradiol (0 (control) to 100 nM) to induce cell proliferation. (A) Because of the autonomous nature of the lux system, bioluminescent production from the same cell population could be measured repeatedly throughout the 6-day period of exposure. (B) Bioluminescent dynamics also responded to 17β-estradiol treatment in a dose-dependent manner, exemplified by the bioluminescent measurements 4 days post exposure (n=3, mean ± standard error).

Figure 16. Dose-dependent response of T-47D/Lux reporter cells to 17β-estradiol exposure.

Constitutively bioluminescent T-47D/Lux cells were grown in RPMI-1640 assay medium containing 10% CD-FBS supplemented with various concentrations of 17β-estradiol (0 (control) to 100 nM) to induce cell proliferation. (A) Because of the autonomous nature of the lux system, bioluminescent production from the same cell population could be measured repeatedly throughout the 6-day period of exposure. (B) Bioluminescent dynamics also responded to 17β-estradiol treatment in a dose-dependent manner, exemplified by the bioluminescent measurements 4 days post exposure (n=3, mean ± standard error).
Table 10. Bioluminescence produced from T-47D/Lux cells in response to 17β-estradiol (E₂) exposure.

Hatched grey boxes indicate bioluminescent output similar to unexposed control ($p > 0.05$ or fold change < 1.2). Orange boxes indicate significantly different bioluminescence compared with unexposed control ($p < 0.05$ and fold change > 1.2).

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>0.1 pM</th>
<th>1 pM</th>
<th>10 pM</th>
<th>100 pM</th>
<th>1 nM</th>
<th>10 nM</th>
<th>100 nM</th>
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bioluminescent production ($p < 0.05$) was observed 3 days after exposure to concentrations of E₂ as low as 1 pM. It was also shown that E₂ stimulated distinctive bioluminescent dynamics in a time- and dose-dependent manner. Treatment with 1 pM E₂ resulted in a significant change in bioluminescence after 3 days of exposure, but the signal was no longer distinguishable from vehicle control after 4 days. Higher concentrations of E₂ were able to elicit light production after 3 days of exposure and were significantly distinguishable from the control for the rest of the exposure period (Table 10). Based on the bioluminescent measurement after 4 days of exposure, it was determined that the EC₅₀ for E₂ in this assay was approximately 10 pM (Figure 16B).

**Development towards an autonomously bioluminescent estrogen reporter**

**Development of a traditional luxAB bioreporter**

A regulatory promoter containing three tandem copies of the ERE (estrogen responsive element) and the adenovirus E₁B minimal TATA box was tested for its capability to mediate estrogen-inducible bacterial luciferase (LuxAB) expression (Figure 17A). This promoter was synthesized *de novo* and cloned into the previously validated pIRES LuxAB vector to replace the constitutive CMV IE promoter. This vector (pERE3-TATA LuxAB) was examined in human breast cancer T-47D cells because they endogenously express both forms of the estrogen receptor (ERα and ERβ) that are essential for estrogen-mediated gene expression.
Transiently transfected T-47D cells were exposed to 10 nM 17β-estradiol (E2) for various times, then lysed and subjected to the cell-lysed based bioluminescence assay to determine luciferase activity. Results showed that expression of the LuxAB proteins was induced by estrogen in a time-dependent manner (Figure 17B). Luciferase activity was increased by ~ 4.5 (± 0.3)-fold over untreated control after 6 h of exposure. Maximal induction within the time frame tested was achieved at 11.3 (± 1.5)-fold after 24 hours of exposure, with peak luminescent expression of 7.64 (± 0.31) × 10^4 RLU/mg total protein. Exposure times longer than 24 h were not attempted because this was the maximal treatment time employed in the conventional literature.

Having demonstrated the effectiveness of this vector for estrogen-inducible expression in transient assays, a T-47D cell line stably expressing the pERE3-TATA LuxAB vector was selected and examined for responsiveness and sensitivity to E2. The reporter cells were exposed to E2 concentrations from 5 pM to 10 nM for 24 hours before proteins were extracted for bioluminescence assays. Data were reported as fold induction over vehicle controls. As shown in Figure 17C, LuxAB activity was induced by E2 in a dose-responsive manner. While 5 pM E2 failed to induce LuxAB expression at a significant level (p > 0.05), treatment with 10 pM E2 increased LuxAB activity significantly (p < 0.05) by ~ 2.00 (± 0.10)-fold. Maximal induction was achieved at 10.10 (± 0.57)-fold by stimulation with 10 nM E2.
Figure 17. 17β-estradiol induced bacterial luciferase (LuxAB) activity.

(A) Schematic representation of the pERE3-TATA LuxAB reporter construct (ERE: estrogen response element, TATA: adenovirus E1B minimal promoter). Bacterial luciferase activity was assayed in (B) T-47D cells transiently transfected with the pERE3-TATA LuxAB reporter vector upon induction with 0 (control) or 10 nM 17β-estradiol (E2) over a 24-hour exposure and (C) in a stable T-47D reporter cell line expressing the same vector upon 24-hour induction within a range of E2 concentrations (n=3, mean ± standard error).
**Bioluminescent measurement of ERE-lux reporter cells in response to estrogen**

Following the successful demonstration of estrogen-responsive LuxAB expression in the pERE3-TATA LuxAB vector, the synthetic ERE3-TATA promoter was tested for its ability to express the full lux cassette for autonomous bioluminescent production using the pERE3-TATA Lux vector. While no background bioluminescence was detected without estrogen induction, treatment with 1 or 10 nM E2 was not able to induce light production to a detectable level. To improve expression, the TATA promoter was replaced by the CMV IE minimal promoter (CMVm), which has been suggested to have a higher basal activity. No detectable bioluminescence was produced in cells expressing the modified vector, regardless of estrogen concentrations.

**Correlating gene expression and bioluminescent production**

The expression of luxC in T-47D cells transiently expressing pERE3-CMVm Lux was evaluated by qRT-PCR and compared with that of stable bioluminescent T-47D cells (Table 11). Two bioluminescent T-47D cell lines stably expressing pCMV Lux, including one with a low level of bioluminescence (~ 1.66-fold over background) and the other with a higher level of light production (~ 90.77-fold over background), were chosen for comparison. While luxC transcription was ~ 52.5% of β-actin transcription in the stable cell line with high levels of constitutive bioluminescence expression, it was only expressed at ~ 6.0% relatively to β-actin
Table 11: Comparison of *luxC* expression level determined by qRT-PCR in various *lux*-expressing T-47D cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th><em>luxC</em> expression level relative to β-actin (%)</th>
<th>Bioluminescence relative to background (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T-47D cells transiently expressing pERE3-CMVm Lux</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-hour treatment with 0 nM E₂</td>
<td>3.15 (2.80 - 3.56)</td>
<td>not significant</td>
</tr>
<tr>
<td>24-hour treatment with 1 nM E₂</td>
<td>6.27 (4.46 - 8.82)</td>
<td>not significant</td>
</tr>
<tr>
<td><strong>T-47D cells stably expressing pCMV Lux</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low bioluminescent expression cell line</td>
<td>6.04 (4.22 - 8.64)</td>
<td>1.66 (± 0.18)</td>
</tr>
<tr>
<td>High bioluminescent expression cell line</td>
<td>52.50 (39.58 - 69.64)</td>
<td>90.77 (± 5.79)</td>
</tr>
</tbody>
</table>
transcription in the low bioluminescent expression cells. In the reporter cells expressing pERE3-CMVm Lux, luxC transcription relative to β-actin transcription increased from ~3.2% to ~6.3% following a 24-hour exposure to 1 nM E₂.

Demonstration of the lux system as a fully autonomous Tet-On reporter

The functionality of the lux cassette as a fully autonomous, real-time reporter for target-regulated gene expression was investigated using a well-characterized Tet-On mammalian expression system. HEK293 Tet-On cells constitutively expressing the Tet-On transactivator were transfected with a tetracycline response element regulated lux reporter construct (pTet Lux) and the dynamics of doxycycline-induced bioluminescent response was determined over a 19 h imaging course (Figure 18). Without doxycycline induction, the Tet-lux reporter cells displayed a background luminescence of ~2.57 × 10⁴ p/s, which decreased gradually over time. Exposure to 100 ng doxycycline/ml induced bioluminescent production that could be differentiated from un-induced control as early as 2.5 hours post treatment (p < 0.05). From this point on, light production continued to increase rapidly with increasing treatment time, reaching a total flux of ~1.33 × 10⁷ p/s after 19 h of exposure.

The expression of the lux genes was also investigated in parallel to determine transcriptional activation following doxycycline induction. Quantitative RT-PCR analysis was performed to measure the expression of the luxC, luxB, and frp genes 19 hours post treatment with 100 ng doxycycline/ml. Compared to
Figure 18. Continuous near-real time monitoring of the bioluminescent response to doxycycline treatment of the HEK293 Tet-*lux* reporter cells.

HEK293 Tet-on cells transiently expressing the pTet Lux reporter construct were treated with 0 (control) or 100 ng doxycycline/ml. Treatment with 100 ng doxycycline/ml increased bioluminescent output as early as 2.5 hours post induction and approached a maximal bioluminescent output of ~ $1.33 \times 10^7$ p/s over the 19-hour exposure period (n=2).
untreated control, the *luxC* and *luxB* genes were up-regulated by ~ 8.6 (range: 7.1 - 10.4)-fold and ~ 8.2 (range: 6.3 - 10.6)-fold, respectively. The *frp* gene was also up-regulated upon doxycycline treatment, but displayed a slightly higher fold change of ~ 13.2 (range: 10.1 - 17.3) compared to control (Figure 19).

**Discussion**

The most distinct advantage of using a *lux*-based human cell bioreporter is its ability to indicate human-relevant effects in a living system and in an autonomous fashion without the requirement of cell destruction or exogenous substrate addition. Thus far, only proof-of-principle demonstrations of *lux* expression in mammalian cells have been provided (Close, Patterson et al. 2010; Close, Hahn et al. 2011), and its application remains limited. The findings in this study represent the use of bioluminescence produced autonomously from human cell lines to directly detect the bioavailability of targets of interest, and facilitate future development of the *lux* system towards high throughput, real time screening systems.

One popular application of bioluminescent reporters is to use the bioluminescent signal to monitor cellular population dynamics. This is made possible because of the quantitative relationship between signal intensity and the number of cells present in a population (Contag and Ross 2002). For instance, cells constitutively expressing firefly luciferase have been widely applied for *in vivo* assessment of tumor growth and response to cancer therapies.
Figure 19. Regulation of lux gene expression in response to doxycycline induction in the Tet-lux reporter cells.

Gene expression was analyzed by qRT-PCR on HEK293 Tet-On cells transiently expressing the pTet Lux reporter vector were treated with 0 (-Dox) or 100 (+Dox) ng doxycycline/ml for 19 hours (n=6 (2 biological replicates each with 3 technical replicates), mean ± standard error).
(Prescher and Contag 2010). However, the firefly luciferase system requires a substrate to be added exogenously prior to data acquisition. To provide a proof-of-principle demonstration of lux-tagged human cell lines for non-invasive monitoring of population dynamics, a constitutively bioluminescent cell line, T-47D/Lux, was developed and evaluated for its potential application as an in vitro bioluminescent cell proliferation assay for estrogenic compounds.

Human breast cancer T-47D cells are estrogen sensitive, meaning that their proliferative behavior is regulated by estrogen (Soto, Murai et al. 1986). Scientific investigations in the past two decades have accumulated evidence suggesting that estrogen regulates cell proliferation by negating the proliferative inhibition exerted by plasma-borne albumins and their interaction with membrane-bound estrogen receptors (Sonnenschein, Soto et al. 1996; Powell, Soto et al. 2001; Powell, Soto et al. 2003). Based on these premises, the E-SCREEN assay was developed 27 years ago for measuring the physiological signature of the action of estrogen (i.e., induction of cell proliferation) and is still widely used for identification of chemicals with potential estrogenic activities (Soto, Sonnenschein et al. 1995). This assay compares the cell yield following 5-day to 6-day incubation in the presence or absence of estrogen. The original method of quantifying cell numbers involved direct nuclei counting, but a higher throughput and less laborious colorimetric MTT assay has been widely adopted as the endpoint measurement of viable cell counts. Regardless of the method used, cell
lysis is required prior to data acquisition, which therefore provides only single time point snapshots.

However, it is demonstrated in this study that by using the autonomous bioluminescence emitted from T-47D/Lux cells as the endpoint of measurement, the same cell population can be monitored repeatedly throughout the course of exposure; therefore, cell proliferation can be captured progressively at any time point of interest. Results show that the bioluminescent signal emitted from these modified T-47D cells responds to estrogen in a dose-dependent manner, exemplified by a typical sigmoidal curve that is similarly obtained using other endpoint measurements (Figure 16B) (Soto, Sonnenschein et al. 1995). $17\beta$-estradiol concentrations to induce maximal and half-maximal ($EC_{50}$) bioluminescence in our assay are approximately 0.1 nM and 10 pM, respectively. These values are comparable to that measured in the traditional E-SCREEN method ($EC_{50} = 7 - 12$ pM using MCF-7 cells) (Soto, Maffini et al. 2006). The minor discrepancies between our results and those reported in other studies are most likely due to logistical factors such as differences between cell line clones and culture conditions (Zacharewski 1997). However, in general, using bioluminescent output as an indicator for cell proliferation, estrogen can be detected in the picomolar range.

It is clear that for these bioluminescent cells to be widely applied as a screening method for estrogenic substances, detailed validation and comparison
with current bioassays will be required. However, during this proof-of-principle demonstration, similar results to those obtained using the traditionally methods following estrogen treatment were acquired. Additionally, this method offers the advantage that data acquisition can be performed in a fully automated fashion since the need for sample destruction or substrate addition is eliminated, making it an ideal candidate for high-throughput analysis. It also permits the tracking of dynamic effects upon the same population over time, which is not feasible using traditional cell lysis-requiring methods. As an example, treatment with low levels of estrogen (0.1 pM) only produced intermittently significant signal over the 6-day course of exposure (Table 10). These types of dynamics would likely be overlooked using the traditional single time point approach. Moreover, these autonomously bioluminescent cells have the potential to be implemented into an in vivo tumor model for basic and preclinical research, as they permit non-invasive interrogation of cellular events in living animals.

Another important application of bioluminescent reporters is for the detection of specific target compounds via monitoring target-regulated expression of their reporter gene. Under this strategy, the expression of the reporter gene is linked to a promoter that is responsive to the target analyte. In this study, it was further investigated whether or not lux expression could be regulated by an estrogen-responsive promoter for estrogenic compound detection. Because the promoter architectures for optimized estrogen regulation are not well studied, a synthetic
promoter was first evaluated using the LuxAB reporter system before being applied for full *lux* cassette expression. This promoter, consisting of three tandem copies of EREs spaced 4 bp apart and a TATA promoter located 8 bp downstream of the EREs, was shown to be capable of functionally regulating LuxAB expression in response to a dynamic range of E2 concentrations (Figure 17C). While a 24-hour exposure to 10 pM E2 induced LuxAB activity significantly, the most rapid increase was observed at E2 concentrations between 100 pM and 10 nM, with a maximal induction of ~10-fold over untreated control. Although this maximal fold change is similar to a previously published study using firefly luciferase as a reporter in T-47D cells, Wilson et al. have previously reported a lower detectable concentration at 0.1 pM, with maximal induction occurring at 100 pM (Wilson, Bobseine et al. 2004). The variation in sensitivity in our system is not unexpected due in part to the relatively low quantum yield of the bacterial luciferase luminescent reaction compared to that of the firefly luciferase reaction. Other factors, including variations in ERE substitution mutations and flanking sequences, the relative position of the TATA promoter to the EREs, and the genetic content linking the promoter and the protein coding region, can also contribute to the disparity in reporter gene expression (Klinge 2001).

Despite the effective regulation of LuxAB expression, this promoter and its CMVm variant were not capable of expressing the full *lux* cassette at the level required to produce detectable bioluminescence in cell culture, regardless of
estrogen induction. However, qRT-PCR analysis did show that expression of the *luxC* gene increased by ~1.99 (1.38 - 2.85)-fold after 24-hour exposure to 1 nM E2, suggesting that the promoter activity did up-regulate gene expression in an estrogen-responsive fashion. Therefore, the discrepancy in the detectability of these endpoint measurements (i.e., enzyme activity and bioluminescent flux) is likely caused by variations in the sensitivity of the detection scheme. To estimate the threshold of *lux* expression for detectable autonomous bioluminescence, we selected two stable bioluminescent T-47D cell lines - one with a minimum level of bioluminescence (~1.66 fold over background) and the other displaying a much greater level of light production (~ 90.77 fold over background) - and compared the transcript level of the *luxC* gene in these two cell lines to that in cells expressing the pERE3-CMVm Lux reporter construct. As expected, higher light output was associated with a significantly higher level of gene expression, expressing *luxC* at ~52.5% of β-actin gene expression compared to ~6.0% in the low expression bioluminescent cells (Table 11). It was also confirmed that the ERE3-minimal promoter was only capable of expressing *luxC* at ~3.2% without and ~6.3% following estrogen induction relative to β-actin gene expression. Therefore, the most probable explanation for the inconsistency of detectability between LuxAB activity and light output measurements is that autonomously bioluminescent detection requires a relatively high level of expression of all six *lux* genes.
Due in part to the poor performance of estrogen-regulated bioluminescent expression, an alternative regulation system was utilized to assess the potential of \textit{lux}-based bioluminescence being used for detection of targets of interest. By using a tetracycline-responsive promoter that has been well characterized and extensively optimized for tight regulation and high levels of expression in mammalian cells, the ability of \textit{lux} expression to function as an autonomously bioluminescent reporter for doxycycline detection has been demonstrated in this study. Unlike other bioluminescent reporters that require substrate addition (and in some cases cell lysis) to produce a detectable signal (therefore providing only single time point measurements), the \textit{lux} reporter system allows for continuous monitoring of the same sample without cell destruction or exogenous substrate addition. This allowed bioluminescent production from cells transiently expressing the pTet Lux vector in response to doxycycline to be recorded in a continuous, near real-time manner. Without doxycycline treatment, these reporter cells displayed a low level of bioluminescent production ($\sim 2.6 \times 10^4$ p/s), which decreased gradually over time, possibly due to compromised cellular metabolism caused by nutrient depletion from the medium and the inability to regulate the CO$_2$ level and humidity in the imaging chamber. In contrast, treatment with 100 ng doxycycline/ml resulted in increasing light production throughout the course of treatment. The minimal time to induce significantly different light production was determined to be $\sim 2.5$ h for the 100 ng doxycycline/ml treatment. It was also demonstrated that an approximate 800-fold change in light production could be
achieved within 19 hours post treatment under the same treatment level (Figure 18). Ultimately, under this expression strategy, the maximal light production induced by doxycycline was greater than that from equal numbers of cells expressing identical genes under the control of a constitutive CMV IE promoter (Chapter II), which was in agreement with previously published studies (Qin, Zhang et al. 2010).

A direct comparison of these values to previously published literature using similarly constructed firefly luciferase reporters would be desirable but is not possible for several reasons. First, most of the published research normally reports fold induction at sporadic time points but not minimal induction time, which is probably prohibited by the extensive amount of laboratory work and sample size that would be required for providing this type of information in the firefly luciferase system. Second, luciferase activity is mostly reported in arbitrary relative light units instead of direct photon counts used in our study, thus the varying sensitivity between detection methods would affect the direct comparison of fold changes. Third, it is known that the activity of the tetracycline-responsive promoter varies in different cell types (Gossen, Freundlieb et al. 1995). Therefore, a relevant comparison would require that the same cell type, transfection method, promoter, and inducing concentration all be maintained across two studies.

Therefore, in order to provide objective interpretations, the lux-based bioluminescence data was compared with gene expression data obtained from
the same cells. It was determined that, while transcription of the \textit{lux} genes increased by approximately 8-fold after a 19-hour exposure to doxycycline at a concentration of 100 ng/ml (Figure 19), the corresponding increase in light production was \~ 800-fold under these same conditions (Figure 18). These results suggest that a relatively small up-regulation in gene expression can significantly amplify the bioluminescent output. Because of the relatively low signal intensity of the \textit{lux} system compared to the firefly luciferase system when both are expressed constitutively (Close, Hahn et al. 2011), there have been concerns over the detectability of the \textit{lux} system under inducible conditions. However, our results demonstrated that a sufficiently high signal-to-noise ratio could be achieved using the Tet-On regulation system. Most importantly, however, these results suggest that real-time, continuous tracking of gene expression regulated by the target of interest (doxycycline in this case) makes \textit{lux}-based bioreporters ideal candidates for detecting the on-set and dynamics of gene expression.

Unlike the synthetic tetracycline-responsive promoter that has been well characterized and extensively optimized for tight regulation and high levels of expression in mammalian cells (Gossen, Freundlieb et al. 1995; Agha-Mohammadi, O'Malley et al. 2004; Loew, Heinz et al. 2010), promoter architectures for optimized estrogen regulation are poorly studied. The literature suggests that the EREs are relatively weak regulatory elements, with most
natural EREs only inducing reporter gene activity by less than 10-fold (reviewed by Klinge (2001)). In contrast, the Tet-On system can induce firefly luciferase activity by several thousand fold (Gossen, Freundlieb et al. 1995), which explains why the Tet-\textit{lux} reporter has enjoyed commercial success. This discrepancy is also affirmed by our data which indicates that doxycycline and estrogen induced \textit{luxC} transcription by ~ 8- and ~ 2-fold, respectively.

For these reasons, it becomes clear that extensive optimizations of the current estrogen-responsive promoter to improve transcriptional activation will be an important route for development towards a functional and autonomous \textit{lux}-based estrogen reporter system. A potential route for optimization could be to modify the flanking regions of the EREs to contain AT-rich sequences since they have been found to improve estrogen receptor binding and transcriptional activation (Klinge, Jernigan et al. 2001). In addition, the copy numbers of ERE and the distance between them have also been shown to mediate synergistic activation (Tyulmenkov, Jernigan et al. 2000) and could be modified accordingly. And finally, because estrogen receptors interact with both general transcriptional factors and other co-regulators (Klinge 2000), the spacer between the EREs and the minimal promoter could be modified to alter the steric hindrance resulting from transcriptional initiation complex formation at the TATA box. This represents a particularly attractive first step for optimization because it has resulted in up to a 100-fold induction of firefly luciferase activity when using a promoter containing
three tandem ERE repeats and a TATA minimal promoter with an ~ 30 bp linker (Legler, van den Brink et al. 1999).

Optimizations can also be made to the configuration of the lux cassette. There have been reports suggesting that splitting the lux cassette into two small transcription units with one being constitutively expressed and the other under regulation can result in improved reporter performance compared to regulating all lux genes at once in E. coli (Yagur-Kroll and Belkin 2011). Mathematical modeling also predicts that induction of the luxC and luxE genes would provide the most rapid control over bioluminescent production (Welham and Stekel 2009). While these predictions remain to be validated in the mammalian cellular background, the Tet-lux reporter system demonstrated in this study will be an ideal candidate for these investigations.
CHAPTER V

Summary and Conclusions
Bioluminescent sensing is an emerging biomedical surveillance strategy that uses external cameras to detect \textit{in vivo} light generated in small animal models of human physiology or \textit{in vitro} light generated in tissue culture or tissue scaffold mimics of human anatomy. At the core of this technology are cells expressing light emitting reporter proteins that reveal the activation dynamics of cellular and molecular functions. As the most recent addition to the bioluminescent reporter protein family for mammalian expression, the \textit{lux} system is the only reporter that permits fully autonomous signal production without the requirement of exogenous excitation or substrate addition, making it an ideal candidate for continuous real-time monitoring of cellular events in cell culture and living animals. However, the signal intensity of the previously characterized \textit{lux} expression system is much lower than that of other bioluminescent reporter systems (Close, Hahn et al. 2011), and thus provides a major limitation preventing its more widespread adoption. In addition, the previously validated \textit{lux} expression strategy requires two vectors for autonomously bioluminescent production (Patterson, Dionisi et al. 2005; Close, Patterson et al. 2010), which complicates the process of generating additional bioluminescent cell lines. For these reasons, the application of the mammalian-adapted \textit{lux} system remains limited. Overall, this dissertation has aimed to 1) develop a more simplistic and efficient format for expression of the \textit{lux} cassette in human cell lines, 2) identify the constraints limiting bioluminescent expression in the previously characterized bioluminescent cells and develop a strategy to improve light output, and 3) validate the capability of \textit{lux}-expressing
human cell lines for monitoring population dynamics and detecting the bioavailability of targets of interest. The following conclusions have been drawn from this investigation in regards to the initial hypotheses:

Hypothesis 1: Through the use of viral 2A peptides as linker regions, it will be possible to simultaneously express up to six genes (the luxCDABEfrp gene cassette) under the regulation of a single promoter for autonomous bioluminescent production in human cell lines.

This investigation has demonstrated, for the first time, the capability of viral 2A peptides to mediate efficient expression of up to six genes. 2A-mediated polycistronic expression of the codon-optimized lux cassette has been shown to be capable of producing constitutive bioluminescence under the regulation of a single promoter in the human cellular background. While the previously characterized two plasmid system is thus far limited to HEK293 cells, due likely to the complicated process of generating cell lines stably expressing two expression vectors, the 2A-based single vector polycistronic expression format has been validated in a panel of human cell lines and reduces both the time and effort required to develop additional bioluminescent cell lines. The coupled transcription and translation of the lux genes, conferred by the use of a single promoter and 2A elements capable of mediating highly efficient stoichiometric protein expression, has been shown to improve bioluminescent production over the IRES-based two plasmid expression system.
Hypothesis 2: The human cellular background is capable of supplying sufficient endogenous substrates for high level \textit{lux}-based bioluminescent production. Therefore, it will be possible to improve bioluminescent expression through increased gene integration.

It has been shown that \textit{lux} gene copy number is a major limiting factor for bioluminescent production in the previously characterized, un-optimized bioluminescent HEK293 cells. While additional copies of the luciferase genes (\textit{luxAB}) or the substrate processing genes (\textit{luxCDEfrp}) increased bioluminescent flux to a certain extent, the largest output gain (~ 80-fold) was achieved following introduction of the full \textit{lux} cassette assembled into a single vector. These results support the initial hypothesis that the availability of endogenous substrates in the human cellular background is not a limiting factor for light production in the un-optimized bioluminescent cells.

The results obtained in this investigation have also lead to the development of a two-step transfection strategy for high level bioluminescent expression. By pre-establishing a stable cell line expressing a partial \textit{lux} sequence (i.e., \textit{luxAB}), it was possible to synergistically improve the expression of the latterly introduced \textit{lux} cassette. Both the data presented in this study, and in the literature, implies that homology-mediated gene integration may play a role in this improved expression, however, detailed mechanisms still remain to be assessed experimentally in order to identify the exact integration locus.
An optimized HEK293 cell line with enhanced bioluminescent production has been constructed using the two-step transfection strategy. The enhanced signal intensity allows bioluminescent detection from a smaller population size (~15 fold lower) in cell culture compared to their un-optimized counterparts. Although their signal intensity is still not as strong as other conventional bioluminescent reporters, the optimized cells offer an improved dynamic detection range, which will certainly provide a substrate-free alternative for desired applications. In addition, due to the improved photonic flux, the enhanced bioluminescent cells have been shown to be compatible with multiple types of detection, including highly sensitive CCD camera-based imaging and the relatively less sensitive, but lower cost and more commonly used, photomultiplier tube (PMT)-based plate reading. The adoption of the autonomously bioluminescent lux-expressing cells for use under these conditions has the capacity to facilitate both high throughout and on-line monitoring applications.

Hypothesis 3: Bioluminescent production dynamics in estrogen-responsive human breast carcinoma T-47D cells can be used as an indicator for estrogen-regulated cell proliferation.

It has been shown here that bioluminescence constitutively emitted from lux-expressing cells can be utilized for monitoring population dynamics in a continuous and non-invasive manner. A constitutively bioluminescent T-47D/Lux cell line has been developed using the polycistronic lux expression vector and
utilized in an estrogen screening assay. Results obtained in this investigation show that the estrogen-induced, dose-dependent cell proliferative response is comparable to that obtained using traditional methods, and can be captured by using the autonomous bioluminescence produced from living cells as an endpoint measurement (Soto, Sonnenschein et al. 1995; Soto, Maffini et al. 2006). It has also been shown that estrogen can be detected in the picomolar range using this method. While detailed validation and comparison with current bioassays will be required before these bioluminescent cells can be widely applied as a screening method for estrogenic compounds, this method has the potential to be implemented as a fully automated, high throughput analysis tool since the need for sample destruction or substrate addition is alleviated. In addition, this system also permits the tracking of dynamic effects upon the same population over time, which is not feasible using traditional cell lysis-requiring methods.

Hypothesis 4: By simultaneously regulating the expression of the $lux$ genes assembled in a polycistronic single vector, it will be possible to construct a bioluminescent reporter capable of responding to target analytes autonomously and in a near real-time manner.

The ability of $lux$ expression to function as an autonomous bioluminescent reporter for the detection of targets of interest has been demonstrated in this investigation. Although the $lux$ system is not currently amenable for autonomously bioluminescent detection of estrogenic compounds due to the
relatively weak transcriptional activation elicited by the estrogen response elements, it has been shown that regulation of the lux genes under the control of a single tetracycline-responsive promoter is capable of governing bioluminescent production in response to administration of the inducing compound doxycycline. The 2A-mediated polycistronic assembly of all six lux genes provides a simplistic means for maintaining low background bioluminescent expression and tightly regulated, increased bioluminescent production upon induction. It has further been shown that lux-based bioluminescent output is highly sensitive, with a relatively small up-regulation in gene expression yielding a sufficiently high signal-to-noise ratio expression for facile detection. Unlike other substrate-requiring bioluminescent reporters that provide only single time point measurements, the autonomous lux reporter system permits doxycycline-regulated bioluminescent production from the same cell population to be recorded in a continuous, near real-time fashion, therefore making it an ideal candidate for detecting the on-set and dynamics of target-regulated gene expression.

Since the estrogen-responsive promoter has not yet enjoyed the same extensive optimization as the synthetic tetracycline-responsive promoter has for high level of expression in mammalian cells, it is suggested that future work be focused on modifying the estrogen-responsive promoter architecture with the aim of enhancing transcriptional activation as an important route for developing an
autonomously bioluminescent estrogen bioreporter.
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VITA

Tingting Xu was born in Jingdezhen, Jiangxi, China on October 10, 1985. She spent the first 17 years of her life in Jingdezhen, where she finished her primary, middle, and high school education. After graduating high school in 2002, she moved to Shanghai, where she spent four years at Shanghai Jiao Tong University for her college studies. She graduated with a Bachelor Degree with a major in bioengineering in 2006. Immediately following graduation, she moved again, this time more than 7,500 miles and across the Pacific Ocean, to Knoxville, TN, USA to attend The University of Tennessee Knoxville for graduate study. She received her PhD in December 2012.