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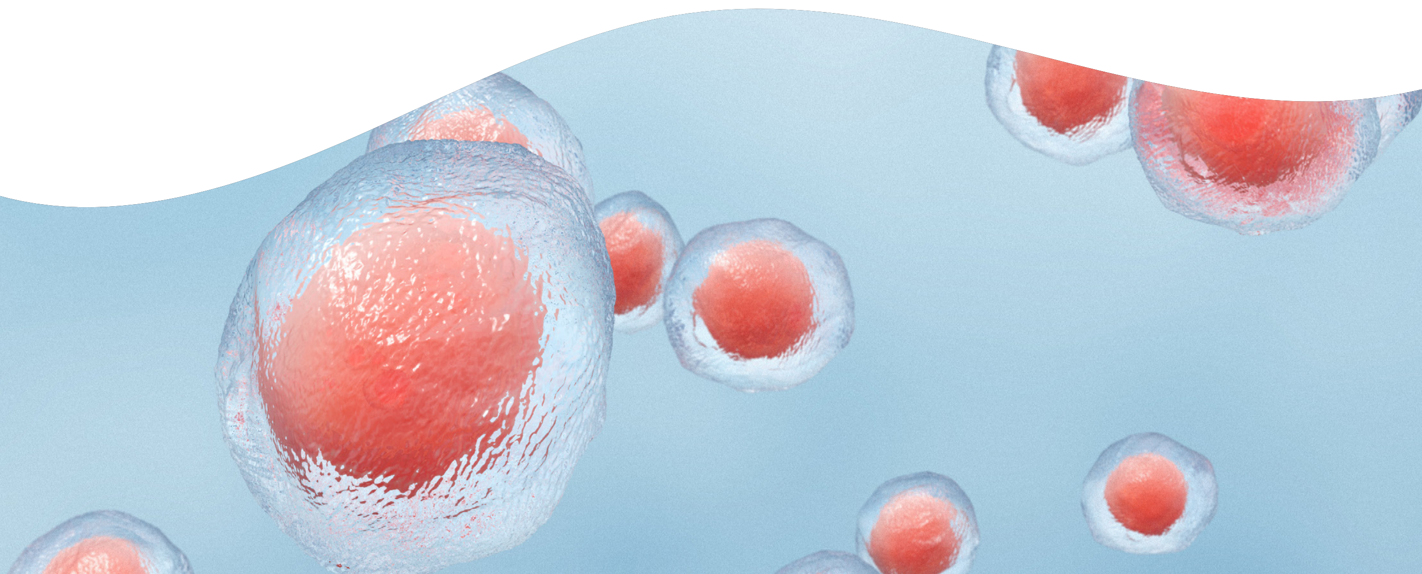
APPLICATION BRIEF

Improved deep-tissue imaging with red-shifted luciferase tumor cell lines.

Widely used for *in vivo* preclinical research and drug development in areas such as cancer and infectious disease, luciferase reporters provide a simple, sensitive means to measure tumor growth and assess drug efficacy through *in vivo* bioluminescence imaging (BLI). Yet it remains an issue that commonly used firefly luciferase generates relatively low bioluminescent signal. Thus, these applications face challenges in the detection of low numbers of luciferase-expressing cells *in vivo*. This low signal makes it especially difficult to detect expressing cells located in deep tissues, a common issue in certain types of aggressive tumors.

To improve luciferase brightness and address the problem of deep tissue imaging, a new codon-optimized luciferase (Red F-luc) from *Luciola italica* was developed which has a red-shifted emission peak wavelength of 617 nm, providing better tissue penetration when compared to peak emissions of 550 nm (Luc) and 590 nm (other commercially available reagents) (Fig. 1). In addition, Red F-luc has approximately 100-fold higher signal intensity compared to the other firefly luciferases. With an emission spectrum red-shifted approximately 20 nm compared to competitor enhanced firefly luciferase genes, and increased brightness, Red F-luc provides improved sensitivity of imaging in deep animal tissues.



Emission spectrum of Red F-luc and competitor luciferases

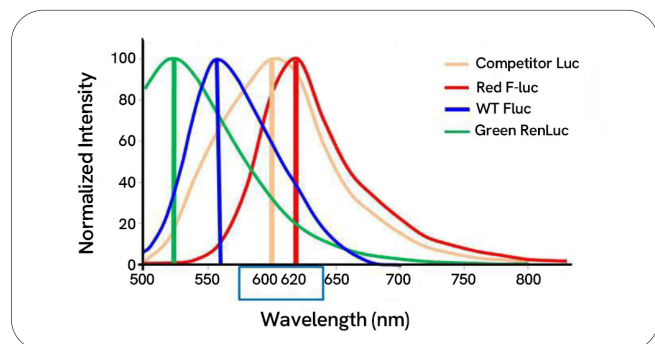


Figure 1: The emission spectrum of Red F-luc is red-shifted approximately 20 nm compared to competitor enhanced firefly luciferase gene for improved sensitivity of imaging in deep animal tissues.

Study design

To generate the construct, a lentiviral vector was developed in which Red F-luc expression is driven by the human ubiquitin C promoter. The Red F-luc gene was fused to GFP or Puromycin resistance gene via T2A linker peptide for selection of transduced cells and *ex vivo* analysis of implanted cells. A total of 20 transgenic cancer cell lines were generated by lentiviral transduction with the Red F-luc construct, selected for puromycin resistance or GFP expression, and subcloned for stable performance (IVISbrite™ tumor cell lines, Revvity) (Fig. 2).

Schematic of lentiviral transfection process

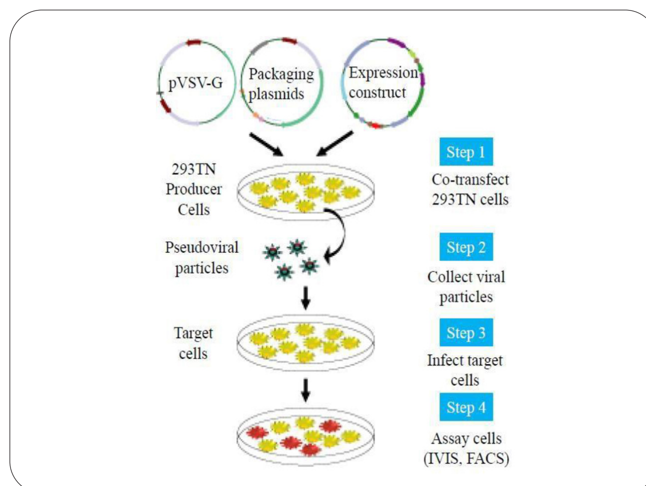


Figure 2: Lentiviral particles are generated by co-transfecting, expressing, packaging, and enveloping vectors into HEK293TN cells. Vesicular Stomatitis Virus G glycoprotein (VSVG) pseudo-typed particles can easily infect a wide variety of mammalian cells including most cancer cell lines, primary, stem, and non-dividing cells.

Once the construct was generated, several studies were conducted, both *in vitro* and *in vivo*, to determine the potential of Red F-luc to address the challenges that limit BLI imaging. First, measurements were taken *in vitro* to compare the brightness of IVISbrite Red F-luc tumor cells against competitor luciferase cells, as well as compare the morphology and growth kinetics against wild type cells. For *in vivo* imaging studies, the bioluminescent signal of Red F-luc transfected cells was measured as tumor volume increased, and the tumor volume and growth rate were compared to wild type cells. Orthotopic tumor imaging was conducted using transfected Red F-luc or competitor-luciferases which were transferred into the brains and lungs of nude mice. Additionally, the range of BLI signal was interrogated via comparative cell dosing experiments. Finally, the stability and bioluminescence quantifiable levels of Red F-luc cell lines were characterized and validated (Figs. 3-6).

Study findings

It was demonstrated that transfected Red F-luc prostate tumor cells had increased brightness when compared to a competitor luciferase, as well as similar morphology and growth kinetics when compared to wild type (WT) cells *in vitro* (Fig. 3A). Detection sensitivity of transgenic Red F-luc prostate tumor cells was confirmed *in vivo* after injection and throughout the timepoints shown (Fig. 3B). BLI signal was found to correlate with tumor volume, as compared to standard luciferase-expressing tumor lines. Tumor volume and growth rate when compared to wild type cells was not affected by the lentiviral transduction.

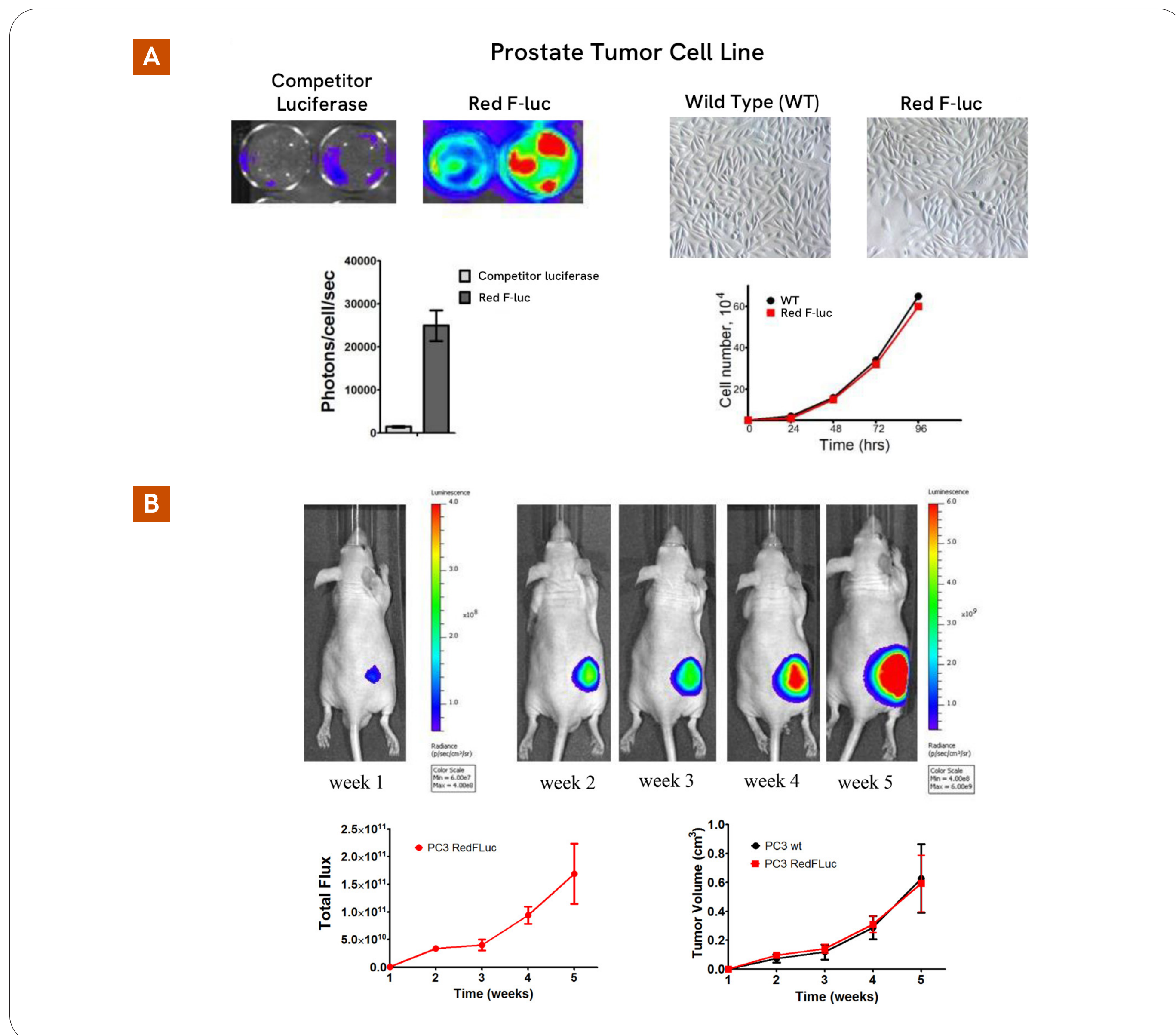


Figure 3: **(3A)** *In vitro* characterization of prostate tumor cells representative of the increased brightness of Red F-luc compared to competitor luciferase as well as the similarity in morphology and growth kinetics of Red F-luc to wild type (WT) cells. **(3B)** The bioluminescent signal of IVISbrite Red F-luc transfected prostate tumor cells, using the IVIS™ SpectrumCT system, correlated with tumor volume, and was found to maintain a growth rate similar to wild type cells *in vivo* (left vs right panels)

When U87MG-Red F-luc cells were injected into the brains of nude mice and the orthotopic tumors were imaged, cells demonstrated sufficient brightness for deep tissue detection and imaging. (Figure 4, upper panel). Red F-luc transduced A549 lung cells were also injected orthotopically into the lungs of nude mice (figure 4, lower panel).

When both 2D and 3D (DLIT) modes of bioluminescence imaging were generated, it was shown that sufficient brightness was produced for 2D detection and improved localization by 3D reconstructions, demonstrating advantage for deep tissue detection and imaging.

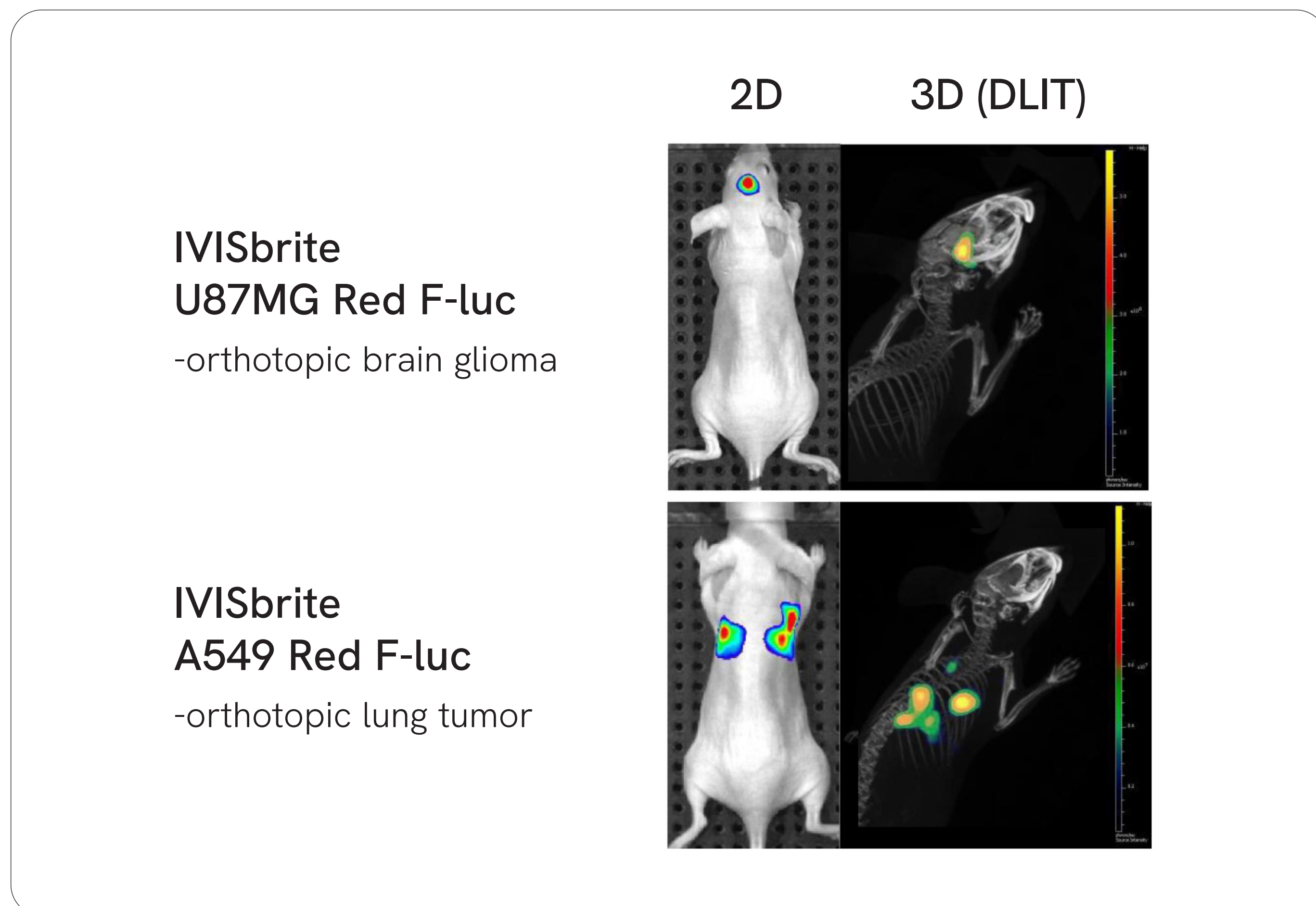


Figure 4: *In vivo* BLI deep tissue detection of orthotopic tumors. For these experiments, 300,000 IVISbrite U87MG-Red F-luc cells were injected into brains of nude mice and the tumors were imaged 2 weeks post injection (upper panel). Red F-luc transduced A549 lung cells were injected orthotopically into lungs of nude mice (lower panel). Both 2D and 3D (DLIT) modes of bioluminescence imaging were generated.

To interrogate the potential range of BLI signal, cell dosing experiments were conducted using both 4T1-competitor and IVISbrite 4T1 Red F-luc cells (Figs. 5A and 5B). The cells were injected intravenously into the tail vein of nude mice, followed by immediate injection with luciferin. Imaging was performed using the IVIS SpectrumCT imaging system and the resulting BLI signal was quantified for different cell doses (1/10th, 1/100th, and 1/1000th of the starting dose of cells, which was 100k for competitor and 70k for Red F-luc. The number of cells was adjusted to reflect the 30% brighter Red F-luc BLI signal compared to the signal of competitor cells). IVISbrite 4T1 Red-F-luc cells, shown on the right in each mouse pair, were able to be visualized in deep tissues, even for lower doses tested (Fig. 5A). Moreover, they exhibited greater brightness than competitor cells when quantified (Fig. 5B). Our findings suggest that even when the cell dose was initially adjusted according to tumor cell brightness and the doses were sequentially decreased, IVISbrite Red F-luc-expressing tumor cells still yielded higher signal from deep tissue *in vivo* as compared to native luciferase-expressing cells, demonstrating that the red-shifted light produced by Red F-luc luciferase more efficiently penetrates tissue at low dosages compared to competitor luciferase.

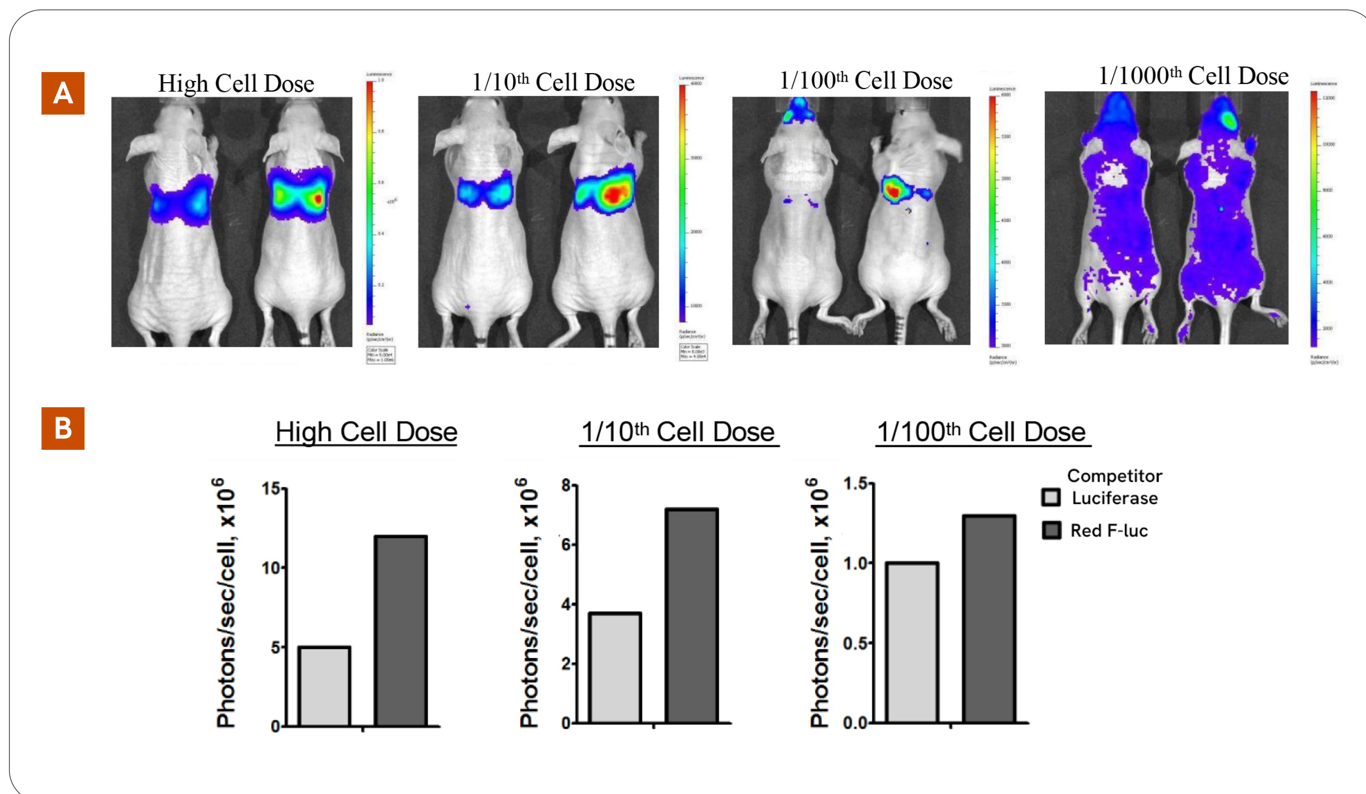


Figure 5: In all mouse images above, 4T1-competitor cells are shown on the left and IVISbrite 4T1 Red F-luc cells are shown on the right. To best assess the impact of spectral differences between the two different luciferases, the number of Red F-luc cells injected in each dose was adjusted to reflect the 30% brighter Red F-luc BLI signal compared to the signal of competitor cells. **(5A)** A "high" cell dose of both 4T1-competitor and IVISbrite 4T1 Red F-luc cells (100,000 versus 70,000 cells, respectively) was injected i.v. into the tail vein of nude mice, and the animals were immediately injected with luciferin and imaged on the IVIS SpectrumCT. Similarly, experiments were performed with 1/10th, 1/100th, and 1/1000th the number of cells for each luciferase. **(5B)** Quantification of the resulting BLI signal suggests that the red-shifted light produced by Red F-luc penetrates tissue much more efficiently compared to competitor luciferase

Photon output of the Red F-luc cloned lines in the previous figures ranged from 8,000 to 40,000 photons/cell/sec, nearly an order of magnitude higher than the brightest bioluminescent cell line reported at the time of the study, with little or no impact on cell morphology or growth *in vitro*, and little or no impact on tumor growth and volume *in vivo*.

A comparison of the full portfolio of IVISbrite Red F-luc tumor cell lines against cells expressing competitor luc showed consistently higher brightness in the Red F-luc expressing cell lines, ranging from 4,000 - 45,000. On average, Red F-luc cell lines were 74-fold brighter than their counterparts (Fig 6). Although both reporter systems showed excellent performance *in vitro* and *in vivo*, the brighter reporter system of Red F-luc would be expected to offer increased sensitivity of detection for small numbers of cells *In vivo*.

Cancer type	Cell line	Competitor luciferase	IVISbrite Red F-luc (photons/cell/second)
Brain	GL261	6190	19000
	U-87MG	1250	10000
Breast	4T1	6500	17500
	AT1 GFP	170	14000
	MCF-7	700	14000
Colorectal	Colo205	200	8600
	HCT-116	1600	28000
	HT29	1590	45000
Fibrosarcoma	HT1080	2200	42000
Leukemia/Lymphoma	K562	1285	4400
Liver	Hep G2	960	43800
Lung	A549	50	20000
	LL/2	10	5900
	NCI-H460	1170	5600
Melanoma	B16F10	450	12300
Ovarian	Skov3	100	14700
Pancreatic	BxPC3	370	17000
Prostate	LNCaP	30	4000
	PC3	1450	39400
	PC3 GFP	750	10000

	Competitor luciferase	IVISbrite Red F-luc
Range of brightness	10 - 6500	4000 - 45000 photons/cell/second
Average Red F-luc:Competitor Luciferase Ratio	74X ± 30	

Figure 6: Comparison of multiple stably cloned tumor lines, expressing either competitor or Red F-luc luciferase, showed consistently higher brightness in Red F-luc expressing lines. On average, Red F-luc lines were 74-fold brighter than their counterparts.

Conclusion

Experimental tumor cell lines serve as important models for human cancer. They allow scientists to conduct experiments that provide insights into neoplasia biology and techniques, agents, and regimens that could enhance cancer detection, prevention, and treatment. With the goal of developing novel reagents and applications for the advancement of *in vivo* imaging, a new codon-optimized luciferase from *Luciola italica* that we call Red F-luc offers the main advantages of a red-shifted emission peak wavelength of 617 nm and approximately 100-fold higher signal intensity compared to the native firefly luciferase.

We have created multiple transgenic cancer cell lines expressing the Red F-luc construct (IVISbrite cell lines) that show extremely high photon output ranging from 4,000 to 45,000 photons/cell/sec. Validation testing of the cells showed no differences in the morphology or growth patterns of transduced cells either *in vitro* or *in vivo* compared to their corresponding parental cell lines. Comparison of multiple cloned tumor lines, expressing either Red F-luc or competitor's equivalent, showed consistently higher brightness in Red F-luc-expressing lines, with marked improvement in tissue penetration at low dosages due to both increased brightness and the spectral properties of the emitted light. On average, our IVISbrite Red F-luc lines were 74-fold brighter than their counterparts. Moreover, due to the red-shifted emission wavelength of the new luciferase, transgenic Red F-luc cells implanted in deep tissues *in vivo* generate about 50% higher BLI signal compared to competitor luciferase.

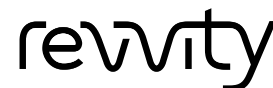
Our *in vivo* imaging reagents are designed for your research in developing molecular and cell-based anti-cancer therapeutics. Here, our results demonstrate the improved efficiency and brightness of our IVISbrite Red F-Luc cell line compared to competitor cell lines and illustrate proof-of-concept of the seamless integration of our IVISbrite bioluminescent reagents and IVIS SpectrumCT imaging platform for *in vivo* imaging studies.

Key takeaways

- Luciferase reporters provide a sensitive means to measure important biological processes in cancer biology and tumor monitoring are useful in assessing drug efficacy through *in vivo* bioluminescence imaging.
- A codon-optimized luciferase (Red F-luc) presents a red-shifted emission peak wavelength of 617 nm and approximately 100-fold higher signal intensity compared to the native firefly luciferase.
- Multiple transgenic cancer cell lines (IVISbrite) were generated by lentiviral transduction with the Red F-luc construct showing high photon output that ranged from 4,000 to 45,000 photons/cell/sec.
- Transgenic Red F-luc cell lines were on average 74-fold brighter than their counterparts. Cells implanted in deep tissues *in vivo* generate around 50% higher BLI signal compared to competitor luciferase. This improvement in tissue penetration allowed for imaging smaller doses of cells in deeper tissues.

References

1. Yevtodiyenko, A Mao, H, Vasquez, KO & Peterson, JD. (2014). "Using a brighter, red-shifted luciferase for *in vivo* tumor bioluminescence imaging." [Poster Presentation]. Presented at the World Molecular Imaging Congress. Seoul, Korea.
2. Branchini, BR et al. (2006). "Luciferase from the Italian firefly *Luciola italica*: molecular cloning and expression." *Comp Biochem Physiol B Biochem Mol Biol,* vol. 145, no. 2, pp. 159-67.



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