A Real-Time Assay of Caspase 3/7 Activity in Cell Culture and Animal Models

by Brock F. Binkowski and Braeden L. Butler

Introduction

In an effort to study protease activity in a more physiologically relevant context, Promega has expanded its patented GloSensor™ technology platform to include luminescent biosensors for the real-time, non-lytic analysis of protease activity in cultured cells or animal models. Here we describe work performed in association with collaborators at the University of Michigan (1) to develop and validate a biosensor for caspase 3/7.

Improved Biosensor Design

Circularly permuted firefly luciferase provides sensitive detection of protease activity, as demonstrated by the Protease-Glo™ Assay (Cat.# G9451). This cell-free approach offers a facile way of generating and screening numerous biosensor constructs containing peptide cleavage sites of variable length and composition, including proteases that require P´ residues (2). Although robust in a biochemical format, the biosensor construct associated with the Protease-Glo™ Assay showed reduced performance in cell-based assays.

We used directed evolution to develop a new firefly luciferase scaffold that shows superior performance for detection of protease activity in living cells. Like previous designs, a protease cleavage site is used to fuse the original N- and C-termini, and circular permutation is used to create new termini elsewhere in the structure (Figure 1). In the absence of cleavage, a steric constraint keeps the biosensor largely inactive. Following protease cleavage, the constraint is lifted, and large increases in luminescence result. A DEVDG cleavage site was used in this context to create a caspase-3/7 biosensor.

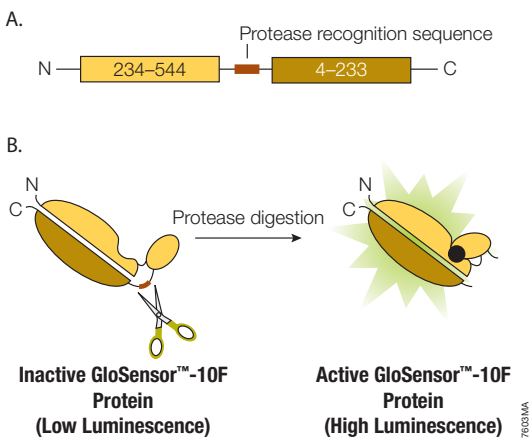

Figure 1. Principle of a real-time biosensor assay for caspase 3/7. A peptide containing the DEVDG sequence was used to fuse the original N- and C-termini of an engineered firefly luciferase, with new termini created via circular permutation. Cleavage at the constrained 'hinge' results in active enzyme.

Monitoring Apoptosis in Cell Culture

Galbán *et al*. stably expressed the caspase-3/7 biosensor in 1833 breast cells and D54 glioma cells (1), which were then used to monitor caspase activation in tissue culture following addition of the cell-permeable substrate (GloSensor™ cAMP Reagent, Cat.# E1290).

Promega has developed a general approach to monitor protease activity in living cells in real-time using genetically encoded biosensors. This approach can be used for cultured cells or in animal models.

Both cell lines showed large increases in luminescence following treatment with inducers of extrinsic and intrinsic apoptotic pathways (maximal induction of 20- to 200-fold), with the kinetics of induction varying between insults. Biosensor activation was also used as a surrogate for apoptosis in a high-throughput screen for anticancer agents, demonstrating the utility of the assay to identify compounds that induce apoptosis with widely different kinetic profiles. Results from Promega also demonstrate the large differences in signal magnitude and kinetics that can be seen between different types of apoptotic insults (Figure 2, Panel A), results that were also seen following addition of Bright-Glo™ reagent (Cat.# E2650) to demonstrate use of an alternative assay format involving cell lysis (Figure 2, Panel B).

Monitoring Apoptosis in vivo

In SCID mice, Galbán *et al*. implanted xenografts of 1833 cells stably expressing caspase-3/7 biosensor and monitored caspase activation following treatment with inducers of apoptosis and interperitoneal injection of 150mg/kg of VivoGlo™ Luciferin (Cat.# P1041, Figure3). As observed with in vitro studies, large increases in luminescence were detected in vivo (maximal fold induction of 30- to 100-fold), and differences were seen

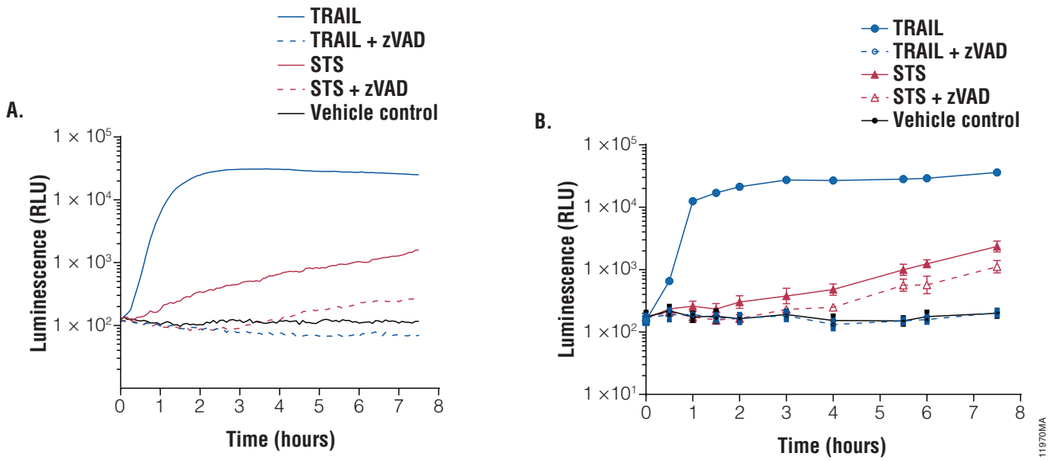

Figure 2. The caspase-3/7 biosensor assay can be used in lytic and non-lytic formats. D54-MG glioma cells stably expressing pGloSensor™-30F DEVDG plasmid and treated with inducers of extrinsic or intrinsic apoptotic pathways. Cells were treated with 200ng/ml TRAIL or 1µM staurosporine (STS), respectively, at time zero. Where indicated, cells were also treated at time zero with 20µM ZVAD-FMK pan-caspase inhibitor. **Panel A.** Cells pre-equilibrated in buffered medium with a 2% v/v dilution of GloSensor™ cAMP Reagent and measured every 5 minutes after treatment on a GloMax® Multi+ luminometer at 37°C. **Panel B.** Replicate plates with cells in buffered medium incubated at 37°C after treatment. Luminescence measured using Bright-Glo™ Luciferase Assay System (Cat.# E2650) in a GloMax® Multi+ luminometer at the indicated time points.

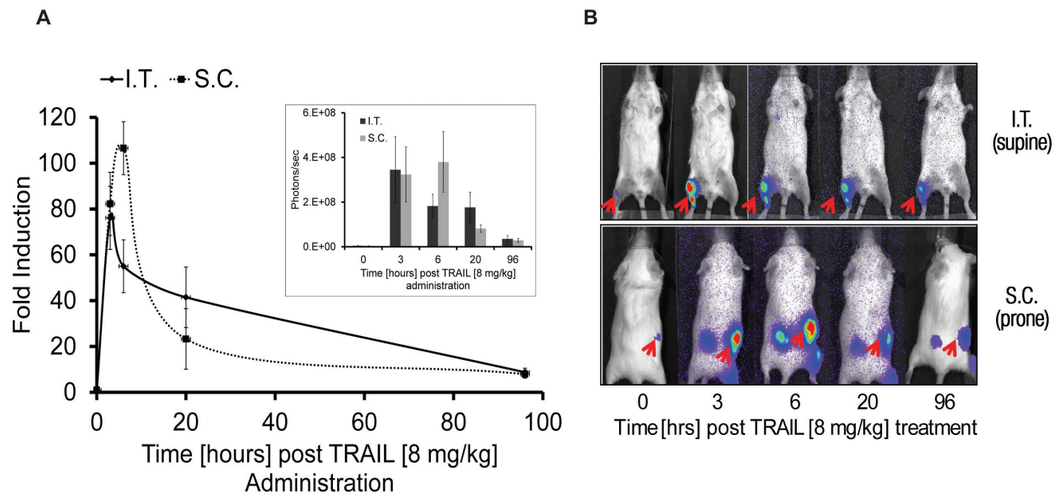

Figure 3. In vivo imaging of apoptosis using caspase-3/7 biosensor (adapted from reference 1). Panel A. Bioluminescence signal intensity and fold induction over pretreatment values are shown for intra tibial (I.T.) or subcutaneous (S.C.) xenografts of 1833 cells stably expressing pGloSensor™-30F DEVDG plasmid into SCID mice (tumor size 50–100mm^3). Luciferase activity was monitored at the indicated time points post-TRAIL treatment and after injection of 150mg/kg of VivoGlo™ Luciferin (Cat.# P1041). **Panel B.** Representative images of mice used in the study. Image credit: Galbán, S. *et al.* (2013) Imaging proteolytic activity in live cells and animal models. *PLOS ONE* **8**, e66248.

in the caspase activation profile for inducers of extrinsic or intrinsic apoptotic pathways. To better facilitate work in vivo, Galbán *et al*. engineered a mouse to allow tissue specific expression of the caspase-3/7 biosensor via the CRE-Lox system, allowing sensitive detection of apoptosis in a tissue specific manner. Tissue or cell type specific expression of the biosensor in this context will allow the sensitive detection of apoptosis in a variety of in vivo model systems of human disease.

Summary

The caspase-3/7 biosensor described here provides sensitive and real-time detection of effector caspase activation in cultured cells. When used in vivo, it enables caspase activation to be monitored in select tissues or xenografts with high sensitivity over a broad dynamic range, thus providing a potentially useful tool for therapeutic development. As with Protease-Glo™ Assay, the potential exists to extend the approach to a wide variety of protease targets both in vivo and in cell-based assays. For the latter, the approach was recently extended to viral proteases (3).

References

1. Galbán, S. *et al.* (2013) Imaging proteolytic activity in live cells and animal models. PLOS ONE **8**, e66248.

2. Binkowski, B. *et al.* (2008) Novel genetically encoded biosensors using firefly luciferase. *ACS Chem. Biol.* **3**, 346–51.

3. Kilianski, A. *et al.* (2013) Assessing activity and inhibition of Middle East Respiratory Syndrome coronavirus papain-like and 3C-like proteases using luciferase-based biosensors. *J. Virol.* **87**, 11955–62.

The caspase-3/7 biosensor is available as a custom research material under the commercial name pGloSensor™-30F DEVDG Plasmid (www.promega.com/products/drug-discovery/custom-assay-materials/). This construct uses a CMV promoter for constitutive expression of the biosensor in mammalian cells together with SV40 mediated expression of a *Renilla* luciferase-neomycin phosphotransferase fusion for selection of stably transfected cells. Unique restriction sites allow users to remove the DEVDG sequence and introduce cleavage sites for their protease of interest.

For more information on GloSensor™ luciferase technology platform and related products, please see: ***www.promega.com/products/pm/glosensor***

Product	Size	Cat.#
Protease-Glo™ Assay	1 each	G9451
pGloSensor™-10F Linear Vector	1µg	G9461
pGloSensor™-30F DEVDG Vector	NA	Please Enquire
Bright-Glo™ Luciferase Assay System	10ml	E2650
VivoGlo™ Luciferin, in vivo Grade	50mg	P1041