

EVROGEN KILLER RED SYSTEM

- First fully genetically-encoded photosensitizer
- No exogenous chemical compounds or cofactors except oxygen required
- Direct expression in cells
- Induction by green light irradiation
- Unique tool for precise protein photoinactivation *in vivo*
- Allows selective light-induced cell killing

Introduction

Photosensitizers are chromophores that generate reactive oxygen species (ROS) upon light irradiation. They find use for precise inactivation of selected proteins in chromophore-assisted light inactivation (CALI) technique and for the light-induced cell killing, for example in photodynamic therapy.

All known to date photosensitizers are chemical compounds that must be introduced into living systems exogenously. This limitation severely constricts the area of their applications.

KillerRed is a first entirely genetically encoded photosensitizer (1). It has been developed on the basis of the Anthomedusae chromoprotein anm2CP (2). Unlike chemical photosensitizers, KillerRed can be directly expressed by target cells, both individually and in fusion with a target protein. It shows no cell toxic effects before light activation. Upon green light irradiation KillerRed generates reactive oxygen species (ROS) that damage the neighboring molecules.

KillerRed features

KillerRed is a dimeric red fluorescent protein with fluorescence excitation/emission maxima peaking at 585/610 nm, extinction coefficient $45,000 \text{ M}^{-1}\text{cm}^{-1}$ at 585 nm, fluorescence quantum yield 0.25 (Fig. 1).

KillerRed demonstrates successful performance in many fusions including that with cytoplasmic β -actin, fibrillarin, dopamine transporter, etc. Moreover, KillerRed has proven availability to create stably transfected cell lines.

KillerRed phototoxicity is induced by green light irradiation at 540-580 nm and depends on light intensity, irradiation time and KillerRed concentration.

In CALI, mild illumination of KillerRed-tagged protein for a limited time results in precise inactivation of this protein only. Upon the more prolonged and intensive irradiation KillerRed can be effectively used for damaging the organelles and killing the target cells.

KillerRed use

KillerRed can be used for directional killing of bacterial or eukaryotic cells and for precise protein photoinactivation *in vivo*. KillerRed or KillerRed-fusion protein can be expressed in cells or cell organelles of interest from KillerRed expression vector. If required, stable KillerRed transformants can be selected using G418 (3).

KillerRed-mediated ROS generation should be induced by green light irradiation at 540-580 nm. Intensity of green light and irradiation time must be individually determined for particular biological system and instrumentation. Following examples (see section "Examples of use") allow rough estimation of these conditions.

KillerRed-mediated ROS production is accompanied by profound KillerRed photobleaching. The resulting cell events (cell fate after irradiation, effect on protein localization) can be monitored using another fluorescent reporter, for example a green fluorescent protein. [Vector sets](#) comprising KillerRed and TurboGFP expression vectors are available from Evrogen.

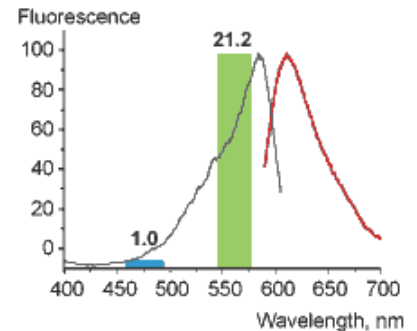


Figure 1. Excitation (black line) and emission (red line) spectra for KillerRed. Blue and green rectangles show relative phototoxic effect from irradiation with blue (460-490 nm) and green (540-580 nm) light of 35 mW/cm^2 . Numbers above the rectangles represent decrease in viable cells after 30-min irradiation (folds).

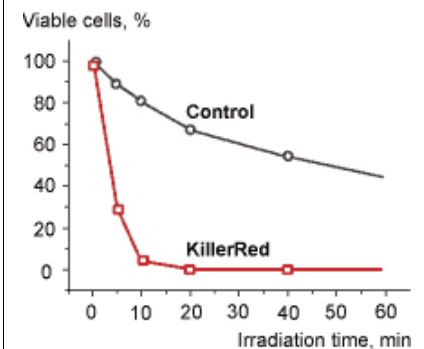


Figure 2. Time-course of light-induced killing of *E. coli* expressing KillerRed.

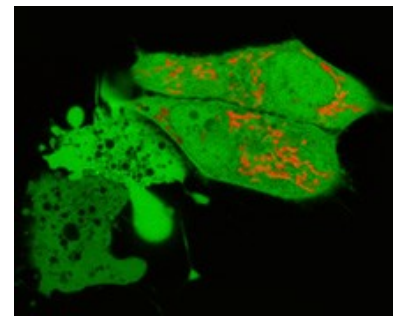


Figure 3. Confocal image of HeLa cells expressing KillerRed-dMito in mitochondria (red) and TurboGFP in cytoplasm (green). Lower left cells were pre-irradiated with green light resulting in KillerRed bleaching and subsequent apoptotic cell death.

Examples of use

KillerRed-mediated killing of bacterial cells

E. coli cells (XL1-Blue strain) were transformed with bacterial expression vector encoding KillerRed. A single *E. coli* colony was picked out, diluted into 1 ml of PBS buffer and divided into two equal portions. One of them was irradiated with white light (1W/cm², light source Fiber-Light from Dolan-Jenner Industries, Inc) for different periods of time, whereas the other was kept in darkness. Both sample aliquots were then plated onto Petri dishes at different dilutions. The number of growing colonies corresponded to the number of bacteria cells surviving after irradiation (i.e. colony forming units, CFU). CFU number for the irradiated *E. coli* portion was compared to non-irradiated one, thus allowing estimation of the relative phototoxic effect for KillerRed. In control experiments, *E. coli* cells expressing different fluorescent and non-fluorescent proteins were used.

KillerRed killed 96% of bacterial cells after 10 minutes and almost all cells after 20 minutes of irradiation (Fig. 2). At the same time, control *E. coli* cells showed almost zero effect on bacterial cell survival.

Note: Although white light illumination was used in this experiment, only the green component of irradiation spectra appeared to be crucial for induction of KillerRed phototoxicity. Strong cell killing effect was achieved upon green (540-580 nm) and almost zero effect upon blue (460-490 nm) light irradiation of equalized power (about 35mW/cm²).

KillerRed-mediated killing of eukaryotic cells

Effects of KillerRed localized in mitochondria

Mitochondrial localization increases the phototoxic effect of photosensitizers (primarily by provoking the apoptosis). Use of KillerRed targeted to mitochondria (pKillerRed-dMito vector is available from Evrogen) allows effective cell killing through apoptotic pathway as has been demonstrated in the following experiments.

HeLa cells expressing cytoplasmic green fluorescent marker, TurboGFP, and mitochondrially localized KillerRed-dMito were generated. Two cells (Fig. 3, on the left) were pre-irradiated with green (515-560 nm, 7W/cm²) light for ten minutes. This led to profound KillerRed photobleaching. Cells are shown in 60 minutes after irradiation. It is clearly visible that irradiated cells have abnormal shape and “bubbles”, characteristic for apoptotic cell death pathway. These cells disrupted within next 30-60 minutes.

In another experiment, nearly 100% of B16 melanoma cells expressing KillerRed-dMito died within 45 min after 15 min irradiation (40x objective, 535-575 nm excitation filter, 3.3 W/cm²).

Noticeably that when preincubated with the pancaspase inhibitor zVAD-fmk (10 μM), the cells survived an identical irradiation treatment and preserved their native shape for at least 1.5 hour after illumination. This indicates that KillerRed-dMito-mediated cell death proceeds via apoptosis, but not necrosis.

Long-term effects of KillerRed

Apart from the immediate phototoxic effect, photosensitizers can mediate postponed cellular responses, such as cell growth arrest or cell death via a long-term apoptotic mechanism.

B16 melanoma cells expressing KillerRed-dMito or EGFP were mixed together and irradiated by green light of low intensity (3.7x objective, 535-575 nm excitation filter, 115 mW/cm²) for 45 minutes. No red fluorescent cells were observed in 16 hr after irradiation, while green fluorescent cells remained viable. It confirms that KillerRed localized in mitochondria can mediate cell death through long-term mechanisms in response to irradiation by low light intensities that are usable for many applications.

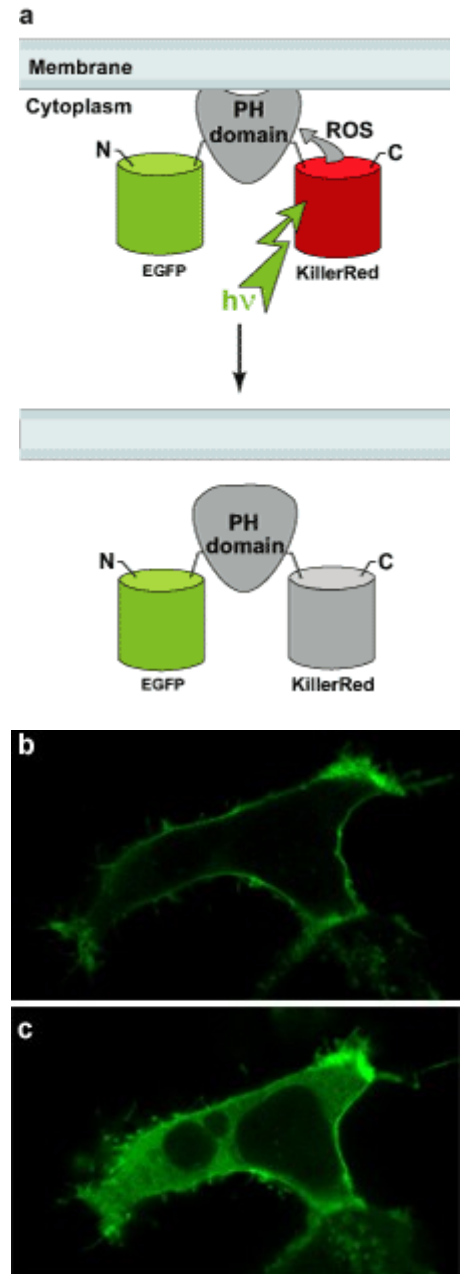


Figure 4. KillerRed-mediated light-induced inactivation of PLC delta-1 PH domain.

(a) Schematic outline of the experimental system. Triple fusion EGFP-PH-KillerRed is localized predominantly at the plasma membrane due to specific affinity of PH domain to phosphatidylinositol 4,5-bisphosphate. Irradiation with intense green light leads to KillerRed-mediated ROS production, PH domain damage and fusion protein dissociation from the membrane. (b,c) A confocal image of a cell expressing EGFP-PH-KillerRed triple fusion (EGFP green fluorescent signal) before (b) and after (c) 10-s irradiation with green light. Note considerable increase in cytoplasmic signal.

Effects of KillerRed localized in cytoplasm

Irradiation of KillerRed localized in cell cytoplasm results in weaker effect on cell survival. In this case effective cell killing requires more intensive light irradiation and high KillerRed concentration in the cell cytoplasm.

In our experiments on 293T human kidney cells, death of irradiated KillerRed expressing cells (10 min, 100x objective, 535-575 nm excitation filter, 5.8 W/cm²) reached 40-60% depending on protein concentration. Thereof, for effective killing of selected cells, we recommend to use mitochondria-targeted KillerRed.

Use of KillerRed for precise protein inactivation

Chromophore-assisted light inactivation in bacterial cells

In the model CALI experiment KillerRed was fused to β -galactosidase (β -gal) enzyme and expressed in *E. coli*. Upon green light irradiation (540-580 nm, 30 min, 360mW/cm²) β -gal activity was effectively suppressed in living *E. coli* streaks. On the contrary, no effect of green light on the enzyme activity was detected in control cells containing unmodified β -gal gene. In vitro test showed that in *E. coli* cell extract β -gal fused to KillerRed lost 99,4% of enzymatic activity within 25 min of white light exposure (1W/cm²), with half inactivation time of about 5 min (Fig. 5). Irradiation of *E. coli* extracts containing unfused β -gal protein alone or β -gal mixed with KillerRed had no effect on enzyme activity. To further verify specificity of KillerRed phototoxic effect, horse-radish peroxidase (HRP) was added to the sample. Upon 15 min illumination (white light, 1W/cm²) only 2% of HRP activity was lost, showing high specificity of the phototoxic effect.

Chromophore-assisted light inactivation in mammalian cells

Pleckstrin homology (PH) domain of phospholipase C delta-1 (PLC delta-1) was used as a model protein for testing the KillerRed-mediated CALI in mammalian cells. The PH domain locates to the inner leaf of plasma membrane as a result of its high affinity for membrane phospholipids. It was anticipated that in the event of direct protein inactivation by dye-generated ROS, PH domain will lose its membrane affinity and become evenly distributed throughout the cell.

In the model experiment a triple EGFP-PH-KillerRed fusion protein that allows both protein visualization and CALI was constructed (Fig. 4a) and transiently expressed in mammalian cell line. Intracellular localization of EGFP signal was evaluated before and after CALI of the PH domain using confocal and fluorescence microscopy. As expected, light-induced damaging of PH domain affected its membrane affinity dramatically. In resting cells, most of the fluorescent signal located to the cell membrane, with the cytoplasm/membrane EGFP signal intensity ratio being ~ 0.2. After 10 sec of green light irradiation (63x objective, mercury lamp, 515-560 nm filter, 7W/cm²) translocation of the PH domain into cytoplasm was clearly visible (Fig. 4b,c). If irradiated for longer times, considerable amount of PH domain translocated into cytoplasm, increasing the cytoplasm-to-membrane green fluorescent signal ratio to 0.5-0.9.

In the negative control experiments, the cellular location of a DsRedExpress (Clontech) containing construct, GFP-PH-DsRedExpress, showed no dependence on green light irradiation. Similarly, no detectable CALI of the PH domain could be achieved when KillerRed was expressed in the cell separately from PH domain, in either the membrane or cytosol.

References

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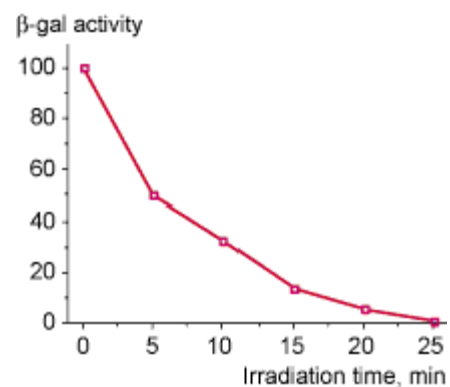


Figure 5. Time course of CALI of β -gal.

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