Published in final edited form as:

Curr Protoc Cytom. ; 69: 12.35.1–12.35.10. doi:10.1002/0471142956.cy1235s69.

The application of KillerRed for acute protein inactivation in living cells

Timothy S. Jarvela^{*} and Adam D. Linstedt

Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh PA 15213

Abstract

Generating loss of protein function is a powerful investigatory tool particularly if carried out at a physiologically relevant timescale in a live-cell fluorescent imaging experiment. KillerRed mediated chromophore assisted light inactivation (CALI) uses genetic encoding for specificity and light for acute inactivation that can also be spatially restricted. This unit provides protocols for setting up and carrying out properly controlled KillerRed experiments during live-cell imaging of cultured cells.

Keywords

KillerRed; CALI; acute inactivation; protein inactivation; ROS

INTRODUCTION

To investigate protein function it is necessary to have methods of specific inhibition and relevant assays in which to assess the effects of the inhibition. The cell biologist's toolkit now contains many approaches. Because cellular processes are typically dynamic and spatially localized, live imaging using fluorescence microscopy has emerged as one of the most powerful readouts of protein function. However, most methods of inhibition including pharmacological and genetic lack the temporal and spatial restriction necessary to properly probe function at a particular time and location during a live imaging experiment. In addition, gene knockout or knockdown techniques occur over long time scales allowing adaptive responses, which can mask the true role of a given protein. Chromophore assisted light inactivation (CALI) allows for targeted inactivation of proteins through generation of reactive oxygen species (ROS) adjacent to the protein of interest. The inhibition occurs within seconds and is spatially limited to the cellular region exposed to light. ROS have a half maximal reactivity radius on the order of 6 nanometers (Linden et al., 1992) and will rapidly react with amino acid side-chains causing changes to the protein structure. CALI was originally performed through introduction of exogenous chromophores attached to antibodies (Jay, 1988), which required antibody preparation and microinjection. The genetically encoded photosensitizer KillerRed (Bulina et al., 2006) allows CALI to be

^{*}Corresponding author: Timothy S. Jarvela, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh PA 15213. tjarvela@andrew.cmu.edu.

performed with ‘plug and play’ ease, making it broadly applicable to many protein targets. KillerRed is useful as a complement to traditional approaches such as knockouts and RNAi. In this unit, we discuss acute inactivation mediated by the fluorophore KillerRed.

Coupled with fluorescent live cell imaging, the KillerRed approach allows for direct observation of the cellular environment immediately before and immediately after the inactivation. For example, inactivation can be carried out at discrete points in development or the cell cycle, or immediately prior or subsequent to exposing cells to an external stimulus. KillerRed can therefore be used to dissect the function of proteins involved in multiple distinct pathways, without confusing accumulated effects. Additionally, KillerRed can be useful for studying proteins that are essential, which is difficult with longer-term inhibition approaches due to loss of viability.

Basic Protocol 1 uses widefield light to inactivate KillerRed tagged proteins in all cells within field of view. For setups equipped with a laser-scanning device, the laser can be used to inactivate specific cells (Alternate Protocol 1). Support Protocol 1 discusses construct design and experimental setup. Though KillerRed is a broadly applicable technique, it requires fine-tuning to properly assay each protein of interest.

Strategic Planning and controls for KillerRed experiments

The following concepts are particularly germane when preparing for KillerRed based experiments. (1) To control for the effects of ROS production that are not specific to the protein of interest a control construct tagged with KillerRed should be expressed at similar levels and activated under the same conditions. If possible, the control construct should localize to the same region of the cell as the experimental construct. If a specific control is not available, cytosolic KillerRed is a suitable generic control for most experimental conditions. (2) The KillerRed control construct should then be used to determine inactivation conditions. That is, excitation conditions are altered to yield bleaching of the KillerRed control construct (indicating ROS production) but without observable effects on the cellular process of interest. (3) KillerRed constructs can be used to replace the endogenous protein by their expression in knockdown/knockout backgrounds. This insures that all copies of the protein of interest are light sensitive and allows a test of the constructs ability to function prior to its inactivation. Significantly, however, if the target protein forms dimers or multimers, it is possible to inactivate endogenous proteins in *trans*, meaning that the transfected construct interacts with its endogenous partner and both are inactivated (Jarvela and Linstedt, 2014, 2012). (4) KillerRed should be paired with fluorescent reporters responding to other wavelengths to readout the physiological activity of interest. The behavior of the reporters is then recorded immediately before and after the KillerRed inactivation event. For simplicity, this protocol assumes the use of GFP-tagged constructs for this purpose.

BASIC PROTOCOL 1

Inactivation of KillerRed using epifluorescent light

The most basic way to inactivate KillerRed, and therefore the most broadly applicable, is to use a strong white light source (e.g., mercury arc lamp) filtered through a 535–585nm excitation filter to expose the samples to green-yellow light. The irradiation time and intensity will need to be adjusted based on the individual setup, but the numbers used in this protocol should serve as a starting point.

Materials

Cells expressing KillerRed and reporter constructs from *Support Protocol 1*

Inverted confocal microscope

High numerical aperture objective

Lasers for desired fluorophores – 561nm or 535nm for KillerRed imaging

Epifluorescent light source (Mercury arc lamp, high powered LEDs)

Excitation filter for KillerRed – between 540–580nm [e.g., Cy3, TRITC, or TexasRed filter sets]

Stage incubator with gas control by injection of CO₂ or flow of 95% air, 5% CO₂ mixture through the incubator (*see critical parameters*)

Gas tank [100% CO₂ or 95% air/5% CO₂ depending on setup]

1. Prepare cells expressing KillerRed and reporter constructs in an imaging chamber, as described in Support Protocol 1.

Note: it is recommended to use carbonate/CO₂ buffered cell media optimized for imaging rather than buffered-media in a sealed chamber. Adequate gas exchange is necessary to maintain optimal oxygen levels for ROS production.
2. Setup the microscope for the experiment. *Set the environmental chamber to 37°C and 5%CO₂. Turn on lasers and start up microscope software. Warm lamp for at least 2 minutes to achieve stable light levels. This improves reproducibility between experiments. Start up microscope acquisition software.*
3. Place imaging chamber on microscope stage and bring sample into focus.

Use a high powered objective (a high NA 60x or 100x) to achieve best resolution as well as highest intensity of illumination light, achieving more efficient inactivation. Do not use KillerRed fluorescence to bring sample into focus, as extra exposure to green light can cause premature inactivation. Keep exposure to green light at a minimum prior to inactivation.
4. Acquire time-lapse images of the GFP reporter during the pre inactivation time period.

It is useful to acquire a time course of pre inactivation images to determine the baseline of cellular activity with which to compare the post inactivation results.

5. Acquire pre inactivation images of KillerRed fluorescence.

Pre inactivation images of the KillerRed construct are useful for comparing between experiments when determining the standard conditions for inactivation and troubleshooting. It is best to acquire these images as close to the inactivation point as possible to minimize the effects caused by KillerRed imaging during the pre inactivation time period.

6. Inactivate KillerRed with green light for 30 seconds.

An exposure for 30 seconds of 540–580nm green light at an intensity of $2\text{W}/\text{cm}^2$ is likely sufficient to bleach the field of view. This is achieved with maximum illumination of an EXFO X-Cite lamp with a mercury bulb rated at 120-watts, with the iris fully opened. Lower intensity bulbs and/or LED systems should be suitable replacements, though inactivation time may need to be extended if the light intensity of the 540–580nm range is lower.

Some microscope setups will allow for automation of this task using either pre-existing settings in the accompanying software or by generating a macro script. If this is not the case, the experimenter will have to adjust the settings manually for inactivation.

7. Optional: Acquire post inactivation image of KillerRed fluorescence.

This step is useful for determining the extent of KillerRed inactivation. Although inactivation does not require bleaching of the fluorophore, the two usually coincide. These images will be useful in determining standard inactivation conditions and for troubleshooting. This step may be omitted to improve temporal resolution of the early post inactivation time period.

8. Acquire time-lapse images of post inactivation cells. After acquisition, analyze the results to assess expression level and bleaching of the KillerRed construct and to determine the effects of the inactivation on the behavior of the GFP-tagged construct.

A successful experiment will achieve inactivation using settings where the control KillerRed does not generate a phenotype.

ALTERNATE PROTOCOL 1 (optional)

Inactivation of KillerRed constructs using a scanning laser capable of photobleaching a region of interest

This protocol is for microscopes with a manipulable laser that can be directed onto a user defined region of interest. Many steps are identical to basic protocol 1. Using a programmable laser allows for the inactivation of a specific cell in a field or even a subcellular region (diffusability of the KillerRed construct must be considered). The important variables to adjust for inactivation are the laser intensity and the speed that the laser beam scans over the region of interest.

Materials

Steerable focused laser system [e.g., FRAPPA (Andor), Direct FRAP (Zeiss), cell[^]frap (Olympus)]

Additional equipment and reagents from Basic Protocol 1

1. Prepare cells expressing KillerRed and reporter constructs in an imaging chamber, as described in support protocol 1.
2. Setup the microscope. Prepare the environmental chamber to 37°C and 5%CO₂. Turn on lasers and start up microscope software.

Note: it is recommended to use a carbonate/CO₂ buffered imaging media rather than buffered media in a sealed chamber. Adequate gas exchange is necessary to maintain optimal oxygen levels for ROS production

3. Place sample on the microscope stage and bring into focus.
4. Define Region of Interest (ROI) for KillerRed inactivation using microscope software. Define laser intensity and speed for bleaching in the software's FRAP settings.

It is important to maintain consistent ROI sizes for inactivation between experiments as larger sizes can lead to significantly longer inactivation times. This can increase deviation in the experiment, especially when observing the initial moments after inactivation. If ROI sizes are too large and inactivation is proceeding too slowly, it is recommended to switch to basic protocol 1.

5. Acquire time-lapse images of the GFP reporter during the pre inactivation time period.
6. Acquire pre inactivation images of KillerRed fluorescence.
7. Inactivate KillerRed in ROI.

Full power of a 25mW 561nm laser at 60μs dwell time repeated 3 times is likely sufficient to inactivate KillerRed tagged proteins and should serve as a good starting point.

8. Optional: Acquire post inactivation image of KillerRed fluorescence.

This step is useful for determining the extent of KillerRed inactivation. Although inactivation does not require bleaching of the fluorophore, the two usually coincide. These images will be useful in determining standard inactivation conditions and for troubleshooting. This step may be omitted to improve temporal resolution of the early post inactivation time period.

9. Acquire time-lapse images of post inactivation cells. Analyze for results of inactivation.

10. Optional: Repeat steps 6 through 8 if performing subcellular targeting of protein inactivation. Repeat inactivation as required to inactivate new protein diffusing into the region of interest.

Due to differing diffusion rates, not all proteins will be amenable to subcellular inactivation. Rapidly diffusing proteins will recover protein activity in an ROI before the loss of the target protein can be analyzed. Therefore, this works best when performed on distinct subcellular compartments, rather than diffuse cytosolic proteins.

SUPPORT PROTOCOL 1 (optional)

Construct generation and cell transfection

KillerRed can be added to most proteins that can be tagged with GFP. For large or lengthy proteins, place KillerRed closest to the functional domain that you are most interested in ablating. Reactive oxygen species have a half maximal effective radius on the order of 6nm (Linden et al., 1992) so placement near the desired region improves inactivation and minimizes off target effects.

Killer Red has a tendency to form dimers, which may interfere with localization and function of the construct. An indication that this is occurring is the formation of aggregates upon expression. One remedy is to insert two tandem copies of KillerRed into the construct. There intramolecular association will create a pseudo-monomer. As with any protein tagging experiment, trial and error may be required to find an optimal position for the tag.

Materials

Cell line of interest (e.g., HeLa cells)

Expression vector containing KillerRed fused in frame with the coding sequence of the protein of interest (see commentary for designing fusion proteins)

Vector containing a relevant reporter construct [e.g., encoding GFP-tagged Golgi protein for Golgi experiments]

Imaging Chamber (Metal chamber with 12mm cover glass or glass bottom dishes (MaTek)

Cell culture media (e.g., MEM with 10% fetal bovine serum) for cell line used

Imaging media (see recipe)

Additional reagents for tissue culture maintenance

Transfection reagent (e.g., JetPRIME (PolyPlus), Oligofectamine, LipofectAMINE2000 (Invitrogen))

Additional reagents for knockdown of endogenous protein (if desired)

1. Design and create construct encoding KillerRed fusion with protein of interest.
2. Seed cells in 60 mm tissue culture dishes according to cell line and transfection reagent specifications
3. Co-transfect cells with KillerRed fusion protein construct and the GFP-tagged reporter construct, according to reagent instructions.
4. After 24 hours, pass the cells from the transfection dish into dishes containing 12mm glass coverslips (for an imaging chamber) or into glass bottom dishes.

The KillerRed fluorophore requires >4 hours to mature. Passing the cells to new dishes after transfection and allowing further growth for 24–48 hours allows adequate levels of KillerRed fluorescence to develop.

If the presence of immature KillerRed is a problem, treatment with translation inhibitors (e.g., cyclohexamide) for a period of 4 hours prior to the start of the experiment should ensure adequate folding time for all fusion proteins.

5. Pre-warm imaging chamber to 37°C. Place coverslip on top of imaging chamber bottom. Carefully screw chamber top with o-ring into bottom chamber. Fill the well with 500µL of imaging media (see recipe). Place lid of 35mm tissue culture dish onto the chamber to prevent excess evaporation but allow for adequate gas exchange.

Be careful to correctly tighten the two halves of the imaging chamber together. Do not over-tighten as this could cause cracking of the glass coverslip. Under-tightening may cause leakage of imaging media onto the objective.

6. For MaTEK glass bottom dishes, replace cell culture media with 1.5ml of pre-warmed imaging media (see recipe).

REAGENTS AND SOLUTIONS

50mM Trolox

Dissolve 125mg Trolox in 10ml of ethanol. Separate into 1ml aliquots and store at –20°C.

Imaging media

Imaging media should be adjusted to what is suitable the cell line being used. HeLa cells use MEM with 10% FBS made fresh for each day of experiments. Final Trolox concentration can be adjusted up or down, to promote or inhibit respectively, KillerRed ROS production.

To 10mls of MEM with 10% fetal bovine serum:

Add 75µl of 50mM Trolox solution (final concentration of 375µM)

COMMENTARY

Background Information

Protein inactivation by CALI was first demonstrated in 1988 by Daniel Jay by using a pulsed laser to excite the fluorophore malachite green either attached to a ligand or antibody which was either bound to the cell surface or microinjected into the cell cytoplasm. In 1994, (Liao et al., 1994) determined that inactivation was primarily through the generation of ROS and free radicals. Exposing a fluorophore to light results in, among other things, the production of radicals and ROS. By changing conditions to favor production of reactive products, fluorophores can be used to cause the inactivation of nearby proteins. This occurs through ROS and radical reactions with exposed side chains (specifically cysteines, histidines, methionines, tryptophans, and tyrosines (Halliwell, 1989)) and results in changes to functional groups, the creation of crosslinks, or even breakage of the peptide backbone (Surrey et al., 1998). Combined, this damage results in conformation changes to the affected proteins and decreases their activity. The high reactivity of the produced species limits their travel to a half maximal distance of around 6 nanometers (Linden et al., 1992), depending on the conditions and the radical involved. Together, these conditions allow for inactivation of proteins in close proximity to a ROS generating fluorophore.

There are many dyes that are suitable photosensitizers. Malachite green (Jay, 1988) and fluorescein (Beck et al., 2002) must be targeted to proteins via specific antibodies. FIAsh (Marek and Davis, 2002) and ReAsH (Tour et al., 2003) are bi-arsenic dyes that can bind to a specific sequence tetra-cysteine motif. Another photosensitizer, miniSOG (Shu et al., 2011), can generate ROS when the bound flavin mononucleotide within miniSOG is irradiated with <500nm light (Lin et al., 2013). Of these, only miniSOG rivals KillerRed for ROS production and ease of use.

KillerRed was generated from the hydrozoan chromoprotein anm2CP (Bulina et al., 2006). The solved crystal structure of KillerRed revealed a coordinated water channel thought to be responsible for the generation of ROS (Carpentier et al., 2009; Pletnev et al., 2009; Roy et al., 2010). It has been used to disrupt pleckstrin homology domain localization (Bulina et al., 2006), block cell cycle progression (Serebrovskaya et al., 2011), inactivate membrane tethers (Jarvela and Linstedt, 2014), block ER exit (Jarvela and Linstedt, 2012), cause double stranded DNA breaks (Waldeck et al., 2011), and target cells for death (Buytaert et al., 2007; Muthiah et al., 2014). A newer version of KillerRed, called SuperNova may have the photosensitive properties of KillerRed without the tendency to dimerize (Takemoto et al.,

2013) and may be a useful alternative for proteins whose localization is disrupted by KillerRed tagging.

Critical Parameters

Among the most challenging aspects of KillerRed experiments will be the optimization of the inactivation conditions, which requires manipulating the following four variables while inactivating a control construct. They can be adjusted in either direction to influence KillerRed inactivation. These are irradiation time, irradiation intensity, antioxidant concentration, and oxygen concentration. Ideally, KillerRed inactivation should be performed at the highest intensity possible to quickly inactivate the protein. If the highest power output causes a phenotype with the control construct, decrease the intensity with neutral density filters or by decreasing the laser power. Start with a 30 s inactivation time (for Basic Protocol 1) or 60 microsec dwell time and 5 repeats (for Alternate protocol 2). If the KillerRed fluorescence is not bleached entirely, increase the time of irradiation. If phototoxicity is observed, decrease the time. Antioxidant concentration will help quench ROS before they react with more distant proteins. Start with 375 μ M concentration of Trolox. If the KillerRed construct is particularly high expressing, increase the Trolox concentration. If the control KillerRed construct requires a long time to bleach, try decreasing the antioxidant concentration. KillerRed requires oxygenated media to function. If KillerRed is not bleaching readily, try using fresh media, or perfuse oxygenated media over the cells.

A successful imaging experiment with acute protein inactivation mediated by KillerRed requires the fulfillment of several criteria. Each of these should be considered prior to designing KillerRed experiments.

1. *Choosing KillerRed targets.* In larger proteins, it is recommended to place the KillerRed tag as close to the functional domain as possible, to promote more effective inactivation.
2. *Suitable KillerRed control constructs.* These constructs should be designed to compliment the experimental constructs as closely as possible. That is, they should have similar expression levels and localization patterns.
3. *Spectral considerations.* The excitation spectra of the fluorophore in KillerRed allows for photoinactivation (as well as excitation) optimally by using wavelengths between 540 and 580nm, with a peak at 585nm. The emission spectra peaks at 610nm (Bulina et al., 2006). This allows for KillerRed to be use with a variety of red filter sets (e.g., Cy3, TRITC, TexasRed) under most confocal setups.
4. *Determining irradiation parameters.* Many newer confocal microscopes have a tunable FRAP or photoactivation setting, which allows for targeting lasers to regions of the field of view by selecting a region of interest. For consistency across experimental replicates, it is a good idea to measure the power output of the laser being used to inactivate KillerRed. This laser can be in the range of 540nm to 560nm for optimal irradiation of KillerRed. For older microscopes, KillerRed inactivation can still be achieved through widefield illumination of the

sample. Indeed, this may be preferable in some cases as it allows for bleaching of a large area in a shorter amount of time compared to a point scanning laser.

5. *Controlling for off target effects.* A number of studies have shown that phototoxicity leading to apoptosis and/or necrosis can occur using KillerRed. Examples include constructs localized to the mitochondria (Buytaert et al., 2007), *plasma membrane* (Buytaert et al., 2007), and *lysosomes* (Serebrovskaya et al., 2014). While this is the desired outcome for certain investigations it must be avoided using the optimization procedure outline above, if the goal is specific protein inactivation. *Inactivation power requirements.* A high powered mercury arc lamp is suggested to quickly inactivate KillerRed. Confocal laser light is generally insufficient to cause rapid inactivation and should be avoided. In our hands, an output of 535–585nm light at 2W/cm² through a 100x objective was efficient at inactivating KillerRed in 30s (Jarvela and Linstedt, 2014, 2012).
6. *Recovery of functional proteins by diffusion or translation.* Loss of function from proteins inactivated by KillerRed will last until functional copies are restored to the inactivated area. In the case of whole cell/field inactivation, this occurs over hours, as it requires the translation and folding of new proteins. For spatially restricted inactivation, the effects will last until functional proteins diffuse into the inactivated zone, which can occur in seconds to minutes. Therefore it is necessary to plan experiments to include multiple rounds of irradiation if the assay time extends past the recovery point.
7. *Limit superfluous light exposure.* Continual exposure of cells expressing KillerRed to low levels of light can cause oxidative stresses and cell death. For best results, minimize light exposure during culturing and prior to the experiment. Brief periods of light exposure (e.g., working in a laminar flow hood or observing cells under a phase contrast microscope) should not be a cause for concern.

Troubleshooting

Negative results from KillerRed inactivation experiments can be caused by a variety of issues. The following is a list to help diagnose common problems.

1. The KillerRed tagged protein does not localize properly. As noted above, improper localization may be due to self-interaction of KillerRed leading to aggregates. To overcome this, create a fusion protein with two copies of KillerRed in tandem to function as a pseudo-monomer (Serebrovskaya et al., 2011). This can help to promote proper folding and decrease aggregation of the fusion proteins.
2. KillerRed fluorescence returns quickly after irradiation. Though functional inactivation may not require loss of KillerRed fluorescence the loss is a good indicator. If fluorescence returns after irradiation it may indicate the presence of a reservoir of immature fluorophores that complete the folding reaction shortly after the inactivation event. Because fluorophores undergoing folding are not photosensitizers these proteins may restore function. The use of a weaker

promoter in the expression vector and longer incubation times after transfection should produce less of a reservoir. Alternatively, a pre treatment with translation inhibitors (e.g. cyclohexamide for >4 h) can be used diminish the reservoir.

3. KillerRed fluorescence does not bleach after irradiation with green light. This may be caused by a lack of oxygen in the media. Prepare fresh media and decrease the amount of media added to the imaging chamber to allow better oxygen diffusion to the adherent cells.

Anticipated Results

Expected results from KillerRed mediated inactivation will vary depending on the individual experimental setup. Collectively, the aim of KillerRed mediated experiments is to achieve protein inactivation with temporal and/or spatial restriction so that dynamic properties can be observed. Therefore, it is expected that many KillerRed experiments will not phenocopy knockdowns or knockouts over the short term. It is possible, and sometimes desirable, to follow KillerRed inactivated cells over long time periods (with repeated photoinactivation as protein levels recover) to observe the effects of chronic inhibition by KillerRed. This can mimic the long term effects of knockouts and knockdowns, which can provide a positive control for inactivation.

An example of KillerRed mediated inactivation is shown in Figure 1. In this experiment, the Golgi receptor for the dynein motor, golgin160 (Yadav et al., 2012) was tagged with KillerRed and inactivated. The Golgi was visualized using GalNAcT2-GFP, a Golgi localized reporter construct, beginning 10 minutes prior to inactivation of KillerRed. To serve as a control for generation of ROS at the Golgi, KillerRed was anchored to the Golgi with the C-terminal trans-membrane domain of the Golgi protein giantin (Jarvela and Linstedt, 2014). Identical conditions were used to inactivate both control and experimental KillerRed constructs and bleaching of the constructs was verified (Fig 1A). As expected, following inactivation of the control KillerRed construct, the GalNAcT2-GFP Golgi reporter showed a stable and low number of Golgi objects that was similar to the period prior to inactivation (Fig 1B&C). A stable Golgi was also apparent for the 10 min prior to inactivation of the golgin160 KillerRed construct; however, within minutes of inactivation, the number of Golgi objects dramatically increased (Fig 1B&C). This Golgi dispersal reflects loss of inward Golgi movement upon inactivation of the motor complex.

Time Considerations

Preparation of cells for imaging—Transfection and expression of KillerRed will require 2 days. If performed in a knockdown background, start knockdown 1–3 days earlier. Imaging chamber and media will require 30 minutes to assemble and warm up to 37°C.

Microscope setup and imaging—The microscope stage and environmental chamber should be started a half hour before the experiment to allow conditions to stabilize. Lasers and arc lamps should be allowed to warm up for greater than 2 minutes to achieve stable light output. The time required to find a suitable expressing cell will vary depending on transfection efficiency and behavior of the construct. Time-lapse imaging length will vary

depending on experimental conditions, but multiple rounds of KillerRed inactivation may be required for experiments longer than 2 hours.

KillerRed inactivation—Inactivation of whole fields of view can be achieved using high powered mercury arc lamps or high powered LED lamps through a green-yellow filter for approximately 30 seconds, depending on the intensity of the light. Using a scanning laser to spatially restrict inactivation could require up to two minutes for large areas.

Acknowledgments

Funding was provided to A.D.L.: RO1S GM0811101, GM095549 and GM56779

LITERATURE CITED

- Beck S, Sakurai T, Eustace BK, Beste G, Schier R, Rudert F, Jay DG. Fluorophore-assisted light inactivation: a high-throughput tool for direct target validation of proteins. *Proteomics*. 2002; 2:247–255. [PubMed: 11921440]
- Bulina ME, Chudakov DM, Britanova OV, Yanushevich YG, Staroverov DB, Chepurnykh TV, Merzlyak EM, Shkrob Ma, Lukyanov S, Lukyanov Ka. A genetically encoded photosensitizer. *Nature Biotechnology*. 2006; 24:95–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16369538>.
- Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochimica et Biophysica Acta*. 2007; 1776:86–107. Available at: <http://dx.doi.org/10.1016/j.bbcan.2007.07.001>. [PubMed: 17693025]
- Carpentier P, Violot S, Blanchoin L, Bourgeois D. Structural basis for the phototoxicity of the fluorescent protein KillerRed. *FEBS Letters*. 2009; 583:2839–42. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19646983>. [PubMed: 19646983]
- Halliwell B. Oxidants and the central nervous system: some fundamental questions. Is oxidant damage relevant to Parkinson's disease, Alzheimer's disease, traumatic injury or stroke? *Acta Neurologica Scandinavica. Supplementum*. 1989; 126:23–33. [PubMed: 2694733]
- Jarvela T, Linstedt AD. Irradiation-induced protein inactivation reveals Golgi enzyme cycling to cell periphery. *Journal of Cell Science*. 2012; 125:973–80. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22421362>. [PubMed: 22421362]
- Jarvela T, Linstedt AD. Isoform-specific tethering links the Golgi ribbon to maintain compartmentalization. *Molecular Biology of the Cell*. 2014; 25:133–144. [PubMed: 24227884]
- Jay DG. Selective Destruction of Protein Function by Chromophore-Assisted Laser Inactivation. *Proceedings of the National Academy of Sciences*. 1988; 85:5454–5458. Available at: <http://www.pnas.org/cgi/doi/10.1073/pnas.85.15.5454>.
- Liao JC, Roider J, Jay DG. Chromophore-assisted laser inactivation of proteins is mediated by the photogeneration of free radicals. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91:2659–2663. [PubMed: 8146171]
- Lin JY, Sann SB, Zhou K, Nabavi S, Proulx CD, Malinow R, Jin Y, Tsien RY. Optogenetic inhibition of synaptic release with chromophore-assisted light inactivation (CALI). *Neuron*. 2013; 79:241–253. Available at: <http://dx.doi.org/10.1016/j.neuron.2013.05.022>. [PubMed: 23889931]
- Linden KG, Liao JC, Jay DG. Spatial specificity of chromophore assisted laser inactivation of protein function. *Biophysical Journal*. 1992; 61:956–962. Available at: [http://dx.doi.org/10.1016/S0006-3495\(92\)81902-1](http://dx.doi.org/10.1016/S0006-3495(92)81902-1). [PubMed: 1581504]
- Marek KW, Davis GW. Transgenically encoded protein photoinactivation (FLaSH-FALI): acute inactivation of synaptotagmin I. *Neuron*. 2002; 36:805–813. [PubMed: 12467585]
- Muthiah M, Park S-H, Nurunnabi M, Lee J, Lee Y, Park H, Lee B, Min J-J, Park I-K. Intracellular delivery and activation of the genetically encoded photosensitizer Killer Red by quantum dots encapsulated in polymeric micelles. *Colloids and Surfaces B: Biointerfaces*. 2014

- Pletnev S, Gurskaya NG, Pletneva NV, Lukyanov Ka, Chudakov DM, Martynov VI, Popov VO, Kovalchuk MV, Wlodawer A, Dauter Z, et al. Structural basis for phototoxicity of the genetically encoded photosensitizer KillerRed. *The Journal of biological chemistry*. 2009; 284:32028–39. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2797274&tool=pmcentrez&rendertype=abstract>. [PubMed: 19737938]
- Roy A, Carpentier P, Bourgeois D, Field M. Diffusion pathways of oxygen species in the phototoxic fluorescent protein KillerRed. *Photochemical and Photobiological Sciences*. 2010; 9:1342–1350. Available at: <http://dx.doi.org/10.1039/c0pp00141d>. [PubMed: 20820672]
- Serebrovskaya EO, Gorodnicheva TV, Ermakova GV, Solovieva Ea, Sharonov GV, Zagaynova EV, Chudakov DM, Lukyanov S, Zaisky AG, Lukyanov Ka. Light-induced blockage of cell division with a chromatin-targeted phototoxic fluorescent protein. *The Biochemical journal*. 2011; 435:65–71. [PubMed: 21214518]
- Serebrovskaya EO, Ryumina AP, Boulina ME, Shirmanova MV, Zagaynova EV, Bogdanova EA, Lukyanov SA, Lukyanov KA. Phototoxic effects of lysosome-associated genetically encoded photosensitizer KillerRed. *Journal of Biomedical Optics*. 2014; 19:71403. Available at: <http://dx.doi.org/10.1117/1.JBO.19.7.071403>.
- Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY. A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. *PLoS Biology*. 2011; 9:e1001041. Available at: <http://dx.doi.org/10.1371/journal.pbio.1001041>. [PubMed: 21483721]
- Surrey T, Elowitz MB, Wolf PE, Yang F, Nédélec F, Shokat K, Leibler S. Chromophore-assisted light inactivation and self-organization of microtubules and motors. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95:4293–8. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=22482&tool=pmcentrez&rendertype=abstract>. [PubMed: 9539730]
- Takemoto K, Matsuda T, Sakai N, Fu D, Noda M, Uchiyama S, Kotera I, Arai Y, Horiuchi M, Fukui K, et al. SuperNova, a monomeric photosensitizing fluorescent protein for chromophore-assisted light inactivation. *Scientific reports*. 2013;3.
- Tour O, Meijer RM, Zacharias DA, Adams SR, Tsien RY. Genetically targeted chromophore-assisted light inactivation. *Nature Biotechnology*. 2003; 21:1505–1508. Available at: <http://dx.doi.org/10.1038/nbt914>.
- Waldeck W, Mueller G, Wiessler M, Tóth K, Braun K. Positioning effects of KillerRed inside of cells correlate with DNA strand breaks after activation with visible light. *International journal of medical sciences*. 2011; 8:97–105. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3030142&tool=pmcentrez&rendertype=abstract>. [PubMed: 21278894]
- Yadav S, Puthenveedu MA, Linstedt AD. Golgin160 recruits the dynein motor to position the Golgi apparatus. *Developmental Cell*. 2012; 23:153–165. Available at: <http://dx.doi.org/10.1016/j.devcel.2012.05.023>. [PubMed: 22814606]

Figure 1.

Example data from a KillerRed inactivation experiment. HeLa cells stably expressing the Golgi marker GalNAcT2-GFP were transfected with a Golgi localized, membrane anchored KillerRed control construct or a KillerRed-tagged version of the dynein motor receptor golgin160. (A) Pre- and post-inactivation images of KillerRed fluorescence are shown for each KillerRed construct. (B) Fluorescence image outtakes of the Golgi reporter (GalNAcT2-GFP) during a time course spanning 10 minutes prior (Pre-inactivation) to and 60 minutes post (Post-inactivation) irradiation for 30s with 535–585nm green light at $2\text{W}/\text{cm}^2$. (C) The number of discrete GFP positive objects is shown for each time point. Time of irradiation is set to zero. Inactivation of the control construct caused no significant change, whereas inactivation of golgin160-KR showed a rapid increase in Golgi objects.