

Novel Method for Site-specific Induction of Oxidative DNA Damage to Study Recruitment of Repair Proteins to Heterochromatin and Euchromatin

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[Abstract] ROS-induced DNA damage is repaired in living cells within a temporal and spatial context, and chromatin structure is critical to a consideration of DNA repair processes *in situ*. It's well known that chromatin remodeling factors participate in many DNA damage repair pathways, indicating the importance of chromatin remodeling in facilitating DNA damage repair. To date, there has been no method to induce site-specific oxidative DNA damage in living cells. Therefore, it is not known whether the DNA repair mechanisms differ within active or condensed chromatin. We recently established a novel method, DTG (Damage Targeted at one Genome-site), to study DNA damage response of reactive oxygen species (ROS)-induced DNA damage in living cell at one genome loci with active or inactive transcription. For this, we integrated a tetracycline responsive elements (TRE) cassette (~90 kb) at X-chromosome in U2OS cells (Lan *et al.*, 2010), then fused KillerRed (KR), a light-stimulated ROS-inducer which can specifically produce ROS-induced DNA damage, to a tet-repressor (tetR-KR, OFF) or a transcription activator (TA-KR, ON) (Lan *et al.*, 2014) (Figure 1). TetR-KR or TA-KR binds to the TRE cassette and induces ROS damage under hetero- or euchromatin states, respectively. How chromatin states regulate the DNA damage response processes can be examined by using this powerful method.

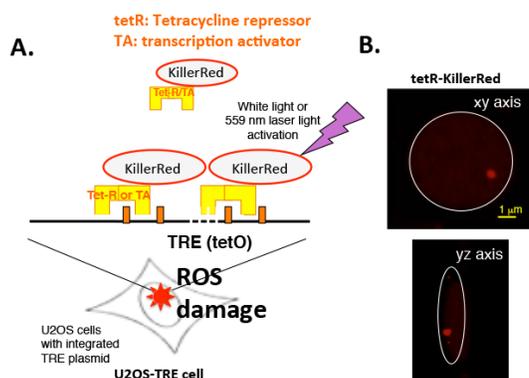


Figure 1. Scheme of the DTG system. A. Scheme of tetR and TA tagged KR expression in the U2OS TRE cell line. To induce ROS-mediated damage at a specific locus in the genome, we fused KR to the tetracycline repressor to induce ROS damage in a 90 kb tetracycline response element (TRE) array (totally 96 repeats) in U2OS cells. B. Expression of tetR-KR in U2OS TRE cell line.

Materials and Reagents

1. U2OS-SCE cell line (made in our laboratory) (Lan *et al.*, 2010)

Notes:

- a. *In this cell line, 200 copies of pTRE / I-SceI were integrated in U2OS cells.*
- b. *Cells are cultured with Dulbecco's Modified Eagle's Medium High glucose with stable L-glutamine (DMEM) (EuroClone S.p.A. P.IVA, catalog number: ECM0103L) with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, catalog number: F9665).*
- c. *For preparation of cells, cells were washed with PBS without Ca²⁺ and Mg²⁺ (EuroClone S.p.A. P.IVA, catalog number: ECB4004L), trypsinized with Trypsin/EDTA without out Ca²⁺ and Mg²⁺ (Thermo Fisher Scientific, catalog number: BW17161E).*

2. Plasmids

- a. pBROAD3/tetR-KR
- b. pBROAD3/tetR-mCherry
- c. pBROAD3/TA-KR
- d. pBROAD3/TA-mCherry

*Note: pBROAD3/tetR-mcherry was provided by Dr. Edith Heard (Masui *et al.*, 2011); pBROAD3/tetR-KR, pBROAD3/TA-mCherry, pBROAD3/TA-KR were made in our laboratory (Lan *et al.*, 2014).*

Sequences are available based on requests.

3. GFP-fusion protein or endogenous protein
4. Lipofectamine 2000 (Life Technologies, catalog number: 12566-014)
5. Opti-MEM (Life Technologies, catalog number: 51985-091)
6. 35 mm glass bottom culture dishes (MatTek, catalog number: P35GC-1.5-14-C)
7. IMMOIL-F30CC (Chip Humphries, catalog number: Z-81225)

Equipment

1. 35 mm glass bottom culture dishes
2. 37 °C, 5% CO₂ cell culture incubator
3. Olympus FV1000 confocal microscopy system (OLYMPUS, model: FV1000- FILTER DETECT; SYS.PACKAGE: IX81-1 405/M_AR/559/635N) with 488, 559 nm lasers
4. PLAPON 60x oil lens (super chromatic abe. corr. obj W/1.4NA FV) (OLYMPUS, catalog number: FM1-U2B990)

5. Thermo-plate (MATS-U52RA26 for IX81/71/51/70/50, metal insert, HQ control) (OLYMPUS, catalog number: OTH-I0126)
6. 15 watt cool white fluorescent bulb (OSRAM SYLVANIA)
7. A stage UVP (Upland, CA)

Procedure

1. Seed U2OS-SCE cells into 35 mm glass bottom culture dishes at an approximate density of 5×10^5 per dish in DMEM with 10% FBS at 37 °C incubator with 5% CO₂ for overnight.
2. For GFP-fusion protein, co-transfect 1 µg GFP-Fusion protein with any 1 µg of pBROAD3/tetR-KR, pBROAD3/tetR-mCherry, pBROAD3/TA-KR and pBROAD3/TA-mCherry using Lipofectamine 2000. For endogenous protein, just transfect anyone of pBROAD3/tetR-KR, pBROAD3/tetR-mCherry, pBROAD3/TA-KR and pBROAD3/TA-mCherry. Follow the transfection protocol of Lipofectamine 2000.
3. Then incubate in the 5% CO₂ incubator at 37 °C for 24 h to 48 h.
4. KR activation (there are two ways for KR activation)
 - a. Local activation of one KR spot

Local activation of one KR spot was performed with a 559 nm laser (1 mW/scan) of Olympus FV1000 confocal microscopy system in a selected area. One scan takes less than 1 sec. The final power (160 mJ) delivered to the KR (around 1 m²) spot is around 6 mJ/m².
 - b. Activation of KR in bulk cells

Activation of KR in bulk cells was done by exposing cells to a 15 watt SYLVANIA cool white fluorescent bulb for 10 min in a stage UVP.
5. The response of either GFP-tagged protein or endogenous protein of authors at the sites of tetR or TA-KR can be monitored in living cell.
6. The response of endogenous protein at the sites of tetR or TA-KR can be monitored by immunostaining with the antibody.

Notes

For calculation of the dose that was delivered to the KillerRed spot:

1. In the case of the 559 nm laser, the laser light was delivered to the selected area (around 25 m²) with 20 mJ/sec for 8 sec. Therefore, the final power (160 mJ) delivered to the KR (around 1 m²) spot is around 6 mJ/ m². For calculation of the dose that was delivered to the KillerRed spot based on the pixel size, the pixel size for irradiation is (0.138 µm/pixel) and the dwell time per pixel is (8 us/pixel). The irradiation is at 1.0 mW (1.0 mJ/s). With a

- dwelling time of 8 us/pixel, this irradiates each pixel with 8.0 nJ/pixel/scan. Multiplying by the number of scans gives the total energy per pixel.
2. In the case of fluorescent light activation, the rate of light is 15 J/m²/sec. With a 10 min light exposure, 9,000 J were delivered to the whole dish; the final power delivered to the KR (around 1 m²) spot is around 9 mJ/ m² upon light exposure. Cells were placed under a water bottle (height to light is 15 cm) to prevent an increase of temperature.

Acknowledgments

This protocol has been adapted from Lan *et al.* (2010) and Lan *et al.* (2014).

References

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