# Use of Tetracysteine-Tag and ReAsH as a Marker for DNA Damage in Correlative Light and Electron Microscopy

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## Introduction

DNA is the most critical biological target of ionizing radiation with DNA double-strand breaks (DSBs) being the most lethal form of DNA damage. The repair protein 53BP1 is readily recruited to DSBs and can be used as a DSB marker. The dynamics of repair processes can be studied in live cell experiments using fluorescence microscopy (FM) utilizing fluorescently tagged repair proteins. During DNA damage repair chromatin has to undergo several structural changes to facilitate damage processing. Direct imaging of these changes is limited by the resolution of FM. Correlative light and electron microscopy can combine the advantages of live imaging of dynamic processes with the high resolution of EM for studying chromatin changes at positions of ongoing repair marked by repair protein aggregates. For this purpose a marker is desirable that can be detected in both microscope types. In the last years several markers were developed: miniSOG, FLIPPER or Tetracysteine in combination with ReAsH [1, 2, 3]. Here we describe the establishment and first results of a cell line stably expressing the Tetracysteine (TC)-Tag coupled to the DSB repair protein 53BP1. Due to its small size, the TC-tag has a lower probability of interference with the protein function compared to classical fluorescent protein tags. Fluorescence is generated by the binding of membrane permeable FlAsH-EDT2 (Fluorescein Arsenical Hairpin Binder, Em528nm) or ReAsH-EDT2 (Resorufin Arsenical Hairpin Binder, Em608nm) to the Tetracysteine motive of the TC-tag. To detect 53BP1 in EM one can make use of the ReAsH mediated photoconversion of diaminobenzidine (DAB) causing polymerization of DAB, which can be subsequently contrasted by uranyl acetate (UA) leading to an electron dense material. Using this method, it will be possible to perform live cell experiments followed by subsequent imaging of the region of interest (DSB or DSB cluster) at high resolution in EM.

## Methods

### Cloning and Cell Culture

U2OS cells were transfected with the plasmid pcDNA6.2/nTC-Tag-DEST_53BP1 (plasmid backbone from Invitrogen). Between the protein of interest and the TC-tag a V5-epitope is inserted, which can serve as a control in Western Blot and immunofluorescence (IF). The transfected cells were selected by blasticidin and a stable cell line was generated.

### Live Imaging

For live experiments the cells were stained with FlAsH and Hoechst 33342. For imaging a FM with an environmental chamber was used. Irradiation was done using a nitrogen (337nm) or diode (405nm) laser. Images were taken several times and samples were fixed afterwards.

### X-ray Irradiation

The cells were stained with FlAsH or ReAsH before irradiation. Cells were irradiated with 1Gy X-ray at 250kV. Samples were fixed 30 minutes after irradiation.

### Immunocytochemical Staining

After irradiation the samples were fixed with 2% paraformaldehyde. Permeabilization was done with 0.5% Triton and blocking of unspecific binding sites using 0.4% BSA. For immunocytochemical staining V5-epitope antibody (AB, Invitrogen) and 53BP1 AB (Abcam) were used as primary AB. The secondary AB was Alexa647 (Life Technologies) for 53BP1, and Alexa488 or Alexa568 (Life Technologies) for V5, depending on whether FlAsH or ReAsH for the TC-staining was used.

### FlAsH/ReAsH Staining

The FlAsH or ReAsH staining was done in living cells using a modified protocol from Invitrogen. The growth medium was removed and the cells were washed with Opti-MEM three times. Subsequently, the staining was done with 0.5µM FlAsH/ReAsH in Opti-MEM for 30 min at 37°C. After staining the cells were washed two times with Opti-MEM and once with 250µM Dimercaprol, also called British anti-Lewisite (BAL) for 10 min. The samples were protected against light.

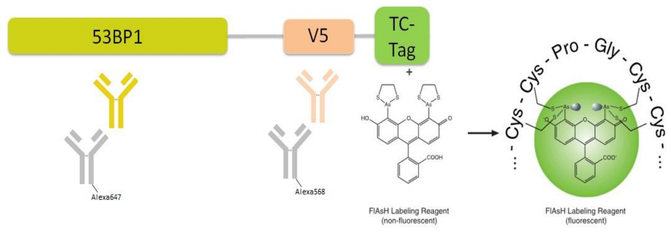


Fig. 1: Schematic representation of IF and FlAsH staining. Figure of FlAsH staining from [4].

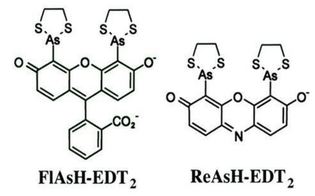


Fig. 2: Structure of FlAsH-EDT$_2$ and ReAsH-EDT$_2$. Figure from [3].

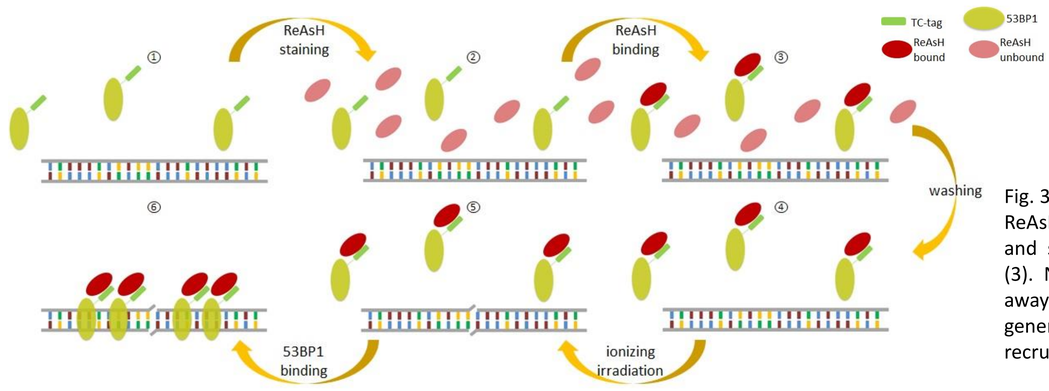


Fig. 3: Schematically staining of ReAsH. ReAsH-EDT$_2$ bind to TC-tagged 53BP1 and start to fluorescent red (ReAsH) (3). Not bound reagent gets washed away (4). By irradiation DSB are generated (5) and 53BP1 gets recruited (d).

## Results

The U2OS-nTC-Tag-53BP1 cells show radiation induced foci (RIF) after X-rays (Fig. 4 and 5). These RIF are visible both using FlAsH/ReAsH (Fig. 4b and 5b) or AB staining against V5 or 53BP1 (Fig 4c,d and 5c,d), respectively, demonstrating functional recruitment of the TC-tagged 53BP1 and proper FlAsH and ReAsH staining. FlAsH and ReAsH staining did not reveal any difference in the quality of the signal (Fig. 4b and 5b).
In the live cell recruitment experiment, the cells were irradiated with a nitrogen (337nm) or diode (405nm) laser. The bleached Hoechst signal (dark streaks through nucleus in Fig. 6c, e, g) indicates sites of laser generated DSBs. The recruitment of 53BP1 was already visible a few minutes after irradiation (Fig. 6d) and stayed till fixation 3 h after irradiation (Fig. 6h). After IF staining, the V5 and 53BP1 signal colocalized with the FlAsH signal of 53BP1 (Fig. 6g-k).

### X-ray Irradiation

FlAsH

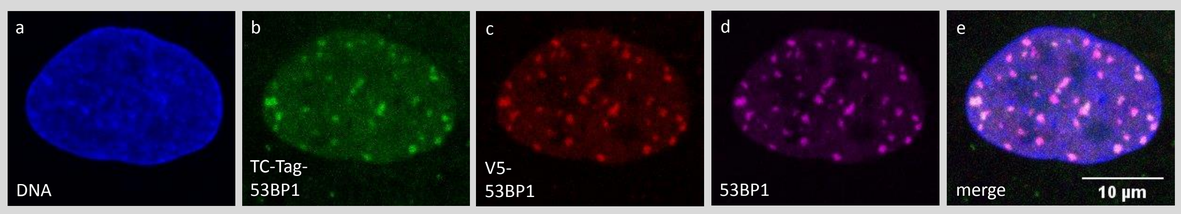


Fig. 4: Fluorescence images after 1Gy X-ray irradiation in fixed cells. U2OS-nTC-Tag-53BP1 cells were stained with FlAsH before irradiation. 30 min post-irradiation cells were fixed with PFA and additionally immunostained against V5-epitope and 53BP1. a: DAPI staining of DNA, b: FlAsH stained TC-Tag, c: AB staining against V5 and d: against 53BP1. e: merged image of a-d. The foci in b-c showed specific colocalization.

ReAsH

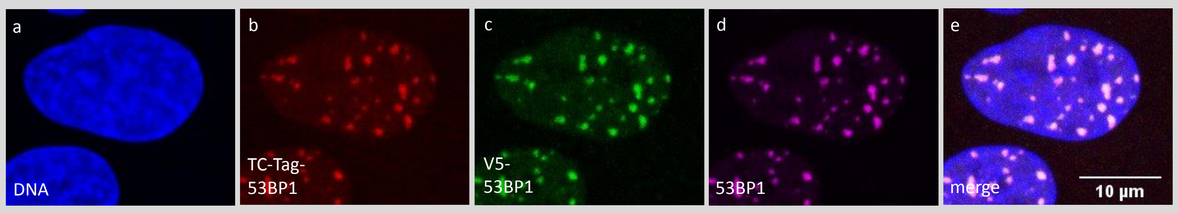


Fig. 5: Fluorescence images after 1Gy X-ray irradiation in fixed cells. U2OS-nTC-Tag-53BP1 cells were stained with ReAsH before irradiation. 30 min post-irradiation cells were fixed with PFA and additionally immunostained against V5-epitope and 53BP1. a: DAPI staining of DNA, b: ReAsH stained TC-Tag, c: AB staining against V5 and d: against 53BP1. e: merged image of a-d. The foci in b-c showed specific colocalization.

### Laser Irradiation

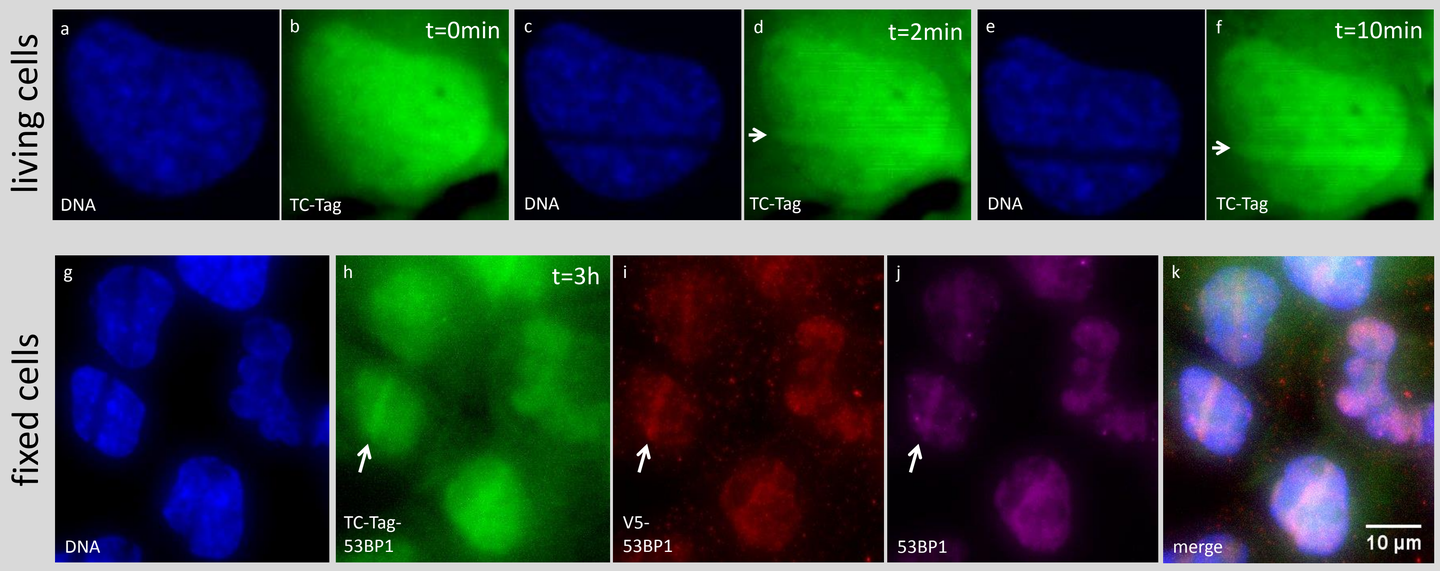


Fig. 6: Fluorescence images of living (a-f) and fixed cells (g-k). Three time points are shown (b, d and f). After imaging different fields, the sample was fixed (3h after irradiation). The fixed cells were stained against V5 (i) and 53BP1 (j). Both signals colocalized with the FlAsH signal of the TC-Tag, which is still clearly visible after fixation.

## Summary and Outlook

- We created a stable cell line (U2OS) with a TC-tagged 53BP1 which could be stained specifically with FlAsH or ReAsH.
- FlAsH and ReAsH show no difference in the quality of the signal (Fig. 4 and 5).
- The live experiment with laser irradiation shows recruitment of the FlAsH stained 53BP1 already after two minutes (Fig 6b).
- We show that Tetracysteine and ReAsH as marker for 53BP1 is a working alternative for GFP when CLEM experiments are performed due to its potential of DAB photoconversion for EM.

For CLEM the polymerization of DAB by photoconversion through illumination of ReAsH, and the DAB contrasted by UA (Fig. 7) will be the next part to be established. Samples will then be imaged using EM.

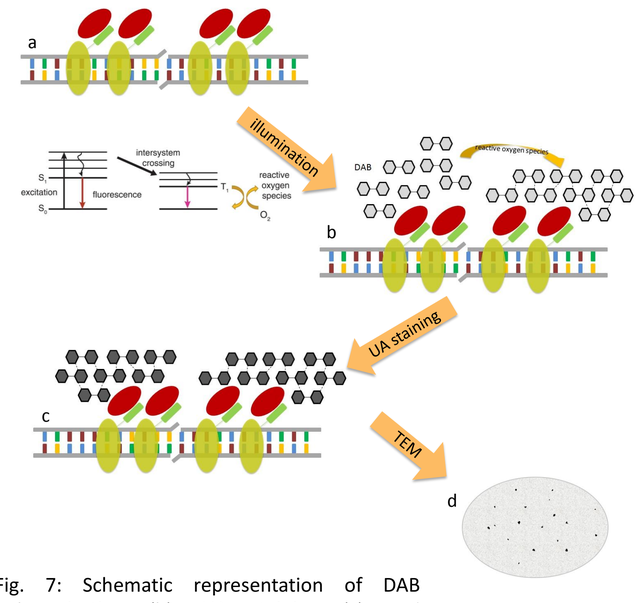


Fig. 7: Schematic representation of DAB polymerization (b), UA staining (c) and schematically picture of a transmission EM image (d). Picture for illumination from [5].

### References

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### Funding

HGS-HIRe for FAIR
Helmholtz Graduate School for Hadron and Ion Research

Bundesministerium für Bildung und Forschung

This work was funded by BMBF grant 02NUK037A