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Imaging of Fluorescently Tagged ATM Kinase at the Sites of DNA Double Strand Breaks

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Abstract

The combination of live cell imaging and laser micro-irradiation is an important technique to investigate the recruitment and kinetics of DNA repair molecules to DNA damage sites. In this chapter, we describe the detailed methods to study the dynamics of fluorescently tagged ATM protein kinase at laser-induced DNA double strand break (DSB) sites. The same protocol can be applied to analyze the recruitment and kinetics of other potential or known DNA repair proteins to DSB sites.

Keywords

ATM kinase; Laser micro-irradiation; DNA double strand breaks; Green fluorescent protein

1 Introduction

A cell's ability to quickly respond to and repair DNA damage is paramount, as destabilization of a cell's genome can result in senescence, apoptosis, or carcinogenesis. The importance of DNA repair pathways is supported by the observation that a host of genetic disorders are found in humans with germline mutations that encode for proteins responsible for the cellular response to DNA damage. One such disorder is ataxia-telangiectasia (A-T), which is a rare, autosomal-recessive disorder, characterized by progressive cellular ataxia, neuro-degeneration, cell cycle checkpoint defects, decreased DNA double strand break (DSB) repair capacity, immune deficiencies, genomic instability, and increased risk of carcinogenesis. The gene responsible for the A-T phenotype encodes a serine/threonine protein kinase termed ataxia-telangiectasia mutated (ATM). ATM's primary function is that it plays a central role in the cellular response to DSBs via its ability to phosphorylate an extensive range of substrates. In this regard, ATM acts like a transducer to relay a strong signal to numerous downstream effectors, which include proteins responsible for modulating the cell cycle, DNA repair, modulating signaling pathways, and apoptosis. Following DSB induction, ATM undergoes catalytic activation, is recruited to DNA damage sites, and is autophosphorylated.

A significant number of studies have focused on analyzing the key players and posttranslational modifications that modulate ATM's recruitment to DSBs and its dynamics/kinetics at DSBs. Traditionally, localization of ATM to DSBs was analyzed via immunofluorescence techniques that visualize nuclear foci formation of ATM at or near

DNA damage sites. A major limitation of this technique is that to observe DSB-induced ATM foci, cells must be fixed and thus determining the real-time dynamics/kinetics of ATM at DNA damage sites is unattainable. To this our end, our group and others utilize live cell imaging of fluorescently tagged ATM to study ATM's DNA damage responses in real time in living cells. Coupling irradiation via a micro-laser with fluorescent microscopy allows for continuous monitoring of real-time recruitment, retention, and dissociation of fluorescent-tagged ATM to DSBs in vivo [1–4]. Further, we have employed this technique to study the recruitment and kinetics of a significant number of other DNA repair proteins to laser-generated DSBs. In this chapter, we will provide the reagents and experimental procedures required to analyze the real-time dynamics of fluorescently tagged ATM at the sites of DSBs and its recovery after photobleaching.

2 Materials

2.1 Plasmids

Full-length wild-type ATM cDNA was obtained from Dr. Michael B. Kastan [5]. The ATM cDNA was subcloned into a modified pcDNA3 vector that carries a FLAG and YFP tag upstream of the multiple clone site to generate a FLAG-YFP-tagged ATM (YFP-ATM) [3]. The QuikChange Site-Directed Mutagenesis kit (Stratagene) was applied to introduce an alanine point substitution at serine 1981 to generate the FLAG-YFP-tagged ATM S1981A mutant expression vector (YFP-ATM S1981A) [3] (*see Note 1*).

2.2 Cell Culture and Transfection

1. ATM-deficient cells such as A-T patient-derived and SV40-transformed AT5BIVA (AT5) fibroblasts [6] to express YFP-FLAG-tagged ATM constructs.
2. Minimum Essential Medium Eagle Alpha Modification supplemented with 10% fetal bovine serum.
3. CO₂-independent medium supplemented with 10% fetal bovine serum.
4. Transfection reagents such as Lipofectamine 2000 reagent (Invitrogen) or electroporation with Nucleofector (Lonza, *see Note 2*).
5. 35-mm glass-bottom culture dishes (MatTek).
6. Digital temperature control system with a heating insert P (PeCon GmbH) and the Tempcontrol 37-2 control unit (PeCon GmbH).

¹Full-length ATM expression constructs are large in size and could recombine during DNA amplification. *E. coli* strains deficient in endonucleases and recombination (e.g., XL10) are recommended to improve the quality of DNA preparation. To amplify the ATM constructs, transform 50–100 ng of plasmid into XL10 cells, and then plate on LB + AMP plate for overnight incubation at 37 °C. Pick a single colony (small in size, not big ones) and grow up in 200 ml LB + AMP in a shaker incubator at 37 °C for 24 h. Next day, purify the plasmid DNA by the Qiagen midi-prep kit according to manufacturer's instructions. A typical yield for the ATM cDNA containing vectors is approximately 100 µg.

²Apply Nucleofector protocol X-001 with solution V to electroporate YFP-FLAG-ATM constructs into AT5BIVA cells.

2.3 Microscopes and Imaging

1. An inverted Axiovert 200M microscope equipped with a Plan-Apochromat 63×/NA 1.40 oil immersion objective and an AxioCam HRm digital camera (Carl Zeiss MicroImaging, Inc.) for time-lapse imaging.
2. A confocal LSM 510 Meta microscope (Carl Zeiss MicroImaging, Inc.) for FRAP analysis. Computer with Carl Zeiss AxioVision 4.8.2. software.

2.4 Laser Micro-Irradiation Unit

Spectra-physics nitrogen laser (Andor technology, VSL-337ND-S): The Spectra-Physics VSL-337ND-S nitrogen laser emits 4 ns pulses in the UV at 337 nm. The pulse repetition rate may be varied from 1 to 60 Hz with a pulse energy of up to 300 µJ. The nitrogen laser is directly coupled to the epifluorescence path of the microscope through a Micropoint Laser Illumination and Ablation System (Photonic Instruments, Inc.) and focused through a Plan-Apochromat 63× oil immersion objective. The nitrogen wavelength can be changed from 337 nm to 365 nm by BPBD 365 dye solution. Power output is controlled by the Micropoint System ranged from 1% to 89%.

3 Methods

3.1 YFP-ATM Accumulation at Laser Micro-Irradiation Damage Site

3.1.1 Transient Expression of ATM

1. For transient expression, transfect the ATM constructs into AT5 cells via Lipofectamine 2000 or Nucleofector following the manufacturer's protocol (*see* Note 3).
2. 24 hours after the transfection, split cells and plate 1×10^5 cells onto a 35-mm glass-bottom culture dish and allow to attach and grow for 24 h (*see* Notes 4 and 5).

3.1.2 Microscope and Laser-Irradiation Setup

1. Tempcontrol—switch on at least 15 min prior to use for live cell imaging (37 °C)
2. Turn on MCU28 motor stage controller
3. Turn on laser via the key switch. Set the repetition rate to 12 or maximum position depending on experiment.
4. Switch on MicroPoint Laser Illumination and Ablation System and set number to 75 for DSB induction.

³Transient expression can be performed in the human cell line HT1080.

⁴Transient expression utilizing Nucleofector typically results in approximately 20% of cells expressing YFP-ATM.

⁵It should be noted that stably expressed of YFP-ATM is normally applied in laser-micro-irradiation assays. To make stable cell lines, linearize the vector with a restriction enzyme and verify the complete digestion with agarose gel electrophoresis. Purify the linearized vector by phenol extraction (twice) followed by ethanol precipitation and wash with 70% ethanol and the pellet allowed to air dry. Resuspend the pellet in TE or water and calculate the DNA concentration. Perform transfection as described. Following transfection, plate 1×10^4 cells on 100 mm (four total dishes) with normal growth medium. 24 h post transfection, add the selection drug G418 (500 µg/ml), and incubate the cells for 10 days. Pick single colonies and check expression of YFP-ATM via microscopy (YFP signal) and Western blot analysis (antibodies that recognize FLAG tag or ATM).

5. Turn on microscope and the EXFO:UV lamp.
6. Select Objective on 63× (oil immersion)
7. Use 70/30 beam splitter for laser damage along with GFP filter on the UV adaptor.
8. Filter set selection: select designed GFP emission filter set for laser damage with GFP excitation filter on the UV adaptor.
9. Replace normal growth medium with heated CO₂-independent medium for micro-irradiation experiment.
10. Apply one drop of immersion oil (Carl Zeiss, 0000001111807) on the objective and lower objective with “focus down” bottom before placing the sample on it.
11. Attach the 35-mm dish onto the heated dish holder to stabilize the dish and then place this onto the heating insert.
12. Focus a cell and put “Cross mark” (only on the right side-eye) in the center of the nucleus.
13. Set up imaging parameter utilizing AxioVision software. The parameters utilized for multidimensional acquisition are:
 - a. “Dye”—YFP
 - b. “Exposure”—“fixed”—“400 ms”
 - c. “Hardware setting”
 - During acquisition—“workgroup: GFP”
 - After acquisition—“workgroup: shutter closed”
 - d. For initial accumulation (up to 10 min) utilize “Time lapse setting”
 - Interval—20 s
 - Cycles—32
 - e. For 2 h kinetics, utilize hand held timer and take images at specific time points (*see* Notes 6 and 7).

3.1.3 DSB Repair Kinetics with Laser Micro-Irradiation

1. Introduce DSBs in the nuclei of cultured cells by micro-irradiation with a pulsed nitrogen laser (365 nm, 10 Hz pulse). Set the output of the laser power at 75% of the maximum.
2. Initial accumulation (10 min) of ATM to laser-generated DSBs. For micro-irradiation and time-lapse imaging of initial accumulation of proteins to DSBs, maintain cells in CO₂-independent medium at 37 °C. Start image acquisition before laser micro-irradiation to obtain an image of the unirradiated cell. After first image acquisition, induce DSBs in cell nuclei by micro-irradiation with pulsed nitrogen laser allowing four laser pulses to hit a defined region of a single

nucleus. As defined in Subheading 3.1.2, **step 13**, the defined parameters in AxioVision software will acquire images before and after irradiation via a 400-ms exposure time. Capture images every 20 s for the duration of the 10 min time course. Convert signal intensities of accumulated YFP at the micro-irradiated site into a numerical value by the use of the Carl Zeiss Axiovision software version 4.8.2 (see below).

3. Kinetics of ATM at laser-induced DSBs (2 h). Apply the same procedure as described above for 2-h micro-irradiation and time-lapse imaging except capture images at defined time points, typically at 3, 10, 20, 30, 60, 90, and 120 min post micro-irradiation. Set a fixed exposure time to capture all images before and after irradiation.

3.1.4 Calculation of Relative Fluorescent Intensity for Protein Recruitment Kinetics

1. Open AxioVision software and appropriate image set of the same cell.
2. Circle damage site (D, white cycles in Fig. 1) and a background area (B, yellow cycles in Fig. 1) at each time point (see Note 8).
3. Obtain densitometric mean (fluorescence of the spot) via the software, which is based on the fluorescence intensity and area size (see Note 9).
4. To eliminate the background fluorescence, laser-induced fluorescence intensity accumulated at the damaged spot D_t is subtracted with the background fluorescence intensity B_t at an undamaged spot in the same nuclei at each time point (Fig. 1). The absolute fluorescence intensity $I_t = D_t - B_t$.
5. Compensate nonspecific photobleaching and UV lamp output fluctuation by normalizing the absolute fluorescence intensity $I_{(t)}$ accumulation at the damaged spot of each time point based on background intensity prior to laser damage using the formula: Normalized fluorescence intensity $NI_t = (I_t/B_t) \times D_0$, where B_t represents the undamaged site background intensity of each time point, D_0 represents the intensity of the damaged spot prior to irradiation.
6. Calculate relative fluorescence intensity (RFI) by using the formula: $RFI_t = (NI_t - NI_0)/(NI_{max} - NI_0)$, where NI_0 means normalized fluorescence intensity of the damaged spot prior to laser irradiation and NI_{max} is the maximum normalized fluorescence intensity of the damaged spot.
7. Each data point is the average of 10 independent measurements.
8. Average the relative intensity to make a graph (Fig. 2).

⁸The circle of the DNA damage site (accumulation site of YFP-tagged ATM) is typically set a 1 micron. But, this is also adjusted based on the size of the spot. The background site is typically 2 micron.

⁹Perform all calculations by utilizing Microsoft Excel. The densitometric means are placed in Excel for calculations.

3.1.5 Enhancement of Laser Micro-Irradiation with DNA Photosensitizer

1. DNA photosensitizer, such as Hoechst 33258 (Sigma-Aldrich B2883) and bromodeoxyuridine (BrdU, Sigma-Aldrich B5002), can also be used to assist in increasing signal (Fig. 3).
2. For sensitization with Hoechst, change to CO₂-independent medium and then add 1 µl of 10 mg/ml Hoechst 33258 in 2.5 ml medium and incubate the cells for minutes before initiating the experiment.
3. For BrdU sensitization, treat cells in regular medium with 10 µM BrdU for 16–24 h and then change to CO₂-independent medium, followed by laser initiation.

3.2 Fluorescence Recovery After Photobleaching (FRAP)

1. Perform FRAP measurements by photobleaching the entire ATM accumulation spot that formed after laser-induced DSBs and by measuring the subsequent recovery of fluorescence signal at the photobleached region.
2. Perform imaging and FRAP measurements on an LSM 510 Meta confocal microscope using a Plan-Apochromat 63×/NA 1.40 oil immersion objective. Acquire images by scanning with the 514-nm line of the argon laser of the LSM 510 Meta microscope using the standard image acquisition function of the LSM 510 software version 4.0SP1.
3. Set the tube current of the argon laser at 6.1 A, and the laser intensity at 0.3%. Set the pinhole at 2.51 Airy units, corresponding to an optical slice of 1.9 µm. Introduce DSBs in the nuclei of cultured cells by laser micro-irradiation with the pulsed nitrogen laser (*see* Subheading 3.1.3).
4. Perform FRAP measurements at 10–20 min after the introduction of DSBs, when accumulation of YFP-ATM reaches maximum.
5. Perform photobleaching with a pulse at 100% laser intensity (514-nm line of an argon laser, tube current set at 6.1 A). Match the dimensions of the circular FRAP area to those of the accumulation spot (~1.9 µm²).
6. Acquire image of the nucleus before photobleaching, immediately after photobleaching and at 2-s time intervals 50 cycles for 180 s.
7. In every image, measure fluorescence of the photobleached accumulation spot (Fas), from which the background fluorescence intensity was subtracted (*see step 4* in Subheading 3.1.4).
8. Normalize Fas intensity to the prebleach value: $F_{\text{norm}} = \text{Fas}_{(t)} / \text{Fas}_{(0)}$.
9. Set the prebleach intensity $\text{Fas}_{(0)}$ as the maximum intensity and normalize to 1. Set the intensity immediately postbleach as 0.
10. Each data point is the average of 10 independent measurements (Fig. 4).

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Fig. 2.

Ablation of autophosphorylation at serine 1981 of ATM accelerates dissociation of ATM from DSBs. Initial accumulation kinetics (a) and 2-h time course (b) of YFP-ATM wt or YFP-ATM S1981A at laser-generated DSBs. *Error bars* represent the SD. © So et al. [3]. Originally published in JOURNAL CELL BIOLOGY. <http://jcb.rupress.org/content/187/7/977>

Fig. 3.

Local accumulation of ATM in cells exposed to laser microirradiation in the presence or absence BrdU. **(a)** AT5BIVA cells stably expressing YFP-ATM wt were treated or untreated with BrdU (10 μ M) for 16 h, and micro-irradiated with indicated laser output. Images were taken at 10 min after micro-irradiation. Bar, 10 μ m. **(b)** Summary of YFP-ATM wt recruitment to irradiated region in AT5BIVA cells under different conditions. © So et al. [3]. Originally published in JOURNAL CELL BIOLOGY. <http://jcb.rupress.org/content/187/7/977>

Fig. 4.

Phosphorylation of ATM influences the exchange rate between DNA-bound and free forms of ATM protein at DNA double strand break sites. FRAP curves of wild-type ATM and S1981A in AT5BIVA cells at DNA double strand break sites. The data show the normalized data from the average of three independent experiments, fitted to an Exponential Rise to Max model. Prebleach intensity levels were normalized to 1 and post-bleach intensity levels were normalized to 0. In each experiment at least 10 cells were measured. ©Davis et al. [4]. Originally published in CELL CYCLE. <http://www.tandfonline.com/doi/full/10.4161/cc.9.13.12148>