Identifyn Standards for Optical Microscopy Methods™
Identifyn® Primary Antibody
Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P)
Cat ID# PA-RR 000012

Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7)
Cat ID# - PA-MM 000002

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')$_2$ (H + L)
Cat ID# - SA 000063

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, F(ab')$_2$ (H + L)
Cat ID# - SA 000019

ATM (ataxia-telangiectasia mutated) is a crucial serine/threonine kinase that plays a central role in the DNA damage response (DDR), particularly in response to DNA double-strand breaks (DSBs). ATM's activation and subsequent phosphorylation cascades are essential for detecting DNA damage and initiating repair processes. Here's a detailed overview of how ATM is activated, its targets, and its role in the DDR:

Activation of ATM

In response to DNA damage, particularly DSBs, ATM is activated through a series of events:

Detection of DNA Breaks: ATM can sense DNA breaks by associating with the MRN complex (MRE11, RAD50, and NBS1). This complex recognizes DSBs, leading to ATM's recruitment to the damaged site.

Autophosphorylation: Upon detection of DNA damage, ATM autophosphorylates at serine 1981 (S1981), thereby activating it. This phosphorylation event is critical for ATM's kinase activity and its ability to phosphorylate downstream targets.

Dimer-to-Monomer Transition: In its inactive form, ATM is a dimer. It transitions to an active monomeric state upon activation, allowing it to interact with and phosphorylate various downstream proteins.

Phosphorylation Targets of ATM

Once activated, ATM phosphorylates many proteins involved in the DNA damage response, cell cycle regulation, and apoptosis. Here are some critical targets of ATM:

CHK2: ATM phosphorylates CHK2, a cell cycle checkpoint control kinase. This phosphorylation helps to halt the cell cycle, allowing time for DNA repair.

p53: ATM phosphorylates p53, activating this tumor suppressor, leading to cell cycle arrest or apoptosis if the damage is irreparable.

H2AX: ATM phosphorylates histone H2AX, forming γ-H2AX, a marker of DNA damage. This modification helps signal the presence of DSBs and recruit DNA repair machinery.

BRCA1: ATM's phosphorylation of BRCA1 is critical for homologous recombination, a key DNA repair pathway for DSBs.

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NBS1: As part of the MRN complex, NBS1 phosphorylation by ATM enhances the complex's DNA repair functions.

53BP1: ATM phosphorylation of 53BP1 helps recruit this protein to sites of DSBs, thereby guiding repair pathways such as non-homologous end joining (NHEJ).

Role of ATM Phosphorylation in DNA Repair

ATM-mediated phosphorylation plays a central role in coordinating the DNA damage response:
Signal Propagation: Phosphorylation cascades initiated by ATM amplify the signal in response to DNA damage, ensuring an efficient response.

Recruitment of Repair Machinery: Phosphorylation of specific proteins helps recruit DNA repair complexes to the sites of DNA damage.

Cell Cycle Regulation: ATM activation can lead to cell cycle checkpoints, preventing cells with damaged DNA from dividing.

Apoptosis: If the DNA damage is too severe to repair, ATM's activation can lead to apoptosis, preventing the propagation of damaged cells

ATM's phosphorylation in response to DNA damage is crucial for detecting DNA double-strand breaks and coordinating the DNA damage response. By phosphorylating a range of critical proteins, ATM orchestrates a complex signaling network that facilitates DNA repair, cell cycle regulation, and apoptosis, thereby maintaining genomic stability.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, DNA damage was induced by treatment with 2 µM Etoposide–spiked complete growth media for 12 hours, followed by -20 °C methanol fixation and blocking with Thermo Fisher Fish Serum Blocker (cat#37527X3). The primary antibody, Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P), was incubated at room temperature for 1 hour at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, $F(ab')_2$ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS.

The second primary antibody, Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.
Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, $F(ab')_2$ (H + L) was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI cat#P36962.

Microscope
Zeiss LSM980 Confocal with **NIR Detection**, Axio Observer.Z1/7, using a Plan-Apochromat 63x/1.40 Oil DIC M27, was used with the following parameters for each dye:

Z Stack - (3D) - *Panel A, Z-Plane 40 of 82*
Z-Stack = 82 Slices (2.43 µm)

Channels = 2
Scaling (Per Pixel) = 35.29 nm X 35.29 nm X 30.00 nm

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Image Size (Pixels) = 945 X 703
Image Size (Scaled) = 33.34 µm X 24.81 µm
Bit Depth = 16 Bit
Image Center Position = X: 92.76 mm, Y: 50.17 mm
Stage Position = X: 92.76 mm, Y: 50.17 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - *Panels B and C, Colocalization Measurement, same Z-Plane.*
Z-Stack = 82 Slices (2.43 µm)

Channels = 2
Scaling (Per Pixel) = 35.29 nm X 35.29 nm X 30.00 nm
Image Size (Pixels) = 154 X 118
Image Size (Scaled) = 5.43 µm X 4.16 µm
Bit Depth = 16 Bit
Image Center Position = X: 92.76 mm, Y: 50.17 mm
Stage Position = X: 92.76 mm, Y: 50.17 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 328 µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS LP 640
Laser Wavelength = **639 nm @ 0.40%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.9
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.04 µs
Frame Time = 19.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 643 - 735
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = Super Resolution: 9.8 (3D, Auto)

488 -

Reflector = None

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Contrast Method = Fluorescence
Pinhole = 5.00 AU / 250 µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 550
Laser Wavelength = **488 nm @ 0.5%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.9
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.04 µs
Frame Time = 19.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 499 - 548
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = Super Resolution: 9.5 (3D, Auto)

2-Dimensional View - *Panel A*

Airyscan optical section acquired at Z-plane 40 of 82 showing Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG – (BLR031P), detected using Identifyn® SRM Alexa Fluor™ 647 affinity-purified Goat Anti-Rabbit $F(ab')_2$ (H + L) (red), and Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7), detected using Identifyn® SRM Alexa Fluor™ 488 affinity-purified Donkey Anti-Mouse $F(ab')_2$ (H + L) (green). A white box denotes the region of interest (ROI) selected for colocalization analysis in Panels B and C.

Colocalization View - *Panels B and C*

Horizontal Axis - AF647, Threshold 4521, Range Auto
Vertical Axis - AF488, Threshold 12040, Range Auto

Image Measurement = Entire Field of Panel B

Colocalization analysis of phospho-ATM (X-axis) and H2Ax (Y-axis) was performed across the entire image acquisition.

Phospho-ATM shows a lack of colocalization with H2Ax, consistent with the known biology of phospho-ATM signaling and total H2Ax distribution.

Summary

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Panel A shows an Airyscan optical section acquired at Z-plane 40 of 82 demonstrating Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG – (BLR031P), detected using Identifyn® SRM Alexa Fluor™ 647 affinity-purified Goat Anti-Rabbit $F(ab')_2$ (H + L) (red), and Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7), detected using Identifyn® SRM Alexa Fluor™ 488 affinity-purified Donkey Anti-Mouse $F(ab')_2$ (H + L) (green). A white box denotes the region of interest (ROI) selected for colocalization analysis in Panels B and C.

Panel B presents an enlarged ROI from the same Airyscan optical section shown in Panel A, demonstrating the spatial relationship between phospho-ATM and H2Ax within the selected nuclear region. Minimal direct signal overlap is observed between phospho-ATM and total H2Ax within the analyzed volume. This finding is biologically consistent with the established role of activated phospho-ATM as an upstream DNA damage response kinase responsible for phosphorylation of H2Ax into γH2AX following induction of DNA double-strand breaks. Because the present dataset measures total H2Ax rather than γH2AX specifically, strong molecular colocalization with phospho-ATM is not expected. Instead, phospho-ATM localizes to active DNA damage signaling domains and checkpoint-associated repair territories, while total H2Ax remains broadly distributed throughout chromatin.

Importantly, this dataset functions as a biological pressure test for the Identifyn® phospho-ATM antibody under super-resolution conditions. If the antibody lacked specificity, substantial nonspecific overlap with broadly distributed H2Ax chromatin signal would be expected. Instead, the reduced overlap observed at Airyscan resolution demonstrates that increased spatial resolution resolves the apparent colocalization previously observed at diffraction-limited widefield scales into structurally distinct signaling territories. The observed segregation of phospho-ATM from total H2Ax strongly supports the specificity of the Identifyn® phospho-ATM antibody, as the staining pattern closely matches the expected biological behavior of activated ATM signaling rather than nonspecific chromatin-wide overlap. Collectively, these findings support the interpretation that the broad overlap observed in lower-resolution imaging modalities primarily reflects shared DNA-damage-associated nuclear environments rather than direct molecular colocalization between phospho-ATM and total H2Ax.

Image Correction

NONE

Image by J.K. Bennett
Image Analysis and Data Presentation by B.T. Bennett

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