

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.

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 μ M 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 Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A, Z-Plane 61 of 120

Z-Stack = 120 Slices (3.57 μ m)

Channels = 2

Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm

Image Size (Pixels) = 1557 X 1805
Image Size (Scaled) = 32.87 μ m X 38.11 μ m
Bit Depth = 16 Bit
Image Center Position = X: 57.77 mm, Y: 35.27 mm
Stage Position = X: 57.77 mm, Y: 35.27 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Z Stack - (3D) - 3-Dimensional Volume Projection, with Orthogonal Cut Plane

Z-Stack = 120 Slices (3.57 μ m)

Channels = 2
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image Size (Pixels) = 376 X 330
Image Size (Scaled) = 7.94 μ m X 6.97 μ m
Bit Depth = 16 Bit
Image Center Position = X: 57.77 mm, Y: 35.27 mm
Stage Position = X: 57.77 mm, Y: 35.27 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

647 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 640
Channel Name = T1-Axiocam 820 -SR
Excitation Wavelength = 640
Emission Wavelength = 729
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 100 ms
Depth of Focus = 1.13 μ m
Binning Mode = 2,2
Laser Wavelength = 640 nm @ 1.0%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 36.5 μ m grating

488 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 488
Channel Name = T2-Axiocam 820-SR
Excitation Wavelength = 488

Emission Wavelength = 525
 Effective NA = 1.4
 Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
 Imaging Device = Axiocam 820
 Camera Adapter = 1X
 Exposure Time = 120 ms
 Depth of Focus = 0.81 μ m
 Binning Mode = 2,2
 Laser Wavelength = 488 nm @ 1.00%
 Frame Time = 1.59 s
 Scan Direction = Unidirectional
 Grating = 27.5 μ m grating

Post Processing - SIM2 Processing

Processing Dimension - 3D
 Result Sampling = 4
 Process Sampling = 4
 Channels = 2
 Algorithm = SIM2
 Input SNR = Low
 Regularization Weight = 0.0000
 Iterations = 3
 Filter = No Filter
 Fitting = Fast Fit
 Normalization = Auto

2-Dimensional View - Panel A

Lattice SIM2 optical section acquired at Z-plane 61 of 120 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 analysis in Panel B.

3D Image Processing - Panel B

3D Volume Projection = Precise
 Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
 Projection = View Angle 45°, Scale Z 35%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Clipping Image Process - Panel B

Clipping planes are introduced in Panel B to highlight the phospho-ATM within the nuclear compartment by adjusting or cutting away portions of the image using a triple-wedge cut, removing parts of the image in the X-Y, X-Z, and Y-Z planes.

X-Y = Activate was selected, textured opaque was chosen, and a white outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip = -20%

Clip X = 0°

Clip Z = 0°

X-Z = Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip = -28%

Clip Y = -45°

Clip Z = 0°

Y-Z = Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = 0%

Clip Y = -45°

Clip Z = 0°

Summary

Panel A shows a Lattice SIM² optical section acquired at Z-plane 61 of 120, demonstrating phospho-ATM and H2Ax distribution following etoposide-induced DNA damage. 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), demonstrate highly organized intranuclear redistribution following genotoxic stress. A white box denotes the region of interest selected for three-dimensional structural analysis in Panel B.

Panel B presents a three-dimensional orthogonal wedge reconstruction of the selected ROI, employing simultaneous X-Y (white), X-Z (green), and Y-Z (red) cut planes to interrogate the organization of the volumetric signal. Prior Airyscan and Lattice SIM datasets demonstrated that phospho-ATM and total H2Ax progressively lose apparent colocalization as spatial resolution increases toward ~60 nm, strongly supporting the biological specificity of the Identifyn® phospho-ATM antibody and the expected segregation of activated ATM signaling from broadly distributed total H2Ax chromatin. Within the Lattice SIM² dataset, however, the increased resolving power approaching ~40 nm XY begins to reveal internal signal heterogeneity and apparent microdomain organization within phospho-ATM-associated aggregates.

In the lower-right orthogonal wedge reconstruction, a yellow-circled region of interest highlights structural patterns that appear superficially reminiscent of the nanoscale subunit architectures resolved by STORM imaging in Panel C. At this resolution scale, SIM² may begin to suggest the presence of subdomains, clustered repair assemblies, or partially separated signaling territories within ATM-associated DNA damage response foci. However, these observations remain highly interpretive. Even at ~40 nm XY resolution, SIM² remains limited by axial resolution constraints and residual signal convolution, such that apparent "subunits" may still represent overlapping molecular populations compressed within the diffraction volume. In contrast, STORM imaging achieves substantially higher localization precision and may be metaphorically viewed as "seeing inside" the broader volumetric signal observed in SIM².

Biologically, activated phospho-ATM functions as a master upstream kinase coordinating the DNA double-strand break response through phosphorylation-dependent checkpoint signaling, chromatin remodeling, and recruitment of downstream repair machinery, including phosphorylation of H2Ax into γ H2AX. Because the present dataset measures total H2Ax rather than γ H2AX specifically, direct molecular colocalization is not expected, and the observed spatial segregation is therefore biologically appropriate. Importantly, the progression from Airyscan through Lattice SIM and ultimately Lattice SIM² demonstrates the power of the Stepdown Methodology™ in progressively resolving apparent colocalization into structurally distinct DNA damage-associated signaling territories.

Nevertheless, the dataset also highlights the fundamental limitations of optical super-resolution when interrogating molecular architecture at biologically relevant scales. ATM itself is a large kinase complex, while nucleosomal and chromatin-associated signaling domains exist at dimensions approaching or below the resolving capacity of SIM². Thus, although super-resolution modalities provide increasingly valuable insights into response, redistribution, aggregate formation, and candidate spatial organization, caution is warranted when interpreting apparent microdomains or presumed molecular subunits without true nanoscale localization methods such as STORM.

Image Correction

Panel B, the 647 channel was changed from its default red to white to improve visualization of the dataset.

Image by J. H. Cheong
Image Analysis and Data Presentation by B.T. Bennett

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