

Identifyn Standards for Optical Microscopy Methods™Identifyn® Primary Antibody**Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P)***Cat ID# - 000013***Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M)***Cat ID# - DC 000003*Identifyn® Secondary Antibody**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000060*

ATR (Ataxia Telangiectasia and Rad3-related) is a critical serine/threonine kinase that plays a central role in the cellular response to DNA damage, particularly in response to replication stress and single-strand breaks (SSBs). Unlike ATM (Ataxia Telangiectasia Mutated), which is primarily activated by double-strand breaks, ATR is activated by persistent single-stranded DNA (ssDNA) regions that arise during DNA replication or repair processes.

Overview of ATR Activation

ATR is activated when extensive ssDNA accumulates, often resulting from stalled replication forks or other forms of replication stress. This activation is usually mediated by RPA (Replication Protein A), which binds to ssDNA and recruits ATR and its key activator, ATRIP (ATR Interacting Protein).

Recruitment to Sites of Damage: RPA-coated ssDNA signals DNA damage or replication stress. ATR, with ATRIP, is recruited to these sites, activating ATR's kinase activity.

Interaction with the 9-1-1 Complex: ATR activation is further facilitated by the 9-1-1 complex (comprising RAD9, RAD1, and HUS1), which is loaded onto DNA by RAD17-RFC at sites of DNA damage. This complex interacts with TOPBP1 (Topoisomerase II Binding Protein 1), a key activator of ATR, thereby fully activating ATR kinase activity.

ATR's Role in Single-Strand Break Repair

ATR plays a vital role in facilitating and coordinating the repair of single-strand breaks through various mechanisms:

Checkpoint Activation: ATR is critical for activating checkpoint signaling pathways, notably through phosphorylation of CHK1. This leads to cell cycle arrest or delay, allowing time for DNA repair before cell division continues. This checkpoint activation is crucial in preventing cells from progressing with unrepaired single-strand breaks.

Coordination of Repair Pathways: ATR activation helps coordinate DNA repair processes by recruiting repair proteins and integrating signals across various pathways. This coordination ensures that repair proteins involved in base excision repair (BER), homologous recombination, and other SSB-related pathways are recruited and activated as needed.

Stabilization of Replication Forks: ATR contributes to the stabilization of stalled replication forks, preventing the collapse into double-strand breaks. This is particularly

important in preventing the progression of single-strand breaks into more severe DNA damage.

ATR in DNA Damage Response

Beyond its direct role in facilitating single-strand break repair, ATR has broader implications in the DNA damage response:

Genomic Stability: By ensuring proper checkpoint activation and coordinating DNA repair, ATR helps maintain genomic stability, reducing the risk of mutations and chromosomal abnormalities.

Response to Replication Stress: ATR is particularly critical in responding to replication stress, which can result in single-strand breaks due to replication fork stalling or collapse. ATR's activation in these contexts ensures that DNA repair processes are promptly initiated.

ATR is a central player in responding to single-strand breaks and replication stress, activating checkpoint pathways, coordinating DNA repair, and stabilizing replication forks. Through these functions, ATR significantly contributes to maintaining genomic stability and ensuring that cells do not progress with unrepaired DNA damage.

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 medium 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 ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3 times in 1X PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5 times in 1X PBS.

The second primary antibody, Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M) was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5 times in 1X PBS and mounted using [ProLong™ Diamond Antifade Mountant with DAPI \(cat# P36962\)](#).

Microscope

Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807 Mono, and X-Cite Xylis Lamp was used with the following parameters for each dye:

Z Stack - (3D) - Panel A

Z-Stack = 85 Slices (8.4 μ m)

Channels = 3

Scaling (Per Pixel) = 0.143 μ m X 0.143 μ m X 0.100 μ m

Image Size (Pixels) = 621 X 500

Image Size (Scaled) = 88.71 μ m X 71.43 μ m

Bit Depth = 14 Bit

Image Center Position = X: 46.89 mm, Y: 50.53 mm

Stage Position = X: 46.89 mm, Y: 50.53 mm

ROI Center Offset = X: 1.14 μ m, Y: 0.00 μ m

Z Stack - (3D) - Panel B, Region of Interest

Z-Stack = 47 Slices (4.6 µm), Z-stack was trimmed via the Gallery Tool, to remove non-data-bearing Z-planes

Channels = 3

Scaling (Per Pixel) = 0.143 µm X 0.143 µm X 0.100 µm

Image Size (Pixels) = 217 X 173

Image Size (Scaled) = 31.00 µm X 24.71 µm

Bit Depth = 14 Bit

Image Center Position = X: 46.89 mm, Y: 50.53 mm

Stage Position = X: 46.89 mm, Y: 50.53 mm

ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

647 -

Reflector = 50 Cy 5

Beam Splitter = 660

Filter Excitation Wavelength = 625 - 655

Filter Emission Wavelength = 665 - 715

Contrast Method = Fluorescence

Light Source = X-Cite Xylis Lamp Intensity @ 25.00%

Exposure Time = 250 ms

Excitation Wavelength = 653

Emission Wavelength = 668

Effective NA = 1.25

Imaging Device = Axiocam 807 Mono

Camera Adapter = 1X

Depth of Focus = 1.30 µm

Binning Mode = 2,2

488 -

Reflector = 38 eGFP

Beam Splitter = 495

Filter Excitation Wavelength = 450 - 490

Filter Emission Wavelength = 500 - 550

Contrast Method = Fluorescence

Light Source = X-Cite Xylis Lamp Intensity @ 10.00%

Exposure Time = 100 ms

Excitation Wavelength = 493

Emission Wavelength = 517

Effective NA = 1.25

Imaging Device = Axiocam 807 Mono

Camera Adapter = 1X

Depth of Focus = 1.00 µm

Binning Mode = 2,2

DAPI - Collected but not Shown in Data Representation

Reflector = 49 DAPI

Beam Splitter = 395

Filter Excitation Wavelength = 335 - 383

Filter Emission Wavelength = 420 - 470

Contrast Method = Fluorescence

Light Source = X-Cite Xylis Lamp Intensity @ 5.00%
 Exposure Time = 15 ms
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.4
 Imaging Device = Axiocam 807 Mono
 Camera Adapter = 1X
 Depth of Focus = 0.72 μm
 Binning Mode = 2,2

Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mode "Optical Sectioning" - enabled correction selected.

Normalization, Phase Corrector, Fourier Filter, and Deconvolution **were NOT used.**

3D Image Processing - Panel A

3D Maximum Projection = Precise
 Appearance = Threshold 13% all channels, Ramp 0% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 90%, Enabled Tone Mapping
 Projection = View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel B

3D Volume Projection = Precise
 Appearance = Threshold 13% all channels, Ramp 50% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
 Projection = View Angle 35°, Scale Z 100%, 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 nuclear colocalization of the phospho-ATR with gamma-H2AX in response to etoposide-induced DNA damage 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 blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -93%

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 = 13%

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 = 3%

Clip Y = -45°

Clip Z = 0°

Panel B shows a wedge-cut view displayed from the top with a slight backward rotation, highlighting the region of wedge removal and revealing the full depth of signal across the z-stack. This orientation provides an alternative view of wedge geometry and confirms nuclear colocalization of the phospho-ATR and gamma-H2AX in response to etoposide-induced DNA damage within the nuclear compartment.

Summary

Panel A presents a three-dimensional Apotome SIM acquisition following etoposide-induced DNA damage, showing Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P), visualized using Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L), together with Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M). Compared with conventional widefield imaging, Apotome SIM optical sectioning improves axial discrimination and reduces out-of-focus fluorescence, enabling enhanced visualization of ATR.p-associated DNA damage response territories within the nuclear volume. Following etoposide exposure, ATR.p exhibited pronounced intranuclear redistribution and punctate damage-associated signaling structures that overlapped with γH2Ax-enriched nuclear regions, consistent with coordinated replication stress and DNA damage checkpoint activation.

Panel B presents a wedge-cut volumetric reconstruction of the same dataset using orthogonal clipping planes to interrogate signal distribution throughout the z-stack. The clipping workflow reveals that ATR.p and γH2Ax signals occupy shared nuclear response territories across multiple focal planes, while simultaneously illustrating the limitations of diffraction-limited optical sectioning approaches. Although Apotome SIM improves volumetric interpretation compared with standard widefield microscopy, individual DNA damage foci, repair subdomains, and nanoscale signaling architectures remain unresolved at this imaging stage. Thus, the observed overlap should be interpreted as biologically coordinated recruitment within DNA damage-associated nuclear territories rather than definitive molecular colocalization.

Importantly, this dataset represents a critical transitional stage within the Identifyn® Stepdown Methodology™, bridging diffraction-limited widefield imaging and higher-resolution confocal and super-resolution modalities. The progression from widefield to Apotome SIM demonstrates how improved optical sectioning progressively refines apparent signal overlap while preserving biologically coherent ATR.p and γH2Ax-associated DNA damage response behavior prior to higher-resolution interrogation by confocal, Airyscan, Lattice SIM, SIM², and STORM imaging workflows.

Image Correction

NONE

Image by E.F.Simon

Image Analysis and Data Presentation by B.T. Bennett

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