

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 by phosphorylating 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 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) - 2D at Z-Plane 58 of 115, Panel A

Z-Stack = 115 Slices (3.42 μ m)

Channels = 3

Scaling (Per Pixel) = 0.021 nm X 0.021 nm X 0.030 nm

Image Size (Pixels) = 1145 X 1654

Image Size (Scaled) = 24.17 μ m X 34.92 μ m

Bit Depth = 16 Bit

Image Center Position = X: 69.03 mm, Y: 39.62 mm

Stage Position = X: 69.03 mm, Y: 39.62 mm

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

Z Stack - (3D) - 3D Volume Projection, Panels B and C

Z-Stack = 115 Slices (3.42 μ m)

Channels = 3
Scaling (Per Pixel) = 0.021 nm X 0.021 nm X 0.030 nm
Image Size (Pixels) = 275 X 268
Image Size (Scaled) = 5.81 μ m X 5.66 μ m
Bit Depth = 16 Bit
Image Center Position = X: 69.03 mm, Y: 39.62 mm
Stage Position = X: 69.03 mm, Y: 39.62 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 = 120 ms
Depth of Focus = 1.13 μ m
Binning Mode = 2,2
Laser Wavelength = 640 nm @ 3.5%
Frame Time = 1.59 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 @ 0.50%
Frame Time = 1.59 s

Scan Direction = Unidirectional
Grating = 27.5 μm grating

Post Processing - SIM Processing

Processing Dimension = 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 2
Algorithm = SIM
SIM Settings = Standard
Auto Sharpness = On
Sharpness = 11.3281 (647), 11.2313 (488)
Fitting = Fast Fit
Normalization = Auto

2-Dimensional View- Panel A

Shows 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) secondary antibody and Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M). A Region of Interest is denoted by a white box to be further examined in the 3-Dimensional Volume Projection in Panel B and C.

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 35°, Scale Z 75%, 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 C to highlight the Rabbit anti-Human Phospho-ATR (Threonine 19189) Recombinant Monoclonal and gamma-H2AX (Serine 139)488 Direct Conjugate 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-Z and Y-Z planes.

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 = -8%
Clip Y = -30°
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 = -27%

Clip Y = 15°
Clip Z = 0°

Panel C 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 distribution across the z-stack. This orientation provides an alternative view of wedge geometry. It supports intranuclear overlap, coordinated recruitment, and improved sub-diffraction structural discrimination of ATR.p and γ H2Ax-associated DNA damage signaling territories relative to preceding imaging modalities.

Storm - Panel C

Imaging from the same product dataset is shown in Panel C to demonstrate the remaining resolution limitations of Lattice SIM microscopy. While Lattice SIM provides improved sub-diffraction localization and structural discrimination relative to Airyscan imaging, STORM further resolves nanoscale organizational features and the underlying DNA damage-associated signaling architectures that are not fully distinguishable in the Lattice SIM dataset. Importantly, the observed signal distribution and structural organization remain highly consistent with the preceding Airyscan data while beginning to approximate the single-molecule localization patterns visualized by STORM imaging more closely.

Summary

Panel A shows a single Lattice SIM optical section (Z-plane 58 of 115) of 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 γ H2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M), following etoposide-induced DNA damage. Relative to conventional widefield, WF-SIM, confocal, and Airyscan imaging, Lattice SIM provides substantially improved sub-diffraction structural discrimination and enhanced visualization of punctate DNA damage-associated signaling architectures within the nuclear compartment. ATR.p demonstrated highly organized intranuclear redistribution patterns and spatial overlap with γ H2Ax-enriched damage territories, consistent with coordinated replication-stress signaling and DNA damage checkpoint activation.

Panels B and C present three-dimensional volume reconstruction and wedge-cut clipping-plane analysis of the region of interest identified in Panel A, allowing improved interrogation of signal organization throughout the z-stack. Compared with preceding modalities, Lattice SIM imaging reveals increased structural separation of resolution-limited signaling aggregates and improved visualization of sub-diffractional organizational features associated with ATR.p and γ H2Ax recruitment. Although the observed overlap and structural refinement strongly support coordinated recruitment within shared DNA damage-associated nuclear territories, definitive nanoscale molecular architecture and precise repair-complex organization remain incompletely resolved at this imaging stage. Accordingly, the observed signal relationship should continue to be interpreted as coordinated recruitment and overlapping damage-associated signaling territories rather than definitive molecular colocalization.

Importantly, this dataset represents a major super-resolution refinement stage within the Identifyn® Stepdown Methodology™, demonstrating progressively improved structural

interpretation as spatial resolution increases across modalities. The transition from widefield to WF-SIM, confocal, Airyscan, and now Lattice SIM imaging illustrates the progressive emergence of increasingly discrete ATR.p and γ H2Ax-associated signaling architectures prior to final nanoscale interrogation using SIM² and single-molecule STORM imaging workflows.

Image Correction

NONE

Image by J. H. Cheong

Image Analysis and Data Presentation by B.T. Bennett

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