

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 56 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) = 1374 X 194
 Image Size (Scaled) = 29.01 μ m X 35.76 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, Panel B**Z-Stack = 115 Slices (3.42 µm)**

Channels = 3

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

Image Size (Pixels) = 250 X 280

Image Size (Scaled) = 5.28 µm X 5.91 µm

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 µm, Y: 0.00 µm

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 µm

Binning Mode = 2,2

Laser Wavelength = 640 nm @ 3.5%

Frame Time = 1.59 s

Scan Direction = Unidirectional

Grating = 36.5 µm grating

488 - Collected in Image Acquisition but not Shown in this Analysis

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 µm

Binning Mode = 2,2

Laser Wavelength = 488 nm @ 0.50%
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

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. A Region of Interest is denoted by a white box to be further examined in the 3-Dimensional Volume Projection 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 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

STORM - Panel C

STORM imaging from the same product dataset is shown in Panel C to demonstrate the remaining resolution limitations of Lattice SIM² microscopy. Using SIM² super-resolution imaging, structures that appear as single foci under confocal and Airyscan conditions become increasingly resolved into spatially distinct signaling architectures, with Lattice SIM² providing additional refinement of sub-diffraction-resolution structural organization compared with conventional Lattice SIM imaging. However, only modest additional structural discrimination is achieved when progressing from SIM to SIM², highlighting the diminishing structural gains prior to the transition to single-molecule localization microscopy.

In contrast, STORM imaging provides substantially enhanced nanoscale structural resolution, revealing fine ATR.p-associated signaling architectures and sub-diffraction organizational features not fully distinguishable within the SIM² dataset. Importantly,

although structural patterns observed in SIM² show reproducible similarities to those visualized by STORM, any apparent correspondence between modalities is inferential, and caution is warranted when extrapolating structural equivalence across imaging scales. Moreover, while STORM substantially improves nanoscale visualization, additional unresolved substructure likely remains below the modality's effective resolution limits.

Summary

Panel A shows a single SIM² optical section (Z-plane 56 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), following etoposide-induced DNA damage. Relative to conventional widefield, WF-SIM, confocal, Airyscan, and Lattice SIM imaging, SIM² provides further refinement of sub-diffraction structural discrimination and improved visualization of punctate ATR.p-associated signaling architectures within the nuclear compartment. Distinct intranuclear signaling structures become increasingly separable from broader resolution-limited aggregates observed in preceding modalities, supporting progressively refined interpretation of ATR.p-associated DNA damage response organization.

Panel B presents a three-dimensional volume reconstruction of the region of interest identified in Panel A, further illustrating the volumetric distribution and structural organization of ATR.p-associated signaling territories throughout the z-stack. Relative to preceding modalities, SIM² imaging demonstrates improved separation and refinement of sub-diffraction signaling structures while simultaneously emphasizing the remaining limitations of diffraction-based super-resolution imaging approaches. Although reproducible similarities emerge between SIM² and STORM-associated structural patterns, the definitive molecular architecture and precise nanoscale organization of repair complexes remain incompletely resolved at this imaging stage. Accordingly, the observed signal organization should continue to be interpreted as progressively refined structural discrimination of ATR.p-associated damage territories rather than definitive molecular equivalence to single-molecule localization datasets.

Importantly, this dataset represents one of the final super-resolution refinement stages within the Identifyn® Stepdown Methodology™, demonstrating the progressive emergence of increasingly discrete ATR.p-associated signaling architectures as imaging resolution advances across modalities. The transition from widefield to WF-SIM, confocal, Airyscan, Lattice SIM, and now SIM² imaging illustrates how incremental improvements in optical sectioning and sub-diffraction structural discrimination progressively refine the interpretation of ATR.p-associated DNA damage response organization prior to final nanoscale interrogation using 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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