

Identifyn Standards for Optical Microscopy Methods™Identifyn Primary Antibody**Rabbit anti-Human RAD50 Polyclonal, IgG***Cat ID# - PA-RP 000007*Identifyn® Secondary Antibodies**Identifyn®SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000057***Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, IgG (H + L)***Cat ID# - SA 000015*

Rad50 recognizes and processes double-strand breaks during DNA repair. It is a component of the MRN complex (Mre11-Rad50-Nbs1), which is crucial for identifying and processing DNA double-strand breaks. Rad50 also provides tethering and bridging of DNA ends. In its homodimeric state, Rad50 contains an ATPase domain at its ends, facilitating the alignment of broken DNA strands. Rad50 is also associated with telomere maintenance. Dysregulation or mutations in Rad50 or the MRN complex can lead to genomic instability, increased cancer susceptibility, and other genetic disorders.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human RAD50 Polyclonal, IgG, was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L), was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 3 times in PBS.

The second primary antibody, [Alpha Tubulin Monoclonal Antibody \(DM1A\)](#), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, IgG (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, 1.25X Tubelens, was used with the following parameters for each dye:

Single Plane (2D) - Z-plane 71 of 185 total - Panel A

Channels = 3

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

Image Size (Pixels) = 3126 X x 2929

Image Size (Scaled) = 66.00 µm X 61.84 µm

Bit Depth = 16 Bit

Image Center Position = X: 60.64 mm, Y: 39.26 mm

Stage Position = X: 60.64 mm, Y: 39.26 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Single Plane (2D) - Z-plane 71 of 185 total - Panel B

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image Size (Pixels) = 1268 X x 1258
Image Size (Scaled) = 26.77 μ m X 25.56 μ m
Bit Depth = 16 Bit
Image Center Position = X: 60.64 mm, Y: 39.26 mm
Stage Position = X: 60.64 mm, Y: 39.26 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Z Stack - (3D) - Panel C**Z-Stack =165 Slices (4.89 μ m)**

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image Size (Pixels) = 522 X x 438
Image Size (Scaled) = 11.02 μ m X 9.25 μ m
Bit Depth = 16 Bit
Image Center Position = X: 60.64 mm, Y: 39.26 mm
Stage Position = X: 60.64 mm, Y: 39.26 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Z Stack - (3D) - Panel D**Z-Stack =165 Slices (4.89 μ m)**

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image Size (Pixels) = 151 X x 135
Image Size (Scaled) = 3.19 μ m X 2.85 μ m
Bit Depth = 16 Bit
Image Center Position = X: 60.64 mm, Y: 39.26 mm
Stage Position = X: 60.64 mm, Y: 39.26 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 μm
Binning Mode = 2,2
Laser Wavelength = 640 nm @1.00%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 27.5 μm 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 = 100 ms
Depth of Focus = 0.81 μm
Binning Mode = 2,2
Laser Wavelength = 488 nm @1.00%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 27.5 μm grating

DAPI -

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 = 405
Channel Name = T3-Axiocam 820 #2-SR
Excitation Wavelength = 405
Emission Wavelength = 445
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 50 ms
Depth of Focus = 0.69 μm
Binning Mode = 2,2

Laser Wavelength = 405 nm @2.00%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 36.5 μ m grating

Post Processing - SIM Processing

Processing Dimension = 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 3
Algorithm = SIM
SIM Settings = Standard
Auto Sharpness = On
Sharpness = 9.6423 (647), 11.5105 (488), 10.1126 (DAPI)
Fitting = Fast Fit
Normalization = Auto

2-Dimensional View - Panels A and B

Panel features Rabbit anti-Human RAD50 Polyclonal, IgG with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L) combined with a Mouse Monoclonal Alpha Tubulin primary, counterstained with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, IgG (H + L) and DAPI,

Panel B: the 647 channel (RAD50) is changed from the default red to white to enhance visual contrast, and tubulin (488) is not shown.

Image was taken from Z-stack Acquisition at Z-plane 71 of 185.

3D Image Processing - Panel C

3D Maximum Projection = Precise
Appearance = Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, 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 D

3D Volume Projection = Precise
Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 120%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 15°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Panel E - STORM

Summary -

Super-resolution lattice structured illumination microscopy (SIM) was used to further characterize the intranuclear distribution of RAD50 following etoposide-induced DNA damage within the Identifyn® Standards for Optical Microscopy Methods™ step-down framework. HeLa cells were labeled with a rabbit anti-human RAD50 polyclonal IgG, detected with Identifyn® SRM Alexa Fluor™ 647 affinity-purified secondary antibodies, and α -tubulin and DAPI served as cytoskeletal and nuclear references, respectively. Across all lattice SIM views, RAD50 signal was strictly confined to the nuclear compartment, consistent with its established role as a core component of the MRN complex involved in DNA double-strand break (DSB) detection and signaling.

Two-dimensional lattice SIM optical sections (Panels A and B) acquired from a 185-slice Z-stack reveal punctate and aggregated intranuclear RAD50 distributions with substantially increased spatial definition relative to Airyscan imaging. Three-dimensional volume reconstructions (Panel C) and higher-magnification region-of-interest analyses (Panel D) further resolve damage-associated RAD50 assemblies throughout the nuclear volume. At lattice SIM lateral resolution (~60–80 nm in the X–Y plane), these signal patterns display reproducible organizational features consistent with RAD50 accumulation at DNA damage sites coordinated by the MRN complex.

Functionally, RAD50 plays a central role in DNA double-strand break sensing, tethering, and signaling through its interaction with MRE11 and NBS1, facilitating ATM activation and repair pathway engagement. While lattice SIM does not resolve all substructures detectable by single-molecule localization microscopy, the improved resolution reduces signal convolution and enables more reliable visualization of damage-associated RAD50 organization within the nuclear volume. Direct comparison with STORM imaging from the same dataset (Panel E) indicates that apparent aggregates at SIM resolution may represent resolution-limited composites of multiple RAD50 sub-assemblies that are separable only at the single-molecule level.

Collectively, these data position lattice SIM as a critical intermediate modality within the step-down workflow, bridging enrichment-level localization (confocal/Airyscan) and definitive structural resolution (SIM²/STORM). Although chromatin-level architectural conclusions require higher-resolution localization approaches, accurate mapping of RAD50 nuclear organization at this stage provides essential context for interpreting DNA damage sensing, MRN complex engagement, and early repair pathway activation following genotoxic stress.

Image Corrections

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast in Panels B, C, and D.

Image by P. Raut

Image Analysis and Data Presentation by B.T. Bennett

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