

Identifyn Standards for Optical Microscopy Methods™Identifyn® Primary Antibody**Rabbit anti-Human RAD50 Recombinant Monoclonal, IgG – (BLR026N)***Cat ID# - PA-RR 000011***Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7)***Cat ID# - PA-MM 000002*Identifyn® Secondary Antibody**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000060***Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)***Cat ID# - SA 000017*

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 the identification and processing of 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 susceptibility to cancer, and other genetic disorders.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 µM etoposide-spiked complete growth media for 24 hours, followed by -20 °C methanol fixation and blocking with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human RAD50 Recombinant Monoclonal, IgG – (BLR026N), 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, Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7), 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™ 488 Affinity Purified Goat Anti-Mouse IgG, F(ab')₂ (H + L) was incubated for 1 hour at room temperature at a dilution of 1:1000. 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, 1.25X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - 2D at Z-Plane 70 of 142, Panel A**Z-Stack = 142 Slices (4.23 µm)**

Channels = 2

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

Image size (Pixels) = 2462 X 2204
Image Size (Scaled) = 51.98 μm X 46.53 μm
Bit Depth = 16 Bit
Image Center Position = X: 66.49 mm, Y: 35.66 mm
Stage Position = X: 66.49 mm, Y: 35.66 mm
ROI Center Offset = X: 0.00 μm , Y: 0.00 μm

Z Stack - (3D) - 3D Volume Projection, Panel B
Z-Stack = 142 Slices (4.23 μm)

Channels = 2
Scaling (Per Pixel) = 0.021 nm X 0.021 nm X 0.030 nm
Image size (Pixels) = 302 X 258
Image Size (Scaled) = 6.38 μm X 5.45 μm
Bit Depth = 16 Bit
Image Center Position = X: 66.49 mm, Y: 35.66 mm
Stage Position = X: 66.49 mm, Y: 35.66 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 @ 2.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 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 = 120 ms
 Depth of Focus = 0.81 μm
 Binning Mode = 2,2
 Laser Wavelength = 488 nm @ 1.50%
 Frame Time = 1.59 s
 Scan Direction = Unidirectional
 Grating = 36.5 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 = 10.5107 (647), 11.1617 (488)
 Fitting = Fast Fit
 Normalization = Auto

2-Dimensional View - Panel A

Shows Rabbit anti-Human RAD50 Recombinant Monoclonal, IgG - (BLR026N), visualized using Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L) secondary antibody and Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7) visualized using Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L). 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 4% all channels, Ramp 96% 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 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

STORM - Panel C

Panel C shows STORM imaging from the same product dataset to demonstrate the resolution limitations of Lattice SIM; however, Lattice SIM provides improved localization precision and structural detail relative to Airyscan imaging. Moreover, the signal pattern is consistent with the Airyscan data and is more closely resolution-limited than that of a single-molecule STORM image.

Summary

Using lattice structured illumination microscopy (SIM), Panel A shows a single optical section (Z-plane 70/142) depicting RAD50 (647) and H2Ax (488) following etoposide-induced DNA damage. RAD50 is visualized with Identifyn® SRM Alexa Fluor™ 647 donkey anti-rabbit secondary antibody, and H2Ax with Identifyn® SRM Alexa Fluor™ 488 goat anti-mouse secondary antibody. Panel B presents a three-dimensional volume projection of the region of interest, with the H2Ax channel omitted to enhance visualization of RAD50 structural organization.

Structures that appear as single foci under confocal and Airyscan imaging are further resolved by SIM into spatially distinct features, revealing increased heterogeneity within RAD50-associated assemblies. However, despite this improvement, the observed structural detail remains constrained by SIM's effective resolution.

In Panel C, single-molecule STORM imaging of RAD50 provides substantially greater structural refinement, resolving these assemblies into smaller, discrete nanoscale substructures. While lattice SIM begins to reveal patterns that show some similarity to STORM-resolved organization, these correspondences remain qualitative and should be interpreted with caution. The apparent similarities may reflect an underlying structural organization, but they are prone to resolution-driven conflation and may also reflect interpretive bias rather than true architectural equivalence.

Importantly, RAD50 (~153 kDa) forms extended coiled-coil dimers and higher-order assemblies, yet the apparent dimensions of SIM-resolved features substantially exceed the expected molecular scale. This reflects the combined effects of the point spread function and labeling geometry (including antibody size and fluorophore displacement), indicating that these structures do not represent true molecular dimensions. As such, SIM captures partially resolved yet resolution-limited aggregates, whereas STORM begins to resolve the underlying nanoscale organization.

Collectively, these data demonstrate that while lattice SIM improves upon confocal and Airyscan and begins to suggest underlying RAD50 organization, it remains insufficient to resolve the nanoscale architecture fully. Observed similarities to STORM may indicate a partially resolved structure, but definitive interpretation requires higher-resolution imaging, as provided by single-molecule approaches.

Image Correction

NONE

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Image Analysis and Data Presentation by B.T. Bennett

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