

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 72 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) = 1374 X 1593
Image Size (Scaled) = 29.01 μ m X 33.63 μ m
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 μ m, Y: 0.00 μ m

Z Stack - (3D) - 3D Transparency Projection, Panel B**Z-Stack = 40 Slices (1.17 μ m)** - Gallery was reduced to the Z-Planes that have RAD50 Signal

Channels = 2
Scaling (Per Pixel) = 0.021 nm X 0.021 nm X 0.030 nm
Image size (Pixels) = 232 X 227
Image Size (Scaled) = 4.90 μ m X 4.75 μ m
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 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 @ 2.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 μ m grating

488 - Acquired but not Shown in the SIM2 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 μ m
 Binning Mode = 2,2
 Laser Wavelength = 488 nm @ 1.50%
 Frame Time = 1.59 s
 Scan Direction = Unidirectional
 Grating = 36.5 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 RAD50 Recombinant Monoclonal, IgG - (BLR026N), 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 Transparency Projection = Precise
 Appearance = Threshold 5% all channels, Ramp 20% all channels, Maximum 100% all channels
 Background = Light Grey
 Light = Brightness 100%, **Disabled** 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

Panel C shows STORM imaging from the same product dataset, illustrating the resolution limitations of Lattice SIM² imaging. While SIM² provides improved localization precision and structural detail relative to conventional SIM, a more direct comparison with STORM demonstrates that the nanoscale organization of RAD50 assemblies remains only partially resolved under SIM² acquisition conditions.

Summary

Panel A shows a single optical section at the corresponding Z-plane used for SIM² acquisition, displaying RAD50 detected in the 647 channel (Identifyn® SRM Alexa Fluor™ 647 donkey anti-rabbit secondary). A white box denotes the region of interest (ROI) selected for subsequent analysis.

Panel B presents a three-dimensional transparency projection (Zeiss ZEN), which enables visualization of signal distribution throughout the z-volume by rendering voxel intensity without full surface occlusion. This approach is used to emphasize internal structure and spatial relationships within RAD50-associated assemblies that are not readily appreciated in 2D projections. Within this representation, multiple RAD50 foci exhibit apparent "doublet" or paired morphologies, which may suggest the presence of DNA break ends or tethered structures. However, these features must be interpreted with caution, as they may reflect resolution-limited separation or convolution of the signal rather than true discrete subunits.

Using SIM², structures that appear as single foci under confocal and Airyscan are partially resolved into more spatially distinct features, revealing increased heterogeneity in RAD50 organization. In several instances, patterns resembling paired or elongated structures are observed. While these may reflect underlying biological architecture—consistent with RAD50's role in bridging DNA ends within the MRN complex—they remain at the limit of SIM² resolution and are susceptible to overinterpretation.

Panel C shows single-molecule STORM imaging of RAD50, which provides substantially greater structural refinement. Although some qualitative similarities in spatial patterning are observed between SIM² and STORM, these correspondences are not definitive. Features appearing as "doublets" in SIM² may represent partially resolved structure, but they may also arise from point spread function effects and labeling geometry. Notably, STORM reveals nanoscale organization that SIM² does not fully capture, and even these structures may remain incompletely resolved.

Importantly, RAD50 (~153 kDa) forms extended coiled-coil dimers and higher-order assemblies spanning tens to hundreds of nanometers, thereby tethering and stabilizing the ends of DNA double-strand breaks. Despite this inherent structural scale, the apparent dimensions of SIM²-resolved features often exceed expected molecular architecture due to the combined effects of optical resolution limits and antibody-based labeling displacement. As such, while SIM² begins to reveal aspects of RAD50 organization beyond the diffraction limit of imaging, it does not provide definitive structural resolution.

Collectively, these data demonstrate that SIM² improves visualization of RAD50 spatial organization and suggest the presence of underlying structural features, including possible paired assemblies. However, accurate interpretation of these features requires higher-resolution approaches, such as STORM, because resolution limits and potential signal conflation constrain SIM².

Image Correction

NONE

Image by J. H. Cheong

Image Analysis and Data Presentation by B.T. Bennett

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