

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 LSM 980 Confocal, Axio Observer.Z1/7, using with a Plan-Apochromat 63x/1.40 Oil DIC M27 was used with the following parameters for each dye:

Z Stack - (3D) - 2D at Z-Plane 68 of 121, Panel A**Z-Stack = 171 Slices (6.0 µm)**

Channels = 2

Scaling (Per Pixel) = $0.035\ \mu\text{m} \times 0.035\ \mu\text{m} \times 0.030\ \mu\text{m}$
Image size (Pixels) = 856×958
Image Size (Scaled) = $30.22\ \mu\text{m} \times 33.85\ \mu\text{m}$
Bit Depth = 16 Bit
Image Center Position = X: 72.54 mm, Y: 53.04 mm
Stage Position = X: 72.54 mm, Y: 53.04 mm
ROI Center Offset = X: $0.00\ \mu\text{m}$, Y: $0.00\ \mu\text{m}$

Z Stack - (3D) - Panel B**Z-Stack = 121 Slices ($3.6\ \mu\text{m}$)**

Channels = 2
Scaling (Per Pixel) = $0.035\ \mu\text{m} \times 0.035\ \mu\text{m} \times 0.030\ \mu\text{m}$
Image size (Pixels) = 149×117
Image Size (Scaled) = $5.26\ \mu\text{m} \times 4.13\ \mu\text{m}$
Bit Depth = 16 Bit
Image Center Position = X: 72.54 mm, Y: 53.04 mm
Stage Position = X: 72.54 mm, Y: 53.04 mm
ROI Center Offset = X: $0.00\ \mu\text{m}$, Y: $0.00\ \mu\text{m}$

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / $328\ \mu\text{m}$
LMS MBS = MBS 488/561/639 & 405/730
LMS SBS = SBS LP 640
Laser Wavelength = **639 nm @ 0.50%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.1
Rotation = 0°
Sampling = 2.00
Pixel Time = $1.15\ \mu\text{s}$
Frame Time = 17.86 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 643 - 745
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = Super Resolution: 10.0 (3D, Auto)

488 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 5.00 AU / 250 μ m
 LMS MBS = MBS 488/561/639 & 405/730
 LMS SBS = SBS SP 550
 Laser Wavelength = **488 nm @ 0.05%**
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.1
 Rotation = 0°
 Sampling = 2.00
 Pixel Time = 1.15 μ s
 Frame Time = 17.86 s
 LSM Scan Speed = 6
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 4
 Excitation Wavelength = 493
 Emission Wavelength = 517
 Effective NA = 1.4
 Detection Wavelength = 499 - 548
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 800 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 Airyscan Mode = Super Resolution: 9.5 (3D, 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, illustrating the resolution limitations of Airyscan imaging. While Airyscan improves localization precision and

structural detail compared with conventional confocal microscopy, it still cannot fully resolve the nanoscale organization of RAD50 assemblies revealed by STORM.

Summary

Panel A shows a single optical section (Z-plane 68/121) depicting RAD50 and H2Ax distribution following etoposide-induced DNA damage. RAD50 is detected with Identifyn® SRM Alexa Fluor™ 647 donkey anti-rabbit secondary antibody (red), and H2Ax is detected with Identifyn® SRM Alexa Fluor™ 488 goat anti-mouse secondary antibody (green). A white box denotes the region of interest (ROI) used for subsequent analysis. Panel B provides a three-dimensional volume projection of this ROI, highlighting the spatial organization of RAD50 within H2Ax-containing chromatin.

At the diffraction-limited scale, RAD50 appears as discrete nuclear foci within regions of H2Ax distribution. This spatial relationship is consistent with established biology: H2Ax is a constitutive histone variant broadly distributed throughout chromatin, whereas RAD50, as part of the MRN complex, is recruited to DNA double-strand breaks to facilitate damage recognition, DNA end tethering, and the initiation of repair signaling. Apparent overlap, therefore, reflects RAD50 localization within chromatin domains rather than specific targeting of H2Ax.

Conventional confocal and Airyscan imaging remain resolution-limited and cannot resolve the underlying substructure of RAD50 assemblies. RAD50 forms extended coiled-coil dimers and higher-order complexes at sites of DNA damage, with structural features occurring at the nanoscale. These subunits and architectural details are not resolved by Airyscan, leading to apparent foci that represent aggregated or convolved signal rather than discrete molecular organization.

Panel C shows STORM imaging of RAD50, revealing substantially greater structural refinement and the beginning of resolution of these assemblies into smaller nanoscale substructures. While Panels A and B capture correct localization and damage-associated recruitment, they lack the resolution required to distinguish this underlying organization. Accordingly, the signal patterns observed in confocal and Airyscan imaging reflect broader spatial distribution. In contrast, STORM provides a more accurate representation of RAD50 nanoscale architecture, although even this may not fully resolve all underlying structure.

Image Correction

NONE

Image by J.K. Bennett
Image Analysis and Data Presentation by B.T. Bennett

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