

Multiplex Confocal Immunofluorescence - 4-Plex**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***Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Donkey Anti-Mouse, IgG (H + L)***Cat ID# - SA 000037*

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 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 first 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 blocked for 30 minutes at room temperature and 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 blocked for 30 minutes at room temperature and washed 5 times in 1X PBS.

The third primary antibody, [Invitrogen Nuclear Pore Complex Monoclonal Antibody \(39C7\)](#), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3 times in 1X PBS. Identifyn® SRM Alexa Fluor™ 555 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 5 times in 1X PBS and mounted using [ProLong™ Diamond Antifade Mountant with DAPI \(cat#P36962\)](#).

Microscope

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

Single Plane (2D) - Panels A and B

Channels = 4
Scaling (Per Pixel) = 0.059 μm X 0.059 μm X 0.030 μm
Image size (Pixels) = 528 X 673
Image Size (Scaled) = 31.06 μm X 39.59 μm
Bit Depth = 16 Bit
Image Center Position = X: 72.53 mm, Y: 52.26 mm
Stage Position = X: 72.53 mm, Y: 52.26 mm
ROI Center Offset = X: 0.00 μm , Y: 0.00 μm

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 50 μm
LMS MBS = MBS 488/561/639 & 405/730
LMS SBS = SBS SP 505
Laser Wavelength = **488 nm @ 0.05%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.28 μs
Frame Time = 5.28 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 511 - 545
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 7.4 (3D, Auto)

647 -

Reflector = None

Contrast Method = Fluorescence
Pinhole = 1.00 AU / 65 μ m
LMS MBS = MBS 488/561/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = **639 nm @ 0.2%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.28 μ s
Frame Time = 5.28 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 645 - 700
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 550 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 8.0 (3D, Auto)

555 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 56 μ m
LMS MBS = MBS 488/561/639 & 405/730
LMS SBS = SBS SP 550
Laser Wavelength = **561 nm @ 0.4%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.28 μ s
Frame Time = 5.28 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 553
Emission Wavelength = 568
Effective NA = 1.4
Detection Wavelength = 560 - 600
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT

Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 7.7 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 44 µm
LMS MBS = MBS 488/561/639 & 405/730
LMS SBS = Mirror
Laser Wavelength = **405 nm @ 0.3%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.28 µs
Frame Time = 5.28 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 435 - 480
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.8 (3D, Auto)

Split View - Panel A

The top-left panel 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. The top-middle panel shows 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). The top-right panel shows Invitrogen Nuclear Pore Complex Monoclonal Antibody (39C7), visualized with Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Donkey Anti-Mouse, IgG (H + L), the lower-left panel shows DAPI staining, and the bottom-middle panel shows the four-channel merged image.

3D Image Processing

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 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph
NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Clipping Image Process - Panel B

Clipping planes are introduced in Panel B to highlight the Rabbit anti-Human RAD50 Rabbit Recombinant Monoclonal, IgG - (BLR026N) and the Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7) within the nuclear compartment by adjusting or cutting away portions of the image using a triple-wedge cut, removing parts of the image in the X-Y, X-Z, and Y-Z planes.

X-Y = Activate was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -2%

Clip X = 0°

Clip Z = 0°

X-Z = Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = 3%

Clip Y = 30°

Clip Z = 0°

Y-Z = Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -16%

Clip Y = -30°

Clip Z = 0°

Summary

Panel A presents a four-channel (4-plex) confocal split view showing RAD50 (647), H2Ax (488), nuclear pore complex (NPC, 555), and DAPI. Each target was acquired under controlled laser power conditions (488: 0.05%, 555: 0.4%, 647: 0.2%, DAPI: 0.3%), enabling balanced signal detection while minimizing cross-talk and saturation. The bottom panel displays the merged image, illustrating the spatial relationship of all four channels within the same field of view.

Panel B shows a three-dimensional volume-intensity projection with triple-wedge clipping (X-Y, X-Z, Y-Z planes), allowing visualization of the signal distribution within the nuclear volume. This processing strategy enables spatial interrogation of RAD50 and H2Ax localization in context, while preserving structural relationships across all axes.

Biologically, RAD50, as a core component of the MRN complex, is recruited to sites of DNA double-strand breaks and plays a key role in damage recognition, DNA end tethering, and initiation of repair signaling. H2Ax, a constitutive histone variant, is distributed throughout chromatin and provides the structural framework within which DNA damage responses occur. As such, observed colocalization reflects RAD50 engagement within H2Ax-containing chromatin domains rather than damage-specific targeting of H2Ax itself.

NPC staining provides a spatial reference for the nuclear envelope, enabling confirmation of intranuclear localization and structural orientation within the cell. DAPI serves as a nuclear counterstain, defining chromatin architecture and supporting segmentation of nuclear regions.

Collectively, this 4-plex dataset demonstrates controlled multiplex acquisition and three-dimensional visualization of DNA damage-associated protein localization within the nuclear environment, integrating structural context with biologically relevant recruitment patterns.

Image Correction

NONE

Image by J.K. Bennett

Image Analysis and Data Presentation by B.T. Bennett

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