

Identifyn Standards for Optical Microscopy Methods™Identifyn® Primary Antibody**Rabbit anti-Human RAD50 Recombinant Monoclonal, IgG – (BLR026N)***Cat ID# - PA-RR 000011*Identifyn® Secondary Antibody**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000057*

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**Plus Damage -**

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 blocked with [Thermo Fisher Fish Serum Blocker cat#37527X3](#). The primary antibody, Rabbit anti-Human RAD50 Recombinant Monoclonal - (BLR026N), was incubated at room temperature for 1 hour at a 1:250 dilution. Cells were washed 3X in 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 5X in PBS and mounted using [ProLong™ Diamond Antifade Mountant with DAPI \(cat# P36962\)](#).

Minus Damage -

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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:

Z Stack - (3D) - 2D at Z-Plane 68 of 111, Plus Etoposide/DNA Damage.

Z-Stack = 111 Slices (3.30 µm)

Channels = 1

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

Z Stack - (3D) - 3D at Z-Plane 76 of 151, No Etaposide/Minus DNA Damage.
Z-Stack = 151 Slices (4.5 μm)

Channels = 1
Scaling (Per Pixel) = 0.059 μm X 0.059 μm X 0.030 μm
Image size (Pixels) = 515 X 560
Image Size (Scaled) = 30.30 μm X 32.94 μm
Bit Depth = 16 Bit
Image Center Position = X: 79.20 mm, Y: 49.34 mm
Stage Position = X: 79.20 mm, Y: 49.34 mm
ROI Center Offset = X: 0.00 μm , Y: 0.00 μm

647 - Plus Damage, from Confocal Data Set

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 65 μm
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 μs
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)

647 - Minus Damage - noting the same laser input power and conditions

Reflector = None
Contrast Method = Fluorescence

Pinhole = 1.00 AU / 64 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 = 11
 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: 8.7 (3D, Auto)

Profile View Display - *Plus DNA Damage, Top*

Profile Definition = Stroke Thickness 1.5, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - **X (Min 0.0 and Max 34.70), Y (Min 0.0 and Max 15829.00)**

This panel measures RAD50 DNA damage-induced foci/aggregates across an arbitrary line, noting that the foci/aggregate signal reached approximately ~15K A.U. at its maximum.

Measured at the Z-plane 68 of 111, Plus Etoposide.

Profile View Display - *Minus DNA Damage, Bottom*

Profile Definition = Stroke Thickness 1.5, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - **X (Min 0.0 and Max 24.90), Y (Min 0.0 and Max 1779)**

This panel measures RAD50 in the absence of DNA damage across an arbitrary line, noting that the foci/aggregate signal reached approximately ~2K A.U. at its maximum.

3D at Z-Plane 76 of 151, No Etoposide.

Histogram Display - *Plus DNA Damage, Top*

Histogram Definition = Normal, Histo Table set to Frequency, Bin Count 256, Bin Size was 256, Lower Threshold 0, Upper Threshold 65535

Histogram View = X and Y axes were set to Auto - X (Min 0.0 and Max 65535), Y (Min 0 and Max 100)

Arithmetic Mean intensity = 4406.031

The histogram was generated from the yellow-boxed region to quantify the overall RAD50 protein response within a defined nuclear area following DNA damage induction. This measurement provides additional support for the observed increase in signal relative to the control condition, complementing the line-profile analysis presented alongside it.

Histogram Display - Minus DNA Damage, Bottom

Histogram Definition = Normal, Histo Table set to Frequency, Bin Count 256, Bin Size was 256, Lower Threshold 0, Upper Threshold 65535

Histogram View = X and Y axes were set to Auto - X (Min 0.0 and Max 65535), Y (Min 0 and Max 500000)

Arithmetic Mean intensity = 430.889

The histogram was generated from the yellow-boxed region to quantify the overall RAD50 protein within a defined nuclear area in the absence of DNA damage induction. This measurement provides additional support for the observed increase in signal relative to the control condition, complementing the line-profile analysis presented alongside it.

Control Conclusion -

In untreated cells, RAD50 displays low-intensity, predominantly diffuse nuclear localization. Following etoposide-induced DNA damage, RAD50 signal intensity increases and redistributes into discrete nuclear foci. This reorganization is consistent with recruitment of RAD50 to DNA double-strand breaks (DSBs) as part of the MRE11–RAD50–NBS1 (MRN) complex.

Quantitative analysis demonstrates a ~10-fold increase in mean fluorescence intensity following damage, supported by line-profile measurements showing an elevated, spatially concentrated signal across nuclear regions. These data confirm both increased abundance and focal organization of RAD50 under genotoxic stress.

Co-localization with H2AX-marked chromatin throughout the dataset indicates association with DNA-containing regions where damage-associated recruitment occurs. At higher resolution, RAD50 foci resolve into discrete puncta, reflecting localized accumulation within chromatin domains.

Together, these data support RAD50 redistribution and increased intensity as indicators of active DNA damage response engagement and MRN complex-mediated processing of DNA breaks within H2AX-containing chromatin.

Image Correction

NONE

Image by J.K. Bennett

Image Analysis and Data Presentation by B.T. Bennett

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