

Identifyfyn Standards for Optical Microscopy Methods™Identifyfyn Primary Antibody**Rabbit anti-Human RAD50 Polyclonal, IgG***Cat ID# - PA-RP 000007*Identifyfyn® Secondary Antibodies**Identifyfyn®SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000057***Identifyfyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, IgG (H + L)***Cat ID# - SA 000015*

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 cancer susceptibility, and other genetic disorders.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human RAD50 Polyclonal, IgG, was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyfyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L), was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 3 times in PBS.

The second primary antibody, [Alpha Tubulin Monoclonal Antibody \(DM1A\)](#), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyfyn® SRM Alexa Fluor™ 488 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 5X in 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) - Z-Plane 59 of 161 - Panels A & E**Z Stack - (3D) - Panel B****Z-Stack = 161 Slices (4.80 µm)**

Channels = 3

Scaling (Per Pixel) = $0.059\ \mu\text{m} \times 0.059\ \mu\text{m} \times 0.030\ \mu\text{m}$
Image Size (Pixels) = 1017 X 1017
Image Size (Scaled) = $59.81\ \mu\text{m} \times 59.81\ \mu\text{m}$
Bit Depth = 16 Bit
Image Center Position = X: 92.75 mm, Y: 50.66 mm
Stage Position = X: 92.75 mm, Y: 50.66 mm
ROI Center Offset = X: $0.00\ \mu\text{m}$, Y: $0.00\ \mu\text{m}$

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / $65\ \mu\text{m}$
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = **639nm @0.10%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.2
Rotation = 0°
Sampling = 1.20
Pixel Time = $0.33\ \mu\text{s}$
Frame Time = 3.36s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging - 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 636 - 732
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 7.2 (3D, Auto)

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / $50\ \mu\text{m}$
LMS MBS = MBS 488/639 & 405/730
LMS SBS = Mirror
Laser Wavelength = **488nm @0.10%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.2
Rotation = 0°
Sampling = 1.20
Pixel Time = $0.33\ \mu\text{s}$

Frame Time = 3.36s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging - 8
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 489 - 550
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.7 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 43µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = Mirror
Laser Wavelength = **405nm @0.2%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.2
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.33µs
Frame Time = 3.36s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging - 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 430 - 470
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.1 (3D, Auto)

2-Dimensional View - Panel A

Panel features Rabbit anti-Human RAD50 Polyclonal, IgG with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L) combined with a Mouse Monoclonal Alpha Tubulin primary, counterstained with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, IgG (H + L).

The image was taken from Z-stack Acquisition at plane 59 of 161.

3D Image Processing - Panel B

3D Maximum Projection = Precise

Appearance = Threshold 2% all channels, Ramp 0% all channels, Maximum 100% all channels

Background = Black

Light = Brightness 100%, 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)

Note that the 53BP1, counterstained with 647, was pseudo-colored white from red in Zeiss Zen, to enhance visual contrast.

LSM 980 Zen Acquisition Info Tab - Panel C

Shown is the acquisition of the 647 channel (RAD50 Rabbit Polyclonal with Identifyn 647 secondary) using the **639nm laser at 0.10%**.

Not shown, the 488 channel (Alpha Tubulin) using the **488nm laser at 0.10%**, and DAPI using the **405nm laser at 0.2%**.

Profile View Display - Panels D & E

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 49.0), Y (Min 0 and Max 21092)

This panel measures RAD50 DNA damage-induced foci/aggregates across an arbitrary line, noting that the foci/aggregate signal reached approximately 2'K A.U. at its maximum.

Measured at Z-plane 59 of 161.

Histogram Display - Panels E & F

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 2000)

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

Summary -

Panel A – Confocal Imaging of RAD50

Confocal imaging of RAD50 following etoposide-induced DNA damage. RAD50 is detected using a rabbit anti-human RAD50 polyclonal antibody conjugated to Identifyn® SRM Alexa Fluor™ 647 (red). α -Tubulin is visualized with Identifyn® SRM Alexa Fluor™ 488 (green) and serves as a cytoplasmic exclusionary control.

Panel B – Nuclear Damage Context

Three-dimensional maximum-intensity projection of rabbit anti-human 53BP1 polyclonal antibody (white) with DAPI nuclear staining (blue), providing confirmation of DNA damage-associated nuclear responses under the same treatment conditions.

Panel C – Acquisition Parameters

Laser power settings for the 639 nm channel (0.10) were used during confocal acquisition, demonstrating low excitation conditions.

Panels D and E – Quantitative Signal Analysis

Line profile and histogram measurements of RAD50 signal intensity under low laser power conditions, yielding an arithmetic mean intensity of 6,693.144 and a maximum signal of 28,623.8, confirming robust signal induction without acquisition-driven amplification.

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Line profile and histogram measurements of RAD50 signal intensity under low laser power conditions, yielding an arithmetic mean intensity of 6,693.144 and a maximum signal of 28,623.8, confirming robust signal induction without acquisition-driven amplification.

Confocal imaging demonstrates etoposide-induced recruitment of RAD50 to the nuclear compartment, where it appears as discrete punctate foci consistent with activation of the DNA damage response. Quantitative intensity measurements obtained under low laser power confirm a genuine biological signal rather than forced amplification. At confocal resolution, the assay provides a clear go/no-go readout for RAD50 recruitment; however, finer structural organization of RAD50 foci is not resolved until crossing the super-resolution threshold using Airyscan, SIM, SIM², and STORM, where sub-focal architecture becomes progressively apparent.

Image Corrections

Identifyfyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast in Panels B and E.

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

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