

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 Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, AxioCam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:

Single Plane (2D)

Channels = 3

Scaling (Per Pixel) = 0.110 µm X 0.110 µm

Image size (Pixels) = 1216 X 1028

Image Size (Scaled) = 133.18 µm X 112.59 µm

Bit Depth = 14 Bit

Image Center Position = X: 0.00 µm, Y: 0.00 µm

ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

647 -

Reflector = 50 Cy 5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10.00%
Exposure Time = 100 ms
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Imaging Device = AxioCam 705
Camera Adapter = 1X
Depth of Focus = 1.03 μ m
Binning Mode = 2,2

488 -

Reflector = 38 eGFP
Beam Splitter = 495
Filter Excitation Wavelength = 450 - 490
Filter Emission Wavelength = 500 - 550
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15.00%
Exposure Time = 150 ms
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Imaging Device = AxioCam 705
Camera Adapter = 1X
Depth of Focus = 0.80 μ m
Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10.00%
Exposure Time = 25 ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = AxioCam 705
Camera Adapter = 1X
Depth of Focus = 0.72 μ m
Binning Mode = 2,2

Split View - Panel A

The top-left panel features Rabbit anti-Human RAD50 Polyclonal, IgG, visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the top-right panel shows Alpha Tubulin visualized with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, IgG (H + L), the bottom-left panel features DAPI nuclear staining, and the bottom-right panel features the merged image of all channels.

Colocalization View - Panel B, and Image Panel C, Measurement was of the entire field

Horizontal Axis - AF647, Threshold 82, Range Auto

Vertical Axis - DAPI, Threshold 254, Range Auto

Zeiss Zen Acquisition Settings - Panel D

Shown are the acquisition settings across all three channels, including LED intensity and exposure times, along with all other relevant parameters, provided for methodological transparency.

Summary

Panel A presents a split-view of the full acquisition field, including α -tubulin detected with a 488-nm secondary antibody used solely as a cytoplasmic exclusionary control. This demonstrates that the RAD50 signal is not cytoplasmic and is instead confined to the nuclear compartment. *α -Tubulin was included exclusively to delineate cytoplasmic boundaries and was not used as a biological marker in the analysis.*

Panels B and C present orthogonal intensity profiles of RAD50 (horizontal axis) and DAPI (vertical axis), confirming exclusive nuclear localization of RAD50 following etoposide-induced DNA damage. No measurable signal is detected within the nucleolus or cytoplasm, indicating compartment-restricted recruitment to the nuclear space.

Panel D provides acquisition transparency, documenting low LED excitation for RAD50/Alexa Fluor 647 with a 100 ms exposure time. These parameters demonstrate that the observed signal is not artificially driven by acquisition settings but instead reflects a robust, specific biological response mediated by a highly specific primary antibody following pharmacologically induced DNA damage.

Conclusion: RAD50 exhibits exclusive nuclear recruitment following etoposide-induced DNA damage. Whole-cell context and orthogonal intensity profiling confirm the absence of RAD50 signal in the cytoplasm and nucleolus, demonstrating compartment-restricted localization. α -Tubulin is used solely as a cytoplasmic exclusionary control to define cellular boundaries and is not interpreted as a biological marker. Acquisition parameters employ low excitation and short exposure times, confirming that the observed RAD50 signal reflects a robust and specific biological response rather than acquisition-driven amplification.

Image Correction

Panel C - Rabbit anti-Human Rad50 Polyclonal counterstained with Identifyn®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.

Image by E.F.Simon

Image Analysis and Data Presentation by B.T. Bennett

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