

Identifyn Standards for Optical Microscopy Methods™Identifyn® Primary Antibody**Rabbit anti-Human RAD51 Recombinant Monoclonal, IgG (BLR017M)***Cat ID# - PA-RR 000002*Identifyn® Secondary Antibodies**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, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific***Cat ID# - SA 000012*

Rad51 is a critical protein in the homologous recombination (HR) process, which is a crucial mechanism for repairing DNA double-strand breaks (DSBs) and maintaining genomic stability. Rad51 foci formation in response to DNA damage is a hallmark of the cellular DNA repair process, particularly Homologous Recombination (HR). When cells encounter DNA Double-Strand Breaks (DSBs) or other forms of DNA damage, Rad51 proteins assemble into discrete nuclear foci at the damage sites. Localization and visualization of Rad51 aggregate via immunofluorescent applications allow for the study of Rad51 response kinetics in response to damage, thereby making the study of Rad51 foci formation critical to the study of the cellular response to DNA damage and how it plays a crucial role in the repair process. Rad51 is essential to recognizing DNA damage, activating the Homologous Recombination (HR) pathway, Rad51 filament formation, and DNA repair and resolution...

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2µM 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 RAD51 Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS, and Identifyn® 647 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody followed this, [Alpha Tubulin Monoclonal Antibody \(DM1A\)](#), which was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 488 secondary antibody 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](#).

Microscope

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

Single Plane (2D) - Split View Panel A

Channels = 2
Scaling (Per Pixel) = $0.110\mu\text{m} \times 0.110\mu\text{m}$
Image size (Pixels) = 1216 X 1028
Image Size (Scaled) = $133.18\mu\text{m} \times 112.59\mu\text{m}$
Bit Depth = 16 Bit
Image Center Position = X: $0.00\mu\text{m}$, Y: $0.00\mu\text{m}$
ROI Center Offset = X: $0.00\mu\text{m}$, Y: $0.00\mu\text{m}$

647 -

Reflector = 50 Cy5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @25%
Exposure Time = 150ms
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Imaging Device = Axiocam 705 mono
Camera Adapter = 1X
Depth of Focus = $1.03\mu\text{m}$
Binning Mode = 2,2

488 -

Reflector = 38 HE eGFP
Beam Splitter = 495
Filter Excitation Wavelength = 450 - 490
Filter Emission Wavelength = 500 - 550
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10%
Exposure Time = 50ms
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Imaging Device = Axiocam 705 mono
Camera Adapter = 1X
Depth of Focus = $0.80\mu\text{m}$
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 @15%
Exposure Time = 40ms
Excitation Wavelength = 353

Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = AxioCam 705 mono
Camera Adapter = 1X
Depth of Focus = 0.72µm
Binning Mode = 2,2

Post Processing

None

Profile View Display - Panels B and C

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

Profile View = X and Y axes were set to Auto - X (Min 0.00 and Max 27.7), Y (Min 80 and Max 1188)

Summary

Panel A presents a split view of the three channels collected using widefield microscopy with an sCMOS camera: RAD51 (shown in red), Tubulin (in green), and DAPI (in blue). Panel B illustrates a line profile across a single nucleus, measuring signal points' fluorescent intensity and distance, which appear as individual foci. However, Panel C highlights the significant limitations of using widefield microscopy to study foci or structural information. In this case, a single focus is observed to have a width of approximately 1 micron. In contrast, STORM data reveals that a single event has a Full Width at Half Maximum (FWHM) of approximately 40-100 nm. Despite these limitations, widefield microscopy is still helpful for studying cellular sub-population protein expression levels and provides valuable population data. Foci traditionally describe a circular localized signal representing protein recruitment to the site of an event, or the minimum sub-cellular structural information available.

A review of the entire RAD51 data set reveals that undiscovered structural details remain, even at STORM resolution (which allows for single-molecule imaging). Additionally, "foci" should be regarded as a non-specific biological term representing an observation defined by diffraction-limited acquisition methods.

Image Correction

None

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Analysis and Presentation by B. T. Bennett

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