

**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™ 555 Affinity Purified Donkey Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000038***Identifyn® SRM Alexa Fluor™ 680 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific***Cat ID# - SA 000067*

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® 555 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® 680 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 Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:

**Z Stack - (3D) - Panel A**

**Z-Stack = 92 Slices (9.10µm)**

Channels = 3  
Scaling (Per Pixel) = 0.143µm X 0.143µm X 0.100µm  
Image Size (Pixels) = 1024 X 1024  
Image Size (Scaled) = 146.29µm X 146.29µm  
Bit Depth = 14 Bit  
Image Center Position = X: 89.99mm, Y: 51.11mm  
Stage Position = X: 89.99mm, Y: 51.11mm  
ROI Center Offset = X: 1.14, Y: 0.00µm

**Single Plane (2D) - Panels B and C**

Z- Plane 47 of 92

**555 -**

Reflector = AF532-49014  
Beam Splitter = 550  
Filter Excitation Wavelength = 515 - 545  
Filter Emission Wavelength = 555 - 595  
Contrast Method = Fluorescence  
Light source = X-Cite Xylis Lamp Intensity @20%  
Exposure Time = 150ms  
Excitation Wavelength = 553  
Emission Wavelength = 568  
Effective NA = 1.25  
Imaging Device = Axiocam 807 mono  
Camera Adapter = 1X  
Depth of Focus = 1.10µm  
Binning Mode = 2,2

**680 -**

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 @20%  
Exposure Time = 350ms  
Excitation Wavelength = 679  
Emission Wavelength = 702  
Effective NA = 1.25  
Imaging Device = Axiocam 807 mono  
Camera Adapter = 1X  
Depth of Focus = 1.36µm  
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 @8.0%  
 Exposure Time = 18ms  
 Excitation Wavelength = 353  
 Emission Wavelength = 465  
 Effective NA = 1.25  
 Imaging Device = AxioCam 807  
 Camera Adapter = 1X  
 Depth of Focus = 0.90µm  
 Binning Mode = 2,2

### Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mode "Optical Sectioning" - enabled correction selected.

Normalization, Phase Correction, Fourier Filter, and Deconvolution **were NOT used**.

### 3D Image Processing - Panel A

3D Maximum Projection = Precise  
 Appearance = Threshold 13% for all channels, Ramp 0% for all channels, and Maximum 100% for 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)

### 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 18.6), Y (Min 2 and Max 4340)

### Summary

Panel A presents a three-dimensional view of the complete image acquisition, displayed as a maximum projection. This panel demonstrates the widespread nature of RAD51 staining across a larger population of cells using diffraction-limited Widefield Structured Illumination Microscopy (SIM).

Panel B focuses on a single nucleus extracted from the Region of Interest (ROI) in Panel A to highlight the specificity of the RAD51 signal and the appearance of foci at this limited resolution. A line profile is also depicted in Panel B across the nucleus, showing several signal points from the foci.

The histogram in Panel C illustrates excellent fluorescent signal intensity, and importantly, it displays the distance over which the foci signals extend. Here, we note that the foci span more than 200 nm, which can be misleading in understanding RAD51 localization following damage. Our super-resolution dataset indicates that the signal observed may contain more than one focus or several "doublets." Furthermore, our STORM data reveals that a single event has a Full Width at Half Maximum (FWHM) of approximately 40-100 nm.

In conclusion, while Widefield SIM allows for the observation of the ubiquitous presence and reproducibility of the RAD51 antibody across a population of cells, it would be incorrect to assume that these observations reflect single events. Such precise identification can only be achieved with sub-diffraction super-resolution techniques.

### Image Corrections

None

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