

Identifyn Standards for Optical Microscopy Methods™Identifyn® Primary Antibody**Rabbit anti-Human NBS1 (P95) Polyclonal, IgG***Cat ID# - PA-RP 000010***Mouse anti-Human PARP1 Monoclonal, IgG1 - (5F10/B7)***Cat ID# - PA-MM 000001*Identifyn® Secondary Antibody**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000060***Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific***Cat ID# - SA 000034*

NBS1 (Nijmegen breakage syndrome 1) plays a crucial part in the cellular response to DNA double-strand breaks in both the Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) pathways and is a component of the MRE11/RAD50/NBS1 complex (MRN). NBS1 acts as a sensor of DNA double-strand breaks and, via its interaction with γH2AX, recruits MRE11 and RAD50 to the site of damage, processes the DNA ends, prepares them for repair, and facilitates the recruitment of other repair factors. NBS1 also plays a role in coordinating the cellular response to DNA damage with the cell cycle. It interacts with various cell cycle checkpoint proteins, such as ATM (ataxia-telangiectasia mutated), to halt the cell cycle progression, allowing time for DNA repair. NBS1 also contributes to telomere maintenance and participates in detecting and repairing telomere damage, helping prevent genomic instability associated with telomere dysfunction. Dysfunction or mutations in NBS1 can lead to genomic instability and predispose individuals to cancer and other genetic disorders.

Methods

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 fixation in -20 °C methanol and blocking with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human NBS1 Polyclonal, IgG, was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3 times in 1X 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 5 times in 1X PBS.

The second primary antibody, Mouse anti-Human PARP1 Monoclonal, IgG1 - (5F10/B7), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3 times in 1X PBS. Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5 times in 1X 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:

Z Stack - (3D) - 2D at Z-Plane 68 of 121, Panel A**Z-Stack = 121 Slices (3.6 µm)**

Channels = 2

Scaling (Per Pixel) = 0.035 nm X 0.035 nm X 0.030 nm

Image size (Pixels) = 830 X 908

Image Size (Scaled) = 29.29 µm X 32.04 µm

Bit Depth = 16 Bit

Image Center Position = X: 66.25 mm, Y: 51.54 mm

Stage Position = X: 66.25 mm, Y: 51.54mm

ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - 3D Volume Projection, Panel B**Z-Stack = 121 Slices (3.6 µm)**

Channels = 2

Scaling (Per Pixel) = 0.035 nm X 0.035 nm X 0.030 nm

Image size (Pixels) = 159 X 137

Image Size (Scaled) = 5.61 µm X 4.83 µm

Bit Depth = 16 Bit

Image Center Position = X: 66.25 mm, Y: 51.54 mm

Stage Position = X: 66.25 mm, Y: 51.54 mm

ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

647 -

Reflector = None

Contrast Method = Fluorescence

Pinhole = 5.00 AU / 328 µm

LMS MBS = MBS 488/561/639 & 405/730

LMS SBS = SBS LP 640

Laser Wavelength = **639 nm @ 0.3%**

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 1.9

Rotation = 0°

Sampling = 2.00

Pixel Time = 1.04 µs

Frame Time = 19.77 s

LSM Scan Speed = 6

Scan Direction = Bidirectional

Line Step = 1

Averaging = 4

Excitation Wavelength = 653

Emission Wavelength = 668

Effective NA = 1.4

Detection Wavelength = 643 - 735

Imaging Device = Airyscan

Detector Type = GaAsP-PMT

Detector Gain = 800 V

Detector Offset = 0

Detector Digital Gain = 1.0

Airyscan Mode = SuperResolution 10.0 (3D, Auto)

555 -

Reflector = None

Contrast Method = Fluorescence

Pinhole = 5.00 AU / 272 μ m

LMS MBS = MBS 488/561/639 & 405/730

LMS SBS = SBS SP 525

Laser Wavelength = **561 nm @ 1.10%**

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 1.9

Rotation = 0°

Sampling = 2.00

Pixel Time = 1.04 μ s

Frame Time = 19.77 s

LSM Scan Speed = 6

Scan Direction = Bidirectional

Line Step = 1

Averaging = 4

Excitation Wavelength = 553

Emission Wavelength = 568

Effective NA = 1.4

Detection Wavelength = 530 - 582

Imaging Device = Airyscan

Detector Type = GaAsP-PMT

Detector Gain = 800 V

Detector Offset = 0

Detector Digital Gain = 1.0

Airyscan Mode = SuperResolution 9.9 (3D, Auto)

DAPI - Not Shown in Data Analysis, but Collected in Image Acquisition

Reflector = None

Contrast Method = Fluorescence

Pinhole = 5.00 AU / 235 μ m

LMS MBS = MBS 488/561/639 & 405/730

LMS SBS = SBS SP 505

Laser Wavelength = **405 nm @ 0.2%**

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 1.9

Rotation = 0°

Sampling = 2.00

Pixel Time = 1.04 μ s

Frame Time = 19.77 s

LSM Scan Speed = 6

Scan Direction = Bidirectional

Line Step = 1

Averaging = 4

Excitation Wavelength = 353

Emission Wavelength = 465

Effective NA = 1.4
Detection Wavelength = 420 - 480, 495 - 499
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 750 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = SuperResolution 8.1 (3D, Auto)

2-Dimensional View - Panel A

Shows Rabbit anti-Human NBS1 (P95) Polyclonal, IgG, visualized using Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L) secondary antibody and Mouse anti-Human PARP1 Monoclonal, IgG1 - (5F10/B7) visualized using Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific. A Region of Interest is denoted by a white box to be further examined in the 3-Dimensional Volume Projection in Panel B.

3D Image Processing - Panel B

3D Volume Projection = Precise
Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Clipping Image Process - Panel B

Clipping planes may be introduced to highlight the NBS1 Polyclonal and the PARP1 Mouse Monoclonal within the nuclear compartment by adjusting or cutting away portions of the image using a triple-wedge cut, or, in this case, removing parts of the image in the X-Z plane.

X-Y = Not Used in Data Analysis

X-Z = Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = 24%

Clip Y = 30°

Clip Z = 0°

Y-Z = Not Used in Data Analysis

STORM - Panel C

Shown from the same product data (NBS1), set to demonstrate the resolution limitations of Airyscan, however, better than confocal, in determining localization and structural details.

Summary

Panel A shows a single optical section (Z-plane 68/121) depicting NBS1 and PARP1 distribution following etoposide-induced DNA damage. NBS1 (rabbit polyclonal) is detected with Identifyn® SRM Alexa Fluor™ 647 donkey anti-rabbit secondary antibody (red), and PARP1 (monoclonal) is detected with Identifyn® SRM Alexa Fluor™ 555 goat anti-mouse secondary antibody, pseudocolored white for visual contrast.

Panel B presents a three-dimensional reconstruction with an X-Z cut plane (outlined in red). A region of interest (ROI) within the cut plane is indicated by a yellow circle, highlighting a highly conflated signal pattern that superficially resembles higher-resolution STORM structures. A second ROI above the cut plane (yellow circle) demonstrates a comparable pattern, again suggesting partial structural resemblance. However, these features remain diffraction-limited and lack the spatial resolution necessary to resolve discrete protein architecture. While Airyscan provides significant improvements over conventional confocal imaging, these data demonstrate that it is insufficient for accurate nanoscale interpretation of protein structure.

At the diffraction-limited scale, NBS1 and PARP1 signals appear partially overlapping; however, this likely reflects resolution limitations rather than direct molecular association. PARP1 functions as an early sensor of DNA strand breaks, rapidly binding damaged DNA and initiating repair signaling through PARylation, whereas NBS1, as a core component of the MRN complex (MRE11–RAD50–NBS1), is recruited to sites of DNA double-strand breaks and mediates downstream damage signaling, including ATM activation. Their distributions are therefore consistent with coordinated, but mechanistically distinct, roles within DNA damage response regions.

Panel C shows STORM imaging of NBS1 alone, revealing refined structural detail and sub-diffraction organization not resolved in lower-resolution modalities. Together, these observations indicate that the apparent structures observed in Panels A and B reflect broader spatial organization, while true nanoscale architecture requires super-resolution approaches.

Image Correction

The PARP1 channel (555) was pseudocolored white to enhance visual contrast between signals.

Image by J.K. Bennett

Image Analysis and Data Presentation by B.T. Bennett

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