

Identifyfyn Standards for Optical Microscopy Methods™Identifyfyn® Primary Antibody**Rabbit anti-Human NBS1 (P95) Polyclonal, IgG***Cat ID# - PA-RP 000010*Identifyfyn® Secondary Antibody**Identifyfyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000060*

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.

Method**Plus Damage -**

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 -20 °C methanol fixation and blocked with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human NBS1 (P95) Polyclonal, was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3 times in 1X PBS. Identifyfyn® 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 and mounted using [ProLong™ Diamond Antifade Mountant with DAPI \(cat# P36962\)](#).

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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) - Panel A, Showing Z-Plane 81 of 161, Plus Etoposide
Z-Stack = 161 Slices (4.8 μm)

Channels = 1
Scaling (Per Pixel) = $0.059\ \mu\text{m} \times 0.059\ \mu\text{m} \times 0.030\ \mu\text{m}$
Image size (Pixels) = 468×514
Image Size (Scaled) = $27.53\ \mu\text{m} \times 30.23\ \mu\text{m}$
Bit Depth = 16 Bit
Image Center Position = X: 74.44 mm, Y: 45.97 mm
Stage Position = X: 74.44 mm, Y: 45.97 mm
ROI Center Offset = X: $0.00\ \mu\text{m}$, Y: $0.00\ \mu\text{m}$

Single Plane (2D) - No Etoposide.

Channels = 1
Scaling (Per Pixel) = $0.059\ \mu\text{m} \times 0.059\ \mu\text{m}$
Image size (Pixels) = 1503×1503
Image Size (Scaled) = $88.38\ \mu\text{m} \times 88.38\ \mu\text{m}$
Bit Depth = 16 Bit
Image Center Position = X: 80.77 mm, Y: 54.03 mm
Stage Position = X: 80.77 mm, Y: 54.03 mm
ROI Center Offset = X: $0.00\ \mu\text{m}$, Y: $0.00\ \mu\text{m}$

647 - Plus Etoposide-Induced DNA Damage, from Confocal Data Set

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / $64\ \mu\text{m}$
LMS MBS = MBS 488/561/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = **639 nm @ 0.2%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.7
Rotation = 0°
Sampling = 1.20
Pixel Time = $0.29\ \mu\text{s}$
Frame Time = 4.97 s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 645 - 700
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 7.5 (3D, Auto)

647 - Minus Damage - noting the same laser input power and conditions

Reflector = None

Contrast Method = Fluorescence
Pinhole = 1.00 AU / 64 μ m
LMS MBS = MBS 488/561/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = **639 nm @ 0.2%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.5
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.26 μ s
Frame Time = 5.63 s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 645 - 700
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 8.3 (3D, Auto)

Profile View Display - Plus DNA Damage, Top

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

Profile View = X and Y axes were set to Auto - **X (Min 0.0 and Max 29.8), Y (Min 0.0 and Max 39373)**

This panel measures NBS1 DNA damage-induced foci/aggregates across an arbitrary line, noting that the foci/aggregate signal reached approximately ~40K A.U. at its maximum.

Measured at Z-plane 81 of 161

Profile View Display - Minus DNA Damage, Bottom

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 53.40), Y (Min 0.0 and Max 13377)**

This panel measures NBS1 in the absence of DNA damage across an arbitrary line, noting that the foci/aggregate signal reached approximately ~12K A.U. at its maximum.

Histogram Display - Plus DNA Damage, Top

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 0.0 and Max 5000)

Arithmetic Mean intensity = 4847.375

The histogram was generated from the yellow-boxed region shown to quantify the overall NBS1 protein response within a defined nuclear area following DNA-damage induction. This measurement provides additional support for the observed increase in signal relative to the control condition, complementing the line-profile analysis presented alongside it.

Histogram Display - *Minus DNA Damage, Bottom*

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 0.0 and Max 5000)

Arithmetic Mean intensity = 795.313

The histogram was generated from the yellow-boxed region shown to quantify the overall NBS1 protein within a defined nuclear area in the absence of DNA-damage induction. This measurement provides additional support for the observed increase in signal relative to the control condition, complementing the line-profile analysis presented alongside it.

Control Conclusion –

In undamaged cells, NBS1 exhibits a low-to-moderate, predominantly diffuse nuclear distribution, consistent with its role in basal genome surveillance. Following etoposide-induced DNA damage, NBS1 shows a marked increase in mean intensity and reorganizes into discrete nuclear foci, with aggregate signal substantially elevated relative to baseline.

This redistribution is consistent with activation of the DNA damage response and rapid recruitment of NBS1 to sites of DNA double-strand breaks as part of the MRN complex (MRE11–RAD50–NBS1). In this context, NBS1 functions early in the damage response, facilitating break recognition, stabilizing the repair complex, and activating downstream signaling pathways, including ATM-dependent checkpoint activation.

The observed focal accumulation reflects the concentration of NBS1 within damage-associated nuclear microenvironments, where multi-protein repair assemblies form. While these foci represent active DNA damage response domains, their appearance at diffraction-limited resolution likely reflects clustered or overlapping repair complexes rather than singular structures.

The increase in signal intensity and organization supports NBS1's role as a critical upstream mediator of double-strand break repair, coordinating both structural and signaling components of the response. Spatial proximity to other repair factors does not

imply direct molecular interaction but rather coordinated engagement within shared DNA damage response pathways.

Collectively, these data support a model in which NBS1 redistribution following genotoxic stress reflects rapid recruitment to DNA damage sites and integration into higher-order repair assemblies, reinforcing its role as an essential regulator of double-strand break recognition and signaling.

Image Corrections

The NBS1 channel (647) was pseudocolored white to enhance visual contrast in displaying measurements.

Image by J.K. Bennett

Image Analysis and Data Presentation by B.T. Bennett

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