

Identifyn Standards for Optical Microscopy Methods™Identifyn Primary Antibody**Rabbit anti-Human γ H2AX (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)***Cat ID# - PA-RR 000005*Identifyn® Secondary Antibodies**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)***Cat ID# - SA 000062***Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (H + L)***Cat ID# - SA 000020*

Gamma H2AX (γ H2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of double-strand breaks (DSBs). When DSBs occur in chromatin, γ H2AX is rapidly phosphorylated specifically at the serine 139 residue (referred to as Ser139 phosphorylation or γ -phosphorylation) near the site of the break. This phosphorylation event forms γ H2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 μ M 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 γ H2AX Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody, [Actin Recombinant Rabbit Monoclonal Antibody \(JF47-01\)](#), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (H + L) 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 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**Z-Stack = 98 Slices (2.91 μ m)**

Channels = 3

Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm

Image Size (Pixels) = 837 X 805

Image Size (Scaled) = 29.54 μ m X 28.41 μ m

Bit Depth = 16 Bit
Image Center Position = X: 83.02 mm, Y: 52.68 mm
Stage Position = X: 83.02 mm, Y: 52.68 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Single Plane (2D) - Z-plane 50 of 98 total - Panel B**Z-Stack = 98 Slices (2.91 μ m)**

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image Size (Pixels) = 560 X 457
Image Size (Scaled) = 19.77 μ m X 16.13 μ m
Bit Depth = 16 Bit
Image Center Position = X: 83.02 mm, Y: 52.68 mm
Stage Position = X: 83.02 mm, Y: 52.68 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Single Plane (2D) - Z-plane 50 of 98 total - Panel C**Z-Stack = 98 Slices (2.91 μ m)**

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image Size (Pixels) = 135 X 149
Image Size (Scaled) = 4.76 μ m X 5.26 μ m
Bit Depth = 16 Bit
Image Center Position = X: 83.02 mm, Y: 52.68 mm
Stage Position = X: 83.02 mm, Y: 52.68 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 328 μ m
LSM MBS = MBS 488/639, 405/730
LSM SBS = SBS LP 640
Laser Wavelength = 639 nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10 μ s
Frame Time = 18.77 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
LSM Plus Mode = 9.7 (3D, Auto)

555 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 261 μ m
LSM MBS = MBS 488/639, 405/730
LSM SBS = SBS SP 615
Laser Wavelength = 488 nm @0.02%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10 μ s
Frame Time = 18.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 380-608
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 9.2 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.46 AU / 235 μ m
LMS MBS = MBS 488/639, 405/730
LSM SBS = SBS SP 505
Laser Wavelength = 405 nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10 μ s
Frame Time = 18.77 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 = 350-499
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 750 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 8.3 (3D, Auto)

3D Image Processing - Panel A

3D Maximum Projection = Precise
 Appearance = Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, Tone Mapping Enabled
 Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Clipping Image Process = Clipping planes are introduced to pronounce regions of special interest.

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

Position of Clip = -42%

Clip X = 0°

Clip Y = 0°

Summary

Panel A - This panel presents a 3-dimensional Maximum Projection of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green), and DAPI nuclear staining (blue). The image employs an X, Y cut plane, the position is -42%, the angle X is at 0 degrees, and the angle Z is 0 degrees (flat). The cut plane allows us to define the γ H2Ax localization clearly from multiple angles within the nuclear compartment. A white box denotes a Region of Interest to be further investigated in Panel B.

Panel B - This panel displays a 2-dimensional view of the ROI from Panel A at z-plane 50 of 98, illustrating the signal and localization of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (white, changed from default red in Zeiss Zen software for better visualization). Here, we note structural details enhanced by the use of super resolution Airyscan. A white box denotes a Region of Interest to be further investigated in Panel C.

Panel C - This panel displays a 2-dimensional view of the ROI from Panel B at z-plane 50 of 98, illustrating the signal and localization of Rabbit anti-Human γ H2Ax Recombinant

Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™647 (white, changed from default red in Zeiss Zen software for better visualization) Here, we extract as much detail as possible at Airyscan resolution regarding the structural localization of the γ H2Ax, before we step down to super-resolution SIM, where visualization of these structures will improve.

We once again observe a reproducible octagonal structure in many of the DNA repair proteins that interact with sites of DNA damage. Whether these structures are optical artifacts, macro-scale representations, or factual representations is not for us to determine. However, we note that across thousands of images, involving many proteins, multiple applications, and increasing variables, these shapes appear to represent some organizational detail related to DNA.

Image Corrections

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(a'')₂ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

Image by J.K. Bennett
Image Analysis by B.T. Bennett
Data Presentation by P.D. Amistoso

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