

Identifyn Standards for Optical Microscopy Methods™Identifyn Primary Antibody**Rabbit anti-Human γ H2AX (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)***Cat ID# - PA-RR-000001*Identifyn® Secondary Antibodies**Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (H + L)***Cat ID# - SA 000020***Identifyn® SRM Alexa Fluor™ 680 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)***Cat ID# - SA 000073*

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 DSBs. When DSBs occur in chromatin, γ H2AX is rapidly explicitly phosphorylated 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, IgG, 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.

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 for 1 hour at room temperature 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 900 AiryScan, Axio Observer.Z1/7, using with 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 = 112 Slices (3.33 μ m)**

Channels = 3

Scaling (Per Pixel) = 0.033 μ m X 0.033 μ m X 0.030 μ m

Image Size (Pixels) = 1024 X 1024

Image Size (Scaled) = 33.80 μ m X 33.80 μ m

Bit Depth = 16 Bit

Image Center Position = X: 58.13mm, Y: 46.34mm

Stage Position = X: 58.13mm, Y: 46.34mm

ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Z Stack - (3D) - Panel D (680/Tubulin Excluded)**Z-Stack = 112 Slices (3.33µm)**

Channels = 2

Scaling (Per Pixel) = 0.033µm X 0.033µm X 0.030µm

Image Size (Pixels) = 732 X 697

Image Size (Scaled) = 24.16µm X 23.01µm

Bit Depth = 16 Bit

Image Center Position = X: 58.13mm, Y: 46.34mm

Stage Position = X: 58.13mm, Y: 46.34mm

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

488 -

Reflector = None

Contrast Method = Fluorescence

Pinhole = 1.00 AU / 43µm

LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)

Laser Wavelength = 488nm @0.10%

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 3.0

Rotation = 0°

Sampling = 2.14

Pixel Time = 0.52µs

Frame Time = 5.06s

LSM Scan Speed = 9

Scan Direction = Bidirectional

Line Step = 1

Averaging = 8

Excitation Wavelength = 493

Emission Wavelength = 517

Effective NA = 1.4

Detection Wavelength = 480-550

Imaging Device = Airyscan

Detector Type = GaAsP-PMT

Detector Gain = 650 V

Detector Offset = 0

Detector Digital Gain = 1.0

680 -

Reflector = None

Contrast Method = Fluorescence

Pinhole = 1.00 AU / 57µm

LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)

Laser Wavelength = 640nm @1.0%

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 3.0

Rotation = 0°

Sampling = 2.14

Pixel Time = 0.52µs
Frame Time = 5.06s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Detection Wavelength = 656 - 700
Imaging Device = GaAsP-Pmt2
Detector Type = GaAsP-PMT
Detector Gain = 700 V
Detector Offset = 0
Detector Digital Gain = 1.0

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 37µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 405nm @0.4%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.0
Rotation = 0°
Sampling = 2.14
Pixel Time = 0.52µs
Frame Time = 5.06s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 410-470
Imaging Device = GaAsP-Pmt1
Detector Type = GaAsP-PMT
Detector Gain = 650 V
Detector Offset = 0
Detector Digital Gain = 1.0

3D Image Processing - Panel A

3D Maximum Projection = Precise
Appearance = Threshold 1% (680), Threshold 1% (488), Threshold 1% (DAPI), Ramp 0% all channels, Maximum 100% 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)

3D Image Processing - Panel D

3D Volume Projection = Precise

Appearance = Threshold 2% (680), Threshold 2% (DAPI), Ramp 98% (647), Ramp 98% (DAPI), 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 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Profile View Display

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 011.63), Y (Min 303 and Max 29482)

Caliper X = Chosen at points displayed herein that represented the Full Width Half Maximum of the Signal

Summary -

Panel A presents a three-dimensional view of the complete image acquisition as a maximum projection. Gamma-H2AX was stained using Identifyn® anti-Human gamma H2AX Rabbit Recombinant Monoclonal antibody and Identifyn® SRM Alexa Fluor™ 488, highlighting its presence within the nuclear compartment, which was also stained with DAPI. Additionally, tubulin was stained with Identifyn® SRM Alexa Fluor™ 680. An X-Y cut plane was introduced at -26% of the stack, comprising 112 planes, to illustrate the three-dimensional localization within the nuclear compartment. This also reveals larger signals, or "foci," which we demonstrate as artifacts from structural studies conducted at diffraction-limited resolutions.

Panels B and C display the use of a line profile to measure the signal from a single focus, noting that the Full Width Half Maximum (FWHM) is approximately 0.71 microns (710 nm). Our analysis of gamma-H2AX, which started with single-molecule resolutions, indicates that diffraction-limited microscopy must be interpreted with the understanding that these signals represent numerous structures that remain unresolved at resolutions above 220 nm in the X and Y dimensions. Our STORM imaging shows that these structures can be defined at approximately 40 nm.

Panel D provides a volume projection of the same cell, excluding the tubulin staining (680) and highlighting a Region of Interest (ROI) around the nucleus. An X-Z and Y-Z wedge was used to emphasize two key points: first, the specificity of the antibody for the DNA damage phosphorylation event, and second, that the limited resolution in the Z-plane of diffraction-limited confocal microscopy can result in larger signals in the X and Y dimensions. This limitation has historically created a misleading impression of colocalization in studies involving multiple protein targets and restricts structural analysis.

Image Corrections –

None

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