

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 = 133 Slices (3.96 μ m)**

Channels = 3

Scaling (Per Pixel) = 29.72nm X 29.72nm X 30.00nm

Image Size (Pixels) = 951 X 951

Image Size (Scaled) = 28.26 μ m X 28.26 μ m

Bit Depth = 16 Bit

Image Center Position = X: 81.32mm, Y: 42.25mm

Stage Position = X: 81.32mm, Y: 42.25mm

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

Z Stack - (3D) - Panel B**Z-Stack = 149 Slices (4.44µm)**

Channels = 3

Scaling (Per Pixel) = 29.72nm X 29.72nm X 30.00nm

Image Size (Pixels) = 225 X 287

Image Size (Scaled) = 6.69µm X 8.53µm

Bit Depth = 16 Bit

Image Center Position = X: 81.32mm, Y: 42.25mm

Stage Position = X: 81.32mm, Y: 42.25mm

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

488 -

Reflector = None

Contrast Method = Fluorescence

Pinhole = 5.00 AU / 217µm

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

Laser Wavelength = 488nm @0.09%

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 3.5

Rotation = 0°

Sampling = 2.38

Pixel Time = 0.54µs

Frame Time = 4.83s

LSM Scan Speed = 9

Scan Direction = Bidirectional

Line Step = 1

Averaging = 8

Excitation Wavelength = 493

Emission Wavelength = 517

Effective NA = 1.4

Detection Wavelength = 480-549

Imaging Device = Airyscan

Detector Type = GaAsP-PMT

Detector Gain = 800 V

Detector Offset = 0

Detector Digital Gain = 1.0

AiryScan Mode = Super Resolution 8.9 (3D, Auto)

680 -

Reflector = None

Contrast Method = Fluorescence

Pinhole = 5.00 AU / 282µm

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

Laser Wavelength = 640nm @1.0%

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 3.05

Rotation = 0°

Sampling = 2.38

Pixel Time = 0.54µs
Frame Time = 4.83s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Detection Wavelength = 650 - 700
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 850 V
Detector Offset = 0
Detector Digital Gain = 1.0
AiryScan Mode = Super Resolution 8.6 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 183µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 405nm @0.5%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.5
Rotation = 0°
Sampling = 2.3
Pixel Time = 0.54µs
Frame Time = 4.83s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 400-472
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
AiryScan Mode = Super Resolution 7.4 (3D, Auto)
Post Processing - AiryScan 3D Processing
Display Mode "SR"
Super Resolution Auto Filter Selected
Process 3D Current Image
Created

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 B

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)

Histogram of Frequency & Intensity

Histogram Definition

Normal

Bind Count = 256

Bin size = 256

Lower Threshold = 0

Upper Threshold = 65535

Histogram View

X Axis = Auto (min 1.11, max 65535)

Y Axis = Auto (min 0, max 10000000)

Channel Transparency = 100%

Summary -

Panel A presents a three-dimensional view of the complete image acquisition as a maximum projection, without the use of tone mapping. Gamma-H2AX was stained with Identifyn® SRM Alexa Fluor™ 488 and pseudo-colored white for better contrast within the nuclear compartment, which was stained with DAPI. Additionally, tubulin was stained using Identifyn® SRM Alexa Fluor™ 647.

Panel B provides a Region of Interest (ROI) extracted from panel A, enhancing the visualization of the gamma H2AX signal within the nuclear compartment and highlighting any structural details that the super-resolution Airyscan technique can provide at ~120 nm resolution (X, Y) in a three-dimensional volume projection.

Panel C presents a histogram measurement of the fluorescent signal of gamma H2AX within the image acquisition. Notably, we observe frequency and intensity values with maximums of 7.0e05 and 5000, respectively.

In conclusion, we note that while the super-resolution technique of Airyscan is powerful, it shows diminished structure visualization compared to SIM, SIM2, and STORM methods for the gamma H2AX dataset.

Image Corrections –

None

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