

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™ 647 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (H + L)***Cat ID# - SA 000065***Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)***Cat ID# - SA 000040*

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 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, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 647 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® 555 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 Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A**Z-Stack = 108 Slices (3.21 μ m)**

Channels = 3

Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm

Image Size (Pixels) = 3368 X 3816

Image Size (Scaled) = 44.44 μ m X 50.35 μ m

Bit Depth = 16 Bit

Image Center Position = X: 59.14mm, Y: 38.63mm

Stage Position = X: 59.14mm, Y: 38.63mm

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

Single Plane (2D) - Panel B

Z-Plane - 48 of 108

Z Stack - (3D) - Panel C

Z-Stack = 71 of 108 Collected Slices (2.10µm)

Channels = 3

Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm

Image Size (Pixels) = 949 X 550

Image Size (Scaled) = 12.52µm X 7.26µm

Bit Depth = 16 Bit

Image Center Position = X: 59.14mm, Y: 38.63mm

Stage Position = X: 59.14mm, Y: 38.63mm

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

Z Stack - (3D) - Panel E

Z-Stack = 71 of 108 Collected Slices (2.10µm)

Channels = 1

Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm

Image Size (Pixels) = 109 X 160

Image Size (Scaled) = 1.44µm X 2.11µm

Bit Depth = 16 Bit

Image Center Position = X: 59.14mm, Y: 38.63mm

Stage Position = X: 59.14mm, Y: 38.63mm

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

647 -

Reflectors = SR HE LBF 405/488/561/642

Beam Splitter = 405, 488, 561, 642

Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642

Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800

Contrast Method = Fluorescence

Illumination Wavelength = 640

Channel Name = T1-Axiocam 820 -SR

Excitation Wavelength = 640

Emission Wavelength = 729

Effective NA = 1.4

Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800

Imaging Device = Axiocam 820

Camera Adapter = 1X

Exposure Time = 120ms

Depth of Focus = 1.13µm

Binning Mode = 2,2

Laser Wavelength = 640nm @1.50%

Frame Time = 1.59s

Scan Direction = Unidirectional

Grating 27.5µm grating

555 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 561
Channel Name = T2-Axiocam 820 #2-SR
Excitation Wavelength = 561
Emission Wavelength = 597
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 120ms
Depth of Focus = 0.92µm
Binning Mode = 2,2
Laser Wavelength = 561nm @1.50%
Frame Time = 1.33s
Scan Direction = Unidirectional
Grating 32.0µm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 405
Channel Name = T3-Axiocam 820 #2-SR
Excitation Wavelength = 405
Emission Wavelength = 445
Effective NA = 1.4
Detection Wavelength = 419-470, 503 - 546, 576 - 617, 657-800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 50ms
Depth of Focus = 0.69µm
Binning Mode = 2,2
Laser Wavelength = 405nm @6.0%
Frame Time = 676.00ms
Scan Direction = Unidirectional
Grating 36.5µm grating

Post Processing - SIM Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 3

Algorithm = SIM
 Sharpness Setting = Standard
 Auto Sharpness = On
 Sharpness = 10.0285 (647), 10.1661 (555), 8.4947 (DAPI)
 Fitting = Fast Fit
 Histogram = Scale to Min/Max
 Baseline = Cut

3D Image Processing - Panel A

3D Maximum Projection = Precise
 Appearance = Threshold 4% (647), Threshold 4% (555), Threshold 4% (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 C

3D Volume Projection = Precise
 Appearance = Threshold 2% (647), Threshold 2% (555), Threshold 2% (DAPI), 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 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel E (Single Channel 647)

3D Surface Projection = Precise
 Appearance = Threshold 32% (647), Ambient Light 0%, Specular Light 71%, Shininess 85%
 Background = Black
 Light = Azimuth 72°, Elongation 71°, Enabled Tone Mapping
 Projection = View Angle 35°, Scale Z 32%, 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 0.93), Y (Min 5523 and Max 26897)

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. Tubulin was stained with Identifyn® SRM Alexa Fluor™ 555 and

pseudo-colored dark grey for better contrast with the nuclear compartment stained with DAPI. Additionally, gamma-H2AX was stained using Identifyn[®] SRM Alexa Fluor[™] 647 to indicate DNA damage.

Panel B shows Z-plane 48 of 108 total planes, with a white box highlighting the Region of Interest (ROI). Panel C illustrates the ROI in three dimensions using a volume projection.

Panel D features a line profile across z-plane 36 of the z-stack in panel B, measured with the Caliper X tool, noting a distance of 0.20 microns (200 nm) between unresolved peaks, as shown in the inset image. Panel E provides a three-dimensional structure model, removing DAPI and 555 channels and compressing the Z scale to enhance clarity.

In conclusion, the gamma-H2AX primary antibody and 647 secondary antibody effectively visualize phosphorylation sites at 640 nm. As seen in all studies, the well-defined structures in STORM images lose detail when the biological resolution is nearly 3- 4X lower from STORM to SIM.

Image Corrections

None

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