Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647 - Direct Conjugate
Cat ID# - DC 000020

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcγ Fragment Specific
Cat ID# - SA 000034

Gamma H2AX (γH2AX) 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, γH2AX 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 γH2AX 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 2μM 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 γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647 - Direct Conjugate, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.
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.

Control Data - Primary Unconjugated Antibody

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)

Z-Stack = 25 Slices (1.44μm)

Channels = 2
Scaling (Per Pixel) = 16.89nm X 16.89nm X 60.00nm
Image Size (Pixels) = 1873 X 2481
Image Size (Scaled) = 31.64μm X 41.90μm

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Bit Depth = 16 Bit
Image Center Position = X: 55.28mm, Y: 38.37mm
Stage Position = X: 55.28mm, Y: 38.37mm
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 = 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 120ms
Depth of Focus = 1.13µm
Binning Mode = 2,2
Laser Wavelength = 640nm @3.0%
Frame Time = 1.59s
Scan Direction = Unidirectional
Grating 36.5µm 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 = T1-Axiocam 820 #2-SR
Excitation Wavelength = 561
Emission Wavelength = 583
Effective NA = 1.4
Detection Wavelength = 503 - 546, 576 - 590
Imaging Device = Axiocam 820#2
Camera Adapter = 1X
Exposure Time = 100ms
Depth of Focus = 0.92µm
Binning Mode = 2,2
Laser Wavelength = 561nm @1.0%
Frame Time = 1.33s
Scan Direction = Unidirectional
Grating 32.0µm grating

Post Processing - SIM2 Processing

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Processing Dimension - 3D
Result Sampling = 4
Process Sampling = 4
Channels = 1
Algorithm = SIM2
SIM Settings = Standard
Auto Sharpness = On
Sharpness = 9.9866
Input SNR = N/A
Regularization Weight = N/A
Iterations = N/A
Filter = N/A
Fitting = N/A
Normalization = Automatic
Experimental PSF = N/A

3D Image Processing

3D Volume Projection = Precise
Appearance = Threshold 4% (647), Threshold 4% (555), Ramp 96% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, 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

X-Axis = Fluorescence Intensity for both the 657 and 555 channels
Y-Axis = Distance in µm

Red represents the signal for Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647 - Direct Conjugate

Yellow represents the signal for Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcγ Fragment Specific - Tubulin

Summary

The Image demonstrates the nuclear localization of gamma H2AX. The line profile shows not only spikes in the fluorescent intensity of the gamma H2AX signal at foci but also a clear separation of the gamma H2AX in the nuclear compartment from the signal intensity shown for the tubulin (555) within the cytoplasmic compartment.

Note that there is a low 555 signal through the defined nuclear area due to the nature of the 3D Image, whereby the tubulin traverses the space above the nuclear compartment.

Image Corrections

None

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Image by P. Raut

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