Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7)
Cat ID# - PA-MM 000002

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, IgG (H + L)
Cat ID# - SA 000022

H2AX, a variant of the histone protein H2A, and its primary function relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ-H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ-H2AX creates a binding site for various proteins involved in DNA repair It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and other These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ-H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ-H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, followed by -20°C methanol fixation and blocked with Thermo Fisher, Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7), 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-

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Mouse, $F(a'')_2$ (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, IgG (H + L) 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, 1.25X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - *Panel A*

Z-Stack = 122 Slices (3.33 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 5056 X 5056
Image Size (Scaled) = 66.72 µm X 66.72 µm
Bit Depth = 16 Bit
Image Center Position = X: 58.45 mm, Y: 36.83 mm
Stage Position = X: 58.45 mm, Y: 36.83 mm
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 = 100 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @0.5 %
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

488 -

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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 = 488
Channel Name = T2-Axiocam 820-SR
Excitation Wavelength = 488
Emission Wavelength = 525
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 100 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @1.50%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 32 µm 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 = 50 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @6.00%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM² Processing

Processing Dimension = 3D
Result Sampling = 4
Process Sampling = 4
Channels = 3
Algorithm = SIM²

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SIM² Settings = Standard
Input SNR = Low
Regularization = None
Regularization Weight = 0.0000
Iterations = 3
Filter = No Filter
Fitting = Fast Fit
Normalization = Auto

3D Image Processing - *Panel A*

3D Maximum Projection = Precise
Appearance = Threshold 4% all channels, 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)

Profile View Display - *Panel B*

Profile Definition = Stroke Thickness 1, Normal View, Grid Distance 1
Profile View = X and Y axes were set to Auto - X (Min 0.0 and Max 34859.2), Y (Min 0 and Max 36134)

Histogram of Frequency & Intensity - *Panel C*

Histogram Definition

Normal
Bin Count = 256
Bin size = 150
Lower Threshold = 0
Upper Threshold = 38186
Show Thresholds Selected

Histogram View

Statistic Table, Int. Measurement, Frequency Table, and Image all selected.
X Axis = Auto (min 0.00, max 38186)
Y Axis = Auto (min 0, max 7668115)
Logarithmic Selected
Channel Transparency = 27%

Summary

Panel A – This panel presents a 3-dimensional Maximum Projection of Mouse anti-HumanH2Ax Monoclonal, IgG1, counterstained with Identifyn® SRM Alexa Fluor™ 647 secondary (red), Actin primary antibody counterstained with Identifyn® SRM Alexa Fluor™ 488 secondary (green), and DAPI staining (blue) at super resolution SIM squared resolution.

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Panel B - This panel presents a line profile across the nucleus using both the Mouse anti-Human H2Ax Monoclonal, IgG1 counterstained with Identifyn® SRM Alexa Fluor™ 647 secondary (red), and the DAPI (blue) channels. The line profile histogram shows both distance (X) and intensity (Y). We note high brightness and colocalization across the distance (X axis), for both H2AX and DAPI, which is expected. We also chose to cross a nucleolus to show that the area is devoid of signal (beyond background), as neither H2AX nor DAPI should be present.

Panel C - This panel displays a Histogram view, demonstrating the fluorescent intensity in the X axis, and the frequency of signal on the Y axis, you can see here that much like the line profile; we see high brightness and notably relative frequency again pointing to the H2AX signal colocalizing as it should within the nuclear compartment with the DAPI staining We have added transparency via Zeiss Zen to the histogram, allowing for the visualization of both channels.

Image Corrections

None

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

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