Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, Human Fc, IgG (BLR014M)
Cat ID# - PA-RR-000004

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Human IgG (H + L)
Cat ID# - SA 000016

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 double-strand breaks (DSBs). When DSBs occur in chromatin, γH2AX 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 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, Human Fc, 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. 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 = 39 Slices (1.52μm)
Channels = 2
Scaling (Per Pixel) = 0.071μm X 0.071μm X 0.071μm
Image Size (Pixels) = 730 X 586
Image Size (Scaled) = 51.54μm X 41.37μm
Bit Depth = 16 Bit
Image Center Position = X: 77.00mm, Y: 53.10mm
Stage Position = X: 77.00mm, Y: 53.10mm
ROI Center Offset = X: 0.00μm, Y: 0.00μm
488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 45μm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)

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Laser Wavelength = 488nm @1.0%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.4
Rotation = 0°
Sampling = 1.00
Pixel Time = 0.51µs
Frame Time = 10.15s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 16
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 484-590
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 650 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 @0.3%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.4
Rotation = 0°
Sampling = 1.00
Pixel Time = 0.51µs
Frame Time = 10.15s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 16
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*

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3D Maximum Projection = Precise
Appearance = Threshold 1% (488), Threshold 1% (405), Ramp 0.0% 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)

Colocalization View - *Panels B and C*

Horizontal Axis - 488, Threshold 1475, Range Auto
Vertical Axis - DAPI, Threshold 384, Range Auto

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 13.4), Y (Min 4854 and Max 53425)

Summary

Panel A presents a three-dimensional view of the complete image acquisition, displayed as a maximum projection. An X-Y cut plane is positioned at Z plane 19 of 39 to emphasize the center of the nucleus and the gamma-H2AX signal within the nuclear compartment. Panels B and C demonstrate that the gamma-H2AX signal is localized within the nuclear compartment, as indicated in the colocalization plot. Panels D and E display a line profile across several foci, highlighting the peak fluorescent intensity and the distance between the foci signals.

It is important to note that a single Foci can be misleading at diffraction-limited confocal resolutions since it measures approximately 1 micron in width. In contrast, more advanced super-resolution techniques, such as STORM, can achieve a resolution of foci down to approximately 40 nm. This suggests our observed signal may represent more complex structures beyond our current resolution.
Image Corrections –

None

Image by P. Raut
Analysis and Presentation by B. T. Bennett

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