

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™ 532 Affinity Purified Goat Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000058*

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, Identifyn® Rabbit anti-Human γ H2Ax Recombinant Monoclonal, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS, and the Identifyn™532 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 Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, AxioCam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:

Z Stack - (3D)**Z-Stack = 23 Slices (4.4 μ m)**

Channels = 2

Scaling (Per Pixel) = 0.143 μ m X 0.200 μ m

Image Size (Pixels) = 217 X 275

Image Size (Scaled) = 31.00 μ m X 39.29 μ m

Bit Depth = 14 Bit

Image Center Position = X: 73.88mm, Y:52.12mm

Stage Position = X: 73.88mm, Y:52.12mm

ROI Center Offset = X: 1.14 μ m, Y: 0.00 μ m

532-

Reflector = AF532-49014

Beam Splitter = 550

Filter Excitation Wavelength = 515 - 545

Filter Emission Wavelength = 555 - 595
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @35%
Exposure Time = 150ms
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.25
Imaging Device = AxioCam 807
Camera Adapter = 1X
Depth of Focus = 1.07µm
Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335-383
Filter Emission Wavelength = 420-470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity = @8%
Exposure Time = 20ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.25
Imaging Device = AxioCam 807
Camera Adapter = 1X
Depth of Focus = 0.90µm
Binning Mode = 2,2

Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mode "Optical Sectioning" - enabled correction selected.

Normalization, Phase Corrector, Fourier Filter, and Deconvolution **were NOT used.**

3D Image Processing

3D Volume Projection = Precise
Appearance = Threshold 13% all channels, Ramp 50% 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)

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 0.0 and Max 23.9), Y (Min 19 and Max 17202)

Summary

A three-dimensional image representation illustrates foci formation within the nuclear compartment following DNA damage. The profile view highlights the signal intensity across punctate foci, noting the absence of signal or presence of Rabbit anti-Human γ H2Ax Recombinant Monoclonal IgG in the nucleoli and outside the nuclear compartment.

Moreover, while Widefield SIM employs a grid methodology to eliminate out-of-focus light and enables z-plane collection, this method has significant limitations in achieving "confocal-like" resolution claims. The "foci" formation appears combined with "pan-nuclei" staining, indicating a loss of resolution. Although the images demonstrate that the antibody responds correctly and that localization is appropriate, any interpretation of biological functions beyond population studies would be misleading. This is particularly true given the structural details encountered when crossing the diffraction limit into super-resolution and single-molecule methods.

Image Corrections

None

Image by B.T. Bennett

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