Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human γH2Ax (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 (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

DNA damage was NOT induced.

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, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS, and 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 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:
Single Plane (2D)
Channels = 2
Scaling (Per Pixel) = 0.143µm X 0.143µm
Image Size (Pixels) = 963 X 855
Image Size (Scaled) = 135.57µm X 122.14µm
Bit Depth = 14 Bit
Image Center Position = X: 75.74mm, Y: 43.47mm
Stage Position = X: 75.74mm, Y: 43.47mm
ROI Center Offset = X: 1.14µm, Y: 0.00µm

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647 -
Reflector = 50 Cy5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @8.0%
Exposure Time = 150ms
Excitation Wavelength = 663
Emission Wavelength = 668
Effective NA = 1.25
Imaging Device = Axiocam 807 mono
Camera Adapter = 1X
Depth of Focus = 1.30µm
Binning Mode = 2,2

555 -
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 @6.0%
Exposure Time = 50ms
Excitation Wavelength = 553
Emission Wavelength = 568
Effective NA = 1.25
Imaging Device = Axiocam 807 mono
Camera Adapter = 1X
Depth of Focus = 1.10µm
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 @6.0%
Exposure Time = 20ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.25
Imaging Device = Axiocam 807 mono
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 Correction, Fourier Filter, and Deconvolution **were NOT used.**

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Summary

Panel A presents a split view featuring the 647 channel (gamma H2AX), the 555 channel (tubulin), the DAPI channel, and a merged channel image displayed in the lower right corner. Panel B shows these three channels in a larger format for better inspection of the signals. Panels C and D utilize a line profile to measure the signal (photon counts) across the three channels. It is noted that both the 555 and DAPI channels exhibit signal, while the 647 channel (gamma H2AX) shows a flat line with minimal signal detectable.

In summary, the control images indicate that, in the absence of Etoposide-induced DNA damage, there is a significant reduction in the presence of gamma H2AX within the nucleus. Conversely, the complete dataset for gamma H2AX demonstrates a strong nuclear aggregation, indicating that Etoposide induces a DNA damage phosphorylation event.

Image Corrections
None

Image by J.K. Bennett
Analysis and Presentation by B. T. Bennett
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