

Identifyn Standards for Optical Microscopy Methods™Identifyn Primary Antibody**Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)***Cat ID# - PA-RR 000005*Identifyn® Secondary Antibody**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)***Cat ID# - SA 000062*

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 double-strand breaks (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, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), 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-Mouse, F(ab')₂ (H + L) 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 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:

Single Plane (2D) - Z-plane 78 of 154 total - Panel A**Z Stack - (3D) - Panels A & B****Z-Stack = 154 Slices (4.59 μ m)**

Channels = 2

Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm

Image Size (Pixels) = 1805 X 2627

Image Size (Scaled) = 23.82 μ m X 34.66 μ m

Bit Depth = 16 Bit

Image Center Position = X: 58.34 mm, Y: 35.81 mm

Stage Position = X: 58.34 mm, Y: 35.81 mm

ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m**Z Stack - (3D) - Panel C**

Z-Stack = 54 Slices (1.59 µm)

Channels = 2

Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm

Image Size (Pixels) = 163 X 192

Image Size (Scaled) = 2.15 µm X 2.53 µm

Bit Depth = 16 Bit

Image Center Position = X: 58.34 mm, Y: 35.81 mm

Stage Position = X: 58.34 mm, Y: 35.81 mm

ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

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 = 120 ms

Depth of Focus = 1.13 µm

Binning Mode = 2,2

Laser Wavelength = 640 nm @2.00%

Frame Time = 1.59 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 = 2
 Algorithm = SIM²
 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 B

3D Maximum Projection = Precise
 Appearance = Threshold 4% all channels, Ramp 0% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, Tone Mapping Enabled
 Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel C

3D Surface Projection = Precise
 Appearance = Threshold 21% (647) and 26% (DAPI), Ambient Light 0% all channels, Specular Light 100% all channels, Shininess 30% all channels
 Background = Black
 Light = 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)

Summary

Panel A - This panel presents a 2-dimensional view from the image acquisition at z-plane 78 out of 154. The panel illustrates the signal and localization of Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™647 (red) and DAPI (blue). A white box denotes a Region of Interest (ROI) to be further investigated in Panel C.

Panel B - This panel displays a 3-dimensional Maximum Projection of the same image acquisition shown in panel A. Shown are Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (white, changed from default red in Zeiss Zen software to enhance visual contrast), followed by DAPI (blue) at super resolution SIM Squared. We note that in all Etoposide-

induced DNA damage samples at super resolution, we begin to see doublets throughout the nucleus. Through hundreds of super resolution samples and numerous STORM experiments, we have demonstrated that they may represent the opposing end of a double-strand break; however, we make no definitive claims.

A white box denotes the same Region of Interest noted in the 2-dimensional image in panel A, here in Panel B, to be further investigated in Panel C.

Panel C - This panel displays a 3-dimensional Surface Projection of the ROI shown in panel B. Shown is the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (white, changed from default red in Zeiss Zen software to enhance visual contrast) and DAPI (blue), deploying super resolution SIM Squared. Here, we utilize the power of SIM, approximately 40-60 nm axially, to investigate one of the doublets mentioned above. We observe in the surface projection that there are two focal points to the signal. Although SIM Squared demonstrates an increase in resolution, it still lacks the detail we see in STORM; however, it is very familiar in its structure, as shown in panel D.

Panel D - Demonstrates and compares the XZ presentation of the signal we observe in STORM for the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, highlighting the striking similarities, despite being quite far apart in resolution, even at the nanometer scale.

Image Corrections

Panels B & C - Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(a'')₂ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

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

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