

**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™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')<sub>2</sub> (H + L)***Cat ID# - SA 000020***Identifyn® SRM Alexa Fluor™ 680 Affinity Purified Goat Anti-Mouse, F(ab')<sub>2</sub> (H + L)***Cat ID# - SA 000073*

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 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, 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.

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® 680 secondary antibody 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, 1X Tubelens, was used with the following parameters for each dye:

**Single Plane (2D) - Panel A****Z-Plane - 52 of 114**

Channels = 3

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

Image Size (Pixels) = 5056 X 5056

Image Size (Scaled) = 66.72μm X 66.72μm

Bit Depth = 16 Bit

Image Center Position = X: 59.08mm, Y: 39.49mm  
Stage Position = X: 59.08mm, Y: 39.49mm  
ROI Center Offset = X: 0.00μm, Y: 0.00μm

**Z Stack - (3D) - Panel D****Z-Stack = 114 Slices (3.39μm)**

Channels = 3  
Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm  
Image Size (Pixels) = 676 X 475  
Image Size (Scaled) = 8.92μm X 6.27μm  
Bit Depth = 16 Bit  
Image Center Position = X: 59.14mm, Y: 38.63mm  
Stage Position = X: 59.14mm, Y: 38.63mm  
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 = 120ms  
Depth of Focus = 1.13μm  
Binning Mode = 2,2  
Laser Wavelength = 640nm @1.50%  
Frame Time = 1.59s  
Scan Direction = Unidirectional  
Grating 27.5μm grating

**555 -**

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 = 561  
Channel Name = T2-Axiocam 820 #2-SR  
Excitation Wavelength = 561  
Emission Wavelength = 597  
Effective NA = 1.4  
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800

Imaging Device = Axiocam 820 #2  
Camera Adapter = 1X  
Exposure Time = 120ms  
Depth of Focus = 0.92µm  
Binning Mode = 2,2  
Laser Wavelength = 561nm @1.50%  
Frame Time = 1.33s  
Scan Direction = Unidirectional  
Grating 32.0µm 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 = 50ms  
Depth of Focus = 0.69µm  
Binning Mode = 2,2  
Laser Wavelength = 405nm @6.0%  
Frame Time = 676.00ms  
Scan Direction = Unidirectional  
Grating 36.5µm grating

**Post Processing - SIM Processing**

Processing Dimension - 3D  
Result Sampling = 4  
Process Sampling = 4  
Channels = 3  
Algorithm = SIM2  
Sharpness Setting = Standard  
Auto Sharpness = On  
Sharpness = 8.8687 (647), 9.4576 (555), 8.0281 (DAPI)  
Order Combination = Weiner Filter  
Regularization = None  
Regularization Weight = 0.000  
Iterations = 3  
Filter - No Filter  
Fitting = Fast Fit  
Histogram = Scale to Min/Max

**3D Image Processing - Panel C**

3D Volume Projection = Precise

Appearance = Threshold 2% (647), Threshold 2% (555), Threshold 2% (DAPI), Ramp 98% 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)

**Orthogonal View, -Panel B** - Uses Image 52 of the Z-Stack 114 Images.

Cut Lines = X 1375, Y 2135, Z 53

Line Width = X 75, Y 75, Z 1

Cut Line Opacity = 11

Maximum Intensity Opacity (MIP) and Measure 3D Distance NOT selected

### Colocalization View

Horizontal Axis - 647, Threshold 42862, Range Auto

Vertical Axis - DAPI, Threshold 4832, Range Auto

### Clipping Image Process Panels C and D -

The Clipping plane is introduced to demonstrate the specificity of the Identifyn® Rabbit anti-Human  $\gamma$ H2AX Recombinant Monoclonal, Human Fc, IgG to the DNA damage-induced gamma-H2AX foci within the nuclear compartment.

**X-Y** = Active was selected, and a textured opaque filter was chosen to remove the obstruction from the image-cut plane and highlight the nuclear compartment and gamma-H2AX foci. A white outline of the clipped perimeter was shown, the Clip Back was selected, and the Clip Front was NOT selected.

Position of Clip = -14%

Clip Y = 0°

Clip Z = 0°

### Summary

Panel A presents a two-dimensional view of the z-stack acquisition, focusing on z-plane 52 of 114. Panel B provides an orthogonal view, positioning the X and Y cut lines over a "doublet" of gamma H2AX signal within the nuclear compartment. Both the XZ and YZ planes demonstrate the signal separation between the two, utilizing super-resolution SIM2.

Panel C features a colocalization histogram that illustrates the binding of the Identifyn® gamma-H2AX Rabbit Recombinant antibody within the nucleus, as expected. The lower image in Panel C shows the entire Region of Interest (ROI) selected for this measurement.

Panel D displays a three-dimensional ROI from the original data set as a volume projection. This projection is further enhanced using an X, Y cut plane at a z-plane

position of -14%. It includes a textured opaque filter with a white outline to improve the visibility of the gamma-H2AX signal. The data set illustrates that while traditional diffraction-limited microscopy would interpret what appears to be a single signal, increased resolution capabilities reveal multiple signals. However, this does not achieve the level of detail seen in single molecule acquisition techniques like STORM, where structural details are observed more clearly.

### Image Corrections

None

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Analysis and Presentation by B. T. Bennett

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