

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

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, Rabbit anti-Human γ H2Ax 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 Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, AxioCam 705 mono, and X-Cite Xylis Lamp, was used with the following parameters for each dye:

Single Plane (2D)

Channels = 3

Scaling (Per Pixel) = 0.110 μ m X 0.110 μ m

Image size (Pixels) = 943 X 1003

Image Size (Scaled) = 103.28 μ m X 109.85 μ m

Bit Depth = 14 Bit

Image Center Position = X: 0.00 μ m, Y: 0.00 μ m

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

488 -

Reflector = 38 HE eGFP
Beam Splitter = 495
Filter Excitation Wavelength = 450 - 490
Filter Emission Wavelength = 500 - 550
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @00%
Exposure Time = 100ms
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Imaging Device = AxioCam 705 mono
Camera Adapter = 1X
Depth of Focus = 0.80 μ m
Binning Mode = 2,2

680 -

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 @25%
Exposure Time = 150ms
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Imaging Device = AxioCam 705 mono
Camera Adapter = 1X
Depth of Focus = 1.09 μ m
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 @15%
Exposure Time = 40ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = AxioCam 705 mono
Camera Adapter = 1X
Depth of Focus = 0.72 μ m
Binning Mode = 2,2

2.5D Image Processing - Panel B

2.5D Display

Render Mode = Surface

Grid distance = 5

Palette and Show Faces at Side are both Selected

2.5D Display Options

Angle X = 80

Angle Y = -92

Z Scaling = 1.0

Ambient = 50

Specular = 50

Shininess = 50

Light Height = 2.0

Histogram of Frequency & Intensity

Histogram Definition

Normal

Bind Count = 256

Bin size = 64

Lower Threshold = 0

Upper Threshold = 16383

Histogram View

X Axis = Auto (min 0.00, max 16383)

Y Axis = Auto (min 0, max 5000000)

Channel Transparency = 100%

Summary

Panel A presents a two-dimensional view of the complete image acquisition. Gamma-H2AX was stained using Identifyn® anti-Human gamma H2AX Rabbit Recombinant Monoclonal antibody and Identifyn® SRM Alexa Fluor™ 488, highlighting its presence within the nuclear compartment, which was also stained with DAPI. Additionally, tubulin was stained with Identifyn® SRM Alexa Fluor™ 680. It's important to note that the Zeiss Axio Imager M1 is equipped with custom filters and an sCMOS camera, which enhances image quality compared to traditional widefield microscopes. This improves the acquisition and appearance of "foci" and "pan-nuclear" staining. However, it is crucial to recognize that this setup is limited in resolution, and the image lacks structural detail that only super-resolution techniques can provide.

Panel B presents a 2.5D view of the same acquisition, providing detailed information about the fluorescent intensities for all three channels and highlighting the robust and specific signaling of the Identifyn® primary and 488 secondary antibodies.

Panels C and D display a histogram view of the same field, excluding the DAPI and 680 channels. In this format, they measure the signal's frequency and intensity, using an approach distinct from the 2.5D measure. In conclusion, this imaging setup, enhanced by custom filtration for optimal collection and spectral separation, along with the sensitivity of the sCMOS camera, serves as a valuable tool for validating sample preparation, antibody specificity, and fluorescent intensity. However, it does not provide structural or biological information beyond a population study.

Image Correction

None

Image by E.F. Simon

Analysis and Presentation by B. T. Bennett

Copyright 2026 GMD12 LLC.

