

IDENTIFYN RESOLUTION STEPDOWN

H2AX

MICROSCOPY EVIDENCE ASSESSMENT

Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 555

WESTERN BLOT -> WIDEFIELD -> WIDEFIELD SIM / APOTOME -> CONFOCAL -> AIRYSCAN
SIM -> SIM²

7

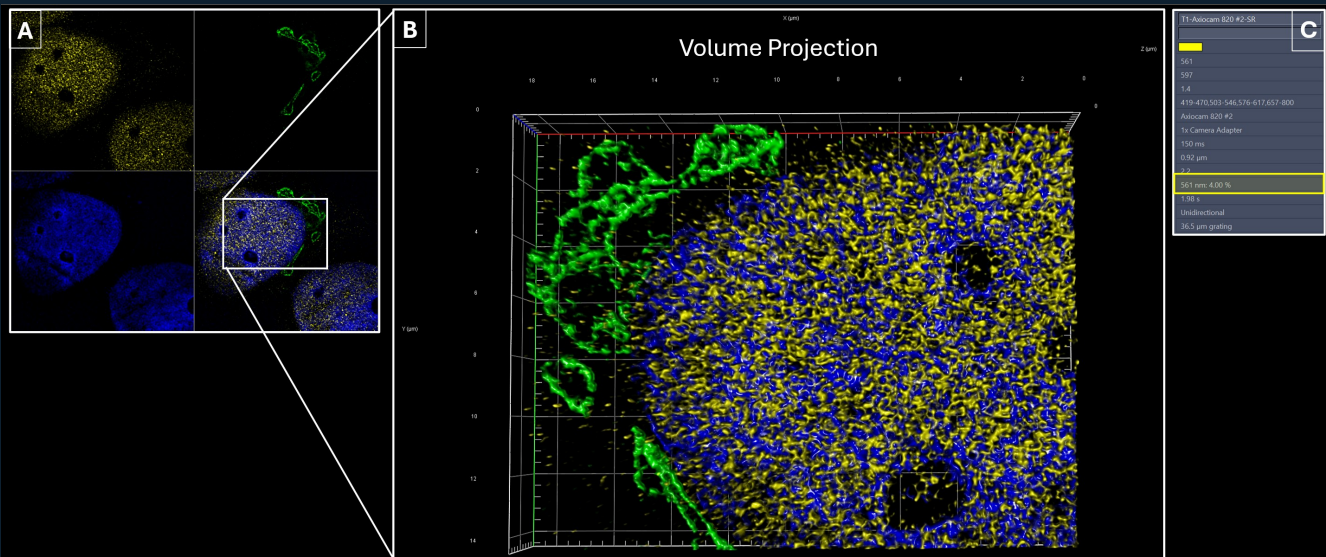
ORDERED EVIDENCE TIERS

0

SUPPORTING CONTROL

PENDING

SCIENTIST REVIEW



Panel A - Split View, this panel illustrates the signal and localization of the Mouse anti-Human H2AX Monoclonal - Identifyn™ SRM Alexa Fluor™ 555, an IgG1 direct conjugate. The upper left section displays the H2AX direct conjugate (yellow), while the upper right shows the Golgi primary antibody labeled with Identifyn™ SRM Alexa Fluor™ 488 (green). The lower left features DAPI nuclear staining (blue), and the lower right presents a merged image of all three channels. A Region of Interest is indicated by the white box, which will be explored further in Panel B.

Panel B - This panel presents a three-dimensional volume projection of the Region of Interest from Panel A. It includes the Mouse anti-Human H2AX Monoclonal - Identifyn™ SRM Alexa Fluor™ 555 (yellow), the Golgi primary antibody with Identifyn™ SRM Alexa Fluor™ 488 (green), and DAPI (blue), utilizing super-resolution SIM Squared with no manipulation of the signal, beyond selecting volume view in Zeiss Zen software.

Panel C - This panel features a clipped image from the Zeiss Zen image acquisition settings for a z-stack comprising 120 planes. **The 561 nm laser power is set at 4.0% to achieve optimal imaging of the direct conjugate over a z-stack distance of 3.57 microns.** Additionally, the Identifyn™ SRM Alexa Fluor™ 488 required a laser power of 1.50%, indicating high brightness for all Identifyn™ products via our patent-pending conjugation chemistry.

REPRESENTATIVE HIGHEST-AVAILABLE VIEW / SIM²

DC-000036 | PRODUCT-LEVEL EVIDENCE DOSSIER

SOURCE-GROUNDED MICROSCOPY + BIOLOGICAL REVIEW / IDENTIFYN EVIDENCE PRESERVED

CRO CAPABILITY DEMONSTRATED

Identifyn performed the experimental and imaging work documented below. BENNETT converts that operating history into a governed, source-bounded scientific record.

- Source-documented sample preparation and antibody/reagent workflow
- Western blot imaging
- Widefield fluorescence microscopy
- Widefield structured illumination / Apotome optical sectioning
- Confocal microscopy
- Airyscan acquisition and reconstruction workflow
- Structured illumination microscopy
- SIM² reconstruction workflow
- Treatment-response experimental workflow
- Z-stack / 3D acquisition
- Multi-channel imaging

SCIENTIFIC QUESTION

What source-bounded experimental and microscopy evidence is preserved for H2AX, and what is the strongest interpretation allowed by the available modalities and controls?

EVIDENCE AVAILABLE

Western blot -> Widefield -> Widefield SIM — Apotome -> Confocal -> Airyscan -> SIM -> SIM²

BENNETT ASSESSMENT

The preserved DC-000036 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

BENNETT / MICROSCOPY AND BIOLOGICAL EVIDENCE REVIEW

SOURCE-GROUNDED RETROSPECTIVE REVIEW / PENDING SCIENTIST REVIEW

SOURCE STATE	SOURCE HASH VERIFIED / EVENT RECORDED
BENNETT EVIDENCE REVIEW	COMPLETED / SOURCE-BOUNDED
NATIVE IMAGE REPROCESSING	NOT PERFORMED
LITERATURE SOURCES	INDEPENDENTLY VERIFIED
SCIENTIST REVIEW	PENDING
PUBLICATION	NOT AUTHORIZED

OVERALL CALL / NIR-BOUNDED PARTIAL STEPDOWN

The preserved DC-000036 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

SETTINGS ASSESSMENT

The record preserves 7 ordered evidence tier(s). Structured source metadata values are reported by stage and remain tied to the original method objects.

BIOLOGICAL SUPPORT

The record supports retrospective, source-bounded microscopy review. Target identity, specificity, treatment effect, binding, interaction and mechanism are not inferred from presentation images alone.

CONTROL ASSESSMENT

No separate control record is preserved; none is inferred from method or template language.

CLAIM CEILING

This draft supports historical capability documentation, source registration, modality ordering, settings review and bounded interpretation. It does not independently validate antibody specificity, quantify a population response, establish binding, interaction, causation or mechanism, or authorize publication.

PROCESSING DISCLOSURE

Retrospective BENNETT architectural evaluation of preserved Identifyn source evidence. BENNETT did not perform native-file reprocessing, new deterministic measurement or the historical image post-processing represented in this record.

REAGENTS AND ACQUIRED CHANNELS

Preparation reagents are reported separately from channels actually documented in the acquisition settings. Fluorophores mentioned in staining are not automatically treated as acquired channels.

EVIDENCE OBJECT	REAGENTS / FLUOROPHORES PRESENT	CHANNELS ACTUALLY ACQUIRED
Western blot	555	Not deterministically stated in the acquisition block
Widefield	405/DAPI; 555; 647	3; AF532-49014; 50 Cy 5; 49 DAPI; 515 - 545; 625 - 655; 335 - 383; 555 - 595
Widefield SIM — Apotome	405/DAPI; 555; 647	3; AF532-49014; 50 Cy 5; 49 DAPI; 515 - 545; 625 - 655; 335 - 383; 555 - 595
Confocal	405/DAPI; 488; 555	3; None; 561 nm @0.3%; 488 nm @0.03%; 405 nm @0.2%; 553; 493; 353
Airyscan	405/DAPI; 488; 555; 750	3; None; 561 nm @0.5%; 488 nm @0.06%; 405 nm @0.2%; 553; 493; 353
SIM	405/DAPI; 488; 555	3; SR HE LBF 405/488/561/642; 200 - 405, 200 - 488, 200 - 561, 200 - 642; 419 - 470, 503 - 546, 576 - 617, 657 - 800; T1-Axiocam 820 #2-SR; T2-Axiocam 820-SR; T3-Axiocam 820 #2-SR; 561
SIM ²	405/DAPI; 488; 555	3; SR HE LBF 405/488/561/642; 200 - 405, 200 - 488, 200 - 561, 200 - 642; 419 - 470, 503 - 546, 576 - 617, 657 - 800; T1-Axiocam 820 #2-SR; T2-Axiocam 820-SR; T3-Axiocam 820 #2-SR; 561

MODALITY ASSESSMENT / PART 1 OF 7

Western blot / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: Azure Sapphire FL. Objective: not explicitly stated. Platform: Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s). Structured source metadata values: 11.

VISIBLE OBSERVATION

A preserved presentation image is available for Western blot. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Western blot, documents platform context as Azure Sapphire FL, and records detected dye/channel descriptors as 555.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

Band position and displayed intensity do not independently establish analytical specificity, target identity, linearity, or treatment effect.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Western blot evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 2 OF 7

Widefield / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Axio Imager.M1. Objective: Plan-Apochromat 63x/1.40 Oil M27. Platform: Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye: Acquisition: Channels: 3; Scaling (Per Pixel): 0.110 μm X 0.110 μm ; Image size (Pixels): 1216 X 1028; Image Size (Pixels): 1216 X 1028; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 705. Timing: Exposure Time: 60 ms; 50 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @8.00%; X-Cite Xylis Lamp Intensity @6.00%. Optical/scan: Effective NA: 1.4. Structured source metadata values: 40.

VISIBLE OBSERVATION

A preserved presentation image is available for Widefield. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Widefield, documents platform context as ZEISS Axio Imager.M1, and records detected dye/channel descriptors as 405/DAPI; 555; 647.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

A presentation image supports gross localization and source-bounded co-occurrence, not direct binding, molecular colocalization, or native quantitative measurement.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Widefield evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 3 OF 7

Widefield SIM — Apotome / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Axio Observer.Z1/7 Apotome. Objective: EC Plan-Neofluar 63X/1.25 oil M27. Platform: 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:. Acquisition: Z-Stack: 85 Slices (8.4 μm); Channels: 3; Scaling (Per Pixel): 0.143 μm X 0.143 μm X 0.100 μm ; Image size (Pixels): 1221 X 641; Image Size (Pixels): 1221 X 641; Bit Depth: 14 Bit. Detector/camera: Imaging Device: 807 mono. Timing: Exposure Time: 150 ms; 40 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @12.00%; X-Cite Xylis Lamp Intensity @6.00%. Optical/scan: Effective NA: 1.25. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 49.

VISIBLE OBSERVATION

A preserved presentation image is available for Widefield SIM — Apotome. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Widefield SIM — Apotome, documents platform context as ZEISS Axio Observer.Z1/7 Apotome, and records detected dye/channel descriptors as 405/DAPI; 555; 647.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

Improved optical sectioning does not by itself establish reconstructed super-resolution or molecular interaction.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Widefield SIM — Apotome evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 4 OF 7

Confocal / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS LSM 980. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:. Acquisition: Z-Stack: 121 Slices (3.6 μm); Channels: 3; Scaling (Per Pixel): 0.055 μm X 0.055 μm X 0.030 μm ; Image size (Pixels): 625 X 563; Image Size (Pixels): 625 X 563; Bit Depth: 16 Bit. Detector/camera: Imaging Device: GaAsP-Pmt3; Detector Type: GaAsP-PMT. Timing: Pixel Time: 0.31 μs ; Frame Time: 3.60 s. Illumination: Laser Wavelength: 561 nm @0.3%; 488 nm @0.03%. Optical/scan: Pinhole: 1.00 AU / 55 μm ; 1.00 AU / 49 μm ; Scan Zoom: 2.2; LSM Scan Speed: 12; Scan Direction: Bidirectional; Averaging: 8; Effective NA: 1.4. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black; LSM Plus Mode: 7.4 (3D, Auto); 8.0 (3D, Auto). Structured source metadata values: 59.

VISIBLE OBSERVATION

A preserved presentation image is available for Confocal. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Confocal, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 555.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

Optical sectioning and source-bounded 3D presentation do not establish binding, mechanism, or population-level effect.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Confocal evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 5 OF 7

Airyscan / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS LSM 980. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:. Acquisition: Z-Stack: 111 Slices (3.3 μm); Channels: 3; Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm; Image size (Pixels): 683 X 536; Image Size (Pixels): 683 X 536; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; Detector Type: GaAsP-PMT. Timing: Pixel Time: 0.74 μs ; Frame Time: 6.95 s. Illumination: Laser Wavelength: 561 nm @0.5%; 488 nm @0.06%. Optical/scan: Pinhole: 5.00 AU / 272 μm ; 5.00 AU / 247 μm ; Scan Zoom: 2.7; LSM Scan Speed: 7; Scan Direction: Bidirectional; Averaging: 4; Effective NA: 1.4. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black; AiryScan Mode: SuperResolution 8.5 (3D, Auto); SuperResolution 9.2 (3D, Auto). Structured source metadata values: 63.

VISIBLE OBSERVATION

A preserved presentation image is available for Airyscan. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Airyscan, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 555; 750.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy guidance that advanced-resolution outputs remain dependent on acquisition, reconstruction or localization quality control.

INTERPRETIVE LIMIT

Displayed feature separation remains reconstruction dependent; no unsupported numerical resolution or molecular architecture claim is permitted.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Airyscan evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 6 OF 7

SIM / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Elyra. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: 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:. Acquisition: Z-Stack: 120 Slices (3.57 μm); 81 Slices (2.4 μm); Channels: 3; Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm; Image size (Pixels): 3453 X 2390; 1514 X 1294; Image Size (Pixels): 3453 X 2390; 1514 X 1294; Bit Depth: 16 Bit. Detector/camera: Imaging Device: AxioCam 820 #2; AxioCam 820. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.98 s; 1.59 s. Illumination: Laser Wavelength: 561 nm @4.00%; 488 nm @1.50%; Illumination Wavelength: 561; 488. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM; Process Sampling: N/A; Result Sampling: 4; Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 66.

VISIBLE OBSERVATION

A preserved presentation image is available for SIM. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as SIM, documents platform context as ZEISS Elyra, and records detected dye/channel descriptors as 405/DAPI; 488; 555.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy guidance that advanced-resolution outputs remain dependent on acquisition, reconstruction or localization quality control.

INTERPRETIVE LIMIT

Reconstruction-dependent structural separation requires native reconstruction QC before numerical resolution or molecular dimensions can be claimed.

CLAIM CEILING

This tier supports a source-bounded statement that the historical SIM evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 7 OF 7

SIM² / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Elyra. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: 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: Acquisition: Z-Stack: 120 Slices (3.57 μ m); Channels: 3; Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm; Image size (Pixels): 1615 X 1410; 891 X 688; Image Size (Pixels): 1615 X 1410; 891 X 688; Bit Depth: 16 Bit. Detector/camera: Imaging Device: AxioCam 820 #2; AxioCam 820. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.98 s; 1.59 s. Illumination: Laser Wavelength: 561 nm @4.00%; 488 nm @1.50%; Illumination Wavelength: 561; 488. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM²; Process Sampling: 4; Result Sampling: 4; Projection: View Angle 35°, Scale Z 77%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 66.

VISIBLE OBSERVATION

A preserved presentation image is available for SIM². BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as SIM², documents platform context as ZEISS Elyra, and records detected dye/channel descriptors as 405/DAPI; 488; 555.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy guidance that advanced-resolution outputs remain dependent on acquisition, reconstruction or localization quality control.

INTERPRETIVE LIMIT

Further reconstruction-dependent segmentation does not establish molecular dimensions, structure identity, or mechanism without independent support.

CLAIM CEILING

This tier supports a source-bounded statement that the historical SIM² evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

CROSS-MODALITY SYNTHESIS

The preserved DC-000036 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

Different acquisitions are not treated as a quantitative intensity ladder. Any continuity statement remains qualitative and source bounded.

BENNETT LITERATURE CORRELATION — SOURCES INDEPENDENTLY VERIFIED

BENNETT uses independently verified primary scientific sources to test whether the interpretation is scientifically consistent and properly bounded. Literature correlation does not retroactively validate the Identifyn dataset or replace scientist review.

- Antibody and microscopy evidence require orthogonal controls and modality-appropriate interpretation; presentation images alone do not establish analytical specificity.
- Advanced-resolution presentation depends on acquisition, reconstruction or localization quality control before numerical resolution, molecular dimensions or cluster identity can be claimed.

REFERENCES

1. Uhlen M, et al. A proposal for validation of antibodies. Nat Methods. 2016;13:823-827. doi:10.1038/nmeth.3995.
2. Gustafsson MGL. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. J Microsc. 2000;198:82-87. doi:10.1046/j.1365-2818.2000.00710.x.
3. Sage D, et al. Quantitative evaluation of software packages for single-molecule localization microscopy. Nat Methods. 2015;12:717-724. doi:10.1038/nmeth.3442.

RELEASE BOUNDARY

This assessment remains a draft pending scientist review. No publication is authorized. Any future native-file analysis must enter BENNETT as a new evidence snapshot with routed engine authority, native-data QA, methods, limitations, and an independently reviewed claim ceiling.

SOURCE RECONCILIATION / QA / PROVENANCE

SOURCE STATUS - PRESERVED / AUTO-RECONCILED

Historical Identifyn source material was subjected to BENNETT retrospective QA. Original source state and correction history remain preserved in provenance.

HIGH CONFIDENCE - AUTO-RECONCILED

Airyscan / Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm -> 0.03530 μm X 0.03530 μm X 0.03000 μm . Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=683 X 536; Image Size (Scaled)=24.11 μm X 18.92 μm ; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.00%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM / Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm -> 0.02111 μm X 0.02111 μm X 0.03000 μm . Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=3453 X 2390; Image Size (Scaled)=72.90 μm X 50.46 μm ; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.01%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM² / Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm -> 0.02111 μm X 0.02111 μm X 0.03000 μm . Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=1615 X 1410; Image Size (Scaled)=34.10 μm X 29.77 μm ; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.02%.

HIGH CONFIDENCE - SCIENTIST-AUTHORIZED CORRECTION

Cross-tier / treatment concentration: 200 μM -> Etoposide = 2 μM . The governing scientist identified every 200 μM occurrence as a technician transcription error. The canonical historical concentration is 2 μM . Supporting evidence: Scientist-authorized correction supplied for the Identifyn historical archive; original technician text remains preserved unchanged. Authority: Dr. Brian T. Bennett. Preservation: Original source text and hashes remain unchanged; only the derivative canonical field is corrected.

BENNETT EVIDENCE REVIEW COMPLETE

INTERPRETATION TO EVIDENCE

BENNETT has completed the bounded retrospective assessment.
The following pages switch ownership to the preserved Identifyn record.



IDENTIFYN PRESERVED SOURCE EVIDENCE

ORIGINAL RECORD BEGINS

Images, source methods, acquisition settings and provenance follow in the locked
Stepdown order. Scientific wording is preserved; missing modalities are not invented.
No separate control record is present in the preserved product record.

PRODUCT-LEVEL STEPDOWN MAP

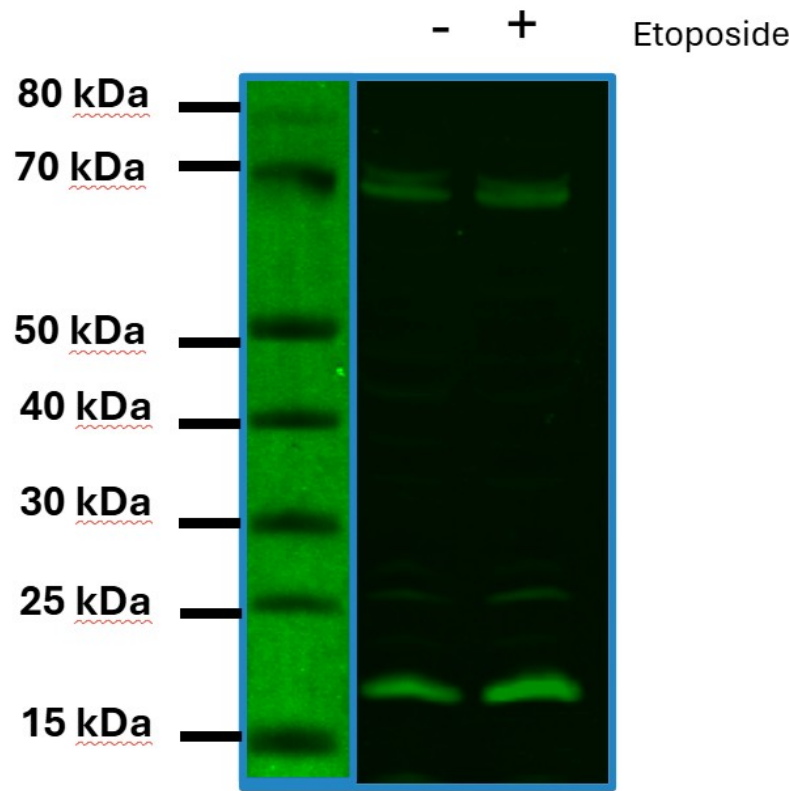
This dossier preserves the complete available evidence progression for DC-000036. Each tier remains tied to its original image, source method, instrument context and cryptographic identity. No separate control record is present in the preserved product record, and none is inferred.

CANONICAL STEPDOWN	EVIDENCE STAGE	INSTRUMENT	STATUS
1	Western blot	Azure Sapphire FL	PRESERVED
2	Widefield	ZEISS Axio Imager.M1	PRESERVED
3	Widefield SIM - Apotome	ZEISS Axio Observer.Z1/7 Apotome	PRESERVED
4	Confocal	ZEISS LSM 980	PRESERVED
5	Airyscan	ZEISS LSM 980	PRESERVED
6	SIM	ZEISS Elyra	PRESERVED
7	SIM ²	ZEISS Elyra	PRESERVED

INTERPRETIVE BOUNDARY

Ordering evidence by resolution tier does not make the modalities interchangeable. Each stage retains its acquisition environment and scientific limitations. This pilot organizes the record; it does not synthesize new biological claims.

ORIGINAL IMAGE EVIDENCE / WESTERN BLOT



EVIDENCE TIER	Western blot
INSTRUMENT	Azure Sapphire FL
CELL MODEL	HeLa
DETECTED DYES	555
METADATA VALUES	11

STRUCTURED ACQUISITION METADATA / WESTERN BLOT

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Scanner	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Detection mode	Fluorescence
Laser	532nm
Emission Filter	572±28nm
Detector	PMT
Intensity	2
Pixel size	100 µm
Scan Speed	High
Quality	High
Focus	Membrane (0 µm in Z-axis)
Image	K. Small

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7)
Cat ID# - DC 000036

Expected Molecular Weight: 15 kDa

HeLa cells were damaged with 200 µM etoposide for 24 hours. Cells were then homogenized using an ultrasonication probe and RIPA lysis buffer, and then centrifuged to pellet the cell debris. The lysate supernatant (100 µg) was mixed with NuPAGE™ LDS Sample Buffer (4X), heated at 80 °C for 10 min, and subjected to electrophoresis using NuPAGE™ 4 to 12% Bis-Tris, 1.0-1.5 mm, Mini Protein Gels in a Mini Gel Tank The Thermo Fisher's PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, was used as a molecular weight marker.

Post electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot™ 3 Starter Kit, PVDF. The transfer membrane was blocked with Blocker™ Casein for 1 hour and then washed five times with PBS. The blocked membrane was incubated with Mouse anti-Human H2AX Monoclonal - Identifyn® SRM Alexa Fluor™ 555 - Direct Conjugate for 2 hours, followed by five washes in 1X PBS. The membrane was dried for 10 minutes at room temperature before imaging.

Scanner = Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)

Scan Settings

Detection mode = Fluorescence

Laser = 532nm

Emission Filter = 572±28nm

Detector = PMT

Intensity = 2

Pixel size = 100 µm

Scan Speed = High

Quality = High

Focus = Membrane (0 µm in Z-axis)

Post Processing

None

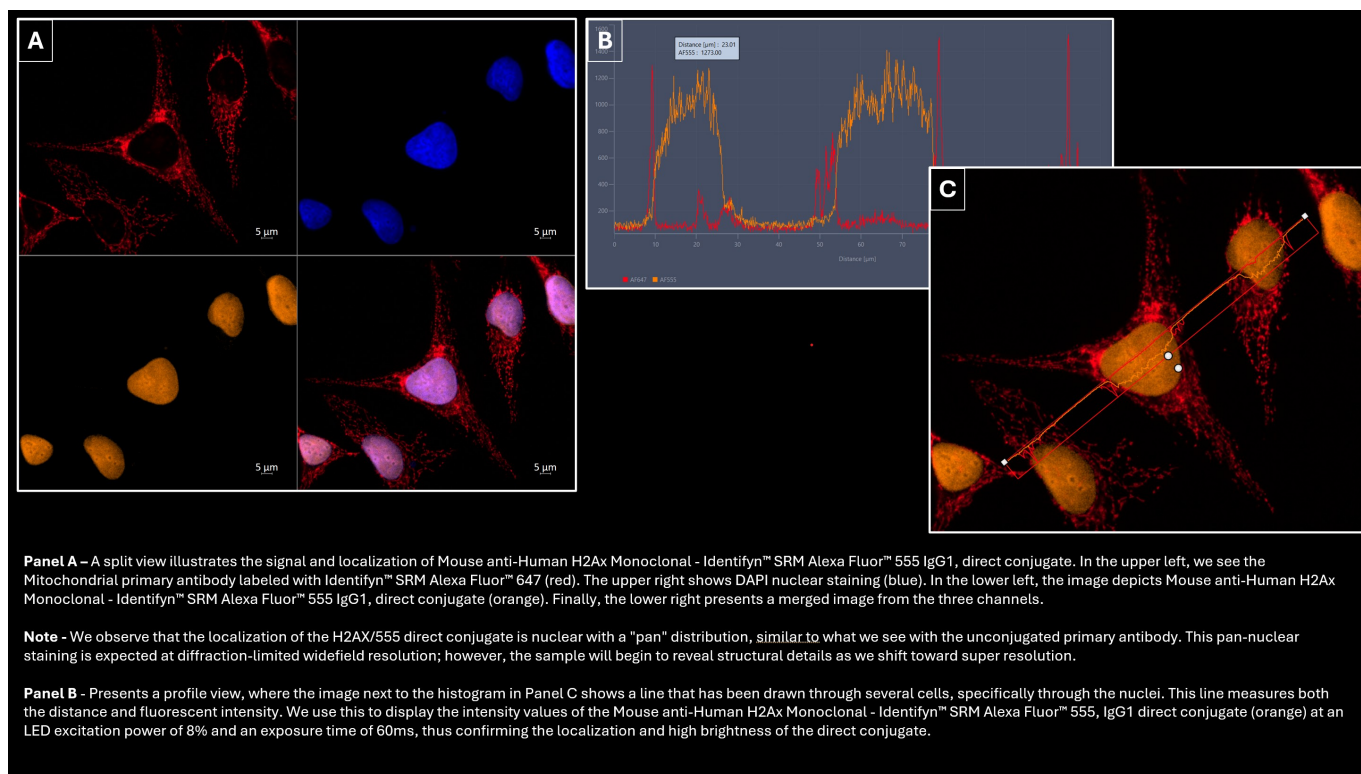
Image: K. Small

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SOURCE PROVENANCE

Catalog	DC-000036
Stage	Western blot
Instrument	Azure Sapphire FL
Microscope configuration	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Structured source metadata values	11
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	460051435699E049B90CB02C393D997CFC3235B826FFFBEB1320635172BCF6CA
Method PDF SHA-256	818D89896C7E5F214943AC8A9277664FCE9F7DC83FC0121CA0FB8CA77C9EF1F7

ORIGINAL IMAGE EVIDENCE / WIDEFIELD



EVIDENCE TIER	Widefield
INSTRUMENT	ZEISS Axio Imager.M1
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 555; 647
METADATA VALUES	40

STRUCTURED ACQUISITION METADATA / WIDEFIELD

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63x/1.40 Oil M27
Channels	3
Scaling (Per Pixel)	0.110 μ m X 0.110 μ m
Image size (Pixels)	1216 X 1028
Image Size (Scaled)	133.18 μ m X 112.59 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
Reflector	AF532-49014 50 Cy 5 49 DAPI
Beam Splitter	550 660 395
Filter Excitation Wavelength	515 - 545 625 - 655 335 - 383
Filter Emission Wavelength	555 - 595 665 - 715 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @8.00% X-Cite Xylis Lamp Intensity @6.00% X-Cite Xylis Lamp Intensity @15.00%
Exposure Time	60 ms 50 ms 20 ms
Excitation Wavelength	553 653 353
Emission Wavelength	568 668 465
Effective NA	1.4
Imaging Device	Axiocam 705
Camera Adapter	1X
Depth of Focus	0.88 μ m 1.03 μ m 0.72 μ m
Binning Mode	2,2
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 118.3), Y (Min 28 and Max 1601)

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7)

Cat ID# - DC 000036

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L)

Cat ID# - SA 000057

H2AX, a variant of the histone protein H2A, has a primary function that relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ -H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ -H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ -H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ -H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

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, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody followed this, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), which 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-Rabbit, IgG (H + L) was incubated for 1 hour at room temperature at a dilution of

1:100 . Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:

Single Plane (2D)

Channels = 3

Scaling (Per Pixel) = 0.110 μm X 0.110 μm

Image size (Pixels) = 1216 X 1028

Image Size (Scaled) = 133.18 μm X 112.59 μm

Bit Depth = 14 Bit

Image Center Position = X: 0.00 μm , Y: 0.00 μm

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

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 @8.00%

Exposure Time = 60 ms

Excitation Wavelength = 553

Emission Wavelength = 568

Effective NA = 1.4

Imaging Device = Axiocam 705

Camera Adapter = 1X

Depth of Focus = 0.88 μm

Binning Mode = 2,2

647 -

Reflector = 50 Cy 5
 Beam Splitter = 660
 Filter Excitation Wavelength = 625 - 655
 Filter Emission Wavelength = 665 - 715
 Contrast Method = Fluorescence
 Light source = X-Cite Xylis Lamp Intensity @6.00%
 Exposure Time = 50 ms
 Excitation Wavelength = 653
 Emission Wavelength = 668
 Effective NA = 1.4
 Imaging Device = Axiocam 705
 Camera Adapter = 1X
 Depth of Focus = 1.03 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 @15.00%
 Exposure Time = 20 ms
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.4
 Imaging Device = Axiocam 705
 Camera Adapter = 1X
 Depth of Focus = 0.72 μ m
 Binning Mode = 2,2

Split View - Panel A

The top-left panel features Mitochondria visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the top-right panel shows DAPI nuclear staining, the bottom-left panel features Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), and the bottom-right panel features the merged image of all channels.

Profile View Display - Panel B & C

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 118.3), Y (Min 28 and Max 1601)

Summary

Panel A - A split view illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal -Identifyn® SRM Alexa Fluor™ 555 IgG1, direct conjugate. In the upper left, we see the Mitochondrial primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red). The upper right shows DAPI nuclear staining (blue). In the lower left, the image depicts Mouse anti-Human H2Ax Monoclonal -Identifyn® SRM Alexa Fluor™ 555 IgG1, direct conjugate

(orange). Finally, the lower right presents a merged image from the three channels.

Note - We observe that the localization of the H2AX/555 direct conjugate is nuclear with a "pan" distribution, similar to what we see with the unconjugated primary antibody. This pan-nuclear staining is expected at diffraction-limited widefield resolution; however, the sample will begin to reveal structural details as we shift toward super resolution.

Panel B - Presents a profile view, where the image next to the histogram in Panel C shows a line that has been drawn through several cells, specifically through the nuclei. This line measures both the distance and fluorescent intensity. We use this to display the intensity values of the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate (orange) at an LED excitation power of 8% and an exposure time of 60ms, thus confirming the localization and high brightness of the direct conjugate.

Image Correction

None

Image by E.F. Simon

Image Analysis by B.T. Bennett

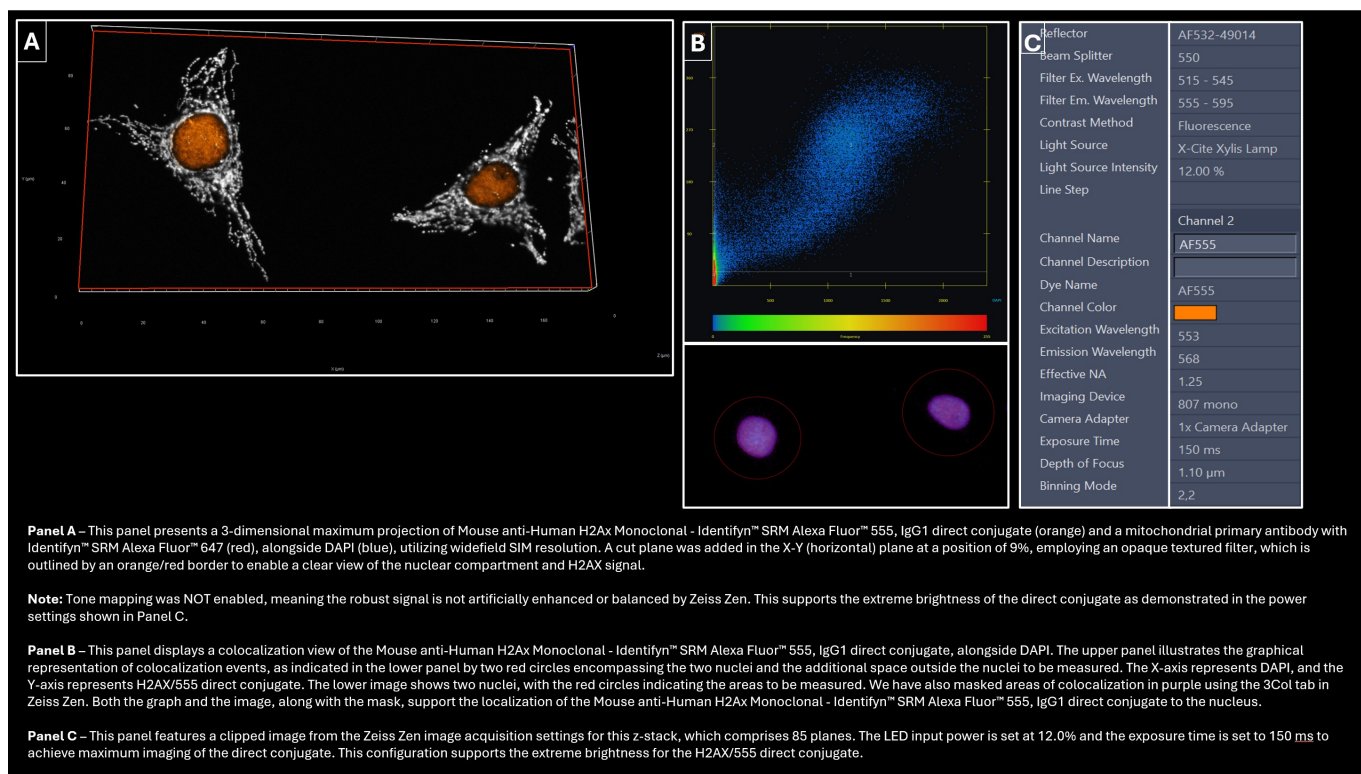
Data Presentation by P.D. Amistoso

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SOURCE PROVENANCE

Catalog	DC-000036
Stage	Widefield
Instrument	ZEISS Axio Imager.M1
Microscope configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylys Lamp, was used with the following parameters for each dye:
Structured source metadata values	40
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	99FFF7C3C87429CFD363DABF388275F3D8C19C55029D1087549F9CD7281199D0
Method PDF SHA-256	FBC935437AC92816CFE45C79D44D8F2A5AE6E9051FEA3973B46097B187A6E6E8

ORIGINAL IMAGE EVIDENCE / WIDEFIELD SIM - APOTOME



EVIDENCE TIER	Widefield SIM - Apotome
INSTRUMENT	ZEISS Axio Observer.Z1/7 Apotome
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 555; 647
METADATA VALUES	49

STRUCTURED ACQUISITION METADATA / WIDEFIELD SIM - APOTOME

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	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:
Objective	EC Plan-Neofluar 63X/1.25 oil M27
Z-Stack	85 Slices (8.4 µm)
Channels	3
Scaling (Per Pixel)	0.143 µm X 0.143 µm X 0.100 µm
Image Size (Pixels)	1221 X 641
Image Size (Scaled)	174.43 µm X 91.57 µm
Bit Depth	14 Bit
Image Center Position	X: 44.19 mm, Y: 49.01 mm
Stage Position	X: 44.19 mm, Y: 49.01 mm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflector	AF532-49014 50 Cy 5 49 DAPI
Beam Splitter	550 660 395
Filter Excitation Wavelength	515 - 545 625 - 655 335 - 383
Filter Emission Wavelength	555 - 595 665 - 715 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @12.00% X-Cite Xylis Lamp Intensity @6.00%
Exposure Time	150 ms 40 ms 20 ms
Excitation Wavelength	553 653 353
Emission Wavelength	568 668 465
Effective NA	1.25
Imaging Device	807 mono
Camera Adapter	1X
Depth of Focus	1.10 µm 1.30 µm 0.90 µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mod" "Optical Sectioning" - enabled correction selected.
3D Maximum Projection	Precise
Appearance	Threshold 13% all channels, Ramp 0% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Tone Mapping was NOT 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)
Z-Position	43

FIELD	SOURCE-DOCUMENTED VALUE
3Col Mask	Purple
Note	Tone mapping was NOT enabled, meaning the robust signal is not artificially enhanced or balanced by Zeiss Zen. This supports the extreme brightness of the direct conjugate as demonstrated in the power settings shown in Panel C.

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7)

Cat ID# - DC 000036

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L)

Cat ID# - SA 000057

H2AX, a variant of the histone protein H2A, has a primary function that relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ -H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ -H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ -H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ -H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

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, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody followed this, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), which 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-Rabbit, IgG (H + L) 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.

Control Data - Primary Unconjugated Antibody

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)- Panel A

Z-Stack = 85 Slices (8.4 µm)

Channels = 3

Scaling (Per Pixel) = 0.143 µm X 0.143 µm X 0.100 µm

Image Size (Pixels) = 1221 X 641

Image Size (Scaled) = 174.43 µm X 91.57 µm

Bit Depth = 14 Bit

Image Center Position = X: 44.19 mm, Y: 49.01 mm

Stage Position = X: 44.19 mm, Y: 49.01 mm

ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

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 @12.00%

Exposure Time = 150 ms

Excitation Wavelength = 553

Emission Wavelength = 568

Effective NA = 1.25

Imaging Device = 807 mono

Camera Adapter = 1X

Depth of Focus = 1.10 µm

Binning Mode = 2,2

647 -

Reflector = 50 Cy 5
 Beam Splitter = 660
 Filter Excitation Wavelength = 625 - 655
 Filter Emission Wavelength = 665 - 715
 Contrast Method = Fluorescence
 Light source = X-Cite Xylis Lamp Intensity @6.00%
 Exposure Time = 40 ms
 Excitation Wavelength = 653
 Emission Wavelength = 668
 Effective NA = 1.25
 Imaging Device = 807 mono
 Camera Adapter = 1X
 Depth of Focus = 1.30 μm
 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 @6.00%
 Exposure Time = 20 ms
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.25
 Imaging Device = 807 mono
 Camera Adapter = 1X
 Depth of Focus = 0.90 μm
 Binning Mode = 2,2

Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mod" "Optical Sectioning" - enabled correction selected.
 Normalization, Phase Corrector, Fourier Filter, and Deconvolution were NOT used.

3D Image Processing - Panel A

3D Maximum Projection = Precise
 Appearance = Threshold 13% all channels, Ramp 0% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, Tone Mapping was NOT 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)

Colocalization View - Panel B

Z-Position = 43

Horizontal Axis - DAPI, Threshold 21, Range Auto

Vertical Axis - AF555, Threshold 24, Range Auto

3Col Mask = Purple

Summary

Panel A - This panel presents a 3-dimensional maximum projection of Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate (orange) and a mitochondrial primary antibody with Identifyn® SRM Alexa Fluor™ 647 (red), alongside DAPI (blue), utilizing widefield SIM resolution. A cut plane was added in the X-Y (horizontal) plane at a position of 9%, employing an opaque textured filter, which is outlined by an orange/red border to enable a clear view of the nuclear compartment and H2AX signal.

Note: Tone mapping was NOT enabled, meaning the robust signal is not artificially enhanced or balanced by Zeiss Zen. This supports the extreme brightness of the direct conjugate as demonstrated in the power settings shown in Panel C.

Panel B - This panel displays a colocalization view of the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate, alongside DAPI. The upper panel illustrates the graphical representation of colocalization events, as indicated in the lower panel by two red circles encompassing the two nuclei and the additional space outside the nuclei to be measured. The X-axis represents DAPI, and the Y-axis represents H2AX/555 direct conjugate. The lower image shows two nuclei, with the red circles indicating the areas to be measured. We have also masked areas of colocalization in purple using the 3Col tab in Zeiss Zen. Both the graph and the image, along with the mask, support the localization of the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate to the nucleus.

Panel C - This panel features a clipped image from the Zeiss Zen image acquisition settings for this z-stack, which comprises 85 planes. The LED input power is set at 12.0% and the exposure time is set to 150 ms to achieve maximum imaging of the direct conjugate. This configuration supports the extreme brightness for the H2AX/555 direct conjugate.

Image Correction

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast between the 555/647 channels.

Image by E.F. Simon

Image Analysis by B.T. Bennett

Data Presentation by P.D. Amistoso

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SOURCE PROVENANCE

Catalog	DC-000036
Stage	Widefield SIM - Apotome
Instrument	ZEISS Axio Observer.Z1/7 Apotome

SOURCE PROVENANCE

Microscope configuration	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:
Structured source metadata values	49
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	6EC235922892FE525179670B1736BCD811A4B0152F25FE82DF6380983743D9D4
Method PDF SHA-256	4A3DF6BC1BDEEF3CE3F86D84855EDFF65BE2BAD603A5300F712E0B921D5AC407

STRUCTURED ACQUISITION METADATA / CONFOCAL

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	121 Slices (3.6 μm)
Channels	3
Scaling (Per Pixel)	0.055 μm X 0.055 μm X 0.030 μm
Image Size (Pixels)	625 X 563
Image Size (Scaled)	34.32 μm X 30.91 μm
Bit Depth	16 Bit
Image Center Position	X: 86.42 mm, Y: 49.59 mm
Stage Position	X: 86.42 mm, Y: 49.59 mm
ROI Center Offset	X: 0.00 μm , Y: 0.00 μm
Reflector	None
Contrast Method	Fluorescence
Pinhole	1.00 AU / 55 μm 1.00 AU / 49 μm 0.72 AU / 49 μm
LSM MBS	MBS 488/561, 405/730
LSM SBS	Mirror
Laser Wavelength	561 nm @0.3% 488 nm @0.03% 405 nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.2
Rotation	0°
Sampling	1.29
Pixel Time	0.31 μs
Frame Time	3.60 s
LSM Scan Speed	12
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	553 493 353
Emission Wavelength	568 517 465
Effective NA	1.4
Detection Wavelength	550-600 480-549 410-470
Imaging Device	GaAsP-Pmt3

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT
Detector Gain	550 V 500 V
Detector Offset	0
Detector Digital Gain	1.0
LSM Plus Mode	7.4 (3D, Auto) 8.0 (3D, Auto) 6.5 (3D, Auto)
3D Maximum Projection	Precise
Appearance	Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, 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)
Clipping Image Process	Clipping planes are introduced to pronounce regions of special interest.
X-Y	Active was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip	-11%
Clip X	0°
Clip Y	0°

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7)

Cat ID# - DC 000036

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L)

Cat ID# - SA 000013

H2AX, a variant of the histone protein H2A, has a primary function that relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ -H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ -H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ -H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ -H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

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, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Golgi GOLPH2 Polyclonal Antibody, was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat

Anti-Rabbit, IgG (H + L) was incubated for 1 hour at room temperature at a dilution of 1:100 . Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:

Single Plane (2D) - Z-plane 57 of 121 total - Panel A

Z Stack - (3D) - Panel A & B

Z-Stack = 121 Slices (3.6 µm)

Channels = 3

Scaling (Per Pixel) = 0.055 µm X 0.055 µm X 0.030 µm

Image Size (Pixels) = 625 X 563

Image Size (Scaled) = 34.32 µm X 30.91 µm

Bit Depth = 16 Bit

Image Center Position = X: 86.42 mm, Y: 49.59 mm

Stage Position = X: 86.42 mm, Y: 49.59 mm

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

555 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 1.00 AU / 55 µm
 LSM MBS = MBS 488/561, 405/730
 LSM SBS = Mirror
 Laser Wavelength = 561 nm @0.3%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.2
 Rotation = 0°
 Sampling = 1.29
 Pixel Time = 0.31 µs
 Frame Time = 3.60 s
 LSM Scan Speed = 12
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 8
 Excitation Wavelength = 553
 Emission Wavelength = 568
 Effective NA = 1.4
 Detection Wavelength = 550-600
 Imaging Device = GaAsP-Pmt3
 Detector Type = GaAsP-PMT
 Detector Gain = 550 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 7.4 (3D, Auto)

488 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 1.00 AU / 49 µm
 LSM MBS = MBS 488/561, 405/730
 LSM SBS = Mirror
 Laser Wavelength = 488 nm @0.03%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.2
 Rotation = 0°
 Sampling = 1.29
 Pixel Time = 0.31 µs
 Frame Time = 3.60 s
 LSM Scan Speed = 12
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 8
 Excitation Wavelength = 493
 Emission Wavelength = 517
 Effective NA = 1.4
 Detection Wavelength = 480-549
 Imaging Device = GaAsP-Pmt3
 Detector Type = GaAsP-PMT
 Detector Gain = 550 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 8.0 (3D, Auto)

DAPI -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 0.72 AU / 49 μ m
 LSM MBS = MBS 488/561, 405/730
 LSM SBS = Mirror
 Laser Wavelength = 405 nm @0.2%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.2
 Rotation = 0°
 Sampling = 1.29
 Pixel Time = 0.31 μ s
 Frame Time = 3.60 s
 LSM Scan Speed = 12
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 8
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.4
 Detection Wavelength = 410-470
 Imaging Device = GaAsP-Pmt3
 Detector Type = GaAsP-PMT
 Detector Gain = 500 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 6.5 (3D, Auto)

Split View - Panel A

The top-left panel shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), the top-right panel shows Golgi visualized with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the bottom-left panel features DAPI nuclear staining, and the bottom-right panel is the merged image of all channels. This split-view image was taken at Z-Position 57 of 121.

3D Image Processing - Panel B

3D Maximum Projection = Precise
 Appearance = Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, 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)
 Clipping Image Process = Clipping planes are introduced to pronounce regions of special interest.
 X-Y = Active was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -11%

Clip X = 0°

Clip Y = 0°

Summary

Panel A - Split View. This panel illustrates the signal and localization of the Mouse anti-Human H2AX Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate. The upper left section displays the H2AX direct conjugate (orange), while the upper right shows the Golgi primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green). The lower left features DAPI nuclear staining (blue), and the lower right presents a merged image of all three channels.

Panel B - This panel presents a three-dimensional maximum projection of the Mouse anti-Human H2AX Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate (orange) and the Golgi primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green) at confocal resolution. The image uses a cut plane on the X-Y axis (blue outline), and both the X and Y angles are set to 0 degrees (flat), while the Z angle is set to -11%.

Panel C - This panel features a clipped image from the Zeiss Zen image acquisition settings for a z-stack comprising 121 planes. The 561 nm laser power is set at 0.3% to achieve optimal imaging of the direct conjugate over a z-stack distance of 3.6 microns. We also note the Identifyn® SRM Alexa Fluor™ 488 required 0.03%, indicating high brightness for all Identifyn® products via our patent-pending conjugation chemistry.

Both images demonstrate the alignment of the nucleolus with the H2AX 555 direct conjugate and the DAPI staining, as expected, since this compartment is generally considered devoid of chromatin material. Consequently, neither DAPI nor H2AX occupies this space, resulting in a negative colocalization of the signal. This confirms the proper localization of the H2AX/555 direct conjugate signal.

Image Corrections

None

Image by J.K. Bennett

Image Analysis by B.T. Bennett

Data Presentation by P.D. Amistoso

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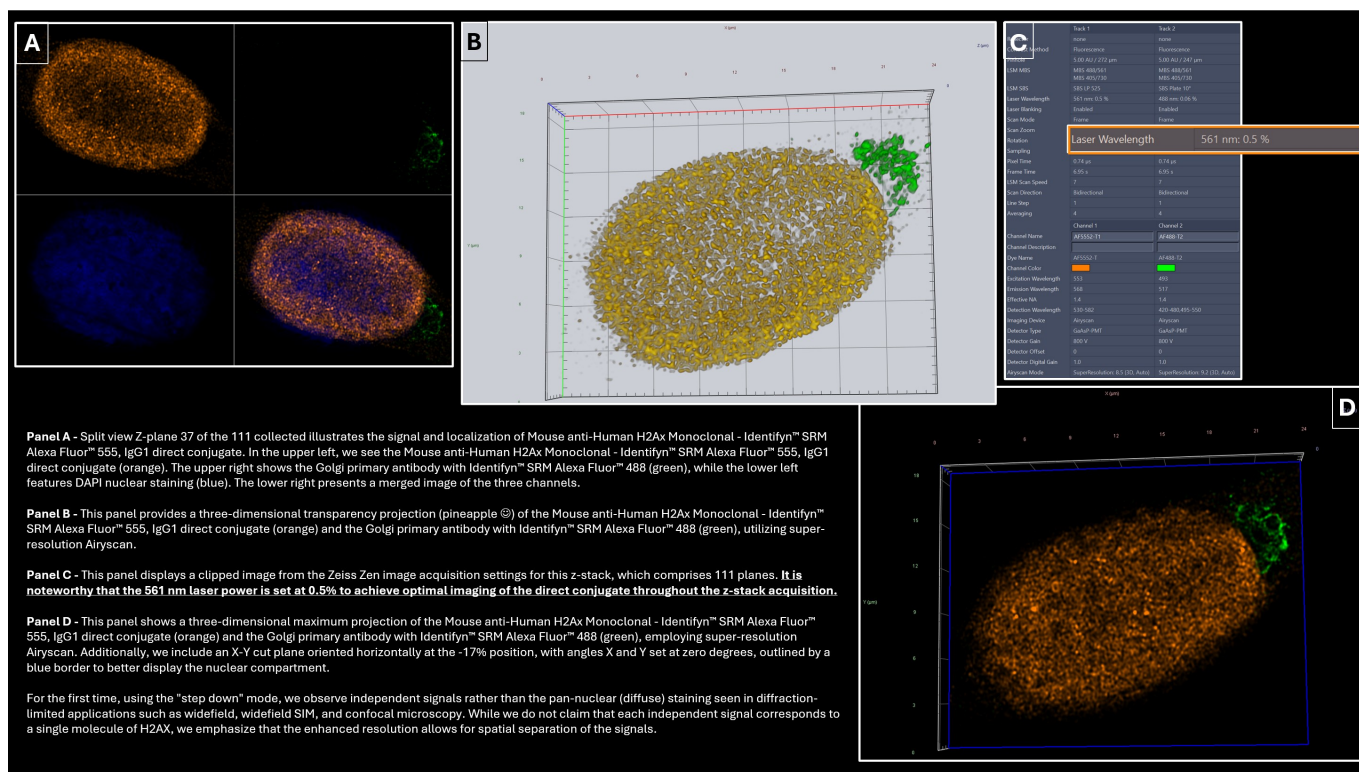
SOURCE PROVENANCE

Catalog	DC-000036
Stage	Confocal
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Structured source metadata values	59
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT

SOURCE PROVENANCE

Cell model	HeLa
Image SHA-256	D18F0982C3C6735BD5C9F81A26A52CBD7FCC34EADC18D5B1175AB65883D459E7
Method PDF SHA-256	14BAADC744E59E48E1632A22FA421ABDD388FA88A6D51D179D1024E0D64C5FD8

ORIGINAL IMAGE EVIDENCE / AIRYSCAN



EVIDENCE TIER	Airyscan
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555; 750
METADATA VALUES	63

STRUCTURED ACQUISITION METADATA / AIRYSCAN

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	111 Slices (3.3 μm)
Channels	3
Scaling (Per Pixel)	0.03530 μm X 0.03530 μm X 0.03000 μm
Image Size (Pixels)	683 X 536
Image Size (Scaled)	24.11 μm X 18.92 μm
Bit Depth	16 Bit
Image Center Position	X: 86.41 mm, Y: 48.00 mm
Stage Position	X: 86.41 mm, Y: 48.00 mm
ROI Center Offset	X: 0.00 μm , Y: 0.00 μm
Reflector	None
Contrast Method	Fluorescence
Pinhole	5.00 AU / 272 μm 5.00 AU / 247 μm 5.46 AU / 235 μm
LSM MBS	MBS 488/561, 405/730
LSM SBS	SBS LP 525 SBS Plate 10° SBS SP 505
Laser Wavelength	561 nm @0.5% 488 nm @0.06% 405 nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.7
Rotation	0°
Sampling	2.00
Pixel Time	0.74 μs
Frame Time	6.95 s
LSM Scan Speed	7
Scan Direction	Bidirectional
Line Step	1
Averaging	4
Excitation Wavelength	553 493 353
Emission Wavelength	568 517 465
Effective NA	1.4
Detection Wavelength	530-582 420-480, 495-550 350-499
Imaging Device	Airyscan

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT
Detector Gain	800 V 750 V
Detector Offset	0
Detector Digital Gain	1.0
Airyscan Mode	SuperResolution 8.5 (3D, Auto) SuperResolution 9.2 (3D, Auto) SuperResolution 7.7 (3D, Auto)
3D Transparency Projection	Precise
Appearance	Threshold 2% all channels, Ramp 20% all channels, Maximum 100% all channels. Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels.
Background	Black
Light	Brightness 100%, 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)
3D Maximum Projection	Precise
Clipping Image Process	Clipping planes are introduced to pronounce regions of special interest.
X-Y	Active was selected, textured opaque was chosen to remove a horizontal portion of the upper Z-Stack of the cell from the image, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip	-17%
Clip X	0°
Clip Y	0°

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7)

Cat ID# - DC 000036

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L)

Cat ID# - SA 000013

H2AX, a variant of the histone protein H2A, has a primary function that relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ -H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ -H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ -H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ -H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

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, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Golgi GOLPH2 Polyclonal Antibody, was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat

Anti-Rabbit, IgG (H + L) 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 DAP, I cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:

Single Plane (2D) - Z-plane 37 of 111 total - Panel A

Z Stack - (3D)- Panel A & B

Z-Stack = 111 Slices (3.3 µm)

Channels = 3

Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm

Image Size (Pixels) = 683 X 536

Image Size (Scaled) = 24.11 µm X 18.92 µm

Bit Depth = 16 Bit

Image Center Position = X: 86.41 mm, Y: 48.00 mm

Stage Position = X: 86.41 mm, Y: 48.00 mm

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

555 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 5.00 AU / 272 μ m
 LSM MBS = MBS 488/561, 405/730
 LSM SBS = SBS LP 525
 Laser Wavelength = 561 nm @0.5%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.7
 Rotation = 0°
 Sampling = 2.00
 Pixel Time = 0.74 μ s
 Frame Time = 6.95 s
 LSM Scan Speed = 7
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 4
 Excitation Wavelength = 553
 Emission Wavelength = 568
 Effective NA = 1.4
 Detection Wavelength = 530-582
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 800 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 Airyscan Mode = SuperResolution 8.5 (3D, Auto)

488 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 5.00 AU / 247 μ m
 LSM MBS = MBS 488/561, 405/730
 LSM SBS = SBS Plate 10°
 Laser Wavelength = 488 nm @0.06%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.7
 Rotation = 0°
 Sampling = 2.00
 Pixel Time = 0.74 μ s
 Frame Time = 6.95 s
 LSM Scan Speed = 7
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 4
 Excitation Wavelength = 493
 Emission Wavelength = 517
 Effective NA = 1.4
 Detection Wavelength = 420-480, 495-550
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 800 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 Airyscan Mode = SuperResolution 9.2 (3D, Auto)

DAPI -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 5.46 AU / 235 μ m
 LSM MBS = MBS 488/561, 405/730
 LSM SBS = SBS SP 505
 Laser Wavelength = 405 nm @0.2%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.7
 Rotation = 0°
 Sampling = 2.00
 Pixel Time = 0.74 μ s
 Frame Time = 6.95 s
 LSM Scan Speed = 7
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 4
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.4
 Detection Wavelength = 350-499
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 750 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 Airyscan Mode = SuperResolution 7.7 (3D, Auto)

Post Processing - AiryScan 3D Processing

Display Mode "SR"
 Super Resolution Auto Filter Selected
 Process 3D Current Image
 Created

Split View - Panel A

The top-left panel shows the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), the top-right panel shows Golgi visualized with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the bottom-left panel features DAPI nuclear staining, and the bottom-right panel is the merged image of all channels. This split-view image was taken at Z-Position 37 of 111.

3D Image Processing - Panel B

3D Transparency Projection = Precise
 Appearance = Threshold 2% all channels, Ramp 20% all channels, Maximum 100% all channels.
 Background = Black
 Light = Brightness 100%, 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)

3D Image Processing - Panel D

3D Maximum Projection = Precise

Appearance = Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels.

Background = Black

Light = Brightness 100%, 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)

Clipping Image Process = Clipping planes are introduced to pronounce regions of special interest.

X-Y = Active was selected, textured opaque was chosen to remove a horizontal portion of the upper Z-Stack of the cell from the image, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -17%

Clip X = 0°

Clip Y = 0°

Summary

Panel A - Split view Z-plane 37 of the 111 collected illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal -Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate. In the upper left, we see the Mouse anti-Human H2Ax Monoclonal - Identifyn™SRM Alexa Fluor™ 555, IgG1 direct conjugate (orange). The upper right shows the Golgi primary antibody with Identifyn® SRM AlexaFluor™ 488 (green), while the lower left features DAPI nuclear staining (blue). The lower right presents a merged image of the three channels.

Panel B - This panel provides a three-dimensional transparency projection of the Mouse anti-Human H2Ax Monoclonal - Identifyn™SRM Alexa Fluor™ 555, IgG1 direct conjugate (orange) and the Golgi primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), utilizing super-resolution Airyscan.

Panel C - This panel displays a clipped image from the Zeiss Zen image acquisition settings for this z-stack, which comprises 111 planes. It is noteworthy that the 561 nm laser power is set at 0.5% to achieve optimal imaging of the direct conjugate throughout the z-stack acquisition.

Panel D - This panel shows a three-dimensional maximum projection of the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRMAlexa Fluor™ 555, IgG1 direct conjugate (orange) and the Golgi primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), employing super-resolution Airyscan. Additionally, we include an X-Y cut plane oriented horizontally at the -17% position, with angles X and Y set at zero degrees, outlined by a blue border to better display the nuclear compartment.

For the first time, using the "step down" mode, we observe independent signals rather than the pan-nuclear (diffuse) staining seen in diffraction-limited applications such as widefield, widefield SIM, and confocal microscopy While we do not claim that each independent signal corresponds to a single molecule of H2AX, we emphasize that the enhanced resolution allows for spatial separation of the signals.

Image Corrections

None

Image by J.K. Bennett

Image Analysis by B.T. Bennett

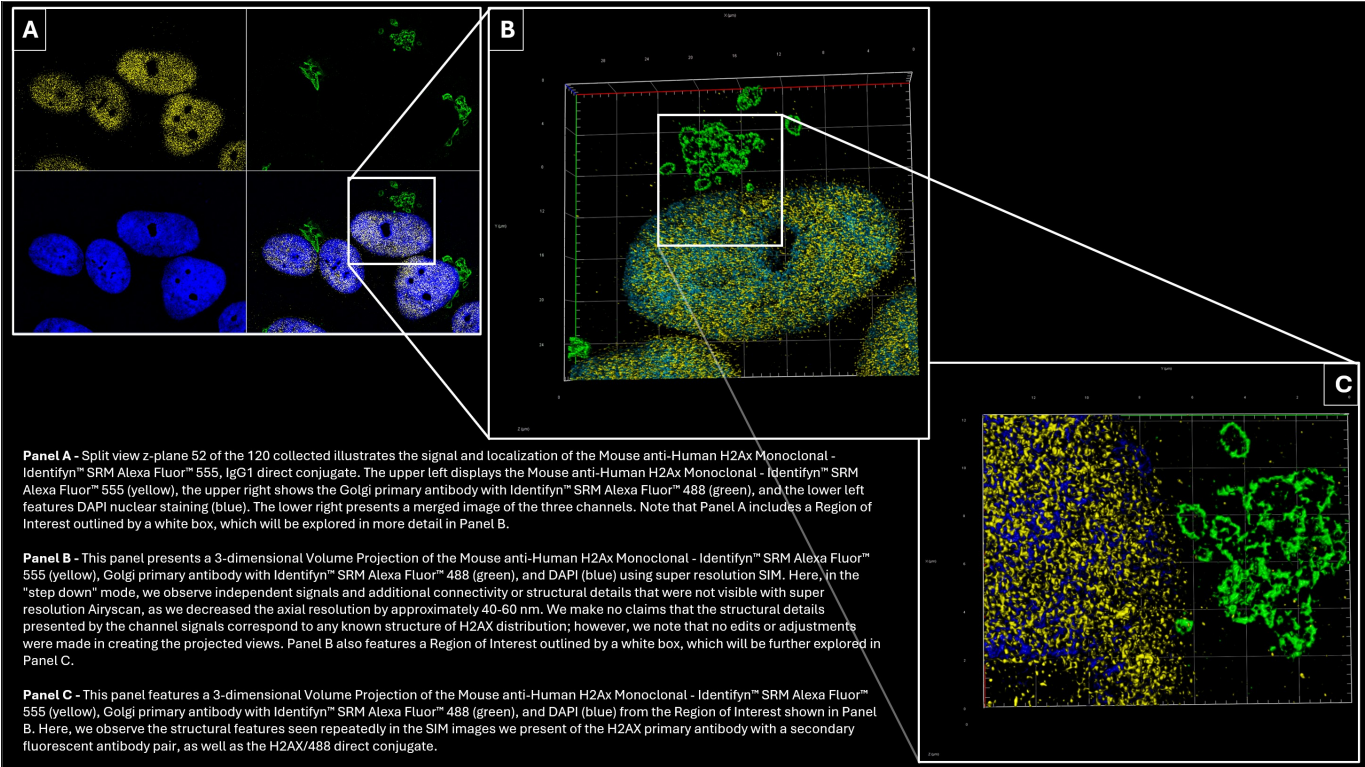
Data Presentation by P.D. Amistoso

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SOURCE PROVENANCE

Catalog	DC-000036
Stage	Airyscan
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Structured source metadata values	63
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	D035BB8C95CC142E85B1EB1C3434FDCAB5710F47A35CC5D879B17C467507EC52
Method PDF SHA-256	62987DDF3D2B24D4983AB356EF3178438391D854EF715EDDBF59F3C051E19BD4

ORIGINAL IMAGE EVIDENCE / SIM



EVIDENCE TIER	SIM
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555
METADATA VALUES	66

STRUCTURED ACQUISITION METADATA / SIM

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	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:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	120 Slices (3.57 μm) 81 Slices (2.4 μm)
Channels	3
Scaling (Per Pixel)	0.02111 μm X 0.02111 μm X 0.03000 μm
Image Size (Pixels)	3453 X 2390 1514 X 1294 580 X 659
Image Size (Scaled)	72.90 μm X 50.46 μm 31.56 μm X 27.32 μm 12.25 μm X 13.91 μm
Bit Depth	16 Bit
Image Center Position	X: 35.56 mm, Y: 42.58 mm
Stage Position	X: 35.56 mm, Y: 42.58 mm
ROI Center Offset	X: 0.00 μm , Y: 0.00 μm
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	561 488 405
Channel Name	T1-Axiocam 820 #2-SR T2-Axiocam 820-SR T3-Axiocam 820 #2-SR
Excitation Wavelength	561 488 405
Emission Wavelength	597 525 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800 419-470, 503-546, 576-617, 657-800
Imaging Device	Axiocam 820 #2 Axiocam 820
Camera Adapter	1X
Exposure Time	150 ms 120 ms 50 ms
Depth of Focus	0.92 μm 0.81 μm 0.69 μm
Binning Mode	2,2
Laser Wavelength	561 nm @4.00% 488 nm @1.50% 405 nm @1.50%
Frame Time	1.98 s 1.59 s 676.00 ms
Scan Direction	Unidirectional
Grating	36.5 μm grating
Result Sampling	4
Process Sampling	N/A

FIELD	SOURCE-DOCUMENTED VALUE
Algorithm	SIM
SIM Settings	Standard
Auto Sharpness	On
Sharpness	11.0768 (555), 11.0661 (488), 9.4255 (DAPI)
Fitting	Fast Fit
Normalization	Auto
3D Volume Projection	Precise
Appearance	Threshold 2% all channels, 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) View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7)

Cat ID# - DC 000036

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L)

Cat ID# - SA 000013

H2AX, a variant of the histone protein H2A, has a primary function that relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ -H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ -H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ -H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ -H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

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, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Golgi GOLPH2 Polyclonal Antibody, was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat

Anti-Rabbit, IgG (H + L) 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.

Control Data - Primary Unconjugated Antibody

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 52 of 120 total - Panel A

Z-Stack = 120 Slices (3.57 µm)

Channels = 3

Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm

Image Size (Pixels) = 3453 X 2390

Image Size (Scaled) = 72.90 µm X 50.46 µm

Bit Depth = 16 Bit

Image Center Position = X: 35.56 mm, Y: 42.58 mm

Stage Position = X: 35.56 mm, Y: 42.58 mm

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

Z Stack - (3D) - Panel B

Z-Stack = 120 Slices (3.57 µm)

Channels = 3

Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm

Image Size (Pixels) = 1514 X 1294

Image Size (Scaled) = 31.56 µm X 27.32 µm

Bit Depth = 16 Bit

Image Center Position = X: 35.56 mm, Y: 42.58 mm

Stage Position = X: 35.56 mm, Y: 42.58 mm

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

Z Stack - (3D) - Panel C

Z-Stack = 81 Slices (2.4 µm)

Channels = 3

Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm

Image Size (Pixels) = 580 X 659

Image Size (Scaled) = 12.25 µm X 13.91 µm

Bit Depth = 16 Bit

Image Center Position = X: 35.56 mm, Y: 42.58 mm

Stage Position = X: 35.56 mm, Y: 42.58 mm

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

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 = T1-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 = 150 ms
 Depth of Focus = 0.92 μ m
 Binning Mode = 2,2
 Laser Wavelength = 561 nm @4.00%
 Frame Time = 1.98 s
 Scan Direction = Unidirectional
 Grating = 36.5 μ m grating

488 -

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 = 488
 Channel Name = T2-Axiocam 820-SR
 Excitation Wavelength = 488
 Emission Wavelength = 525
 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 = 0.81 μ m
 Binning Mode = 2,2
 Laser Wavelength = 488 nm @1.50%
 Frame Time = 1.59 s
 Scan Direction = Unidirectional
 Grating = 36.5 μ m 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 μ m
 Binning Mode = 2,2
 Laser Wavelength = 405 nm @1.50%
 Frame Time = 676.00 ms
 Scan Direction = Unidirectional
 Grating = 36.5 μ m grating

Post Processing - SIM Processing

Processing Dimension - 3D
 Result Sampling = 4
 Process Sampling = N/A
 Channels = 3
 Algorithm = SIM
 SIM Settings = Standard
 Auto Sharpness = On
 Sharpness = 11.0768 (555), 11.0661 (488), 9.4255 (DAPI)
 Fitting = Fast Fit
 Normalization = Auto

Split View - Panel A

The top-left panel features Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), the top-right panel shows Golgi visualized with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the bottom-left panel features DAPI nuclear staining, and the bottom-right panel features the merged image of all channels. This image was taken at Z-Position 52 of 120.

3D Image Processing - Panel B

3D Volume Projection = Precise
 Appearance = Threshold 2% all channels, 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)

3D Image Processing - Panel C

3D Volume Projection = Precise

Appearance = Threshold 2% all channels, 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 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A - Split view z-plane 52 of the 120 collected illustrates the signal and localization of the Mouse anti-HumanH2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 direct conjugate. The upper left displays the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555 (yellow), the upper right shows the Golgi primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), and the lower left features DAPI nuclear staining (blue). The lower right presents a merged image of the three channels. Note that Panel A includes a Region of Interest outlined by a white box, which will be explored in more detail in Panel B.

Panel B - This panel presents a 3-dimensional Volume Projection of the Mouse anti-Human H2Ax Monoclonal -Identifyn® SRM Alexa Fluor™ 555 (yellow), Golgi primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), and DAPI (blue) using super resolution SIM. Here, in the "step down" mode, we observe independent signals and additional connectivity or structural details that were not visible with super resolution Airyscan, as we decreased the axial resolution by approximately 40-60 nm. We make no claims that the structural details presented by the channel signals correspond to any known structure of H2AX distribution; however, we note that no edits or adjustments were made in creating the projected views. Panel B also features a Region of Interest outlined by a white box, which will be further explored in Panel C.

Panel C - This panel features a 3-dimensional Volume Projection of the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555 (yellow), Golgi primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), and DAPI (blue) from the Region of Interest shown in Panel B. Here, we observe the structural features seen repeatedly in the SIM images we present of the H2AX primary antibody with a secondary fluorescent antibody pair, as well as the H2AX/488 direct conjugate.

Note that we collapse the Z Scale to 10% to provide a better visual representation of the structural details.

Image Corrections

None

Image by P. Raut

Image Analysis by B.T. Bennett

Data Presentation by P.D. Amistoso

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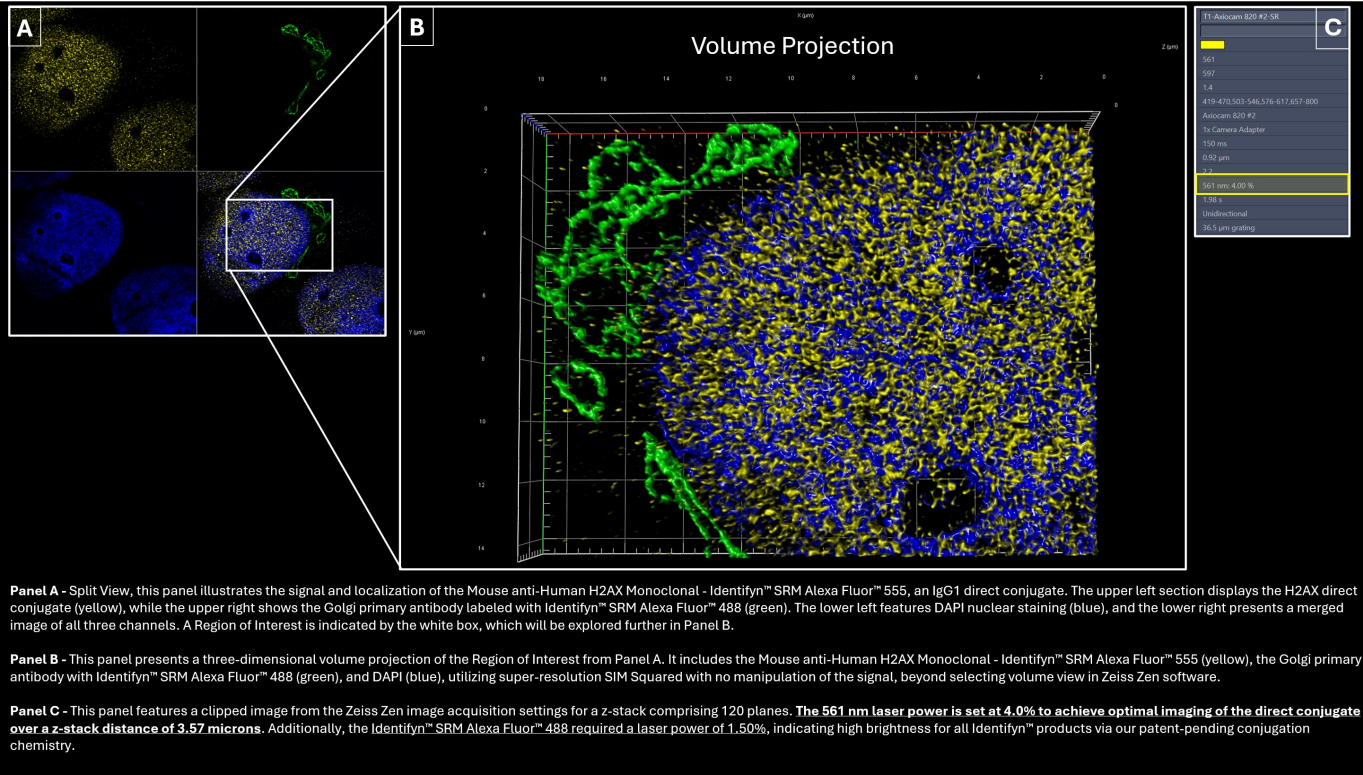
SOURCE PROVENANCE

Catalog	DC-000036
Stage	SIM

SOURCE PROVENANCE

Instrument	ZEISS Elyra
Microscope configuration	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:
Structured source metadata values	66
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	A516301756A98B2F30C7E7A1177667A1E6816CF9FB135414189DF8C3EF65CC21
Method PDF SHA-256	FC73E01195EA1EBD049EAEE7B679C969BD07B806D2E96486C2CF9192BAC31C70

ORIGINAL IMAGE EVIDENCE / SIM²



EVIDENCE TIER	SIM ²
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555
METADATA VALUES	66

STRUCTURED ACQUISITION METADATA / SIM²

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	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:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	120 Slices (3.57 µm)
Channels	3
Scaling (Per Pixel)	0.02111 µm X 0.02111 µm X 0.03000 µm
Image Size (Pixels)	1615 X 1410 891 X 688
Image Size (Scaled)	34.10 µm X 29.77 µm 18.81 µm X 14.53 µm
Bit Depth	16 Bit
Image Center Position	X: 35.56 mm, Y: 42.58 mm
Stage Position	X: 35.56 mm, Y: 42.58 mm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
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	561 488 405
Channel Name	T1-Axiocam 820 #2-SR T2-Axiocam 820-SR T3-Axiocam 820 #2-SR
Excitation Wavelength	561 488 405
Emission Wavelength	597 525 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800 419-470, 503-546, 576-617, 657-800
Imaging Device	Axiocam 820 #2 Axiocam 820
Camera Adapter	1X
Exposure Time	150 ms 120 ms 50 ms
Depth of Focus	0.92 µm 0.81 µm 0.69 µm
Binning Mode	2,2
Laser Wavelength	561 nm @4.00% 488 nm @1.50% 405 nm @3.50%
Frame Time	1.98 s 1.59 s 676.00 ms
Scan Direction	Unidirectional
Grating	36.5 µm grating
Processing Dimension	3D
Result Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
Process Sampling	4
Algorithm	SIM ²
SIM ² Settings	Standard
Input SNR	Low
Regularization	None
Regularization Weight	0.0000
Iterations	3
Filter	No Filter
Fitting	Fast Fit
Normalization	Auto
3D Volume Projection	Precise
Appearance	Threshold 2% all channels, 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 77%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7)

Cat ID# - DC 000036

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L)

Cat ID# - SA 000013

H2AX, a variant of the histone protein H2A, has a primary function that relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ -H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ -H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ -H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ -H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

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, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Golgi GOLPH2 Polyclonal Antibody, was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat

Anti-Rabbit, IgG (H + L) 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.

Control Data - Primary Unconjugated Antibody

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 61 of 120 total - Panel A

Z-Stack = 120 Slices (3.57 µm)

Channels = 3

Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm

Image Size (Pixels) = 1615 X 1410

Image Size (Scaled) = 34.10 µm X 29.77 µm

Bit Depth = 16 Bit

Image Center Position = X: 35.56 mm, Y: 42.58 mm

Stage Position = X: 35.56 mm, Y: 42.58 mm

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

Z Stack - (3D) - Panel B

Z-Stack = 120 Slices (3.57 µm)

Channels = 3

Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm

Image Size (Pixels) = 891 X 688

Image Size (Scaled) = 18.81 µm X 14.53 µm

Bit Depth = 16 Bit

Image Center Position = X: 35.56 mm, Y: 42.58 mm

Stage Position = X: 35.56 mm, Y: 42.58 mm

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

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 = T1-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 = 150 ms
 Depth of Focus = 0.92 μ m
 Binning Mode = 2,2
 Laser Wavelength = 561 nm @4.00%
 Frame Time = 1.98 s
 Scan Direction = Unidirectional
 Grating = 36.5 μ m grating

488 -

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 = 488
 Channel Name = T2-Axiocam 820-SR
 Excitation Wavelength = 488
 Emission Wavelength = 525
 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 = 0.81 μ m
 Binning Mode = 2,2
 Laser Wavelength = 488 nm @1.50%
 Frame Time = 1.59 s
 Scan Direction = Unidirectional
 Grating = 36.5 μ m 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 μ m
 Binning Mode = 2,2
 Laser Wavelength = 405 nm @3.50%
 Frame Time = 676.00 ms
 Scan Direction = Unidirectional
 Grating = 36.5 μ m grating

Post Processing - SIM² Processing

Processing Dimension = 3D
 Result Sampling = 4
 Process Sampling = 4
 Channels = 3
 Algorithm = SIM²
 SIM² Settings = Standard
 Input SNR = Low
 Regularization = None
 Regularization Weight = 0.0000
 Iterations = 3
 Filter = No Filter
 Fitting = Fast Fit
 Normalization = Auto

Split View - Panel A

The top-left panel features Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 555, IgG1 - (1H1D9/B7), the top-right panel shows Golgi visualized with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the bottom-left panel features DAPI nuclear staining, and the bottom-right panel features the merged image of all channels. This image was taken at Z-Position 61 of 120.

3D Image Processing - Panel C

3D Volume Projection = Precise

Appearance = Threshold 2% all channels, 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 77%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera

Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A - Split View, this panel illustrates the signal and localization of the Mouse anti-Human H2AX Monoclonal -Identifyn® SRM Alexa Fluor™ 555, an IgG1 direct conjugate. The upper left section displays the H2AX direct conjugate (yellow), while the upper right shows the Golgi primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green). The lower left features DAPI nuclear staining (blue), and the lower right presents a merged image of all three channels. A Region of Interest is indicated by the white box, which will be explored further in Panel B.

Panel B - This panel presents a three-dimensional volume projection of the Region of Interest from Panel A. It includes the Mouse anti-Human H2AX Monoclonal - Identifyn® SRM Alexa Fluor™ 555 (yellow), the Golgi primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), and DAPI (blue), utilizing super-resolution SIM Squared with no manipulation of the signal, beyond selecting volume view in Zeiss Zen software.

Panel C - This panel features a clipped image from the Zeiss Zen image acquisition settings for a z-stack comprising 120 planes. The 561 nm laser power is set at 4.0% to achieve optimal imaging of the direct conjugate over a z-stack distance of 3.57 microns. Additionally, the Identifyn® SRM Alexa Fluor™ 488 required a laser power of 1.50%, indicating high brightness for all Identifyn® products via our patent-pending conjugation chemistry.

Image Corrections

None

Image by P. Raut

Image Analysis by B.T. Bennett

Data Presentation by P.D. Amistoso

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SOURCE PROVENANCE

Catalog	DC-000036
Stage	SIM²
Instrument	ZEISS Elyra
Microscope configuration	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:
Structured source metadata values	66
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa

SOURCE PROVENANCE

Image SHA-256	03CA6E37803601AA8DC4B016576926EFF1689F25750343EF108FF743CEA7A0FB
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