BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

IDENTIFYN RESOLUTION STEPDOWN

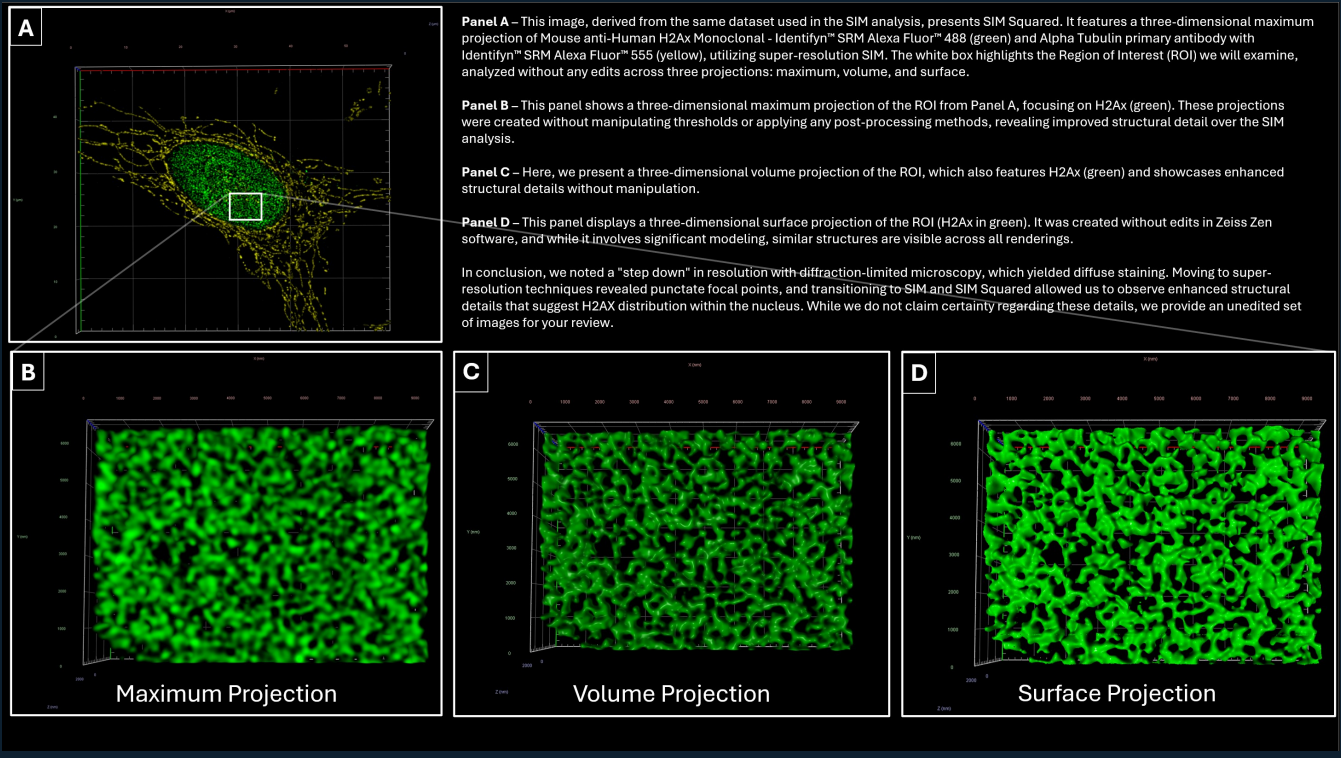
H2AX

MICROSCOPY EVIDENCE ASSESSMENT

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488

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

7	0	PENDING
ORDERED EVIDENCE TIERS	SUPPORTING CONTROL	SCIENTIST REVIEW



Panel A – This image, derived from the same dataset used in the SIM analysis, presents SIM Squared. It features a three-dimensional maximum projection of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488 (green) and Alpha Tubulin primary antibody with Identifyn™ SRM Alexa Fluor™ 555 (yellow), utilizing super-resolution SIM. The white box highlights the Region of Interest (ROI) we will examine, analyzed without any edits across three projections: maximum, volume, and surface.

Panel B – This panel shows a three-dimensional maximum projection of the ROI from Panel A, focusing on H2Ax (green). These projections were created without manipulating thresholds or applying any post-processing methods, revealing improved structural detail over the SIM analysis.

Panel C – Here, we present a three-dimensional volume projection of the ROI, which also features H2Ax (green) and showcases enhanced structural details without manipulation.

Panel D – This panel displays a three-dimensional surface projection of the ROI (H2Ax in green). It was created without edits in Zeiss Zen software, and while it involves significant modeling, similar structures are visible across all renderings.

In conclusion, we noted a "step down" in resolution with diffraction-limited microscopy, which yielded diffuse staining. Moving to super-resolution techniques revealed punctate focal points, and transitioning to SIM and SIM Squared allowed us to observe enhanced structural details that suggest H2AX distribution within the nucleus. While we do not claim certainty regarding these details, we provide an unedited set of images for your review.

REPRESENTATIVE HIGHEST-AVAILABLE VIEW / SIM²

DC-000034 | 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-000034 record contains Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². Missing modalities are not represented or inferred.

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 / PARTIAL STEPDOWN / SOURCE-BOUNDED REVIEW

The preserved DC-000034 record contains Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². Missing modalities are not represented or inferred.

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	488	Not deterministically stated in the acquisition block
Widefield	405/DAPI; 488; 647	3; 38 eGFP; 50 Cy 5; 49 DAPI; 450 - 490; 625 - 655; 335 - 383; 500 - 550
Widefield SIM — Apotome	405/DAPI; 488; 555	3; 38 HE eGFP; AF532-49014; 49 DAPI; 450 - 490; 515 - 545; 335 - 383; 500 - 550
Confocal	405/DAPI; 488; 647	3; None; 488 nm @0.3%; 639 nm @0.09%; 405 nm @0.2%; 493; 653; 353
Airyscan	405/DAPI; 488; 647	3; None; 488 nm @0.6%; 639nm @0.1%; 405 nm @0.2%; 493; 653; 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; T2-Axiocam 820-SR; T1-Axiocam 820 #2-SR; T3-Axiocam 820 #2-SR; 488
SIM²	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; T2-Axiocam 820-SR; T1-Axiocam 820 #2-SR; T3-Axiocam 820 #2-SR; 488

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 488.

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: 200 ms; 50 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @15.00%; X-Cite Xylis Lamp Intensity @8.15%. Optical/scan: Effective NA: 1.4. Structured source metadata values: 38.

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; 488; 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: 84 Slices (8.3 µm); Channels: 3; Scaling (Per Pixel): 0.143 µm X 0.143 µm X 0.100 µm; Image size (Pixels): 1295 X 833; Image Size (Pixels): 1295 X 833; Bit Depth: 14 Bit. Detector/camera: Imaging Device: 807 mono. Timing: Exposure Time: 200 ms; 80 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @12.00%; X-Cite Xylis Lamp Intensity @10.00%. Optical/scan: Effective NA: 1.25. Structured source metadata values: 43.

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; 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

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: 181 Slices (5.4 µm); Channels: 3; Scaling (Per Pixel): 0.059 µm X 0.059 µm X 0.030 µm; Image size (Pixels): 613 X 500; 857 X 857; Image Size (Pixels): 613 X 500; 857 X 857; Bit Depth: 16 Bit. Detector/camera: Imaging Device: GaAsP-Pmt3; Detector Type: GaAsP-PMT. Timing: Pixel Time: 0.39 µs; Frame Time: 2.85 s. Illumination: Laser Wavelength: 488 nm @0.3%; 639 nm @0.09%. Optical/scan: Pinhole: 1.00 AU / 50 µm; 1.00 AU / 65 µm; Scan Zoom: 2.6; 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: 6.5 (3D, Auto); 7.0 (3D, Auto). Structured source metadata values: 66.

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; 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

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: 201 Slices (6 µm); Channels: 3; Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm; Image size (Pixels): 1657 X 1013; Image Size (Pixels): 1657 X 1013; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; Detector Type: GaAsP-PMT. Timing: Pixel Time: 1.04 µs; Frame Time: 19.77 s. Illumination: Laser Wavelength: 488 nm @0.6%; 639nm @0.1%. Optical/scan: Pinhole: 5.00 AU / 268 µm; 5.00 AU / 328 µm; Scan Zoom: 1.9; LSM Scan Speed: 6; 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 8.9 (3D, Auto); SuperResolution 10.0 (3D, Auto). Structured source metadata values: 55.

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; 647.

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: 119 Slices (3.54 µm); Channels: 3; Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm; Image size (Pixels): 5056 X 5056; 2279 X 1715; Image Size (Pixels): 5056 X 5056; 2279 X 1715; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820; Axiocam 820 #2. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.98 s; 676.00 ms. Illumination: Laser Wavelength: 488 nm @2.00%; 561 nm @1.50%; Illumination Wavelength: 488; 561. 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); Background: Black. Structured source metadata values: 63.

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: 119 Slices (3.54 µm); Channels: 3; Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm; Image size (Pixels): 4458 X 3761; 728 X 512; Image Size (Pixels): 4458 X 3761; 728 X 512; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820; Axiocam 820 #2. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.98 s; 676.00 ms. Illumination: Laser Wavelength: 488 nm @2.00%; 561 nm @1.50%; Illumination Wavelength: 488; 561. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM²; Process Sampling: 4; 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); Background: Black. Structured source metadata values: 72.

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-000034 record contains Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². Missing modalities are not represented or inferred.

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)=1657 X 1013; Image Size (Scaled)=58.47 µm X 35.74 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.05%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM / Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm -> 0.01320 µm X 0.01320 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)=5056 X 5056; Image Size (Scaled)=66.72 µm X 66.72 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.03%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM² / Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm -> 0.01320 µm X 0.01320 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)=4458 X 3761; Image Size (Scaled)=58.83 µm X 49.63 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.03%.

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

AI-POWERED BIOLOGICAL INTELLIGENCE

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®

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

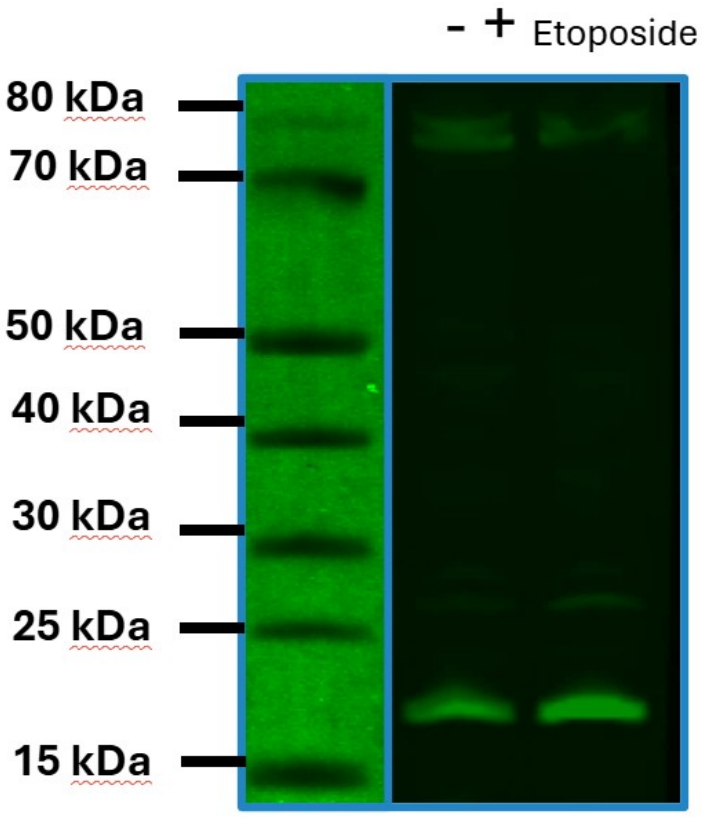
This dossier preserves the complete available evidence progression for DC-000034. Each tier remains tied to its original image, source method, instrument context and cryptographic identity. No separate control record is present in the preserved product record, and none is inferred.

CANONICAL STEPDOWN TIER	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	SIM²	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	488
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	488nm
Emission Filter	513±17nm
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™ 488, IgG1 - (1H1D9/B7)
Cat ID# - DC 000034

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™ 488 - 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 = 488nm
Emission Filter = 513±17nm
Detector = PMT
Intensity = 2
Pixel size = 100 µm
Scan Speed = High
Quality = High
Focus = Membrane (0 µm in Z-axis)

Post Processing

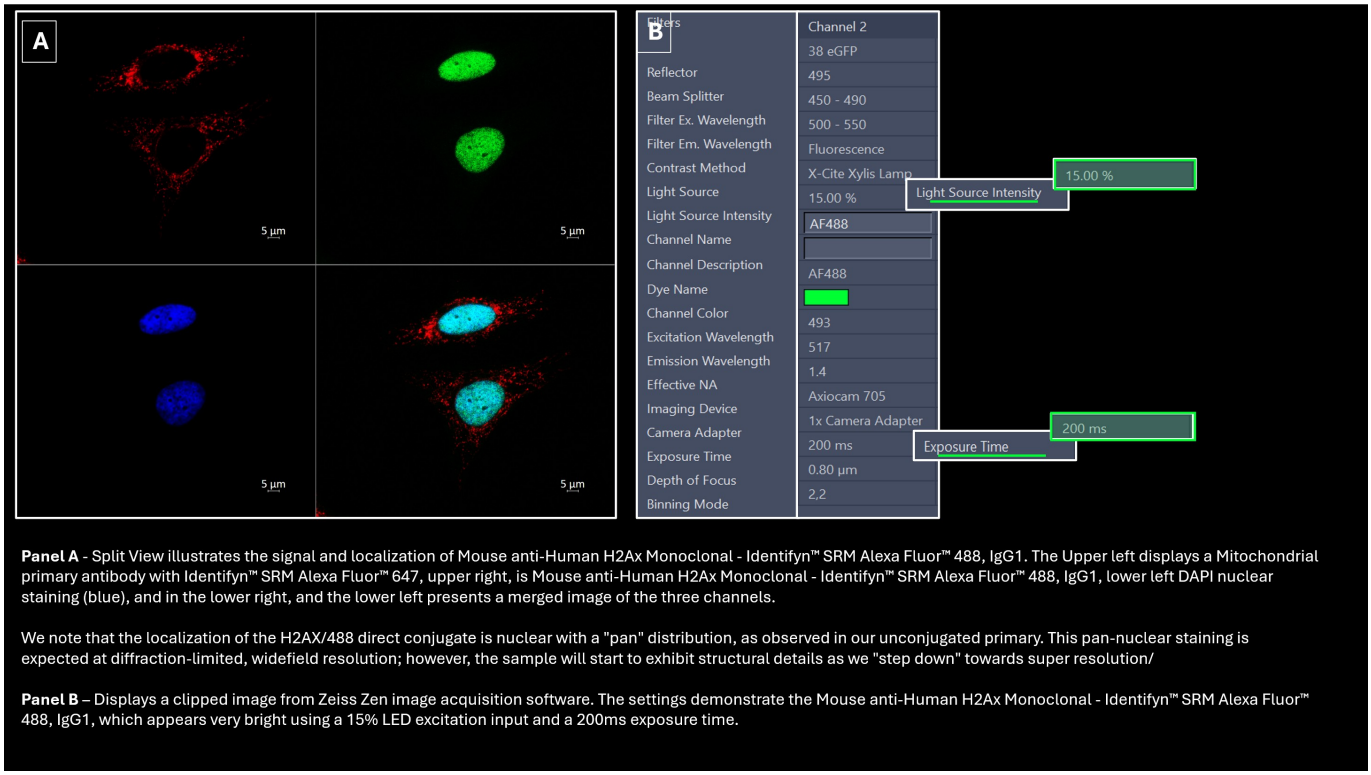
None

Image: K. Small
Data Presentation by P.D. Amistoso

SOURCE PROVENANCE

Catalog	DC-000034
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	6396C6A777BCFF4E0057F414CBBF98D66023A1FBFA1FC8241D71072D90A5B226
Method PDF SHA-256	BB54D638C0835C83B2A54AAED10C6AAAA6806E645627E1658BBE08E3FBAAE154

ORIGINAL IMAGE EVIDENCE / WIDEFIELD



Panel A - Split View illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1. The Upper left displays a Mitochondrial primary antibody with Identifyn™ SRM Alexa Fluor™ 647, upper right, is Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1, lower left DAPI nuclear staining (blue), and in the lower right, and the lower left presents a merged image of the three channels.

We note that the localization of the H2AX/488 direct conjugate is nuclear with a "pan" distribution, as observed in our unconjugated primary. This pan-nuclear staining is expected at diffraction-limited, widefield resolution; however, the sample will start to exhibit structural details as we "step down" towards super resolution/

Panel B – Displays a clipped image from Zeiss Zen image acquisition software. The settings demonstrate the Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1, which appears very bright using a 15% LED excitation input and a 200ms exposure time.

EVIDENCE TIER	Widefield
INSTRUMENT	ZEISS Axio Imager.M1
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647
METADATA VALUES	38

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 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
Reflector	38 eGFP | 50 Cy 5 | 49 DAPI
Beam Splitter	495 | 660 | 395
Filter Excitation Wavelength	450 - 490 | 625 - 655 | 335 - 383
Filter Emission Wavelength	500 - 550 | 665 - 715 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @15.00% | X-Cite Xylis Lamp Intensity @8.15%
Exposure Time	200 ms | 50 ms | 20 ms
Excitation Wavelength	493 | 653 | 353
Emission Wavelength	517 | 668 | 465
Effective NA	1.4
Imaging Device	Axiocam 705
Camera Adapter	1X
Depth of Focus	0.80 µm | 1.03 µm | 0.72 µm
Binning Mode	2,2

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™ 488, IgG1 - (1H1D9/B7)
Cat ID# - DC 000034

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, and its primary function 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 other. 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™ 488, 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, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), 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™ 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 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

488 -

Reflector = 38 eGFP
Beam Splitter = 495
Filter Excitation Wavelength = 450 - 490
Filter Emission Wavelength = 500 - 550
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15.00%
Exposure Time = 200 ms
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 0.80 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 @8.15%
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 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 @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 µm
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 Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 - (1H1D9/B7), the bottom-left panel features DAPI nuclear staining, and the bottom-right panel features the merged image of all channels.

Summary

Panel A - Split View illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1. The Upper left displays a Mitochondrial primary antibody with Identifyn® SRM Alexa Fluor™ 647, upper right, is Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1, lower left DAPI nuclear staining (blue), and in the lower right, and the lower left presents a merged image of the three channels.

We note that the localization of the H2AX/488 direct conjugate is nuclear with a "pan" distribution, as observed in our unconjugated primary. This pan-nuclear staining is expected at diffraction-limited, widefield resolution; however, the sample will start to exhibit structural details as we "step down" towards super resolution.

Panel B - Displays a clipped image from Zeiss Zen image acquisition software. The settings demonstrate the Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1, which appears very bright using a 15% LED excitation input and a 200ms exposure time.

Image Correction

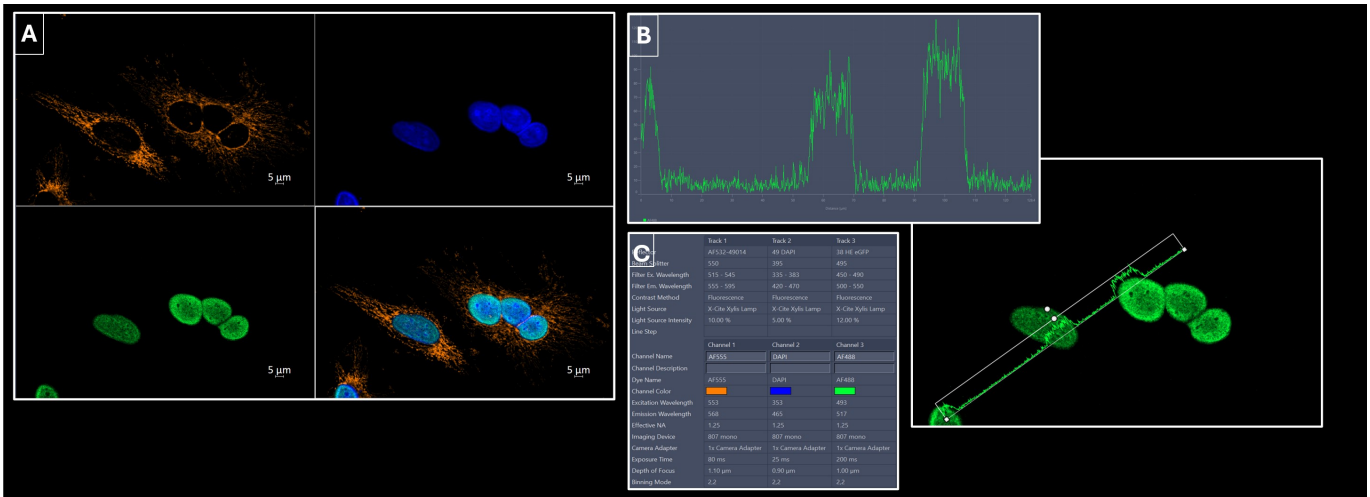
None

Image by E.F. Simon
Image Analysis by B.T. Bennett
Data Presentation by P.D. Amistoso

SOURCE PROVENANCE

Catalog	DC-000034
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 Xylis Lamp, was used with the following parameters for each dye:
Structured source metadata values	38
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	16B2CBF2856F400814033BA141EA557089D3563FE81FB9770A4A7055D665AAA4
Method PDF SHA-256	A656BAAD62D277C6BD95BA9472B29E96543987043691102ACC8EF6BC9BF8675F

ORIGINAL IMAGE EVIDENCE / WIDEFIELD SIM - APOTOME



Panel A - Split View. Z-pane 43 of the 84 collected, illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1. The Upper left displays a Mitochondrial primary antibody with Identifyn™ SRM Alexa Fluor™ 555 (orange), upper right, is DAPI nuclear staining (blue), lower left is Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 (green), in the lower right, presents a merged image of the three channels.

We note that the localization of the H2AX/488 direct conjugate is nuclear with a "pan" distribution, as observed in our unconjugated primary. This pan-nuclear staining is expected at diffraction-limited resolution; here, widefield SIM resolution provides a slight improvement over the widefield, as pan-nuclear staining is still apparent with no expected structural or special details.

Panel B - Presents a profile view, whereby the image next to the histogram shows a line that has been drawn through several nuclei. The line measures both distance and fluorescent intensity. We use this to display the intensity values at the power settings shown in Panel C, indicating that the localization of the signal is confined to the nuclear space and robust.

Panel C– Displays a clipped image from Zeiss Zen image acquisition software. The settings demonstrate the Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1, displays high brightness using a 12% LED excitation input and a 200ms exposure time.

The line profile combined with the transparency of our acquisition values leads to the conclusion that the Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 displays high brightness.

EVIDENCE TIER	Widefield SIM - Apotome
INSTRUMENT	ZEISS Axio Observer.Z1/7 Apotome
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555
METADATA VALUES	43

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	84 Slices (8.3 µm)
Channels	3
Scaling (Per Pixel)	0.143 µm X 0.143 µm X 0.100 µm
Image Size (Pixels)	1295 X 833
Image Size (Scaled)	185.00 µm X 119.00 µm
Bit Depth	14 Bit
Image Center Position	X: 52.47 mm, Y: 49.81 mm
Stage Position	X: 52.47 mm, Y: 49.81 mm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflector	38 HE eGFP | AF532-49014 | 49 DAPI
Beam Splitter	495 | 550 | 395
Filter Excitation Wavelength	450 - 490 | 515 - 545 | 335 - 383
Filter Emission Wavelength	500 - 550 | 555 - 595 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @12.00% | X-Cite Xylis Lamp Intensity @10.00% | X-Cite Xylis Lamp Intensity @5.00%
Exposure Time	200 ms | 80 ms | 25 ms
Excitation Wavelength	493 | 553 | 353
Emission Wavelength	517 | 568 | 465
Effective NA	1.25
Imaging Device	807 mono
Camera Adapter	1X
Depth of Focus	1.00 µm | 1.10 µm | 0.90 µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mod" "Optical Sectioning" - enabled correction selected.
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 128.4), Y (Min 0 and Max 132)

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™ 488, IgG1 - (1H1D9/B7)
Cat ID# - DC 000034

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Rabbit, IgG (H + L)
Cat ID# - SA 000035

H2AX, a variant of the histone protein H2A, and its primary function 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 other. 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™ 488, 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, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), 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™ 555

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 Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:

Single Plane (2D) - Z-plane 43 of 84 total - Panel A

Z-Stack = 84 Slices (8.3 µm)

Channels = 3
Scaling (Per Pixel) = 0.143 µm X 0.143 µm X 0.100 µm
Image Size (Pixels) = 1295 X 833
Image Size (Scaled) = 185.00 µm X 119.00 µm
Bit Depth = 14 Bit
Image Center Position = X: 52.47 mm, Y: 49.81 mm
Stage Position = X: 52.47 mm, Y: 49.81 mm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

488 -

Reflector = 38 HE eGFP
Beam Splitter = 495
Filter Excitation Wavelength = 450 - 490
Filter Emission Wavelength = 500 - 550
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @12.00%
Exposure Time = 200 ms
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.25
Imaging Device = 807 mono
Camera Adapter = 1X
Depth of Focus = 1.00 µm
Binning Mode = 2,2

555 -

Reflector = AF532-49014
Beam Splitter = 550
Filter Excitation Wavelength = 515 - 545
Filter Emission Wavelength = 555 - 595
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10.00%
Exposure Time = 80 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

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 @5.00%
Exposure Time = 25 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.

Split View - Panel A

The top-left panel shows Mitochondria visualized with Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the top-right panel features DAPI nuclear staining, the bottom-left panel shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 - (1H1D9/B7), and the bottom-right panel is the merged image of all channels. This split-view image was taken at Z-Position 43 of 84.

Profile View Display - Panel 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 128.4), Y (Min 0 and Max 132)

Image Correction

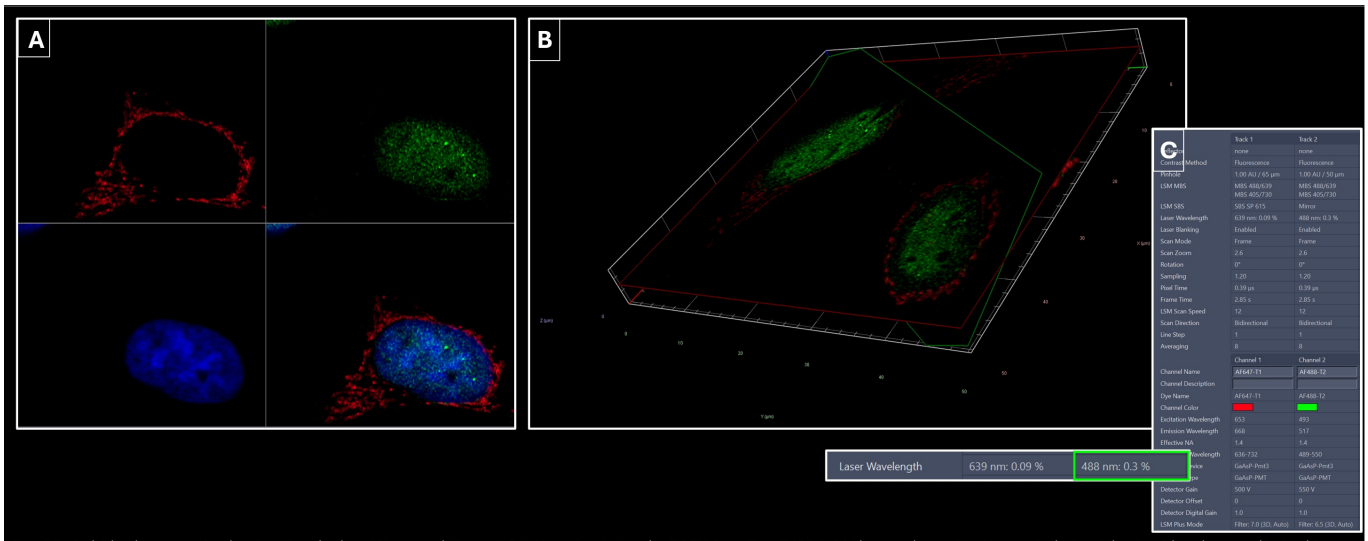
None

Image by E.F. Simon
Image Analysis by B.T. Bennett
Data Presentation by P.D. Amistoso

SOURCE PROVENANCE

Catalog	DC-000034
Stage	Widefield SIM - Apotome
Instrument	ZEISS Axio Observer.Z1/7 Apotome
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	43
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	E793A6A63424F649883CCADC3744F729A997F58697342F700945A49756215E32
Method PDF SHA-256	5D7535A439C7D875DAE3F8E8CAE08ED0A7E0EB2E7EF95453C89F85FBEBE8599D

ORIGINAL IMAGE EVIDENCE / CONFOCAL



Panel A - Split View illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 direct conjugate. The Upper left displays Mitochondrial primary antibody with Identifyn™ SRM Alexa Fluor™ 647 (red), upper right, is Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 direct conjugate (green), lower left DAPI nuclear staining (light blue), and in the lower right presents a merged image of the three channels.

Panel B – This panel presents a 3-dimensional Maximum Projection of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 direct conjugate (green) and Mitochondrial primary antibody with Identifyn™ SRM Alexa Fluor™ 647 (red) at confocal resolution. The image employs a double "wedge" or a double cut plane on the X-Y (red outline) and Y-Z planes (green outline). In the X-Y cut plane, the angles X and Y are set to 0 degrees (flat), and in the Y-Z cut plane, the Y is at =75 degrees and the Z at -34 degrees, to provide the cross-cut angle.

Panel C - A clipped image from Zeiss Zen image acquisition settings for this z-stack comprising 181 planes. **We note that the 488nm laser power is set at 0.3% to obtain maximal imaging of the direct conjugate.**

Both images show the alignment of the nucleolus in both the H2AX, 488 direct conjugate and the DAPI staining, as one would expect, since this compartment is, to date, assumed to be devoid of chromatin material; therefore, neither DAPI nor H2AX should occupy this space. Thereby, we have a negative "colocalization" of signal, not necessarily components, as colocalization at confocal resolutions does not present substantial or grounded science.

EVIDENCE TIER	Confocal
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647
METADATA VALUES	66

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	181 Slices (5.4 µm)
Channels	3
Scaling (Per Pixel)	0.059 µm X 0.059 µm X 0.030 µm
Image Size (Pixels)	613 X 500 | 857 X 857
Image Size (Scaled)	36.04 µm X 29.40 µm | 50.39 µm X 50.39 µm
Bit Depth	16 Bit
Image Center Position	X: 91.97 mm, Y: 53.97 mm
Stage Position	X: 91.97 mm, Y: 53.97 mm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	1.00 AU / 50 µm | 1.00 AU / 65 µm | 1.00 AU / 44 µm
LSM MBS	MBS 488/639, 405/730
LSM SBS	Mirror | SBS SP 615
Laser Wavelength	488 nm @0.3% | 639 nm @0.09% | 405 nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.6
Rotation	0°
Sampling	1.20
Pixel Time	0.39 µs
Frame Time	2.85 s
LSM Scan Speed	12
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	493 | 653 | 353
Emission Wavelength	517 | 668 | 465
Effective NA	1.4
Detection Wavelength	489-550 | 636-732 | 430-487
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	6.5 (3D, Auto) | 7.0 (3D, Auto) | 5.9 (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	Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip	11% | -1%
Clip X	0°
Clip Y	0° | -75°
Y-Z	Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Clip Z	-34°

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™ 488, IgG1 - (1H1D9/B7)
Cat ID# - DC 000034

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, and its primary function 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™ 488, 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, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), 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™ 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 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 69 of 181 total - Panel A

Z-Stack = 181 Slices (5.4 µm)

Channels = 3
Scaling (Per Pixel) = 0.059 µm X 0.059 µm X 0.030 µm
Image Size (Pixels) = 613 X 500
Image Size (Scaled) = 36.04 µm X 29.40 µm
Bit Depth = 16 Bit
Image Center Position = X: 91.97 mm, Y: 53.97 mm
Stage Position = X: 91.97 mm, Y: 53.97 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B

Z-Stack = 181 Slices (5.4 µm)

Channels = 3
Scaling (Per Pixel) = 0.059 µm X 0.059 µm X 0.030 µm
Image Size (Pixels) = 857 X 857
Image Size (Scaled) = 50.39 µm X 50.39 µm
Bit Depth = 16 Bit
Image Center Position = X: 91.97 mm, Y: 53.97 mm
Stage Position = X: 91.97 mm, Y: 53.97 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 50 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = Mirror
Laser Wavelength = 488 nm @0.3%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.39 µs
Frame Time = 2.85 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 = 489-550
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 550 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 6.5 (3D, Auto)

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 65 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = SBS SP 615
Laser Wavelength = 639 nm @0.09%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.39 µs
Frame Time = 2.85 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 636-732
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 7.0 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 44 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = Mirror
Laser Wavelength = 405 nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.39 µs
Frame Time = 2.85 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 = 430-487
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 5.9 (3D, Auto)

Split View - Panel A

The top-left panel shows the Mitochondria visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the top-right panel shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 - (1H1D9/B7), and the bottom-left panel features DAPI nuclear staining. The bottom-right panel is the merged image of all channels. This split-view image was taken at Z-Position 69 of 181.

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 = Activate was selected, textured opaque was chosen, and a red 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°

Y-Z = Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -1%
Clip Y = -75°
Clip Z = -34°

Summary

Panel A - Split View illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate. The Upper left displays Mitochondrial primary antibody with Identifyn® SRM Alexa Fluor™ 647 (red), upper right, is Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate (green), lower left DAPI nuclear staining (light blue), and in the lower right presents a merged image of the three channels.

Panel B - This panel presents a 3-dimensional Maximum Projection of Mouse anti-Human H2Ax Monoclonal -Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate (green) and Mitochondrial primary antibody with Identifyn® SRM Alexa Fluor™ 647 (red) at confocal resolution. The image employs a double "wedge" or a double cut plane on the X-Y (red outline) and Y-Z planes (green outline) In the X-Y cut plane, the angles X and Y are set to 0 degrees (flat), and in the Y-Z cut plane, the Y is at -75 degrees and the Z at -34 degrees, to provide the cross-cut angle.

Panel C - A clipped image from Zeiss Zen image acquisition settings for this z-stack comprising 181 planes. We note that the 488nm laser power is set at 0.3% to obtain maximal imaging of the direct conjugate.

Both images show the alignment of the nucleolus in both the H2AX, 488 direct conjugate and the DAPI staining, as one would expect, since this compartment is, to date, assumed to be devoid of chromatin material; therefore, neither DAPI nor H2AX should occupy this space. Thereby, we have a negative "colocalization" of signal, not necessarily components, as colocalization at confocal resolutions does not present substantial or grounded science.

Image Corrections

None

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

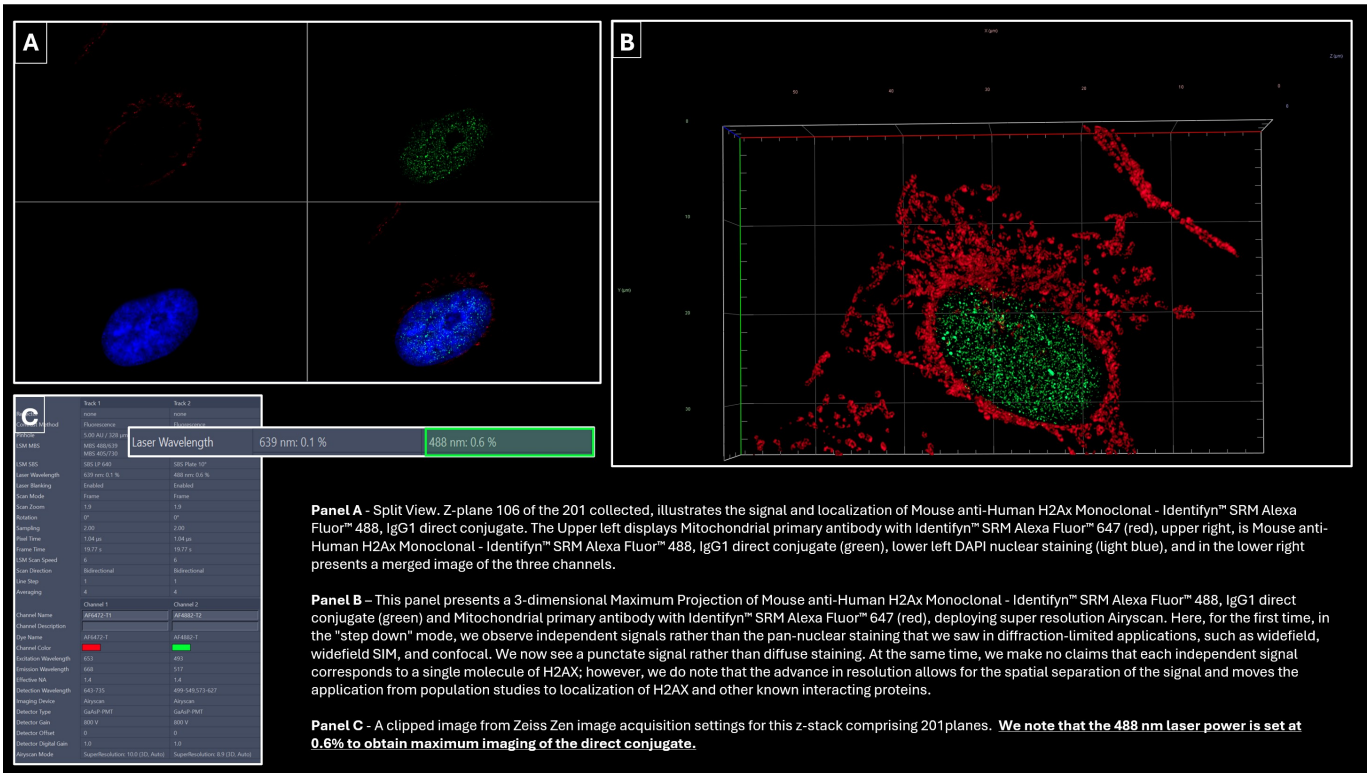
SOURCE PROVENANCE

Catalog	DC-000034
Stage	Confocal
Instrument	ZEISS LSM 980

SOURCE PROVENANCE

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	66
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	5E410800DDC1E2AC7D1B7F94CD6C8306612030892E6D0EB53E651C73AB8D0BE4
Method PDF SHA-256	EB2AE1F5475962C9C428EDB2F80D26CE129878FBC1D2B899BDE5E85EBDE65CE7

ORIGINAL IMAGE EVIDENCE / AIRYSCAN



Panel A - Split View. Z-plane 106 of the 201 collected, illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 direct conjugate. The Upper left displays Mitochondrial primary antibody with Identifyn™ SRM Alexa Fluor™ 647 (red), upper right, is Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 direct conjugate (green), lower left DAPI nuclear staining (light blue), and in the lower right presents a merged image of the three channels.

Panel B – This panel presents a 3-dimensional Maximum Projection of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488, IgG1 direct conjugate (green) and Mitochondrial primary antibody with Identifyn™ SRM Alexa Fluor™ 647 (red), deploying super resolution Airyscan. Here, for the first time, in the "step down" mode, we observe independent signals rather than the pan-nuclear staining that we saw in diffraction-limited applications, such as widefield, widefield SIM, and confocal. We now see a punctate signal rather than diffuse staining. At the same time, we make no claims that each independent signal corresponds to a single molecule of H2AX; however, we do note that the advance in resolution allows for the spatial separation of the signal and moves the application from population studies to localization of H2AX and other known interacting proteins.

Panel C - A clipped image from Zeiss Zen image acquisition settings for this z-stack comprising 201planes. **We note that the 488 nm laser power is set at 0.6% to obtain maximum imaging of the direct conjugate.**

EVIDENCE TIER	Airyscan
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647
METADATA VALUES	55

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	201 Slices (6 µm)
Channels	3
Scaling (Per Pixel)	0.03530 µm X 0.03530 µm X 0.03000 µm
Image Size (Pixels)	1657 X 1013
Image Size (Scaled)	58.47 µm X 35.74 µm
Bit Depth	16 Bit
Image Center Position	X: 89.38 mm, Y: 52.52 mm
Stage Position	X: 89.38 mm, Y: 52.52 mm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	5.00 AU / 268 µm | 5.00 AU / 328 µm | 5.46 AU / 235 µm
LSM MBS	MBS 488/639, 405/730
LSM SBS	SBS Plate 10° | SBS LP 640 | SBS SP 505
Laser Wavelength	488 nm @0.6% | 639nm @0.1% | 405 nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	1.9
Rotation	0°
Sampling	2.00
Pixel Time	1.04 µs
Frame Time	19.77 s
LSM Scan Speed	6
Scan Direction	Bidirectional
Line Step	1
Averaging	4
Excitation Wavelength	493 | 653 | 353
Emission Wavelength	517 | 668 | 465
Effective NA	1.4
Detection Wavelength	499-549, 573-627 | 643-735 | 350-499
Imaging Device	Airyscan

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT
Detector Gain	800 V
Detector Offset	0
Detector Digital Gain	1.0
Airyscan Mode	SuperResolution 8.9 (3D, Auto) | SuperResolution 10.0 (3D, Auto) | SuperResolution 7.8 (3D, Auto)
3D Maximum Projection	Precise
Appearance	Threshold 2% 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)

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™ 488, IgG1 - (1H1D9/B7)
Cat ID# - DC 000034

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, and its primary function 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 other 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™ 488, 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, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), 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™ 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 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 106 of 201 total - Panel A

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

Z-Stack = 201 Slices (6 µm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image Size (Pixels) = 1657 X 1013
Image Size (Scaled) = 58.47 µm X 35.74 µm
Bit Depth = 16 Bit
Image Center Position = X: 89.38 mm, Y: 52.52 mm
Stage Position = X: 89.38 mm, Y: 52.52 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 268 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = SBS Plate 10°
Laser Wavelength = 488 nm @0.6%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.9
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.04 µs
Frame Time = 19.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 499-549, 573-627
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = SuperResolution 8.9 (3D, Auto)

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 328 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = SBS LP 640
Laser Wavelength = 639nm @0.1%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.9
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.04 µs
Frame Time = 19.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 643-735
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = SuperResolution 10.0 (3D, Auto)

DAPI -

Reflector - None
Contrast Method - Fluorescence
Pinhole = 5.46 AU / 235 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = SBS SP 505
Laser Wavelength = 405 nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.9
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.04 µs
Frame Time = 19.77 s
LSM Scan Speed = 6
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 = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = SuperResolution 7.8 (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 Mitochondria visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the top-right panel shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 - (1H1D9/B7), and the bottom-left panel features DAPI nuclear staining. The bottom-right panel is the merged image of all channels. This split-view image was taken at Z-Position 106 of 201.

3D Image Processing - Panel B

3D Maximum Projection = Precise
Appearance = Threshold 2% 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)

Summary

Panel A - Split View. Z-plane 106 of the 201 collected illustrates the signal and localization of Mouseanti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate The Upper left displays Mitochondrial primary antibody with Identifyn® SRM Alexa Fluor™ 647 (red), upper right, is Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate (green), lower left DAPI nuclear staining (light blue), and in the lower right presents a merged image of the three channels.

Panel B - This panel presents a 3-dimensional Maximum Projection of Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate (green) and Mitochondrial primary antibody with Identifyn® SRM Alexa Fluor™ 647 (red), deploying super resolution Airyscan. Here, for the first time, in the "step down" mode, we observe independent signals rather than the pan-nuclear (diffuse) staining that we saw in diffraction-limited applications, such as widefield, widefield SIM, and confocal. We now see a punctate signal rather than diffuse staining. At the same time, we make no claims that each independent signal corresponds to a single molecule of H2AX; however, we do note that the advance in resolution allows for the spatial separation of the signal and moves the application from population studies to localization of H2AX and other known interacting proteins.

Panel C - A clipped image from Zeiss Zen image acquisition settings for this z-stack comprising 201 planes. Note that the 488 nm laser power is set at 0.6% to obtain maximum imaging of the direct conjugate.

Image Corrections

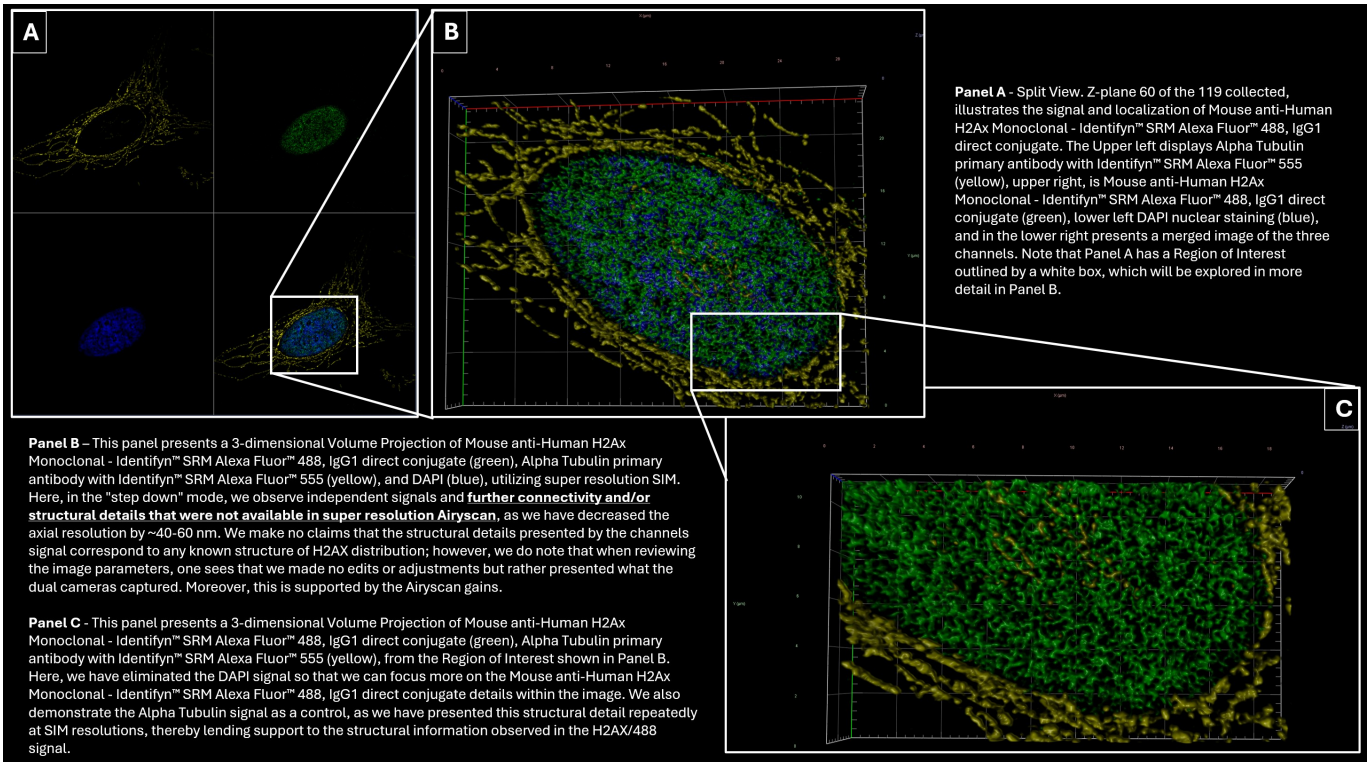
None

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

SOURCE PROVENANCE

Catalog	DC-000034
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	55
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	C1ED76178D6AC612349ACAE28F3381AEA3ED26DD90F4EAB6AB80B8C7A48B7152
Method PDF SHA-256	1A79AFD5EF7CC9728F1660931AA3CC200299FB5FB61C20A699913F7A68A60E04

ORIGINAL IMAGE EVIDENCE / SIM



EVIDENCE TIER	SIM
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555
METADATA VALUES	63

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	119 Slices (3.54 µm)
Channels	3
Scaling (Per Pixel)	0.01320 µm X 0.01320 µm X 0.03000 µm
Image Size (Pixels)	5056 X 5056 | 2279 X 1715 | 1457 X 843
Image Size (Scaled)	66.72 µm X 66.72 µm | 30.07 µm X 22.63 µm | 19.23 µm X 11.12 µm
Bit Depth	16 Bit
Image Center Position	X: 56.75 mm, Y: 37.80 mm
Stage Position	X: 56.75 mm, Y: 37.80 mm
ROI Center Offset	X: 0.00µm, Y: 0.00µm | 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	488 | 561 | 405
Channel Name	T2-Axiocam 820-SR | T1-Axiocam 820 #2-SR | T3-Axiocam 820 #2-SR
Excitation Wavelength	488 | 561 | 405
Emission Wavelength	525 | 567 | 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device	Axiocam 820 | Axiocam 820 #2
Camera Adapter	1X
Exposure Time	150 ms | 120 ms | 50 ms
Depth of Focus	0.81 µm | 0.92 µm | 0.69 µm
Binning Mode	2,2
Laser Wavelength	488 nm @2.00% | 561 nm @1.50% | 405 nm @3.50%
Frame Time	1.98 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	10.2082 (555), 9.6147 (488), 8.5848 (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)

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™ 488, IgG1 - (1H1D9/B7)
Cat ID# - DC 000034

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Rabbit, IgG (H + L)
Cat ID# - SA 000035

H2AX, a variant of the histone protein H2A, and its primary function 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 other 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™ 488, 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, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), 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™ 555

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 60 of 119 total - Panel A

Z-Stack = 119 Slices (3.54 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 5056 X 5056
Image Size (Scaled) = 66.72 µm X 66.72 µm
Bit Depth = 16 Bit
Image Center Position = X: 56.75 mm, Y: 37.80 mm
Stage Position = X: 56.75 mm, Y: 37.80 mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

Z Stack - (3D) - Panel B

Z-Stack = 119 Slices (3.54 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 2279 X 1715
Image Size (Scaled) = 30.07 µm X 22.63 µm
Bit Depth = 16 Bit
Image Center Position = X: 56.75 mm, Y: 37.80 mm
Stage Position = X: 56.75 mm, Y: 37.80 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel C

Z-Stack = 119 Slices (3.54 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 1457 X 843
Image Size (Scaled) = 19.23 µm X 11.12 µm
Bit Depth = 16 Bit
Image Center Position = X: 56.75 mm, Y: 37.80 mm
Stage Position = X: 56.75 mm, Y: 37.80 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

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 = 150 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @2.00%
Frame Time = 1.98 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

555 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 561
Channel Name = T1-Axiocam 820 #2-SR
Excitation Wavelength = 561
Emission Wavelength = 567
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 120 ms
Depth of Focus = 0.92 µm
Binning Mode = 2,2
Laser Wavelength = 561 nm @1.50%
Frame Time = 1.98 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 405
Channel Name = T3-Axiocam 820 #2-SR
Excitation Wavelength = 405
Emission Wavelength = 445
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 50 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.50%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 36.5 µm 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 = 10.2082 (555), 9.6147 (488), 8.5848 (DAPI)
Fitting = Fast Fit
Normalization = Auto

Split View - Panel A

The top-left panel shows the Mitochondria visualized with Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Rabbit, IgG (H + L), the top-right panel shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 - (1H1D9/B7), and the bottom-left panel features DAPI nuclear staining. The bottom-right panel is the merged image of all channels. This split-view image was taken at Z-Position 60 of 119.

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 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A - Split View. Z-plane 60 of the 119 collected, illustrates the signal and localization of Mouse anti-Human H2Ax Monoclonal -Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate The Upper left displays Alpha Tubulin primary antibody with Identifyn® SRM Alexa Fluor™ 555 (yellow), upper right, is Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate (green), lower left DAPI nuclear staining (blue), and in the lower right presents a merged image of the three channels. Note that Panel A has 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 Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™488, IgG1 direct conjugate (green), Alpha Tubulin primary antibody with Identifyn® SRM Alexa Fluor™ 555 (yellow), and DAPI (blue), utilizing super resolution SIM. Here, in the "step down" mode, we observe independent signals and further connectivity and/or structural details that were not available in super resolution Airyscan, as we have decreased the axial resolution by ~40-60 nm. We make no claims that the structural details presented by the channels signal correspond to any known structure of H2AX distribution; however, we do note that when reviewing the image parameters, one sees that we made no edits or adjustments but instead presented what the dual cameras captured. Moreover, this is supported by the Airyscan gains.

Panel C - This panel presents a 3-dimensional Volume Projection of Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate (green), Alpha Tubulin primary antibody with Identifyn® SRM Alexa Fluor™ 555 (yellow), from the Region of Interest shown in Panel B. Here, we have eliminated the DAPI signal so that we can focus more on the Mouse anti-Human H2AxMonoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG1 direct conjugate details within the image. We also demonstrate the Alpha Tubulin signal as a control, as we have presented this structural detail repeatedly at SIM resolutions, thereby lending support to the structural information observed in the H2AX/488 signal.

Image Corrections

None

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

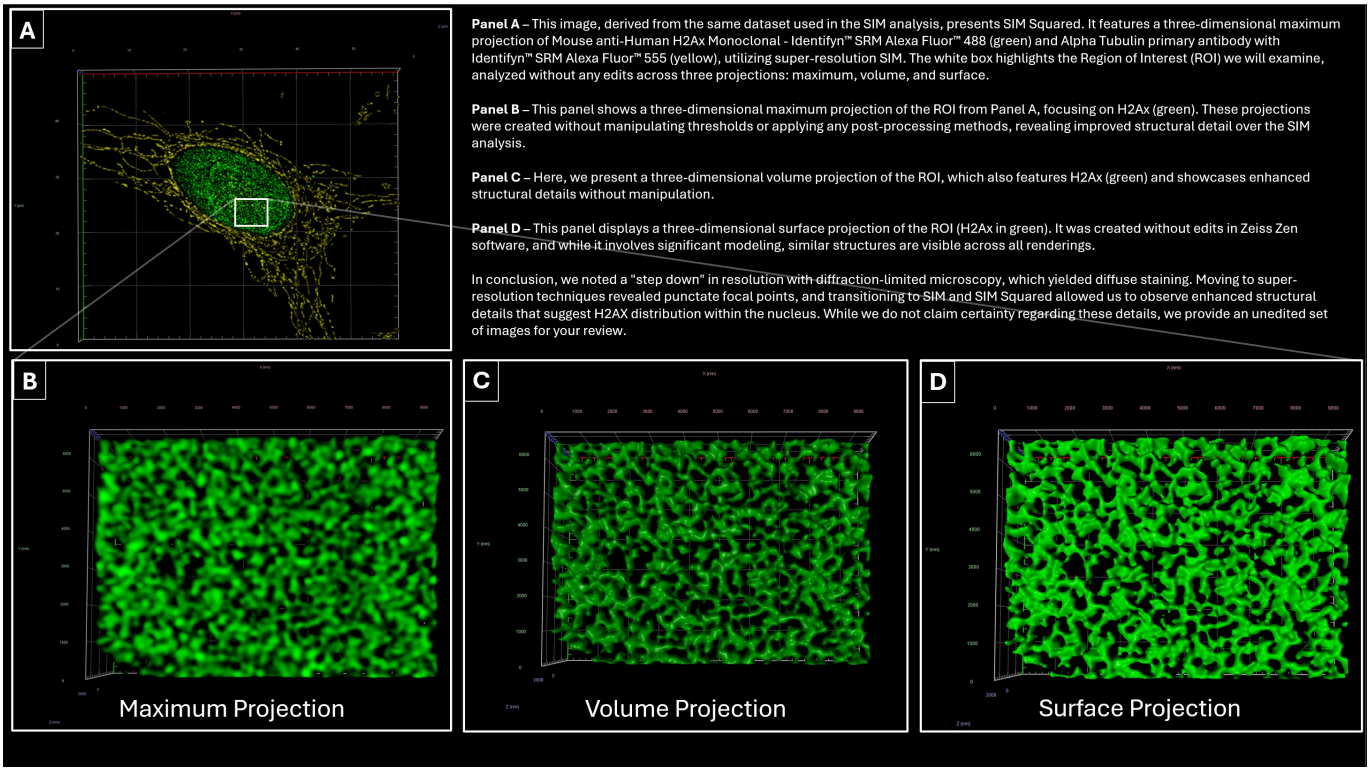
SOURCE PROVENANCE

Catalog	DC-000034
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	63
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	61DD05CF88BB204BD336BAC53D5324162CB490A3821C9EA6CA8763A25DDF3A18
Method PDF SHA-256	3742F48D6B66FDDDAB2FA1CCAFCCDFDDCEAECB10B830D567646B7DD5E370F1E1

ORIGINAL IMAGE EVIDENCE / SIM²



Panel A – This image, derived from the same dataset used in the SIM analysis, presents SIM Squared. It features a three-dimensional maximum projection of Mouse anti-Human H2Ax Monoclonal - Identifyn™ SRM Alexa Fluor™ 488 (green) and Alpha Tubulin primary antibody with Identifyn™ SRM Alexa Fluor™ 555 (yellow), utilizing super-resolution SIM. The white box highlights the Region of Interest (ROI) we will examine, analyzed without any edits across three projections: maximum, volume, and surface.

Panel B – This panel shows a three-dimensional maximum projection of the ROI from Panel A, focusing on H2Ax (green). These projections were created without manipulating thresholds or applying any post-processing methods, revealing improved structural detail over the SIM analysis.

Panel C – Here, we present a three-dimensional volume projection of the ROI, which also features H2Ax (green) and showcases enhanced structural details without manipulation.

Panel D – This panel displays a three-dimensional surface projection of the ROI (H2Ax in green). It was created without edits in Zeiss Zen software, and while it involves significant modeling, similar structures are visible across all renderings.

In conclusion, we noted a "step down" in resolution with diffraction-limited microscopy, which yielded diffuse staining. Moving to super-resolution techniques revealed punctate focal points, and transitioning to SIM and SIM Squared allowed us to observe enhanced structural details that suggest H2AX distribution within the nucleus. While we do not claim certainty regarding these details, we provide an unedited set of images for your review.

EVIDENCE TIER	SIM²
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555
METADATA VALUES	72

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	119 Slices (3.54 µm)
Channels	3
Scaling (Per Pixel)	0.01320 µm X 0.01320 µm X 0.03000 µm
Image Size (Pixels)	4458 X 3761 | 728 X 512
Image Size (Scaled)	58.83 µm X 49.63 µm | 9.61 µm X 6.76 µm
Bit Depth	16 Bit
Image Center Position	X: 56.75 mm, Y: 37.80 mm
Stage Position	X: 56.75 mm, Y: 37.80 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	488 | 561 | 405
Channel Name	T2-Axiocam 820-SR | T1-Axiocam 820 #2-SR | T3-Axiocam 820 #2-SR
Excitation Wavelength	488 | 561 | 405
Emission Wavelength	525 | 597 | 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 | Axiocam 820 #2
Camera Adapter	1X
Exposure Time	150 ms | 120 ms | 50 ms
Depth of Focus	0.81 µm | 0.92 µm | 0.69 µm
Binning Mode	2,2
Laser Wavelength	488 nm @2.00% | 561 nm @1.50% | 405 nm @3.50%
Frame Time	1.98 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	SIM²
SIM² Settings	Standard
Input SNR	Low
Regularization	None
Regularization Weight	0.0000
Iterations	3
Filter	No Filter
Fitting	Fast Fit
Normalization	Auto
3D Maximum Projection	Precise
Appearance	Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels | Threshold 1%, Ramp 0%, Maximum 100% | Threshold 2%, Ramp 98%, Maximum 100% | Threshold 20%, Ambient Light 0%, Specular Light 100%, Shininess 35%
Background	Black
Light	Brightness 100%, Enabled Tone Mapping | Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping | Azimuth 80°, Elongation -130°, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
3D Volume Projection	Precise
3D Surface Projection	Precise

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™ 488, IgG1 - (1H1D9/B7)
Cat ID# - DC 000034

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Rabbit, IgG (H + L)
Cat ID# - SA 000035

H2AX, a variant of the histone protein H2A, and its primary function 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 other. 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™ 488, 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, Mitochondria Rabbit Recombinant Antibody (MTC02,2860R), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 555 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:

Z Stack - (3D) - Panel A

Z-Stack = 119 Slices (3.54 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 4458 X 3761
Image Size (Scaled) = 58.83 µm X 49.63 µm
Bit Depth = 16 Bit
Image Center Position = X: 56.75 mm, Y: 37.80 mm
Stage Position = X: 56.75 mm, Y: 37.80 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B, C, & D

Z-Stack = 119 Slices (3.54 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 728 X 512
Image Size (Scaled) = 9.61 µm X 6.76 µm
Bit Depth = 16 Bit
Image Center Position = X: 56.75 mm, Y: 37.80 mm
Stage Position = X: 56.75 mm, Y: 37.80 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

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 = 150 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @2.00%
Frame Time = 1.98 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

555 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 561
Channel Name = 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 = 120 ms
Depth of Focus = 0.92 µm
Binning Mode = 2,2
Laser Wavelength = 561 nm @1.50%
Frame Time = 1.98 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 405
Channel Name = T3-Axiocam 820 #2-SR
Excitation Wavelength = 405
Emission Wavelength = 445
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 50 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.50%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM² Processing

Processing Dimension = 3D
Result Sampling = 4
Process Sampling = 4
Channels = 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

3D Image Processing - Panel A

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)

3D Image Processing - Panel B

3D Maximum Projection = Precise
Appearance = Threshold 1%, Ramp 0%, Maximum 100%
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 C

3D Volume Projection = Precise
Appearance = Threshold 2%, Ramp 98%, Maximum 100%
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 D

3D Surface Projection = Precise
Appearance = Threshold 20%, Ambient Light 0%, Specular Light 100%, Shininess 35%
Background = Black
Light = Azimuth 80°, Elongation -130°, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A - This image, derived from the same dataset used in the SIM analysis, presents SIM Squared. It features a three-dimensional maximum projection of Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 488 (green) and Alpha Tubulin primary antibody with Identifyn® SRM Alexa Fluor™ 555 (yellow), utilizing super-resolution SIM. The white box highlights the Region of Interest (ROI) we will examine, analyzed without any edits across three projections: maximum, volume, and surface.

Panel B - This panel shows a three-dimensional maximum projection of the ROI from Panel A, focusing on H2Ax (green). These projections were created without manipulating thresholds or applying any post-processing methods, revealing improved structural detail over the SIM analysis.

Panel C - Here, we present a three-dimensional volume projection of the ROI, which also features H2Ax (green) and showcases enhanced structural details without manipulation.

Panel D - This panel displays a three-dimensional surface projection of the ROI (H2Ax in green). It was created without edits in Zeiss Zen software, and while it involves significant modeling, similar structures are visible across all renderings.

In conclusion, we noted a "step down" in resolution with diffraction-limited microscopy, which yielded diffuse staining. Moving to super-resolution techniques revealed punctate focal points, and transitioning to SIM and SIM Squared allowed us to observe enhanced structural details that suggest H2AX distribution within the nucleus. While we do not claim certainty regarding these details, we provide an unedited set of images for your review.

Image Corrections

None

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

SOURCE PROVENANCE

Catalog	DC-000034
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	72
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	68910B767DF569BBB41920893C7196F93ADEE953079CCC52CAE05D1C0FC362E8
Method PDF SHA-256	994F1EBDF90604E61F3C69D03050613A6E32269E86223A1F9C01103BC386C9BA