BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

IDENTIFYN RESOLUTION STEPDOWN

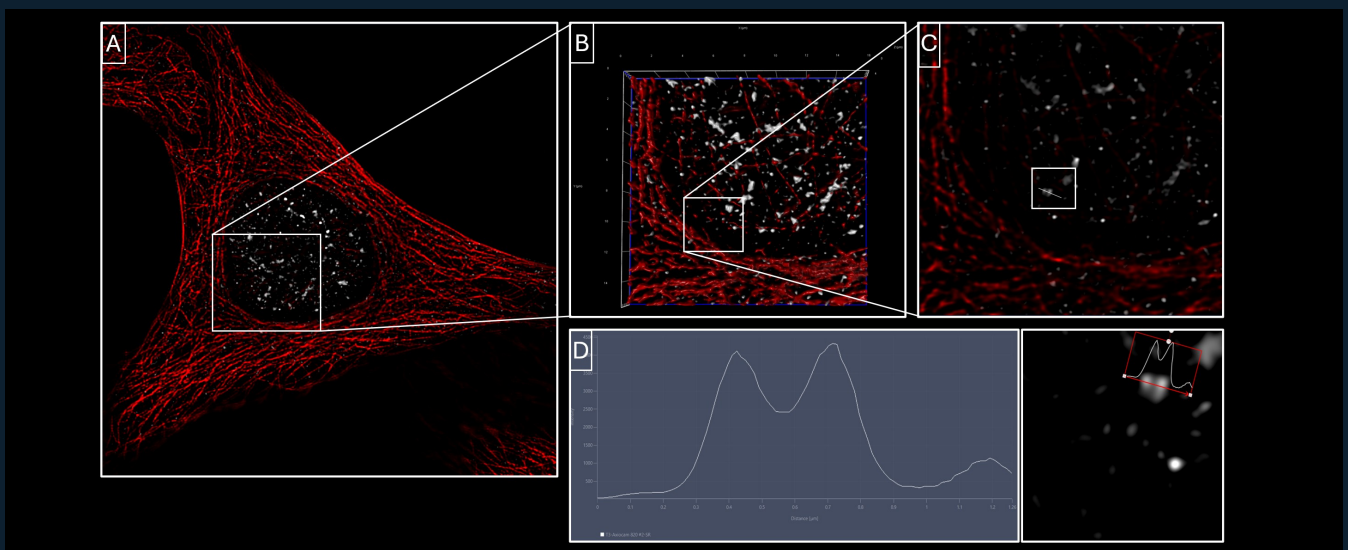
H2AX

MICROSCOPY EVIDENCE ASSESSMENT

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405

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

7	1	PENDING
ORDERED EVIDENCE TIERS	SUPPORTING CONTROL	SCIENTIST REVIEW



Panel A - Presents a two-dimensional view of the image acquisition at z-plane 71 of 146 of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG (default blue changed to white to enhance visualization), alongside Alpha-Tubulin with an Identifyn™ SRM Alexa Fluor™ 647 (red). The white box indicates a Region of Interest to be examined in panel B.

Panel B - Utilizes the Region of Interest (ROI) from the image in Panel A. Here, we observe gamma-H2AX foci, and through maximum projection and we identify a repeatable aggregate formation at the site of Etoposide induced DNA damage and with the use of super resolution SIM2, we observe better detail of the signal. Although this spacing remains unresolved at super-resolution SIM2 imaging, it is evident that two signals are present in the unresolved resolution, as in STORM, where we see two closely separated ends of the signal. The white box indicates a Region of Interest to be examined in panel C.

Panel C - Presents a two-dimensional view of the image acquisition again, as in panel A, at z-plane 71 of 146 of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG (default blue changed to white to enhance visualization) with a white box, with a line showing the area to be examined in panel D via the use of the line profile tool within Zeiss Zen software.

Panel D - Shows a line profile we have selected that crosses two unresolved visual aggregates. The line profile histogram reveals two separate signals (the two peaks); however, as one can see, they are again unresolved at super resolution SIM2, approximately 40nm X, Y.

REPRESENTATIVE HIGHEST-AVAILABLE VIEW / SIM²

DC-000040 | 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
- Preserved control experiment
- 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 -> Control

BENNETT ASSESSMENT

The preserved DC-000040 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

BENNETT / MICROSCOPY AND BIOLOGICAL EVIDENCE REVIEW

SOURCE-GROUNDED RETROSPECTIVE REVIEW / PENDING SCIENTIST REVIEW

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

OVERALL CALL / NIR-BOUNDED PARTIAL STEPDOWN

The preserved DC-000040 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

SETTINGS ASSESSMENT

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

BIOLOGICAL SUPPORT

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

CONTROL ASSESSMENT

A true preserved control record is present and appears last.

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	405/DAPI	Not deterministically stated in the acquisition block
Widefield	405/DAPI; 750	2; AF405; AF750; 372 - 408; 672 - 748; 417 - 445; 765 - 855; 401
Widefield SIM — Apotome	405/DAPI; 750	2; AF405; AF750; 372 - 408; 672 - 748; 417 - 445; 765 - 855; 401
Confocal	405/DAPI; 750	2; None; 405 nm @0.3%; 730nm @0.2%; 401; 752; 422; 779
Airyscan	405/DAPI	3; None; 405 nm @0.4%; 401; 422
SIM	405/DAPI; 488; 647	2; SR HE LBF 405/488/561/642; 200 - 405, 200 - 488, 200 - 561, 200 - 642; 419 - 470, 503 - 546, 576 - 617, 657 - 800; T3-Axiocam 820 #2-SR; T1-Axiocam 820-SR; 405; 640
SIM²	405/DAPI; 488; 647; 680	2; SR HE LBF 405/488/561/642; 200 - 405, 200 - 488, 200 - 561, 200 - 642; 419 - 470, 503 - 546, 576 - 617, 657 - 800; T3-Axiocam 820 #2-SR; T1-Axiocam 820-SR; 405; 640
Control	405/DAPI; 555; 647	2; 50 Cy5; AF532-49014; 49 DAPI; 625 - 655; 515 - 545; 335 - 383; 665 - 715

MODALITY ASSESSMENT / PART 1 OF 8

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 405/DAPI.

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 8

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 MR R3, and X-Cite Xylis Lamp, was used with the following parameters for each dye:. Acquisition: Channels: 2; 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: 200ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @15.00%. Optical/scan: Effective NA: 1.25. Structured source metadata values: 30.

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; 750.

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 8

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 705, and X-Cite Xylis Lamp was used with the following parameters for each dye:. Acquisition: Z-Stack: 33 Slices (3.2 µm) - Panel A; Channels: 2; Scaling (Per Pixel): 0.110 µm X 0.110 µm X 0.100 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. Illumination: Light source: X-Cite Xylis Lamp Intensity @15.00%. Optical/scan: Effective NA: 1.25. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 35.

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; 750.

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 8

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: 221 Slices (6.30 µm) - Panels A & B; 75 Slices (2.22 µm) - Panel C; Channels: 2; Scaling (Per Pixel): 0.059 µm X 0.059 µm X 0.030 µm; 58.81 nm X 58.81 nm X 30.00 nm; Image size (Pixels): 1017 X 1017; 320 X 360; Image Size (Pixels): 1017 X 1017; 320 X 360; Bit Depth: 16 Bit. Detector/camera: Imaging Device: GaAs-Pmt3; NIR Ch2; Detector Type: GaAs-PMT. Timing: Pixel Time: 0.33 µs; Frame Time: 3.36 s. Illumination: Laser Wavelength: 405 nm @0.3%; 730nm @0.2%. Optical/scan: Pinhole: 1.00 AU / 41 µm; 1.00AU / 79µm; Scan Zoom: 2.2; LSM Scan Speed: 12; Scan Direction: Bidirectional; Averaging: 8; Effective NA: 1.4. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black; LSM Plus Mode: Filter: 6.3 (3D, Auto); Filter: 6.9 (3D, Auto). Structured source metadata values: 58.

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; 750.

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 8

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: 121 Slices (3.6 µm); Channels: 3; Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm; Image size (Pixels): 1793 X 1793; 161 X 140; Image Size (Pixels): 1793 X 1793; 161 X 140; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; Detector Type: GaAsP-PMT. Timing: Pixel Time: 1.15 µs; Frame Time: 17.86 s. Illumination: Laser Wavelength: 405 nm @0.4%. Optical/scan: Pinhole: 5.00 AU / 215 µm; Scan Zoom: 2.1; 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; LSM Plus Mode: 8.4 (3D, Auto). Structured source metadata values: 46.

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.

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 8

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: 146 Slices (4.35 µm); Channels: 2; Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm; Image size (Pixels): 5056 X 5056; 1254 X 1196; Image Size (Pixels): 5056 X 5056; 1254 X 1196; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820 #2; Axiocam 820. Timing: Exposure Time: 100 ms; Frame Time: 676.00 ms; 1.33 s. Illumination: Laser Wavelength: 405 nm @3.00%; 640 nm @2.00%; Illumination Wavelength: 405; 640. 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: 58.

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; 647.

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 8

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: 146 Slices (4.35 µm); Channels: 2; Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm; Image size (Pixels): 5056 X 5056; 1220 X 1188; Image Size (Pixels): 5056 X 5056; 1220 X 1188; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820 #2; Axiocam 820. Timing: Exposure Time: 100 ms; Frame Time: 676.00 ms; 1.33 s. Illumination: Laser Wavelength: 405 nm @3.00%; 640 nm @2.00%; Illumination Wavelength: 405; 680. 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: 60.

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; 647; 680.

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.

MODALITY ASSESSMENT / PART 8 OF 8

Control / 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: Channels: 2; Scaling (Per Pixel): 0.143µm X 0.143µm; Image size (Pixels): 963 X 855; Image Size (Pixels): 963 X 855; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 807 mono. Timing: Exposure Time: 150ms; 50ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @8.0%; X-Cite Xylis Lamp Intensity @6.0%. Optical/scan: Effective NA: 1.25. Structured source metadata values: 40.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

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

LITERATURE CORRELATION

Consistent with peer-reviewed antibody-control guidance that a no-primary or related control bounds background but is not a complete specificity system.

INTERPRETIVE LIMIT

A control bounds the recorded comparison only; it does not establish universal specificity, zero background, zero cross-reactivity, or absence of free dye.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Control 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-000040 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

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

BENNETT LITERATURE CORRELATION — SOURCES INDEPENDENTLY VERIFIED

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

- Antibody and microscopy evidence require orthogonal controls and modality-appropriate interpretation; presentation images alone do not establish analytical specificity.

- Advanced-resolution presentation depends on acquisition, reconstruction or localization quality control before numerical resolution, molecular dimensions or cluster identity can be claimed.

REFERENCES

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

RELEASE BOUNDARY

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

SOURCE RECONCILIATION / QA / PROVENANCE

SOURCE STATUS - PRESERVED / AUTO-RECONCILED

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

HIGH CONFIDENCE - AUTO-RECONCILED

Confocal / Scaling (Per Pixel): 58.81 nm X 58.81 nm X 30.00 nm -> 0.05881 µm X 0.05881 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)=1017 X 1017; Image Size (Scaled)=59.81 µm X 59.81 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.00%.

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)=1793 X 1793; Image Size (Scaled)=63.29 µm X 63.29 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.00%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM / Scaling (Per Pixel): 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)=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 - SCIENTIST-AUTHORIZED CORRECTION

Cross-tier / treatment concentration: 200 µM; 2µM -> 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.

HIGH CONFIDENCE - SOURCE-DERIVED METADATA CANONICALIZATION

Product identity / phosphosite: Blank structured canonical field -> Serine 139. The phosphorylation site is explicitly stated in the preserved source product identity. Supporting evidence: Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405

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.
A preserved control follows all available Stepdown tiers as supporting evidence.

PRODUCT-LEVEL STEPDOWN MAP

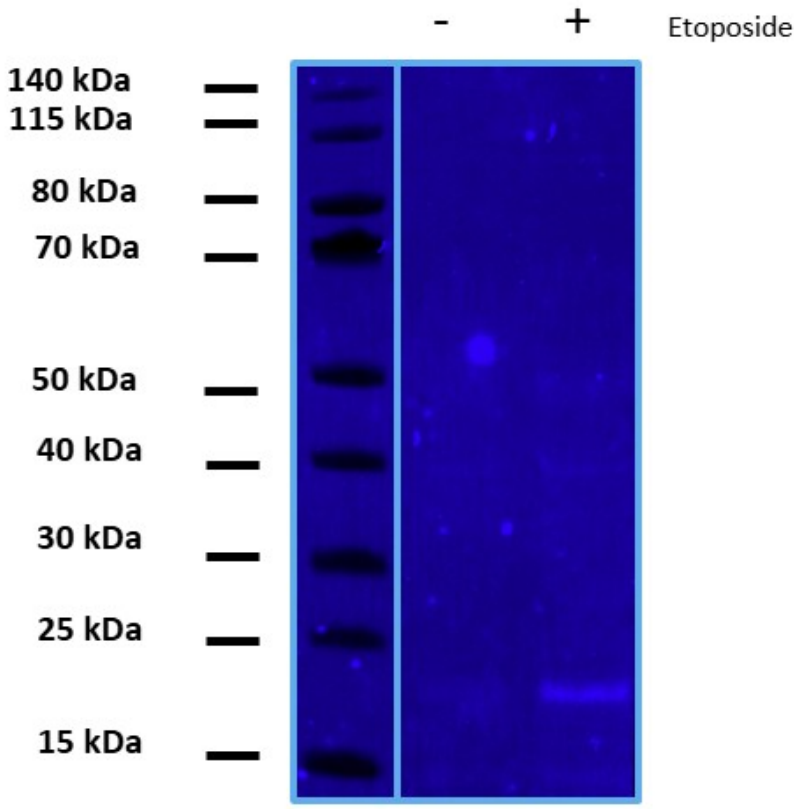
This dossier preserves the complete available evidence progression for DC-000040. Each tier remains tied to its original image, source method, instrument context and cryptographic identity. A preserved control is retained as supporting evidence and appears after every resolution tier.

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	405/DAPI
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	375nm
Emission Filter	452±45nm
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

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M)
Cat ID# - DC 000040

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 down 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 washed 5X with PBS. The blocked membrane was incubated with Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405 - Direct Conjugate for 2 hours, followed by 5X PBS wash. 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 = 375nm
Emission Filter = 452±45nm
Detector = PMT
Intensity = 2
Pixel size = 100 µm
Scan Speed = High
Quality = High
Focus = Membrane (0 µm in Z-axis)

Post Processing

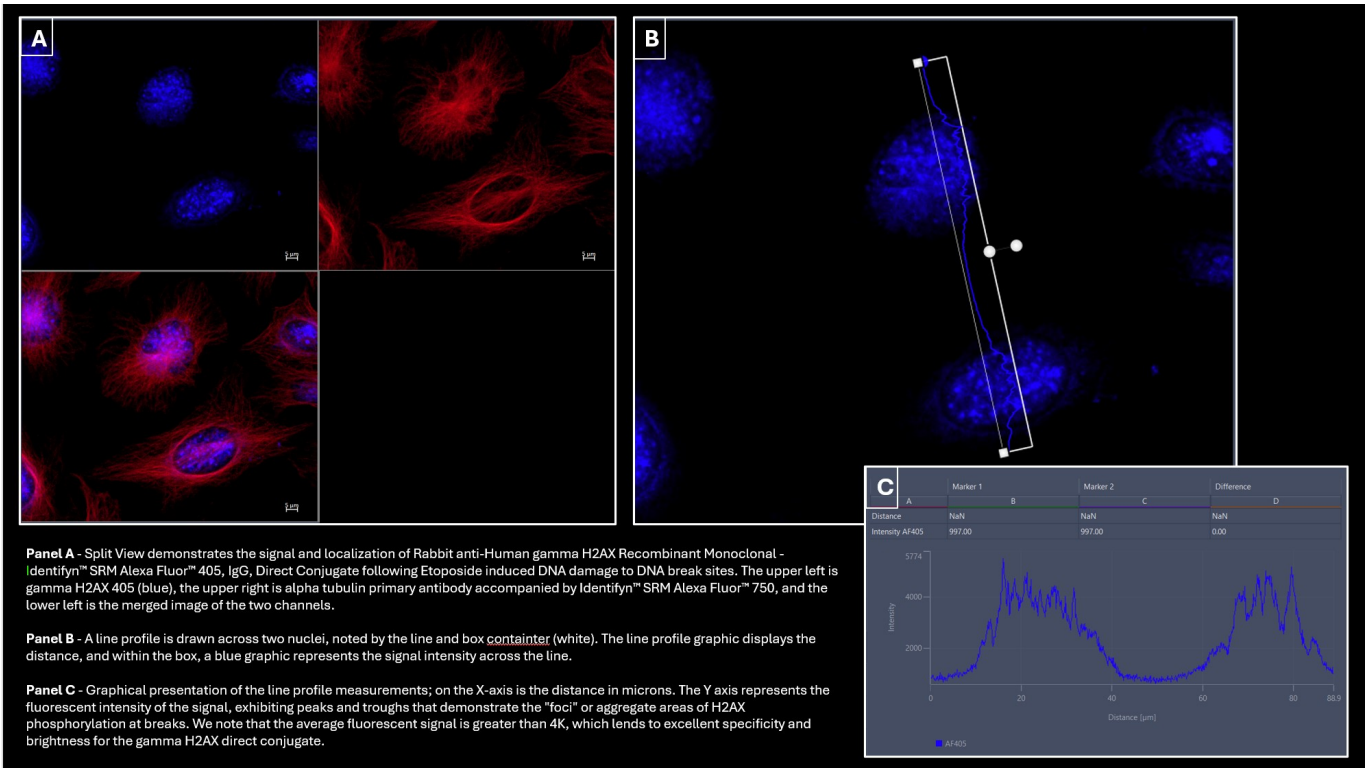
None

Image: K. Small

SOURCE PROVENANCE

Catalog	DC-000040
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	25D15D6E37E3BFA680335542A1C9A29E25E37DE66C77F403F556BB245F3DBA07
Method PDF SHA-256	C1281D51459CDD7BA1A27BD2C43262CA47FBC2B8B5490CCBDF7D8611FB08857E

ORIGINAL IMAGE EVIDENCE / WIDEFIELD



Panel A - Split View demonstrates the signal and localization of Rabbit anti-Human gamma H2AX Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG, Direct Conjugate following Etoposide induced DNA damage to DNA break sites. The upper left is gamma H2AX 405 (blue), the upper right is alpha tubulin primary antibody accompanied by Identifyn™ SRM Alexa Fluor™ 750, and the lower left is the merged image of the two channels.

Panel B - A line profile is drawn across two nuclei, noted by the line and box containter (white). The line profile graphic displays the distance, and within the box, a blue graphic represents the signal intensity across the line.

Panel C - Graphical presentation of the line profile measurements; on the X-axis is the distance in microns. The Y axis represents the fluorescent intensity of the signal, exhibiting peaks and troughs that demonstrate the "foci" or aggregate areas of H2AX phosphorylation at breaks. We note that the average fluorescent signal is greater than 4K, which lends to excellent specificity and brightness for the gamma H2AX direct conjugate.

EVIDENCE TIER	Widefield
INSTRUMENT	ZEISS Axio Imager.M1
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 750
METADATA VALUES	30

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 MR R3, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63x/1.40 Oil M27
Channels	2
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: 0.00µm, Y: 0.00µm
ROI Center Offset	X: 0.00µm, Y: 0.00µm
Reflector	AF405 | AF750
Beam Splitter	412 | 760
Filter Excitation Wavelength	372 - 408 | 672 - 748
Filter Emission Wavelength	417 - 445 | 765 - 855
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @15.00%
Exposure Time	200ms
Excitation Wavelength	401 | 752
Emission Wavelength	422 | 779
Effective NA	1.25
Imaging Device	Axiocam 705
Camera Adapter	1X
Depth of Focus	0.82 µm | 1.51 µm
Binning Mode	2,2
Profile Definition	Stroke Thickness 1, Caliper X, Grid Distance 1
Profile View	X and Y axes were set to Auto - X (Min 0.0 and Max 88.9), Y (Min 576 and Max 5774)

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

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M)
Cat ID# - DC 000040

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 750 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000078

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly and explicitly phosphorylated at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PPBS. dentifyn™ SRM Alexa Fluor™ 750 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated at room temperature for 1 hour at a dilution of 1:100 . Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant cat#P36961.

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam MR R3, and X-Cite Xylis Lamp, was used with the following parameters for each dye:

Single Plane (2D)

Channels = 2
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: 0.00μm, Y: 0.00μm
ROI Center Offset = X: 0.00μm, Y: 0.00μm

405 -

Reflector = AF405
Beam Splitter = 412
Filter Excitation Wavelength = 372 - 408
Filter Emission Wavelength = 417 - 445
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15.00%
Exposure Time = 200ms
Excitation Wavelength = 401
Emission Wavelength = 422
Effective NA = 1.25
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 0.82 µm
Binning Mode = 2,2

750 -

Reflector = AF750
Beam Splitter = 760
Filter Excitation Wavelength = 672 - 748
Filter Emission Wavelength = 765 - 855
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15.00%
Exposure Time = 200ms
Excitation Wavelength = 752
Emission Wavelength = 779
Effective NA = 1.25
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 1.51 µm
Binning Mode = 2,2

Split View - Panel A

The top-left panel shows the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M) after DNA damage, the top-right panel features Alpha Tubulin visualized with Identifyn® SRM Alexa Fluor™ 750 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific, and the bottom-left panel is the merged image of both channels.

Profile View Display - Panel B & C

Profile Definition = Stroke Thickness 1, Caliper X, Grid Distance 1
Profile View = X and Y axes were set to Auto - X (Min 0.0 and Max 88.9), Y (Min 576 and Max 5774)

Summary

Panel A - Split View demonstrates the signal and localization of Rabbit anti-Human gamma H2AXRecombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG, Direct Conjugate following Etoposide-induced DNA damage to DNA break sites. The upper left is gamma H2AX 405 (blue), the upper right is alpha tubulin primary antibody accompanied by Identifyn® SRM Alexa Fluor™ 750, and the lower left is the merged image of the two channels.

Panel B - A line profile is drawn across two nuclei, noted by the line and box container (white). The line profile graphic displays the distance, and within the box, a blue graphic represents the signal intensity across the line.

Panel C - Graphical presentation of the line profile measurements; on the X-axis is the distance in microns. The Y axis represents the fluorescent intensity of the signal, exhibiting peaks and troughs that demonstrate the "foci" or aggregate areas of H2AX phosphorylation at breaks. We note that the average fluorescence signal is greater than 4K, which lends to excellent specificity and brightness for the gammaH2AX direct conjugate.

Image Correction

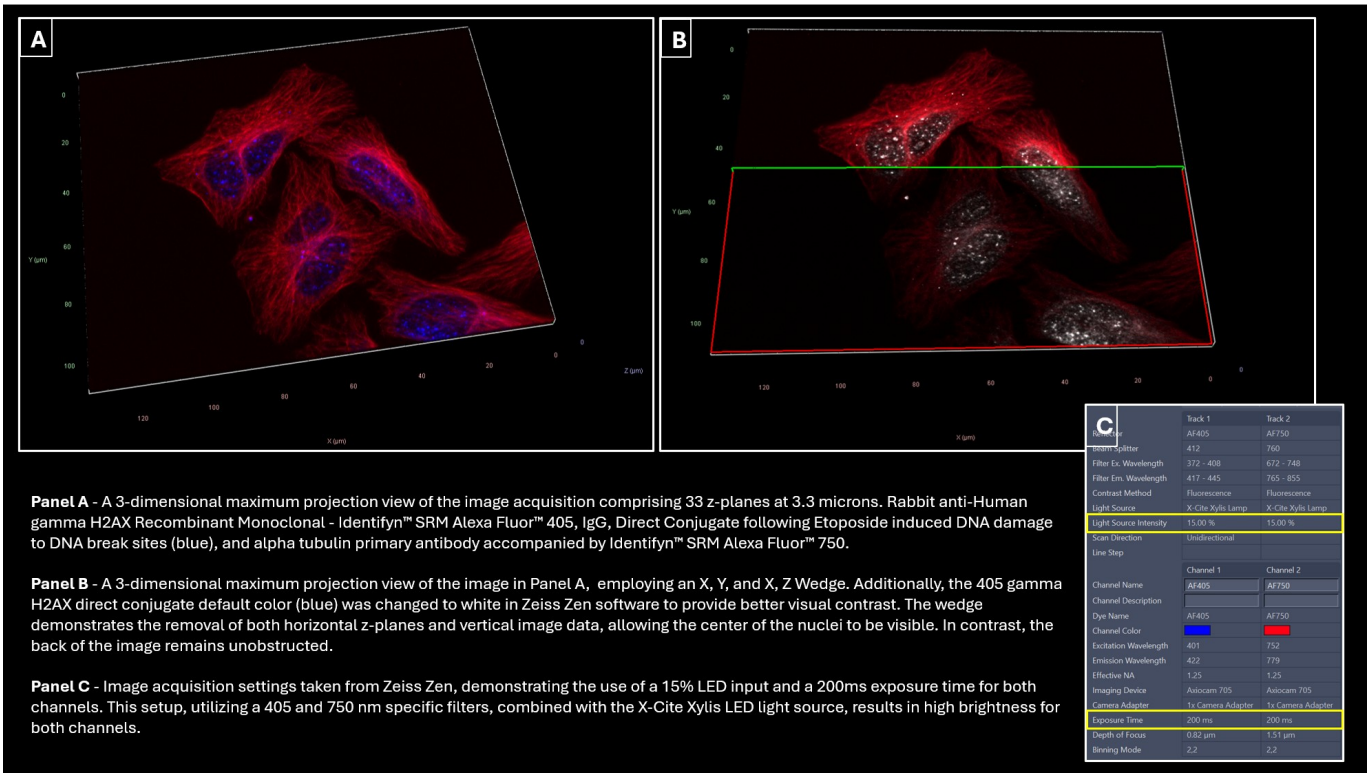
None

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

SOURCE PROVENANCE

Catalog	DC-000040
Stage	Widefield
Instrument	ZEISS Axio Imager.M1
Microscope configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam MR R3, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Structured source metadata values	30
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	18482EA9E74D6595608FB329303848599CF569EA8B5E2E990F83186694A71C76
Method PDF SHA-256	6D15C66A5C4F63A6092C6CC75D0B026CE0CA689C509E09A2929B066855A7FA8B

ORIGINAL IMAGE EVIDENCE / WIDEFIELD SIM - APOTOME



Panel A - A 3-dimensional maximum projection view of the image acquisition comprising 33 z-planes at 3.3 microns. Rabbit anti-Human gamma H2AX Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG, Direct Conjugate following Etoposide induced DNA damage to DNA break sites (blue), and alpha tubulin primary antibody accompanied by Identifyn™ SRM Alexa Fluor™ 750.

Panel B - A 3-dimensional maximum projection view of the image in Panel A, employing an X, Y, and X, Z Wedge. Additionally, the 405 gamma H2AX direct conjugate default color (blue) was changed to white in Zeiss Zen software to provide better visual contrast. The wedge demonstrates the removal of both horizontal z-planes and vertical image data, allowing the center of the nuclei to be visible. In contrast, the back of the image remains unobstructed.

Panel C - Image acquisition settings taken from Zeiss Zen, demonstrating the use of a 15% LED input and a 200ms exposure time for both channels. This setup, utilizing a 405 and 750 nm specific filters, combined with the X-Cite Xylis LED light source, results in high brightness for both channels.

EVIDENCE TIER	Widefield SIM - Apotome
INSTRUMENT	ZEISS Axio Observer.Z1/7 Apotome
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 750
METADATA VALUES	35

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 705, 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	33 Slices (3.2 µm) - Panel A
Channels	2
Scaling (Per Pixel)	0.110 µm X 0.110 µm X 0.100 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	AF405 | AF750
Beam Splitter	412 | 760
Filter Excitation Wavelength	372 - 408 | 672 - 748
Filter Emission Wavelength	417 - 445 | 765 - 855
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @15.00%
Exposure Time	200 ms
Excitation Wavelength	401 | 752
Emission Wavelength	422 | 779
Effective NA	1.25
Imaging Device	Axiocam 705
Camera Adapter	1X
Depth of Focus	0.82 µm | 1.51 µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mode "Optical Sectioning" - enabled correction selected.
3D Maximum Projection	Precise
Appearance	Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels.
Background	Black
Light	Brightness 100%, Tone Mapping was NOT Enabled
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

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Identifyn® Primary Antibody

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Cat ID# - DC 000040

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Identifyn® SRM Alexa Fluor™ 750 Affinity Purified Goat Anti Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000078

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly explicitly phosphorylated at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 750 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant cat#P36961.

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 705, and X-Cite Xylis Lamp was used with the following parameters for each dye:

Z Stack - (3D)

Z-Stack = 33 Slices (3.2 µm) - Panel A

Channels = 2
Scaling (Per Pixel) = 0.110 µm X 0.110 µm X 0.100 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

405 -

Reflector = AF405
Beam Splitter = 412
Filter Excitation Wavelength = 372 - 408
Filter Emission Wavelength = 417 - 445
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15.00%
Exposure Time = 200 ms
Excitation Wavelength = 401
Emission Wavelength = 422
Effective NA = 1.25
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 0.82 µm
Binning Mode = 2,2

750 -

Reflector = AF750
Beam Splitter = 760
Filter Excitation Wavelength = 672 - 748
Filter Emission Wavelength = 765 - 855
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15.00%
Exposure Time = 200 ms
Excitation Wavelength = 752
Emission Wavelength = 779
Effective NA = 1.25
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 1.51 µm
Binning Mode = 2,2

Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mode "Optical Sectioning" - enabled correction selected.

Normalization, Phase Correction, Fourier Filter, and Deconvolution were NOT used.

3D Image Processing - Panel A & B

3D Maximum Projection = Precise
Appearance = Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels.
Background = Black
Light = Brightness 100%, Tone Mapping was NOT Enabled
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

X-Y and X-Z Wedge selected

Depth Coding NOT selected

Summary

Panel A - A 3-dimensional maximum projection view of the image acquisition comprising 33 z-planes at 3.3 microns. Rabbit anti-Human gamma H2AX Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™405, IgG, Direct Conjugate following Etoposide induced DNA damage to DNA break sites (blue), and alpha tubulin primary antibody accompanied by Identifyn® SRM Alexa Fluor™ 750.

Panel B - A 3-dimensional maximum projection view of the image employing an X, Y, and X, Z Wedge. Additionally, the 405 gamma H2AX direct conjugate default color (blue) was changed to white in Zeiss Zen software to provide better visual contrast. The wedge demonstrates the removal of both horizontal planes and vertical image data, allowing the center of the nuclei to be visible. In contrast, the back of the image remains unobstructed.

Panel C - Image acquisition settings taken from Zeiss Zen, demonstrating the use of a 15% LED input and a 200ms exposure time for both channels. This setup, utilizing a 405 and 750 nm specific filter, combined with the X-Cite Xylis LED light source, results in high brightness for both channels.

Image Correction

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M) was pseudo-colored in Zen Software from blue, the default color, to violet.

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

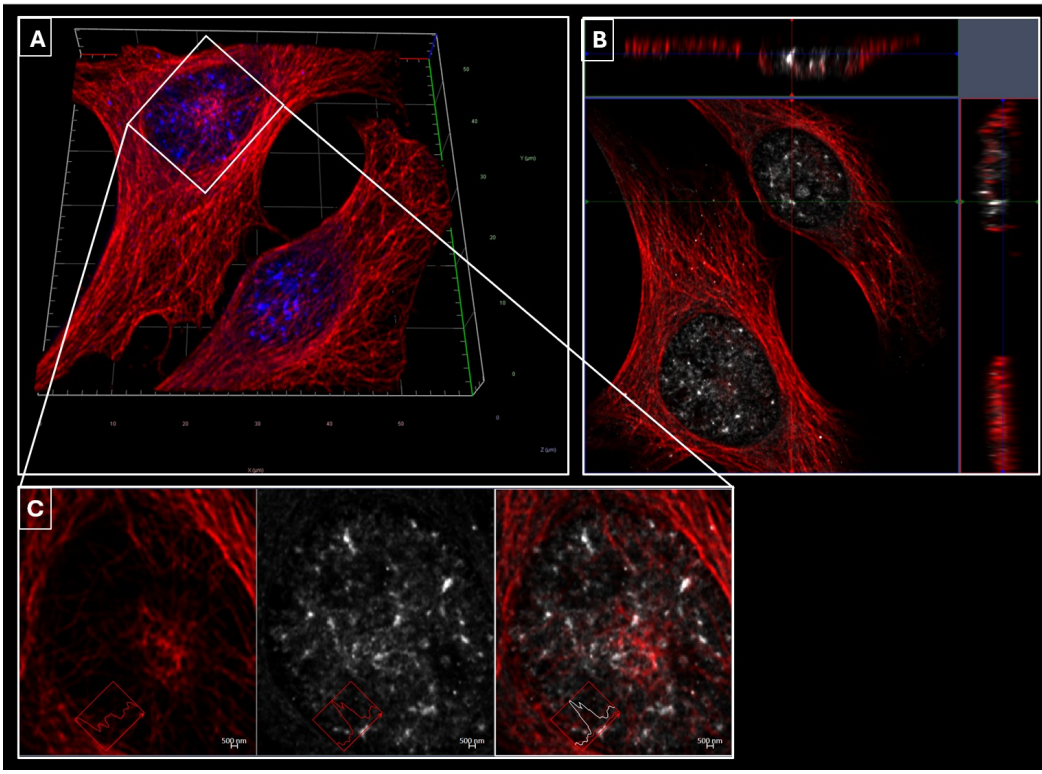
SOURCE PROVENANCE

Catalog	DC-000040
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 705, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Structured source metadata values	35

SOURCE PROVENANCE

Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	D29200C5F1B11B83FEC673E99699F4E396F55B1280C28478EF6F4B5839563871
Method PDF SHA-256	18F0E6C57AF7EE784E2FC44972F53C01CEC9C1E6462D491730DBDB0631C9D50E

ORIGINAL IMAGE EVIDENCE / CONFOCAL



Panel A - This panel presents a 3-dimensional maximum projection view of the image acquisition, comprising 211 z-planes at a resolution of 6.3 microns. The image features a Rabbit anti-Human gamma H2AX Recombinant Monoclonal antibody Identifyn™ SRM Alexa Fluor™ 405, IgG, Direct Conjugate highlighting DNA break sites (shown in blue) following Etoposide-induced DNA damage, alongside an alpha tubulin primary antibody labeled with Identifyn™ SRM Alexa Fluor™ 750.

Panel B - This panel displays an orthogonal view that demonstrates the localization of the gamma H2AX 405 Direct Conjugate within the nuclear compartment across all axes. For enhanced visual contrast, the 405 channel color has been adjusted from its default blue to white using Zeiss Zen software.

Panel C - This split view is combined with a Profile View. The left panel shows alpha tubulin labeled with Identifyn™ SRM Alexa Fluor™ 750, the center panel features the Rabbit anti-Human gamma H2AX Recombinant Monoclonal Identifyn™ SRM Alexa Fluor™ 405, IgG, Direct Conjugate; the default color has been changed to white, and the right panel displays the merged image. Across all panels, a line profile is drawn over the gamma H2AX signal. At the confocal resolution, as noted in other data sets, spatial localization between the two foci begins to appear, while super-resolution clearly reveals this localization more distinctly.

EVIDENCE TIER	Confocal
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 750
METADATA VALUES	58

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	221 Slices (6.30 µm) - Panels A & B | 75 Slices (2.22 µm) - Panel C
Channels	2
Scaling (Per Pixel)	0.059 µm X 0.059 µm X 0.030 µm | 0.05881 µm X 0.05881 µm X 0.03000 µm
Image Size (Pixels)	1017 X 1017 | 320 X 360
Image Size (Scaled)	59.81 µm X 59.81 µm | 18.82 µm X 21.17 µm
Bit Depth	16 Bit
Image Center Position	X: 71.23 mm, Y: 48.53 mm
Stage Position	X: 71.23 mm, Y: 48.53 mm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	None
Pinhole	1.00 AU / 41 µm | 1.00AU / 79µm
LSM MBS	Plate MBS 405/730
Laser Wavelength	405 nm @0.3% | 730nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.2
Rotation	0°
Sampling	1.20
Pixel Time	0.33 µs
Frame Time	3.36 s
LSM Scan Speed	12
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	401 | 752
Emission Wavelength	422 | 779
Effective NA	1.4
Detection Wavelength	395 - 460 | 755 - 900
Imaging Device	GaAs-Pmt3 | NIR Ch2
Detector Type	GaAs-PMT
Detector Gain	550 V | 650 V

FIELD	SOURCE-DOCUMENTED VALUE
Detector Offset	0
Detector Digital Gain	1.0
LSM Plus Mode	Filter: 6.3 (3D, Auto) | Filter: 6.9 (3D, Auto)
Contrast Method	Fluorescence
LSM SBS	SBS LP 740
3D Maximum Projection	Precise
Appearance	Threshold 2% (740), Threshold 4% (405), Ramp 0% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Tone Mapping was NOT Enabled
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
Cut Lines	X 564, Y 280, Z 97
Line Width	1 for all axes
Cut Line Opacity	50
Profile Definition	Stroke Thickness 1, Normal, Grid Distance 1
Profile View	X and Y axes were set to Auto - X (Min 0.0 and Max 4324.9), Y (Min 865 and Max 26680)

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

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Cat ID# - DC 000040

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 750 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000078

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly and explicitly phosphorylated at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), 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™ 750 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant cat#P36961.

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:

Z Stack - (3D)

Z-Stack = 221 Slices (6.30 µm) - Panels A & B

Channels = 2
Scaling (Per Pixel) = 0.059 µm X 0.059 µm X 0.030 µm
Image Size (Pixels) = 1017 X 1017
Image Size (Scaled) = 59.81 µm X 59.81 µm
Bit Depth = 16 Bit
Image Center Position = X: 71.23 mm, Y: 48.53 mm
Stage Position = X: 71.23 mm, Y: 48.53 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z-Stack = 75 Slices (2.22 µm) - Panel C

Channels = 2
Scaling (Per Pixel) = 58.81 nm X 58.81 nm X 30.00 nm
Image Size (Pixels) = 320 X 360
Image Size (Scaled) = 18.82 µm X 21.17 µm
Bit Depth = 16 Bit
Image Center Position = X: 71.23 mm, Y: 48.53 mm
Stage Position = X: 71.23 mm, Y: 48.53 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

405 -

Reflector = None
Contrast Method - Fluorescence
Pinhole = 1.00 AU / 41 µm
LSM MBS = Plate MBS 405/730
Laser Wavelength = 405 nm @0.3%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.2
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.33 µs
Frame Time = 3.36 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 401
Emission Wavelength = 422
Effective NA = 1.4
Detection Wavelength = 395 - 460
Imaging Device = GaAs-Pmt3
Detector Type = GaAs-PMT
Detector Gain = 550 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.3 (3D, Auto)

750 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 79µm
LSM MBS = Plate MBS 405/730
LSM SBS = SBS LP 740
Laser Wavelength = 730nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.2
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.33 µs
Frame Time = 3.36 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 752
Emission Wavelength = 779
Effective NA = 1.4
Detection Wavelength = 755 - 900
Imaging Device = NIR Ch2
Detector Type = GaAs-PMT
Detector Gain = 650 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.9 (3D, Auto)

3D Image Processing - Panel A

3D Maximum Projection = Precise
Appearance = Threshold 2% (740), Threshold 4% (405), Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Tone Mapping was NOT Enabled
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Orthogonal View - Panel B

Maximum Intensity Projection (MIP)
Cut Lines = X 564, Y 280, Z 97
Line Width = 1 for all axes
Cut Line Opacity = 50

Profile View Display - Panel C

Profile Definition = Stroke Thickness 1, Normal, Grid Distance 1
Profile View = X and Y axes were set to Auto - X (Min 0.0 and Max 4324.9), Y (Min 865 and Max 26680)

Split View - Panel C

The left panel features Alpha Tubulin visualized with Identifyn® SRM Alexa Fluor™ 750 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific, the middle panel shows the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M) after DNA damage, the right panel is the merged image of both channels.

Summary

Panel A - This panel presents a 3-dimensional maximum projection view of the image acquisition, comprising 211 z-planes at a resolution of 6.3 microns. The image features a Rabbit anti-Human gammaH2AX Recombinant Monoclonal antibody Identifyn® SRM Alexa Fluor™ 405, IgG, Direct Conjugate highlighting DNA break sites (shown in blue) following Etoposide-induced DNA damage, alongside alpha tubulin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 750.

Panel B - This panel displays an orthogonal view that demonstrates the localization of the gamma H2AX405 Direct Conjugate within the nuclear compartment across all axes, or enhanced visual contrast; the 405 channel color has been adjusted from its default blue to white using Zeiss Zen software.

Panel C - This split view is combined with a Profile View. The left panel shows alpha tubulin labeled with Identifyn® SRM Alexa Fluor™ 750, the center panel features the Rabbit anti-Human gamma H2AX Recombinant Monoclonal Identifyn® SRM Alexa Fluor™ 405, IgG, Direct Conjugate; the default color has been changed to white, and the right panel displays the merged image. Across all panels, a line profile is drawn over the gamma H2AX signal. At the confocal resolution, as noted in other data sets, spatial localization between the two foci begins to appear, while super-resolution clearly reveals this localization more distinctly.

Image Corrections

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M) was pseudo-colored in Zen Software from blue, the default color, to violet.

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

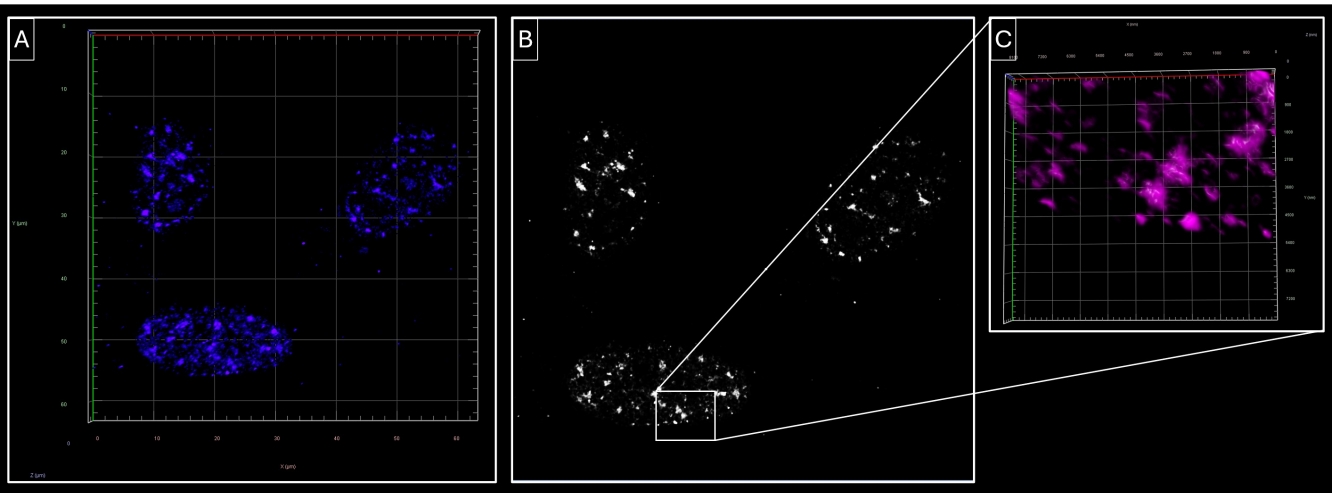
SOURCE PROVENANCE

Catalog	DC-000040
Stage	Confocal
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Structured source metadata values	58
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa

SOURCE PROVENANCE

Image SHA-256	A246BF12BD2868CB90CC18CCE86E997B41DC72612629025075A3677D46423382
Method PDF SHA-256	FDC0A2BFE8AE345E315B5790D8FACAA0B59649B39021274235B7C0756480092A

ORIGINAL IMAGE EVIDENCE / AIRYSCAN



Panel A - This panel presents a three-dimensional maximum projection view of the total image acquisition, consisting of 121 z-planes captured at a thickness of 3.6 microns. It features the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG, displayed in its default blue color. No image edits were performed, and the maximum projection was generated using Zeiss Zen software. Image aquired us Zeiss LSM980 with Airyscan

Panel B - This panel demonstrates a single z-plane, specifically the 64th of the 121 collected, to illustrate the structural details of the gamma H2AX aggregate. The color was altered in Zeiss Zen software from blue to white to enhance visualization, and a region of interest is highlighted by a white box for further examination in Panel C.

Panel C - This panel focuses on the Region of Interest from Panel B, specifically examining the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG signal. It employs a volume projection, to investigate structural details at super-resolution SIM capabilities, enabling resolution of axial objects down to approximately 100-120 nm in the X and Y dimensions.

The Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG displays what has historically been referred to as "foci" in all samples for both the primary antibody alone and the direct conjugates across various microscopes, ranging from diffraction-limited to super-resolution. However, our findings using super-resolution techniques consistently suggest that the term "foci" may be outdated, as we now observe that the previously unresolved "foci" represent defined structures. As we progress through our patent pending "step-down" method of super-resolution, culminating in STORM imaging, we clearly observe what appear to be breaks or opposing ends. While we make no biological claims, the evidence strongly supports this hypothesis.

EVIDENCE TIER	Airyscan
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI
METADATA VALUES	46

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	121 Slices (3.6 µm)
Channels	3
Scaling (Per Pixel)	0.03530 µm X 0.03530 µm X 0.03000 µm
Image Size (Pixels)	1793 X 1793 | 161 X 140
Image Size (Scaled)	63.29 µm X 63.29 µm | 5.68 µm X 4.94 µm
Bit Depth	16 Bit
Image Center Position	X: 77.20 mm, Y: 45.59 mm
Stage Position	X: 77.20 mm, Y: 45.59 mm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	5.00 AU / 215 µm
LSM MBS	Plate MBS 405/730
LSM SBS	SBS SP 505
Laser Wavelength	405 nm @0.4%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.1
Rotation	0°
Sampling	2.00
Pixel Time	1.15 µs
Frame Time	17.86 s
LSM Scan Speed	6
Scan Direction	Bidirectional
Line Step	1
Averaging	4
Excitation Wavelength	401
Emission Wavelength	422
Effective NA	1.4
Detection Wavelength	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
LSM Plus Mode	8.4 (3D, Auto)
3D Maximum Projection	Precise
Appearance	Threshold 2% all channels, Ramp 0% all channels, Maximum 100% all channels | Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Tone Mapping Enabled | 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 Volume 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

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M)
Cat ID# - DC 000040

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly and explicitly phosphorylated at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong Diamond Antifade Mountant (cat#P36961).

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 64 of 121 total - Panel B

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

Z-Stack = 121 Slices (3.6 μm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image Size (Pixels) = 1793 X 1793
Image Size (Scaled) = 63.29 μm X 63.29 μm
Bit Depth = 16 Bit
Image Center Position = X: 77.20 mm, Y: 45.59 mm
Stage Position = X: 77.20 mm, Y: 45.59 mm
ROI Center Offset = X: 0.00 μm, Y: 0.00 μm

Z Stack - (3D) - Panel C

Z-Stack = 121 Slices (3.6 µm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image Size (Pixels) = 161 X 140
Image Size (Scaled) = 5.68 µm X 4.94 µm
Bit Depth = 16 Bit
Image Center Position = X: 77.20 mm, Y: 45.59 mm
Stage Position = X: 77.20 mm, Y: 45.59 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

405 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 215 µm
LSM MBS = Plate MBS 405/730
LSM SBS = SBS SP 505
Laser Wavelength = 405 nm @0.4%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.1
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.15 µs
Frame Time = 17.86 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 401
Emission Wavelength = 422
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
LSM Plus Mode = 8.4 (3D, Auto)

3D Image Processing - Panel A

3D Maximum Projection = Precise
Appearance = Threshold 2% all channels, Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Tone Mapping Enabled
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel C

3D 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)

Panel A - This panel presents a three-dimensional maximum projection view of the total image acquisition, consisting of 121 z-planes captured at a thickness of 3.6 microns. It features the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG, displayed in its default blue color. No image edits were performed, and the maximum projection was generated using Zeiss Zen software. Image acquired using Zeiss LSM980 with Airyscan.

Panel B - This panel demonstrates a single z-plane, specifically the 64th of the 121 collected, to illustrate the structural details of the gamma H2AX aggregate. The color was altered in Zeiss Zen software from blue to white to enhance visualization, and a white box highlights a region of interest for further examination in Panel C.

Panel C - This panel focuses on the Region of Interest from Panel B, specifically examining the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG signal. t employs a volume projection to investigate structural details at super-resolution SIM capabilities, enabling resolution of axial objects down to approximately 100-120 nm in the X and Y dimensions.

The Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG displays what has historically been referred to as "foci" in all samples for both the primary antibody alone and the direct conjugates across various microscopes, ranging from diffraction-limited to super-resolution. However, our findings using super-resolution techniques consistently suggest that the term "foci" may be outdated, as we now observe that the previously unresolved "foci" represent defined structures. As we progress through our patent-pending "step-down" method of super-resolution, culminating in STORM imaging, we observe what appear to be breaks or opposing ends. While we make no biological claims, the evidence strongly supports this hypothesis.

Image Corrections

Panel C - Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M) was pseudo-colored in Zen Software from blue, the default color, to violet.

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

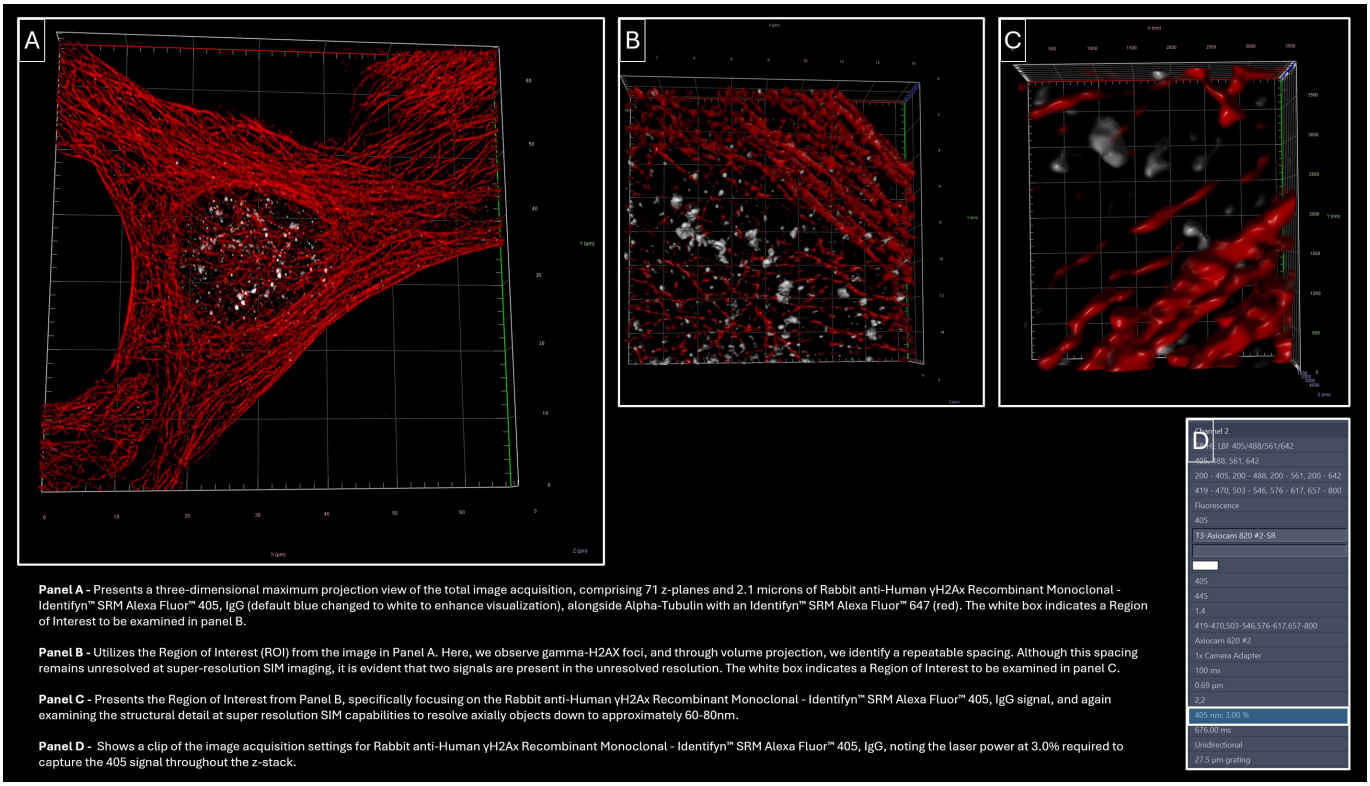
SOURCE PROVENANCE

Catalog	DC-000040
Stage	Airyscan
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	46
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	0F805AD8CAFD7D342C3E3EBC888193E374908DFF7DF43094DD520F6C381F1580
Method PDF SHA-256	84B303B5E4E0A56928B3E208AF6D1E678D5589533D4E8BA4C06E43E46F7D384B

ORIGINAL IMAGE EVIDENCE / SIM



Panel A - Presents a three-dimensional maximum projection view of the total image acquisition, comprising 71 z-planes and 2.1 microns of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG (default blue changed to white to enhance visualization), alongside Alpha-Tubulin with an Identifyn™ SRM Alexa Fluor™ 647 (red). The white box indicates a Region of Interest to be examined in panel B.

Panel B - Utilizes the Region of Interest (ROI) from the image in Panel A. Here, we observe gamma-H2AX foci, and through volume projection, we identify a repeatable spacing. Although this spacing remains unresolved at super-resolution SIM imaging, it is evident that two signals are present in the unresolved resolution. The white box indicates a Region of Interest to be examined in panel C.

Panel C - Presents the Region of Interest from Panel B, specifically focusing on the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG signal, and again examining the structural detail at super resolution SIM capabilities to resolve axially objects down to approximately 60-80nm.

Panel D - Shows a clip of the image acquisition settings for Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG, noting the laser power at 3.0% required to capture the 405 signal throughout the z-stack.

EVIDENCE TIER	SIM
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647
METADATA VALUES	58

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	146 Slices (4.35 µm)
Channels	2
Scaling (Per Pixel)	0.01320 µm X 0.01320 µm X 0.03000 µm
Image Size (Pixels)	5056 X 5056 | 1254 X 1196 | 272 X 298
Image Size (Scaled)	66.72 µm X 66.72 µm | 16.55 µm X 15.78 µm | 3.59 µm X 3.93 µm
Bit Depth	16 Bit
Image Center Position	X: 59.90 mm, Y: 35.40 mm
Stage Position	X: 59.90 mm, Y: 35.40 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	405 | 640
Channel Name	T3-Axiocam 820 #2-SR | T1-Axiocam 820-SR
Excitation Wavelength	405 | 640
Emission Wavelength	445 | 729
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device	Axiocam 820 #2 | Axiocam 820
Camera Adapter	1X
Exposure Time	100 ms
Depth of Focus	0.69 µm | 1.13 µm
Binning Mode	2,2
Laser Wavelength	405 nm @3.00% | 640 nm @2.00%
Frame Time	676.00 ms | 1.33 s
Scan Direction	Unidirectional
Grating	27.5 µm grating | 36.5 µm grating
Processing Dimension	3D
Result Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
Process Sampling	N/A
Algorithm	SIM
SIM Settings	Standard
Auto Sharpness	On
Sharpness	10.0136 (647), 8.5717 (405)
Fitting	Fast Fit
Normalization	Auto
3D Maximum Projection	Precise
Appearance	Threshold 4% all channels, Ramp 0% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Tone Mapping Enabled | 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 Volume 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

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M)
Cat ID# - DC 000040

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Mouse, IgG (H + L)
Cat ID# - SA 000059

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly and explicitly phosphorylated at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), 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 Donkey Anti-Mouse, IgG (H + L) was incubated at room temperature for 1 hour at a dilution of 1:100 . Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant, cat#P36961.

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) - Panels A

Z-Stack = 146 Slices (4.35 µm)

Channels = 2
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: 59.90 mm, Y: 35.40 mm
Stage Position = X: 59.90 mm, Y: 35.40 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B

Z-Stack = 146 Slices (4.35 µm)

Channels = 2
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 1254 X 1196
Image Size (Scaled) = 16.55 µm X 15.78 µm
Bit Depth = 16 Bit
Image Center Position = X: 59.90 mm, Y: 35.40 mm
Stage Position = X: 59.90 mm, Y: 35.40 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel C

Z-Stack = 146 Slices (4.35 µm)

Channels = 2
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 272 X 298
Image Size (Scaled) = 3.59 µm X 3.93 µm
Bit Depth = 16 Bit
Image Center Position = X: 59.90 mm, Y: 35.40 mm
Stage Position = X: 59.90 mm, Y: 35.40 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

405 -

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 = 100 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.00%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 27.5 µm grating

647 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 640
Channel Name = T1-Axiocam 820-SR
Excitation Wavelength = 640
Emission Wavelength = 729
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 100 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @2.00%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM Processing

Processing Dimension = 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 2
Algorithm = SIM
SIM Settings = Standard
Auto Sharpness = On
Sharpness = 10.0136 (647), 8.5717 (405)
Fitting = Fast Fit
Normalization = Auto

3D Image Processing - Panel A

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

3D Image Processing - Panel B

3D Volume Projection = Precise
Appearance = Threshold 4% all channels, Ramp 0% 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 A

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

Panel A - Presents a three-dimensional maximum projection view of the total image acquisition, comprising 71 z-planes and 2.1 microns of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™405, IgG (default blue changed to white to enhance visualization), alongside Alpha-Tubulin with an Identifyn® SRM Alexa Fluor™ 647 (red). The white box indicates a Region of Interest to be examined in panel B.

Panel B - Utilizes the Region of Interest (ROI) from the image in Panel A. Here, we observe gamma-H2AX foci, and through volume projection, we identify a repeatable spacing. Although this spacing remains unresolved at super-resolution SIM imaging, it is evident that two signals are present in the unresolved resolution. The white box indicates a Region of Interest to be examined in panel C.

Panel C - Presents the Region of Interest from Panel B, specifically focusing on the Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG signal, and again examining the structural detail at

super resolution SIM capabilities to resolve axially objects down to approximately 60-80nm.

Panel D - Shows a clip of the image acquisition settings for Rabbit anti-Human γH2Ax Recombinant Monoclonal-Identifyn® SRM Alexa Fluor™ 405, IgG, noting the laser power at 3.0% required to capture the 405 signal throughout the z-stack.

Image Corrections

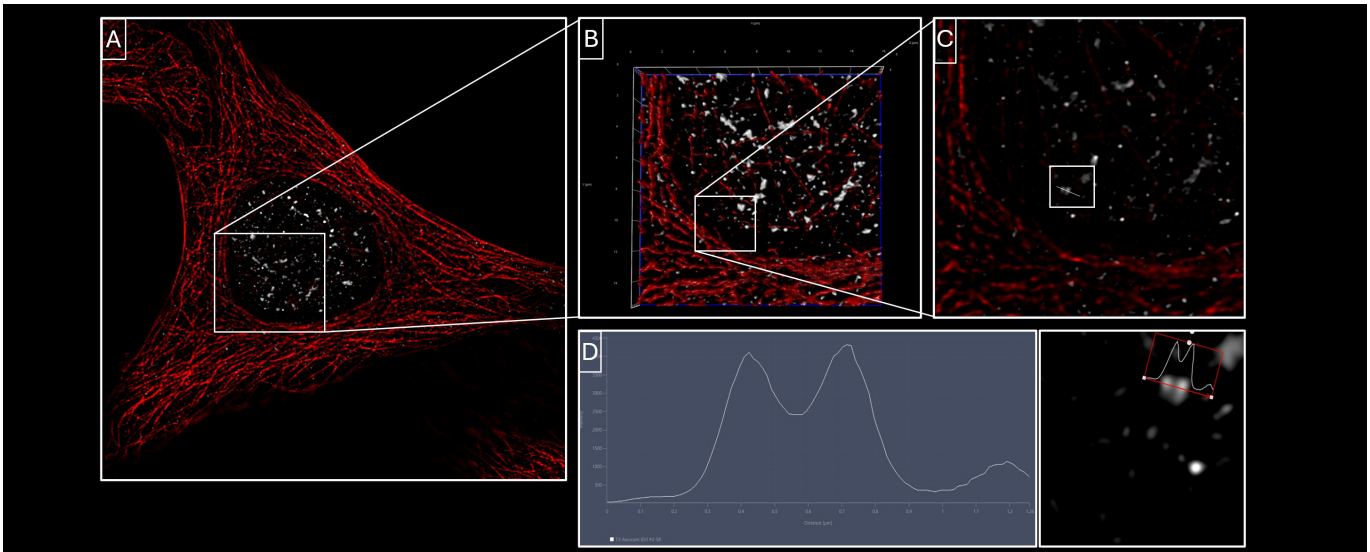
Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M) was pseudo-colored in Zen Software from blue, the default color, to white to provide visual contrast.

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

SOURCE PROVENANCE

Catalog	DC-000040
Stage	SIM
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	58
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	7A1C3408183D10DF42FBE13DA2E6D71F85211053C34A9B7CAB9BB478EDDEF4C2
Method PDF SHA-256	0D336CF1AE3A5C605FEE4CDD14458F01CCFE6F34D898C560202B89E526D582E6

ORIGINAL IMAGE EVIDENCE / SIM²



Panel A - Presents a two-dimensional view of the image acquisition at z-plane 71 of 146 of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG (default blue changed to white to enhance visualization), alongside Alpha-Tubulin with an Identifyn™ SRM Alexa Fluor™ 647 (red). The white box indicates a Region of Interest to be examined in panel B.

Panel B - Utilizes the Region of Interest (ROI) from the image in Panel A. Here, we observe gamma-H2AX foci, and through maximum projection and we identify a repeatable aggregate formation at the site of Etoposide induced DNA damage and with the use of super resolution SIM2, we observe better detail of the signal. Although this spacing remains unresolved at super-resolution SIM2 imaging, it is evident that two signals are present in the unresolved resolution, as in STORM, where we see two closely separated ends of the signal. The white box indicates a Region of Interest to be examined in panel C.

Panel C - Presents a two-dimensional view of the image acquisition again, as in panel A, at z-plane 71 of 146 of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn™ SRM Alexa Fluor™ 405, IgG (default blue changed to white to enhance visualization) with a white box, with a line showing the area to be examined in panel D via the use of the line profile tool within Zeiss Zen software.

Panel D - Shows a line profile we have selected that crosses two unresolved visual aggregates. The line profile histogram reveals two separate signals (the two peaks); however, as one can see, they are again unresolved at super resolution SIM2, approximately 40nm X, Y.

EVIDENCE TIER	SIM²
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647; 680
METADATA VALUES	60

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	146 Slices (4.35 µm)
Channels	2
Scaling (Per Pixel)	0.01320 µm X 0.01320 µm X 0.03000 µm
Image Size (Pixels)	5056 X 5056 | 1220 X 1188 | 287 X 347
Image Size (Scaled)	66.72 µm X 66.72 µm | 16.60 µm X 15.68 µm | 3.79 µm X 4.58 µm
Bit Depth	16 Bit
Image Center Position	X: 59.90 mm, Y: 35.40 mm
Stage Position	X: 59.90 mm, Y: 35.40 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	405 | 680
Channel Name	T3-Axiocam 820 #2-SR | T1-Axiocam 820-SR
Excitation Wavelength	405 | 640
Emission Wavelength	445 | 729
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device	Axiocam 820 #2 | Axiocam 820
Camera Adapter	1X
Exposure Time	100 ms
Depth of Focus	0.69 µm | 1.13 µm
Binning Mode	2,2
Laser Wavelength	405 nm @3.00% | 640 nm @2.00%
Frame Time	676.00 ms | 1.33 s
Scan Direction	Unidirectional
Grating	27.5 µm grating | 36.5 µm grating
Result Sampling	4
Process Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
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 Volume Projection	Precise
Appearance	Threshold 4% all channels, Ramp 0% 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)
Z-Position	71 of 146
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 1258.1), Y (Min 11 and Max 4538)

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.

Bennett Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M)
Cat ID# - DC 000040

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Mouse, IgG (H + L)
Cat ID# - SA 000059

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly and explicitly phosphorylated at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), 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 Donkey Anti-Mouse, IgG (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant,c at#P36961.

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 71 of 146 total - Panel A

Z Stack - (3D)

Z-Stack = 146 Slices (4.35 µm)

Channels = 2
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: 59.90 mm, Y: 35.40 mm
Stage Position = X: 59.90 mm, Y: 35.40 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B

Z-Stack = 146 Slices (4.35 µm)

Channels = 2
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 1220 X 1188
Image Size (Scaled) = 16.60 µm X 15.68 µm
Bit Depth = 16 Bit
Image Center Position = X: 59.90 mm, Y: 35.40 mm
Stage Position = X: 59.90 mm, Y: 35.40 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Single Plane (2D) - Z-plane 71 of 146 total - Panel C

Z Stack - (3D)

Z-Stack = 146 Slices (4.35 µm)

Channels = 2
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 287 X 347
Image Size (Scaled) = 3.79 µm X 4.58 µm
Bit Depth = 16 Bit
Image Center Position = X: 59.90 mm, Y: 35.40 mm
Stage Position = X: 59.90 mm, Y: 35.40 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

405 -

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 = 100 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.00%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 27.5 µm grating

647 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 680
Channel Name = T1-Axiocam 820-SR
Excitation Wavelength = 640
Emission Wavelength = 729
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 100 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @2.00%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM² Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = 4
Channels = 2
Algorithm = SIM²
SIM² Settings = Standard
Input SNR = Low
Regularization = None
Regularization Weight = 0.0000
Iterations = 3
Filter = No Filter
Fitting = Fast Fit
Normalization = Auto

3D Image Processing - Panel B

3D Volume Projection = Precise
Appearance = Threshold 4% all channels, Ramp 0% 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)

Profile View Display - Panel D

Z-Position = 71 of 146
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 1258.1), Y (Min 11 and Max 4538)

Summary

Panel A - Presents a two-dimensional view of the image acquisition at z-plane 71 of 146 of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG (default blue changed to white to enhance visualization), alongside Alpha-Tubulin with an Identifyn® SRM Alexa Fluor™ 647 (red). The white box indicates a Region of Interest to be examined in panel B.

Panel B - Utilizes the Region of Interest (ROI) from the image in Panel A. Here, we observe gamma-H2AX foci, and through maximum projection, we identify a repeatable aggregate formation at the site of Etoposide-induced DNA damage, and with the use of super resolution SIM², we observe better detail of the signal. Although this spacing remains unresolved at super-resolution SIM² imaging, it is evident that two signals are present in the unresolved resolution, as in STORM, where we see two closely separated ends of the signal. The white box indicates a Region of Interest to be examined in panel C.

Panel C - Presents a two-dimensional view of the image acquisition again, as in panel A, at z-plane 71 of 146 of Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG (default blue changed to white to enhance visualization) with a white box showing the area to be examined in panel D via the use of the line profile tool within Zeiss Zen software.

Panel D - Shows a line profile we have selected that crosses two unresolved visual aggregates. The line profile histogram reveals two separate signals (the two peaks); however, as one can see, they are again unresolved at super resolution SIM², approximately 40nm X, Y.

Image Corrections

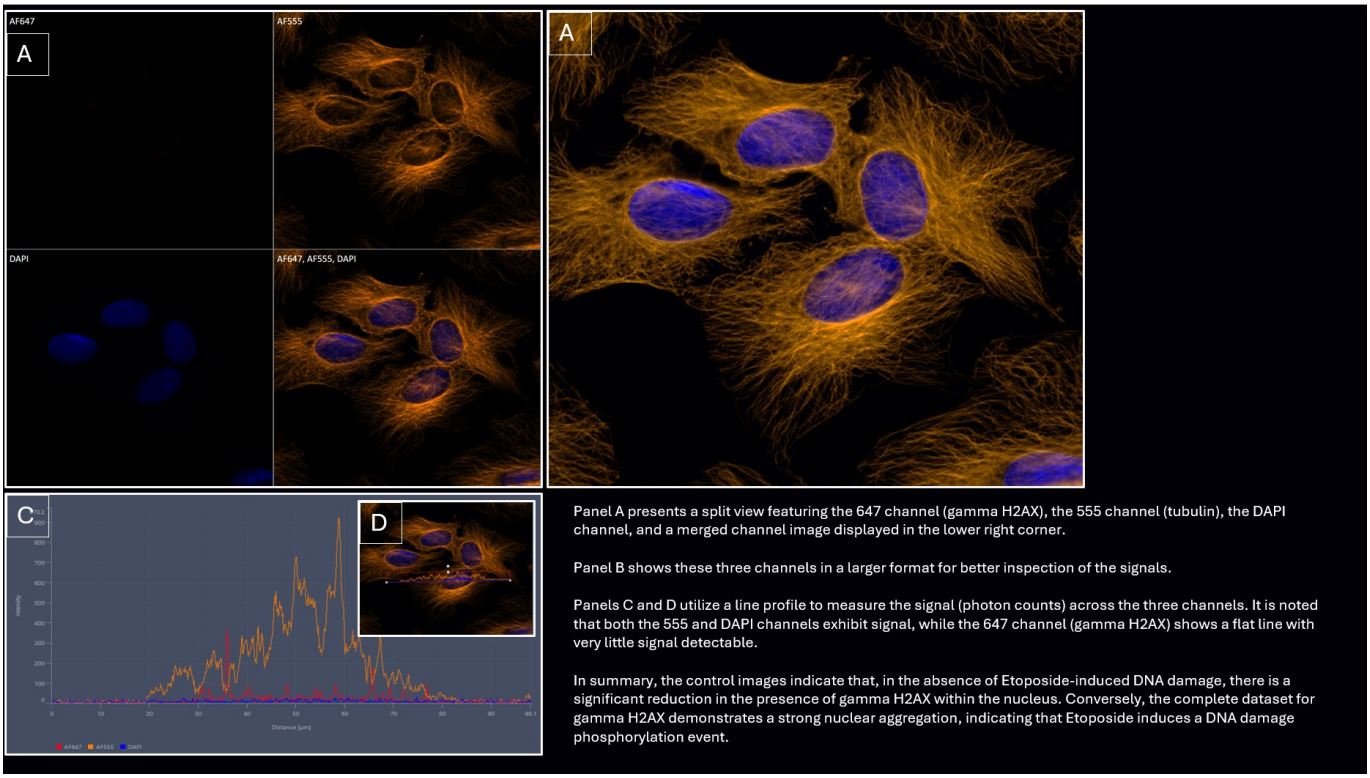
Rabbit anti-Human γH2Ax Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 405, IgG - (BLR014M) was pseudo-colored in Zen Software from blue, the default color, to white to provide visual contrast.

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

SOURCE PROVENANCE

Catalog	DC-000040
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	60
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	DAF7ECFC3A1DEF1AE17DAD6F868CA08F77ACBF7AC861C4A772E49F1F92D354B1
Method PDF SHA-256	1592EB700EDEC4A1888DD1821ED59BDC50688735627B33009236912F7F46CEEF

ORIGINAL IMAGE EVIDENCE / CONTROL



EVIDENCE TIER	Control
INSTRUMENT	ZEISS Axio Observer.Z1/7 Apotome
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 555; 647
METADATA VALUES	40

STRUCTURED ACQUISITION METADATA / CONTROL

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
Channels	2
Scaling (Per Pixel)	0.143µm X 0.143µm
Image Size (Pixels)	963 X 855
Image Size (Scaled)	135.57µm X 122.14µm
Bit Depth	14 Bit
Image Center Position	X: 75.74mm, Y: 43.47mm
Stage Position	X: 75.74mm, Y: 43.47mm
ROI Center Offset	X: 1.14µm, Y: 0.00µm
Reflector	50 Cy5 | AF532-49014 | 49 DAPI
Beam Splitter	660 | 550 | 395
Filter Excitation Wavelength	625 - 655 | 515 - 545 | 335 - 383
Filter Emission Wavelength	665 - 715 | 555 -595 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @8.0% | X-Cite Xylis Lamp Intensity @6.0%
Exposure Time	150ms | 50ms | 20ms
Excitation Wavelength	663 | 553 | 353
Emission Wavelength	668 | 568 | 465
Effective NA	1.25
Imaging Device	Axiocam 807 mono
Camera Adapter	1X
Depth of Focus	1.30µm | 1.10µm | 0.90µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mode “Optical Sectioning" - enabled correction selected.

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

Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, F(ab')□ (H + L)
Cat ID# - SA 000065

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, F(ab')□ (H + L)
Cat ID# - SA 000040

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly explicitly phosphorylated at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

DNA damage was NOT induced.

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, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS, and Identifyn® 647 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 555 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Microscope

Zeiss 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)

Channels = 2
Scaling (Per Pixel) = 0.143µm X 0.143µm
Image Size (Pixels) = 963 X 855
Image Size (Scaled) = 135.57µm X 122.14µm
Bit Depth = 14 Bit
Image Center Position = X: 75.74mm, Y: 43.47mm
Stage Position = X: 75.74mm, Y: 43.47mm
ROI Center Offset = X: 1.14µm, Y: 0.00µm

647 -

Reflector = 50 Cy5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @8.0%
Exposure Time = 150ms
Excitation Wavelength = 663
Emission Wavelength = 668
Effective NA = 1.25
Imaging Device = Axiocam 807 mono
Camera Adapter = 1X
Depth of Focus = 1.30µm
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 @6.0%
Exposure Time = 50ms
Excitation Wavelength = 553
Emission Wavelength = 568
Effective NA = 1.25
Imaging Device = Axiocam 807 mono
Camera Adapter = 1X
Depth of Focus = 1.10µm
Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @6.0%
Exposure Time = 20ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.25
Imaging Device = Axiocam 807 mono
Camera Adapter = 1X
Depth of Focus = 0.90µm
Binning Mode = 2,2

Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mode “Optical Sectioning" - enabled correction selected.

Normalization, Phase Correction, Fourier Filter, and Deconvolution were NOT used.

Summary

Panel A presents a split view featuring the 647 channel (gamma H2AX), the 555 channel (tubulin), the DAPI channel, and a merged channel image displayed in the lower right corner. Panel B shows these three channels in a larger format for better inspection of the signals. Panels C and D utilize a line profile to measure the signal (photon counts) across the three channels. It is noted that both the 555 and DAPI channels exhibit signal, while the 647 channel (gamma H2AX) shows a flat line with minimal signal detectable.

In summary, the control images indicate that, in the absence of Etoposide-induced DNA damage, there is a significant reduction in the presence of gamma H2AX within the nucleus. Conversely, the complete dataset for gamma H2AX demonstrates a strong nuclear aggregation, indicating that Etoposide induces a DNA damage phosphorylation event.

Image Corrections

None

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

SOURCE PROVENANCE

Catalog	DC-000040
Stage	Control
Instrument	ZEISS Axio Observer.Z1/7 Apotome

SOURCE PROVENANCE

Microscope configuration	Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Structured source metadata values	40
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	693352B829D585A85341B417AF9FEE694B830498561FDCF68DF1D48921F873C9
Method PDF SHA-256	662B365ABA686B879FA42AA20C5F118FE9468709E00A7C467FFBA6F6749AA72B