

IDENTIFYN RESOLUTION STEPDOWN

H2AX

MICROSCOPY EVIDENCE ASSESSMENT

Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc

WESTERN BLOT -> WIDEFIELD -> WIDEFIELD SIM / APOTOME -> CONFOCAL -> AIRYSCAN
SIM -> SIM² -> SINGLE-MOLECULE STORM

8

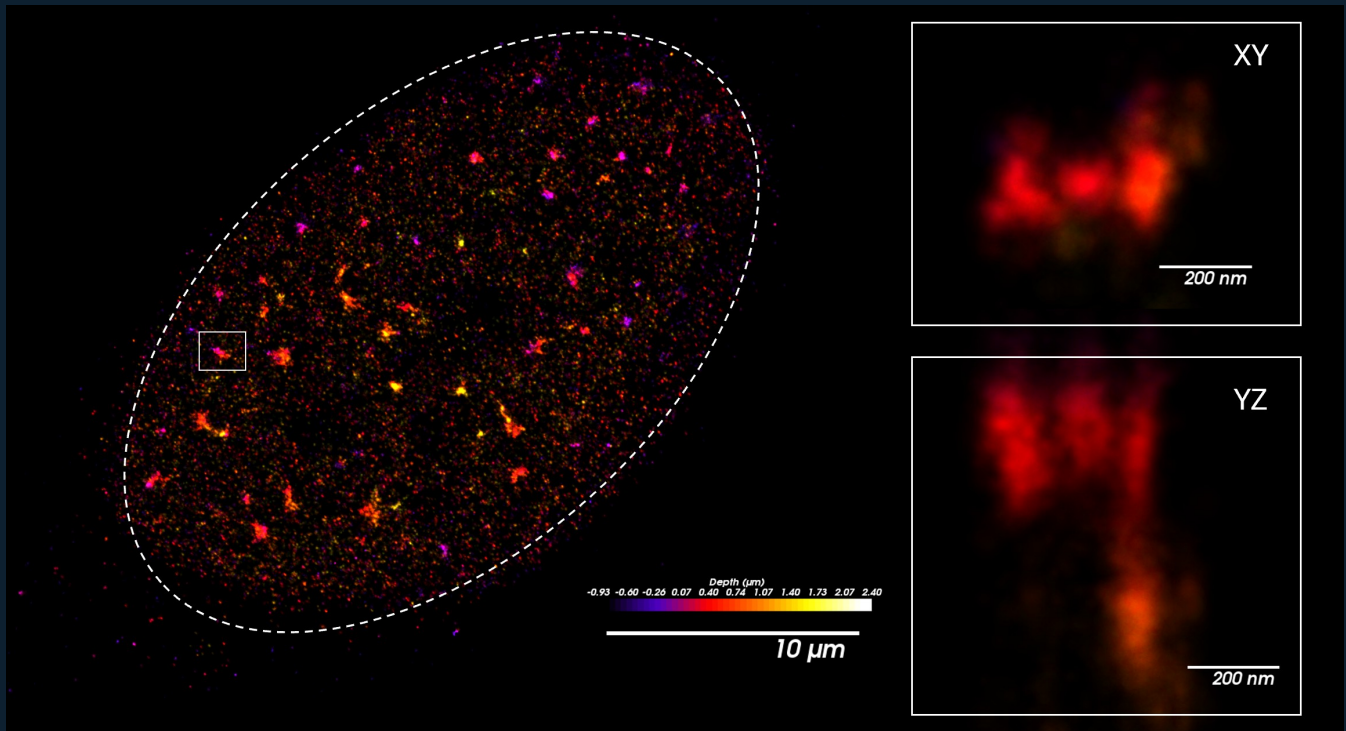
ORDERED EVIDENCE TIERS

1

SUPPORTING CONTROL

PENDING

SCIENTIST REVIEW



REPRESENTATIVE HIGHEST-AVAILABLE VIEW / SINGLE-MOLECULE STORM

PA-RR-000005 | 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
- Single-molecule STORM presentation 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 -> Single-molecule STORM
-> Control

BENNETT ASSESSMENT

The preserved PA-RR-000005 record contains the full ordered evidence sequence from Western blot through single-molecule STORM. Each modality retains its own acquisition and processing boundary.

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 / FULL STEPDOWN / SOURCE-BOUNDED REVIEW

The preserved PA-RR-000005 record contains the full ordered evidence sequence from Western blot through single-molecule STORM. Each modality retains its own acquisition and processing boundary.

SETTINGS ASSESSMENT

The record preserves 8 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	488	Not deterministically stated in the acquisition block
Widefield	405/DAPI; 647	2; 50 Cy 5; 49 DAPI; 625 - 655; 335 - 383; 665 - 715; 420 - 470; 653
Widefield SIM — Apotome	405/DAPI; 647	2; 50 Cy 5; 49 DAPI; 625 - 655; 335 - 383; 665 - 715; 420 - 470; 653
Confocal	405/DAPI; 488; 555; 647	3; None; 639 nm @0.02%; 561 nm @0.007%; 405 nm @0.2%; 653; 553; 353
Airyscan	405/DAPI; 488; 555; 647; 750	3; None; 639 nm @0.2%; 488 nm @0.02%; 405 nm @0.2%; 653; 493; 353
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; T1-Axiocam 820-SR; T3-Axiocam 820 #2-SR; 640; 405
SIM ²	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; T1-Axiocam 820-SR; T3-Axiocam 820 #2-SR; 640; 405
Single-molecule STORM	647	Both Channels
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 9

Western blot / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

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

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as Western blot, documents platform context as Azure Sapphire FL, and records detected dye/channel descriptors as 488.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 2 OF 9

Widefield / 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): 0.143 μm X 0.143 μm ; Image size (Pixels): 919 X 805; Image Size (Pixels): 919 X 805; Bit Depth: 14 Bit. Detector/camera: Imaging Device: 807 mono. Timing: Exposure Time: 200 ms; 12 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @20.00%; X-Cite Xylis Lamp Intensity @6.00%. Optical/scan: Effective NA: 1.25. Structured source metadata values: 40.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

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

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 3 OF 9

Widefield SIM — Apotome / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Axio Observer.Z1/7 Apotome. Objective: EC Plan-Neofluar 63X/1.25 oil M27. Platform: Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:. Acquisition: Z-Stack: 86 Slices (8.5 μm); Channels: 2; Scaling (Per Pixel): 0.143 μm X 0.143 μm X 0.100 μm ; Image size (Pixels): 1283 X 860; Image Size (Pixels): 1283 X 860; Bit Depth: 14 Bit. Detector/camera: Imaging Device: 807 mono. Timing: Exposure Time: 150 ms; 25 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @15.00%; X-Cite Xylis Lamp Intensity @10.00%. Optical/scan: Effective NA: 1.25. 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; 647.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 4 OF 9

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: 147 Slices (4.38 μm); Channels: 3; Scaling (Per Pixel): 0.030 μm X 0.030 μm X 0.030 μm ; Image size (Pixels): 1476 X 1476; Image Size (Pixels): 1476 X 1476; Bit Depth: 16 Bit. Detector/camera: Imaging Device: GaAsP-Pmt3; Detector Type: GaAsP-PMT. Timing: Pixel Time: 0.23 μs ; Frame Time: 4.84 s. Illumination: Laser Wavelength: 639 nm @0.02%; 561 nm @0.007%. Optical/scan: Pinhole: 1.00 AU / 64 μm ; 1.00 AU / 57 μm ; Scan Zoom: 3.0; LSM Scan Speed: 12; Scan Direction: Bidirectional; Averaging: 8; Effective NA: 1.4. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black; LSM Plus Mode: 7.9 (3D, Auto); 7.4 (3D, Auto). Structured source metadata values: 66.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as Confocal, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 555; 647.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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 9

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: 98 Slices (2.91 μm); Channels: 3; Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm; Image size (Pixels): 837 X 805; 560 X 457; Image Size (Pixels): 837 X 805; 560 X 457; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; Detector Type: GaAsP-PMT. Timing: Pixel Time: 1.10 μs ; Frame Time: 18.77 s. Illumination: Laser Wavelength: 639 nm @0.2%; 488 nm @0.02%. Optical/scan: Pinhole: 5.00 AU / 328 μm ; 5.00 AU / 261 μm ; Scan Zoom: 2.0; 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: 9.7 (3D, Auto); 9.2 (3D, Auto). Structured source metadata values: 66.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as Airyscan, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 555; 647; 750.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 6 OF 9

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: 154 Slices (4.59 μ m); Channels: 2; Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm; Image size (Pixels): 1801 X 2570; 321 X 198; Image Size (Pixels): 1801 X 2570; 321 X 198; Bit Depth: 16 Bit. Detector/camera: Imaging Device: AxioCam 820; AxioCam 820 #2. Timing: Exposure Time: 120 ms; 50 ms; Frame Time: 1.59 s; 676.00 ms. Illumination: Laser Wavelength: 640 nm @2.00%; 405 nm @6.00%; Illumination Wavelength: 640; 405. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM; Process Sampling: N/A; Result Sampling: 4; Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); View Angle 10°, Scale Z 15%, 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 9

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: 154 Slices (4.59 μ m); 54 Slices (1.59 μ m); Channels: 2; Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm; Image size (Pixels): 1805 X 2627; 163 X 192; Image Size (Pixels): 1805 X 2627; 163 X 192; Bit Depth: 16 Bit. Detector/camera: Imaging Device: AxioCam 820; AxioCam 820 #2. Timing: Exposure Time: 120 ms; 50 ms; Frame Time: 1.59 s; 676.00 ms. Illumination: Laser Wavelength: 640 nm @2.00%; 405 nm @6.00%; Illumination Wavelength: 640; 405. 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: 61.

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.

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 9

Single-molecule STORM / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: Bruker Vutara VXL. Objective: Olympus 60X/1.30 silicon oil objective. Platform: Bruker Vutara VXL Biplane Single Molecule Microscope, using an Olympus 60X/1.30 silicon oil objective, was used with the following parameters:.

Detector/camera: Camera: Both Cameras. Timing: Exposure Time: 20ms Selected; Super Resolution Camera Exposure Time: 20ms; Widefield Camera Exposure Time: 100ms. Illumination: Photoactivation/Imaging Laser: 0% Selected.

Structured source metadata values: 80.

VISIBLE OBSERVATION

A preserved presentation image is available for Single-molecule STORM. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Single-molecule STORM, documents platform context as Bruker Vutara VXL, and records detected dye/channel descriptors as 647.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

Without localization tables, uncertainty, blinking, drift, and independent cluster evidence, the presentation cannot establish molecule count, cluster truth, exact precision, or molecular architecture.

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 9 OF 9

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 Xylys 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 Xylys Lamp Intensity @8.0%; X-Cite Xylys 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 PA-RR-000005 record contains the full ordered evidence sequence from Western blot through single-molecule STORM. Each modality retains its own acquisition and processing boundary.

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

BENNETT LITERATURE CORRELATION — SOURCES INDEPENDENTLY VERIFIED

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

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

REFERENCES

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

RELEASE BOUNDARY

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

SOURCE RECONCILIATION / QA / PROVENANCE

SOURCE STATUS - PRESERVED / AUTO-RECONCILED

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

HIGH CONFIDENCE - AUTO-RECONCILED

Airyscan / Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm -> 0.03530 μ m X 0.03530 μ m X 0.03000 μ m. 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)=837 X 805; Image Size (Scaled)=29.54 μ m X 28.41 μ m; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.02%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM / Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm -> 0.01320 μ m X 0.01320 μ m X 0.03000 μ m. 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)=1801 X 2570; Image Size (Scaled)=23.76 μ m X 33.91 μ m; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.06%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM² / Scaling (Per Pixel): 13.20 nm X 13.20 nm X 30.00 nm -> 0.01320 μ m X 0.01320 μ m X 0.03000 μ m. 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)=1805 X 2627; Image Size (Scaled)=23.82 μ m X 34.66 μ m; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.05%.

HIGH CONFIDENCE - SCIENTIST-AUTHORIZED CORRECTION

Cross-tier / treatment concentration: 200 μ M; 2 μ M -> Etoposide = 2 μ M. The governing scientist identified every 200 μ M occurrence as a technician transcription error. The canonical historical concentration is 2 μ M. 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 γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc

BENNETT EVIDENCE REVIEW COMPLETE

INTERPRETATION TO EVIDENCE

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



IDENTIFYN PRESERVED SOURCE EVIDENCE

ORIGINAL RECORD BEGINS

Images, source methods, acquisition settings and provenance follow in the locked
Stepdown order. Scientific wording is preserved; missing modalities are not invented.
A preserved control follows all available Stepdown tiers as supporting evidence.

PRODUCT-LEVEL STEPDOWN MAP

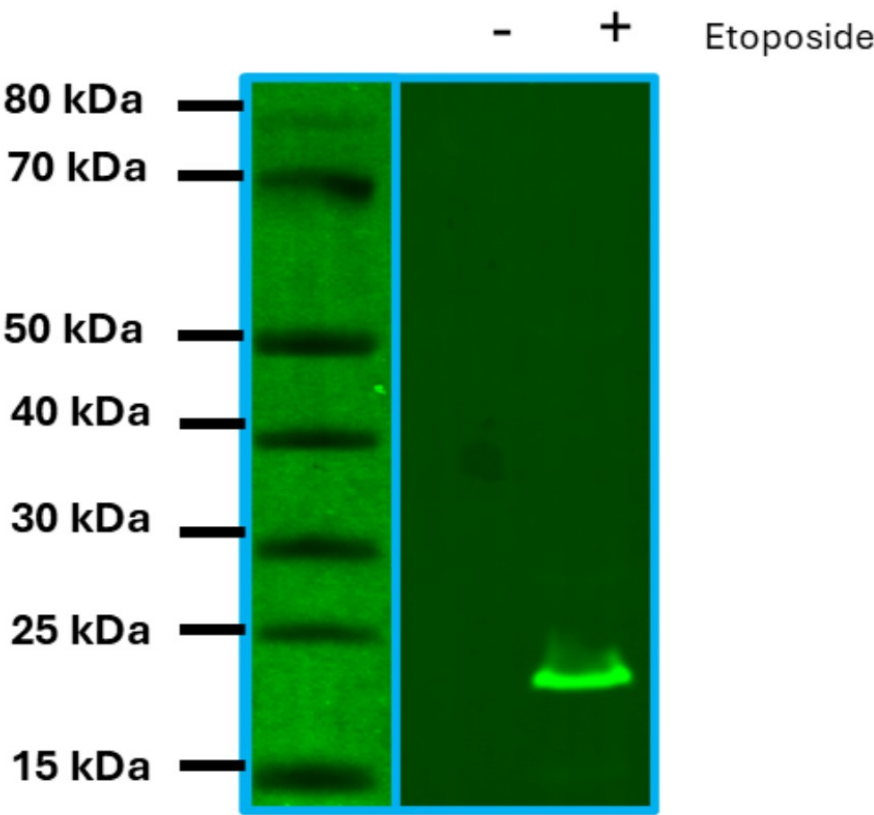
This dossier preserves the complete available evidence progression for PA-RR-000005. Each tier remains tied to its original image, source method, instrument context and cryptographic identity. A preserved control is retained as supporting evidence and appears after every resolution tier.

CANONICAL STEPDOWN	EVIDENCE STAGE	INSTRUMENT	STATUS
1	Western blot	Azure Sapphire FL	PRESERVED
2	Widefield	ZEISS Axio Observer.Z1/7 Apotome	PRESERVED
3	Widefield SIM - Apotome	ZEISS Axio Observer.Z1/7 Apotome	PRESERVED
4	Confocal	ZEISS LSM 980	PRESERVED
5	Airyscan	ZEISS LSM 980	PRESERVED
6	SIM	ZEISS Elyra	PRESERVED
7	SIM ²	ZEISS Elyra	PRESERVED
8	Single-molecule STORM	Bruker Vutara VXL	PRESERVED

INTERPRETIVE BOUNDARY

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

ORIGINAL IMAGE EVIDENCE / WESTERN BLOT



EVIDENCE TIER	Western blot
INSTRUMENT	Azure Sapphire FL
CELL MODEL	HeLa
DETECTED DYES	488
METADATA VALUES	11

STRUCTURED ACQUISITION METADATA / WESTERN BLOT

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Scanner	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Detection mode	Fluorescence
Laser	488nm
Emission Filter	513±17nm
Detector	PMT
Intensity	2
Pixel size	100 µm
Scan Speed	High
Quality	High
Focus	Membrane (0 µm in Z-axis)
Image	K. Small

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG2a

Cat ID - PA-RR 000005

Expected MW: 15 kDa

HeLa cells were treated with 200 μ M etoposide for 24 hours to induce DNA damage Cells were then homogenized using an ultrasonication probe and RIPA lysis buffer and then centrifuged to pellet the cell debris The lysate supernatant (100 μ g) 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 ThermoFisher's PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, was used as a molecular weight marker.

Post electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot™ 3 Starter Kit PVDF The transfer membrane was blocked with Blocker™ Casein for 1 hour and then washed five times with PBS The blocked membrane was incubated with Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a, for 2 hours, followed by five washes in 1X PBS Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, IgG (H + L) was incubated for 1 hour and washed 5X with PBS The membrane was dried for 10 minutes at room temperature before imaging.

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

Scan Settings

Detection mode = Fluorescence

Laser = 488nm

Emission Filter = 513 $\pm$ 17nm

Detector = PMT

Intensity = 2

Pixel size = 100 μ m

Scan Speed = High

Quality = High

Focus = Membrane (0 μ m in Z-axis)

Post Processing

None

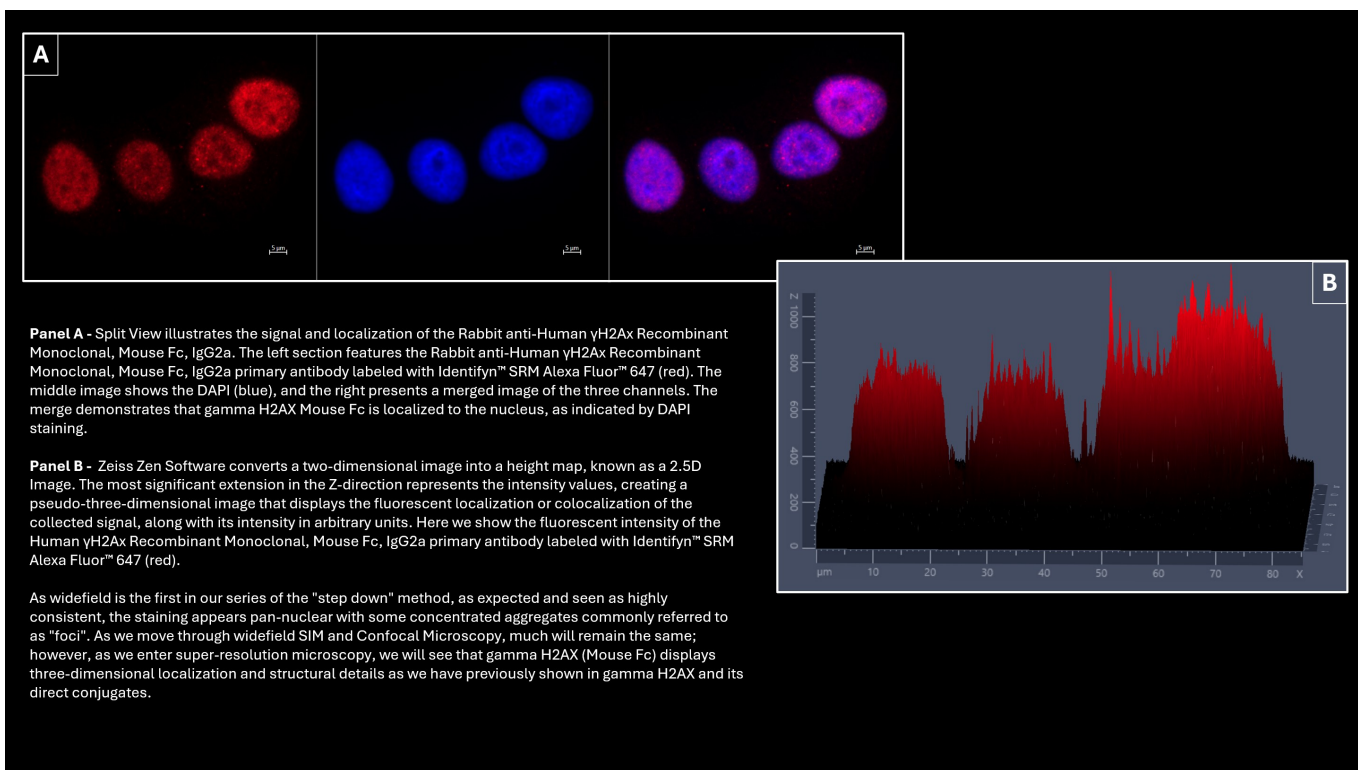
Image: K. Small

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SOURCE PROVENANCE

Catalog	PA-RR-000005
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	D1A6CDC882F6726BCD314D24689B41D422602B8AA0616A3D036F0B36AC9F9B04
Method PDF SHA-256	37622E566B119E3F49ADE41414D3C3B0A5C1CE81BEA535BA93E333889049FA27

ORIGINAL IMAGE EVIDENCE / WIDEFIELD



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

STRUCTURED ACQUISITION METADATA / WIDEFIELD

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Axio 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)	0.143 μ m X 0.143 μ m
Image size (Pixels)	919 X 805
Image Size (Scaled)	131.29 μ m X 115.00 μ m
Bit Depth	14 Bit
Image Center Position	X: 62.87 μ m, Y: 42.95 μ m
Stage Position	X: 62.86 μ m, Y: 42.95 μ m
ROI Center Offset	X: 1.14 μ m, Y: 0.00 μ m
Reflector	50 Cy 5 49 DAPI
Beam Splitter	660 395
Filter Excitation Wavelength	625 - 655 335 - 383
Filter Emission Wavelength	665 - 715 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @20.00% X-Cite Xylis Lamp Intensity @6.00%
Exposure Time	200 ms 12 ms
Excitation Wavelength	653 353
Emission Wavelength	668 465
Effective NA	1.25
Imaging Device	807 mono
Camera Adapter	1X
Depth of Focus	1.30 μ m 0.90 μ m
Binning Mode	2,2
Render Mode	Surface
Grid distance	1
Angle X	63
Angle Y	-0
Z Scaling	1.0
Ambient	50
Specular	20
Shininess	50
Light Height	2.0

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)

Cat ID# - SA 000062

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 μ M Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (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 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) = 0.143 μ m X 0.143 μ m

Image size (Pixels) = 919 X 805

Image Size (Scaled) = 131.29 μ m X 115.00 μ m

Bit Depth = 14 Bit

Image Center Position = X: 62.87 μ m, Y: 42.95 μ m

Stage Position = X: 62.86 μ m, Y: 42.95 μ m

ROI Center Offset = X: 1.14 μ m, Y: 0.00 μ m

647 -

Reflector = 50 Cy 5
 Beam Splitter = 660
 Filter Excitation Wavelength = 625 - 655
 Filter Emission Wavelength = 665 - 715
 Contrast Method = Fluorescence
 Light source = X-Cite Xylis Lamp Intensity @20.00%
 Exposure Time = 200 ms
 Excitation Wavelength = 653
 Emission Wavelength = 668
 Effective NA = 1.25
 Imaging Device = 807 mono
 Camera Adapter = 1X
 Depth of Focus = 1.30 μ m
 Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
 Beam Splitter = 395
 Filter Excitation Wavelength = 335 - 383
 Filter Emission Wavelength = 420 - 470
 Contrast Method = Fluorescence
 Light source = X-Cite Xylis Lamp Intensity @6.00%
 Exposure Time = 12 ms
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.25
 Imaging Device = 807 mono
 Camera Adapter = 1X
 Depth of Focus = 0.90 μ m
 Binning Mode = 2,2

Split View - Panel A

The first panel on the left features Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M) visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(a'')₂ (H + L), the middle panel features DAPI nuclear staining, and the last panel on the right features the merged image of all channels.

2.5D Image Processing

Zeiss Zen Software converts a two-dimensional image into a height map, known as a 2.5D Image. The most significant extension in the Z-direction represents the intensity values, creating a pseudo-three-dimensional image that displays the fluorescent localization or colocalization of the collected signal, along with its intensity in arbitrary units. Here we show the fluorescent intensity of the Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red)

2.5D Display

Render Mode = Surface

Grid distance = 1
 Palette and Show Faces at Side are both Selected

2.5D Display Options

Angle X = 63
 Angle Y = -0
 Z Scaling = 1.0
 Ambient = 50
 Specular = 20
 Shininess = 50
 Light Height = 2.0

Summary

Panel A - Split View illustrates the signal and localization of the Rabbit anti-Human γ H2AX Recombinant Monoclonal, Mouse Fc, IgG2. The left section features the Rabbit anti-Human γ H2AX Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red). The middle image shows the DAPI (blue), and the right presents a merged image of the three channels. The merge demonstrates that gamma H2AX Mouse Fc is localized to the nucleus, as indicated by DAPI staining.

Panel B - Zeiss Zen Software converts a two-dimensional image into a height map, known as a 2.5D Image. The most significant extension in the Z-direction represents the intensity values, creating a pseudo-three-dimensional image that displays the fluorescent localization or colocalization of the collected signal, along with its intensity in arbitrary units. Here we show the fluorescent intensity of the Human γ H2AX Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red).

As widefield is the first in our series of the "step down" method, as expected and seen as highly consistent, the staining appears pan-nuclear with some concentrated aggregates commonly referred to as "foci. As we move through widefield SIM and Confocal Microscopy, much will remain the same; however, as we enter super-resolution microscopy, we will see that gamma H2AX (Mouse Fc) displays three-dimensional localization and structural details, as we have previously shown in gamma H2AX and its direct conjugates.

Image Correction

None

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

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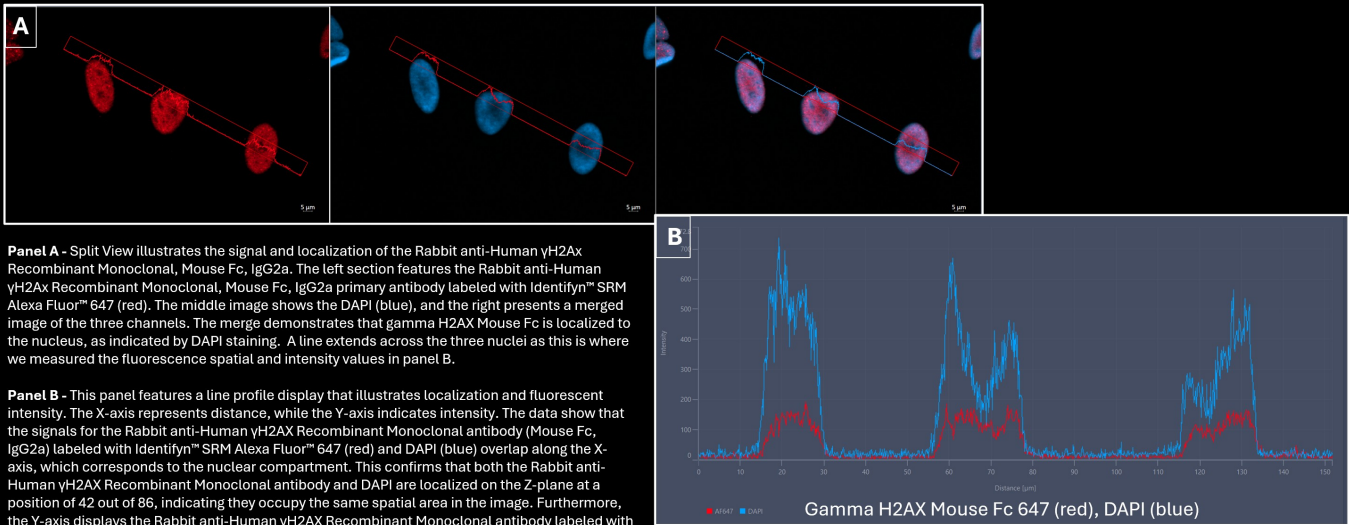
SOURCE PROVENANCE

Catalog	PA-RR-000005
Stage	Widefield

SOURCE PROVENANCE

Instrument	ZEISS Axio Observer.Z1/7 Apotome
Microscope configuration	Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Structured source metadata values	40
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	70DD3E9F621290792E23636CC2C9E4A06F8E8DC6711AB942858FA0EE5DB4393E
Method PDF SHA-256	1649AC2784221A4C2B65C4909B790BE5BCE74D07B968D0AA4C27DC9CE2443415

ORIGINAL IMAGE EVIDENCE / WIDEFIELD SIM - APOTOME



EVIDENCE TIER	Widefield SIM - Apotome
INSTRUMENT	ZEISS Axio Observer.Z1/7 Apotome
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 647
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 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Objective	EC Plan-Neofluar 63X/1.25 oil M27
Z-Stack	86 Slices (8.5 µm)
Channels	2
Scaling (Per Pixel)	0.143 µm X 0.143 µm X 0.100 µm
Image Size (Pixels)	1283 X 860
Image Size (Scaled)	183.29 µm X 122.86 µm
Bit Depth	14 Bit
Image Center Position	X: 59.86 mm, Y: 44.39 mm
Stage Position	X: 59.86 mm, Y: 44.39 mm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflector	50 Cy 5 49 DAPI
Beam Splitter	660 395
Filter Excitation Wavelength	625 - 655 335 - 383
Filter Emission Wavelength	665 - 715 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @15.00% X-Cite Xylis Lamp Intensity @10.00%
Exposure Time	150 ms 25 ms
Excitation Wavelength	653 353
Emission Wavelength	668 465
Effective NA	1.25
Imaging Device	807 mono
Camera Adapter	1X
Depth of Focus	1.30 µm 0.90 µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mod" "Optical Sectioning" - enabled correction selected.
Z-Position	42 of 86
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 151.8), Y (Min 0 and Max 1718)

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 γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)

Cat ID# - SA 000062

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 μ M Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (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 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) - Z-plane 42 of 86 total - Panel A

Z Stack - (3D)

Z-Stack = 86 Slices (8.5 μ m)

Channels = 2

Scaling (Per Pixel) = 0.143 μ m X 0.143 μ m X 0.100 μ m

Image Size (Pixels) = 1283 X 860

Image Size (Scaled) = 183.29 μ m X 122.86 μ m

Bit Depth = 14 Bit

Image Center Position = X: 59.86 mm, Y: 44.39 mm

Stage Position = X: 59.86 mm, Y: 44.39 mm

ROI Center Offset = X: 1.14 μ m, Y: 0.00 μ m

647 -

Reflector = 50 Cy 5
 Beam Splitter = 660
 Filter Excitation Wavelength = 625 - 655
 Filter Emission Wavelength = 665 - 715
 Contrast Method = Fluorescence
 Light source = X-Cite Xylis Lamp Intensity @15.00%
 Exposure Time = 150 ms
 Excitation Wavelength = 653
 Emission Wavelength = 668
 Effective NA = 1.25
 Imaging Device = 807 mono
 Camera Adapter = 1X
 Depth of Focus = 1.30 μ m
 Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
 Beam Splitter = 395
 Filter Excitation Wavelength = 335 - 383
 Filter Emission Wavelength = 420 - 470
 Contrast Method = Fluorescence
 Light source = X-Cite Xylis Lamp Intensity @10.00%
 Exposure Time = 25 ms
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.25
 Imaging Device = 807 mono
 Camera Adapter = 1X
 Depth of Focus = 0.90 μ m
 Binning Mode = 2,2

Post Processing - SIM Processing

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

Split View - Panel A

The first panel on the left features Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M) visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L), the middle panel features DAPI nuclear staining, and the last panel on the right features the merged image of all channels. This image was taken at Z-Position 42 of 86.

Profile View Display - Panels A & B

Z-Position = 42 of 86
 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 151.8), Y (Min 0 and Max 1718)

Summary

Panel A - Split View illustrates the signal and localization of the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a. The left section features the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red). The middle image shows the DAPI (blue), and the right presents a merged image of the three channels. The merge demonstrates that gamma H2AX Mouse Fc is localized to the nucleus, as indicated by DAPI staining. A line extends across the three nuclei, as this is where we measured the fluorescence spatial and intensity values in panel B.

Panel B - This panel features a line profile display that illustrates localization and fluorescent intensity. The X-axis represents distance, while the Y-axis indicates intensity. The data show that the signals for the Rabbit anti-Human γ H2AX Recombinant Monoclonal antibody (Mouse Fc, IgG2a) labeled with Identifyn® SRM Alexa Fluor™ 647 (red) and DAPI (blue) overlap along the X-axis, which corresponds to the nuclear compartment. This confirms that both the Rabbit anti-Human γ H2AX Recombinant Monoclonal antibody and DAPI are localized on the Z-plane at a position of 42 out of 86, indicating they occupy the same spatial area in the image. Furthermore, the Y-axis displays the Rabbit anti-Human γ H2AX Recombinant Monoclonal antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), which demonstrates strong fluorescent intensity following Etoposide-induced DNA damage.

Image Correction

None

Image by E.F. Simon

Image Analysis by B.T. Bennett

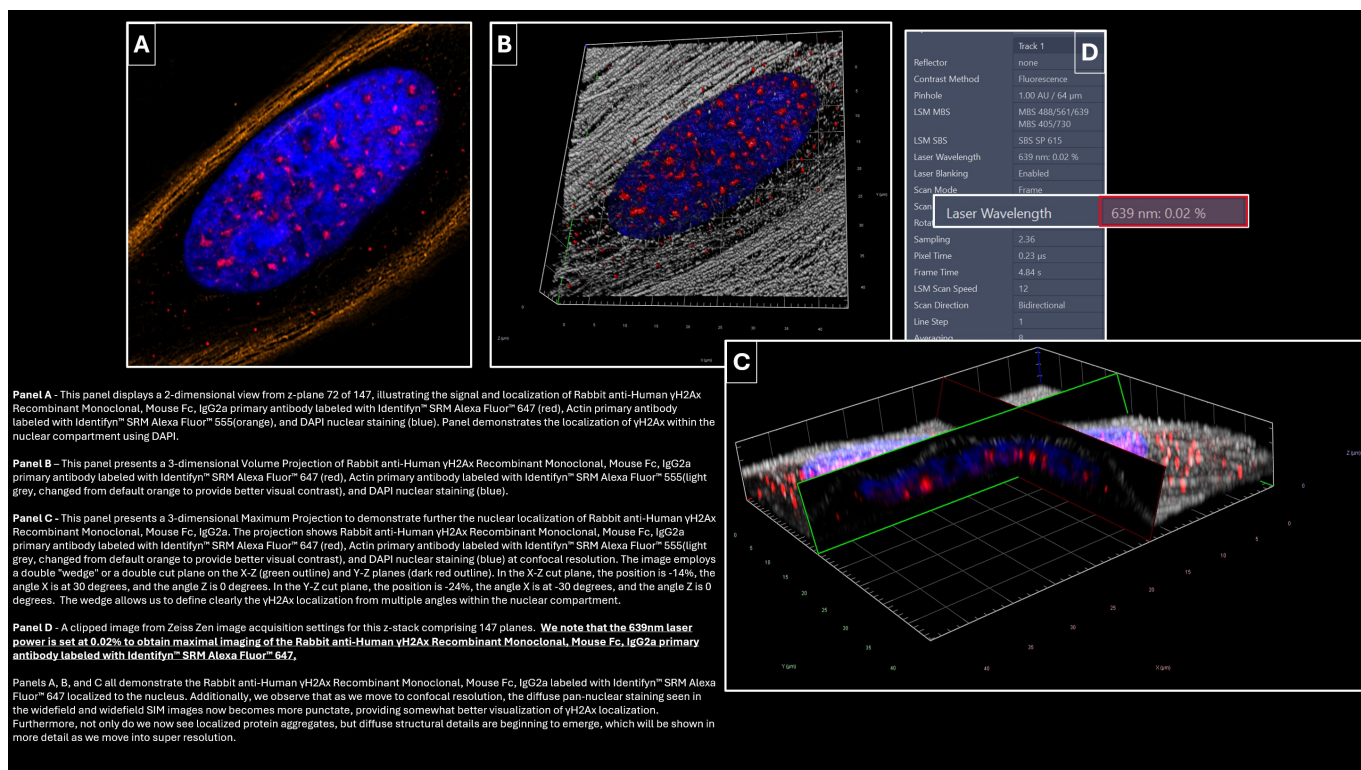
Data Presentation by P.D. Amistoso

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SOURCE PROVENANCE

Catalog	PA-RR-000005
Stage	Widefield SIM - Apotome
Instrument	ZEISS Axio Observer.Z1/7 Apotome
Microscope configuration	Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Structured source metadata values	35
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	89CEB3ED6C2DB24C1EE10D384F1897079F5B3E482051A994189E93C00BE7342A
Method PDF SHA-256	43A45975D68A60C8B0E19F91897EF9666EC91734D2B1053D8B39315B64999F0F

ORIGINAL IMAGE EVIDENCE / CONFOCAL



EVIDENCE TIER	Confocal
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555; 647
METADATA VALUES	66

STRUCTURED ACQUISITION METADATA / CONFOCAL

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	147 Slices (4.38 μm)
Channels	3
Scaling (Per Pixel)	0.030 μm X 0.030 μm X 0.030 μm
Image Size (Pixels)	1476 X 1476
Image Size (Scaled)	44.18 μm X 44.18 μm
Bit Depth	16 Bit
Image Center Position	X: 71.24 mm, Y: 48.14 mm
Stage Position	X: 71.24 mm, Y: 48.14 mm
ROI Center Offset	X: 0.00 μm , Y: 0.00 μm
Reflector	None
Contrast Method	Fluorescence
Pinhole	1.00 AU / 64 μm 1.00 AU / 57 μm 1.00 AU / 41 μm
LSM MBS	MBS 488/561/639, 405/730
LSM SBS	SBS SP 615 Mirror
Laser Wavelength	639 nm @0.02% 561 nm @0.007% 405 nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	3.0
Rotation	0°
Sampling	2.36
Pixel Time	0.23 μs
Frame Time	4.84 s
LSM Scan Speed	12
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	653 553 353
Emission Wavelength	668 568 465
Effective NA	1.4
Detection Wavelength	645-700 565-625 430-460
Imaging Device	GaAsP-Pmt3

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT
Detector Gain	650 V 500 V
Detector Offset	0
Detector Digital Gain	1.0
LSM Plus Mode	7.9 (3D, Auto) 7.4 (3D, Auto) 6.8 (3D, Auto)
3D Volume Projection	Precise
Appearance	Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels Threshold 1% 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 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 Maximum Projection	Precise
Clipping Image Process	A clipping plane was introduced to pronounce regions of special interest.
X-Z	Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip	-14% -24%
Clip X	30°
Clip Z	0°
Y-Z	Activate was selected, textured opaque was chosen, and a dark red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Clip Y	-30°

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 γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific

Cat ID# - SA 000056

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (H + L)

Cat ID# - SA 000043

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 μ M Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-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.

The second primary antibody, Actin Recombinant Rabbit Monoclonal Antibody (JF47-01), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (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 with DAPI cat#P36962.

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 72 of 147 total - Panel A

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

Z-Stack = 147 Slices (4.38 µm)

Channels = 3

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

Image Size (Pixels) = 1476 X 1476

Image Size (Scaled) = 44.18 µm X 44.18 µm

Bit Depth = 16 Bit

Image Center Position = X: 71.24 mm, Y: 48.14 mm

Stage Position = X: 71.24 mm, Y: 48.14 mm

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

647 -

Reflector = None

Contrast Method = Fluorescence

Pinhole = 1.00 AU / 64 µm

LSM MBS = MBS 488/561/639, 405/730

LSM SBS = SBS SP 615

Laser Wavelength = 639 nm @0.02%

Laser Blanking = Enabled

Scan Mode = Frame

Scan Zoom = 3.0

Rotation = 0°

Sampling = 2.36

Pixel Time = 0.23 µs

Frame Time = 4.84 s

LSM Scan Speed = 12

Scan Direction = Bidirectional

Line Step = 1

Averaging = 8

Excitation Wavelength = 653

Emission Wavelength = 668

Effective NA = 1.4

Detection Wavelength = 645-700

Imaging Device = GaAsP-Pmt3

Detector Type = GaAsP-PMT

Detector Gain = 650 V

Detector Offset = 0

Detector Digital Gain = 1.0

LSM Plus Mode = 7.9 (3D, Auto)

555 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 1.00 AU / 57 μ m
 LSM MBS = MBS 488/561/639, 405/730
 LSM SBS = Mirror
 Laser Wavelength = 561 nm @0.007%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 3.0
 Rotation = 0°
 Sampling = 2.36
 Pixel Time = 0.23 μ s
 Frame Time = 4.84 s
 LSM Scan Speed = 12
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 8
 Excitation Wavelength = 553
 Emission Wavelength = 568
 Effective NA = 1.4
 Detection Wavelength = 565-625
 Imaging Device = GaAsP-Pmt3
 Detector Type = GaAsP-PMT
 Detector Gain = 650 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 7.4 (3D, Auto)

DAPI -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 1.00 AU / 41 µm
 LSM MBS = MBS 488/561/639, 405/730
 LSM SBS = Mirror
 Laser Wavelength = 405 nm @0.2%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 3.0
 Rotation = 0°
 Sampling = 2.36
 Pixel Time = 0.23 µs
 Frame Time = 4.84 s
 LSM Scan Speed = 12
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 8
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.4
 Detection Wavelength = 430-460
 Imaging Device = GaAsP-Pmt3
 Detector Type = GaAsP-PMT
 Detector Gain = 500 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 6.8 (3D, Auto)

3D Image Processing - Panel B

3D Volume Projection = Precise
 Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
 Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel C

3D Maximum Projection = Precise
 Appearance = Threshold 1% 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)

Clipping Image Process = A clipping plane was introduced to pronounce regions of special interest.

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

Position of Clip = -14%

Clip X = 30°

Clip Z = 0°

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

Position of Clip = -24%

Clip Y = -30°

Clip Z = 0°

Summary

Panel A - This panel displays a 2-dimensional view from z-plane 72 of 147, illustrating the signal and localization of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 555 (orange), and DAPI nuclear staining (blue). Panel demonstrates the localization of γ H2Ax within the nuclear compartment using DAPI.

Panel B - This panel presents a 3-dimensional Maximum Projection of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 555 (light grey, changed from default orange to provide better visual contrast), and DAPI nuclear staining (blue).

Panel C - This panel presents a 3-dimensional Volume Projection to demonstrate further the nuclear localization of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a. The projection shows Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 555 (light grey, changed from default orange to provide better visual contrast), and DAPI nuclear staining (blue) at confocal resolution. The image employs a double "wedge" or a double cut plane on the X-Z (green outline) and Y-Z planes (dark red outline). In the X-Z cut plane, the position is -14%, the angle X is at 30 degrees, and the angle Z is 0 degrees. In the Y-Z cut plane, the position is -24%, the angle X is at -30 degrees, and the angle Z is 0 degrees. The wedge allows us to define clearly the γ H2Ax localization from multiple angles within the nuclear compartment.

Panel D - A clipped image from Zeiss Zen image acquisition settings for this z-stack comprising 147 planes. We note that the 639nm laser power is set at 0.02% to obtain maximal imaging of the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647.

Panels A, B, and C all demonstrate the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a labeled with Identifyn® SRM Alexa Fluor™ 647 localized to the nucleus. Additionally, we observe that as we move to confocal resolution, the diffuse pan-nuclear staining seen in the widefield and widefield SIM images now becomes more punctate, providing somewhat better visualization of γ H2Ax localization. Furthermore, not only do we now see localized protein aggregates, but diffuse structural details are beginning to emerge, which will be shown in more detail as we move into super resolution.

Image Corrections

Panel A - Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

Panel B - Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Donkey Anti-Mouse, F(ab')₂ (H + L) was pseudo-colored in Zen Software from orange, the default color, to white to provide visual contrast.

Panel C - Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

Image by J.K. Bennett

Image Analysis by B.T. Bennett

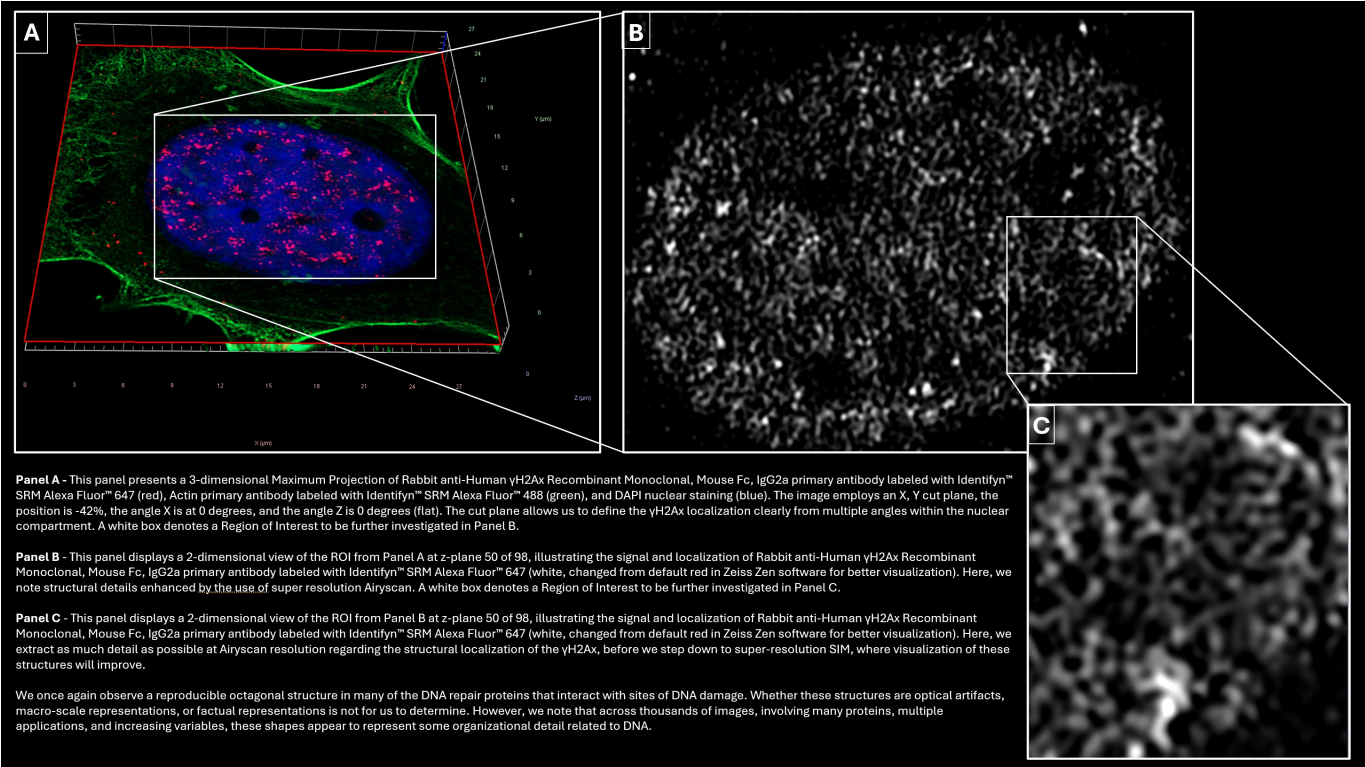
Data Presentation by P.D. Amistoso

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SOURCE PROVENANCE

Catalog	PA-RR-000005
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	66
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	077217842F68EBCEB56D309E7B570407E76450136BD21490BB97160CFBACD1DA
Method PDF SHA-256	0D4FCD70FE192BE9517346F443678E092F57B554EA1AA602A4F8A9F4ABA1A07D

ORIGINAL IMAGE EVIDENCE / AIRYSCAN



EVIDENCE TIER	Airyscan
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555; 647; 750
METADATA VALUES	66

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	98 Slices (2.91 μm)
Channels	3
Scaling (Per Pixel)	0.03530 μm X 0.03530 μm X 0.03000 μm
Image Size (Pixels)	837 X 805 560 X 457 135 X 149
Image Size (Scaled)	29.54 μm X 28.41 μm 19.77 μm X 16.13 μm 4.76 μm X 5.26 μm
Bit Depth	16 Bit
Image Center Position	X: 83.02 mm, Y: 52.68 mm
Stage Position	X: 83.02 mm, Y: 52.68 mm
ROI Center Offset	X: 0.00 μm , Y: 0.00 μm
Reflector	None
Contrast Method	Fluorescence
Pinhole	5.00 AU / 328 μm 5.00 AU / 261 μm 5.46 AU / 235 μm
LSM MBS	MBS 488/639, 405/730
LSM SBS	SBS LP 640 SBS SP 615 SBS SP 505
Laser Wavelength	639 nm @0.2% 488 nm @0.02% 405 nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.0
Rotation	0°
Sampling	2.00
Pixel Time	1.10 μs
Frame Time	18.77 s
LSM Scan Speed	6
Scan Direction	Bidirectional
Line Step	1
Averaging	4
Excitation Wavelength	653 493 353
Emission Wavelength	668 517 465
Effective NA	1.4
Detection Wavelength	643-735 380-608 350-499
Imaging Device	Airyscan

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT
Detector Gain	800 V 750 V
Detector Offset	0
Detector Digital Gain	1.0
LSM Plus Mode	9.7 (3D, Auto) 9.2 (3D, Auto) 8.3 (3D, Auto)
LMS MBS	MBS 488/639, 405/730
3D Maximum Projection	Precise
Appearance	Threshold 1% 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)
Clipping Image Process	Clipping planes are introduced to pronounce regions of special interest.
X-Y	Activate was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip	-42%
Clip X	0°
Clip Y	0°

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)

Cat ID# - SA 000062

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (H + L)

Cat ID# - SA 000020

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 μ M Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody, Actin Recombinant Rabbit Monoclonal Antibody (JF47-01), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (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 with DAPI cat#P36962.

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) - Panel A

Z-Stack = 98 Slices (2.91 µm)

Channels = 3

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

Image Size (Pixels) = 837 X 805

Image Size (Scaled) = 29.54 µm X 28.41 µm

Bit Depth = 16 Bit

Image Center Position = X: 83.02 mm, Y: 52.68 mm

Stage Position = X: 83.02 mm, Y: 52.68 mm

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

Single Plane (2D) - Z-plane 50 of 98 total - Panel B

Z-Stack = 98 Slices (2.91 µm)

Channels = 3

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

Image Size (Pixels) = 560 X 457

Image Size (Scaled) = 19.77 µm X 16.13 µm

Bit Depth = 16 Bit

Image Center Position = X: 83.02 mm, Y: 52.68 mm

Stage Position = X: 83.02 mm, Y: 52.68 mm

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

Single Plane (2D) - Z-plane 50 of 98 total - Panel C

Z-Stack = 98 Slices (2.91 µm)

Channels = 3

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

Image Size (Pixels) = 135 X 149

Image Size (Scaled) = 4.76 µm X 5.26 µm

Bit Depth = 16 Bit

Image Center Position = X: 83.02 mm, Y: 52.68 mm

Stage Position = X: 83.02 mm, Y: 52.68 mm

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

647 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 5.00 AU / 328 μ m
 LSM MBS = MBS 488/639, 405/730
 LSM SBS = SBS LP 640
 Laser Wavelength = 639 nm @0.2%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.0
 Rotation = 0°
 Sampling = 2.00
 Pixel Time = 1.10 μ s
 Frame Time = 18.77 s
 LSM Scan Speed = 6
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 4
 Excitation Wavelength = 653
 Emission Wavelength = 668
 Effective NA = 1.4
 Detection Wavelength = 643-735
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 800 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 9.7 (3D, Auto)

555 -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 5.00 AU / 261 μ m
 LSM MBS = MBS 488/639, 405/730
 LSM SBS = SBS SP 615
 Laser Wavelength = 488 nm @0.02%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.0
 Rotation = 0°
 Sampling = 2.00
 Pixel Time = 1.10 μ s
 Frame Time = 18.77 s
 LSM Scan Speed = 6
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 4
 Excitation Wavelength = 493
 Emission Wavelength = 517
 Effective NA = 1.4
 Detection Wavelength = 380-608
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 800 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 9.2 (3D, Auto)

DAPI -

Reflector = None
 Contrast Method = Fluorescence
 Pinhole = 5.46 AU / 235 μ m
 LMS MBS = MBS 488/639, 405/730
 LSM SBS = SBS SP 505
 Laser Wavelength = 405 nm @0.2%
 Laser Blanking = Enabled
 Scan Mode = Frame
 Scan Zoom = 2.0
 Rotation = 0°
 Sampling = 2.00
 Pixel Time = 1.10 μ s
 Frame Time = 18.77 s
 LSM Scan Speed = 6
 Scan Direction = Bidirectional
 Line Step = 1
 Averaging = 4
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.4
 Detection Wavelength = 350-499
 Imaging Device = Airyscan
 Detector Type = GaAsP-PMT
 Detector Gain = 750 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = 8.3 (3D, Auto)

3D Image Processing - Panel A

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

Position of Clip = -42%**Clip X = 0°****Clip Y = 0°**

Summary

Panel A - This panel presents a 3-dimensional Maximum Projection of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green), and DAPI nuclear staining (blue). The image employs an X, Y cut plane, the position is -42%, the angle X is at 0 degrees, and the angle Z is 0 degrees (flat). The cut plane allows us to define the γ H2Ax localization clearly from multiple angles within the nuclear compartment. A white box denotes a Region of Interest to be further investigated in Panel B.

Panel B - This panel displays a 2-dimensional view of the ROI from Panel A at z-plane 50 of 98, illustrating the signal and localization of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (white, changed from default red in Zeiss Zen software for better visualization). Here, we note structural details enhanced by the use of super resolution Airyscan. A white box denotes a Region of Interest to be further investigated in Panel C.

Panel C - This panel displays a 2-dimensional view of the ROI from Panel B at z-plane 50 of 98, illustrating the signal and localization of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (white, changed from default red in Zeiss Zen software for better visualization). Here, we extract as much detail as possible at Airyscan resolution regarding the structural localization of the γ H2Ax, before we step down to super-resolution SIM, where visualization of these structures will improve.

We once again observe a reproducible octagonal structure in many of the DNA repair proteins that interact with sites of DNA damage. Whether these structures are optical artifacts, macro-scale representations, or factual representations is not for us to determine. However, we note that across thousands of images, involving many proteins, multiple applications, and increasing variables, these shapes appear to represent some organizational detail related to DNA.

Image Corrections

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(a") $\square$ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

Image by J.K. Bennett

Image Analysis by B.T. Bennett

Data Presentation by P.D. Amistoso

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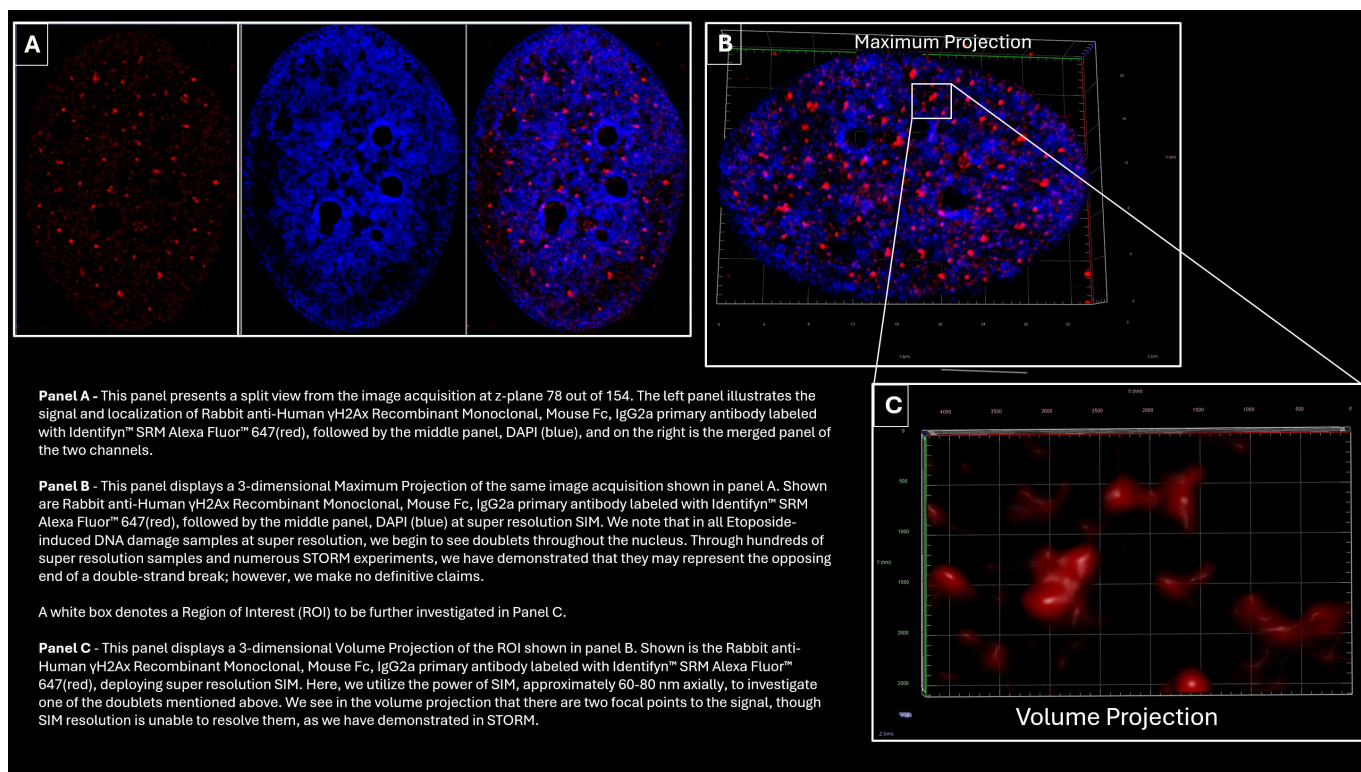
SOURCE PROVENANCE

Catalog	PA-RR-000005
Stage	Airyscan
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:

SOURCE PROVENANCE

Structured source metadata values	66
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	C669C6F03E7E37566BC29B20B69AABC56FCBBD11890AF360ED813178CCDC1670
Method PDF SHA-256	87634DF86D46B06B463D91A8230D426812DB58E07F1970FCA2F95F4D356EADA8

ORIGINAL IMAGE EVIDENCE / SIM



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	154 Slices (4.59 μm)
Channels	2
Scaling (Per Pixel)	0.01320 μm X 0.01320 μm X 0.03000 μm
Image Size (Pixels)	1801 X 2570 321 X 198
Image Size (Scaled)	23.76 μm X 33.91 μm 4.24 μm X 2.61 μm
Bit Depth	16 Bit
Image Center Position	X: 58.34 mm, Y: 35.81 mm
Stage Position	X: 58.34 mm, Y: 35.81 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	640 405
Channel Name	T1-Axiocam 820-SR T3-Axiocam 820 #2-SR
Excitation Wavelength	640 405
Emission Wavelength	729 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device	Axiocam 820 Axiocam 820 #2
Camera Adapter	1X
Exposure Time	120 ms 50 ms
Depth of Focus	1.13 μm 0.69 μm
Binning Mode	2,2
Laser Wavelength	640 nm @2.00% 405 nm @6.00%
Frame Time	1.59 s 676.00 ms
Scan Direction	Unidirectional
Grating	32 μm grating 36.5 μm grating
Result Sampling	4
Process Sampling	N/A

FIELD	SOURCE-DOCUMENTED VALUE
Algorithm	SIM
SIM Settings	Standard
Auto Sharpness	On
Sharpness	9.6564 (647), 8.5127 (DAPI)
Fitting	Fast Fit
Normalization	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) View Angle 10°, Scale Z 15%, 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 γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)

Cat ID# - SA 000062

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 μ M Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PB. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (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 with DAPI cat#P36962.

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 78 of 154 total - Panel A

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

Z-Stack = 154 Slices (4.59 μ m)

Channels = 2

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

Image Size (Pixels) = 1801 X 2570

Image Size (Scaled) = 23.76 μ m X 33.91 μ m

Bit Depth = 16 Bit

Image Center Position = X: 58.34 mm, Y: 35.81 mm

Stage Position = X: 58.34 mm, Y: 35.81 mm

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

Z Stack - (3D) - Panel C

Z-Stack = 154 Slices (4.59 µm)

Channels = 2

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

Image Size (Pixels) = 321 X 198

Image Size (Scaled) = 4.24 µm X 2.61 µm

Bit Depth = 16 Bit

Image Center Position = X: 58.34 mm, Y: 35.81 mm

Stage Position = X: 58.34 mm, Y: 35.81 mm

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

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 = 120 ms

Depth of Focus = 1.13 µm

Binning Mode = 2,2

Laser Wavelength = 640 nm @2.00%

Frame Time = 1.59 s

Scan Direction = Unidirectional

Grating = 32 µm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
 Beam Splitter = 405, 488, 561, 642
 Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
 Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
 Contrast Method = Fluorescence
 Illumination Wavelength = 405
 Channel Name = T3-Axiocam 820 #2-SR
 Excitation Wavelength = 405
 Emission Wavelength = 445
 Effective NA = 1.4
 Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
 Imaging Device = Axiocam 820 #2
 Camera Adapter = 1X
 Exposure Time = 50 ms
 Depth of Focus = 0.69 μ m
 Binning Mode = 2,2
 Laser Wavelength = 405 nm @6.00%
 Frame Time = 676.00 ms
 Scan Direction = Unidirectional
 Grating = 36.5 μ m grating

Post Processing - SIM Processing

Processing Dimension - 3D
 Result Sampling = 4
 Process Sampling = N/A
 Channels = 2
 Algorithm = SIM
 SIM Settings = Standard
 Auto Sharpness = On
 Sharpness = 9.6564 (647), 8.5127 (DAPI)
 Fitting = Fast Fit
 Normalization = Auto

Split View - Panel A

The first panel on the left features Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M) visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(a'')₂ (H + L), the middle panel features DAPI nuclear staining, and the last panel on the right features the merged image of all channels. This image was taken at Z-Position 78 of 154.

3D Image Processing - Panel B

3D Maximum Projection = Precise
 Appearance = Threshold 2% all channels, Ramp 0% all channels, Maximum 100% all channels
 Background = Black
 Light = Brightness 100%, 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 10°, Scale Z 15%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A - This panel presents a split view from the image acquisition at z-plane 78 out of 154. The left panel illustrates the signal and localization of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), followed by the middle panel, DAPI (blue), and on the right is the merged panel of the two channels.

Panel B - This panel displays a 3-dimensional Maximum Projection of the same image acquisition shown in panel A. Shown are Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), followed by the middle panel, DAPI (blue) at super resolution SIM. We note that in all Etoposide-induced DNA damage samples at super resolution, we begin to see doublets throughout the nucleus. Through hundreds of super resolution samples and numerous STORM experiments, we have demonstrated that they may represent the opposing end of a double-strand break; however, we make no definitive claims.

A white box denotes a Region of Interest (ROI) to be further investigated in Panel C.

Panel C - This panel displays a 3-dimensional Volume Projection of the ROI shown in panel B. Shown is the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), deploying super resolution SIM. Here, we utilize the power of SIM, approximately 60-80 nm axially, to investigate one of the doublets mentioned above. We observe in the volume projection that there are two focal points to the signal, though SIM resolution is unable to resolve them, as we have demonstrated in STORM.

Image Corrections

None

Image by P. Raut

Image Analysis by B.T. Bennett

Data Presentation by P.D. Amistoso

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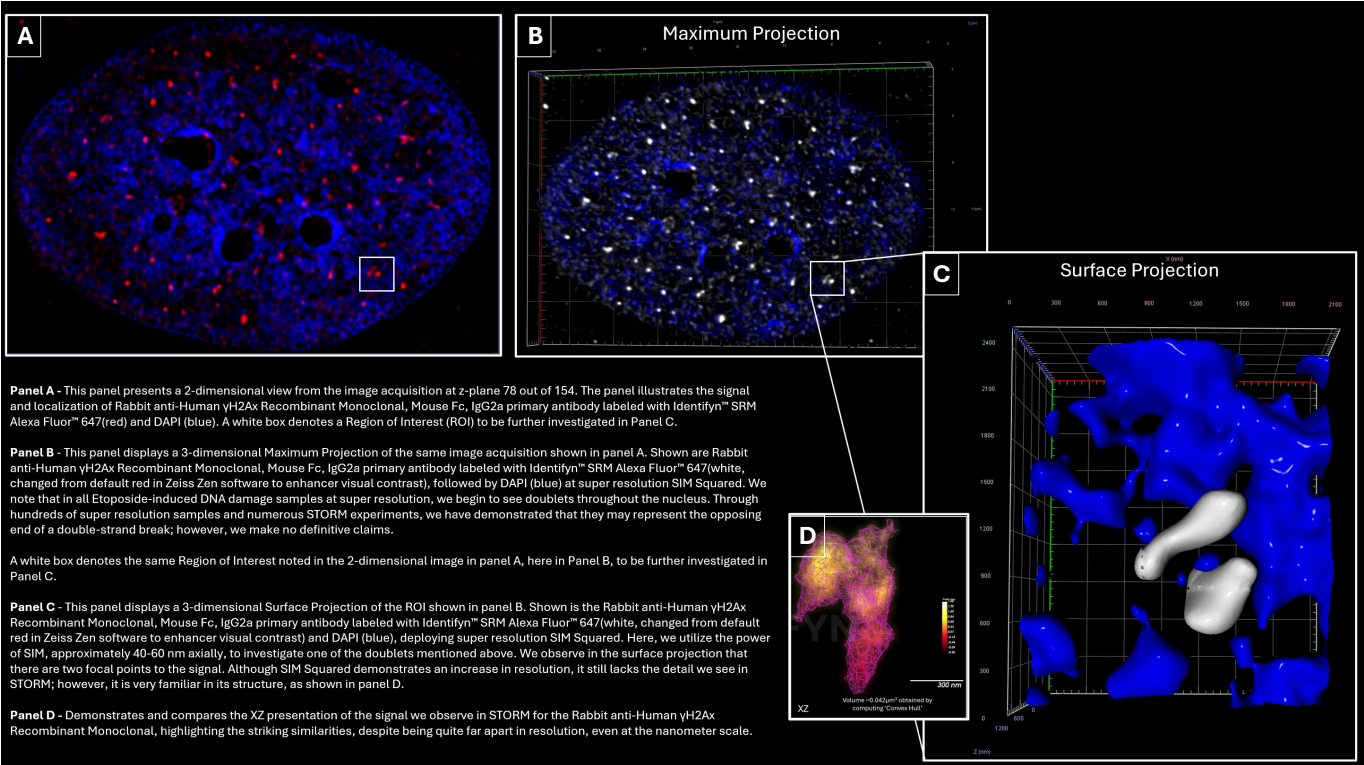
SOURCE PROVENANCE

Catalog	PA-RR-000005
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:

SOURCE PROVENANCE

Structured source metadata values	58
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	1E3B59F3CA7B909A07C60FE0516C11FFF1F3B817D162091E1FF3DE1122A614D5
Method PDF SHA-256	ED8EFE4BEE26E4E0CBE475B3AB888934A0F174D0B813B292A825F65B8373E13C

ORIGINAL IMAGE EVIDENCE / SIM²



EVIDENCE TIER	SIM ²
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647
METADATA VALUES	61

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	154 Slices (4.59 µm) 54 Slices (1.59 µm)
Channels	2
Scaling (Per Pixel)	0.01320 µm X 0.01320 µm X 0.03000 µm
Image Size (Pixels)	1805 X 2627 163 X 192
Image Size (Scaled)	23.82 µm X 34.66 µm 2.15 µm X 2.53 µm
Bit Depth	16 Bit
Image Center Position	X: 58.34 mm, Y: 35.81 mm
Stage Position	X: 58.34 mm, Y: 35.81 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	640 405
Channel Name	T1-Axiocam 820-SR T3-Axiocam 820 #2-SR
Excitation Wavelength	640 405
Emission Wavelength	729 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device	Axiocam 820 Axiocam 820 #2
Camera Adapter	1X
Exposure Time	120 ms 50 ms
Depth of Focus	1.13 µm 0.69 µm
Binning Mode	2,2
Laser Wavelength	640 nm @2.00% 405 nm @6.00%
Frame Time	1.59 s 676.00 ms
Scan Direction	Unidirectional
Grating	32 µm grating 36.5 µm grating
Result Sampling	4
Process Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
Algorithm	SIM ²
SIM ² Settings	Standard
Input SNR	Low
Regularization	None
Regularization Weight	0.0000
Iterations	3
Filter	No Filter
Fitting	Fast Fit
Normalization	Auto
3D Maximum Projection	Precise
Appearance	Threshold 4% all channels, Ramp 0% all channels, Maximum 100% all channels Threshold 21% (647) and 26% (DAPI), Ambient Light 0% all channels, Specular Light 100% all channels, Shininess 30% all channels
Background	Black
Light	Brightness 100%, Tone Mapping Enabled Azimuth 80°, Elongation -130°, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
3D Surface Projection	Precise

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)

Cat ID# - SA 000062

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2 μ M Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(ab')₂ (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 with DAPI cat#P36962.

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 78 of 154 total - Panel A

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

Z-Stack = 154 Slices (4.59 μ m)

Channels = 2

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

Image Size (Pixels) = 1805 X 2627

Image Size (Scaled) = 23.82 μ m X 34.66 μ m

Bit Depth = 16 Bit

Image Center Position = X: 58.34 mm, Y: 35.81 mm

Stage Position = X: 58.34 mm, Y: 35.81 mm

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

Z Stack - (3D) - Panel C

Z-Stack = 54 Slices (1.59 µm)

Channels = 2

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

Image Size (Pixels) = 163 X 192

Image Size (Scaled) = 2.15 µm X 2.53 µm

Bit Depth = 16 Bit

Image Center Position = X: 58.34 mm, Y: 35.81 mm

Stage Position = X: 58.34 mm, Y: 35.81 mm

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

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 = 120 ms

Depth of Focus = 1.13 µm

Binning Mode = 2,2

Laser Wavelength = 640 nm @2.00%

Frame Time = 1.59 s

Scan Direction = Unidirectional

Grating = 32 µm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
 Beam Splitter = 405, 488, 561, 642
 Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
 Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
 Contrast Method = Fluorescence
 Illumination Wavelength = 405
 Channel Name = T3-Axiocam 820 #2-SR
 Excitation Wavelength = 405
 Emission Wavelength = 445
 Effective NA = 1.4
 Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
 Imaging Device = Axiocam 820 #2
 Camera Adapter = 1X
 Exposure Time = 50 ms
 Depth of Focus = 0.69 μ m
 Binning Mode = 2,2
 Laser Wavelength = 405 nm @6.00%
 Frame Time = 676.00 ms
 Scan Direction = Unidirectional
 Grating = 36.5 μ m grating

Post Processing - SIM² Processing

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

3D Surface Projection = Precise

Appearance = Threshold 21% (647) and 26% (DAPI), Ambient Light 0% all channels, Specular Light 100% all channels, Shininess 30% all channels

Background = Black

Light = Azimuth 80°, Elongation -130°, Enabled Tone Mapping

Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A - This panel presents a 2-dimensional view from the image acquisition at z-plane 78 out of 154. The panel illustrates the signal and localization of Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red) and DAPI (blue). A white box denotes a Region of Interest (ROI) to be further investigated in Panel C.

Panel B - This panel displays a 3-dimensional Maximum Projection of the same image acquisition shown in panel A. Shown are Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (white, changed from default red in Zeiss Zen software to enhance visual contrast), followed by DAPI (blue) at super resolution SIM Squared. We note that in all Etoside-induced DNA damage samples at super resolution, we begin to see doublets throughout the nucleus. Through hundreds of super resolution samples and numerous STORM experiments, we have demonstrated that they may represent the opposing end of a double-strand break; however, we make no definitive claims.

A white box denotes the same Region of Interest noted in the 2-dimensional image in panel A, here in Panel B, to be further investigated in Panel C.

Panel C - This panel displays a 3-dimensional Surface Projection of the ROI shown in panel B. Shown is the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (white, changed from default red in Zeiss Zen software to enhance visual contrast) and DAPI (blue), displaying super resolution SIM Squared. Here, we utilize the power of SIM, approximately 40-60 nm axially, to investigate one of the doublets mentioned above. We observe in the surface projection that there are two focal points to the signal. Although SIM Squared demonstrates an increase in resolution, it still lacks the detail we see in STORM; however, it is very familiar in its structure, as shown in panel D.

Panel D - Demonstrates and compares the XZ presentation of the signal we observe in STORM for the Rabbit anti-Human γ H2Ax Recombinant Monoclonal, highlighting the striking similarities, despite being quite far apart in resolution, even at the nanometer scale.

Image Corrections

Panels B & C - Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, F(a") $\square$ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

Image by P. Raut

Image Analysis by B.T. Bennett

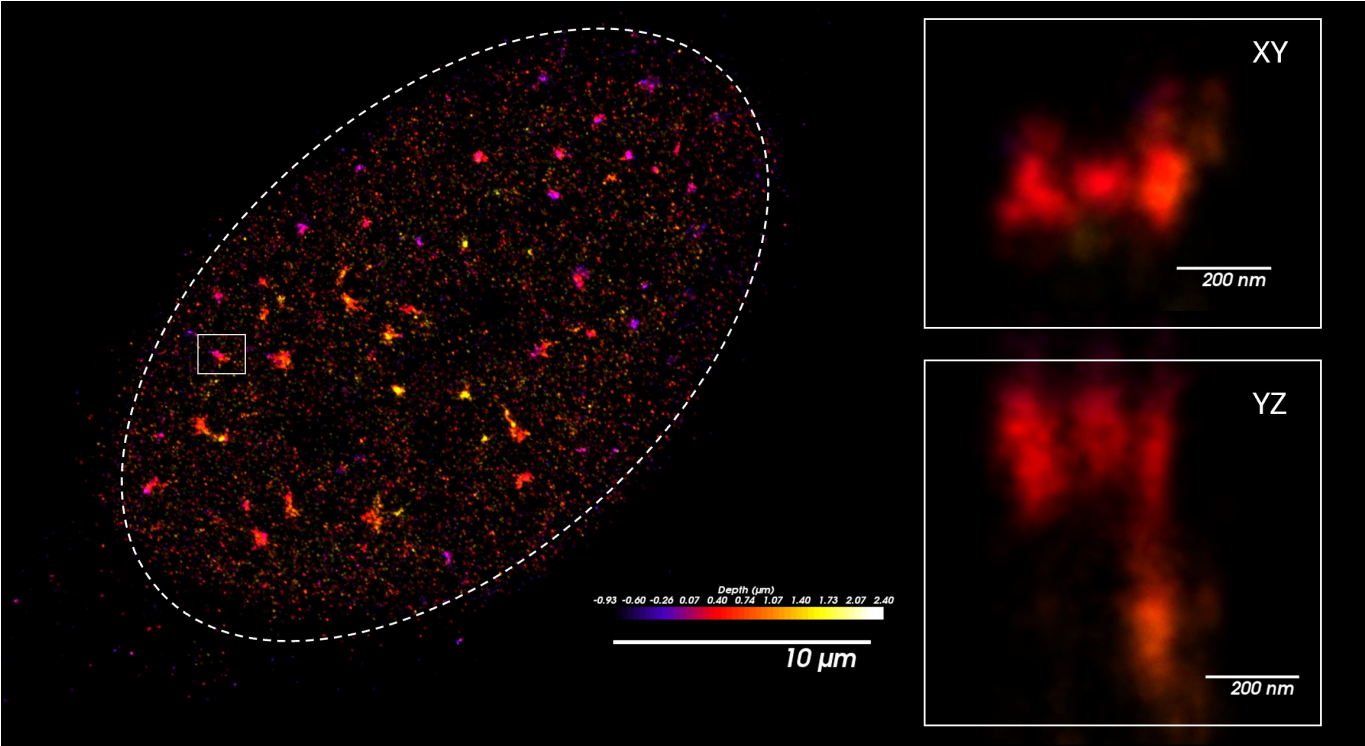
Data Presentation by P.D. Amistoso

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SOURCE PROVENANCE

Catalog	PA-RR-000005
Stage	SIM ²
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	61
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	DE220E05B94264E7341410078E76463AAA5E173B5AF253ED6AE11F374349DAA1
Method PDF SHA-256	6CC12849F983E9C9FAD84158F2795324E76C1EE2616D4818E65D18ADE7564941

ORIGINAL IMAGE EVIDENCE / SINGLE-MOLECULE STORM



EVIDENCE TIER	Single-molecule STORM
INSTRUMENT	Bruker Vutara VXL
CELL MODEL	HeLa
DETECTED DYES	647
METADATA VALUES	80

STRUCTURED ACQUISITION METADATA / SINGLE-MOLECULE STORM

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	Bruker Vutara VXL Biplane Single Molecule Microscope, using an Olympus 60X/1.30 silicon oil objective, was used with the following parameters:
Objective	Olympus 60X/1.30 silicon oil objective
Number of Probes	1
Number of Simultaneous Probes	1
Number of Reference Probes	1
Camera	Both Cameras
Color Channel	Both Channels
Probe Capture Mode	Interleaved
Z -Stack Capture Mode	Interleaved
Multiple Location Capture	Not selected
Post Record Reference	Not Selected
Fluidics	Not Selected
Autofocus Drift Correction	On
Biplane Module	635nm Dichroic
Objective	Silicon Selected
Stage Insert	Rectangular Selected
Course Focus Position	8-Well Chamber Selected
Dichroic	SuperRes
PSF	(Red, Orange)
Heater	Current: 26
Recording Vertical Field of View	100%
Widefield Camera Exposure Time	100ms 20ms
Super Resolution Camera Exposure Time	20ms
Widefield Camera Binning	1
Per-Probe Exposure Time	Selected
Predefined Probe	First probe: 640 nm (635 nm Dichroic); First reference probe: 640 nm (635 nm Dichroic)
Photoactivation/Imaging Laser	0% Selected
Exposure Time	20ms Selected
Record Workflow	Selected Options
View Mode	Widefield 200µm
Capture Mode	Live
Piezo Current	179.5 µm

FIELD	SOURCE-DOCUMENTED VALUE
Widefield 640nm	0.1X Neutral Density Filter: Selected, @0.2% Laser Input
Reference Probe 1 Laser control (640 nm (640 nm Dichroic))	Not Selected
Widefield camera Exposure Time	100ms
Widefield Laser	"Widefield 640nm" was deselected, and "calibrate" under Autofocus was selected.
Current Intensity	4.5
Calibration Value	-5070 nm/unit
Autofocus Mode	Pre-Capture
SuperRes	50µm is Selected
Piezo Record	Begin: 180; End: 181.6
SuperRes 640nm	0.1X Neutral Density Filter: Selected, @0.02% Laser Input
Record Workflow - Record Tab	Capture Parameters
Frames	Probe 1: 200
Z Positions	8 using Steps: 0.2µm (200nm)
Cycles	20
Time Points	1
Pause Between Cycles	Not Selected
Automatic Photoactivation Laser Control	Not Selected
Laser Power	50%
Cutout Width	16px
Max Frame Accumulation	1 frame
Max Accumulation Offset	1px
Fiducial at Max Accumulation	Not selected
Max Particles Per Frame	No Max
Background SLPE Removal	Not Selected
Cutout/Background Removal	Not Selected
Preview Cutout Positions	Selected
Probe1	BG threshold: 15
Sizing Mode	Radial Precision
Particle size	4nm
Color by	Depth
Color Table	Single
Opacity Mode	Constant; Opacity 1: 08
Front-to-back Attenuation	Not Selected
Method	Particle (direct)
Time Intervals	10 Intervals

FIELD	SOURCE-DOCUMENTED VALUE
Pixel Size	50nm
Smoothing	8.00
PSF Z offset	-500 to +500
Radial precision	25
Axial precision	60
Clustering Algorithm	DBSCAN
Maximum Particle Distance	0.1µm (1000nm)
Maximum Particle Count to Form Cluster	10
Computer Hulls	Not Selected
Compute Density Isosurfaces	Not Selected
Edit Settings	All Probes
Hull Alpha shape Radius	Not Selected
Compute	Selected
Following Cluster Analysis Computation, under the Results Tab, ID Unassigned	Deselected

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

Identifyn® Secondary Antibody

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

Cat ID# - SA 000057

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

Method

HeLa cells were grown on μ -Slide 8 Well high Glass Bottom Ibidi chamber, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Mouse Fc, IgG2a, was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 3X in Identifyn's™ proprietary Wash Buffer, and Identifyn® 647 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:50. Cells were washed 5X in Identifyn's™ proprietary Wash Buffer followed by the addition of Identifyn's™ proprietary STORM Buffer to the sample chamber enabling image acquisition.

Microscope

Bruker Vutara VXL Biplane Single Molecule Microscope, using an Olympus 60X/1.30 silicon oil objective, was used with the following parameters:

Calibration -

Calibration follows Identifyn's Calibration Standard Operating Procedure, which can be found here.

<https://identifyn.com/storm-calibration>

Image Acquisition -

SRX Software - Under the 'Single Molecule Localization' tab, 'Record' is selected and contains the following workflow tabs and parameters.

Configuration Tab - Capture Configuration

Number of Probes: 1
 Number of Simultaneous Probes: 1
 Number of Reference Probes: 1
 Camera: Both Cameras
 Color Channel: Both Channels
 Probe Capture Mode: Interleaved
 Z -Stack Capture Mode: Interleaved
 Multiple Location Capture: Not selected
 Post Record Reference: Not Selected
 Fluidics: Not Selected
 Autofocus Drift Correction: On

Microscope configuration

Biplane Module: 635nm Dichroic
 Objective: Silicon Selected
 Stage Insert: Rectangular Selected
 Course Focus Position: 8-Well Chamber Selected
 Dichroic: SuperRes
 PSF: (Red, Orange)
 Heater: Current: 26

Camera Configuration

Recording Vertical Field of View: 100%
 Widefield Camera Exposure Time: 100ms
 Super Resolution Camera Exposure Time: 20ms
 Widefield Camera Binning: 1
 Per-Probe Exposure Time: Selected

Probe Configuration

Predefined Probe: First probe: 640 nm (635 nm Dichroic); First reference probe: 640 nm (635 nm Dichroic)
 Photoactivation/Imaging Laser: 0% Selected
 Exposure Time: 20ms Selected

Experiment Configuration

Record Workflow: Selected Options
 View Mode: Widefield 200µm
 Capture Mode: Live

Stage and Piezo Selection

Examination of the Sample

Cell(s) or Cellular Structure Localization - The red-bordered box identifies the area of the SuperRes view and defines a suitable cell(s) or cellular structure. The cell(s) or structures are optimally focused.

Piezo Current: 179.5 μ m

Probe 1 Laser control (640 nm (635 nm Dichroic)

Widefield 640nm: 0.1X Neutral Density Filter: Selected, @0.2% Laser Input
Reference Probe 1 Laser control (640 nm (640 nm Dichroic): Not Selected

Advanced Tools Option

Widefield camera Exposure Time: 100ms
Default values are maintained unless otherwise noted.

Widefield Laser: "Widefield 640nm" was deselected, and "calibrate" under Autofocus was selected.

Autofocus - Calibration Values

Current Position" -0.979
Current Intensity: 4.5
Calibration Value: -5070 nm/unit
Autofocus Mode: Pre-Capture

View Mode

SuperRes: 50 μ m is Selected

Capture Mode

Live

Stage and Piezo Selection

Cell(s) or Cellular Structure Localization - Positioning of the target Cell or Subsection of the Target Cell is optimized and optimally focused.

Piezo Record: Begin: 180; End: 181.6

Probe 1 Laser control (640 nm (635 nm Dichroic)

SuperRes 640nm: 0.1X Neutral Density Filter: Selected, @0.02% Laser Input

Reference Probe 1 Laser control (640 nm (635 nm Dichroic)

Not Selected

Advanced Tools

Widefield Camera Exposure Time: 20ms
Default values are maintained unless otherwise noted.

Record Workflow - Record Tab: Capture Parameters

Frames: Probe 1: 200

Z Positions: 8 using Steps: 0.2µm (200nm)

Cycles: 20

Time Points: 1

Pause Between Cycles: Not Selected

Automatic Photoactivation Laser Control: Not Selected

Probe 1 Laser control (640 nm (640 nm Dichroic))

Laser Power: 50%

Expert Configuration Options - The Localization Tab

Beyond the values shown, all other parameters were set to default values.

Localization

Cutout Width: 16px

Max Frame Accumulation: 1 frame

Max Accumulation Offset: 1px

Fiducial at Max Accumulation: Not selected

Max Particles Per Frame: No Max

Background SLPE Removal: Not Selected

Cutout/Background Removal: Not Selected

Preview Cutout Positions: Selected

Record Workflow - Localization Tab

Data Collection

The folder is created automatically with the localization file for this image acquisition.

Recording Summary

All parameters were carried over from previous tabs and are kept except:

Probe1: BG threshold: 15

Region of Interest -

Not selected

Display

Was deployed to inspect if the BG threshold applied chooses localizations in an expected manner

Record Workflow - View Tab

Region of Interest

Default values

Visualization Parameters

Point Splatting

Sizing Mode: Radial Precision

Particle size: 4nm

Color by: Depth

Color Table: Single

Opacity Mode: Constant; Opacity 1: 08

Front-to-back Attenuation: Not Selected

Post Acquisition analysis - View Tab

Drift Correction - Computed with the following parameters:

Method: Particle (direct)

Time Intervals: 10 Intervals

Pixel Size: 50nm

Smoothing: 8.00

Advanced Particle Filters - Applied to the drift-corrected data with the following parameters

PSF Z offset: -500 to +500

Radial precision: 25

Axial precision: 60

The other parameters were not applied.

Statistical analysis - Computed to clean low background signals.

Statistics Dialog - Cluster Analysis

Clustering Algorithm: DBSCAN

Parameters

Maximum Particle Distance: 0.1 μ m (1000nm)

Maximum Particle Count to Form Cluster: 10

Computer Hulls: Not Selected

Compute Density Isosurfaces: Not Selected

Edit Settings: All Probes

Hull Alpha shape Radius: Not Selected

Compute: Selected

Following Cluster Analysis Computation, under the Results Tab, ID Unassigned: Deselected

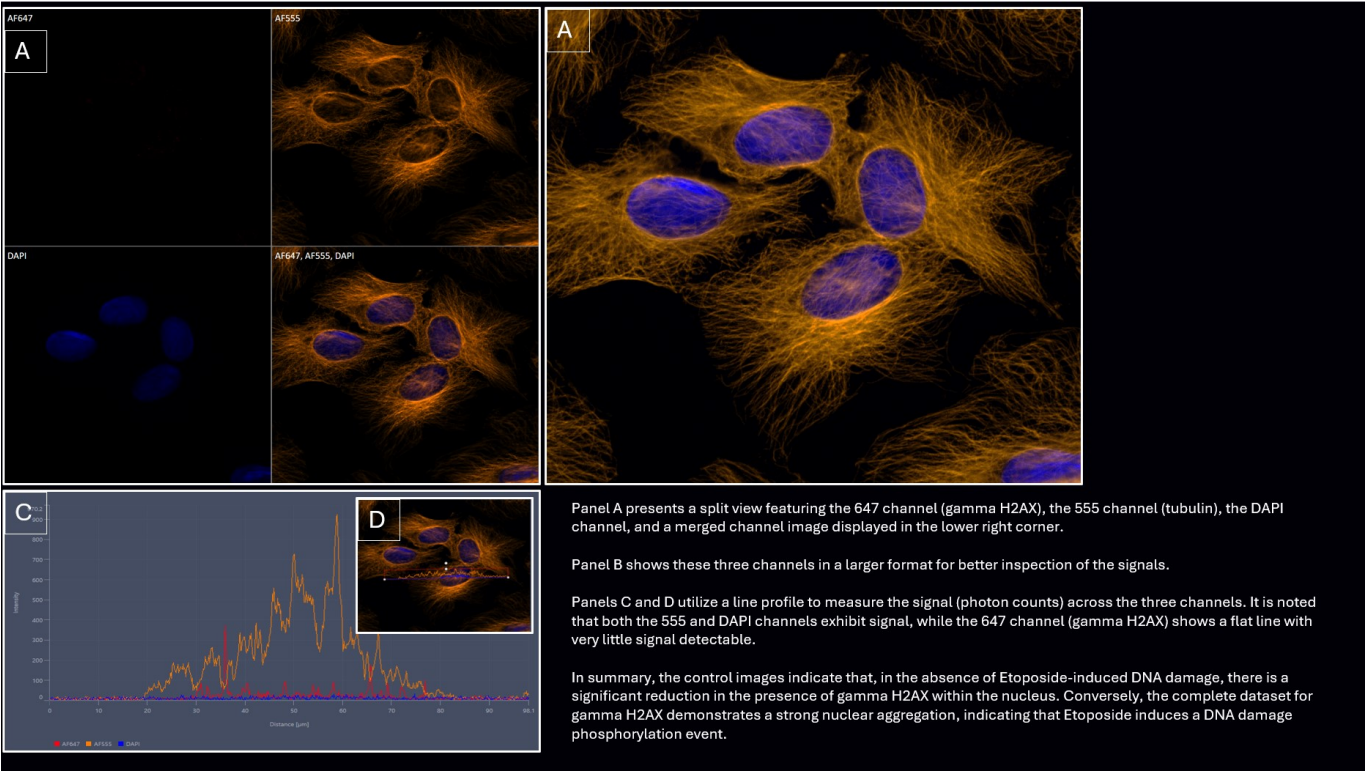
Image by P. Raut

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SOURCE PROVENANCE

Catalog	PA-RR-000005
Stage	Single-molecule STORM
Instrument	Bruker Vutara VXL
Microscope configuration	Bruker Vutara VXL Biplane Single Molecule Microscope, using an Olympus 60X/1.30 silicon oil objective, was used with the following parameters:
Structured source metadata values	80
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	29031517F5FEBADD428F79FCB53277CDFAD416034A1C883592A9B63E8B8C7D19
Method PDF SHA-256	BA65BE4F35A1540A7810E2C9A45607730DE0B4745E886657CF7E422856FA93CB

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 γ H2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)

Cat ID# - PA-RR 000005

NOTE- Control shows Rabbit anti-Human γ H2Ax Recombinant Monoclonal, IgG, (BLR014M)

Cat ID# - PA-RR-000001, as the FC switch is negligible to the results.

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, F(ab')₂ (H + L)

Cat ID# - SA 000065

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Goat Anti-Mouse, F(ab')₂ (H + L)

Cat ID# - SA 000040

Gamma H2AX (γ H2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γ H2AX is rapidly 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 γ H2AX 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 γ H2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS, 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

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SOURCE PROVENANCE

Catalog	PA-RR-000005
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	30D1D696EACC96D5861334F5880C7864FD49FFDE2979E1AAC9A1F73503078597