BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

IDENTIFYN RESOLUTION STEPDOWN

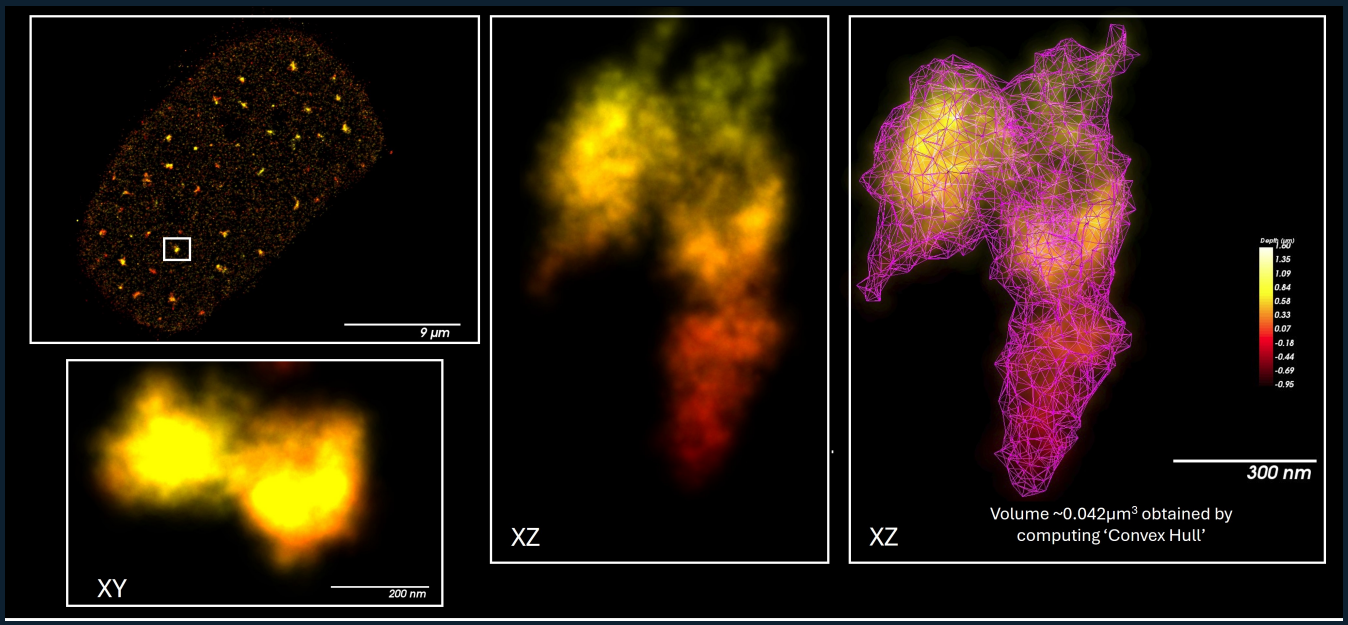
H2AX

MICROSCOPY EVIDENCE ASSESSMENT

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal

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

8	1	PENDING
ORDERED EVIDENCE TIERS	SUPPORTING CONTROL	SCIENTIST REVIEW



REPRESENTATIVE HIGHEST-AVAILABLE VIEW / SINGLE-MOLECULE STORM

PA-RR-000001 | 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-000001 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-000001 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	647	Not deterministically stated in the acquisition block
Widefield	405/DAPI; 488; 680	3; 38 HE eGFP; 50 Cy5; 49 DAPI; 450 - 490; 625 - 655; 335 - 383; 500 - 550
Widefield SIM — Apotome	405/DAPI; 532; 555	2; AF532-49014; 49 DAPI; 515 - 545; 335-383; 555 - 595; 420-470; 528
Confocal	405/DAPI; 488; 647; 680	3; 2; None; 488nm @0.10%; 640nm @1.0%; 405nm @0.4%; 493; 679
Airyscan	405/DAPI; 488; 647; 680	3; None; 488nm @0.09%; 640nm @1.0%; 405nm @0.5%; 493; 679; 353
SIM	405/DAPI; 488; 555; 647	3; 1; 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; T2-Axiocam 820 #2-SR; T3-Axiocam 820 #2-SR
SIM²	405/DAPI; 488; 555; 647; 680	3; SR HE LBF 405/488/561/642; 200 - 405, 200 - 488, 200 - 561, 200 - 642; 419 - 470, 503 - 546, 576 - 617, 657 - 800; T1-Axiocam 820 -SR; T2-Axiocam 820 #2-SR; T3-Axiocam 820 #2-SR; 640
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 using Confocal scanning with lasers and PMT detectors. Structured source metadata values: 12.

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 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

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 Imager.M1. Objective: Plan-Apochromat 63x/1.40 Oil M27. Platform: Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705 mono, and X-Cite Xylis Lamp, was used with the following parameters for each dye:. Acquisition: Channels: 3; Scaling (Per Pixel): 0.110µm X 0.110µm; Image size (Pixels): 943 X 1003; Image Size (Pixels): 943 X 1003; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 705 mono. Timing: Exposure Time: 100ms; 150ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @00%; X-Cite Xylis Lamp Intensity @25%. Optical/scan: Effective NA: 1.4. Structured source metadata values: 55.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as Widefield, documents platform context as ZEISS Axio Imager.M1, and records detected dye/channel descriptors as 405/DAPI; 488; 680.

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: 23 Slices (4.4µm); Channels: 2; Scaling (Per Pixel): 0.143µm X 0.200µm; Image size (Pixels): 217 X 275; Image Size (Pixels): 217 X 275; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 807. Timing: Exposure Time: 150ms; 20ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @35%; X-Cite Xylis Lamp Intensity = @8%. Optical/scan: Effective NA: 1.25. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 40.

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; 532; 555.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 4 OF 9

Confocal / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS LSM 900. Objective: with a Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 900 AiryScan, Axio Observer.Z1/7, using with a Plan-Apochromat 63X/1.40 oil DIC M27 was used with the following parameters for each dye:. Acquisition: Z-Stack: 112 Slices (3.33µm); Channels: 3; 2; Scaling (Per Pixel): 0.033µm X 0.033µm X 0.030µm; Image size (Pixels): 1024 X 1024; 732 X 697; Image Size (Pixels): 1024 X 1024; 732 X 697; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; GaAsP-Pmt2; Detector Type: GaAsP-PMT. Timing: Pixel Time: 0.52µs; Frame Time: 5.06s. Illumination: Laser Wavelength: 488nm @0.10%; 640nm @1.0%. Optical/scan: Pinhole: 1.00 AU / 43µm; 1.00 AU / 57µm; Scan Zoom: 3.0; LSM Scan Speed: 9; 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. Structured source metadata values: 61.

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 900, and records detected dye/channel descriptors as 405/DAPI; 488; 647; 680.

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 900. Objective: with a Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 900 AiryScan, Axio Observer.Z1/7, using with a Plan-Apochromat 63X/1.40 oil DIC M27 was used with the following parameters for each dye:. Acquisition: Z-Stack: 133 Slices (3.96µm); 149 Slices (4.44µm); Channels: 3; Scaling (Per Pixel): 29.72nm X 29.72nm X 30.00nm; Image size (Pixels): 951 X 951; 225 X 287; Image Size (Pixels): 951 X 951; 225 X 287; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; Detector Type: GaAsP-PMT. Timing: Pixel Time: 0.54µs; Frame Time: 4.83s. Illumination: Laser Wavelength: 488nm @0.09%; 640nm @1.0%. Optical/scan: Pinhole: 5.00 AU / 217µm; 5.00 AU / 282µm; Scan Zoom: 3.5; 3.05; LSM Scan Speed: 9; 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; AiryScan Mode: Super Resolution 8.9 (3D, Auto); Super Resolution 8.6 (3D, Auto). Structured source metadata values: 68.

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 900, and records detected dye/channel descriptors as 405/DAPI; 488; 647; 680.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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, 1X Tubelens, was used with the following parameters for each dye:. Acquisition: Z-Stack: 108 Slices (3.21µm); 71 of 108 Collected Slices (2.10µm); Channels: 3; 1; Scaling (Per Pixel): 13.20nm X 13.20nm X 30.00nm; Image size (Pixels): 3368 X 3816; 949 X 550; Image Size (Pixels): 3368 X 3816; 949 X 550; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820; Axiocam 820 #2. Timing: Exposure Time: 120ms; 50ms; Frame Time: 1.59s; 1.33s. Illumination: Laser Wavelength: 640nm @1.50%; 561nm @1.50%; Illumination Wavelength: 640; 561. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM; Process Sampling: N/A; Result Sampling: 4; Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); View Angle 35°, Scale Z 32%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 75.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as SIM, documents platform context as ZEISS Elyra, and records detected dye/channel descriptors as 405/DAPI; 488; 555; 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, 1X Tubelens, was used with the following parameters for each dye:. Acquisition: Z-Stack: 114 Slices (3.39µm); Channels: 3; Scaling (Per Pixel): 13.20nm X 13.20nm X 30.00nm; Image size (Pixels): 5056 X 5056; 676 X 475; Image Size (Pixels): 5056 X 5056; 676 X 475; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820; Axiocam 820 #2. Timing: Exposure Time: 120ms; 50ms; Frame Time: 1.59s; 1.33s. Illumination: Laser Wavelength: 640nm @1.50%; 561nm @1.50%; Illumination Wavelength: 640; 561. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM2; Process Sampling: 4; Result Sampling: 4; Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 73.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as SIM², documents platform context as ZEISS Elyra, and records detected dye/channel descriptors as 405/DAPI; 488; 555; 647; 680.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 8 OF 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 Xylis Lamp was used with the following parameters for each dye:. Acquisition: Channels: 2; Scaling (Per Pixel): 0.143µm X 0.143µm; Image size (Pixels): 963 X 855; Image Size (Pixels): 963 X 855; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 807 mono. Timing: Exposure Time: 150ms; 50ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @8.0%; X-Cite Xylis Lamp Intensity @6.0%. Optical/scan: Effective NA: 1.25. Structured source metadata values: 40.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

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

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

CROSS-MODALITY SYNTHESIS

The preserved PA-RR-000001 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): 29.72nm X 29.72nm X 30.00nm -> 0.02972 µm X 0.02972 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=951 X 951; Image Size (Scaled)=28.26µm X 28.26µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.01%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM / Scaling (Per Pixel): 13.20nm X 13.20nm X 30.00nm -> 0.01320 µm X 0.01320 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=3368 X 3816; Image Size (Scaled)=44.44µm X 50.35µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.04%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM² / Scaling (Per Pixel): 13.20nm X 13.20nm X 30.00nm -> 0.01320 µm X 0.01320 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=5056 X 5056; Image Size (Scaled)=66.72µm X 66.72µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.03%.

UNRESOLVED - PRESERVED AS CONFLICT

Cross-tier / treatment duration: {"Western blot": ["24 hours"], "Widefield": ["24 hours"], "Widefield SIM — Apotome": ["24 hours"], "Confocal": ["24 hours"], "Airyscan": ["24 hours"], "SIM": ["24 hours"], "SIM²": ["24 hours"], "Single-molecule STORM": ["6 hours"]} -> Preserved as modality-specific historical duration values. Genuinely different treatment durations are present across evidence stages, and no source or scientist correction resolves them. Supporting evidence: Only duration values from explicit etoposide-treatment sentences were classified; concentration, wavelength, resolution and pixel-size values were excluded.

HIGH CONFIDENCE - SOURCE-DERIVED METADATA CANONICALIZATION

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

BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

BENNETT EVIDENCE REVIEW COMPLETE

INTERPRETATION TO EVIDENCE

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

IDENTiFYN®

IDENTIFYN PRESERVED SOURCE EVIDENCE

ORIGINAL RECORD BEGINS

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

PRODUCT-LEVEL STEPDOWN MAP

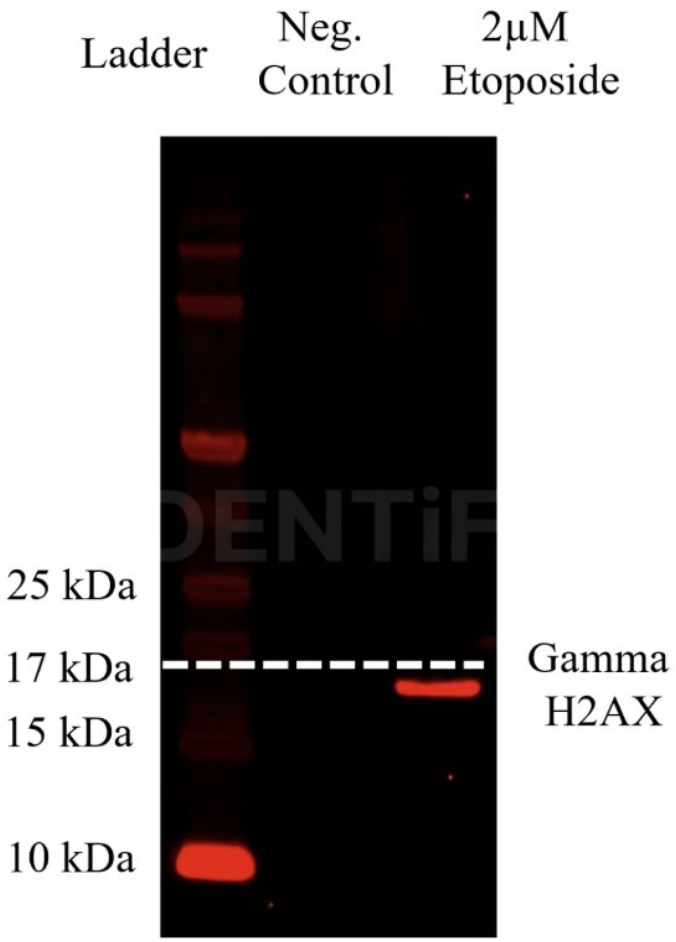
This dossier preserves the complete available evidence progression for PA-RR-000001. Each tier remains tied to its original image, source method, instrument context and cryptographic identity. A preserved control is retained as supporting evidence and appears after every resolution tier.

CANONICAL STEPDOWN TIER	EVIDENCE STAGE	INSTRUMENT	STATUS
1	Western blot	Azure Sapphire FL	PRESERVED
2	Widefield	ZEISS Axio Imager.M1	PRESERVED
3	Widefield SIM - Apotome	ZEISS Axio Observer.Z1/7 Apotome	PRESERVED
4	Confocal	ZEISS LSM 900	PRESERVED
5	Airyscan	ZEISS LSM 900	PRESERVED
6	SIM	ZEISS Elyra	PRESERVED
7	SIM²	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	647
METADATA VALUES	12

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 using Confocal scanning with lasers and PMT detectors
Scanner	Azure Sapphire FL using Confocal scanning with lasers and PMT detectors
Detection mode	Fluorescence
Laser	638 / 676BP37
Intensity	2
Pixel size	100 µm
Scan Speed	High
Quality	High
Focus	Membrane (0 µm in Z-axis)
Black	6957
White	24239
Gamma	1.14
Image	Identifyn® LLC

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.

Methods

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

HeLa cells, induced with 2μM Etoposide over 24 hours for DNA damage and control non-damaged, were lysed and homogenized with an ultrasonicator probe. The supernatant of these lysates was mixed with NuPAGE™ LDS Sample Buffer (4X) and subjected to NuPAGE™ 4 to 12%, Bis-Tris, 1.0-1.5 mm, Mini Protein Gels in a Mini Gel Tank. The Thermo Fisher's PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, was used as a molecular weight marker.

Post electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot™ 3 Starter Kit, PVDF. The transfer membrane was blocked with Blocker™ Casein for 1 hour and washed 5X with PBS. The blocked membrane was incubated with Rabbit anti-Human γH2Ax Recombinant Monoclonal for 2 hours, followed by 5X PBS washes. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat anti-Rabbit, F(ab')2 (H+L) was incubated for 1 hour and washed 5X with PBS. The membrane was dried for 10 min at room temperature before imaging.

Scanner = Azure Sapphire FL using Confocal scanning with lasers and PMT detectors

Scan Settings

Detection mode = Fluorescence
Laser = 638 / 676BP37
Intensity = 2
Pixel size = 100 μm
Scan Speed = High
Quality = High
Focus = Membrane (0 μm in Z-axis)

Post Processing

Light Adjustment
Black = 6957
White = 24239
Gamma = 1.14

Summary

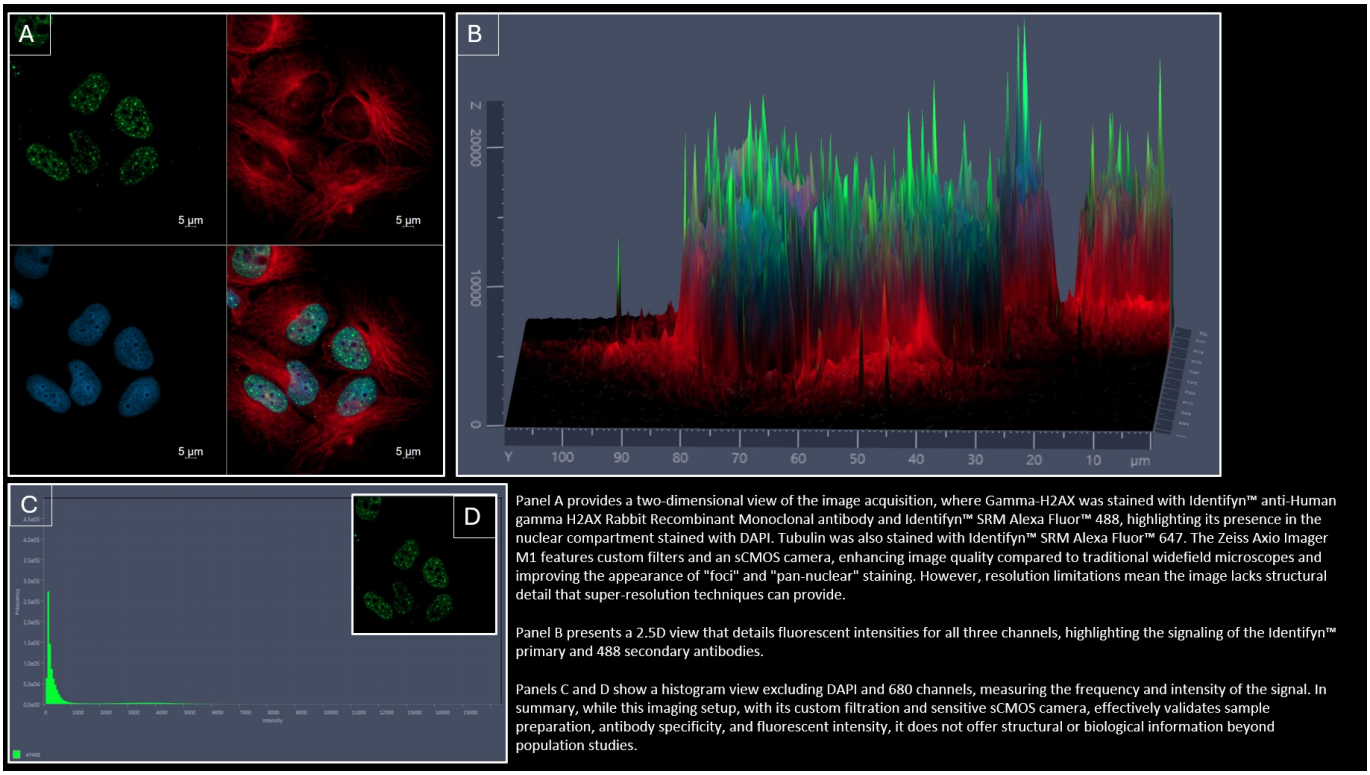
Immunofluorescent Western Blot demonstrates a sparse protein band before etoposide-induced DNA damage, with a robust signal following treatment, confirming Rabbit anti-Human γH2Ax Recombinant Monoclonal ID# - PA-RR 000001 is highly target specific.

Image: Identifyn® LLC

SOURCE PROVENANCE

Catalog	PA-RR-000001
Stage	Western blot
Instrument	Azure Sapphire FL
Microscope configuration	Azure Sapphire FL using Confocal scanning with lasers and PMT detectors
Structured source metadata values	12
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	1548E40FB53BD1BFAD17A04E1555D8D522673E89ECC501C240B65B630711D968
Method PDF SHA-256	5E9D691E85F2D848DCD6C2FA3442A2A7B1F53613CA9D171DB02368A85B175012

ORIGINAL IMAGE EVIDENCE / WIDEFIELD



Panel A provides a two-dimensional view of the image acquisition, where Gamma-H2AX was stained with Identifyn™ anti-Human gamma H2AX Rabbit Recombinant Monoclonal antibody and Identifyn™ SRM Alexa Fluor™ 488, highlighting its presence in the nuclear compartment stained with DAPI. Tubulin was also stained with Identifyn™ SRM Alexa Fluor™ 647. The Zeiss Axio Imager M1 features custom filters and an sCMOS camera, enhancing image quality compared to traditional widefield microscopes and improving the appearance of "foci" and "pan-nuclear" staining. However, resolution limitations mean the image lacks structural detail that super-resolution techniques can provide.

Panel B presents a 2.5D view that details fluorescent intensities for all three channels, highlighting the signaling of the Identifyn™ primary and 488 secondary antibodies.

Panels C and D show a histogram view excluding DAPI and 680 channels, measuring the frequency and intensity of the signal. In summary, while this imaging setup, with its custom filtration and sensitive sCMOS camera, effectively validates sample preparation, antibody specificity, and fluorescent intensity, it does not offer structural or biological information beyond population studies.

EVIDENCE TIER	Widefield
INSTRUMENT	ZEISS Axio Imager.M1
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 680
METADATA VALUES	55

STRUCTURED ACQUISITION METADATA / WIDEFIELD

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705 mono, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63x/1.40 Oil M27
Channels	3
Scaling (Per Pixel)	0.110µm X 0.110µm
Image size (Pixels)	943 X 1003
Image Size (Scaled)	103.28µm X 109.85µm
Bit Depth	14 Bit
Image Center Position	X: 0.00µm, Y: 0.00µm
ROI Center Offset	X: 0.00µm, Y: 0.00µm
Reflector	38 HE eGFP | 50 Cy5 | 49 DAPI
Beam Splitter	495 | 660 | 395
Filter Excitation Wavelength	450 - 490 | 625 - 655 | 335 - 383
Filter Emission Wavelength	500 - 550 | 665 - 715 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @00% | X-Cite Xylis Lamp Intensity @25% | X-Cite Xylis Lamp Intensity @15%
Exposure Time	100ms | 150ms | 40ms
Excitation Wavelength	493 | 679 | 353
Emission Wavelength	517 | 702 | 465
Effective NA	1.4
Imaging Device	Axiocam 705 mono
Camera Adapter	1X
Depth of Focus	0.80µm | 1.09µm | 0.72µm
Binning Mode	2,2
Render Mode	Surface
Grid distance	5
Angle X	80
Angle Y	-92
Z Scaling	1.0
Ambient	50
Specular	50
Shininess	50
Light Height	2.0
Bind Count	256

FIELD	SOURCE-DOCUMENTED VALUE
Bin size	64
Lower Threshold	0
Upper Threshold	16383
X Axis	Auto (min 0.00, max 16383)
Y Axis	Auto (min 0, max 5000000)
Channel Transparency	100%

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')□ (H + L)
Cat ID# - SA 000020

Identifyn® SRM Alexa Fluor™ 680 Affinity Purified Goat Anti-Mouse, F(ab')□ (H + L)
Cat ID# - SA 000073

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly 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 γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 488 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody followed this, Alpha Tubulin Monoclonal Antibody (DM1A), which was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 680 secondary antibody was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI cat#P36962.

Microscope

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

Single Plane (2D)

Channels = 3
Scaling (Per Pixel) = 0.110μm X 0.110μm
Image size (Pixels) = 943 X 1003
Image Size (Scaled) = 103.28μm X 109.85μm
Bit Depth = 14 Bit
Image Center Position = X: 0.00μm, Y: 0.00μm
ROI Center Offset = X: 0.00μm, Y: 0.00μm

488 -

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

680 -

Reflector = 50 Cy5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @25%
Exposure Time = 150ms
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Imaging Device = Axiocam 705 mono
Camera Adapter = 1X
Depth of Focus = 1.09µm
Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15%
Exposure Time = 40ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = Axiocam 705 mono
Camera Adapter = 1X
Depth of Focus = 0.72µm
Binning Mode = 2,2

2.5D Image Processing - Panel B

2.5D Display

Render Mode = Surface
Grid distance = 5
Palette and Show Faces at Side are both Selected

2.5D Display Options

Angle X = 80
Angle Y = -92
Z Scaling = 1.0
Ambient =50
Specular = 50
Shininess = 50
Light Height = 2.0

Histogram of Frequency & Intensity

Histogram Definition

Normal
Bind Count = 256
Bin size = 64
Lower Threshold = 0
Upper Threshold = 16383

Histogram View

X Axis = Auto (min 0.00, max 16383)
Y Axis = Auto (min 0, max 5000000)
Channel Transparency = 100%

Summary

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

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

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

Image Correction

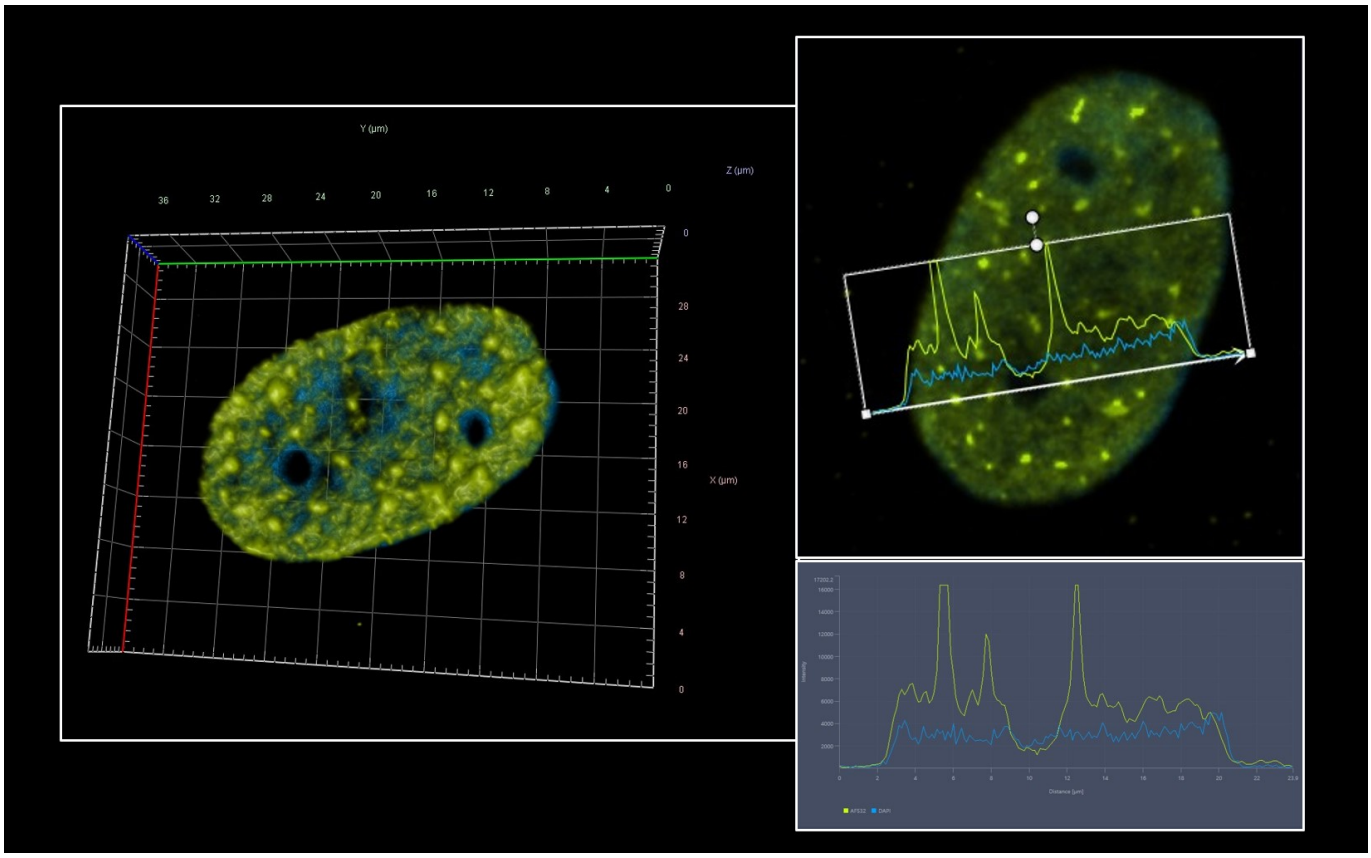
None

Image by E.F. Simon
Analysis and Presentation by B. T. Bennett

SOURCE PROVENANCE

Catalog	PA-RR-000001
Stage	Widefield
Instrument	ZEISS Axio Imager.M1
Microscope configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705 mono, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Structured source metadata values	55
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	F76D67C603059809886B1C672BC890F48401D9FD593DFE7879F991BFB5DDABDF
Method PDF SHA-256	D92C158F039327B0564CCFC11F77AC323E0A6193A74CFEE5C6FFCD0BE999DC90

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; 532; 555
METADATA VALUES	40

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	23 Slices (4.4µm)
Channels	2
Scaling (Per Pixel)	0.143µm X 0.200µm
Image Size (Pixels)	217 X 275
Image Size (Scaled)	31.00µm X 39.29µm
Bit Depth	14 Bit
Image Center Position	X: 73.88mm, Y:52.12mm
Stage Position	X: 73.88mm, Y:52.12mm
ROI Center Offset	X: 1.14µm, Y: 0.00µm
Reflector	AF532-49014 | 49 DAPI
Beam Splitter	550 | 395
Filter Excitation Wavelength	515 - 545 | 335-383
Filter Emission Wavelength	555 - 595 | 420-470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @35% | X-Cite Xylis Lamp Intensity = @8%
Exposure Time	150ms | 20ms
Excitation Wavelength	528 | 353
Emission Wavelength	553 | 465
Effective NA	1.25
Imaging Device	Axiocam 807
Camera Adapter	1X
Depth of Focus	1.07µm | 0.90µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mode "Optical Sectioning" - enabled correction selected.
3D Volume Projection	Precise
Appearance	Threshold 13% all channels, Ramp 50% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
Profile Definition	Stroke Thickness 1, Normal View, Grid Distance 1

FIELD	SOURCE-DOCUMENTED VALUE
Profile View	X and Y axes were set to Auto - X (Min 0.0 and Max 23.9), Y (Min 19 and Max 17202)

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Rabbit, IgG (H + L)
Cat ID# - SA 000058

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly 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 γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Identifyn® Rabbit anti-Human γH2Ax Recombinant Monoclonal, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS, and the Identifyn™532 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:

Z Stack - (3D)

Z-Stack = 23 Slices (4.4μm)

Channels = 2
Scaling (Per Pixel) = 0.143μm X 0.200μm
Image Size (Pixels) = 217 X 275
Image Size (Scaled) = 31.00μm X 39.29μm
Bit Depth = 14 Bit
Image Center Position = X: 73.88mm, Y:52.12mm
Stage Position = X: 73.88mm, Y:52.12mm
ROI Center Offset = X: 1.14μm, Y: 0.00μm

532-

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 @35%
Exposure Time = 150ms
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.25
Imaging Device = Axiocam 807
Camera Adapter = 1X
Depth of Focus = 1.07µm
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 = @8%
Exposure Time = 20ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.25
Imaging Device = Axiocam 807
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 Corrector, Fourier Filter, and Deconvolution were NOT used.

3D Image Processing

3D Volume Projection = Precise
Appearance = Threshold 13% all channels, Ramp 50% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Profile View Display

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 23.9), Y (Min 19 and Max 17202)

Summary

A three-dimensional image representation illustrates foci formation within the nuclear compartment following DNA damage. The profile view highlights the signal intensity across punctate foci, noting the absence of signal or presence of Rabbit anti-Human γH2Ax Recombinant Monoclonal IgG in the nucleoli and outside the nuclear compartment.

Moreover, while Widefield SIM employs a grid methodology to eliminate out-of-focus light and enables z-plane collection, this method has significant limitations in achieving "confocal-like" resolution claims. The "foci" formation appears combined with" pan-nuclei" staining, indicating a loss of resolution. Although the images demonstrate that the antibody responds correctly and that localization is appropriate, any interpretation of biological functions beyond population studies would be misleading. This is particularly true given the structural details encountered when crossing the diffraction limit into super-resolution and single-molecule methods.

Image Corrections

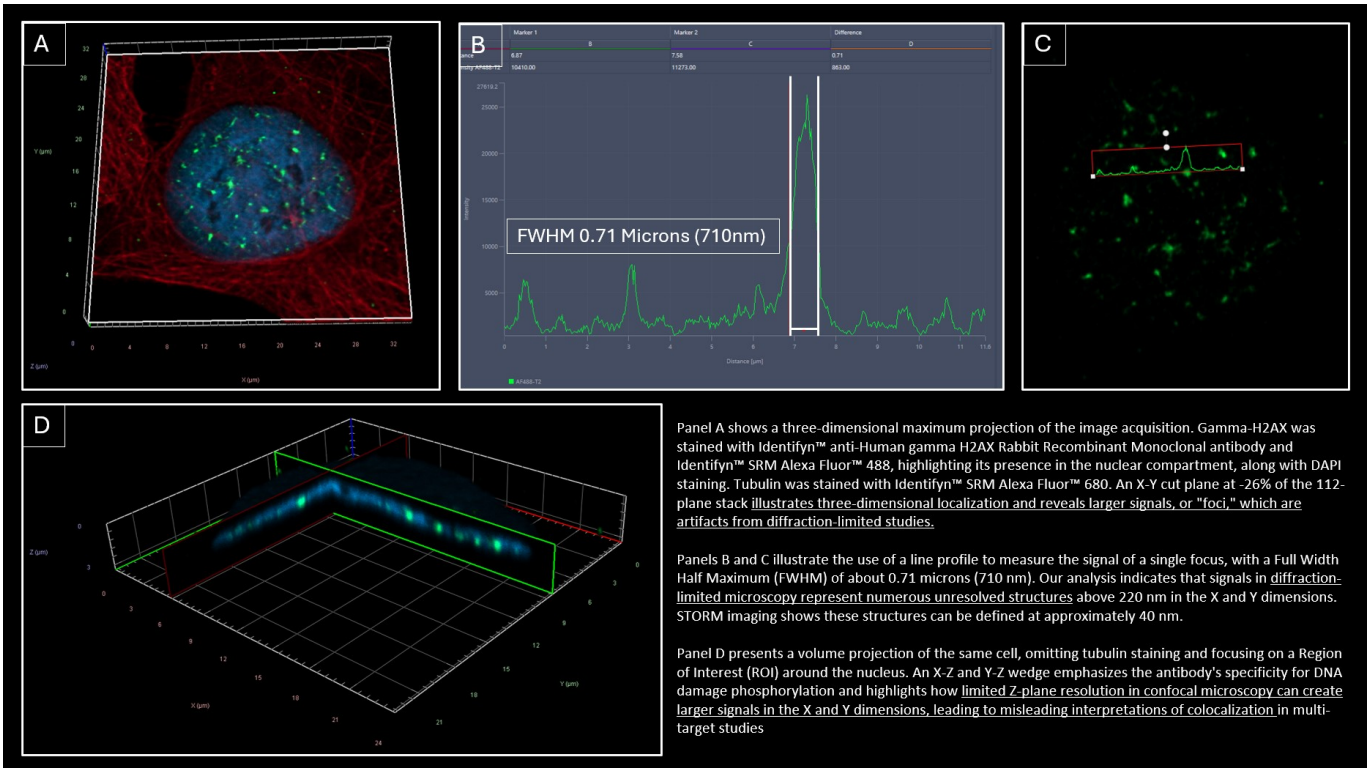
None

Image by B.T. Bennett

SOURCE PROVENANCE

Catalog	PA-RR-000001
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	40
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	0F1611553B6516F7B37EB24AE0D22A079E02F8D7897DEAD8853CC4EDE57AFB7C
Method PDF SHA-256	DD6D0AA56F924198F97A606E932AE15FCA44763AB3DDAE9BA29A02DAA79D2790

ORIGINAL IMAGE EVIDENCE / CONFOCAL



Panel A shows a three-dimensional maximum projection of the image acquisition. Gamma-H2AX was stained with Identifyn™ anti-Human gamma H2AX Rabbit Recombinant Monoclonal antibody and Identifyn™ SRM Alexa Fluor™ 488, highlighting its presence in the nuclear compartment, along with DAPI staining. Tubulin was stained with Identifyn™ SRM Alexa Fluor™ 680. An X-Y cut plane at -26% of the 112-plane stack illustrates three-dimensional localization and reveals larger signals, or "foci," which are artifacts from diffraction-limited studies.

Panels B and C illustrate the use of a line profile to measure the signal of a single focus, with a Full Width Half Maximum (FWHM) of about 0.71 microns (710 nm). Our analysis indicates that signals in diffraction-limited microscopy represent numerous unresolved structures above 220 nm in the X and Y dimensions. STORM imaging shows these structures can be defined at approximately 40 nm.

Panel D presents a volume projection of the same cell, omitting tubulin staining and focusing on a Region of Interest (ROI) around the nucleus. An X-Z and Y-Z wedge emphasizes the antibody's specificity for DNA damage phosphorylation and highlights how limited Z-plane resolution in confocal microscopy can create larger signals in the X and Y dimensions, leading to misleading interpretations of colocalization in multi-target studies

EVIDENCE TIER	Confocal
INSTRUMENT	ZEISS LSM 900
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647; 680
METADATA VALUES	61

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 900 AiryScan, Axio Observer.Z1/7, using with a Plan-Apochromat 63X/1.40 oil DIC M27 was used with the following parameters for each dye:
Objective	with a Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	112 Slices (3.33µm)
Channels	3 | 2
Scaling (Per Pixel)	0.033µm X 0.033µm X 0.030µm
Image Size (Pixels)	1024 X 1024 | 732 X 697
Image Size (Scaled)	33.80µm X 33.80µm | 24.16µm X 23.01µm
Bit Depth	16 Bit
Image Center Position	X: 58.13mm, Y: 46.34mm
Stage Position	X: 58.13mm, Y: 46.34mm
ROI Center Offset	X: 0.00µm, Y: 0.00µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	1.00 AU / 43µm | 1.00 AU / 57µm | 1.00 AU / 37µm
LMS MBS	MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength	488nm @0.10% | 640nm @1.0% | 405nm @0.4%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	3.0
Rotation	0°
Sampling	2.14
Pixel Time	0.52µs
Frame Time	5.06s
LSM Scan Speed	9
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	493 | 679 | 353
Emission Wavelength	517 | 702 | 465
Effective NA	1.4
Detection Wavelength	480-550 | 656 - 700 | 410-470
Imaging Device	Airyscan | GaAsP-Pmt2 | GaAsP-Pmt1
Detector Type	GaAsP-PMT

FIELD	SOURCE-DOCUMENTED VALUE
Detector Gain	650 V | 700 V
Detector Offset	0
Detector Digital Gain	1.0
3D Maximum Projection	Precise
Appearance	Threshold 1% (680), Threshold 1% (488), Threshold 1% (DAPI), Ramp 0% all channels, Maximum 100% all channels | Threshold 2% (680), Threshold 2% (DAPI), Ramp 98% (647), Ramp 98% (DAPI), Maximum 100% all channels
Background	Black
Light	Brightness 100%, Enabled Tone Mapping | Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
3D Volume Projection	Precise
Profile Definition	Stroke Thickness 1, Normal View, Grid Distance 1
Profile View	X and Y axes were set to Auto - X (Min 0.00 and Max 011.63), Y (Min 303 and Max 29482)
Caliper X	Chosen at points displayed herein that represented the Full Width Half Maximum of the Signal

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')□ (H + L)
Cat ID# - SA 000020

Identifyn® SRM Alexa Fluor™ 680 Affinity Purified Goat Anti-Mouse, F(ab')□ (H + L)
Cat ID# - SA 000073

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 488 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody followed this, Alpha Tubulin Monoclonal Antibody (DM1A), which was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 680 secondary antibody was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI cat#P36962.

Microscope

Zeiss LSM 900 AiryScan, Axio Observer.Z1/7, using with 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 = 112 Slices (3.33µm)

Channels = 3
Scaling (Per Pixel) = 0.033µm X 0.033µm X 0.030µm
Image Size (Pixels) = 1024 X 1024
Image Size (Scaled) = 33.80µm X 33.80µm
Bit Depth = 16 Bit
Image Center Position = X: 58.13mm, Y: 46.34mm
Stage Position = X: 58.13mm, Y: 46.34mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

Z Stack - (3D) - Panel D (680/Tubulin Excluded)

Z-Stack = 112 Slices (3.33µm)

Channels = 2
Scaling (Per Pixel) = 0.033µm X 0.033µm X 0.030µm
Image Size (Pixels) = 732 X 697
Image Size (Scaled) = 24.16µm X 23.01µm
Bit Depth = 16 Bit
Image Center Position = X: 58.13mm, Y: 46.34mm
Stage Position = X: 58.13mm, Y: 46.34mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 43µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 488nm @0.10%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.0
Rotation = 0°
Sampling = 2.14
Pixel Time = 0.52µs
Frame Time = 5.06s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 480-550
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 650 V
Detector Offset = 0
Detector Digital Gain = 1.0

680 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 57µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 640nm @1.0%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.0
Rotation = 0°
Sampling = 2.14
Pixel Time = 0.52µs
Frame Time = 5.06s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Detection Wavelength = 656 - 700
Imaging Device = GaAsP-Pmt2
Detector Type = GaAsP-PMT
Detector Gain = 700 V
Detector Offset = 0
Detector Digital Gain = 1.0

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 37µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 405nm @0.4%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.0
Rotation = 0°
Sampling = 2.14
Pixel Time = 0.52µs
Frame Time = 5.06s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 410-470
Imaging Device = GaAsP-Pmt1
Detector Type = GaAsP-PMT
Detector Gain = 650 V
Detector Offset = 0
Detector Digital Gain = 1.0

3D Image Processing - Panel A

3D Maximum Projection = Precise
Appearance = Threshold 1% (680), Threshold 1% (488), Threshold 1% (DAPI), Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel D

3D Volume Projection = Precise
Appearance = Threshold 2% (680), Threshold 2% (DAPI), Ramp 98% (647), Ramp 98% (DAPI), Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Profile View Display

Profile Definition = Stroke Thickness 1, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - X (Min 0.00 and Max 011.63), Y (Min 303 and Max 29482)

Caliper X = Chosen at points displayed herein that represented the Full Width Half Maximum of the Signal

Summary -

Panel A presents a three-dimensional view of the complete image acquisition as a maximum projection. Gamma-H2AX was stained using Identifyn® anti-Human gamma H2AX Rabbit Recombinant Monoclonal antibody and Identifyn® SRM Alexa Fluor™ 488, highlighting its presence within the nuclear compartment, which was also stained with DAPI. Additionally, tubulin was stained with Identifyn® SRM Alexa Fluor™ 680. An X-Y cut plane was introduced at -26% of the stack, comprising 112 planes, to illustrate the three-dimensional localization within the nuclear compartment. This also reveals larger signals, or "foci," which we demonstrate as artifacts from structural studies conducted at diffraction-limited resolutions.

Panels B and C display the use of a line profile to measure the signal from a single focus, noting that the Full Width Half Maximum (FWHM) is approximately 0.71 microns (710 nm). Our analysis of gamma-H2AX, which started with single-molecule resolutions, indicates that diffraction-limited microscopy must be interpreted with the understanding that these signals represent numerous structures that remain unresolved at resolutions above 220 nm in the X and Y dimensions. Our STORM imaging shows that these structures can be defined at approximately 40 nm.

Panel D provides a volume projection of the same cell, excluding the tubulin staining (680) and highlighting a Region of Interest (ROI) around the nucleus. An X-Z and Y-Z wedge was used to emphasize two key points: first, the specificity of the antibody for the DNA damage phosphorylation event, and second, that the limited resolution in the Z-plane of diffraction-limited confocal microscopy can result in larger signals in the X and Y dimensions. This limitation has historically created a misleading impression of colocalization in studies involving multiple protein targets and restricts structural analysis.

Image Corrections -

None

Image by P. Raut
Analysis and Presentation by B. T. Bennett

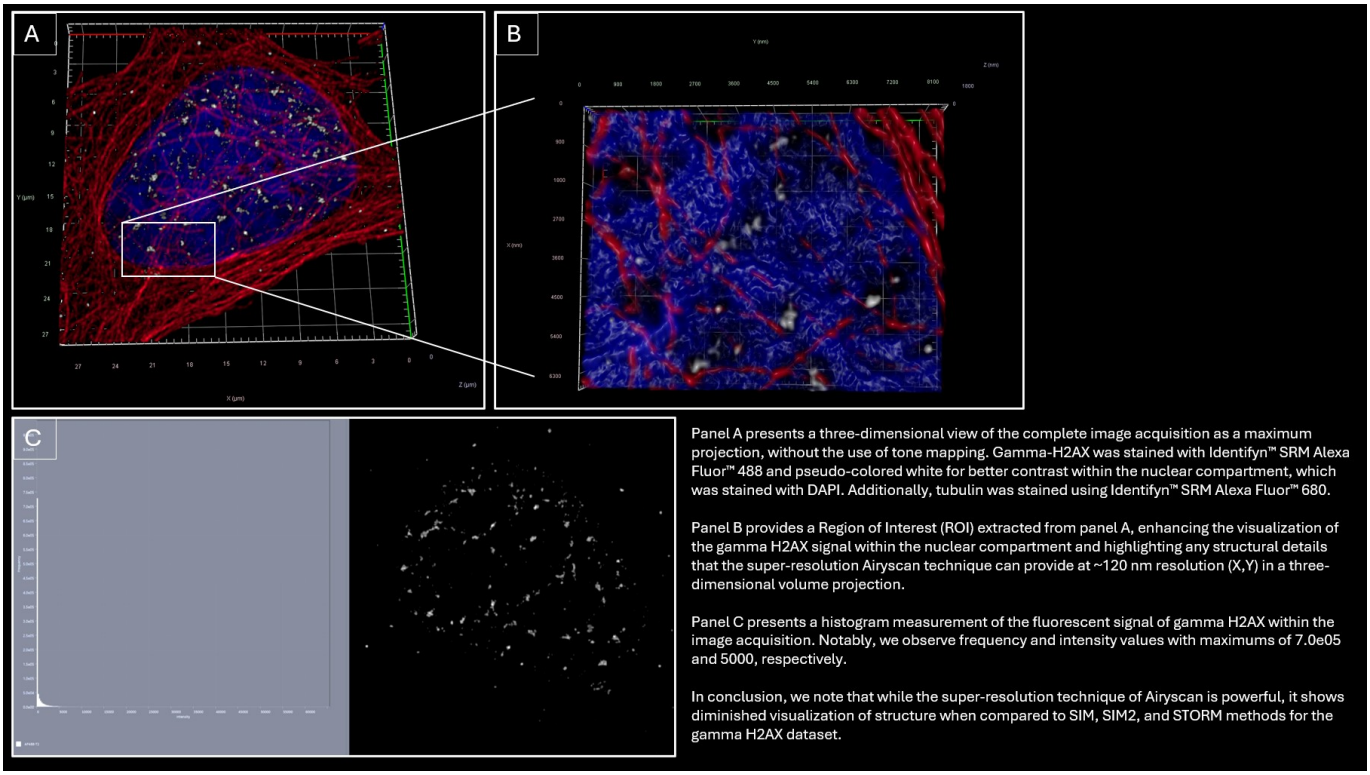
SOURCE PROVENANCE

Catalog	PA-RR-000001
Stage	Confocal
Instrument	ZEISS LSM 900
Microscope configuration	Zeiss LSM 900 AiryScan, Axio Observer.Z1/7, using with a Plan-Apochromat 63X/1.40 oil DIC M27 was used with the following parameters for each dye:
Structured source metadata values	61
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT

SOURCE PROVENANCE

Cell model	HeLa
Image SHA-256	54256385BA03B12952EBB0312C0745326BD8EE3BA09CB8791421FF9ECFCEEC30
Method PDF SHA-256	9F787CA3E09DF6EAB6D7F564CE8817C8550DED5C8D2C4922BF114AEB0F568FAA

ORIGINAL IMAGE EVIDENCE / AIRYSCAN



Panel A presents a three-dimensional view of the complete image acquisition as a maximum projection, without the use of tone mapping. Gamma-H2AX was stained with Identifyn™ SRM Alexa Fluor™ 488 and pseudo-colored white for better contrast within the nuclear compartment, which was stained with DAPI. Additionally, tubulin was stained using Identifyn™ SRM Alexa Fluor™ 680.

Panel B provides a Region of Interest (ROI) extracted from panel A, enhancing the visualization of the gamma H2AX signal within the nuclear compartment and highlighting any structural details that the super-resolution Airyscan technique can provide at ~120 nm resolution (X,Y) in a three-dimensional volume projection.

Panel C presents a histogram measurement of the fluorescent signal of gamma H2AX within the image acquisition. Notably, we observe frequency and intensity values with maximums of 7.0e05 and 5000, respectively.

In conclusion, we note that while the super-resolution technique of Airyscan is powerful, it shows diminished visualization of structure when compared to SIM, SIM2, and STORM methods for the gamma H2AX dataset.

EVIDENCE TIER	Airyscan
INSTRUMENT	ZEISS LSM 900
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 647; 680
METADATA VALUES	68

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 900 AiryScan, Axio Observer.Z1/7, using with a Plan-Apochromat 63X/1.40 oil DIC M27 was used with the following parameters for each dye:
Objective	with a Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	133 Slices (3.96µm) | 149 Slices (4.44µm)
Channels	3
Scaling (Per Pixel)	0.02972 µm X 0.02972 µm X 0.03000 µm
Image Size (Pixels)	951 X 951 | 225 X 287
Image Size (Scaled)	28.26µm X 28.26µm | 6.69µm X 8.53µm
Bit Depth	16 Bit
Image Center Position	X: 81.32mm, Y: 42.25mm
Stage Position	X: 81.32mm, Y: 42.25mm
ROI Center Offset	X: 0.00µm, Y: 0.00µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	5.00 AU / 217µm | 5.00 AU / 282µm | 5.00 AU / 183µm
LMS MBS	MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength	488nm @0.09% | 640nm @1.0% | 405nm @0.5%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	3.5 | 3.05
Rotation	0°
Sampling	2.38 | 2.3
Pixel Time	0.54µs
Frame Time	4.83s
LSM Scan Speed	9
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	493 | 679 | 353
Emission Wavelength	517 | 702 | 465
Effective NA	1.4
Detection Wavelength	480-549 | 650 - 700 | 400-472
Imaging Device	Airyscan
Detector Type	GaAsP-PMT

FIELD	SOURCE-DOCUMENTED VALUE
Detector Gain	800 V | 850 V
Detector Offset	0
Detector Digital Gain	1.0
AiryScan Mode	Super Resolution 8.9 (3D, Auto) | Super Resolution 8.6 (3D, Auto) | Super Resolution 7.4 (3D, Auto)
3D Maximum Projection	Precise
Appearance	Threshold 1% (680), Threshold 1% (488), Threshold 1% (DAPI), Ramp 0% all channels, Maximum 100% all channels | Threshold 2% (680), Threshold 2% (DAPI), Ramp 98% (647), Ramp 98% (DAPI), Maximum 100% all channels
Background	Black
Light	Brightness 100%, Enabled Tone Mapping | Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
3D Volume Projection	Precise
Bind Count	256
Bin size	256
Lower Threshold	0
Upper Threshold	65535
X Axis	Auto (min 1.11, max 65535)
Y Axis	Auto (min 0, max 10000000)
Channel Transparency	100%

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')□ (H + L)
Cat ID# - SA 000020

Identifyn® SRM Alexa Fluor™ 680 Affinity Purified Goat Anti-Mouse, F(ab')□ (H + L)
Cat ID# - SA 000073

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

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 488 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody followed this, Alpha Tubulin Monoclonal Antibody (DM1A), which was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 680 secondary antibody was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI cat#P36962.

Microscope

Zeiss LSM 900 AiryScan, Axio Observer.Z1/7, using with 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 = 133 Slices (3.96µm)

Channels = 3
Scaling (Per Pixel) = 29.72nm X 29.72nm X 30.00nm
Image Size (Pixels) = 951 X 951
Image Size (Scaled) = 28.26µm X 28.26µm
Bit Depth = 16 Bit
Image Center Position = X: 81.32mm, Y: 42.25mm
Stage Position = X: 81.32mm, Y: 42.25mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

Z Stack - (3D) - Panel B

Z-Stack = 149 Slices (4.44µm)

Channels = 3
Scaling (Per Pixel) = 29.72nm X 29.72nm X 30.00nm
Image Size (Pixels) = 225 X 287
Image Size (Scaled) = 6.69µm X 8.53µm
Bit Depth = 16 Bit
Image Center Position = X: 81.32mm, Y: 42.25mm
Stage Position = X: 81.32mm, Y: 42.25mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 217µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 488nm @0.09%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.5
Rotation = 0°
Sampling = 2.38
Pixel Time = 0.54µs
Frame Time = 4.83s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 480-549
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
AiryScan Mode = Super Resolution 8.9 (3D, Auto)

680 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 282µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 640nm @1.0%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.05
Rotation = 0°
Sampling = 2.38
Pixel Time = 0.54µs
Frame Time = 4.83s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Detection Wavelength = 650 - 700
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 850 V
Detector Offset = 0
Detector Digital Gain = 1.0
AiryScan Mode = Super Resolution 8.6 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 183µm
LMS MBS = MBS 405 + 488 + 561 + 640(T10/R90)
Laser Wavelength = 405nm @0.5%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.5
Rotation = 0°
Sampling = 2.3
Pixel Time = 0.54µs
Frame Time = 4.83s
LSM Scan Speed = 9
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 400-472
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
AiryScan Mode = Super Resolution 7.4 (3D, Auto)

Post Processing - AiryScan 3D Processing

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

3D Image Processing - Panel A

3D Maximum Projection = Precise
Appearance = Threshold 1% (680), Threshold 1% (488), Threshold 1% (DAPI), Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel B

3D Volume Projection = Precise
Appearance = Threshold 2% (680), Threshold 2% (DAPI), Ramp 98% (647), Ramp 98% (DAPI), 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)

Histogram of Frequency & Intensity

Histogram Definition

Normal
Bind Count = 256
Bin size = 256
Lower Threshold = 0
Upper Threshold = 65535

Histogram View

X Axis = Auto (min 1.11, max 65535)
Y Axis = Auto (min 0, max 10000000)
Channel Transparency = 100%

Summary -

Panel A presents a three-dimensional view of the complete image acquisition as a maximum projection, without the use of tone mapping. Gamma-H2AX was stained with Identifyn® SRM Alexa Fluor™ 488 and pseudo-colored white for better contrast within the nuclear compartment, which was stained with DAPI. Additionally, tubulin was stained using Identifyn® SRM Alexa Fluor™ 647.

Panel B provides a Region of Interest (ROI) extracted from panel A, enhancing the visualization of the gamma H2AX signal within the nuclear compartment and highlighting any structural details that the super-resolution Airyscan technique can provide at ~120 nm resolution (X, Y) in a three-dimensional volume projection.

Panel C presents a histogram measurement of the fluorescent signal of gamma H2AX within the image acquisition. Notably, we observe frequency and intensity values with maximums of 7.0e05 and 5000, respectively.

In conclusion, we note that while the super-resolution technique of Airyscan is powerful, it shows diminished structure visualization compared to SIM, SIM2, and STORM methods for the gamma H2AX dataset.

Image Corrections -

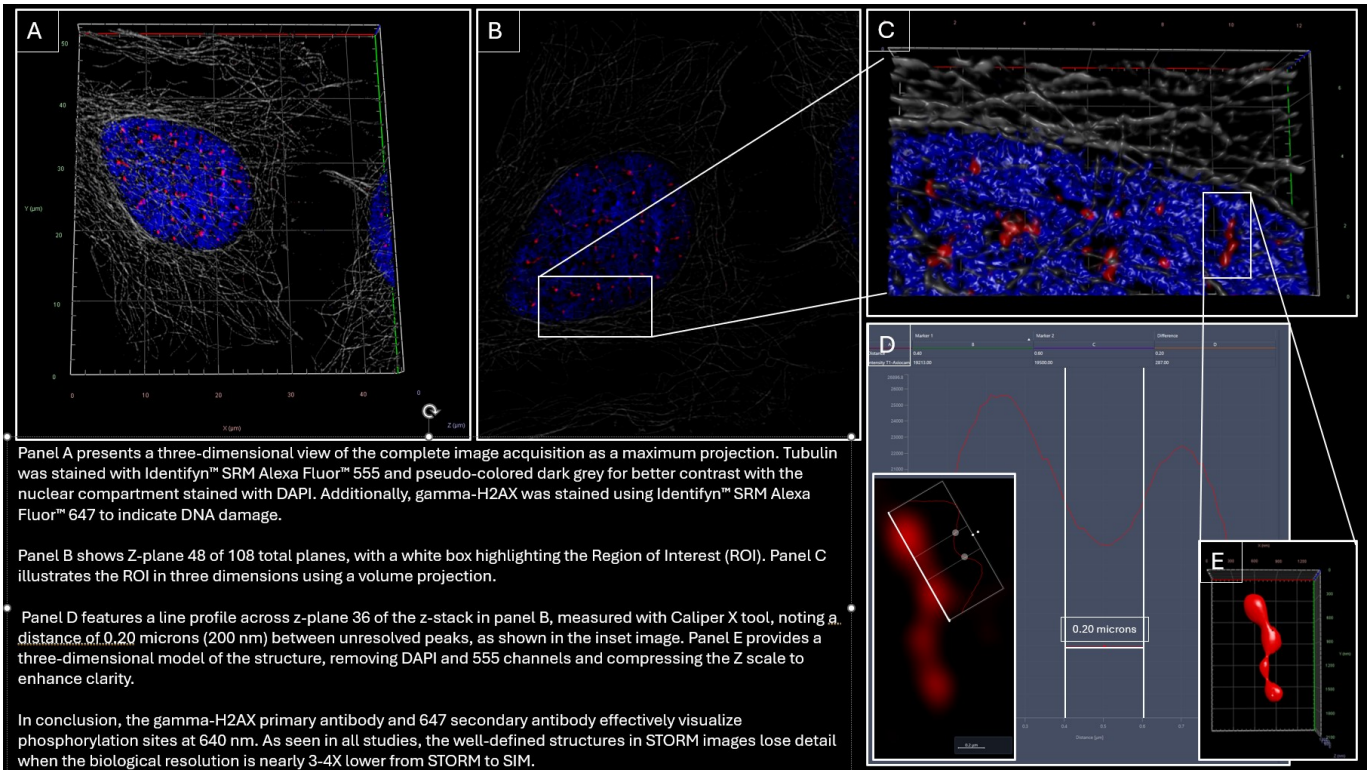
None

Image by P. Raut
Analysis and Presentation by B. T. Bennett

SOURCE PROVENANCE

Catalog	PA-RR-000001
Stage	Airyscan
Instrument	ZEISS LSM 900
Microscope configuration	Zeiss LSM 900 AiryScan, Axio Observer.Z1/7, using with a Plan-Apochromat 63X/1.40 oil DIC M27 was used with the following parameters for each dye:
Structured source metadata values	68
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	F1365A09035E747D44093FA84D8EC00BE46681A963206FED81A878E782C70146
Method PDF SHA-256	2D91B792C79EE5355F0AF9427AF3D42D25212694A1C9A53F3678E4F17300B9F4

ORIGINAL IMAGE EVIDENCE / SIM



Panel A presents a three-dimensional view of the complete image acquisition as a maximum projection. Tubulin was stained with Identifyn™ SRM Alexa Fluor™ 555 and pseudo-colored dark grey for better contrast with the nuclear compartment stained with DAPI. Additionally, gamma-H2AX was stained using Identifyn™ SRM Alexa Fluor™ 647 to indicate DNA damage.

Panel B shows Z-plane 48 of 108 total planes, with a white box highlighting the Region of Interest (ROI). Panel C illustrates the ROI in three dimensions using a volume projection.

Panel D features a line profile across z-plane 36 of the z-stack in panel B, measured with Caliper X tool, noting a distance of 0.20 microns (200 nm) between unresolved peaks, as shown in the inset image. Panel E provides a three-dimensional model of the structure, removing DAPI and 555 channels and compressing the Z scale to enhance clarity.

In conclusion, the gamma-H2AX primary antibody and 647 secondary antibody effectively visualize phosphorylation sites at 640 nm. As seen in all studies, the well-defined structures in STORM images lose detail when the biological resolution is nearly 3-4X lower from STORM to SIM.

EVIDENCE TIER	SIM
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555; 647
METADATA VALUES	75

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, 1X Tubelens, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	108 Slices (3.21µm) | 71 of 108 Collected Slices (2.10µm)
Channels	3 | 1
Scaling (Per Pixel)	0.01320 µm X 0.01320 µm X 0.03000 µm
Image Size (Pixels)	3368 X 3816 | 949 X 550 | 109 X 160
Image Size (Scaled)	44.44µm X 50.35µm | 12.52µm X 7.26µm | 1.44µm X 2.11µm
Bit Depth	16 Bit
Image Center Position	X: 59.14mm, Y: 38.63mm
Stage Position	X: 59.14mm, Y: 38.63mm
ROI Center Offset	X: 0.00µm, Y: 0.00µm
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 | 561 | 405
Channel Name	T1-Axiocam 820 -SR | T2-Axiocam 820 #2-SR | T3-Axiocam 820 #2-SR
Excitation Wavelength	640 | 561 | 405
Emission Wavelength	729 | 597 | 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800 | 419-470, 503 - 546, 576 - 617, 657-800
Imaging Device	Axiocam 820 | Axiocam 820 #2
Camera Adapter	1X
Exposure Time	120ms | 50ms
Depth of Focus	1.13µm | 0.92µm | 0.69µm
Binning Mode	2,2
Laser Wavelength	640nm @1.50% | 561nm @1.50% | 405nm @6.0%
Frame Time	1.59s | 1.33s | 676.00ms
Scan Direction	Unidirectional
Result Sampling	4
Process Sampling	N/A
Algorithm	SIM

FIELD	SOURCE-DOCUMENTED VALUE
Sharpness Setting	Standard
Auto Sharpness	On
Sharpness	10.0285 (647), 10.1661 (555), 8.4947 (DAPI)
Fitting	Fast Fit
Histogram	Scale to Min/Max
Baseline	Cut
3D Maximum Projection	Precise
Appearance	Threshold 4% (647), Threshold 4% (555), Threshold 4% (DAPI), Ramp 0% all channels, Maximum 100% all channels | Threshold 2% (647), Threshold 2% (555), Threshold 2% (DAPI), Ramp 98% all channels, Maximum 100% all channels | Threshold 32% (647), Ambient Light 0%, Specular Light 71%, Shininess 85%
Background	Black
Light	Brightness 100%, Enabled Tone Mapping | Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping | Azimuth 72°, Elongation 71°, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected) | View Angle 35°, Scale Z 32%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
3D Volume Projection	Precise
3D Surface Projection	Precise
Profile Definition	Stroke Thickness 1, Normal View, Grid Distance 1
Profile View	X and Y axes were set to Auto - X (Min 0.00 and Max 0.93), Y (Min 5523 and Max 26897)
Caliper X	Chosen at points displayed herein that represented the Full Width Half Maximum of the Signal

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

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

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

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly 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 γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 647 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

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

Microscope

Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A

Z-Stack = 108 Slices (3.21µm)

Channels = 3
Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm
Image Size (Pixels) = 3368 X 3816
Image Size (Scaled) = 44.44µm X 50.35µm
Bit Depth = 16 Bit
Image Center Position = X: 59.14mm, Y: 38.63mm
Stage Position = X: 59.14mm, Y: 38.63mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

Single Plane (2D) - Panel B

Z-Plane - 48 of 108

Z Stack - (3D) - Panel C

Z-Stack = 71 of 108 Collected Slices (2.10µm)

Channels = 3
Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm
Image Size (Pixels) = 949 X 550
Image Size (Scaled) = 12.52µm X 7.26µm
Bit Depth = 16 Bit
Image Center Position = X: 59.14mm, Y: 38.63mm
Stage Position = X: 59.14mm, Y: 38.63mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

Z Stack - (3D) - Panel E

Z-Stack = 71 of 108 Collected Slices (2.10µm)

Channels = 1
Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm
Image Size (Pixels) = 109 X 160
Image Size (Scaled) = 1.44µm X 2.11µm
Bit Depth = 16 Bit
Image Center Position = X: 59.14mm, Y: 38.63mm
Stage Position = X: 59.14mm, Y: 38.63mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

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 = 120ms
Depth of Focus = 1.13µm
Binning Mode = 2,2
Laser Wavelength = 640nm @1.50%
Frame Time = 1.59s
Scan Direction = Unidirectional
Grating 27.5µm grating

555 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 561
Channel Name = T2-Axiocam 820 #2-SR
Excitation Wavelength = 561
Emission Wavelength = 597
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 120ms
Depth of Focus = 0.92µm
Binning Mode = 2,2
Laser Wavelength = 561nm @1.50%
Frame Time = 1.33s
Scan Direction = Unidirectional
Grating 32.0µm 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 = 50ms
Depth of Focus = 0.69µm
Binning Mode = 2,2
Laser Wavelength = 405nm @6.0%
Frame Time = 676.00ms
Scan Direction = Unidirectional
Grating 36.5µm grating

Post Processing - SIM Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 3
Algorithm = SIM
Sharpness Setting = Standard
Auto Sharpness = On
Sharpness = 10.0285 (647), 10.1661 (555), 8.4947 (DAPI)
Fitting = Fast Fit
Histogram = Scale to Min/Max
Baseline = Cut

3D Image Processing - Panel A

3D Maximum Projection = Precise
Appearance = Threshold 4% (647), Threshold 4% (555), Threshold 4% (DAPI), Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel C

3D Volume Projection = Precise
Appearance = Threshold 2% (647), Threshold 2% (555), Threshold 2% (DAPI), 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 E (Single Channel 647)

3D Surface Projection = Precise
Appearance = Threshold 32% (647), Ambient Light 0%, Specular Light 71%, Shininess 85%
Background = Black
Light = Azimuth 72°, Elongation 71°, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 32%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Profile View Display

Profile Definition = Stroke Thickness 1, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - X (Min 0.00 and Max 0.93), Y (Min 5523 and Max 26897)

Caliper X = Chosen at points displayed herein that represented the Full Width Half Maximum of the Signal

Summary

Panel A presents a three-dimensional view of the complete image acquisition as a maximum projection. Tubulin was stained with Identifyn® SRM Alexa Fluor™ 555 and pseudo-colored dark grey for better contrast with the nuclear compartment stained with DAPI. Additionally, gamma-H2AX was stained using Identifyn® SRM Alexa Fluor™ 647 to indicate DNA damage.

Panel B shows Z-plane 48 of 108 total planes, with a white box highlighting the Region of Interest (ROI). Panel C illustrates the ROI in three dimensions using a volume projection.

Panel D features a line profile across z-plane 36 of the z-stack in panel B, measured with the Caliper X tool, noting a distance of 0.20 microns (200 nm) between unresolved peaks, as shown in the inset image. Panel E provides a three-dimensional structure model, removing DAPI and 555 channels and compressing the Z scale to enhance clarity.

In conclusion, the gamma-H2AX primary antibody and 647 secondary antibody effectively visualize phosphorylation sites at 640 nm. As seen in all studies, the well-defined structures in STORM images lose detail when the biological resolution is nearly 3- 4X lower from STORM to SIM.

Image Corrections

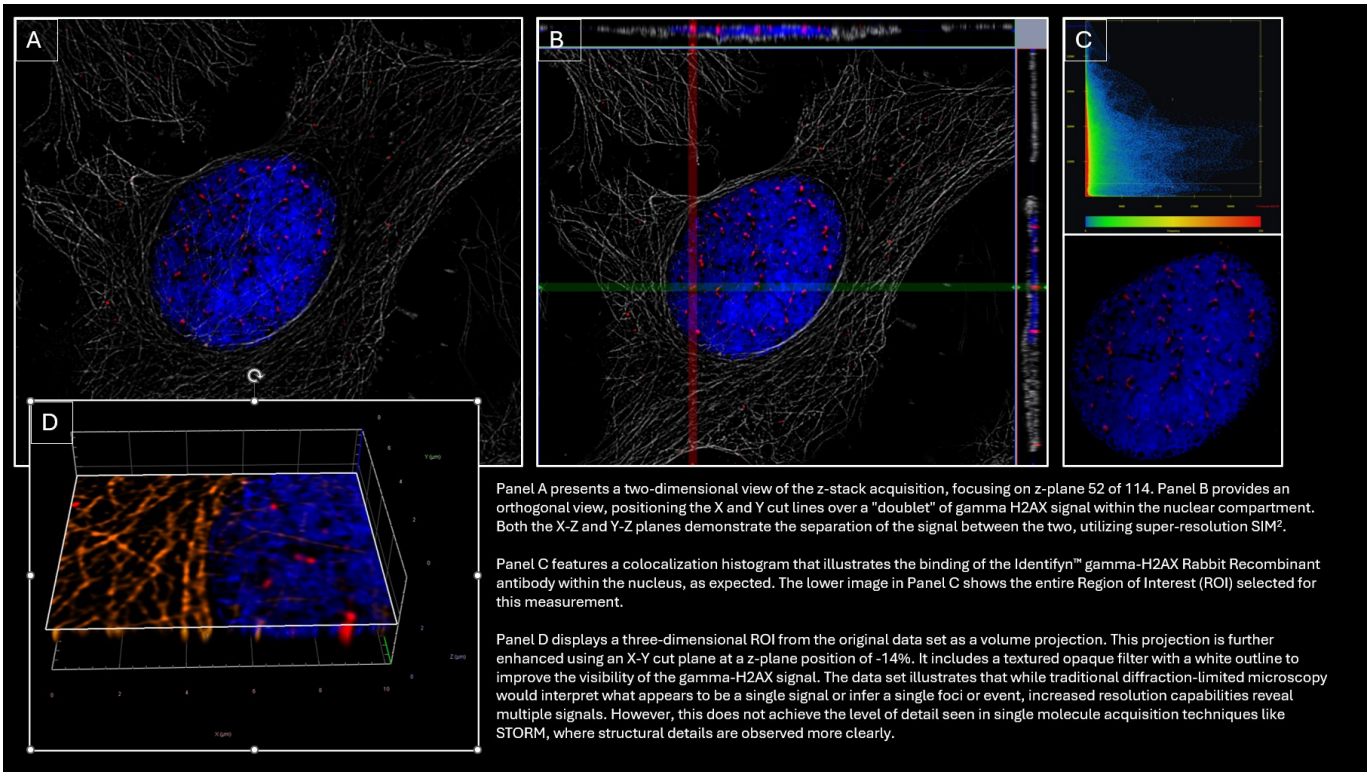
None

Image by P. Raut
Analysis and Presentation by B. T. Bennett

SOURCE PROVENANCE

Catalog	PA-RR-000001
Stage	SIM
Instrument	ZEISS Elyra
Microscope configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1X Tubelens, was used with the following parameters for each dye:
Structured source metadata values	75
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	3584D6EA610C476E5BDC3E143CD0E755DB16222E80196384D4C8852543C02572
Method PDF SHA-256	733D6D58D659540FF544166E639F2EC8D324B6E3B2A9FA51B7D163470C05C7BB

ORIGINAL IMAGE EVIDENCE / SIM²



EVIDENCE TIER	SIM²
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 555; 647; 680
METADATA VALUES	73

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, 1X Tubelens, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Channels	3
Scaling (Per Pixel)	0.01320 µm X 0.01320 µm X 0.03000 µm
Image Size (Pixels)	5056 X 5056 | 676 X 475
Image Size (Scaled)	66.72µm X 66.72µm | 8.92µm X 6.27µm
Bit Depth	16 Bit
Image Center Position	X: 59.08mm, Y: 39.49mm | X: 59.14mm, Y: 38.63mm
Stage Position	X: 59.08mm, Y: 39.49mm | X: 59.14mm, Y: 38.63mm
ROI Center Offset	X: 0.00µm, Y: 0.00µm
Z-Stack	114 Slices (3.39µm)
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 | 561 | 405
Channel Name	T1-Axiocam 820 -SR | T2-Axiocam 820 #2-SR | T3-Axiocam 820 #2-SR
Excitation Wavelength	640 | 561 | 405
Emission Wavelength	729 | 597 | 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800 | 419-470, 503 - 546, 576 - 617, 657-800
Imaging Device	Axiocam 820 | Axiocam 820 #2
Camera Adapter	1X
Exposure Time	120ms | 50ms
Depth of Focus	1.13µm | 0.92µm | 0.69µm
Binning Mode	2,2
Laser Wavelength	640nm @1.50% | 561nm @1.50% | 405nm @6.0%
Frame Time	1.59s | 1.33s | 676.00ms
Scan Direction	Unidirectional
Result Sampling	4
Process Sampling	4
Algorithm	SIM2

FIELD	SOURCE-DOCUMENTED VALUE
Sharpness Setting	Standard
Auto Sharpness	On
Sharpness	8.8687 (647), 9.4576 (555), 8.0281 (DAPI)
Order Combination	Weiner Filter
Regularization	None
Regularization Weight	0.000
Iterations	3
Fitting	Fast Fit
Histogram	Scale to Min/Max
3D Volume Projection	Precise
Appearance	Threshold 2% (647), Threshold 2% (555), Threshold 2% (DAPI), 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)
Cut Lines	X 1375, Y 2135, Z 53
Line Width	X 75, Y 75, Z 1
Cut Line Opacity	11
X-Y	Active was selected, and a textured opaque filter was chosen to remove the obstruction from the image-cut plane and highlight the nuclear compartment and gamma-H2AX foci. A white outline of the clipped perimeter was shown, the Clip Back was selected, and the Clip Front was NOT selected.
Position of Clip	-14%
Clip Y	0°
Clip Z	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 γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Rabbit, F(ab')□ (H + L)
Cat ID# - SA 000020

Identifyn® SRM Alexa Fluor™ 680 Affinity Purified Goat Anti-Mouse, F(ab')□ (H + L)
Cat ID# - SA 000073

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly 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 γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 488 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody followed this, Alpha Tubulin Monoclonal Antibody (DM1A), which was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 680 secondary antibody was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI cat#P36962.

Microscope

Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1X Tubelens, was used with the following parameters for each dye:

Single Plane (2D) - Panel A

Z-Plane - 52 of 114

Channels = 3
Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm
Image Size (Pixels) = 5056 X 5056
Image Size (Scaled) = 66.72µm X 66.72µm
Bit Depth = 16 Bit
Image Center Position = X: 59.08mm, Y: 39.49mm
Stage Position = X: 59.08mm, Y: 39.49mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

Z Stack - (3D) - Panel D

Z-Stack = 114 Slices (3.39µm)

Channels = 3
Scaling (Per Pixel) = 13.20nm X 13.20nm X 30.00nm
Image Size (Pixels) = 676 X 475
Image Size (Scaled) = 8.92µm X 6.27µm
Bit Depth = 16 Bit
Image Center Position = X: 59.14mm, Y: 38.63mm
Stage Position = X: 59.14mm, Y: 38.63mm
ROI Center Offset = X: 0.00µm, Y: 0.00µm

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 = 120ms
Depth of Focus = 1.13µm
Binning Mode = 2,2
Laser Wavelength = 640nm @1.50%
Frame Time = 1.59s
Scan Direction = Unidirectional
Grating 27.5µm grating

555 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 561
Channel Name = T2-Axiocam 820 #2-SR
Excitation Wavelength = 561
Emission Wavelength = 597
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 120ms
Depth of Focus = 0.92µm
Binning Mode = 2,2
Laser Wavelength = 561nm @1.50%
Frame Time = 1.33s
Scan Direction = Unidirectional
Grating 32.0µm 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 = 50ms
Depth of Focus = 0.69µm
Binning Mode = 2,2
Laser Wavelength = 405nm @6.0%
Frame Time = 676.00ms
Scan Direction = Unidirectional
Grating 36.5µm grating

Post Processing - SIM Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = 4
Channels = 3
Algorithm = SIM2
Sharpness Setting = Standard
Auto Sharpness = On
Sharpness = 8.8687 (647), 9.4576 (555), 8.0281 (DAPI)
Order Combination = Weiner Filter
Regularization = None
Regularization Weight = 0.000
Iterations = 3
Filter - No Filter
Fitting = Fast Fit
Histogram = Scale to Min/Max

3D Image Processing - Panel C

3D Volume Projection = Precise
Appearance = Threshold 2% (647), Threshold 2% (555), Threshold 2% (DAPI), 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)

Orthogonal View, -Panel B - Uses Image 52 of the Z-Stack 114 Images.

Cut Lines = X 1375, Y 2135, Z 53
Line Width = X 75, Y 75, Z 1
Cut Line Opacity = 11

Maximum Intensity Opacity (MIP) and Measure 3D Distance NOT selected

Colocalization View

Horizontal Axis - 647, Threshold 42862, Range Auto
Vertical Axis - DAPI, Threshold 4832, Range Auto

Clipping Image Process Panels C and D -

The Clipping plane is introduced to demonstrate the specificity of the Identifyn® Rabbit anti-Human γH2Ax Recombinant Monoclonal, Human Fc, IgG to the DNA damage-induced gamma-H2AX foci within the nuclear compartment.

X-Y = Active was selected, and a textured opaque filter was chosen to remove the obstruction from the image-cut plane and highlight the nuclear compartment and gamma-H2AX foci. A white outline of the clipped perimeter was shown, the Clip Back was selected, and the Clip Front was NOT selected.

Position of Clip = -14%
Clip Y = 0°
Clip Z =0°

Summary

Panel A presents a two-dimensional view of the z-stack acquisition, focusing on z-plane 52 of 114. Panel B provides an orthogonal view, positioning the X and Y cut lines over a "doublet" of gamma H2AX signal within the nuclear compartment. Both the XZ and YZ planes demonstrate the signal separation between the two, utilizing super-resolution SIM2.

Panel C features a colocalization histogram that illustrates the binding of the Identifyn® gamma-H2AX Rabbit Recombinant antibody within the nucleus, as expected. The lower image in Panel C shows the entire Region of Interest (ROI) selected for this measurement.

Panel D displays a three-dimensional ROI from the original data set as a volume projection. This projection is further enhanced using an X, Y cut plane at a z-plane position of -14%. It includes a textured opaque filter with a white outline to improve the visibility of the gamma-H2AX signal. The data set illustrates that while traditional diffraction-limited microscopy would interpret what appears to be a single signal, increased resolution capabilities reveal multiple signals. However, this does not achieve the level of detail seen in single molecule acquisition techniques like STORM, where structural details are observed more clearly.

Image Corrections

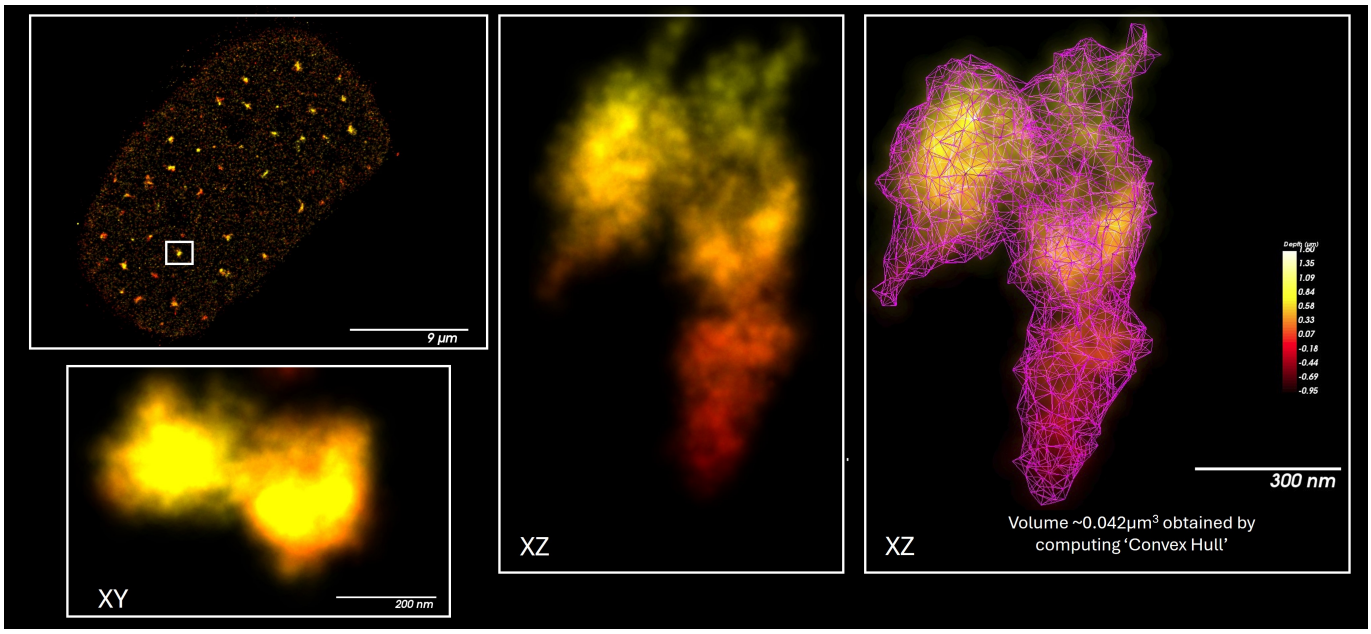
None

Image by P. Raut
Analysis and Presentation by B. T. Bennett

SOURCE PROVENANCE

Catalog	PA-RR-000001
Stage	SIM²
Instrument	ZEISS Elyra
Microscope configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1X Tubelens, was used with the following parameters for each dye:
Structured source metadata values	73
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	0DE84E6EE49D499A3BFA4E9C0C5CB1524070A77CD1C2766CB13E57BE5AD11B1C
Method PDF SHA-256	22BE207D658A85AF0306DFEB6E88F8568BAC9AD23691A00C9E6BD0DAF27F59F6

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	182.5 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: 180.0; End: 181.2
SuperRes 640nm	0.1X Neutral Density Filter: Selected, @0.02% Laser Input
Record Workflow - Record Tab	Capture Parameters
Frames	Probe 1: 500
Z Positions	6 using Steps: 0.2µm (200nm)
Cycles	10
Time Points	1
Pause Between Cycles	Not Selected
Automatic Photoactivation Laser Control	Not Selected
Laser Power	30%
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: 20
Sizing Mode	Constant
Particle size	20nm
Color by	Depth
Color Table	Single
Opacity Mode	Constant; Opacity 1: 0.3
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	-450 to +450
Radial precision	25
Axial precision	60
Clustering Algorithm	DBSCAN
Maximum Particle Distance	0.04µm (400nm)
Maximum Particle Count to Form Cluster	20
Computer Hulls	Selected
Compute Density Isosurfaces	Not Selected
Edit Settings	All Probes
Hull Alpha shape Radius	400nm
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 γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, IgG (H + L)
Cat ID# - SA 000057

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of DSBs. When DSBs occur in chromatin, γH2AX is rapidly 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 γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

Method

Hela cells were grown on a µ-Slide 8 Well high Glass Bottom Ibidi chamber. DNA damage was induced via treatment with 2µM Etoposide-spiked complete growth media for 6 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, 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: 182.5 µm

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: 50µm 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: 180.0; End: 181.2

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: 500
Z Positions: 6 using Steps: 0.2µm (200nm)
Cycles: 10
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: 30%

Expert Configuration Options - The Localization Tab

Beyond the values shown, all other parameters were 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: 20

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: Constant
Particle size: 20nm
Color by: Depth
Color Table: Single
Opacity Mode: Constant; Opacity 1: 0.3
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: -450 to +450
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: 0.04µm (400nm)
Maximum Particle Count to Form Cluster: 20
Computer Hulls: Selected
Compute Density Isosurfaces: Not Selected
Edit Settings: All Probes
Hull Alpha shape Radius: 400nm
Compute: Selected
Following Cluster Analysis Computation, under the Results Tab, ID Unassigned: Deselected

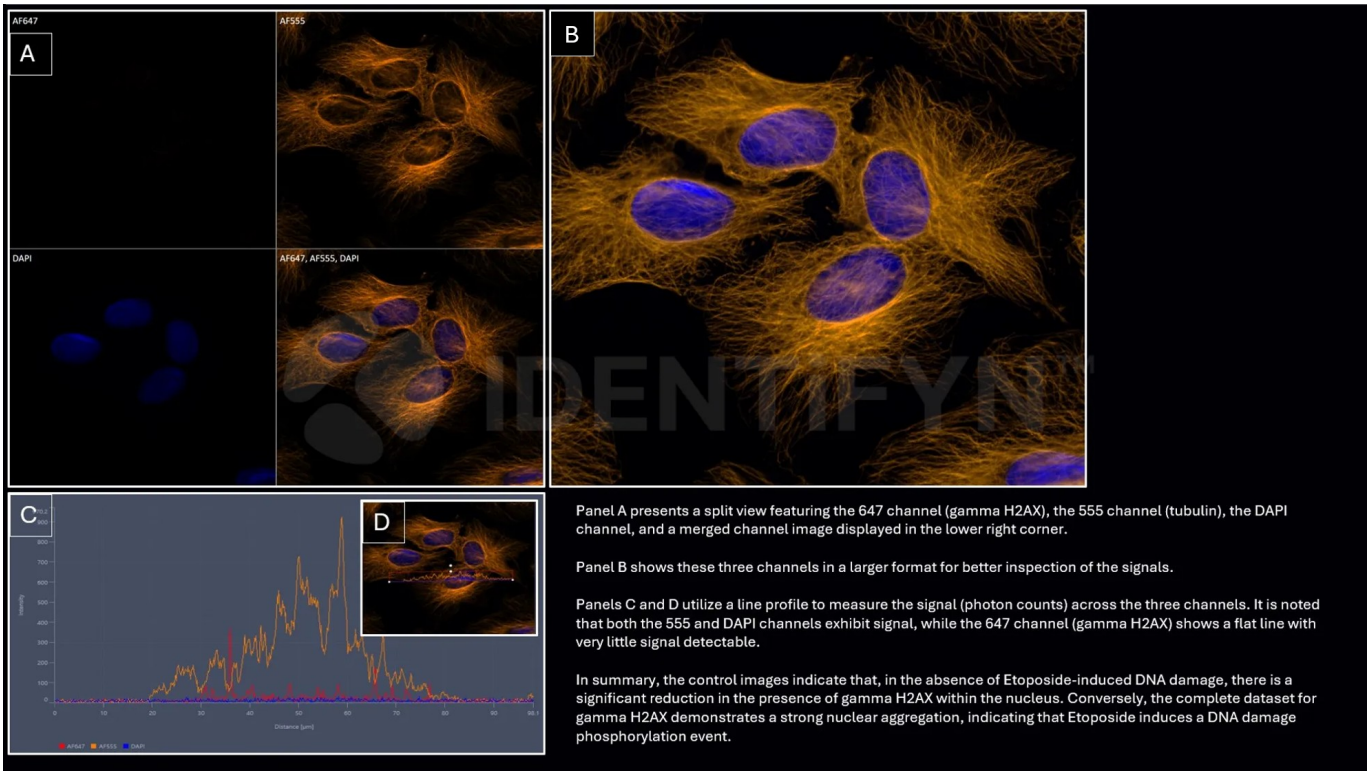
---

Image by S. Thakur

SOURCE PROVENANCE

Catalog	PA-RR-000001
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	9E882AFFE00D297754D080CC6D26993AD45751A33AF8F4D6D6218A0838CD9E65
Method PDF SHA-256	FEA129696348ABC29544547CA92043C131CF68EBADCF5811950BFBCF36541BD8

ORIGINAL IMAGE EVIDENCE / CONTROL



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

STRUCTURED ACQUISITION METADATA / CONTROL

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Objective	EC Plan-Neofluar 63X/1.25 oil M27
Channels	2
Scaling (Per Pixel)	0.143µm X 0.143µm
Image Size (Pixels)	963 X 855
Image Size (Scaled)	135.57µm X 122.14µm
Bit Depth	14 Bit
Image Center Position	X: 75.74mm, Y: 43.47mm
Stage Position	X: 75.74mm, Y: 43.47mm
ROI Center Offset	X: 1.14µm, Y: 0.00µm
Reflector	50 Cy5 | AF532-49014 | 49 DAPI
Beam Splitter	660 | 550 | 395
Filter Excitation Wavelength	625 - 655 | 515 - 545 | 335 - 383
Filter Emission Wavelength	665 - 715 | 555 -595 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @8.0% | X-Cite Xylis Lamp Intensity @6.0%
Exposure Time	150ms | 50ms | 20ms
Excitation Wavelength	663 | 553 | 353
Emission Wavelength	668 | 568 | 465
Effective NA	1.25
Imaging Device	Axiocam 807 mono
Camera Adapter	1X
Depth of Focus	1.30µm | 1.10µm | 0.90µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mode “Optical Sectioning” - enabled correction selected.

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, IgG, (BLR014M)
Cat ID# - PA-RR-000001

Identifyn® Secondary Antibodies

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

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

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

Method

DNA damage was NOT induced.

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human γH2Ax Recombinant Monoclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS, and Identifyn® 647 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody followed this, Alpha Tubulin Monoclonal Antibody (DM1A), which was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® 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
Analysis and Presentation by B. T. Bennett

SOURCE PROVENANCE

Catalog	PA-RR-000001
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	00070BF86A09EEB44D48E241BD88DEEA13EECAAB05D3377A8683C0B615730CA0
Method PDF SHA-256	DD1D4C0EA7A7CC0F7CEFD0CA48F5F5E71562F40746D8A783CA40BC2A9F946EA8