BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

IDENTIFYN RESOLUTION STEPDOWN

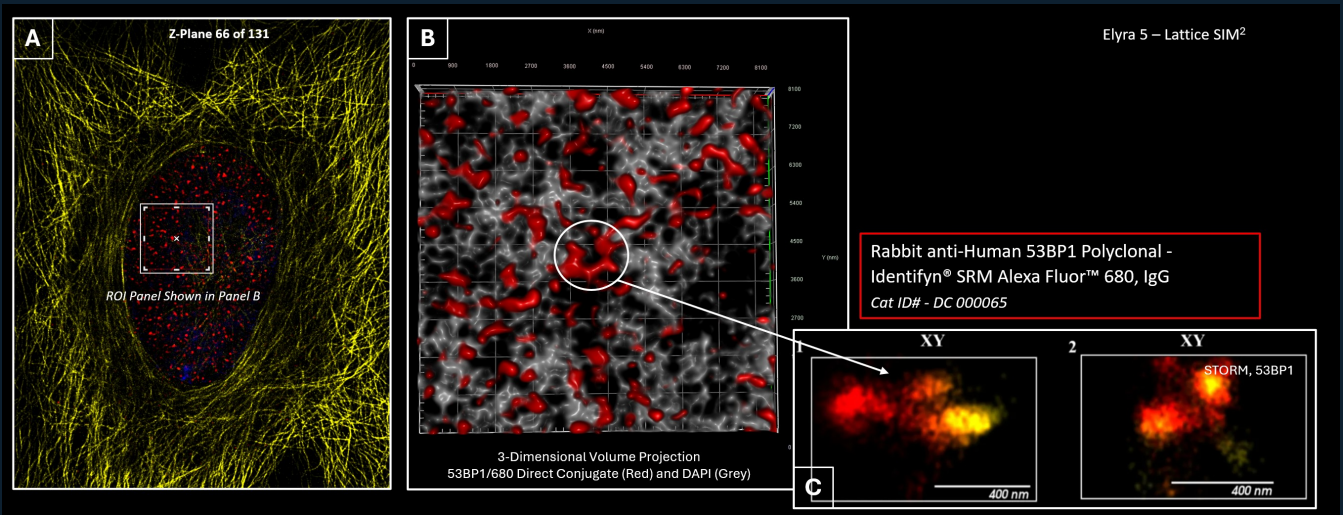
53BP1

MICROSCOPY EVIDENCE ASSESSMENT

Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680

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

7	0	PENDING
ORDERED EVIDENCE TIERS	SUPPORTING CONTROL	SCIENTIST REVIEW



Panel A presents a representative two-dimensional lattice SIM² optical section (Z-plane 66 of 131) of 53BP1 polyclonal Alexa Fluor™ 680 direct conjugate following etoposide-induced DNA damage. α-Tubulin, visualized using an Alexa Fluor™ 532 goat anti-mouse secondary antibody, serves as an exclusionary structural marker defining cytoplasmic boundaries. At the same time, DAPI nuclear counterstaining provides chromatin context and confirms nuclear confinement of the 53BP1 signal. Relative to lattice SIM, SIM² reveals increased spatial refinement of damage-associated 53BP1 structures within the same protein and labeling context.

Panel B shows a three-dimensional volume projection of 53BP1 (Alexa Fluor™ 680 direct conjugate) with DAPI. At SIM² resolution, 53BP1 exhibits a higher degree of structural organization than observed at the SIM gate. With lateral resolution improved to approximately 40–60 nm, effectively halving SIM resolution, architectural features that appeared ordered but indistinct at SIM now become more apparent. 53BP1 assemblies exhibit enhanced spatial definition and organization within and around DAPI-defined chromatin domains.

Panel C presents the corresponding STORM image of 53BP1 for comparison. At single-molecule resolution, discrete subunit-level organization becomes evident. Structural patterns observed in SIM² appear broadly consistent with aspects of the organization resolved by STORM; however, this correspondence remains interpretive. While SIM² provides substantially improved spatial information and reveals globular and organized 53BP1 structures relative to SIM, definitive assignment of molecular subunits requires single-molecule localization microscopy.

Together, these panels demonstrate that lattice SIM² represents a critical intermediate resolution gate within the CCR CDx Step-Down framework. SIM² advances 53BP1 visualization from ordered but composite structures at SIM to more refined, spatially organized assemblies, while still stopping short of molecular-scale resolution. These findings support continued progression to STORM for definitive subunit-level structural interpretation.

REPRESENTATIVE HIGHEST-AVAILABLE VIEW / SIM²

DC-000065 | PRODUCT-LEVEL EVIDENCE DOSSIER

SOURCE-GROUNDED MICROSCOPY + BIOLOGICAL REVIEW / IDENTIFYN EVIDENCE PRESERVED

CRO CAPABILITY DEMONSTRATED

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

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

SCIENTIFIC QUESTION

What source-bounded experimental and microscopy evidence is preserved for 53BP1, and what is the strongest interpretation allowed by the available modalities and controls?

EVIDENCE AVAILABLE

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

BENNETT ASSESSMENT

The preserved DC-000065 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

BENNETT / MICROSCOPY AND BIOLOGICAL EVIDENCE REVIEW

SOURCE-GROUNDED RETROSPECTIVE REVIEW / PENDING SCIENTIST REVIEW

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

OVERALL CALL / NIR-BOUNDED PARTIAL STEPDOWN

The preserved DC-000065 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

SETTINGS ASSESSMENT

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

BIOLOGICAL SUPPORT

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

CONTROL ASSESSMENT

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

CLAIM CEILING

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

PROCESSING DISCLOSURE

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

REAGENTS AND ACQUIRED CHANNELS

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

EVIDENCE OBJECT	REAGENTS / FLUOROPHORES PRESENT	CHANNELS ACTUALLY ACQUIRED
Western blot	680	Not deterministically stated in the acquisition block
Widefield	405/DAPI; 680	2; 50 Cy 5; 49 DAPI; 625 - 655; 335 - 383; 665 - 715; 420 - 470; 679
Widefield SIM — Apotome	405/DAPI; 532; 555; 680	3; 50 Cy 5; AF532-49014; 49 DAPI; 625 - 655; 515 - 545; 335 - 383; 665 - 715
Confocal	405/DAPI; 488; 532; 680	3; None; 639nm @1.50%; 488nm @0.2%; 405nm @0.2%; 679; 528; 353
Airyscan	405/DAPI; 488; 532; 680; 750	3; None; 639nm @2.40%; 488nm @0.10%; 405nm @0.2%; 679; 528; 353
SIM	405/DAPI; 488; 532; 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
SIM²	405/DAPI; 488; 532; 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

MODALITY ASSESSMENT / PART 1 OF 7

Western blot / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

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

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

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

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 2 OF 7

Widefield / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Axio Imager.M1. Objective: Plan-Apochromat 63x/1.40 Oil M27. Platform: Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:. Acquisition: Channels: 2; Scaling (Per Pixel): 0.110 µm X 0.110 µm; Image size (Pixels): 578 X 574; Image Size (Pixels): 578 X 574; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 705. Timing: Exposure Time: 300 ms; 20 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @30.00%; X-Cite Xylis Lamp Intensity @10.00%. Optical/scan: Effective NA: 1.4. Structured source metadata values: 30.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as Widefield, documents platform context as ZEISS Axio Imager.M1, and records detected dye/channel descriptors as 405/DAPI; 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 7

Widefield SIM — Apotome / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Axio Observer.Z1/7 Apotome. Objective: EC Plan-Neofluar 63X/1.25 oil M27. Platform: Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:. Acquisition: Z-Stack: 64 Slices (6.3 µm); Channels: 3; Scaling (Per Pixel): 0.143 µm X 0.143 µm X 0.100 µm; Image size (Pixels): 1600 X 1104; 400 X 358; Image Size (Pixels): 1600 X 1104; 400 X 358; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 807 Mono. Timing: Exposure Time: 250 ms; 100 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @25.00%; X-Cite Xylis Lamp Intensity @10.00%. Optical/scan: Effective NA: 1.25; 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 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; 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

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 4 OF 7

Confocal / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS LSM 980. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:. Acquisition: Z-Stack: 181 Slices (5.4 µm); Channels: 3; Scaling (Per Pixel): 0.059 µm X 0.059 µm X 0.030 µm; Image size (Pixels): 1612 X 1612; Image Size (Pixels): 1612 X 1612; Bit Depth: 16 Bit. Detector/camera: Imaging Device: GaAsP-Pmt3; GaAsp-Pmt3; Detector Type: GaAsP-PMT; GaAsp-PMT. Timing: Pixel Time: 0.24µs; Frame Time: 6.04s. Illumination: Laser Wavelength: 639nm @1.50%; 488nm @0.2%. Optical/scan: Pinhole: 1.00AU / 68µm; 1.00AU / 53µm; Scan Zoom: 1.4; LSM Scan Speed: 11; Scan Direction: Bidirectional; Averaging: 8; Effective NA: 1.4. Processing: LSM Plus Mode: Filter: 8.0 (3D, Auto); Filter: 8.8 (3D, Auto). Structured source metadata values: 55.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as Confocal, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 532; 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 7

Airyscan / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS LSM 980. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:. Acquisition: Z-Stack: 95 Slices (2.82 µm); Channels: 3; Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm; Image size (Pixels): 1793 X 1793; 318 X 304; Image Size (Pixels): 1793 X 1793; 318 X 304; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; Detector Type: GaAsP-PMT. Timing: Pixel Time: 1.15µs; Frame Time: 17.86s. Illumination: Laser Wavelength: 639nm @2.40%; 488nm @0.10%. Optical/scan: Pinhole: 5.00AU / 328µm; 5.00AU / 265µm; Scan Zoom: 2.1; LSM Scan Speed: 6; Scan Direction: Bidirectional; Averaging: 4; Effective NA: 1.4. Processing: LSM Plus Mode: Filter: 8.4 (3D, Auto). Structured source metadata values: 58.

VISIBLE OBSERVATION

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

SOURCE-REPORTED RESULT

The source identifies this object as Airyscan, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 532; 680; 750.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 6 OF 7

SIM / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Elyra. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:. Acquisition: Z-Stack: 145 Slices (4.32 µm); Channels: 3; Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm; Image size (Pixels): 5056 X 5056; 621 X 577; Image Size (Pixels): 5056 X 5056; 621 X 577; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820; Axiocam 820 #2. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.98 s; 1.59 s. Illumination: Laser Wavelength: 640 nm @4.0%; 488 nm @3.0%; Illumination Wavelength: 640; 488. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM; Process Sampling: N/A; Result Sampling: 4; Projection: View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 64.

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; 532; 680.

LITERATURE CORRELATION

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

INTERPRETIVE LIMIT

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

CLAIM CEILING

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

MODALITY ASSESSMENT / PART 7 OF 7

SIM² / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Elyra. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:. Acquisition: Z-Stack: 131 Slices (3.90 µm); Channels: 3; Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm; Image size (Pixels): 3401 X 2979; 401 X 387; Image Size (Pixels): 3401 X 2979; 401 X 387; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820; Axiocam 820 #2. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.98 s; 1.59 s. Illumination: Laser Wavelength: 640 nm @4.0%; 488 nm @3.0%; Illumination Wavelength: 640; 488. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM2; Process Sampling: 4; Result Sampling: 4; Projection: View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 62.

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; 532; 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.

CROSS-MODALITY SYNTHESIS

The preserved DC-000065 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

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

BENNETT LITERATURE CORRELATION — SOURCES INDEPENDENTLY VERIFIED

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

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

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

REFERENCES

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

RELEASE BOUNDARY

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

SOURCE RECONCILIATION / QA / PROVENANCE

SOURCE STATUS - PRESERVED / AUTO-RECONCILED

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

HIGH CONFIDENCE - AUTO-RECONCILED

Airyscan / Scaling (Per Pixel): 35.30 nm X 35.30 nm X 30.00 nm -> 0.03530 µm X 0.03530 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=1793 X 1793; Image Size (Scaled)=63.29 µm X 63.29 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.00%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM / Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm -> 0.02111 µm X 0.02111 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=5056 X 5056; Image Size (Scaled)=106.75 µm X 106.75 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.02%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM² / Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm -> 0.02111 µm X 0.02111 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=401 X 387; Image Size (Scaled)=8.47 µm X 8.17 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.06%.

HIGH CONFIDENCE - SCIENTIST-AUTHORIZED CORRECTION

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

BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

BENNETT EVIDENCE REVIEW COMPLETE

INTERPRETATION TO EVIDENCE

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

IDENTiFYN®

IDENTIFYN PRESERVED SOURCE EVIDENCE

ORIGINAL RECORD BEGINS

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

PRODUCT-LEVEL STEPDOWN MAP

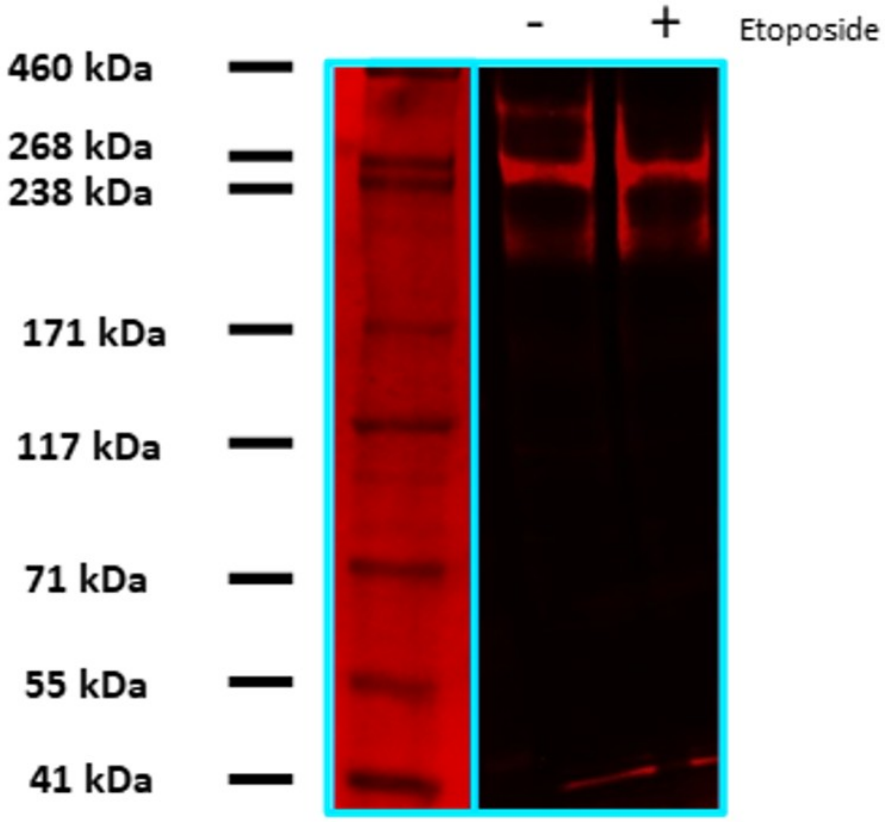
This dossier preserves the complete available evidence progression for DC-000065. Each tier remains tied to its original image, source method, instrument context and cryptographic identity. No separate control record is present in the preserved product record, and none is inferred.

CANONICAL STEPDOWN TIER	EVIDENCE STAGE	INSTRUMENT	STATUS
1	Western blot	Azure Sapphire FL	PRESERVED
2	Widefield	ZEISS Axio Imager.M1	PRESERVED
3	Widefield SIM - Apotome	ZEISS Axio Observer.Z1/7 Apotome	PRESERVED
4	Confocal	ZEISS LSM 980	PRESERVED
5	Airyscan	ZEISS LSM 980	PRESERVED
6	SIM	ZEISS Elyra	PRESERVED
7	SIM²	ZEISS Elyra	PRESERVED

INTERPRETIVE BOUNDARY

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

ORIGINAL IMAGE EVIDENCE / WESTERN BLOT



EVIDENCE TIER	Western blot
INSTRUMENT	Azure Sapphire FL
CELL MODEL	HeLa
DETECTED DYES	680
METADATA VALUES	11

STRUCTURED ACQUISITION METADATA / WESTERN BLOT

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Scanner	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Detection mode	Fluorescence
Laser	685nm
Emission Filter	720±24nm
Detector	PMT
Intensity	3
Pixel size	100 µm
Scan Speed	High
Quality	High
Focus	Membrane (0 µm in Z-axis)
Image	K. Small

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG
Cat ID# - DC 000065

Expected MW: 214 kDa

HeLa cells were treated with 200 µM etoposide for 20 hours to induce DNA damage. Cells were then homogenized using an ultrasonication probe and nuclear lysis buffer, and then centrifuged to pellet the cell debris. The lysate supernatant (100 µg) was mixed with NuPAGE™ LDS Sample Buffer (4X), heated at 80 °C for 10 min, and subjected to electrophoresis using NuPAGE™ Tris-Acetate Mini Protein Gels, 3 to 8%, 1.0 mm in a Mini Gel Tank. The Thermo Fisher's HiMark™ Pre-stained Protein Standard 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, then washed three times with PBS. The blocked membrane was incubated with Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG for 20 hours, followed by five washes in 1X PBS. The membrane was dried for 10 minutes at room temperature before imaging.

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

Scan Settings

Detection mode = Fluorescence
Laser = 685nm
Emission Filter = 720±24nm
Detector = PMT
Intensity = 3
Pixel size = 100 µm
Scan Speed = High
Quality = High
Focus = Membrane (0 µm in Z-axis)

Post Processing

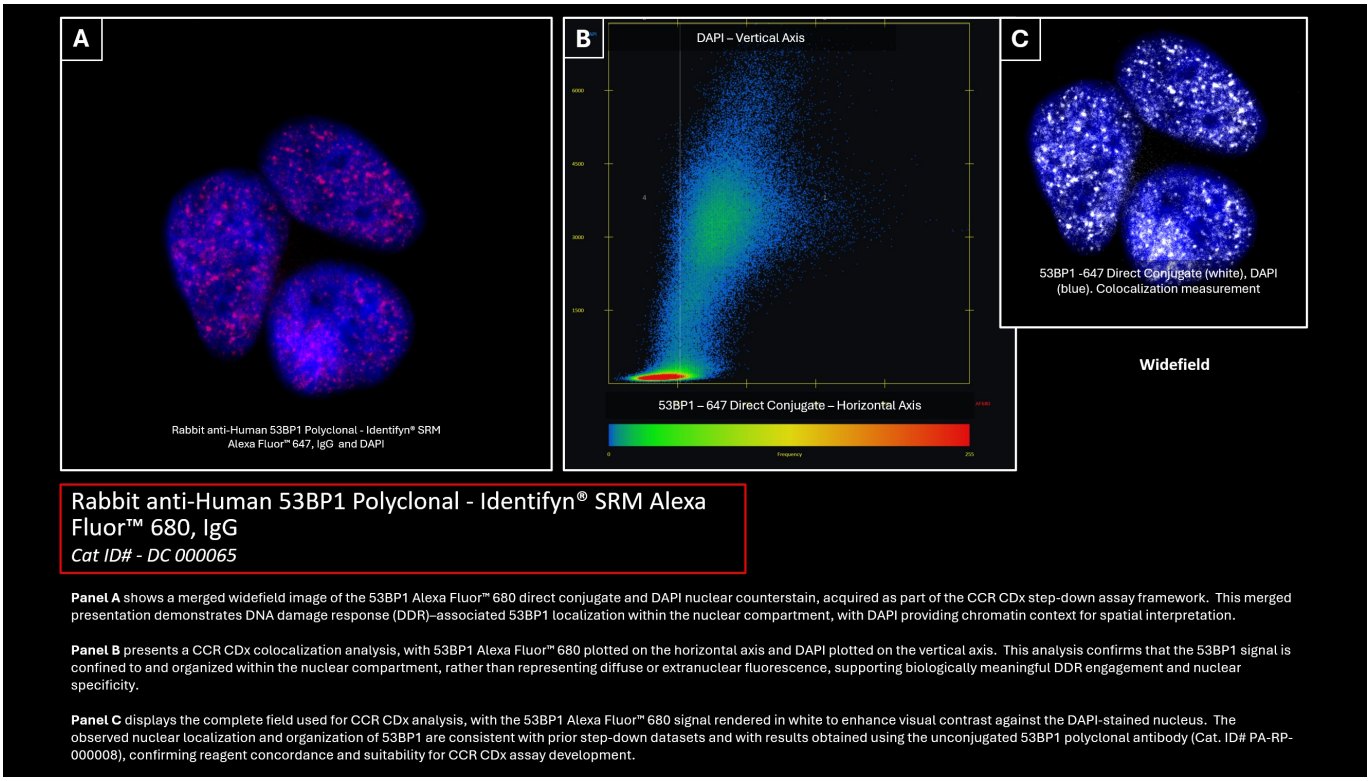
None

Image: K. Small

SOURCE PROVENANCE

Catalog	DC-000065
Stage	Western blot
Instrument	Azure Sapphire FL
Microscope configuration	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Structured source metadata values	11
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	F5441C2C41C940476B7F680AE0660CF0B5E10FC070F5E89C28C0C7572A2D072F
Method PDF SHA-256	0549DDC2FCC5EB985AF6ED99485998172B59097C36BCB8119519FAB301E1E49B

ORIGINAL IMAGE EVIDENCE / WIDEFIELD



EVIDENCE TIER	Widefield
INSTRUMENT	ZEISS Axio Imager.M1
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 680
METADATA VALUES	30

STRUCTURED ACQUISITION METADATA / WIDEFIELD

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63x/1.40 Oil M27
Channels	2
Scaling (Per Pixel)	0.110 µm X 0.110 µm
Image size (Pixels)	578 X 574
Image Size (Scaled)	68.30 µm X 62.87 µm
Bit Depth	14 Bit
Image Center Position	X: 0.00 µm, Y: 0.00 µm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	50 Cy 5 | 49 DAPI
Beam Splitter	660 | 395
Filter Excitation Wavelength	625 - 655 | 335 - 383
Filter Emission Wavelength	665 - 715 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @30.00% | X-Cite Xylis Lamp Intensity @10.00%
Exposure Time	300 ms | 20 ms
Excitation Wavelength	679 | 353
Emission Wavelength	702 | 465
Effective NA	1.4
Imaging Device	Axiocam 705
Camera Adapter	1X
Depth of Focus	1.09 µm | 0.72 µm
Binning Mode	2,2

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG
Cat ID# - DC 000065

53BP1 (P53-binding protein 1) is a crucial player in the DNA damage response (DDR), particularly in the repair of double-strand breaks (DSBs). It has a range of functions that contribute to genomic stability and an appropriate response to DNA damage. The following are the prominent roles and functions of 53BP1 in DNA repair:

DNA Damage Sensing: 53BP1 is among the early proteins recruited to DNA damage sites. It recognizes and binds to the phosphorylated form of H2AX (γ-H2AX), a histone variant phosphorylated at double-strand break sites. This ability to localize to DNA damage sites helps to recruit other proteins involved in DNA repair and signaling.

Non-Homologous End Joining (NHEJ): 53BP1 significantly promotes NHEJ, a pathway to repair double-strand breaks, especially in the G1 phase of the cell cycle when a homologous template is unavailable. 53BP1 promotes this pathway by:

Facilitating Synapsis: 53BP1 helps align the broken DNA ends, preparing them for ligation by the NHEJ machinery.

Restricting End Resection: By inhibiting the end resection process (a step in homologous recombination), 53BP1 ensures that NHEJ remains the primary repair mechanism for DSBs in cells not in the S or G2 phase.

Interaction with Other DNA Repair Proteins: 53BP1 interacts with other proteins to promote NHEJ, such as RIF1 (Rap1 Interacting Factor 1) and the Shieldin complex. These interactions help determine the repair pathway choice, favoring NHEJ over homologous recombination when appropriate.

DNA Damage Signaling: 53BP1 is involved in amplifying DNA damage signaling, contributing to the recruitment of additional repair proteins and activating cell cycle checkpoints. This signaling function helps maintain genomic stability by ensuring that cells do not progress through the cell cycle with unrepaired DNA damage.

Tumor Suppression and Genome Stability: 53BP1 has tumor suppressive properties, given its role in promoting accurate repair and maintaining genomic stability. Its ability to ensure proper repair of DSBs and its interaction with other DDR proteins make it an essential player in preventing mutations and chromosomal aberrations that can lead to cancer.

In summary, 53BP1 is a multifaceted protein in the DNA damage response, promoting non-homologous end joining, facilitating DNA damage signaling, and contributing to genomic stability. Its interactions with other DNA repair proteins and its role in regulating the choice of repair pathways are crucial for maintaining cellular integrity and preventing tumorigenesis.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with Thermo Fisher Fish Serum Blocker (cat#37527X3). The primary antibody, Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG, was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat# P36962).

Control Data - Primary Unconjugated Antibody

Microscope

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

Single Plane (2D)

Channels = 2
Scaling (Per Pixel) = 0.110 µm X 0.110 µm
Image size (Pixels) = 578 X 574
Image Size (Scaled) = 68.30 µm X 62.87 µm
Bit Depth = 14 Bit
Image Center Position = X: 0.00 µm, Y: 0.00 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

680 -

Reflector = 50 Cy 5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @30.00%
Exposure Time = 300 ms
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 1.09 µm
Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10.00%
Exposure Time = 20 ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 0.72 µm
Binning Mode = 2,2

2- Dimensional View - Panel A

Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG (red), and DAPI nuclear counterstain is shown in blue.

Colocalization View - Panel B, and Image Panel C, measurement was the entire image acquisition.

Horizontal Axis - AF680, Threshold 206, Range Auto
Vertical Axis - DAPI, Threshold 738, Range Auto

Summary

CCR CDx Widefield Assessment of 53BP1 Nuclear Compartmentalization

Panel A shows a merged widefield image of the 53BP1 Alexa Fluor™ 680 direct conjugate and DAPI nuclear counterstain, acquired as part of the CCR CDx step-down assay framework. This merged presentation demonstrates DNA damage response (DDR)-associated 53BP1 localization within the nuclear compartment, with DAPI providing chromatin context for spatial interpretation.

Panel B presents a CCR CDx colocalization analysis, with 53BP1 Alexa Fluor™ 680 plotted on the horizontal axis and DAPI plotted on the vertical axis. This analysis confirms that the 53BP1 signal is confined to and organized within the nuclear compartment, rather than representing diffuse or extranuclear fluorescence, supporting biologically meaningful DDR engagement and nuclear specificity.

Panel C displays the complete field used for CCR CDx analysis, with the 53BP1 Alexa Fluor™ 680 signal rendered in white to enhance visual contrast against the DAPI-stained nucleus. The observed nuclear localization and organization of 53BP1 are consistent with prior step-down datasets and with results obtained using the unconjugated 53BP1 polyclonal antibody (Cat. ID# PA-RP-000008), confirming reagent concordance and suitability for CCR CDx assay development.

Image Correction

None

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

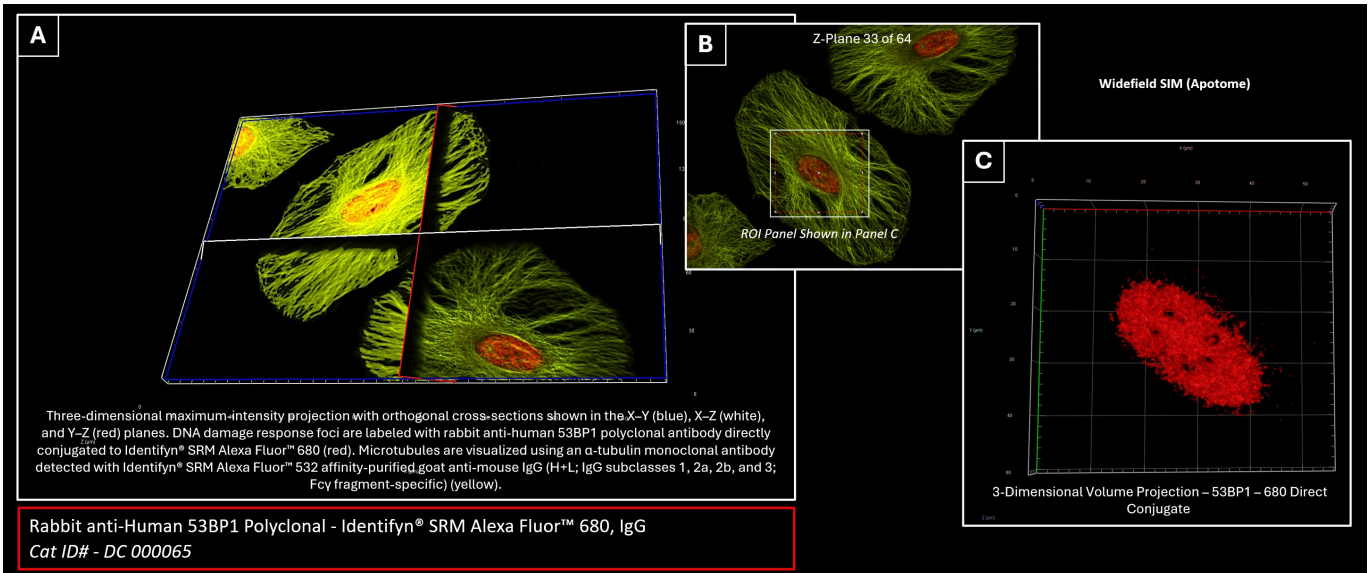
SOURCE PROVENANCE

Catalog	DC-000065
Stage	Widefield
Instrument	ZEISS Axio Imager.M1
Microscope configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Structured source metadata values	30
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa

SOURCE PROVENANCE

Image SHA-256	B5686F5AD9E8B63785DE0FE93F61423AB9871B5910A85ED03E53B7DE0B406604
Method PDF SHA-256	D5F553695C811DB12C1B4C678519DACF2198039EAD1ACBA40259573F54C29363

ORIGINAL IMAGE EVIDENCE / WIDEFIELD SIM - APOTOME



Panel A shows a three-dimensional wedge view acquired at this resolution gate, combining 53BP1 detected with an Alexa Fluor™ 680 direct conjugate and α-tubulin serving as an exclusionary structural marker. Tubulin defines cytoplasmic architecture and nuclear boundaries, confirming that the observed 53BP1 signal is confined to the nuclear compartment and is not associated with cytoskeletal structures. Signal specificity at this gate is supported by concordance with the unconjugated Rabbit anti-Human 53BP1 Polyclonal (PA-RP-000008).

Panel B presents a two-dimensional optical section taken at Z-plane 33 of 64. The white boxed region denotes the region of interest advanced for further evaluation in Panel C, maintaining spatial continuity across panels.

Panel C shows a three-dimensional volume projection of the 53BP1 Alexa Fluor™ 680 direct conjugate alone. At this resolution gate, a strong etoposide-induced 53BP1 response is observed, confirming pathway engagement. However, in volume projection, subnuclear localization, individual foci separation, and structural detail cannot be resolved. Consistent with prior Widefield and Apotome gates, this panel establishes signal presence but does not meet criteria for localization or structural interpretation, requiring advancement in the Step-Down workflow to higher-resolution modalities.

EVIDENCE TIER	Widefield SIM - Apotome
INSTRUMENT	ZEISS Axio Observer.Z1/7 Apotome
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 532; 555; 680
METADATA VALUES	61

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	64 Slices (6.3 µm)
Channels	3
Scaling (Per Pixel)	0.143 µm X 0.143 µm X 0.100 µm
Image size (Pixels)	1600 X 1104 | 400 X 358
Image Size (Scaled)	228.57 µm X 157.71 µm | 57.14 µm X 51.14 µm
Bit Depth	14 Bit
Image Center Position	X: 61.60 µm, Y: 47.52 µm
Stage Position	X: 61.60 µm, Y: 47.52 µm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflector	50 Cy 5 | 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 @25.00% | X-Cite Xylis Lamp Intensity @10.00%
Exposure Time	250 ms | 100 ms | 25 ms
Excitation Wavelength	679 | 528 | 353
Emission Wavelength	702 | 553 | 465
Effective NA	1.25 | 1.4
Imaging Device	Axiocam 807 Mono
Camera Adapter	1X
Depth of Focus	1.36 µm | 1.07 µm | 0.72 µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mode "Optical Sectioning" - enabled correction selected.
3D Maximum Projection	Precise
Appearance	Threshold 1% all channels, Ramp 0% all channels, Maximum 100% all channels | Threshold 13% all channels, Ramp 50% all channels, 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)

FIELD	SOURCE-DOCUMENTED VALUE
X-Y	Activate was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip	17% | -17% | 9%
Clip X	0°
Clip Z	0°
X-Z	Activate was selected, textured opaque was chosen, and a white outline of the clipped perimeter was shown; Clip Front was selected, and Clip Back was NOT selected.
Clip Y	-75°
Y-Z	Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
3D Volume Projection	Precise

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG
Cat ID# - DC 000065

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000023

53BP1 (P53-binding protein 1) is a crucial player in the DNA damage response (DDR), particularly in the repair of double-strand breaks (DSBs). It has a range of functions that contribute to genomic stability and an appropriate response to DNA damage. The following are the prominent roles and functions of 53BP1 in DNA repair:

DNA Damage Sensing: 53BP1 is among the early proteins recruited to DNA damage sites. It recognizes and binds to the phosphorylated form of H2AX (γ-H2AX), a histone variant phosphorylated at double-strand break sites. This ability to localize to DNA damage sites helps to recruit other proteins involved in DNA repair and signaling.

Non-Homologous End Joining (NHEJ): 53BP1 significantly promotes NHEJ, a pathway to repair double-strand breaks, especially in the G1 phase of the cell cycle when a homologous template is unavailable. 53BP1 promotes this pathway by:

Facilitating Synapsis: 53BP1 helps align the broken DNA ends, preparing them for ligation by the NHEJ machinery.

Restricting End Resection: By inhibiting the end resection process (a step in homologous recombination), 53BP1 ensures that NHEJ remains the primary repair mechanism for DSBs in cells not in the S or G2 phase.

Interaction with Other DNA Repair Proteins: 53BP1 interacts with other proteins to promote NHEJ, such as RIF1 (Rap1 Interacting Factor 1) and the Shieldin complex. These interactions help determine the repair pathway choice, favoring NHEJ over homologous recombination when appropriate.

DNA Damage Signaling: 53BP1 is involved in amplifying DNA damage signaling, contributing to the recruitment of additional repair proteins and activating cell cycle checkpoints. This signaling function helps maintain genomic stability by ensuring that cells do not progress through the cell cycle with unrepaired DNA damage.

Tumor Suppression and Genome Stability: 53BP1 has tumor suppressive properties, given its role in promoting accurate repair and maintaining genomic stability. Its ability to ensure proper repair of DSBs and its interaction with other DDR proteins make it an essential player in preventing mutations and chromosomal aberrations that can lead to cancer.

In summary, 53BP1 is a multifaceted protein in the DNA damage response, promoting non-homologous end joining, facilitating DNA damage signaling, and contributing to genomic stability. Its interactions with other DNA repair proteins and its role in regulating the choice of repair pathways are crucial for maintaining cellular integrity and preventing tumorigenesis.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with Thermo Fisher Fish Serum Blocker (cat#37527X3). The primary antibody, Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat#P36962).

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

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

Z Stack - (3D) - Panel A, 3-Dimensional Maximum Projection, Panel B, Indicating the ROI

Z-Stack = 64 Slices (6.3 µm)

Channels = 3
Scaling (Per Pixel) = 0.143 µm X 0.143 µm X 0.100 µm
Image size (Pixels) = 1600 X 1104
Image Size (Scaled) = 228.57 µm X 157.71 µm
Bit Depth = 14 Bit
Image Center Position = X: 61.60 µm, Y: 47.52 µm
Stage Position = X: 61.60 µm, Y: 47.52 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

Z Stack - (3D) - Panel C, 3-Dimensional Volume Projection

Z-Stack = 64 Slices (6.3 µm)

Channels = 3
Scaling (Per Pixel) = 0.143 µm X 0.143 µm X 0.100 µm
Image size (Pixels) = 400 X 358
Image Size (Scaled) = 57.14 µm X 51.14 µm
Bit Depth = 14 Bit
Image Center Position = X: 61.60 µm, Y: 47.52 µm
Stage Position = X: 61.60 µm, Y: 47.52 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

680 -

Reflector = 50 Cy 5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @25.00%
Exposure Time = 250 ms
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.25
Imaging Device = Axiocam 807 Mono
Camera Adapter = 1X
Depth of Focus = 1.36 µm
Binning Mode = 2,2

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 @10.00%
Exposure Time = 100 ms
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.4
Imaging Device = Axiocam 807 Mono
Camera Adapter = 1X
Depth of Focus = 1.07 µm
Binning Mode = 2,2

DAPI - (Not shown in Data Analysis)

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10.00%
Exposure Time = 25 ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = Axiocam 807 Mono
Camera Adapter = 1X
Depth of Focus = 0.72 µm
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 - Panel A

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

Clipping Image Process - Panel A

Clipping planes are introduced in Panel B to highlight the 53BP1, 680 Direct Conjugate within the nuclear compartment by adjusting or cutting away portions of the image using a triple-wedge cut, removing parts of the image in the X-Y, X-Z, and Y-Z planes.

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

Position of Clip = 17%
Clip X = 0°
Clip Z = 0°

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

Position of Clip = -17%
Clip Y = -75°
Clip Z = 0°

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

Position of Clip = 9%
Clip Y = -75°
Clip Z = 0°

2- Dimensional View - Panel B

Panel shows Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG and Alpha Tubulin, with Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific at Z-plane 33 of 64, a white box denotes the region of interest to be investigated in Panel C.

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)

Summary

Three-dimensional imaging at this resolution gate includes a 3D wedge view (Panel A), a single two-dimensional optical section (Panel B), and a 3D volume projection (Panel C). In Panel A, 53BP1 is visualized using an Alexa Fluor™ 680 direct conjugate, with α-tubulin serving as an exclusionary structural marker to define cytoplasmic architecture and nuclear boundaries. This confirms that the observed 53BP1 signal is restricted to the nuclear compartment and is not associated with cytoskeletal structures. Signal specificity is supported by concordance with the corresponding unconjugated Rabbit anti-Human 53BP1 Polyclonal antibody.

Panel B presents a single optical section acquired at Z-plane 33 of 64, with the indicated region of interest advanced for higher-dimensional evaluation. Panel C shows a three-dimensional volume projection of the 53BP1 Alexa Fluor™ 680 direct conjugate alone.

At this gate, a strong etoposide-induced 53BP1 response is observed, satisfying CCR CDx criteria for DNA damage pathway engagement. However, three-dimensional volume projection does not allow reliable separation of subnuclear localization, individual foci, or higher-order structural organization. Consistent with prior Widefield and Apotome gates, signal presence alone does not meet localization or structural resolution requirements for clinical interpretation.

Accordingly, this resolution gate meets pathway engagement criteria but fails localization and structural discrimination criteria under CCR CDx performance standards. Advancement within the Step-Down workflow to higher-resolution imaging tiers is required to resolve 53BP1 organization and enable clinically actionable assessment.

Image Correction

NONE

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

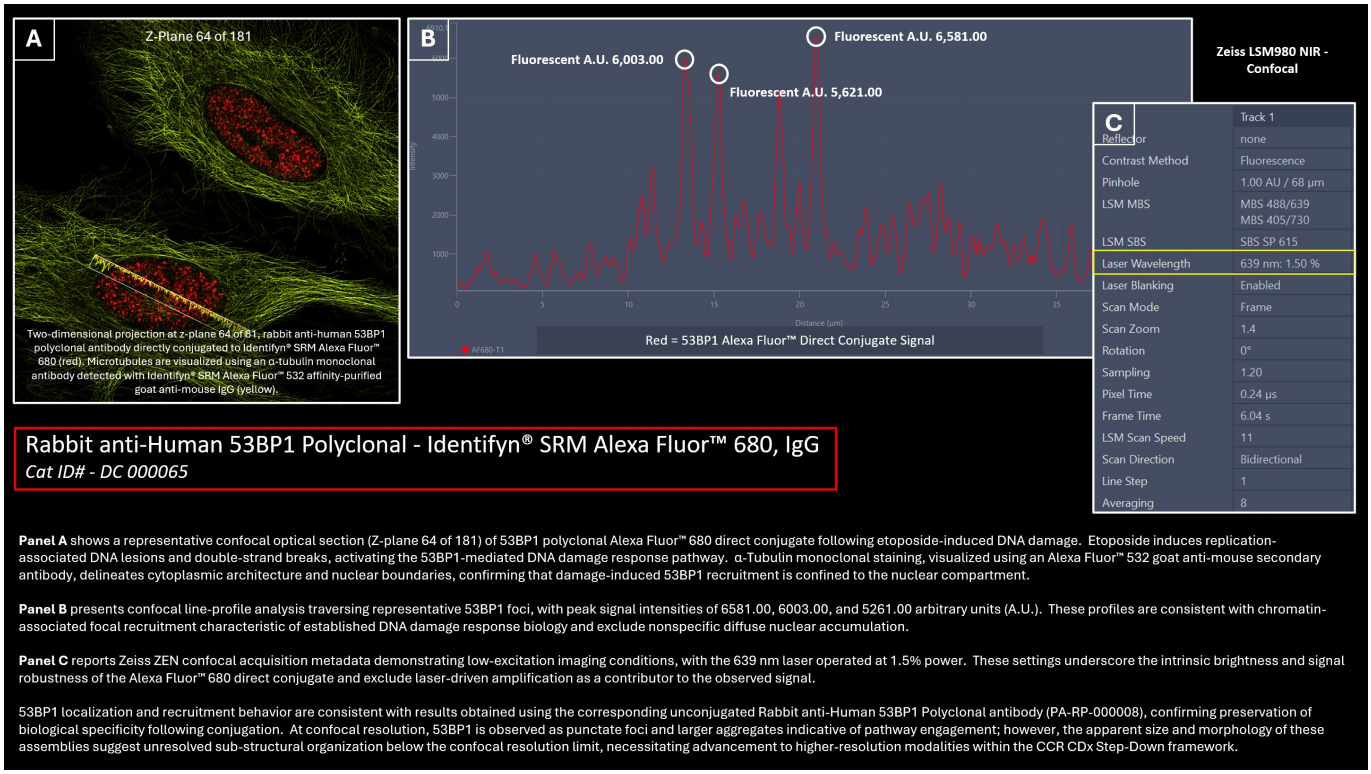
SOURCE PROVENANCE

Catalog	DC-000065
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	61
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT

SOURCE PROVENANCE

Cell model	HeLa
Image SHA-256	94932DFADFC4766DCDB568F68E91E28E0AF7E7DA12F5B25401B16DFB77951CA2
Method PDF SHA-256	201DB7D7F4E694B6354E6985BE708DCE885FC5A693722E70FA638C5EC82FB164

ORIGINAL IMAGE EVIDENCE / CONFOCAL



EVIDENCE TIER	Confocal
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 680
METADATA VALUES	55

STRUCTURED ACQUISITION METADATA / CONFOCAL

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	181 Slices (5.4 µm)
Channels	3
Scaling (Per Pixel)	0.059 µm X 0.059 µm X 0.030 µm
Image size (Pixels)	1612 X 1612
Image Size (Scaled)	94.79 µm X 94.79 µm
Bit Depth	16 Bit
Image Center Position	X: 93.26 µm, Y: 46.93 µm
Stage Position	X: 93.26 µm, Y: 46.93 µm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	1.00AU / 68µm | 1.00AU / 53µm | 1.00AU / 44µm
LMS MBS	MBS 488/639 & 405/730
LMS SBS	SBS SP 615 | SBS SP 505 | Mirror
Laser Wavelength	639nm @1.50% | 488nm @0.2% | 405nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	1.4
Rotation	0°
Sampling	1.20
Pixel Time	0.24µs
Frame Time	6.04s
LSM Scan Speed	11
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	679 | 528 | 353
Emission Wavelength	702 | 553 | 465
Effective NA	1.4
Detection Wavelength	678 - 745 | 526 - 580 | 439 - 480
Imaging Device	GaAsP-Pmt3 | GaAsp-Pmt3

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT | GaAsp-PMT
Detector Gain	650 V | 500 V
Detector Offset	0
Detector Digital Gain	1.0
LSM Plus Mode	Filter: 8.0 (3D, Auto) | Filter: 8.8 (3D, Auto) | Filter: 7.1 (3D, Auto)
Profile Definition	Stroke Thickness 1.5, Normal View, Grid Distance 1
Profile View	X and Y axes were set to Auto - X (Min 0.0 and Max 42.3), Y (Min 61 and Max 6910)

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 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG
Cat ID# - DC 000065

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000023

53BP1 (P53-binding protein 1) is a crucial player in the DNA damage response (DDR), particularly in the repair of double-strand breaks (DSBs). It has a range of functions that contribute to genomic stability and an appropriate response to DNA damage. The following are the prominent roles and functions of 53BP1 in DNA repair:

DNA Damage Sensing: 53BP1 is among the early proteins recruited to DNA damage sites. It recognizes and binds to the phosphorylated form of H2AX (γ-H2AX), a histone variant phosphorylated at double-strand break sites. This ability to localize to DNA damage sites helps to recruit other proteins involved in DNA repair and signaling.

Non-Homologous End Joining (NHEJ): 53BP1 significantly promotes NHEJ, a pathway to repair double-strand breaks, especially in the G1 phase of the cell cycle when a homologous template is unavailable. 53BP1 promotes this pathway by:

Facilitating Synapsis: 53BP1 helps align the broken DNA ends, preparing them for ligation by the NHEJ machinery.

Restricting End Resection: By inhibiting the end resection process (a step in homologous recombination), 53BP1 ensures that NHEJ remains the primary repair mechanism for DSBs in cells not in the S or G2 phase.

Interaction with Other DNA Repair Proteins: 53BP1 interacts with other proteins to promote NHEJ, such as RIF1 (Rap1 Interacting Factor 1) and the Shieldin complex. These interactions help determine the repair pathway choice, favoring NHEJ over homologous recombination when appropriate.

DNA Damage Signaling: 53BP1 is involved in amplifying DNA damage signaling, contributing to the recruitment of additional repair proteins and activating cell cycle checkpoints. This signaling function helps maintain genomic stability by ensuring that cells do not progress through the cell cycle with unrepaired DNA damage.

Tumor Suppression and Genome Stability: 53BP1 has tumor suppressive properties, given its role in promoting accurate repair and maintaining genomic stability. Its ability to ensure proper repair of DSBs and its interaction with other DDR proteins make it an essential player in preventing mutations and chromosomal aberrations that can lead to cancer.

In summary, 53BP1 is a multifaceted protein in the DNA damage response, promoting non-homologous end joining, facilitating DNA damage signaling, and contributing to genomic stability. Its interactions with other DNA repair proteins and its role in regulating the choice of repair pathways are crucial for maintaining cellular integrity and preventing tumorigenesis.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with Thermo Fisher Fish Serum Blocker (cat#37527X3). The primary antibody, Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat#P36962).

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

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

Z Stack - (3D) - Panel A, Z-plane 64 of 181, Line Profile Measurement

Z-Stack =181 Slices (5.4 µm)

Channels = 3
Scaling (Per Pixel) = 0.059 µm X 0.059 µm X 0.030 µm
Image size (Pixels) = 1612 X 1612
Image Size (Scaled) = 94.79 µm X 94.79 µm
Bit Depth = 16 Bit
Image Center Position = X: 93.26 µm, Y: 46.93 µm
Stage Position = X: 93.26 µm, Y: 46.93 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

680 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 68µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = 639nm @1.50%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.4
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.24µs
Frame Time = 6.04s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Detection Wavelength = 678 - 745
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 650 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 8.0 (3D, Auto)

532 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 53µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 505
Laser Wavelength = 488nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.4
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.24µs
Frame Time = 6.04s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.4
Detection Wavelength = 526 - 580
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 8.8 (3D, Auto)

DAPI - (Not shown in Data Analysis)

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 44µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = Mirror
Laser Wavelength = 405nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.4
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.24µs
Frame Time = 6.04s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 439 - 480
Imaging Device = GaAsp-Pmt3
Detector Type = GaAsp-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 7.1 (3D, Auto)

2- Dimensional View - Panel A and C (C, omits the Alpha Tubulin)

Panel shows Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG and Alpha Tubulin, with Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific.

Image from Z-plane 64 of 181 and the Line Profile graphic showing the location where the measurement was taken.

Profile View Display - Panels B & C

Profile Definition = Stroke Thickness 1.5, Normal View, Grid Distance 1
Profile View = X and Y axes were set to Auto - X (Min 0.0 and Max 42.3), Y (Min 61 and Max 6910)

Shown are 3 different measurements of foci intensity.

Zen Acquisition Info Tab -

Shown is the acquisition of the 680 channel (53BP1 680 direct conjugate) using the 639 nm laser at 1.50%, demonstrating that image acquisition settings do not artificially produce the signal.

Summary

Panel A shows a representative confocal optical section (Z-plane 64 of 181) of 53BP1 polyclonal Alexa Fluor™ 680 direct conjugate following etoposide-induced DNA damage. Etoposide induces replication-associated DNA lesions and double-strand breaks, activating the 53BP1-mediated DNA damage response pathway. α-Tubulin monoclonal staining, visualized using an Alexa Fluor™ 532 goat anti-mouse secondary antibody, delineates cytoplasmic architecture and nuclear boundaries, confirming that damage-induced 53BP1 recruitment is confined to the nuclear compartment.

Panel B presents confocal line-profile analysis traversing representative 53BP1 foci, with peak signal intensities of 6581.00, 6003.00, and 5261.00 arbitrary units (A.U.). These profiles are consistent with chromatin-associated focal recruitment characteristic of established DNA damage response biology and exclude nonspecific diffuse nuclear accumulation.

Panel C reports Zeiss ZEN confocal acquisition metadata demonstrating low-excitation imaging conditions, with the 639 nm laser operated at 1.5% power. These settings underscore the intrinsic brightness and signal robustness of the Alexa Fluor™ 680 direct conjugate and exclude laser-driven amplification as a contributor to the observed signal.

53BP1 localization and recruitment behavior are consistent with results obtained using the corresponding unconjugated Rabbit anti-Human 53BP1 Polyclonal antibody (PA-RP-000008), confirming preservation of biological specificity following conjugation. At confocal resolution, 53BP1 is observed as punctate foci and larger aggregates indicative of pathway engagement; however, the apparent size and morphology of these assemblies suggest unresolved sub-structural organization below the confocal resolution limit, necessitating advancement to higher-resolution modalities within the CCR CDx Step-Down framework.

Image Correction

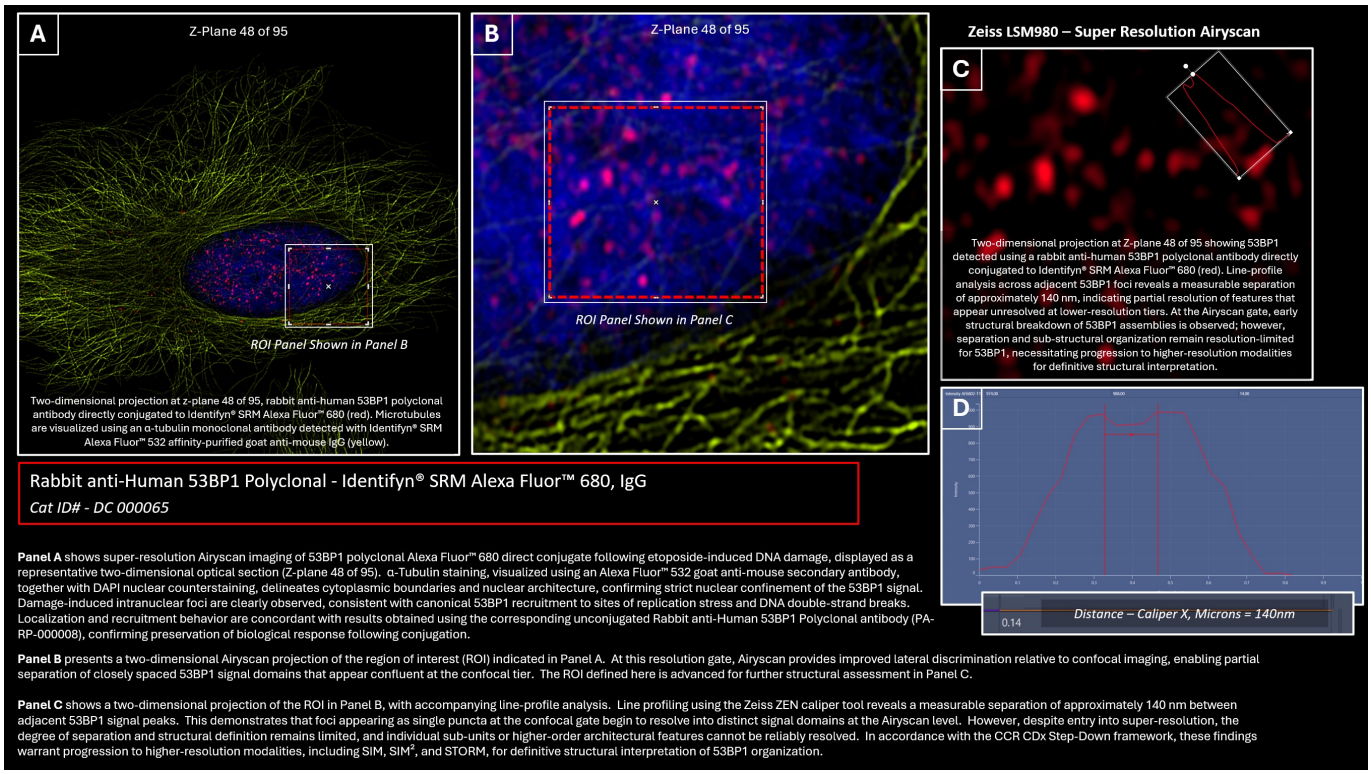
NONE

Image by J.K. Bennett
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000065
Stage	Confocal
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Structured source metadata values	55
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	DC31BF4A71682512BA7519693D3A9B8940E5B77497BB21A21CE6FFE193DA53FC
Method PDF SHA-256	F3A5E0A4EC8C780B5775E10692B590A0338308EB2E9394B667AD1E52B176879D

ORIGINAL IMAGE EVIDENCE / AIRYSCAN



EVIDENCE TIER	Airyscan
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 680; 750
METADATA VALUES	58

STRUCTURED ACQUISITION METADATA / AIRYSCAN

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	95 Slices (2.82 µm)
Channels	3
Scaling (Per Pixel)	0.03530 µm X 0.03530 µm X 0.03000 µm
Image size (Pixels)	1793 X 1793 | 318 X 304 | 147 X 131
Image Size (Scaled)	63.29 µm X 63.29 µm | 11.22 µm X 10.73 µm | 5.19 µm X 4.62 µm
Bit Depth	16 Bit
Image Center Position	X: 93.29 µm, Y: 48.96 µm
Stage Position	X: 93.29 µm, Y: 48.96 µm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	5.00AU / 328µm | 5.00AU / 265µm | 5.00AU / 235µm
LMS MBS	MBS 488/639 & 405/730
LMS SBS	SBS SP 640 | SBS LP 525 | SBS SP 505
Laser Wavelength	639nm @2.40% | 488nm @0.10% | 405nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.1
Rotation	0°
Sampling	2.00
Pixel Time	1.15µs
Frame Time	17.86s
LSM Scan Speed	6
Scan Direction	Bidirectional
Line Step	1
Averaging	4
Excitation Wavelength	679 | 528 | 353
Emission Wavelength	702 | 553 | 465
Effective NA	1.4
Detection Wavelength	643 - 735 | 530 - 582 | 350 - 499
Imaging Device	Airyscan

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT
Detector Gain	875 V | 800 V | 750 V
Detector Offset	0
Detector Digital Gain	1.0
Super Resolution	9.9 (3D, Auto) | 9.3 (3D, Auto)
LSM Plus Mode	Filter: 8.4 (3D, Auto)
Profile Definition	Stroke Thickness 1.0, Normal View, Grid Distance 1
Profile View	X and Y axes were set to Auto - X (Min 0.0 and Max 999.7), Y (Min 61 and Max 1037)

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 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG
Cat ID# - DC 000065

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000023

53BP1 (P53-binding protein 1) is a crucial player in the DNA damage response (DDR), particularly in the repair of double-strand breaks (DSBs). It has a range of functions that contribute to genomic stability and an appropriate response to DNA damage. The following are the prominent roles and functions of 53BP1 in DNA repair:

DNA Damage Sensing: 53BP1 is among the early proteins recruited to DNA damage sites. It recognizes and binds to the phosphorylated form of H2AX (γ-H2AX), a histone variant phosphorylated at double-strand break sites. This ability to localize to DNA damage sites helps to recruit other proteins involved in DNA repair and signaling.

Non-Homologous End Joining (NHEJ): 53BP1 significantly promotes NHEJ, a pathway to repair double-strand breaks, especially in the G1 phase of the cell cycle when a homologous template is unavailable. 53BP1 promotes this pathway by:

Facilitating Synapsis: 53BP1 helps align the broken DNA ends, preparing them for ligation by the NHEJ machinery.

Restricting End Resection: By inhibiting the end resection process (a step in homologous recombination), 53BP1 ensures that NHEJ remains the primary repair mechanism for DSBs in cells not in the S or G2 phase.

Interaction with Other DNA Repair Proteins: 53BP1 interacts with other proteins to promote NHEJ, such as RIF1 (Rap1 Interacting Factor 1) and the Shieldin complex. These interactions help determine the repair pathway choice, favoring NHEJ over homologous recombination when appropriate.

DNA Damage Signaling: 53BP1 is involved in amplifying DNA damage signaling, contributing to the recruitment of additional repair proteins and activating cell cycle checkpoints. This signaling function helps maintain genomic stability by ensuring that cells do not progress through the cell cycle with unrepaired DNA damage.

Tumor Suppression and Genome Stability: 53BP1 has tumor suppressive properties, given its role in promoting accurate repair and maintaining genomic stability. Its ability to ensure proper repair of DSBs and its interaction with other DDR proteins make it an essential player in preventing mutations and chromosomal aberrations that can lead to cancer.

In summary, 53BP1 is a multifaceted protein in the DNA damage response, promoting non-homologous end joining, facilitating DNA damage signaling, and contributing to genomic stability. Its interactions with other DNA repair proteins and its role in regulating the choice of repair pathways are crucial for maintaining cellular integrity and preventing tumorigenesis.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with Thermo Fisher Fish Serum Blocker (cat#37527X3). The primary antibody, Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat#P36962).

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

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

Z Stack - (3D) - Panel A, Z-plane 48 of 95

Z-Stack =95 Slices (2.82 µm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image size (Pixels) = 1793 X 1793
Image Size (Scaled) = 63.29 µm X 63.29 µm
Bit Depth = 16 Bit
Image Center Position = X: 93.29 µm, Y: 48.96 µm
Stage Position = X: 93.29 µm, Y: 48.96 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B, Z-plane 48 of 95

Z-Stack = 95 Slices (2.82 µm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image size (Pixels) = 318 X 304
Image Size (Scaled) = 11.22 µm X 10.73 µm
Bit Depth = 16 Bit
Image Center Position = X: 93.29 µm, Y: 48.96 µm
Stage Position = X: 93.29 µm, Y: 48.96 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel C, Z-plane 48 of 95, Line Profile

Z-Stack = 95 Slices (2.82 µm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 30.00 nm
Image size (Pixels) = 147 X 131
Image Size (Scaled) = 5.19 µm X 4.62 µm
Bit Depth = 16 Bit
Image Center Position = X: 93.29 µm, Y: 48.96 µm
Stage Position = X: 93.29 µm, Y: 48.96 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

680 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00AU / 328µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 640
Laser Wavelength = 639nm @2.40%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.1
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.15µs
Frame Time = 17.86s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 679
Emission Wavelength = 702
Effective NA = 1.4
Detection Wavelength = 643 - 735
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 875 V
Detector Offset = 0
Detector Digital Gain = 1.0
Super Resolution = 9.9 (3D, Auto)

532 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00AU / 265µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS LP 525
Laser Wavelength = 488nm @0.10%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.1
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.15µs
Frame Time = 17.86s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.4
Detection Wavelength = 530 - 582
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Super Resolution = 9.3 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00AU / 235µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 505
Laser Wavelength = 405nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.1
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.15µs
Frame Time = 17.86s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 350 - 499
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 750 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 8.4 (3D, Auto)

2- Dimensional View - Panel A, B, and C (C omits the Alpha Tubulin and DAPI)

Panel shows Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG and Alpha Tubulin, with Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific and DAPI.

Image from Z-plane 48 of 95 and the Line Profile graphic showing the location where the measurement was taken.

Profile View Display - Panel C

Profile Definition = Stroke Thickness 1.0, Normal View, Grid Distance 1
Profile View = X and Y axes were set to Auto - X (Min 0.0 and Max 999.7), Y (Min 61 and Max 1037)

Deploys Caliber X to measure the distance between the two unresolved peaks. As we begin to observe foci separation, or a sub-signal beyond confocal, we note that the measurement is 140nm between the peak signals.

Summary

Panel A shows super-resolution Airyscan imaging of 53BP1 polyclonal Alexa Fluor™ 680 direct conjugate following etoposide-induced DNA damage, displayed as a representative two-dimensional optical section (Z-plane 48 of 95). α-Tubulin staining, visualized using an Alexa Fluor™ 532 goat anti-mouse secondary antibody, together with DAPI nuclear counterstaining, delineates cytoplasmic boundaries and nuclear architecture, confirming strict nuclear confinement of the 53BP1 signal. Damage-induced intranuclear foci are clearly observed, consistent with canonical 53BP1 recruitment to sites of replication stress and DNA double-strand breaks. Localization and recruitment behavior are concordant with results obtained using the corresponding unconjugated Rabbit anti-Human 53BP1 Polyclonal antibody (PA-RP-000008), confirming preservation of biological response following conjugation.

Panel B presents a two-dimensional Airyscan projection of the region of interest (ROI) indicated in Panel A. At this resolution gate, Airyscan provides improved lateral discrimination relative to confocal imaging, enabling partial separation of closely spaced 53BP1 signal domains that appear confluent at the confocal tier. The ROI defined here is advanced for further structural assessment in Panel C.

Panel C shows a two-dimensional projection of the ROI in Panel B, with accompanying line-profile analysis. Line profiling using the Zeiss ZEN caliper tool reveals a measurable separation of approximately 140 nm between adjacent 53BP1 signal peaks. This demonstrates that foci appearing as single puncta at the confocal gate begin to resolve into distinct signal domains at the Airyscan level. However, despite entry into super-resolution, the degree of separation and structural definition remains limited, and individual sub-units or higher-order architectural features cannot be reliably resolved. In accordance with the CCR CDx Step-Down framework, these findings warrant progression to higher-resolution modalities, including SIM, SIM², and STORM, for definitive structural interpretation of 53BP1 organization.

Image Correction

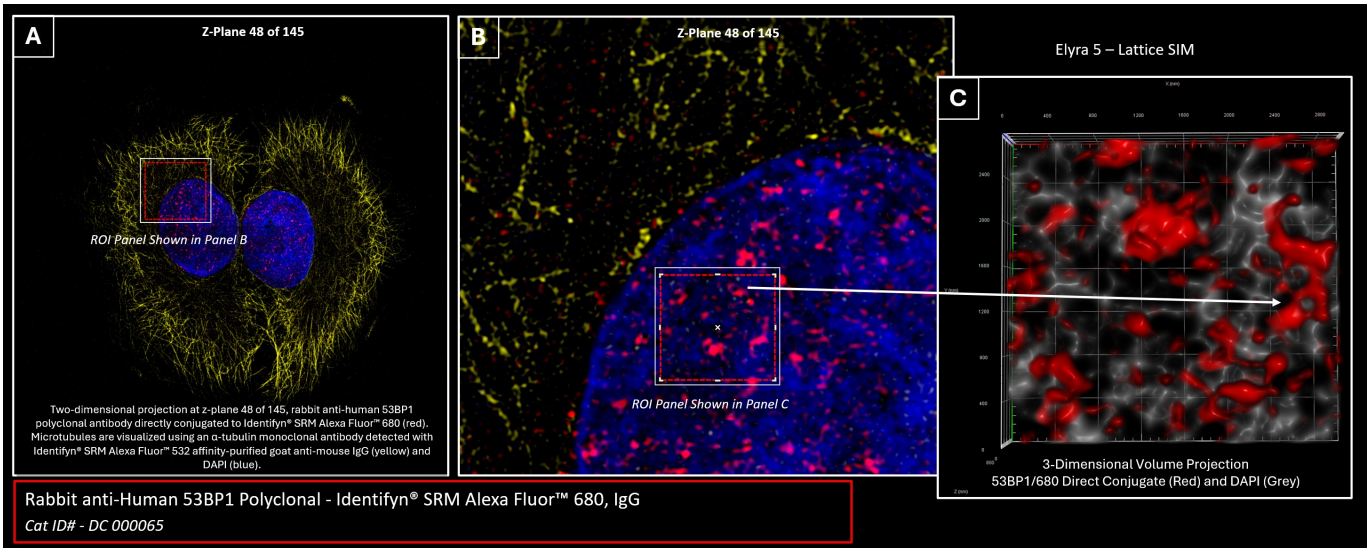
NONE

Image by J.K. Bennett
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000065
Stage	Airyscan
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Structured source metadata values	58
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	59CCBD7AA443E1208D79E9D64C6051449F6463B07203252952954066F63C1897
Method PDF SHA-256	3D1257D45446996EC449CBACD4B05902F07CF4AFC01F4A7E34BC261199B36E3C

ORIGINAL IMAGE EVIDENCE / SIM



Panel A presents a representative two-dimensional lattice SIM optical section (Z-plane 48 of 145) of 53BP1 polyclonal Alexa Fluor™ 680 direct conjugate following etoposide-induced DNA damage. α-Tubulin is visualized using an Alexa Fluor™ 532 goat anti-mouse secondary antibody and serves as an exclusionary structural marker defining cytoplasmic architecture. At the same time, DAPI nuclear counterstaining provides chromatin context and confirms nuclear confinement of the 53BP1 signal. The white box denotes the region of interest (ROI) advanced for higher-resolution evaluation in subsequent panels.

Panel B shows a higher-magnification two-dimensional view of the same Z-plane focused on the ROI indicated in Panel A. At lattice SIM resolution, the 53BP1 signal that appeared as partially separated or weakly resolved foci at the Airyscan gate now resolves into clearly distinct, spatially organized structures. This transition reflects ordered recruitment of 53BP1 along damaged chromatin domains rather than diffuse or confluent aggregation.

Panel C displays a three-dimensional volume projection of the ROI shown in Panel B, visualized with 53BP1 (Alexa Fluor™ 680) and DAPI. At this resolution tier, 53BP1 architecture demonstrates further organization relative to Airyscan, progressing from modestly separated foci to coherent, structured assemblies. However, despite improved spatial definition, individual subunit-level features and molecular-scale architecture remain unresolved.

At lattice SIM lateral resolution (~60–80 nm), 53BP1 structural organization is substantially more defined than at confocal or Airyscan gates, confirming that apparent aggregates observed at lower resolution represent composite, chromatin-associated repair assemblies. Nevertheless, SIM resolution remains insufficient to resolve the underlying subunit architecture of these assemblies, necessitating continued progression within the CCR CDx Step-Down workflow to SIM^2 and single-molecule localization microscopy (STORM) for definitive structural interpretation.

53BP1 localization and recruitment behavior are consistent with results obtained using the corresponding unconjugated Rabbit anti-Human 53BP1 Polyclonal antibody (PA-RP-000008), confirming preservation of biological specificity following conjugation.

EVIDENCE TIER	SIM
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 680
METADATA VALUES	64

STRUCTURED ACQUISITION METADATA / SIM

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	145 Slices (4.32 µm)
Channels	3
Scaling (Per Pixel)	0.02111 µm X 0.02111 µm X 0.03000 µm
Image size (Pixels)	5056 X 5056 | 621 X 577 | 143 X 131
Image Size (Scaled)	106.75 µm X 106.75 µm | 13.11 µm X 12.18 µm | 3.02 µm X 2.77 µm
Bit Depth	16 Bit
Image Center Position	X: 64.53 µm, Y: 38.17 µm
Stage Position	X: 64.53 µm, Y: 38.17 µm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflectors	SR HE LBF 405/488/561/642
Beam Splitter	405, 488, 561, 642
Filter Excitation Wavelength	200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method	Fluorescence
Illumination Wavelength	640 | 488 | 405
Channel Name	T1-Axiocam 820 -SR | T2-Axiocam 820 #2-SR | T3-Axiocam 820 #2-SR
Excitation Wavelength	640 | 488 | 405
Emission Wavelength	729 | 525 | 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device	Axiocam 820 | Axiocam 820 #2
Camera Adapter	1X
Exposure Time	150 ms | 120 ms | 50 ms
Depth of Focus	1.13 µm | 0.81 µm | 0.69 µm
Binning Mode	2,2
Laser Wavelength	640 nm @4.0% | 488 nm @3.0% | 405 nm @3.0%
Frame Time	1.98 s | 1.59 s | 1.33 s
Scan Direction	Unidirectional
Grating	36.5 µm grating
Processing Dimension	3D
Result Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
Process Sampling	N/A
Algorithm	SIM
SIM Settings	Standard
Auto Sharpness	On
Sharpness	11.2460 (680), 11.2272 (532), 9.8155 (DAPI)
Fitting	Fast Fit
Normalization	Auto
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 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG
Cat ID# - DC 000065

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000023

53BP1 (P53-binding protein 1) is a crucial player in the DNA damage response (DDR), particularly in the repair of double-strand breaks (DSBs). It has a range of functions that contribute to genomic stability and an appropriate response to DNA damage. The following are the prominent roles and functions of 53BP1 in DNA repair:

DNA Damage Sensing: 53BP1 is among the early proteins recruited to DNA damage sites. It recognizes and binds to the phosphorylated form of H2AX (γ-H2AX), a histone variant phosphorylated at double-strand break sites. This ability to localize to DNA damage sites helps to recruit other proteins involved in DNA repair and signaling.

Non-Homologous End Joining (NHEJ): 53BP1 significantly promotes NHEJ, a pathway to repair double-strand breaks, especially in the G1 phase of the cell cycle when a homologous template is unavailable. 53BP1 promotes this pathway by:

Facilitating Synapsis: 53BP1 helps align the broken DNA ends, preparing them for ligation by the NHEJ machinery.

Restricting End Resection: By inhibiting the end resection process (a step in homologous recombination), 53BP1 ensures that NHEJ remains the primary repair mechanism for DSBs in cells not in the S or G2 phase.

Interaction with Other DNA Repair Proteins: 53BP1 interacts with other proteins to promote NHEJ, such as RIF1 (Rap1 Interacting Factor 1) and the Shieldin complex. These interactions help determine the repair pathway choice, favoring NHEJ over homologous recombination when appropriate.

DNA Damage Signaling: 53BP1 is involved in amplifying DNA damage signaling, contributing to the recruitment of additional repair proteins and activating cell cycle checkpoints. This signaling function helps maintain genomic stability by ensuring that cells do not progress through the cell cycle with unrepaired DNA damage.

Tumor Suppression and Genome Stability: 53BP1 has tumor suppressive properties, given its role in promoting accurate repair and maintaining genomic stability. Its ability to ensure proper repair of DSBs and its interaction with other DDR proteins make it an essential player in preventing mutations and chromosomal aberrations that can lead to cancer.

In summary, 53BP1 is a multifaceted protein in the DNA damage response, promoting non-homologous end joining, facilitating DNA damage signaling, and contributing to genomic stability. Its interactions with other DNA repair proteins and its role in regulating the choice of repair pathways are crucial for maintaining cellular integrity and preventing tumorigenesis.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with Thermo Fisher Fish Serum Blocker (cat#37527X3). The primary antibody, Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat#P36962).

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A, Z-plane 48 of 145

Z-Stack =145 Slices (4.32 µm)

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 5056 X 5056
Image Size (Scaled) = 106.75 µm X 106.75 µm
Bit Depth = 16 Bit
Image Center Position = X: 64.53 µm, Y: 38.17 µm
Stage Position = X: 64.53 µm, Y: 38.17 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B, Z-plane 48 of 145

Z-Stack =145 Slices (4.32 µm)

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 621 X 577
Image Size (Scaled) = 13.11 µm X 12.18 µm
Bit Depth = 16 Bit
Image Center Position = X: 64.53 µm, Y: 38.17 µm
Stage Position = X: 64.53 µm, Y: 38.17 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel C, 3-Dimensional Volume View

Z-Stack 145 Slices (4.32 µm)

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 143 X 131
Image Size (Scaled) = 3.02 µm X 2.77 µm
Bit Depth = 16 Bit
Image Center Position = X: 64.53 µm, Y: 38.17 µm
Stage Position = X: 64.53 µm, Y: 38.17 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

680 -

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 = 150 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @4.0%
Frame Time = 1.98 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

532 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 488
Channel Name = T2-Axiocam 820 #2-SR
Excitation Wavelength = 488
Emission Wavelength = 525
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 120 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @3.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 405
Channel Name = T3-Axiocam 820 #2-SR
Excitation Wavelength = 405
Emission Wavelength = 445
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 50 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.0%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM Processing

Processing Dimension = 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 3
Algorithm = SIM
SIM Settings = Standard
Auto Sharpness = On
Sharpness = 11.2460 (680), 11.2272 (532), 9.8155 (DAPI)
Fitting = Fast Fit
Normalization = Auto

2- Dimensional View - Panel A, B, and C (C omits the Alpha Tubuliln)

Panel shows Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG and Alpha Tubulin, with Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific and DAPI.

Image from Z-plane 48 of 145.

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 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A presents a representative two-dimensional lattice SIM optical section (Z-plane 48 of 145) of 53BP1 polyclonal Alexa Fluor™ 680 direct conjugate following etoposide-induced DNA damage. α-Tubulin is visualized using an Alexa Fluor™ 532 goat anti-mouse secondary antibody and serves as an exclusionary structural marker defining cytoplasmic architecture. At the same time, DAPI nuclear counterstaining provides chromatin context and confirms nuclear confinement of the 53BP1 signal. The white box denotes the region of interest (ROI) advanced for higher-resolution evaluation in subsequent panels.

Panel B shows a higher-magnification two-dimensional view of the same Z-plane focused on the ROI indicated in Panel A. At lattice SIM resolution, the 53BP1 signal that appeared as partially separated or weakly resolved foci at the Airyscan gate now resolves into clearly distinct, spatially organized structures. This transition reflects ordered recruitment of 53BP1 along damaged chromatin domains rather than diffuse or confluent aggregation.
Panel C displays a three-dimensional volume projection of the ROI shown in Panel B, visualized with 53BP1 (Alexa Fluor™ 680) and DAPI. At this resolution tier, 53BP1 architecture demonstrates further organization relative to Airyscan, progressing from modestly separated foci to coherent, structured assemblies. However, despite improved spatial definition, individual subunit-level features and molecular-scale architecture remain unresolved.

At lattice SIM lateral resolution (~60-80 nm), 53BP1 structural organization is substantially more defined than at confocal or Airyscan gates, confirming that apparent aggregates observed at lower resolution represent composite, chromatin-associated repair assemblies. Nevertheless, SIM resolution remains insufficient to resolve the underlying subunit architecture of these assemblies, necessitating continued progression within the CCR CDx Step-Down workflow to SIM² and single-molecule localization microscopy (STORM) for definitive structural interpretation.

53BP1 localization and recruitment behavior are consistent with results obtained using the corresponding unconjugated Rabbit anti-Human 53BP1 Polyclonal antibody (PA-RP-000008), confirming preservation of biological specificity following conjugation.

Image Correction

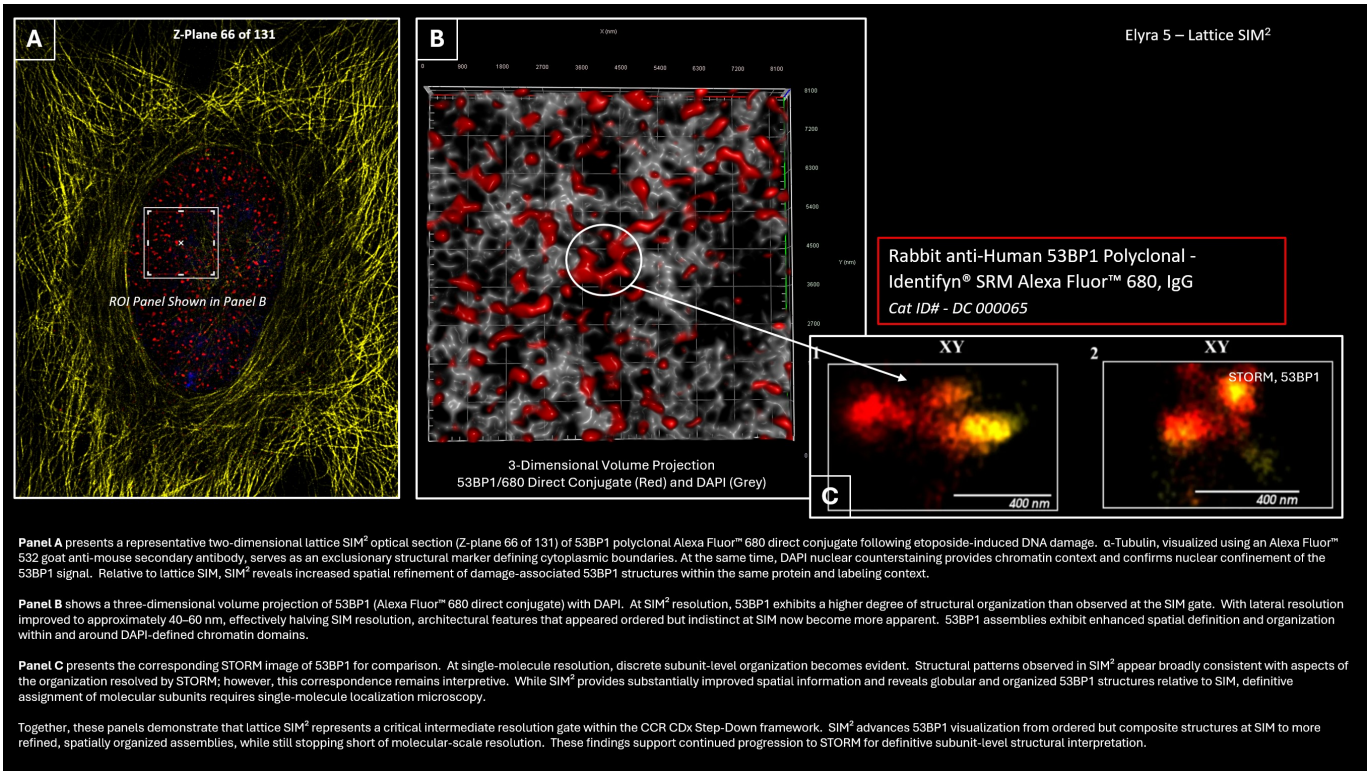
NONE

Image by J. H. Cheong
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000065
Stage	SIM
Instrument	ZEISS Elyra
Microscope configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Structured source metadata values	64
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	85DA1768F90FC5F15CAA0D3EC1B3DF371DBE7462536925FD65BC9CF4F32154B0
Method PDF SHA-256	35F819AEE648E2F756EED9148E654573BCC065B72260A93A6BBB9F4151380872

ORIGINAL IMAGE EVIDENCE / SIM²



Panel A presents a representative two-dimensional lattice SIM² optical section (Z-plane 66 of 131) of 53BP1 polyclonal Alexa Fluor™ 680 direct conjugate following etoposide-induced DNA damage. α-Tubulin, visualized using an Alexa Fluor™ 532 goat anti-mouse secondary antibody, serves as an exclusionary structural marker defining cytoplasmic boundaries. At the same time, DAPI nuclear counterstaining provides chromatin context and confirms nuclear confinement of the 53BP1 signal. Relative to lattice SIM, SIM² reveals increased spatial refinement of damage-associated 53BP1 structures within the same protein and labeling context.

Panel B shows a three-dimensional volume projection of 53BP1 (Alexa Fluor™ 680 direct conjugate) with DAPI. At SIM² resolution, 53BP1 exhibits a higher degree of structural organization than observed at the SIM gate. With lateral resolution improved to approximately 40–60 nm, effectively halving SIM resolution, architectural features that appeared ordered but indistinct at SIM now become more apparent. 53BP1 assemblies exhibit enhanced spatial definition and organization within and around DAPI-defined chromatin domains.

Panel C presents the corresponding STORM image of 53BP1 for comparison. At single-molecule resolution, discrete subunit-level organization becomes evident. Structural patterns observed in SIM² appear broadly consistent with aspects of the organization resolved by STORM; however, this correspondence remains interpretive. While SIM² provides substantially improved spatial information and reveals globular and organized 53BP1 structures relative to SIM, definitive assignment of molecular subunits requires single-molecule localization microscopy.

Together, these panels demonstrate that lattice SIM² represents a critical intermediate resolution gate within the CCR CDx Step-Down framework. SIM² advances 53BP1 visualization from ordered but composite structures at SIM to more refined, spatially organized assemblies, while still stopping short of molecular-scale resolution. These findings support continued progression to STORM for definitive subunit-level structural interpretation.

EVIDENCE TIER	SIM²
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 680
METADATA VALUES	62

STRUCTURED ACQUISITION METADATA / SIM²

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

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	131 Slices (3.90 µm)
Channels	3
Scaling (Per Pixel)	0.02111 µm X 0.02111 µm X 0.03000 µm
Image size (Pixels)	3401 X 2979 | 401 X 387
Image Size (Scaled)	50.69 µm X 62.89 µm | 8.47 µm X 8.17 µm
Bit Depth	16 Bit
Image Center Position	X: 65.31 µm, Y: 40.20 µm
Stage Position	X: 65.31 µm, Y: 40.20 µm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflectors	SR HE LBF 405/488/561/642
Beam Splitter	405, 488, 561, 642
Filter Excitation Wavelength	200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method	Fluorescence
Illumination Wavelength	640 | 488 | 405
Channel Name	T1-Axiocam 820 -SR | T2-Axiocam 820 #2-SR | T3-Axiocam 820 #2-SR
Excitation Wavelength	640 | 488 | 405
Emission Wavelength	729 | 525 | 445
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device	Axiocam 820 | Axiocam 820 #2
Camera Adapter	1X
Exposure Time	150 ms | 120 ms | 50 ms
Depth of Focus	1.13 µm | 0.81 µm | 0.69 µm
Binning Mode	2,2
Laser Wavelength	640 nm @4.0% | 488 nm @3.0% | 405 nm @3.0%
Frame Time	1.98 s | 1.59 s | 1.33 s
Scan Direction	Unidirectional
Grating	36.5 µm grating
Result Sampling	4
Process Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
Algorithm	SIM2
Input SNR	Low
Regularization Weight	0.0000
Iterations	3
Filter	No Filter
Fitting	Fast Fit
Normalization	Auto
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 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

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

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG
Cat ID# - DC 000065

Identifyn® Secondary Antibody

Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000023

53BP1 (P53-binding protein 1) is a crucial player in the DNA damage response (DDR), particularly in the repair of double-strand breaks (DSBs). It has a range of functions that contribute to genomic stability and an appropriate response to DNA damage. The following are the prominent roles and functions of 53BP1 in DNA repair:

DNA Damage Sensing: 53BP1 is among the early proteins recruited to DNA damage sites. It recognizes and binds to the phosphorylated form of H2AX (γ-H2AX), a histone variant phosphorylated at double-strand break sites. This ability to localize to DNA damage sites helps to recruit other proteins involved in DNA repair and signaling.

Non-Homologous End Joining (NHEJ): 53BP1 significantly promotes NHEJ, a pathway to repair double-strand breaks, especially in the G1 phase of the cell cycle when a homologous template is unavailable. 53BP1 promotes this pathway by:

Facilitating Synapsis: 53BP1 helps align the broken DNA ends, preparing them for ligation by the NHEJ machinery.

Restricting End Resection: By inhibiting the end resection process (a step in homologous recombination), 53BP1 ensures that NHEJ remains the primary repair mechanism for DSBs in cells not in the S or G2 phase.

Interaction with Other DNA Repair Proteins: 53BP1 interacts with other proteins to promote NHEJ, such as RIF1 (Rap1 Interacting Factor 1) and the Shieldin complex. These interactions help determine the repair pathway choice, favoring NHEJ over homologous recombination when appropriate.

DNA Damage Signaling: 53BP1 is involved in amplifying DNA damage signaling, contributing to the recruitment of additional repair proteins and activating cell cycle checkpoints. This signaling function helps maintain genomic stability by ensuring that cells do not progress through the cell cycle with unrepaired DNA damage.

Tumor Suppression and Genome Stability: 53BP1 has tumor suppressive properties, given its role in promoting accurate repair and maintaining genomic stability. Its ability to ensure proper repair of DSBs and its interaction with other DDR proteins make it an essential player in preventing mutations and chromosomal aberrations that can lead to cancer.

In summary, 53BP1 is a multifaceted protein in the DNA damage response, promoting non-homologous end joining, facilitating DNA damage signaling, and contributing to genomic stability. Its interactions with other DNA repair proteins and its role in regulating the choice of repair pathways are crucial for maintaining cellular integrity and preventing tumorigenesis.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20°C methanol and blocked with Thermo Fisher Fish Serum Blocker (cat#37527X3). The primary antibody, Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat#P36962).

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was incubated for 1 hour at room temperature at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI, cat#P36962.

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A, Z-plane 66 of 131

Z-Stack = 131 Slices (3.90 µm)

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 3401 X 2979
Image Size (Scaled) = 50.69 µm X 62.89 µm
Bit Depth = 16 Bit
Image Center Position = X: 65.31 µm, Y: 40.20 µm
Stage Position = X: 65.31 µm, Y: 40.20 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel C, 3-Dimensional Volume View

Z-Stack = 131 Slices (3.90 µm)

Channels = 3
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 401 X 387
Image Size (Scaled) = 8.47 µm X 8.17 µm
Bit Depth = 16 Bit
Image Center Position = X: 65.31 µm, Y: 40.20 µm
Stage Position = X: 65.31 µm, Y: 40.20 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

680 -

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 = 150 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @4.0%
Frame Time = 1.98 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

532 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 488
Channel Name = T2-Axiocam 820 #2-SR
Excitation Wavelength = 488
Emission Wavelength = 525
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 120 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @3.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 405
Channel Name = T3-Axiocam 820 #2-SR
Excitation Wavelength = 405
Emission Wavelength = 445
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 50 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.0%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM2 Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = 4
Channels = 3
Algorithm = SIM2
Input SNR = Low
Regularization Weight = 0.0000
Iterations = 3
Filter = No Filter
Fitting = Fast Fit
Normalization = Auto

2- Dimensional View - Panel A

Panel shows Rabbit anti-Human 53BP1 Polyclonal - Identifyn® SRM Alexa Fluor™ 680, IgG and Alpha Tubulin, with Identifyn® SRM Alexa Fluor™ 532 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific and DAPI.

Image from Z-plane 66 of 131.

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 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A presents a representative two-dimensional lattice SIM² optical section (Z-plane 58 of 131) of 53BP1 polyclonal Alexa Fluor™ 680 direct conjugate following etoposide-induced DNA damage. α-Tubulin, visualized using an Alexa Fluor™ 532 goat anti-mouse secondary antibody, serves as an exclusionary structural marker defining cytoplasmic boundaries. At the same time, DAPI nuclear counterstaining provides chromatin context and confirms nuclear confinement of the 53BP1 signal. Relative to lattice SIM, SIM² reveals increased spatial refinement of damage-associated 53BP1 structures within the same protein and labeling context.

Panel B shows a three-dimensional volume projection of 53BP1 (Alexa Fluor™ 680 direct conjugate) with DAPI. At SIM² resolution, 53BP1 exhibits a higher degree of structural organization than observed at the SIM gate. With lateral resolution improved to approximately 40-60 nm, effectively halving SIM resolution, architectural features that appeared ordered but indistinct at SIM now become more apparent. 53BP1 assemblies exhibit enhanced spatial definition and organization within and around DAPI-defined chromatin domains.

Panel C presents the corresponding STORM image of 53BP1 for comparison. At single-molecule resolution, discrete subunit-level organization becomes evident. Structural patterns observed in SIM² appear broadly consistent with aspects of the organization resolved by STORM; however, this correspondence remains interpretive. While SIM² provides substantially improved spatial information and reveals globular and organized 53BP1 structures relative to SIM, definitive assignment of molecular subunits requires single-molecule localization microscopy.

Together, these panels demonstrate that lattice SIM² represents a critical intermediate resolution gate within the CCR CDx Step-Down framework. SIM² advances 53BP1 visualization from ordered but composite structures at SIM to more refined, spatially organized assemblies, while still stopping short of molecular-scale resolution. These findings support continued progression to STORM for definitive subunit-level structural interpretation.

Image Correction

NONE

Image by J. H. Cheong
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000065
Stage	SIM²
Instrument	ZEISS Elyra

SOURCE PROVENANCE

Microscope configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Structured source metadata values	62
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	4E90A1BEAD07C2452463BAFB618ACCCDD1CF3D5F08170C652626EB1228A24B97
Method PDF SHA-256	C0E9A665FD898719714E90F89CC15766937E80F1FC24218BF293665A0F876360