

Identifyfyn Standards for Optical Microscopy Methods™Identifyfyn® Primary Antibody**Rabbit anti-Human γ H2Ax (Serine 139) Recombinant Monoclonal, Human Fc, IgG (BLR014M)***Cat ID# - PA-RR-000004*Identifyfyn® Secondary Antibodies**Identifyfyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Human, IgG (H + L)***Cat ID# - SA 000058*

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

Method

HeLa cells were grown on a μ -Slide 8 Well high Glass Bottom [ibidi chamber](#). DNA damage was induced via treatment with 2 μ M Etoposide-spiked complete growth media for 6 hours, followed by -20°C methanol fixation and blocked with [Thermo Fisher Fish Serum Blocker cat#37527X3](#). The primary antibody, Rabbit anti-Human γ H2Ax Recombinant Monoclonal, Human Fc, IgG, was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 3X in Identifyfyn's™ proprietary Wash Buffer and Identifyfyn® 647 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:5. Cells were washed 5X in Identifyfyn's™ proprietary Wash Buffer followed by the addition of Identifyfyn's™ proprietary STORM Buffer to the sample chamber enabling image acquisition.

Microscope

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

Calibration -

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

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

Image Acquisition -

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

Configuration Tab - Capture Configuration

Number of Probes: 1

Number of Simultaneous Probes: 1

Number of Reference Probes: 1

Camera: Both Cameras

Color Channel: Both Channels

Probe Capture Mode: Interleaved

Z -Stack Capture Mode: Interleaved

Multiple Location Capture: Not selected

Post Record Reference: Not Selected

Fluidics: Not Selected

Autofocus Drift Correction: On

Microscope configuration

Biplane Module: 635nm Dichroic

Objective: Silicon Selected

Stage Insert: Rectangular Selected

Course Focus Position: 8-Well Chamber Selected

Dichroic: SuperRes

PSF: (Red, Orange)

Heater: Current: 26

Camera Configuration

Recording Vertical Field of View: 100%

Widefield Camera Exposure Time: 100ms

Super Resolution Camera Exposure Time: 20ms

Widefield Camera Binning: 1

Per-Probe Exposure Time: Selected

Probe Configuration

Predefined Probe: First probe: 640 nm (635 nm Dichroic); First reference probe: 640 nm (635 nm Dichroic)

Photoactivation/Imaging Laser: 0% Selected

Exposure Time: 20ms Selected

Experiment Configuration

Record Workflow: Selected Options

View Mode: Widefield 200µm

Capture Mode: Live

Stage and Piezo Selection

Examination of the Sample

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

Piezo Current: 182.0 µm

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

Widefield 640nm: 0.1X Neutral Density Filter: Selected, @0.2% Laser Input

Reference Probe 1 Laser control (640 nm (640 nm Dichroic)): Not Selected

Advanced Tools Option

Widefield camera Exposure Time: 100ms

Default values are maintained unless otherwise noted.

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

Autofocus - Calibration Values

Current Position" -0.979

Current Intensity: 4.5

Calibration Value: -5070 nm/unit

Autofocus Mode: Pre-Capture

View Mode

SuperRes: 50µm is selected

Capture Mode

Live

Stage and Piezo Selection

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

Piezo Record: Begin: 180.1; End: 181.3

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

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

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

Not Selected

Advanced Tools

Widefield Camera Exposure Time: 20ms

Default values are maintained unless otherwise noted.

Record Workflow - Record Tab: Capture Parameters

Frames: Probe 1: 500

Z Positions: 6 using Steps: 0.2µm (200nm)

Cycles: 10

Time Points: 1

Pause Between Cycles: Not Selected

Automatic Photoactivation Laser Control: Not Selected

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

Laser Power: 30%

Expert Configuration Options - The Localization Tab

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

Localization

Cutout Width: 16px

Max Frame Accumulation: 1 frame

Max Accumulation Offset: 1px

Fiducial at Max Accumulation: Not selected

Max Particles Per Frame: No Max

Background SLPE Removal: Not Selected

Cutout/Background Removal: Not Selected

Preview Cutout Positions: Selected

Record Workflow - Localization Tab

Data Collection

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

Recording Summary

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

Probe1: BG threshold: 20

Region of Interest -

Not selected

Display

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

Record Workflow - View Tab

Region of Interest

Default values

Visualization Parameters

Point Splatting

Sizing Mode: Constant

Particle size: 20nm

Color by: Depth

Color Table: Single

Opacity Mode: Constant; Opacity 1: 0.3

Front-to-back Attenuation: Not Selected

Post Acquisition analysis - View Tab**Drift Correction** - Computed with the following parameters:

Method: Particle (direct)

Time Intervals: 10 Intervals

Pixel Size: 50nm

Smoothing: 8.00

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

PSF Z offset: -450 to +450

Radial precision: 25

Axial precision: 60

The other parameters were not applied.

Statistical analysis - Computed to clean low background signals.**Statistics Dialog - Cluster Analysis**

Clustering Algorithm: DBSCAN

ParametersMaximum Particle Distance: 0.05 μ m (500nm)

Maximum Particle Count to Form Cluster: 20

Computer Hulls: Selected

Compute Density Isosurfaces: Not Selected

Edit Settings: All Probes

Hull Alpha shape Radius: 600nm

Compute: Selected

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

The upper left panel displays the entire nucleus captured using STORM imaging methodology. The lower left image shows the X-Y projection, which resembles what is typically seen in diffraction-limited microscopy and is commonly referred to as a single foci. However, our data reveal the presence of two foci, which we refer to as a "doublet." The center panel presents the Y-Z projections, illustrating the structure that appears to be a single foci or termination point involved in the damage-induced phosphorylation event. Additionally, it suggests further phosphorylation extending along the chromatin from this focus or termination point.

The right panel demonstrates the volume rendering of the single foci in three dimensions by calculating convex hulls, resulting in a total signal volume of 0.14 cubic microns (140 cubic nanometers).

Image by S. Thakur

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