**Identifyn Standards for Optical Microscopy Methods™**

Identifyn® Primary Antibody

**Rabbit anti-Human RAD51 Recombinant Monoclonal, IgG (BLR017M)**
*Cat ID# - PA-RR 000002*

Identifyn® Secondary Antibodies

**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')$_2$ (H + L)**
*Cat ID# - SA 000063*

Rad51 is a critical protein in the homologous recombination (HR) process, which is a crucial mechanism for repairing DNA double-strand breaks (DSBs) and maintaining genomic stability. Rad51 foci formation in response to DNA damage is a hallmark of the cellular DNA repair process, particularly Homologous Recombination (HR). When cells encounter DNA Double-Strand Breaks (DSBs) or other forms of DNA damage, Rad51 proteins assemble into discrete nuclear foci at the damage sites. Localization and visualization of Rad51 aggregate via immunofluorescent applications allow for the study of Rad51 response kinetics in response to damage, thereby making the study of Rad51 foci formation critical to the study of the cellular response to DNA damage and how it plays a crucial role in the repair process. Rad51 is essential to recognizing DNA damage, activating the Homologous Recombination (HR) pathway, Rad51 filament formation, and DNA repair and resolution...

## Method

Hela cells were grown on a µ-Slide 8 Well high Glass Bottom Ibidi chamber. DNA damage was induced via treatment with 2µM Etoposide–spiked complete growth media for 6 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human RAD51 Recombinant Monoclonal, was incubated for 1 hour at room temperature at a dilution of 1:100. Cells were washed 3X in Identifyn's™ proprietary Wash Buffer, and Identifyn® 647 secondary antibody was incubated at room temperature for 1 hour at a dilution of 1:50. Cells were washed 5X in Identifyn's™ proprietary Wash Buffer followed by the addition of Identifyn's™ proprietary STORM Buffer to the sample chamber enabling image acquisition.

## Microscope

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

## Calibration -

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

https://identifyn.com/storm-calibration

## Image Acquisition -

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**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**

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**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: 182.3 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: 179.0; End: 181.1

**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**

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

**Record Workflow - Record Tab: Capture Parameters**

Frames: Probe 1: 500
Z Positions: 6 using Steps: 0.2µm (200nm)
Cycles: 10
Time Points: 1
Pause Between Cycles: Not Selected
Automatic Photoactivation Laser Control: Not Selected

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

Laser Power: 30%

**Expert Configuration Options - The Localization Tab**

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

**Localization**

Cutout Width: 16 px
Max Frame Accumulation: 1 frame
Max Accumulation Offset: 1px
Fiducial at Max Accumulation: Not selected
Max Particles Per Frame: No Max
Background SLPE Removal: Not Selected
Cutout/Background Removal: Not Selected
Preview Cutout Positions: Selected

**Record Workflow - Localization Tab**

**Data Collection**

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

**Recording Summary**

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

Probe1: BG threshold: 15

**Region of Interest -**

Not selected

**Display**

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

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**Record Workflow - View Tab**

**Region of Interest**

Default values

**Visualization Parameters**

Point Splatting
Sizing Mode: Constant
Particle size: 20nm
Color by: Depth
Color Table: Single
Opacity Mode: Constant; Opacity 1: 0.3
Front-to-back Attenuation: Not Selected

**Post Acquisition analysis - View Tab**

**Drift Correction -** Computed with the following parameters:

Method: Particle (direct)
Time Intervals: 10 Intervals
Pixel Size: 50nm
Smoothing: 8.00

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

PSF Z offset: -450 to +450
Radial precision: 25
Axial precision: 60

The other parameters were not applied.

**Statistical analysis -** Computed to clean low background signals.

**Statistics Dialog - Cluster Analysis**

Clustering Algorithm: DBSCAN

**Parameters**

Maximum Particle Distance: 0.05µm (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

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## Summary

The upper left panel (A) displays the X-Y and X-Z views, emphasizing individual foci selected using the STORM Imaging Methodology, which is depicted in the lower left corner (Whole Nucleus), capturing the entire nucleus. The middle left panels (B and C) illustrate the "Doublet" and "Triplet" signals in the X-Y view, respectively. Meanwhile, the upper right panel (D) presents the X-Y view, and the lower right panel (E) shows the X-Z view, both providing volumetric measurements of the "Triplet" image. Notably, these measurements reveal some unresolved yet interesting structures.

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Image by S. Thakur

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