Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human Ku70 Polyclonal, IgG
Cat ID# - PA-RP 000006

Identifyn® Secondary Antibodies

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

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, F(ab')$_2$ (H + L)
Cat ID# - SA 000042

Ku70 is a crucial protein involved in the Non-Homologous End Joining (NHEJ) pathway, one of DNA's central mechanisms for repairing double-strand breaks (DSBs).

Recognition and Binding to DNA Breaks: Ku70 and Ku80 form the Ku heterodimer. This heterodimer has a high affinity for DNA ends and is responsible for recognizing and binding to DSBs. Its structure allows it to slide onto the DNA strand and form a ring around the break, stabilizing it.

Recruitment of Additional NHEJ Proteins: After binding to the DNA break, the Ku heterodimer acts as a scaffold, recruiting and assembling other proteins essential for the NHEJ pathway. This includes DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which are crucial in further processing the break.

Protection of DNA Ends: By binding to the ends of the DNA at the site of the break, Ku70/80 helps protect the DNA from further degradation or unwanted processing, ensuring that the ends are preserved for the repair process.

Alignment of DNA Ends: Ku70/80 helps align the broken DNA ends, facilitating the ligation step in NHEJ. This alignment is crucial for accurate repair, even though NHEJ does not require extensive sequence homology.

Participation in End Processing and Ligation: Once other NHEJ factors have been recruited, Ku70/80 can assist in processing DNA ends if necessary. Ultimately, it helps bring the DNA ends together for ligation by the ligase complex (such as DNA Ligase IV, XRCC4, and XLF), completing the repair process.

In summary, Ku70, as part of the Ku heterodimer, plays a central role in the NHEJ pathway by recognizing and binding to DNA breaks, recruiting and organizing other repair proteins, protecting DNA ends, and facilitating the alignment and ligation necessary to repair double-strand breaks.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2µM Etoposide–spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Rabbit anti-Human Ku70 Polyclonal, IgG, was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F('b')$_2$ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000.

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The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, $F('b')_2$ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI cat#P36962.

Microscope

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

Single Plane (2D) - Z-plane 54 of 160 total - *Panel A*

Z Stack - (3D)

Z-Stack = 160 Slices (4.77 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 3431 X 4860
Image Size (Scaled) = 45.27 µm X 64.13 µm
Bit Depth = 16 Bit
Image Center Position = X: 55.92 mm, Y: 39.25 mm
Stage Position = X: 55.92 mm, Y: 39.25 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Single Plane (2D) - Z-plane 54 of 160 total - *Panel B*

Z Stack - (3D)

Z-Stack = 160 Slices (4.77 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 1583 X 2118
Image Size (Scaled) = 20.89 µm X 27.95 µm
Bit Depth = 16 Bit
Image Center Position = X: 55.92 mm, Y: 39.25 mm
Stage Position = X: 55.92 mm, Y: 39.25 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - *Panel C*

Z-Stack = 65 Slices (1.92 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 454 X 455
Image Size (Scaled) = 5.99 µm X 6.00 µm
Bit Depth = 16 Bit

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Image Center Position = X: 55.92 mm, Y: 39.25 mm
Stage Position = X: 55.92 mm, Y: 39.25 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

647 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 640
Channel Name = T1-Axiocam 820-SR
Excitation Wavelength = 640
Emission Wavelength = 729
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 100 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @1.00%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 27.5 µm grating

488 -

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-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
Camera Adapter = 1X
Exposure Time = 120 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @1.50%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

DAPI -

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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 = 100 ms
Depth of Focus = 0.69 µm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.00%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 27.5 µm grating

Post Processing - SIM² Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = 4
Channels = 3
Algorithm = SIM²
SIM² Settings = Standard
Input SNR = Low
Regularization = None
Regularization Weight = 0.0000
Iterations = 3
Filter = No Filter
Fitting = Fast Fit
Normalization = Auto

3D Image Processing - *Panel C*

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

Summary

Here, we present the **same image as in the Ku70 super-resolution SIM dataset** to facilitate a comparison of halving the resolution and revealing additional structural

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details.

Panel A - This panel presents a 2-dimensional view from the image acquisition at z-plane 54 out of 160, illustrating the signal and localization of Rabbit anti-Human Ku70 Polyclonal IgG, labeled with Identifyn® SRM Alexa Fluor™ 647 (white; the default red color has been changed in Zeiss Zen software to enhancevisual contrast), Alpha Tubulin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green), and DAPI nuclear staining (blue). A white box indicates a region of interest (ROI) for further investigation in Panel B.

Panel B - This panel presents a 2-dimensional view from the ROI in Panel A at the same z-plane, 54 The image illustrates the signal and localization of Rabbit anti-Human Ku70 Polyclonal IgG, labeled with Identifyn® SRM Alexa Fluor™ 647 (white; the default red color has been changed in Zeiss Zen software to enhance visual contrast), Alpha Tubulin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green). DAPI nuclear staining (blue). **Here we see similar structural details to those captured in the SIM resolution**. A white box indicates a region of interest (ROI) for further investigation in Panel C.

Panel C- This panel provides a three-dimensional volume projection of Rabbit anti-Human Ku70 Polyclonal IgG from the ROI identified in Panel B. Ku70 is counterstained with Identifyn® SRM Alexa Fluor™ 647 secondary antibody (white; the default red color has been changed in Zeiss Zen software to enhance visual contrast), and DAPI (blue). **No adjustments to thresholds or manipulations of any kind were made; the image represents the image acquisition, in a Volume Projection, at default values, and represents nothing more than what the cameras saw.**

NOTE - Using **super-resolution Structured Illumination Microscopy Squared** with the Elyra 5, **we observe enhanced structural details of Ku70 that were previously seen as "pan-nuclear"** or diffuse under diffraction-limited techniques such as widefield imaging and confocal microscopy. Notably, the **structures appear more defined in SIM Squared compared to standard SIM, as the axial resolution improves from approximately 80 nm to 40 nm. However, the interpretation of these details can be subjective, and we encourage you to draw your conclusions.**

What is clear is that at the SIM Squared resolution, we not only see organized structural details for Ku70, but also for DAPI. While DAPI is often considered merely a counterstain for the nucleus, this level of super-resolution reveals that the previously diffuse blue nucleus now shows distinct structures. These structures exhibit similar patterns and interactions with Ku70 and other nuclear proteins that we regularly image.

Is it possible that DAPI may be a more significant tool in super-resolution than previously recognized?

Image Corrections

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, $F(a'')_2$ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

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