Materials and Methods

Cell Culture and Immunofluorescence Staining

Cells were seeded at a density of xxx × 10³ cells/cm² onto 18 mm diameter round glass coverslips (Fisher Scientific, Waltham, MA, USA) placed in sterile 6-well tissue culture plates. Cultures were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Gibco), at 37°C in a humidified incubator containing 5% CO₂ for 24–48 hours to allow adhesion and spreading.

After incubation, cells were washed three times with phosphate-buffered saline (PBS; pH 7.4, Gibco) at room temperature and fixed in 3–4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15–20 minutes at room temperature. Following fixation, samples were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature.

To quench residual free aldehydes and reduce autofluorescence, samples were incubated with 0.25 M glycine (Sigma-Aldrich) in PBS for 10 minutes.

Nonspecific antibody binding sites were blocked by incubating cells with 5% normal goat serum or a commercial protein blocking solution (e.g., Protein Block Serum-Free; Dako) for 30 minutes at room temperature.

Primary antibodies (either directly conjugated or detected via secondary antibodies) were diluted in appropriate antibody diluent (e.g., Dako Antibody Diluent with Background Reducing Components) and incubated overnight at 4°C in a humidified chamber. For multiplex immunofluorescence, primary antibodies conjugated to Alexa Fluor dyes spanning multiple spectra (e.g., Alexa Fluor 405, 488, 555, 594, and 647) were used. Negative controls included matched isotype IgG antibodies at corresponding concentrations.

Following primary antibody incubation, coverslips were washed three times with PBS and incubated for 1 hour at room temperature in the dark with fluorescently labeled secondary antibodies or phalloidin conjugates as needed. Cellular nuclei were optionally counterstained with 0.5 ng/ml DAPI (Sigma-Aldrich).

After final washes, samples were mounted with an antifade reagent (e.g., ProLong Gold or VECTASHIELD, Thermo Fisher or Vector Laboratories) to preserve fluorescence.

Confocal Microscopy and Image Acquisition (Leica TCS SP8)

Imaging was performed on a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DMi8 inverted stand and a motorized XYZ stage including a Super Z autofocus drive with 1500 µm travel range for precise focus maintenance during Z-stack acquisition.

An environmental chamber integrated with the system maintained live-cell imaging conditions at 37°C and 5% CO₂ when required.

Laser Excitation Sources

405 nm diode laser (continuous wave, 50 mW) for excitation of blue fluorophores (e.g., Alexa Fluor 405, DAPI)

Pulsed white light laser (WLL) tunable from 470 to 670 nm with 1 nm increments, operating at 80 MHz repetition rate with up to 8 selectable acousto-optic tunable filter (AOTF) channels, each delivering ~1.5 mW

Fixed laser lines at 458 nm, 488 nm, 514 nm, 561 nm, and 594 nm

STED Depletion Lasers

Continuous-wave (CW) STED lasers at 592 nm and 660 nm

Pulsed STED laser at 775 nm

Laser power settings were optimized per fluorophore and sample, typically using depletion laser powers of 20–50% (approximately 3–7 MW/cm² at the focal plane) and excitation powers between 1% and 20% (0.7 to 17 kW/cm²).

Objectives and Imaging Parameters

Images were acquired using high numerical aperture oil immersion objectives such as HC PL APO 63×/1.40 NA or 100×/1.40 NA oil immersion objectives (Leica) offering high resolution and light collection efficiency.

Frame sizes typically at 512 × 512 pixels

Pixel sizes set to ~40–60 nm for confocal mode, and ~18–20 nm for STED mode to oversample enhanced resolution

Pixel dwell times ranged from 1–5 µs per pixel, adjusted per sample brightness and photobleaching risk

Line averaging between 2–4 applied for noise reduction

Z-stack images collected with step sizes matching Nyquist sampling for axial resolution (typically 0.15–0.3 μm)

Detection System

Fluorescence emission was separated spectrally using an Acousto-Optical Beam Splitter (AOBS) and acquired via:

Two Hybrid Detectors (HyD) (GaAsP-based) with high quantum efficiency (~45% at 530 nm), working in photon counting mode (ideal for confocal and STED imaging to increase sensitivity and suppress background)

Two standard Photomultiplier Tubes (PMTs) for conventional fluorescence detection

One transmitted light PMT detector coupled with a differential interference contrast (DIC) polarizer for transmitted light imaging

Spectral detection windows were set carefully to minimize crosstalk between fluorophores, examples:

Fluorochrome Excitation (nm) Emission Detection Range (nm)

Alexa Fluor 405 405 420–450

Alexa Fluor 488 488 500–540

Alexa Fluor 555 561 (or 555) 560–585

Alexa Fluor 594 594 600–630

Alexa Fluor 647 640 660–700

Sequential scanning was employed during multi-color imaging to prevent spectral bleed-through and enable proper fluorophore detection.

In STED imaging, time-gated detection was applied on the HyD detectors, with time gates typically set to 1.5–6.5 ns after excitation to reduce background and improve resolution.

Live-Cell Time-Lapse Imaging

Time-lapse imaging was performed with environmental conditions maintained by a motorized stage and enclosed incubation system (37°C, 5% CO₂, humidified atmosphere).

Dynamic series of images (50 frames) were acquired continuously in confocal or STED modes with variable dwell times (approximately 1.5, 2.5, and 5 µs per pixel) to evaluate fluorescence signal stability and photobleaching during prolonged imaging.

Image Processing and Quantitative Analysis

Image datasets were initially visualized and processed using Leica LAS X software for 3D rendering, Z-projections, and basic corrections.

Quantitative analyses, including fluorescence intensity measurements and colocalization metrics, were conducted using Fiji/ImageJ software with the “Colocalization Finder” plugin for pixel overlap quantification.

Fluorescence intensity profiles, intensity change curves, and colocalization coefficients were plotted and statistically analyzed in OriginPro 2023b or equivalent statistical software.

Data represent averages ± standard error (SE) from at least five cells per experimental condition, with experiments independently repeated at least three times.

Statistical significance was determined using two-sided Student’s t-tests or ANOVA, with a significance threshold of p < 0.05.

Image adjustments for brightness and contrast were applied linearly and consistently across samples to preserve quantitative integrity.