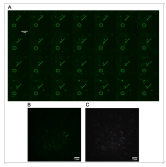

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Microscopy

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1 *Works for me* dx.doi.org/10.17504/protocols.io.77ghrjw

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ABSTRACT

Live-cell images of U251 cells were acquired at Instituto Gulbenkian de Ciência on a commercial widefield Nikon High Content Screening microscope, equipped with a 100x/1.45 plan-apo oil-immer- sion objective and an Andor Zyla 4.2 Scientific complementary metal-oxide-semiconductor (sCMOS) camera. To image eGFP/Venus–fused proteins, a 470 nm laser line, and GFP fluorescence filtersets were used and for the imaging of HaloTag-fused proteins, a 635 nm laser line and Cy5 fluorescent filtersets were used. An exposure time of 80 ms was used and the camera readout bandwidth was set to 200MHz.The resulting pixel size was 65 nm using a 1024x1024 pixel as field of view. All acquisition were done at room temperature.The microscope, cameras, and hardware were controlled through Nikon Elements software. Images were analyzed by means of the ImageJ free software.
Single-molecule imaging and Super-Resolution Radial Fluctuations (SRRF) imaging were performed on a Leica DMI6000 inverted microscope using a 100x/1.46 a-plan apochromat oil immersion objective. A 561 nm excitation laser (Coherent Sapphire) was set to 50 mW resulting in an effective power density of ~2.3kW/cm^2. The laser beam was passed through a custom filter cube (Chroma Technology) with a zet405/561x excitation filter, a zt405/561/657rpc-uf2 dichroic beam splitter, and an et610/75m emission filter. Fluorescent light was imaged on an Evolve512 electron multiplying charge-coupled device (EM-CCD) camera (Photometrics) after additional 1.6x magnification. An EM gain of 300 was used and the camera read out band width was set to 10 MHz. The resulting pixel size was 100 nm using a 512 x 512 pixel as field of view. The incubation chamber was maintained at 37°C. The microscope , cameras, and hardware were controlled through MetaMorph software (Molecular Devices). For single-molecule experiments and for each cell, 1000 frames were acquired at 33 ms exposure time with no interval between frames. No photoactivation was necessary as spontaneous photoactivation of PA-JF549 combined with photobleaching by 561 nm illumination gave a reasonable density of single molecules.The Trackmate ImageJ plugin was used to detect, fit and track individual GFAP molecules in living cells (A-C). For the detection of GFAP molecules, we selected a LoG detector and an estimated blob diameter of 0,4 mm and a threshold of 1500. For the tracking, we used a simple LAP tracker with a linking maximum distance of 0,5 mm, a gap-closing maximum distance of 0,5 mm and a gap-closing max frame gapof0.The average diffusion of GFAP molecules and its fractions, per individual cell, were calculated by fitting the SpotOn 2-state(bound-free) kinetic model (0,05mm^2/s as maximum D_{bound} and 0,02 mm^2/s as minimum D_{free}) to the distribution of translocations for individual molecules, obtained with TrackMate. For SRRF imaging, 100 frames were acquired for each cell. SRRF images were generated by running the NanoJ-SRRF ImageJ plugin on groups of 100 diffraction-limited images using the default settings.

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