Video Article

EVALUATION OF A KNOCK-DOWN PROTEIN BY FLUORESCENCE DENSITY QUANTIFICATION IN NEURONAL PRIMARY CULTURES

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Abstract

I. Fluorescence signal detection Obtaining a specific and reproducible silencing of a gene using RNAi requires accurate design and standardization1. A protein of interest is down-regulate using transfection of an RNAi carried in plasmid vectors expressing GFP as reporter2 and a non silencing RNAi as control (Figure 1). Forty-eight hours after transfection the neuronal cultures are fixed with paraformaldehyde 4% prepared on cytoskeleton buffer solution (CBS) 3,4. The quantification relies on the identification of neurons in the green channel (488 nm) by GFP transfection-positive cells and the fluorescence density of the labeling of a particular protein detected by immunofluorescence. The target protein is labeled with a specific monoclonal primary antibody and detected by the incubation with a secondary antibody conjugated to a fluorophore emiting to 594 nm. Also Hoechst staining is used for nuclei detection (Figure 1). II. Image acquisition - As a first step check by direct observation the general viability of the fixed cells, nuclear morphology, the homogeneity of the population, gel mount conditions and cell density at 10X magnification. This is best achieved under UV filter observation. If the samples displays gel mount bubbles, low viability by condensed nuclei or evident neuronal affection, replicate assays must be done. - Start a field scan, identifying the neurons that are prone for quantification (i.e viable and with no overlapping label with other cells), the suggested scan procedure is going from top left, downwards to bottom right on the coverslips. This scan must be performed quickly and in a different length-wave of the target protein dye. In this particular case, the observations are done to 488 nm for GFP. - Some additional coverslips are suggested to adjust the capture parameters in the image analyses software, such as gain, offset, time of exposure. During the scan make a stop on a field with neurons transfected and non-transfected neurons, for doing internal comparison of the fluorescence intensity. - Capture the images at 40x field, a) in the length-wave of the fluorophore labeling the target protein, in this study 594nm. b) Capture the images of the neuronal basal identification, in this case 488nm for the GFP reporter. c) For additional fluorophores capture following from less to more stable. d) Repeat the captures for every single neuron in the coverslips as much as necessary, at least 30 per replicate per group of 3 independent assays, in order to obtain representative images for the statistical analyses. III. Image analyses - Separate the image folders for double-blind groups and replicates. - Use the neuronal basal image for starting the quantification, in this study we use the GFP image per each neuron. Use the tool irregular AOI (area of interest) and delineate an area around the zone to be quantified, in this study, we use the soma area (Figure 2a). This procedure should be repeated in the untransfected neuron as internal control reference. - Paste the delineated AOI on the corresponding image captured from the specific immunolabeled neuron, which will be quantified (fluorophore emiting to 594nm as in this case) (Figure 2b). This procedure must be repeated in the canvas of the image without cells, for measuring the fluorescence background (Figure 2c). - Go to “count and measure objects” and convert these areas into quantification data areas by using the tool “convert AOI to object”. An image with at least 3 numbered areas corresponding to the somas areas of transfected, untransfected cells and its background will be quantified (Figure 2b). - Using the “count and measure objects/select measurements” option in the menu, to obtain the quantification of the area in “density” and “object pixels” (Figure 2c). Export the measures to a data matrix for statistical analysis (table 1). All those values must be compared to the total control (non silencing RNAi) of the each assay for obtaining the statistical significance. - Additional, measures can be used for supporting the silencing effect by the RNAi treatment, such as: a) Neurites extension: An usual biological effect evaluated in neuronal primary cultures is the neurite morphogenesis, therefore to evidence an RNAi action may be the neurite extension and branching 5. The GFP signal is used to quantify the number and extension of primary neurites. Images are acquired at 40X fields and processed with “large spectral filter” parameter for improving the resolution. Length and number of neurites are measured using the tool “manual measurement/spatial trace feature”, previously calibrated to micrometers (Figure 3a,b). b) Western blotting: To quantify the protein levels of the target protein in neuronal primary cultures after RNAi treatment by Western blotting 6, it is necessary to transduction the neuronal cultu

Disclosures

No conflicts of interest declared.