Biophysical Journal, Volume *117*

Supplemental Information

Flow Arrest in the Plasma Membrane

Michael Chein, Eran Perlson, and Yael Roichman

Supplementary material

Methods

All methods were carried out in accordance with Tel-Aviv University guidelines and regulations. All animal experimentations were approved by Tel-Aviv University Animal Ethics Committee.

Dissociated spinal cord culture

Neuronal cell culture was performed according to "Rapid purification of embryonic rat motor neurons: an in vitro model for studying MND/ALS pathogenesis" (1). Briefly, spinal cords were collected from C57BL/6 or ICR mice at E12.5. In all experiments, mice of both sexes were used. Spinal cords were then dissociated using trypsin and repeated trituration. Cells were collected through centrifugation followed by Optiprep (Sigma-Aldrich) gradient centrifugation to achieve a motor neuron-enriched cell culture. Neurons were then plated on glass bottom dishes (10,000 cells per insert) coated with complete Neurobasal medium as describe before (1). All neuronal cultures were grown for 4 DIV.

Preparation of glass bottom culture plate samples

Cells were plated on round coverslip-bottom 35mm dishes with #1.5 thickness (FD35-100, WPI). Before use, the plates were cleaned as follows: Plates were treated with 20% NaOH for 45', washed in DDW, then treated with 30% sulfuric acid for 30', rinsed in DDW and cleaned with 70% ethanol then washed with sterile DDW and left to dry in a sterile hood. Before plating cells, PDMS was mixed and cast in round plates, then cut to fit on the glass bottom dishes. Wells for plating the cells were punched using 6mm (4 wells / plate) puncher. PDMS wells cast was cleaned with adhesive tape and 70% ethanol, and left to dry inside a biological hood. The cast was firmly attached to the glass surface and the plate was incubated for 5-10' in 60°C. PDMS was pressed against the glass to ensure tight bondage. For plating spinal cord neurons, wells were treated over night with poly-ornithine and 2 hours of laminin. For plating HEK293T cells, wells were treated with 0.1% poly-L-lysine (P4707, Sigma Aldrich) for 45' before plating. HEK293T cells were maintained in DMEM (Biological Industries, Israel) supplemented with 1% Glutamax (Gibco), 10% Fetal Bovine Serum and 1% Penicilin-Streptomycin (Biological Industries, Israel). Neurons, were plated at a density of 10,000 cells/6mm PDMS well. HEK293T were plated on 8mm wells at a density of 15,000/well.

Preparation of expression plasmids for the study of membrane receptors

TrkB-GFP plasmid, encoding rat full-length TrkB fused to EGFP at the C'-terminus under a CMV promoter was gifted by Rosalind Segal (Harvard University). p75-GFP plasmid, encoding rat p75NTR fused to EGFP was a gift by Francisca C Bronfman(Pontifical Catholic University of Chile). The pLL3.7- CMV-EGFP 3rd generation lentiviral vector (Addgene #11795) was gifted by Uri Ashery (Tel Aviv University). LV-TrkB-GFP and LV-p75-GFP were cloned by inserting TrkB and p75 from TrkB-GFP and p75-GFP into pLL3.7-CMV-EGFP downstream of the CMV promoter. LV- TrkB-ACP was subcloned by inserting the ACP sequence from pACP-tagm2 (NEB) immediately after the TrkB signal sequence and cloning into pLL-3.7-CMV whose EGFP reporter was excised.

Plasmid expression in HEK293T and spinal cord neurons

Lentiviral particles were produced in HEK cells using a 2nd generation packaging system based on Gag-Pol helper and VSVG coat constructs with selected Lentiviral (LV) vector construct. For lentiviral production, HEK cells grown on 60mm dish at 70-80% confluence were transfected 10µg of LV-vector together with 7.5µg of pGag-Pol and 2.5µg of pVSVG. Plasmids were mixed in Calcium-Phosphate transfection mix: 25mM Hepes, 5mM Kcl, 140mM NaCl, 0.75mM Na_2PO_4 with 125mM $CaCl_2$ immediately before addition to cells, in a volume of 0.5mL per plate. Culture supernatants were harvested at 2 days post-transfection, and concentrated ×10 using the PEG virus precipitation kit (Abcam, ab102538). Final pellet was re-suspended in Neurobasal media, aliquoted and kept in -80℃ until use. For SCN transduction, 2-10µl of concentrated LV suspension was used per well containing 10,000 neurons. LV were added 1-2 hours after plating of the neurons, and were washed out three times in CNB 24 hours later. For HEK live and fixed cell imaging experiments, cells were plated and transfected 1 day post plating with Fugene 6 reagent in DMEM.

Total Internal Reflection Fluorescence (TIRF) and Oblique fluorescence microscopy

Live and fixed cell TIRF imaging was done on a FEI-Munich iMic-42 digital microscope equipped with fast 360° spinning beam scanner to allow even illumination of the entire diameter of the back focal plane of the objective. A 100x Olympus 1.49 numerical aperture TIRF objective was used for objective-based TIR. As illumination source, 4 solid-state laser lines at 405, 488, 561 and 640nm were used with maximum output power of 50mW each. Control of stage, excitation and acquisition parameters were via Live Acquisition 2 software. Images were captured using Ixon897 EMCCD camera (Andor). In all live imaging experiments, a 37°C, 5% CO2 and humidity conditions were kept using a custom environmental control system (Live Imaging Services), and gain was set to 300 to maximize signal capture and minimize exposure of the sample. For SPT experiments, exposure time was 25 milliseconds with 1 millisecond delay. Imaging area was cropped prior to imaging to allow fast imaging times. Laser intensities used were 40% and 70% for 561 and 488 respectively. TIRF angle varied for each plate but was between 2.480-2.500. all movies acquired were 1500 frames long.

Surface ACP-CoA labeling

Freshly prepared mixture of ACP-CoA labeling, New England Biolabs (NEB), was used for single-dye ACP labeling. 2µM fluorescent CoA (488, 547 or 647), 1mM MgCl2, 0.8µM SFP-synthase enzyme in either DMEM with 1% Glutamax (for HEK cells) or Neurobasal 2% B27, 1% Glutamax (for spinal cord neurons) supplemented with 0.5% BSA. A volume of 25 µl was added for 6 mm PDMS wells, and incubated 30' in the cell-culture incubator. Cells were then washed 3 times with the media.

Single particle tracking

Fluorescently labeled protein receptors imaged by TIRF (see details above) were identified, characterized and followed using the well known protocol of Crocker and Grier (2) implemented in the MATLAB software (MathWorks). In this algorithm each protein in an image is assigned a position (x and y coordinates) and a brightness (sum of the intensity of pixels in a window around the motor cluster). proteins in consecutive snapshots are linked using the least squares method (2). Each identified protein was then assigned a life-time, i.e. the duration time since it appeared in the field of view till it left it by one of three processes: it was internalized, or the movie ended, or it bleached. We used all proteins for the correlated diffusion studies that report on almost instantaneous hydrodynamic interactions, but only 3 s (~120 frames) long trajectories for quantifying the compartment size (see below). The localization error of the localization algorithm is a tenth of a pixel, in optimal conditions. These would corresponde to a 8nm localization eror. However, due to the proteins are smaller than the diffraction limit and are not very bright. We, therefore, estimate the localization error from imaging proteins immobilized on a glass substrate, resulting in the value of 50nm.

Mean square displacement analysis

Single particle trajectories were extracted from the TIRF movies by conventional video microscopy (2). For MSD analysis we used all mobile particle trajectories, defined so that their maximum displacement from their original position was larger than 0.5 μm. In addition, we use only trajectories longer than 3s to ensure a proper estimation of the cage size.

The ensemble average mean squared displacement of tracer particles is defined as the average over all particles of the squared displacement of each particle at time τ from its position at $\tau = 0$:

$$MSD(\tau) = 1/N \sum_{i=1}^{N} \left(x_i(\tau) - x_i(0)\right)^2$$

where $x_i(\tau)$ is time the position of particle i at time τ, and $N$is the total number of tracked particles.

The ensemble and time average mean squared displacement of tracer particles is defined as the average over all particles of the squared displacement of each particle at time τ from its position at $\tau = 0$:

$$MSD(\tau) = \frac{1}{N}\sum_{i=1}^{N} \frac{1}{M_i}\sum_{j=1}^{M_i} \left(x_i(t_j + \tau) - x_i(t_j)\right)^2$$

where t_j is the acquisition time series, τ is the lag time (the time over which the squared displacement is measured), and M_i is the total number of trajectory segments of length τ extracted for particle i. To obtain the plots in **Error! Reference source not found.**C,D we averaged particles in different locations within the cell membrane, in different cells, and for different cultures.

Correlated diffusion analysis

To calculate the correlated diffusion according to Eq. (1) of the main text we use all the single particle trajectories obtained, mobile and immobile. We do so to increase our statistical sample for calculating these correlations. This is justified, since the correlated diffusion at short lag times reflects only the

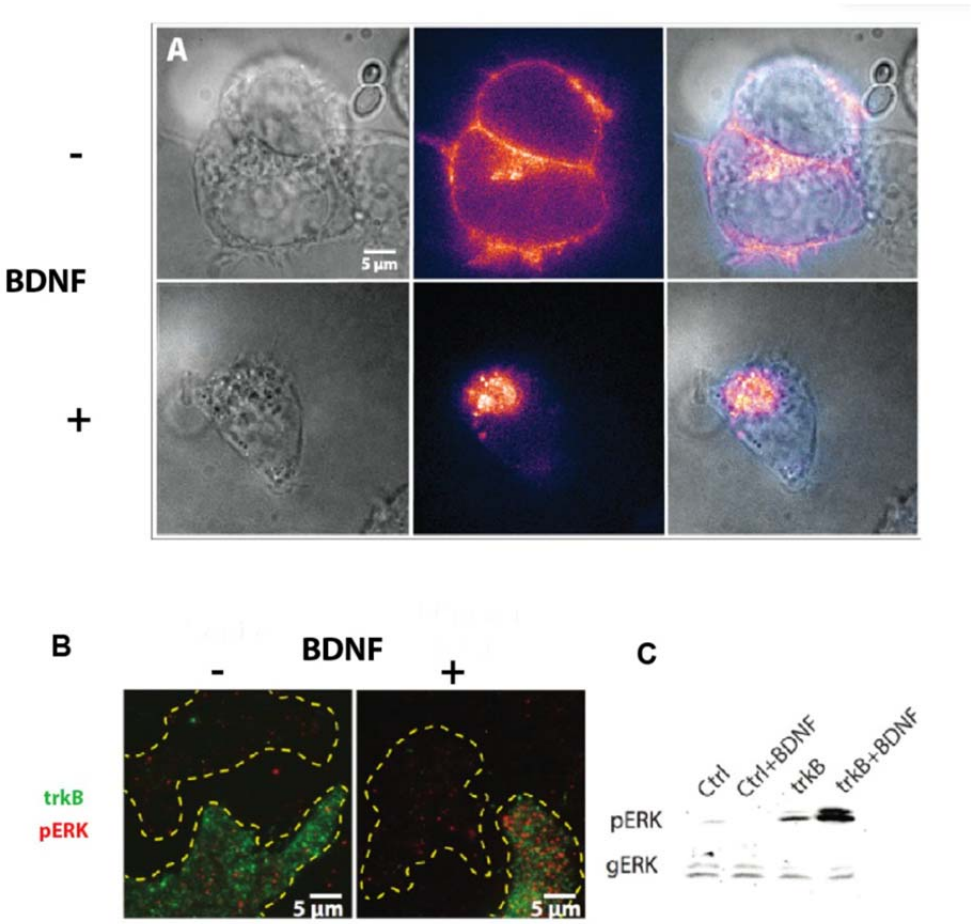
instantaneous flow field a protein creates due to thermal motion, irrespective of its long time behavior (confined motion or free). Localization errors are averaged out when measuring correlated motion, as long as they do not dominate the motion. Here, our localization error is of the order of 6 nm, and the proteins are moving with a diffusion constant of D=0.375 μm^2/s resulting in meaningful measured correlation when averaging over 40,000 trajectories.

In order to fit the functional dependence of the obtained correlated diffusion we first subtract the long-range constant correlation value, which is positive and equal for the longitudinal and transverse correlations. This correlation, which is two orders of magnitude smaller that the maximal correlation value is a result of center of mass motion of the sample.

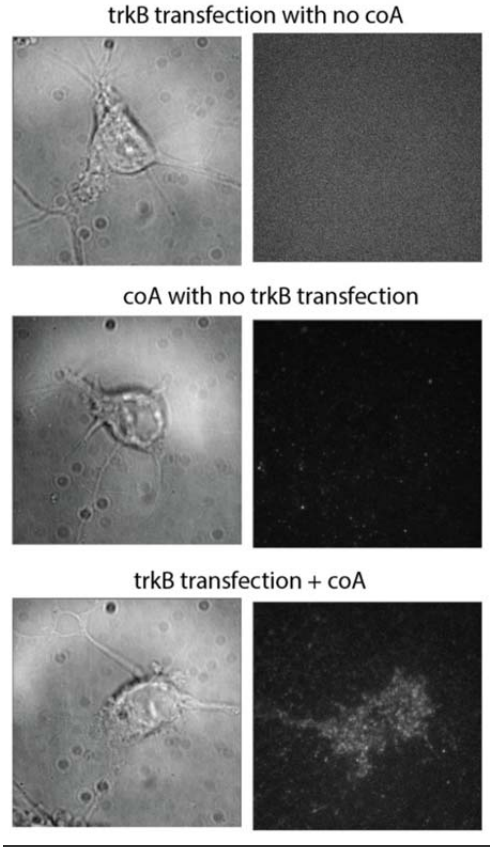
Methods references:

1. Camu W, Henderson CE (1994) Rapid purification of embryonic rat motoneurons: An in vitro model for studying MND/ALS pathogenesis. *J Neurol Sci* 124:73–74.

2. Crocker J (1996) Methods of Digital Video Microscopy for Colloidal Studies. *J Colloid Interface Sci* 179(1):298–310.



Supplementary figure 1: TrkB ACP is biologically active. A) *TrkB ACP expressing motor neurons were imaged 30 minutes after the addition of 50 ng/ml BDNF or control media. Results indicate that TrkB-ACP is internalized in response to 50 ng/ml BDNF within 30 minutes. Image was acquired using pseudoTIRF (Cui et al. 2007).* ***B)*** *TIRF images showing HEK cells that express TrkB ACP (green) respond to 50 ng/ml BDNF treatment by activation of pERK (red) more strongly then non ACP expressing cells (non-green cells marked by yellow outline). Cells were fixed 30 minutes after treatment, permibilized using triton and stained with Sigma's mouse anti pERK antibody (M8159) at 1:5000 concentration.* ***C)*** *Immunoblots demonstrating the activation of pERK in HEK cells with/out TrkB ACP transfection and with/out 50 ng/ml BDNF treatment. Cells were lysed 30 min after treatment, and prepared for blotting. Antibodies used were Sigma's mouse anti pERK antibody (M8159) at 1:5000 concentration, and Sigma's rabbit anti general ERK antibody (M5670) at 1:10000 concentration.*



Supplementary figure 2: The specificity of the ACP tag *– Left panel shows bright field images of motor neuron, while he right panel shows the same field of view in TIRF, with the 488 laser set to 70%. As shown, the background noise is very minute, and only the condition in which cells were transfected and the dye was added, show specific signal.*