Supporting Material:

Netrin-1 regulated distribution of UNC5B and DCC in live cells revealed by TICCS

Angelica A. Gopal,[1,2] Benjamin Rappaz,[2,3] Vincent Rouger,[1] Iain B. Martyn,[3]

Peter D. Dahlberg,[3] Rachel J. Meland,[1] Ian V. Beamish,[2,4]

Timothy E. Kennedy,[2,4] Paul W. Wiseman[1,2,3,*]

[1]Department of Chemistry, McGill University, Montreal, Quebec, Canada

[2]McGill Program in Neuroengineering

[3]Department of Physics, McGill University, Montreal, Quebec, Canada

[4]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada

*Corresponding author: paul.wiseman@mcgill.ca

Supporting Material and Methods

Cell culture

Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.1 mM nonessential amino acids (Gibco, Carlsbad, CA). Cells were maintained in a humidified, 5% CO_2 atmosphere at 37°C. Cells were lifted with trypsin and plated on 35 mm microwell dishes (MatTek Corp., Ashland, MA) and grown for 2 days before being imaged. At 24 h post plating, plasmids encoding full-length DCC-EGFP and full-length UNC5B-mCherry were transfected into the cells using LTX Lipofectamine with the Plus Reagent, as specified by the manufacturer (Life Technologies, Carlsbad, CA). During imaging cells were maintained at ~37°C in a 5% CO_2 environment in an incubator. The cells were allowed to equilibrate in the imaging incubator for at least 10 min before data acquisition to avoid thermal drift of the stage.

Fixed cells

HEK293T cells were rinsed twice with 37°C PBS, then incubated with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 30 min at room temperature. Cells were rinsed twice with PBS at room temperature and stored in PBS at 4°C until microscopy imaging the next day. Cells were imaged in an incubator at ~37 °C in a 5% CO_2 environment to match collection conditions for the live cells.

Cell Surface Biotinylation and Western Blot Analysis

HEK293T cells were grown to a density of ~95k cells/ cm^2, then transfected to express DCC-EGFP. After culturing for 24 hrs, expression was confirmed using the EGFP reporter. Cells were then treated with 200 ng/mL of netrin-1 for 5 min, washed twice for 5 min with ice-cold phosphate-buffered saline containing 0.1 mM calcium chloride and 1 mM magnesium chloride (pH 7.4) to halt protein trafficking.

Surface proteins were biotinylated by incubation with 0.75 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL) in PBS for 30 min at 4°C. The reaction was then quenched by 2 x 5 min washes with ice-cold 10 mM glycine in PBS and then washed twice in ice-cold PBS before lysis in phosphate buffered RIPA supplemented with protease inhibitors (150 mM NaCl, 10 mM phosphate, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 µg/mL aprotinin, 1 mM EDTA, pH 7.4, 5 µg/mL leupeptin, 1 mM PMSF). Biotinylated proteins were precipitated

with streptavidin-agarose beads (Thermo Scientific, Rockford, IL) and separated by SDS-PAGE on 10% gels.

Following electrophoresis, proteins were transferred to a nitrocellulose membrane and blocked for 1 hr in 5% non-fat dry milk in tris-buffered saline containing 0.1% Tween-20. Western blot analysis was performed using anti-DCCin (1:1000) and anti-GAPDH (1:5000) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were incubated for 45 min at room temperature and bands were visualized using Amersham ECL Western Blotting Detection Reagents (GE Healthcare, UK). Developed blots were scanned using a Canon MF4600 Series PCL6 scanner.

Supporting Results

DCC-EGFP and UNC5B-mCherry expression versus autofluorescence control

Fluorescence confocal microscopy images of live HEK293T cells expressing DCC-EGFP and UNC5B-mCherry on the plasma membrane, compared to images of non-transfected live cells (autofluorescence control), in Fig. S1. The images have not been contrast adjusted. Autofluorescence in non-transfected cells is negligible compared to fluorescence generated by the expression of fluorescent plasmids, and intensity traces from Fig. S1 are shown in Fig. S2.

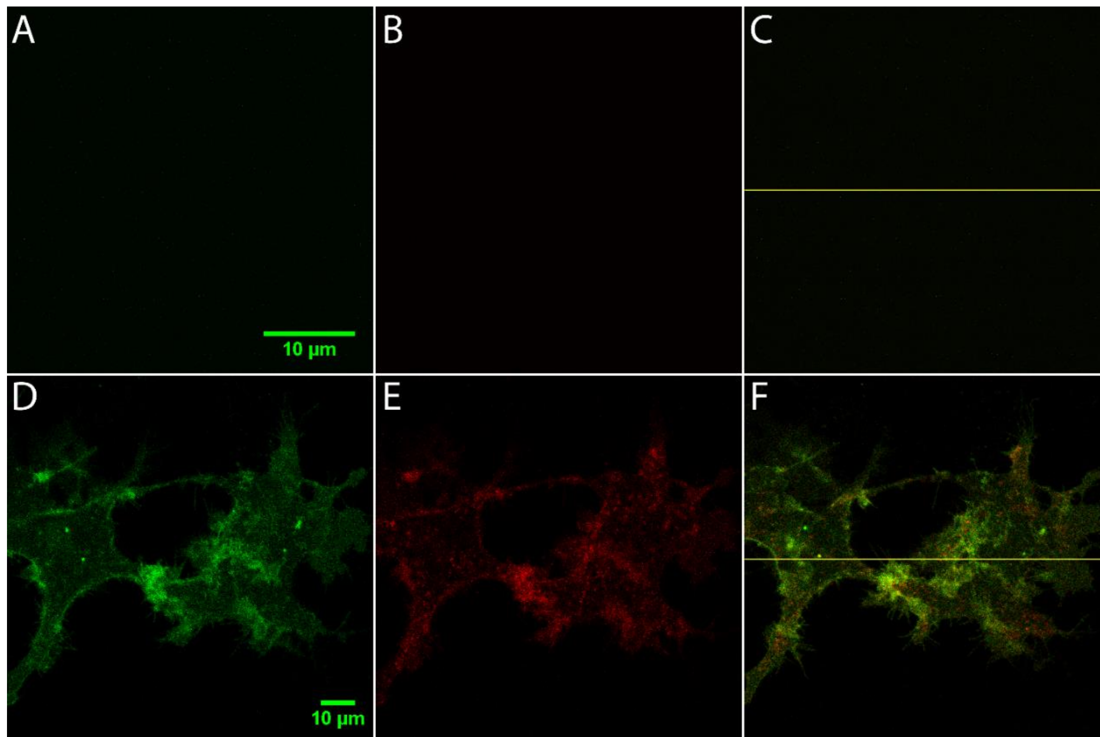

Figure S1: Fluorescence emission of DCC-EGFP and UNC5B-mCherry is readily distinguished from autofluorescence background. **(A)** Channel 1: Non-transfected live HEK293T cells in EGFP channel, **(B)** Channel 2: Non-transfected live HEK293T cells in mCherry channel, **(C)** Composite overlay image of (A) and (B). **(D)** Channel 1: EGFP channel showing distribution of DCC in transfected live HEK293T cells, **(E)** Channel 2: mCherry channel showing distribution of UNC5B in transfected live HEK293T cells, **(F)** Composite overlay of (D) and (E). Yellow line in (C) and (F) was selected for the intensity traces shown in Fig. S2. DCC-EGFP was excited by a 488 nm Ar laser, whilst UNC5B-mCherry was excited by a 594 nm He-Ne laser.

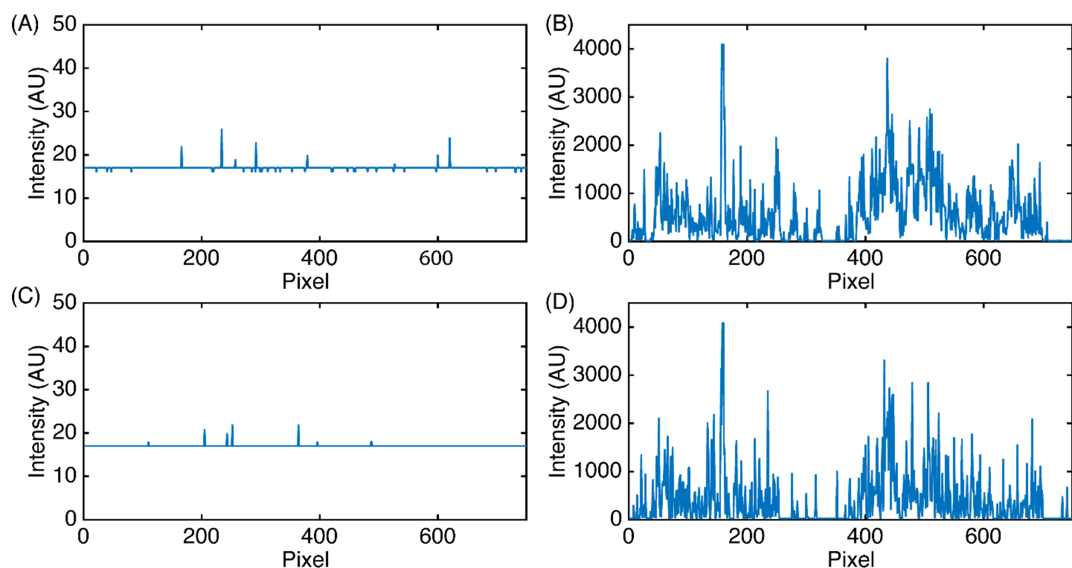

Figure S2: Intensity traces taken from the yellow lines indicated in Fig. S1 for non-transfected control cells and transfected expressing cells. **(A)** Intensity trace extracted from the EGFP channel in Fig. S1A. **(B)** Intensity trace extracted from the mCherry channel in Fig. S1B. **(C)** Intensity trace extracted from the EGFP channel in Fig. S1D. **(D)** Intensity trace extracted from the mCherry channel in Fig. S1E.

Detector cross talk is comparable to background fluorescence intensity levels

Detector cross talk was measured in both channels by exciting cells expressing only EGFP constructs with the 594 nm laser, and cells expressing only mCherry constructs with the 488 nm line, where images are shown in Fig. S3. The images have not been contrast adjusted. Measured image intensities were no different from background fluorescence intensity levels. Intensity traces from Fig. S3 are shown in Fig. S4.

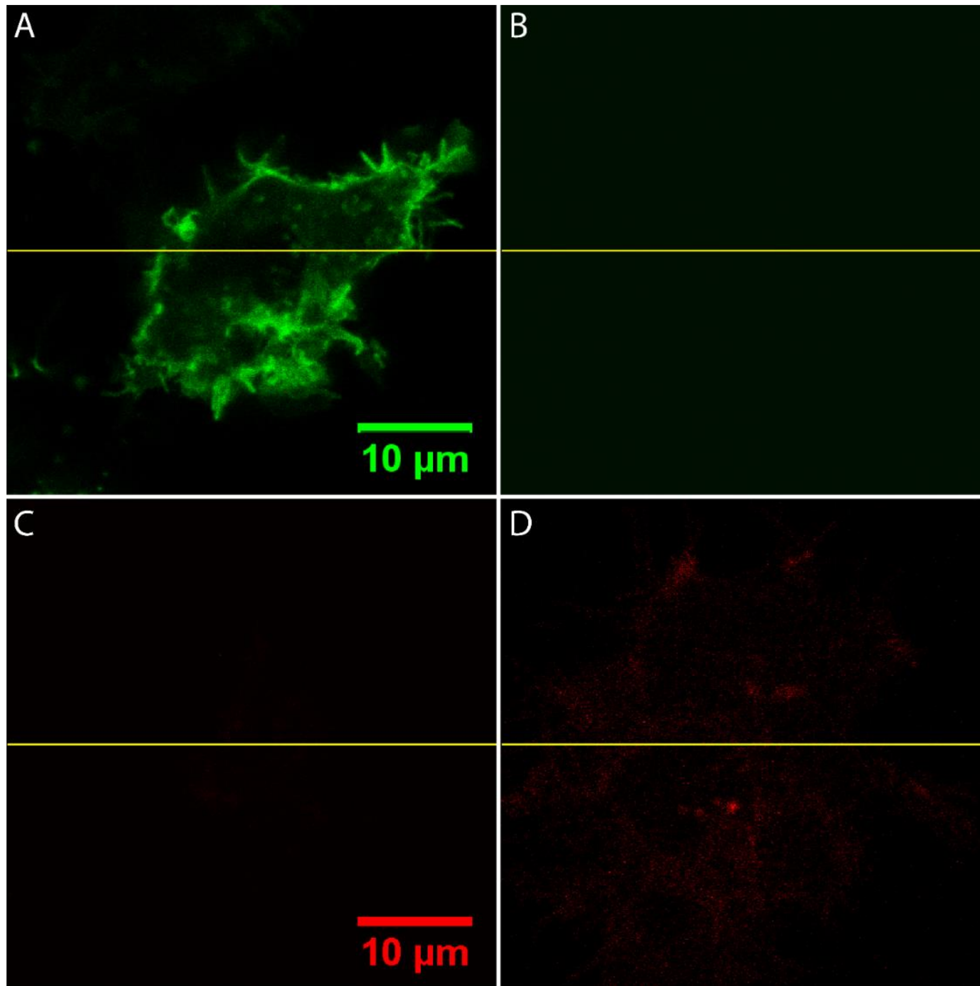

Figure S3: Fluorescence expression of DCC-EGFP or UNC5B-mCherry is readily distinguished from resulting crosstalk. **(A)** HEK293T cells transfected with DCC-EGFP excited by a 488 nm Ar laser, **(B)** excited by a 594 nm He-Ne laser. **(C)** HEK293T cell transfected with UNC5B-mCherry excited by a 488 nm Ar laser, **(D)** excited by a 594 nm He-Ne laser. Yellow lines are selected for intensity traces shown in Fig. S4.

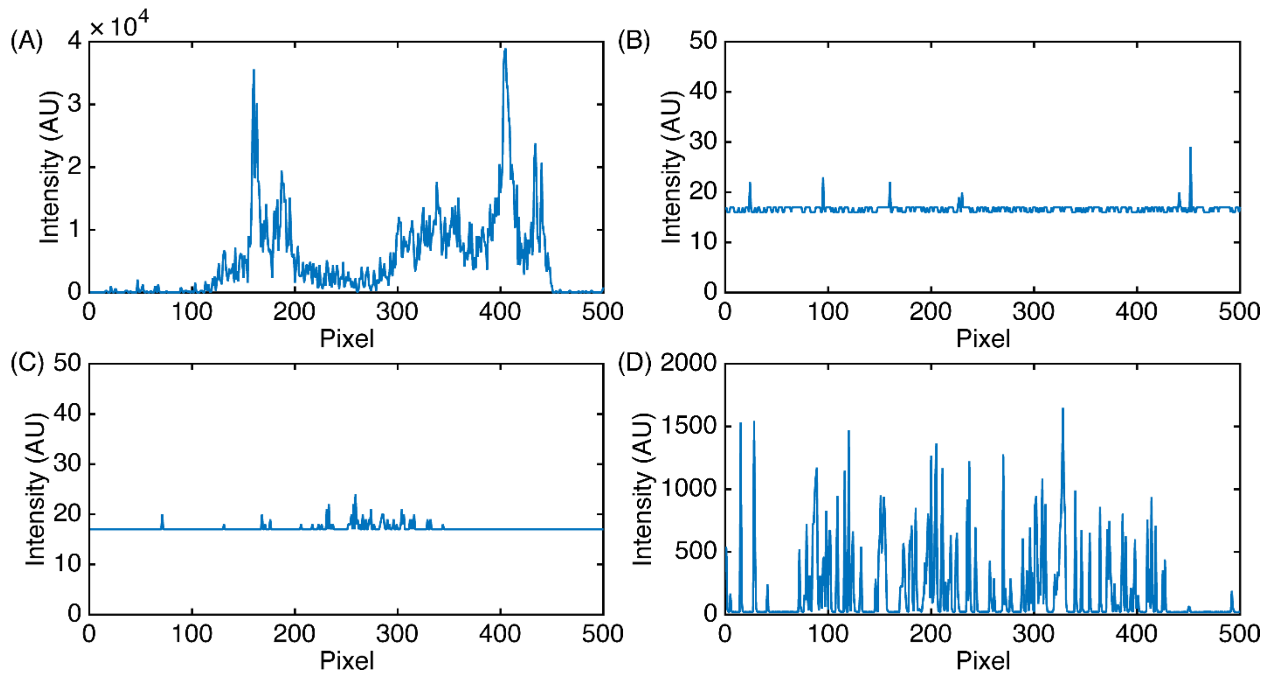

Figure S4: Intensity traces taken from the yellow lines indicated in Fig. S3 for evaluation of detector cross talk. **(A)** Intensity trace extracted from the EGFP channel in Fig. S1A, excited by a 488 nm Ar laser. **(B)** Intensity trace extracted from the EGFP channel in Fig. S1B, excited by a 594 nm He-Ne laser. **(C)** Intensity trace extracted from the mCherry channel in Fig. S1C, excited by a 488 nm Ar laser. **(D)** Intensity trace extracted from the mCherry channel in Fig. S1D, excited by a 594 nm He-Ne laser.

Netrin-1 Recruits DCC to the Plasma Membrane

To independently determine if application of netrin-1 recruits DCC to the plasma membrane, recombinant DCC-EGFP was expressed in HEK293T cells. Proteins exposed on the extracellular face of the plasma membrane were then selectively labelled by cell surface biotinylation. The biotinylated proteins were then isolated using streptavidin coated beads, and assessed by western blot analysis for DCC. Fig. S5 reveals an increase in biotinylated cell surface DCC 5 minutes after exposure to netrin-1, supporting the conclusion that DCC is rapidly recruited to the plasma membrane following stimulation with netrin-1.

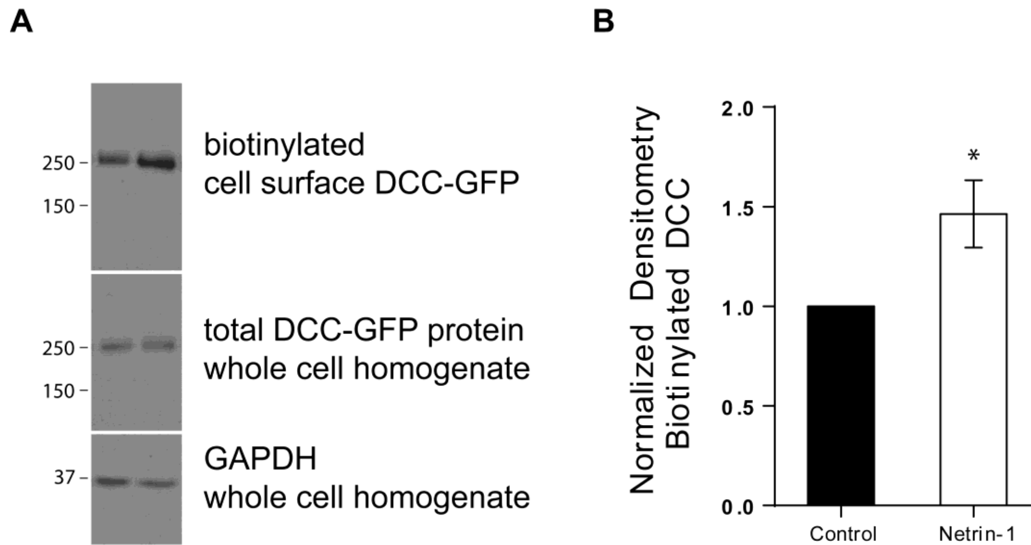

Figure S5: **(A)** Western blot analysis of recombinant DCC-EGFP expressed in HEK293T cells showing total DCC protein in whole cell homogenates and increased levels of biotinylated cell surface DCC 5 min following application of 200 ng/mL netrin-1. Immunoreactivity for GAPDH in the whole cell homogenates is presented as a control. The molecular weight markers correspond to 250, 150 and 37 kDa as marked. **(B)** Quantification of immunoreactive band optical density reveals a significant increase in the amount of biotinylated cell surface DCC following application of netrin-1. * indicates significance at a 95% CL via a two-tailed t-test. N=4.

Fixed cell analysis:

As a negative control for the sliding window TICCS analysis, cells fixed with 4% paraformaldehyde after transfection with DCC-EGFP and UNC5B-mCherry were imaged and analyzed in a similar manner to transfected live cells. A representative set of correlation functions from a TICCS analysis is shown in Fig. S6. The correlation functions for these fixed cells could not be fit with a diffusion decay model as expected.

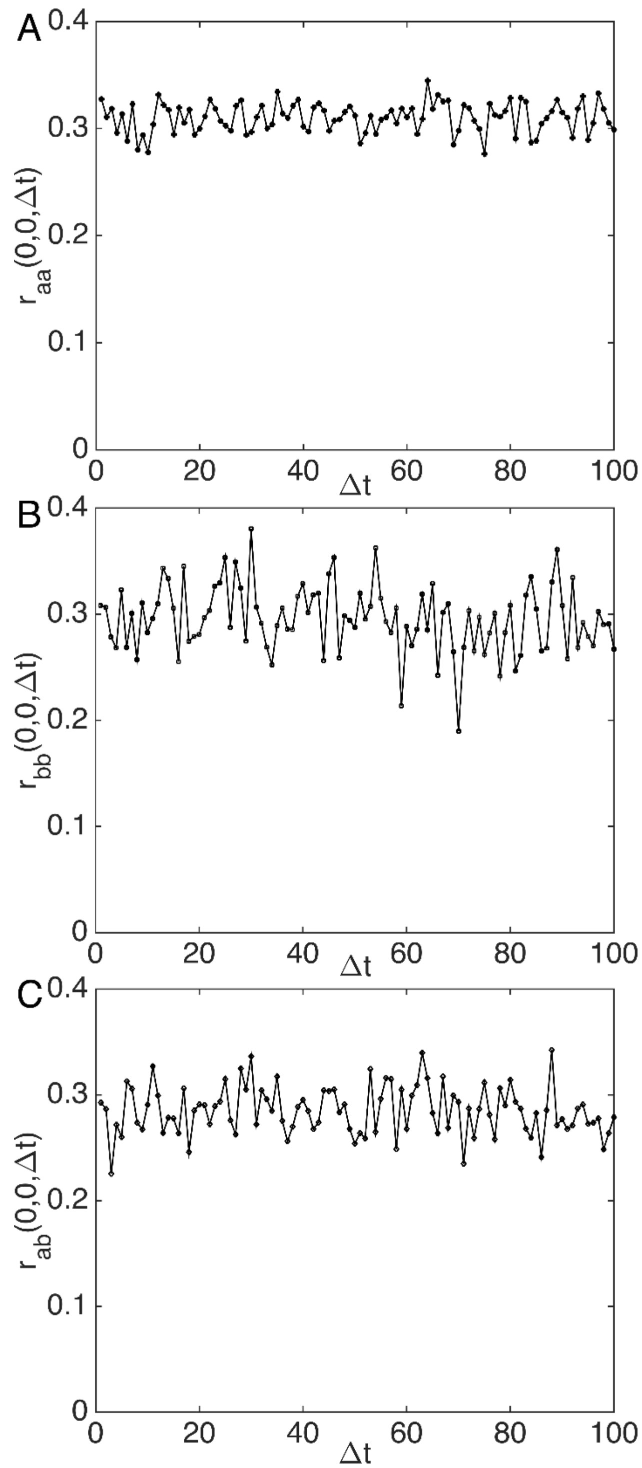

Figure S6: Typical TICCS correlation functions from imaging of fixed HEK293T cells expressing DCC-EGFP and UNC5B-mCherry. (A) Autocorrelation function for channel 1 DCC-EGFP. (B) Autocorrelation function for channel 2 UNC5B-mCherry. (C) Cross correlation function for channel 1x2. $\Delta t = 0.97s$.