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Spatially Selective Dissection of Signal Transduction in Neurons Grown on Netrin-1 Printed Nanoarrays via Segmented Fluorescence Fluctuation Analysis

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Abstract

Axonal growth cones extend during neural development in response to precise distributions of extracellular cues. Deleted in Colorectal Cancer (DCC), a receptor for the chemotropic guidance cue netrin-1, directs F-actin reorganization and is essential for mammalian neural development. To elucidate how the extracellular distribution of netrin-1 influences the distribution of DCC and F-actin within axonal growth cones, we patterned nanoarrays of substrate bound netrin-1 using lift off nanocontact printing. The distribution of DCC and F-actin in embryonic rat cortical neuron growth cones was then imaged using total internal reflection fluorescence (TIRF) microscopy.

Fluorescence fluctuation analysis via Image Cross-Correlation Spectroscopy (ICCS) was applied to extract the molecular density and aggregation state of DCC and F-actin, identifying the fraction of DCC and F-actin colocalizing with the patterned netrin-1 substrate. ICCS measurement of spatially segmented images based on the substrate nanodot patterns revealed distinct molecular distributions of F-actin and DCC in regions directly overlying the nanodots compared to over the reference surface surrounding the nanodots. Quantifiable variations between the populations of DCC and F-actin on and off the nanodots reveal specific responses to the printed protein substrate.

We report that nanodots of substrate-bound netrin-1 locally recruit and aggregate DCC and direct F-actin organization. These effects were blocked by tetanus toxin, consistent with netrin-1 locally recruiting DCC to the plasma membrane via a VAMP2 dependent mechanism. Our findings demonstrate the utility of segmented ICCS image analysis, combined with precisely patterned immobilized ligands, to reveal local receptor distribution and signaling within specialized subcellular compartments.

Keywords: fluorescence fluctuation analysis, image correlation spectroscopy, nanopatterned cell substrates, chemotropic guidance, deleted in colorectal cancer, receptor aggregation
Migrating cells and axons may traverse distances of many cell diameters during development, directed by spatial distributions of extracellular guidance cues. Netrins are a small family of laminin related secreted proteins that function as attractants and repellents to direct cell and axon migration during neural development.\textsuperscript{1} Loss-of-function mutations in netrin-1 or the netrin receptor deleted in colorectal cancer (DCC) are lethal in mice, indicating that these proteins are essential for mammalian development.\textsuperscript{2, 3}

Netrin-1 is a \textasciitilde75 kDa protein, composed of three domains: N-terminal domains VI and V are named after homologous domains in laminins, while the positively charged “domain C” or netrin-like module at the carboxyl terminus has sequence similarity with the tissue inhibitor of metalloproteinases family. The C domain binds heparin sulphate, likely contributing to localizing netrin-1 in the extracellular matrix (ECM).\textsuperscript{1} Netrin-1 immobilization is required to transduce the force required to direct axon guidance,\textsuperscript{4, 5, 6} and netrin-1 multimerizes DCC to activate intracellular signaling cascades, consistent with recent crystallographic studies.\textsuperscript{7, 8}

DCC is a \textasciitilde180 kDa single-pass type 1 transmembrane protein, composed extracellularly of 4 immunoglobulin (Ig) domains and 6 fibronectin type III domains, an overall structure shared with many members of the Ig cell adhesion molecule superfamily. The intracellular domain of DCC lacks any obvious catalytic domain, but netrin-1 binding and DCC multimerization activates several signaling pathways, including focal adhesion kinase and Src family kinase signaling, leading to activation of the Rho GTPases, Cdc42 and Rac1, and the reorganization of filamentous (F)-actin.\textsuperscript{1} Together these findings support a model where netrin-1, decorating the ECM, binds to and aggregates DCC, providing the capacity to transduce force across the plasma membrane and locally activate signal transduction to direct cytoskeletal reorganization.

A migrating axonal growth cone makes specialized cell-extracellular matrix adhesions called point-contacts. These are similar to focal adhesions in migrating cells and are thought to function as a force transducing component of a molecular “clutch” between the ECM and cytoskeleton that promotes the extension of a cell’s leading edge by locally restraining the retrograde flow of F-actin.\textsuperscript{9, 10, 11} Studies of glial tumor cell migration revealed DCC colocalized with markers of focal adhesions and determined that netrin-1 promotes focal adhesion maturation;\textsuperscript{12} however, how netrin-1 influences the localization and distribution of DCC within neuronal growth cones is not well understood.

To study the molecular mechanisms that direct cell motility, we previously developed low-cost lift-off nanocontact printing to create precise patterns of substrate bound protein, such as digital nanodot gradients of guidance cues and demonstrated their capacity to direct C2C12 myoblast migration.\textsuperscript{13, 14} Here we apply nanocontact printing to determine how nanodots of netrin-1 locally direct the distribution and re-organization of DCC and F-actin within axonal growth cones.

Fluorescence fluctuation techniques have been used to measure binding kinetics, densities, clustering states and transport properties of proteins in membranes and within cells via analysis of signal fluctuations, which arise due to changes in local concentration of fluorescent molecules within a microscopic focal volume. One of the earliest of the fluctuation methods was fluorescence correlation spectroscopy (FCS), which was first applied to study chemical kinetics
for DNA intercalating dyes. This method was followed by two color extensions; fluorescence cross-correlation spectroscopy (FCCS) and scanning FCS (SFCS). Image correlation spectroscopy (ICS) was then developed as an imaging analog of FCS in the spatial domain to measure densities and aggregation states of membrane receptors from single images acquired with a confocal or two-photon laser scanning microscope, or using a total internal reflection fluorescence (TIRF) microscope. The multicolor extension, image cross-correlation spectroscopy (ICCS) was developed to measure colocalization between two independent, spectrally separated sets of fluorophores imaged in two detection channels.

Here, we combine the precise spatial localization of nanodot protein printing with quantitative ICCS readout to examine the mechanism underlying the regulated distribution of the netrin receptor DCC and F-actin in axon growth cones of embryonic rat cortical neurons in response to spatially restricted extracellular distributions of netrin-1. We used low-cost lift-off nanocontact printing to pattern cell culture substrates with protein arrays composed of 200 nm diameter nanodots, and then immunostained neurons grown on the arrays to assess DCC and F-actin distributions relative to the printed netrin-1 (Fig. 1). TIRF microscopy images were analyzed by ICCS to extract the number, density, and aggregation state of DCC and F-actin, and the fraction colocalizing with the patterned surface.

Within each image, regions corresponding to the nanodots were segmented, and ICCS was applied separately to the segmented on and off dot areas, providing specific characterization of the DCC and F-actin sub-populations associated with (on) the nanodots and with the reference surface surrounding (off) the nanodot array. Nanodot printing combined with spatially segmented ICCS allowed us to effectively spatially dissect distinct responses to netrin-1 made by the two sub-populations of DCC and F-actin in and proximal to the neuronal plasma membrane.

Our findings reveal that nanodots of substrate-bound netrin-1 locally recruit and aggregate DCC, while IgG nanodots, patterned as a negative control, do not. Furthermore, we provide evidence that nanodots of netrin-1 locally recruit DCC via a mechanism dependent on the vesicle SNARE protein VAMP2, consistent with netrin-1 induced recruitment of an intracellular pool of cargo vesicles containing DCC.

**Theory**

The fluorescence fluctuation image analysis methods image correlation spectroscopy (ICS) and image cross-correlation spectroscopy (ICCS) have been previously described. We provide the basic theory of these techniques needed to understand the results presented here.

**Fluorescence fluctuation and Image Correlation Spectroscopy (ICS) analysis**

Fluorescence intensity fluctuations sampled within an image time series arise from variations in the number of fluorescent molecules detected within focal spots in the images as a function of space and time. If the fluorescently tagged biomolecules are expressed at concentrations where we can assume ideality (i.e. no energy transfer or signal saturation), the molecules are dispersed as monomers and the detected intensity varies linearly with the concentration of the emitting fluorophores, as shown in Fig. 2A-B. The square relative fluctuation (intensity variance/mean
intensity) is the mean number of detected fluorescent molecules per focal spot, since the molecules will obey Poisson statistics within the small volume.

\[
\frac{\langle (\delta i)^2 \rangle}{\langle i \rangle^2} = \frac{1}{\langle n_f \rangle} \quad \text{Eq. 1}
\]

In Eq. 1, \(\delta i = i - \langle i \rangle\) is the intensity fluctuation, where \(\langle i \rangle\) is the mean intensity, and \(\langle n_f \rangle\) is the mean number of independent fluorescent units \textit{(i.e. monomers)} detected within the focal spot.

If the fluorescent molecules are aggregated or clustered, they will still not be spatially resolved by diffraction limited optical microscopy, unless very large clusters are present. Instead, the aggregation will manifest itself globally as larger relative intensity fluctuations arising from what appear to be fewer but brighter fluorescent units per focal spot. If the total number of emitting monomeric subunits does not change, the overall mean intensity will be constant. In other words, the number of independent fluorescent “units” will decrease, while the mean intensity stays the same following aggregation (Fig. 2C).

In principle, this information could be directly measured from the intensity information encoded in the pixels as the variance to mean intensity ratio in Eq. 1, but only if there is no white noise present, and all signal fluctuations were due to molecular/particle number variations. In practice, white noise will always be present, \textit{i.e.} shot noise. Consequently, most fluorescence fluctuation methods employ correlation function analysis as a filter for white noise. The zero lags amplitude is extrapolated from an autocorrelation function fit since white noise will only correlate at zero lags in a correlation function. The goal of the correlation analysis employed here is to obtain the molecular number/aggregation information from the extrapolated amplitudes of correlation functions, with separate segregated analysis for cellular regions on and off nanopatterned substrates.

In these studies, we employed ICS to extract this information from fluorescence microscopy images, which records the fluorescence intensities in an image matrix with pixel intensity values \(i(x,y,t)\).

Most versions of ICS can be generalized \textit{via} a spatio-temporal correlation function of the fluorescence intensity fluctuations from the images, as displayed in Eq. 2. The fluctuations, \(\delta i\), are defined as the difference between the intensity at a given point and the mean intensity.

\[
r_{ab}(\xi, \eta, \tau) = \frac{\langle \delta i_a (x, y, t) \delta i_b (x + \xi, y + \eta, t + \tau) \rangle}{\langle i_a \rangle_t \langle i_b \rangle_{t+\tau}} \quad \text{Eq. 2}
\]

This correlation function has independent spatial lag variables, \(\xi\) and \(\eta\), along with the temporal lag variable, \(\tau\), and calculates the average correlation between pairs of intensity fluctuations separated by shifts in space (\(\xi\) and \(\eta\)) as well as in time (\(\tau\)). The function is normalized to the mean intensities in order to recover the relative fluctuation (variance divided by the mean) when extrapolated to zero lags.
In addition, Eq. 2 is generalized to represent correlation between two different fluorescence wavelength detection channels (a and b) collected on the microscope. If \( a = a \) or \( b = b \), we have an autocorrelation function for channel a or b respectively, while \( a \neq b \) defines a cross-correlation between detection channels, that can extract densities and fractions of interacting hetero-labeled molecular species (see below, & Fig. 2D-F). For the current studies, we only employed spatial correlation function analysis of regions of interest (ROIs), from images collected at a single time point since the neurons were chemically fixed. The spatial correlation function is then simply obtained by taking the limit of Eq. 2 as the time lag, \( \tau \), goes to zero as we are correlating within single image frames:

\[
l_r(\xi, \eta, 0) = \frac{\langle \delta i_a(x, y, t) \delta i_b(x + \xi, y + \eta, t) \rangle}{\langle i_a \rangle_t \langle i_b \rangle_t} \tag{Eq. 3}
\]

For pixelated images, the spatial correlation function is discrete and the spatial lag variables, \( \xi \) and \( \eta \), are integer pixel shifts along the x and y image directions. In practice the discrete spatial correlation functions are calculated using Fourier methods,\(^{21}\) as shown in Eq. 4;

\[
l_r(\xi, \eta, 0) = \frac{\mathcal{F}^{-1}[F(i_a(x, y, t))F^*(i_b(x, y, t))] \rangle}{\langle i_a \rangle_t \langle i_b \rangle_t} - 1 \tag{Eq. 4}
\]

where \( \mathcal{F} \) denotes the discrete 2D spatial fast Fourier transform of an image ROI, \( \mathcal{F}^* \) is the complex conjugate, and \( \mathcal{F}^{-1} \) is the inverse discrete 2D spatial fast Fourier transform.

Mean intensity padding should be applied to the ROI before calculating the correlation function to avoid discrete calculation artifacts. This is done by maintaining an outer boundary around the ROI, where each pixel in the boundary is equal to the ROI mean intensity. The boundary is usually extended to half the number of pixels for a given dimension of a rectangular ROI. As the mean intensity padding is done, it will not contribute to the numerator of the correlation function as it contributes zero magnitude fluctuations. However, mean intensity padding will decrease the amplitude of the normalized correlation function, by a proportional factor of the number of pixels added to the padded image, since we are artificially adding more pixels at the boundary. To recover the correct amplitude, the correlation function should be multiplied by the ratio of the total number of pixels/number of pixels in the original ROI.\(^{26}\) Lastly, we drop the \( t \) index in our notation, and recall that a given spatial correlation analysis will be indexed to a given image frame (without time dependence).

**Fluorescence fluctuation correlation function fitting and output parameters**

The calculated spatial intensity fluctuation correlation function will be well approximated by a Gaussian function in many cases for standard fluorescence microscopy imaging. Hence, we fit our calculated correlation functions with a 2D Gaussian using a nonlinear least squares algorithm that does not weigh the zero lags point in the fit, with three fitting parameters highlighted in bold below:
\[ r_{ab}(\xi,\eta) = g_{ab}(0,0) \exp \left[ -\frac{\xi^2 + \eta^2}{\omega_{0ab}^2} \right] + g_{x\cdot ab} \]  

Eq. 5

The output fit parameters are (for detection channel/labeled species \( x \), where \( x = a \) or \( b \)) \( g_{xx}(0,0) \), the zero lags amplitude; \( \omega_{0xx} \), the e\(^2\) radius of the Gaussian best fit; and \( g_{xx,xx} \), the long-spatial lag offset, which accounts for the incomplete decay of the correlation function. The \( \omega_{0xx} \) is also independently determined empirically by imaging static samples of sub-diffraction limit size fluorescent beads of similar emission wavelengths to the fluorophores. The value of \( \omega_{0xx} \) is an indicator of the quality of the correlation function fit. If the fitted value of \( \omega_{0xx} \) for an ROI correlation function differs by more than 30% from the experimentally measured correlation radius from a microsphere sample, then the fit is discarded.\(^{27}\)

Examples of spatial correlation functions from simulated and segmented images are shown in Fig. 3 as the discrete points, while the mesh is the best fit Gaussian function (Eq. 5). White noise was included in the simulation of Fig. 3A, so the zero spatial lags point was not weighted in the fitting of the correlation function.

The zero spatial lags amplitude of the autocorrelation function is the key molecular parameter from the autocorrelation function \((a=b)\) since this reduces to the square relative fluctuation (Eq. 1) and is, therefore, simply the inverse of the mean number of independent emitting fluorescent particles/units per focal spot area:

\[ \langle n_{xx} \rangle = \frac{1}{g_{xx}(0,0)} \]  

Eq. 6

A cluster density \((CD_{xx}, \text{number of fluorescent particles per } \mu m^2)\) is determined via a simple conversion by knowing the area of a focal spot from the best fit radius:

\[ CD_{xx} = \frac{\langle n_{xx} \rangle}{\pi \omega_{0xx}^2} \]  

Eq. 7

The oligomerization state of the system cannot be determined without an estimate of the total number of monomers present or additional calibration measurements. However, the average intensity of the ROI is proportional to the total number of fluorophores per focal spot area (Fig. 2A-C), after the background autofluorescence is corrected for. In this limit, the ratio of the average intensity to the cluster density will provide a measure of the degree of aggregation \((DA_{xx})\) for a system with a distribution of oligomers/clusters:

\[ DA = \frac{\langle i \rangle_{xx}}{CD_{xx}} = c \frac{\langle n_{m,xx} \rangle}{\langle n_{xx} \rangle} \]  

Eq. 8
The proportionality constant, c, is an optical/spectroscopic constant that relates the measured average intensity to the number of monomers, $<n_{m,xx}>$, present in the focal spot. The constant can be experimentally calculated by imaging a monomeric control under conditions identical to the samples.

**Image Cross-Correlation Spectroscopy and Interaction Fractions**

In spatial ICCS, the cross-correlation function ($a \neq b$ in Eqs. 2-5) statistically averages spatially coincident fluctuations across the two image detection channels (Fig. 2D-E) arising from hetero-labeled molecular species that are part of a common multi-molecular complex (Fig. 2F). By averaging cross fluctuations over an ROI of sufficient size, the non-random hetero species can be measured above background, within concentration dependent detection limits, from the amplitude fits of the cross-correlation function ($ab$: coincident species carrying both $a$ and $b$ fluorophores), and the two autocorrelation functions ($aa$: all $a$ fluorophore carrying species and $bb$: all $b$ fluorophore carrying species). The average number of independent colocalized (i.e. hetero) fluorescent particles in a focal spot, $<n_{ab}>$, is calculated using the amplitudes of the cross correlation function, $g_{ab}(0,0)$, the amplitudes of the autocorrelation functions, $g_{aa}(0,0)$ and $g_{bb}(0,0)$, and the ratio of the effective focal spot areas for the two detection channels ($A_b = \pi \omega_{bb}^2 > A_a = \pi \omega_{aa}^2$):\(^{19}\)

$$
\langle n_{ab} \rangle = \frac{g_{ab}(0,0)}{g_{aa}(0,0) g_{bb}(0,0)} \frac{A_b}{A_a}
$$

Eq. 9

It is important to distinguish the colocalization measured via correlation of sampled fluctuations from simple colocalization traditionally applied by direct overlap of images (visual intensity based colocalization). Two different emission wavelength fluorophores present in the same focal volume will show an intensity overlap between images and hence visual colocalization in the image even if they are not part of a common macromolecular complex (due to the inherent spatial resolution limits of standard optical microscopy). However, given sufficient sampling, cross-correlation analysis of fluctuations between detection channels can distinguish randomly distributed fluorescent probes from fluorophores that are part of a common macromolecular complex (and hence non-randomly associated in space). However, the cross-correlation methods cannot determine if the colocalization is a direct molecular interaction within the common complex (unlike Förster resonance energy transfer (FRET) methods which can distinguish direct molecular interactions).

The interaction fractions, historically labeled "M1" and "M2", are calculated via Eq. 9 and Eq. 10, and are the ratios of the number of colocalized particles to the total number of independent particles for each detection channel. These fractions provide a measure of the relative colocalization within the image ROI.

$$
M1 = \frac{g_{ab}(0,0)}{g_{aa}(0,0)} \frac{\langle n_{ab} \rangle}{\langle n_{bb} \rangle}
$$

Eq. 10
In this study of neurons on nanopatterned surfaces, channel $a$ will be the fluorophores associated with the immunostained neurons (green channel, Alexa Fluor 488) while channel $b$ is the nanopatterned surface (red channel, Alexa Fluor 546). The CD and DA of the neuronal population were evaluated with the M2 interaction fraction.

In each case, ROIs have had a background intensity correction applied, by identifying an area of the image with no cells and subtracting the mean value of the background area from the ROI, and setting negative pixel values to zero.

**Nanodot Segmentation ICCS**

Nanodots in images were segmented with the MATLAB function, *im2bw*, which converts a grayscale image to binary dependent on a threshold value (automatically determined by the function *graythresh*). Fig. 3D shows a typical segmentation of Fig. 3B, the nanodot simulated image.

ICCS analysis was performed on only the pixels segmented in the ROI by using mean intensity padding to mask the pixels that were not included within the segmentation. This step was performed to restrict the analysis only on the molecular receptor distribution of the neuron mapping onto the nanodots or, conversely, that mapping onto off dot reference surface areas. Simulated images after application of the segmentation and padding are shown in Fig. 3E and 3F (the simulated particles and simulated nanodots respectively), and correlation functions were only calculated on images containing 10+ segmented pixels. The autocorrelation function calculated from Fig. 3E is displayed in Fig. 3G. The full set of autocorrelation functions and cross-correlation functions from the computer simulated images in Fig. 3 are shown in Fig. S1-3 in the supporting information and show convergence of the measurements to the set particle density values. The correlation function amplitude was corrected to take into account the artificially added spatial lags from mean intensity padding as detailed above.

However, segmentation of the original image alters the $\omega_{0xx}$ for areas associated with the nanodots as the optical intensity profile is changed. Therefore, it will no longer match the actual $\omega_{0xx}$ of the imaging system (as was shown previously for ICS with image scrambling). However, the $g_{xx}(0,0)$ amplitude is unaffected, so accurate density output is preserved. The cluster densities were calculated with the actual measured $\omega_{0xx}$ of the imaging system for ICCS analysis on segmented images.

To analyze the molecular receptor distribution of the neuron off the nanodots, the segmentation image was inverted (Fig. 3H), and the areas associated with the nanodot were mean intensity padded instead (Fig. 3I, Fig. 3J). Only the autocorrelation functions of the neuron images were

\[
M_2 = \frac{g_{ab}(0,0)}{g_{bb}(0,0)} = \frac{\langle n_{ab} \rangle}{\langle n_{aa} \rangle}\]

Eq. 11
calculated (Fig. 3K), as there is almost no fluorescence intensity from the nanodots in the other detection channel. In this case, autocorrelation analysis of the nanodots and cross-correlation analysis were not performed.

Results and Discussion

Here, through the combined application of lift-off nanocontact printing, primary mammalian neuronal cell culture, immunohistochemistry, TIRF imaging and ICCS, we quantify interactions between DCC, F-actin and a nanocontact printed array of the DCC ligand, netrin-1. Non-segmented ICCS analysis outputs the cluster density (CD - number of particles per μm²), the degree of aggregation (DA) of the molecules of interest, and the interaction fraction (M2), quantifying the fraction of molecules of interest that colocalize with the nanopatterned substrate. Since conditions change in different experimental sessions (i.e. extent of staining, neuronal cultures etc.), we limited results to comparisons within single studies. Fig. 4 displays typical TIRF microscopy images resulting from immunohistochemical staining of neurons cultured on nanopatterned substrates. Quantitative results from non-segmented ICCS analyses are shown in Fig. 5.

Implementing segmentation ICCS analysis (examples using simulated images shown in Fig. 3), we quantified the distribution of plasma membrane DCC and F-actin directly overlying the patterned protein in the nanodot array, in addition to quantifying the adjacent regions of membrane overlying the reference surface surrounding the printed nanodots. This image segmentation relative to the printed pattern revealed details previously masked when carrying out whole image (non-segmented) ICCS analysis. Consistent with a cellular response to the patterned protein, segmentation of the nanodots revealed two distinct populations of F-actin and DCC. As the growth cones cover a limited area, and there are a finite number of nanodots for each selected cell ROI, we average over multiple cells for cell population distribution statistics.

Increased F-actin at netrin-1 nanodots

To examine the cellular responses made to cell culture substrates patterned with netrin-1 compared to IgG as a negative control, embryonic rat cortical neurons were cultured on patterned substrates of either protein, followed by fixation and phalloidin staining to visualize F-actin (Fig. 4). Phalloidin specifically binds F-actin, a polymer of monomeric G-actin, which is a critical component of the cytoskeleton required for directional motility.

Non-segmented ICCS analysis detected no significant differences in the cluster density, degree of aggregation and interaction fraction for F-actin associated with either IgG control or netrin-1 patterned surfaces, shown in Fig. 5. In contrast, segmented ICCS analysis revealed significant differences in the distribution of F-actin overlying and off the nanodot array (Fig. 6).

For both patterned IgG or netrin-1, significantly more units of F-actin (increased cluster density) were detected overlying nanodots than off (Fig. 6A); however, no significant difference in the number of F-actin units was found between IgG and netrin-1. In contrast to the lack of change in the number of units, phalloidin staining overlying netrin-1 nanodots exhibited a higher intensity
than on IgG nanoarrays (Fig. 6B), consistent with an increase in the local concentration of F-actin on the netrin-1 nanodots. For IgG nanodots, significantly less aggregated F-actin was detected on the nanodot than off the nanodot (Fig. 6C), along with more units of F-actin detectable on the IgG nanodots (Fig. 6A), whereas no significant difference in aggregation was found in response to the netrin-1 nanodot array. The fraction of colocalized F-actin on the nanodots was unchanged between IgG and netrin-1 nanoarrays (Fig. 6D).

The difference in the cluster density (Fig. 6A) and degree of aggregation (Fig. 6B) of phalloidin on the IgG nanodots suggests that the reference surface has a negative effect on actin polymerization. Netrin-1 promotes the polymerization of F-actin in multiple cell types. Here, the higher intensity of phalloidin staining associated with netrin-1 nanodots compared to the IgG control suggests that more F-actin is either locally recruited or polymerized in response to the netrin-1 nanodots.

We did not observe colocalization between netrin-1 and F-actin (Fig. 6D), as netrin-1 nanodots were immobilized on the glass substrate via nanocontact printing, while phalloidin stains intracellular F-actin. DCC is not known to directly couple to F-actin, and F-actin radiates outward as polymerized filaments in 3D, while the receptor is confined to the membrane. However, netrin-1 activation of DCC may result in changes in the distribution of F-actin some distance away from the immobilized netrin-1, such as the increase in phalloidin intensity (Fig. 6B) on netrin-1 nanodots.

**Netrin-1 aggregates and colocalizes DCC**

Multiple studies have provided evidence for netrin-1 recruiting and aggregating DCC in the plasma membrane of different cell types, including cultured mammalian cortical neurons. Here, first using non-segmented ICCS analysis, we assess the organization of DCC in the growth cones of embryonic cortical neurons in response to netrin-1 nanodots compared to control IgG nanodots (Fig. 5). DCC cluster density decreased by ~66% on netrin-1 nanodots, compared to the IgG control pattern (Fig. 5A), while the degree of DCC aggregation increased by more than 500% (Fig. 5C). Both findings are consistent with substantial reorganization of DCC to a higher aggregation state in response to netrin-1 nanodots. Furthermore, netrin-1 nanodots evoked a local increase in DCC intensity of more than 120% compared to control (Fig. 5B), and the fraction of DCC colocalized with the printed substrate increased by 45% on netrin-1 nanodots (Fig. 5D). These findings, derived from non-segmented ICCS analysis, reveal increased DCC recruitment, aggregation, and colocalization to netrin-1 nanodots.

In the segmented ICCS analysis we detected a highly significant change between netrin-1 and IgG (Fig. 7), concurrent with the non-segmented ICCS analysis (Fig. 5), with all on/off nanodot netrin-1 measurements being significantly different than the IgG control measurements. Cluster density decreased (Fig. 7A), while there was an increase in intensity and degree of aggregation (Fig. 7B, Fig. 7C), and increased M2 on netrin-1 nanodots (Fig. 7D), as seen with the non-segmented ICCS analysis (Fig. 5).
The IgG control reveals slight differences between the DCC population on the nanodot and that off the nanodot (Fig. 7A, Fig. 7C), similar to the F-actin segmented ICCS analysis (Fig. 6A, Fig. 6C), likely due to the IgG substrate masking a negative influence of the reference surface.

**TeTx blocks the aggregation and colocalization of DCC with netrin-1**

To investigate the mechanism underlying the netrin-1 induced increases in local DCC aggregation, colocalization, and reduced cluster density, embryonic rat cortical neurons cultured on nanodot arrays were treated with TeTx 1 day prior to fixation. TeTx is an inhibitor of regulated vesicle recruitment to the plasma membrane that cleaves the soluble vesicle-associated N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) VAMP2.\(^39\) In embryonic rat spinal commissural neurons and neocortical neurons, activation of protein kinase A (PKA) triggers a VAMP2 dependent mechanism that increases the sensitivity of the chemoattractant response to netrin-1 by recruiting DCC to the growth cone plasma membrane.\(^34\) Application of exogenous netrin-1 also rapidly increases exocytosis in neuronal growth cones through mechanisms that require SNARE function and the activation of Erk1/2 and Src family kinases.\(^41\) Interactions between DCC, the t-SNARE syntaxin-1, and v-SNARE tetanus neurotoxin-insensitive (TI)-VAMP are implicated in this increased vesicle trafficking and membrane insertion, in response to netrin-1.\(^34, 44, 45\)

Treatment of cortical neurons with TeTx, which blocks VAMP2 dependent vesicle insertion but does not alter baseline levels of axon extension,\(^46, 47\) blocked the netrin-1 nanodot induced changes in DCC cluster density, intensity and degree of aggregation, remaining unchanged compared to control IgG nanodots in the non-segmented ICCS analysis (Fig. 5). However, in the presence of TeTx, the fraction of DCC colocalizing with the printed substrate decreased by ~36% on the netrin-1 nanodots compared to control. These findings indicate that TeTx blocked both the recruitment and aggregation of DCC in response to netrin-1. In fact, TeTx disrupted the response to such an extent that DCC exhibited higher colocalization with IgG nanodots than with netrin-1 nanodots. Quantification of growth cone surface area on netrin-1 nanopatterns, revealed that TeTx reduced the size of the growth cone by ~44% (N=24/18), consistent with the recruitment of DCC containing vesicles contributing to both cytoskeletal reorganization and membrane insertion that underlie netrin-1 induced growth cone expansion.\(^31, 45, 48\)

Segmented ICCS analysis of neurons treated with TeTx revealed differences between the distributions of DCC in plasma membrane overlying netrin-1 nanodots and DCC in plasma membrane adjacent to the nanodots overlying the reference surface (Fig. 8). DCC overlying the netrin-1 nanodots exhibited a significantly higher cluster density (Fig. 8A), but a lower degree of aggregation than DCC overlying the reference surface (Fig. 8C). Fig. 9 shows typical ROIs from the DCC population on netrin-1 nanodots, exposed to TeTx, with the subsequent segmentation, and autocorrelation functions of the DCC populations on and off the nanodot array. This suggests that blocking vesicle insertion into the plasma membrane via the application of TeTx completely inhibits the recruitment of DCC into oligomers in response to netrin-1 nanodots.

The DCC cluster density was significantly lower for netrin-1 compared to the IgG control for both populations (Fig. 8A), on and off the nanodots, a finding previously masked using the non-
segmented ICCS analysis. Aggregation of DCC off the nanodot array is not seen in the IgG control, where there is a significant difference between the highly aggregated DCC off the netrin-1 array and the DCC in a lower aggregation state off the IgG array (Fig. 8C). Netrin-1 is a bifunctional guidance cue, with cells expressing both DCC and an UNC5 homologue, like cortical neurons, potentially generating chemoattractant or chemorepellent responses, although the mechanisms of response switching is not well understood.1 Our findings here suggest that blocking DCC recruitment to the plasma membrane may contribute to switching the neurons from an attractant to a repellent response, perhaps driving the residual plasma membrane DCC off the nanodot. Although an intriguing possibility, additional studies are required to investigate the underlying mechanism and the functional consequences of this redistribution of DCC in the plasma membrane.

Conclusion

Our findings, using embryonic rat cortical neurons, provide evidence that TeTx mediated cleavage of the v-SNARE VAMP2 completely disrupts DCC multimerization and colocalization in the response of cultured cortical neuron growth cones to immobilized netrin-1, and consequently inhibits growth cone expansion. These findings support the conclusion that the recruitment of intracellular cargo vesicles containing DCC is essential for locally concentrating DCC within the plasma membrane in response to netrin-1.

Previous studies have been dedicated to characterizing the behavior of a population of cells,49, 50 whereas here we have characterized molecular responses in single cells at defined locations, via correlation analysis and nano-patterning. The combination of segmentation ICCS with nano-patterning has enabled measurements of localized nanoscale interactions between proteins patterned as a printed substrate and molecules of interest, including cytoskeletal components and membrane receptors. Previous implementations of ICS methods used bulk cell treatments of ligands or agonists in solution so the exact locations of ligands binding to membrane receptors across a population of cells could not be known.24, 35 With the nanodot presentation of active ligand and segmented ICCS analysis, we can localize where the expected ligand/receptor interaction occurs and this is a refinement on the image correlation technique. The application of TIRF microscopy to illuminate a thin optical section with nanodot patterning has allowed characterization of molecular populations at a nanoscale level, determining the relative distributions of receptors with immobilized extracellular proteins, and cytoskeletal elements proximal to the plasma membrane. Our approach could also be potentially applied to live cell imaging and combined with temporal ICCS, to study receptor recruitment and dynamics to protein nanopatterns in live cells, although care must be taken to monitor time dependent changes in the mean intensities for segmentation padding over the analysis time windows selected. In practice, such time windows would likely need to be shorter than membrane protein turn over times for the receptor of interest.

Using segmentation ICCS in combination with nanocontact printing, we have investigated the distribution of F-actin and DCC, in the axonal growth cones of embryonic rat cortical neurons, cultured on substrates patterned with nanodot arrays of netrin-1 and IgG. We demonstrate that nanodots of netrin-1 locally recruit and aggregate DCC. This local response to netrin-1 was
blocked by TeTx, supporting the conclusion that netrin-1 recruits DCC from a pool of intracellular cargo vesicles via a mechanism dependent on the v-SNARE VAMP2.
Materials and Methods

Electron-Beam Lithography

A 4” Si wafer was coated with a poly(methyl methacrylate) (PMMA) resist and a nanodot array of 200 nm diameter dots with 1 µm pitch was patterned by e-beam lithography (VB6 UHR EWF, Vistec, Montreal, QC, Canada), followed by reactive-ion etching (System100 ICP380, Plasmalab, Everett, WA, USA) to a depth of 100 nm into the Si wafer.

Stamp Fabrication

After cleaning, the Si wafer was coated with perfluorooctyltriethoxysilane (Sigma-Aldrich, Oakville, ON, Canada) by vapor phase deposition. An accurate polymer copy of the wafer was obtained after double replication using polydimethylsiloxane (PDMS) and a UV-sensitive polyurethane as previously described.13 First, a 6 mm layer of 1:10 PDMS (Dow Corning, Corning, NY, USA) was poured on the wafer inside a Petri dish, followed by degassing under vacuum in a desiccator for 10 min. Next, the PDMS was cured in an oven for 24 h at 60 °C (VWR, Montreal, QC, Canada) then peeled from the wafer. To remove uncured monomers and other extractables, the PDMS replica was submersed in 70% ethanol for 24 h then baked at 60°C for 4 h. Next, a drop of UV sensitive polyurethane (Norland Optical Adhesive 63 (NOA 63), Norland Products, Cranbury, NJ) was applied to the PDMS replica and cured by 600 W of UV light (Uvitron International, Inc., West Springfield, MA) for 30 s. The PDMS was then removed yielding an NOA replica of the original Si wafer pattern with 200 nm diameter holes.

Nanocontact Printing

A flat PDMS stamp was cured against a perfluorooctyltriethoxysilane treated flat Si wafer. Following removal of the extractables as described above, the flat PDMS stamp was inked with a 10 µL drop of either fluorescently labelled IgG (25 µg/mL, chicken, Invitrogen, Burlington, ON, Canada), or recombinant purified netrin-1 (12.5 µg/mL, produced and purified as described51). A fluorescently tagged secondary IgG was mixed into the protein ink as a fiducial marker to facilitate the localization of the nanodots. A plasma activated hydrophilic coverslip was then placed on the drop to spread the solution evenly across the surface of the hydrophobic PDMS stamp during a 5 min incubation period. After rinsing with PBS and double distilled water for 15 s each, the inked stamps were briefly dried under a stream of N2 and immediately brought into contact with a plasma activated (PlasmaEtch PE-50, PlasmaEtch, Carson City, NV, USA) Norland Optical Adhesive (NOA) master for 5 s. The PDMS and NOA were separated and proteins in the contact regions were transferred to the NOA. The remaining proteins on the PDMS were transferred to the final substrate by printing the PDMS stamp for 5 s onto a plasma activated glass coverslip. We have not attempted to orient the netrin-1 binding; however, positively charged amino acids enriched at the carboxyl terminus of netrin-1 likely interact with the glass surface. DCC binds amino acid clusters in the amino terminal half of netrin-1, distant from the positively charged carboxyl terminus. This may result in a relatively large proportion of the printed netrin-1 being oriented such that it retains the capacity to interact with DCC.
Backfilling

A reference surface (RS) consisting of 75% by volume poly-L-lysine grafted polyethylene glycol (10 µg/mL, PLL (20 KDa)-g[3.5]-PEG(2 KDa), abbreviated as PEG, (Surface Solutions, Dübendorf, Switzerland)) and 25% poly-D-lysine (10 µg/mL, PDL, 70-150 kDa, (Sigma-Aldrich, Oakville, ON, Canada)) was incubated on the coverslip on a rocking plate for 15 min before washing off unbound PEG and PDL with 1xPBS.\textsuperscript{52}

Cell culture

All studies were conducted in accordance with the guidelines of the Canadian Council for Animal Care and approved by the McGill University Animal Care Committee. Pregnant Sprague-Dawley rats were obtained from Charles River Canada (Sherbrooke, QC, Canada). Cultures of embryonic rat neocortical neurons were prepared from Charles River Canada (Sherbrooke, QC, Canada). Cortices were dissected in ice cold Hank’s Balanced Salt Solution, diced with a scalpel, and then digested with 0.25% Trypsin and 0.05% DNAseI in Minimal Essential Media for suspension cultures with 0.02 M HEPES at pH 7.4. Dissociated cells were washed with Neurobasal media containing 10% fetal bovine serum and a single cell suspension obtained by trituration using flame glass pipettes. Neurons were seeded at 50,000 cells per coverslip and grown at 37°C in 5% CO\textsubscript{2} for 2 DIV in Neurobasal media, 1% B27, 0.5% N2, 1% Penn/Strep, 0.2% Fungizone, and 0.25% L-glutamine. All reagents were obtained from Invitrogen, Burlington, ON, Canada. 1.6 nM tetanus toxin (TeTx) was added to the plated neurons after 1 DIV and left in the growth medium for the remainder of the experiment.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde with gluteraldehyde for 1 min, permeabilized with 0.1% triton-X 100 for 5 min, and blocked with 1% horse serum overnight at 4°C. Cells were labelled with phalloidin conjugated to Alexa Fluor 488 (1:250, Invitrogen, Burlington, ON, Canada) or immunostained with a goat anti-DCC (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA) and detected with a secondary fluorescent chicken anti-goat antibody conjugated to Alexa Fluor 488 (polyclonal, 1:500, Invitrogen, Burlington, ON, Canada). Protein patterns were mixed with a secondary chicken anti-goat antibody conjugated to Alexa Fluor 546 (polyclonal, 1:20, Invitrogen, Burlington, ON, Canada).

Total internal reflection fluorescence microscopy

Fluorescence imaging of membranes for fixed and stained samples was conducted on a Nikon Ti-E inverted microscope (Nikon, Melville, NY, USA) equipped with a 100X/N.A 1.49 oil immersion objective lens. Alexa Fluor 488 was excited with a 488 nm diode laser, while the Alexa 546 was excited with a 561 nm diode laser. Fluorescence filter cubes of FITC/Cy2/GFP and TRITC/Cy3/RFP were used for the respective emissions.

Image analysis

The ICS/ ICCS image analysis programs were written using MATLAB 2013a (The MathWorks, Natick, MA) and run on a PC computer with a 3.4 GHz processor and 16 GB of random-access...
memory. Segmented ICCS was performed on TIRF images to extract number densities, intensities and aggregation measurements of neuronal populations, in addition to the colocalization between the neuronal population and the nanodot array (theory detailed above). Segmentation for ICCS was guided by the imaged nanodot locations which established imaged cellular regions on and off the nanodot for separate analysis (see above for details). Each region of interest was treated as a single “window” for ICCS analysis.

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Supporting Information
Additional figures relating to segmentation ICCS.
References


Figure 1: Experimental methodology schematic. (A) Protein nanopatterns are patterned by lift-off nanocontact printing. A flat PDMS stamp is coated with protein, and applied to a lift off stamp with nanoholes, with nanodots of protein remaining on the PDMS stamp. The PDMS stamp is then pressed onto a glass surface, to apply the protein nanodot array to the glass surface. (B) The glass surface is prepared by backfilling with a reference surface of 75% PEG: 25% PDL to yield a surface affinity similar to netrin-1. Embryonic rat cortical neurons were then seeded and grown for 48 h prior to fixation, immunostaining, and imaging using TIRF microscopy with two detection channels: immunostained neuron (green, channel 1) and nanodot patterns (red, channel 2). The green channel samples the stained neuronal macromolecules of interest, while the red channel images the nanoarray which guides the segmentation. (C) Regions of interest (ROI) were selected for image segmentation and ICS/ICCS analysis. Outputs are molecular densities, \( \langle n_x \rangle \), from the inverse of intensity normalized spatial correlation function amplitudes (see Theory and Fig. 2). A schematic zoom of the nanodots shows the diffraction limited focal spot (yellow) relative to the size of the nanodot. Red dots in the schematic represent fluorophores within the nanodot for imaging and localization.
Figure 2: Schematics of molecular distributions in a diffraction limited focal spot sampled for ICCS. (A) Ideal monomeric fluorescent molecules, where emitting molecules (green) are in a diffraction limited excitation focus (blue). (B) An increase in the number of monomers results in an increase in intensity detected from the diffraction limited spot. Average intensity will remain the same if there is no change in the number of subunits (assuming no energy transfer). (C) The monomers from (B) aggregate into dimers, and the square relative intensity fluctuation will increase following aggregation, therefore the variance (numerator of the correlation function) increases, while the mean intensity will stay constant (denominator). The total number of units is the same as (A). ICS analysis will extract the average number of independent fluorescent units per area. In combination with image intensity, the average aggregation state can be measured. (D-F) In two color imaging, the number of fluorescent molecules of each color can be detected, where (D) shows the green population in a blue diffraction limited spot and (E) shows the red population of a nanodot in a yellow diffraction limited spot (corresponding to Fig. 1C). Cross-correlating the green image with the red image can detect the number of colocalized particles (F), through ICCS. ICS/ICCS analysis averages information collected from multiple focal spots in the selected image region of interest (ROI), to obtain statistical means for fluctuation derived density and aggregation parameters.
Figure 3: Schematic of simulations showing ICCS analysis. (A) Computer simulated image with 44 fluorescent particles per beam area with white noise added. (B) Simulated image of a nanodot array. (C) Autocorrelation function, calculated from (A) using Eq. 4, shown as discrete points; colored mesh indicates the best fit, via Eq. 5, to the correlation function. The central zero spatial-lags point was not included in the fitting. \( g(0,0) = 0.023, \omega_0 = 0.32, R^2 = 0.998 \). (D) Segmentation of nanodots from (A). (E) Segmentation using (D) of simulated image from (A), applied with mean intensity padding outside of segmented areas. Note that the off dot locations appear as zero fluctuation contrast areas as they are mean intensity. (F) Segmentation using (D) of simulated nanodot image from (B), applied with mean intensity padding to non-segmented areas. (G) Autocorrelation function and fit calculated from (E). \( g(0,0) = 0.022, \omega_0 = 0.25, R^2 = 0.998 \). (H) Inverse image of nanodot segmentation from (D). (I) Segmentation using (H) of simulated image from (A), applied with mean intensity padding of non-segmented (i.e. on dot) areas. Note that dot locations appear as zero fluctuation contrast disks of mean intensity. (J) Segmentation using (H) of simulated image from (B), applied with mean intensity padding of non-segmented areas. (K) Autocorrelation function and fit calculated from (I). \( g(0,0) = 0.023, \omega_0 = 0.31, R^2 = 0.998 \). The number of particles from the simulated images (E) and (I), previously set in (A), was recovered from the \( g(0,0) \) value in (G) and (K).
Figure 4: Patterned protein nanoarrays facilitate visualizing interactions between neuronal growth cone proteins with substrate-bound ligand. Netrin-1 nanopatterns marked with Alexa Fluor 546 (red) were printed as 200 nm dots using lift-off nanocontact printing, the reference surface backfilled, and embryonic rat cortical neurons cultured on the patterns for 48 h, then fixed and stained with Alexa Fluor 488 (green) for either F-actin or DCC. (A) Composite images showing phalloidin stained neurons for visualization of F-actin, (B) DCC stained neurons, and (C) DCC stained neurons when exposed to TeTx. The white box indicated on the composite images (A-C) is magnified in the green channel (D-F), the red channel (G-I), and the composite (J-L) to visualize the influence of netrin-1 on the distribution of F-actin, and between netrin-1 and DCC in the growth cone. Scale bar is 10 µm for full size images and 2 µm for the magnified images.

206x160mm (300 x 300 DPI)
Figure 5: Results from non-segmented ICCS analysis on neurons labeled for F-actin (using phalloidin) and DCC +/- TeTx, grown on control IgG and netrin-1 nanodot arrays. (A) Cluster density (number of fluorescent units per $\mu m^2$) for the neuronal population in each study. (B) Intensity associated with the neuronal population for each study. (C) Degree of aggregation of the neuronal population for each study. (D) Fraction of colocalized units of the neuronal population with the nanodot print for each study. N = 23/26 ROIs for a sample of 10 neurons stained for actin, N = 58/42 ROIs for a sample of 11/15 neurons stained for DCC, N = 57/66 ROIs for a sample of 11/15 neurons stained for DCC treated with TeTx. Error bars are standard error of the mean. ** p<0.01, *** p<0.001 compared to IgG control evaluated by one-way ANOVA.
Figure 6: Results from segmented ICCS analysis on neurons labeled with phalloidin for F-actin staining, grown on control IgG and netrin-1 nanodot arrays. (A) Cluster density (number of fluorescent units per µm²) for the neuronal population on and off the nanodot array. (B) Intensity associated with the neuronal population on and off the nanodot array. (C) Degree of aggregation of the neuronal population on and off the nanodot array. (D) Fraction of colocalized units of the neuronal population with the nanodot print on the nanodot array for each study. N = 32/30 ROIs for a sample of 10 neurons. Error bars are standard error of the mean. * p<0.05, ** p<0.01, *** p<0.001 evaluated by one-way ANOVA.
Figure 7: Results of segmented ICCS analyses on neurons immunolabeled for DCC following culture on control IgG and netrin-1 nanodot arrays. (A) Cluster density (number of fluorescent units per $\mu m^2$) for the neuronal population on and off the nanodot array. (B) Intensity associated with the neuronal population on and off the nanodot array. (C) Degree of aggregation of the neuronal population on and off the nanodot array. (D) Fraction of colocalized units of the neuronal population with the nanodot print on the nanodot array for each study. N =48/42 ROIs for a sample of 11/15 neurons. Error bars are standard error of the mean. * p<0.05, ** p<0.01, *** p<0.001 evaluated by one-way ANOVA.

105x74mm (300 x 300 DPI)
Figure 8: Segmented ICCS analysis of neurons treated with TeTx, immunolabeled for DCC, cultured on control IgG and netrin-1 nanodot arrays. (A) Cluster density (number of fluorescent units per μm²) for the neuronal population on and off the nanodot array. (B) Intensity associated with the neuronal population on and off the nanodot array. (C) Degree of aggregation of the neuronal population on and off the nanodot array. (D) Fraction of colocalized units of the neuronal population with the nanodot print on the nanodot array for each study. N = 92/96 ROIs for a sample of 11/15 neurons. Error bars are standard error of the mean. * p<0.05, *** p<0.001 evaluated by one-way ANOVA.

100x66mm (300 x 300 DPI)
Figure 9: Typical segmented ICCS analysis of neurons treated with TeTx, immunolabeled for DCC, cultured on netrin-1 nanodot arrays. (A) Typical ROI of an imaged neuron in the green channel, showing the stained DCC population. Intensity has been adjusted for visualization of the stained population. Scale bar is 1 μm. (B) Corresponding ROI from the red channel, displaying the netrin-1 nanodots. (C) Composite image of both channels. (D) Segmentation of (B) to show the pixels selected to be analyzed for nanodot measurement. 91 pixels were selected to be associated with the nanodot array, out of the ROI comprised of 32X44 pixels. (E) Autocorrelation function calculated from the DCC population associated with the nanodot array, calculated using Eq. 4, shown with discrete points. The colored mesh indicates the autocorrelation fitting (Eq. 5). The zero spatial-lags point was not included in the fitting. g(0,0) = 0.047, \langle n \rangle = 21 DCC particles per focal spot area, R^2 = 0.80. (F) Autocorrelation function calculated from the DCC population off the nanodot array. g(0,0) = 0.14, \langle n \rangle = 7 DCC particles per focal spot area, R^2 = 0.91.

285x128mm (300 x 300 DPI)