Single-molecule imaging of Wnt3A protein diffusion on living cell membranes

Anna Lippert, §\* Agnieszka A. Janeczek,†§ Alexandre Fürstenberg, ¶♦ Aleks Ponjavic, \* W.E. Moerner, ¶ Roel Nusse$, Jill A. Helms¥, \* Nicholas D. Evans, †‡ and Steven F. Lee, ¶\*‡

\* Department of Chemistry, University of Cambridge, Cambridge, CB2 1EW, UK † Centre for Human Development, Stem Cells and Regeneration, Insititute for Life Sciences, University of Southampton, Southampton, SO16 6YD, UK; ¶ Department of Chemistry, Stanford University, Stanford CA, USA; ♦ Institute for Physical and Theoretical Chemistry, Goethe University Frankfurt am Main, Max-von-Laue Str. 7, 60438 Frankfurt am Main; $ Department of Developmental Biology, Howard Hughes Medical Institute, Stanford University, Stanford CA, USA ¥ Division of Plastic and Reconstructive Surgery, Stanford University Stanford CA, USA.

§These authors contributed equally, ‡Corresponding authors

ABSTRACT Wnt proteins are secreted, hydrophobic, lipidated proteins found in all animals that play essential roles in development and disease. Lipid modification is thought to facilitate the interaction of the protein with its receptor, Frizzled, but may also regulate the transport of Wnt protein and its localisation at the cell membrane. Here, by employing single-molecule fluorescence techniques, we show that Wnt proteins associate with and diffuse on the plasma membranes of living cells in the absence of any receptor binding. We find that labelled Wnt3A transiently and dynamically associates with the membranes of Drosophila S2 cells, diffuses with Brownian kinetics on flattened membranes and on cellular protrusions, and does not transfer between cells in close contact. In S2R+ cells, which express Frizzled receptors, membrane diffusion rate is reduced and membrane residency time is increased. These results provide direct evidence of Wnt3A interaction with living cell membranes and represent a new system for investigating the dynamics of Wnt transport.

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Wnt proteins are a family of secreted growth factors that are conserved throughout the animal kingdom (1, 2). This long evolutionary history is reflected in their involvement in a wide range of mammalian developmental and disease processes. Wnt signalling is necessary for normal development (3), whereas dysregulated Wnt signalling can cause cancer (4).

While the intracellular trafficking of Wnt proteins and downstream effects of receptor engagement are well studied, much less is known about their extracellular transport. All Wnt proteins (apart from Drosophila WntD) have been found to be lipidated (5, 6); a modification that is necessary for the intracellular transport, secretion and activity of Wnt proteins (7). Lipid modifications are unusual in secreted proteins - lipidation might be expected to prevent their movement in the aqueous extracellular space. But in contrast to this, Wnt proteins have been shown to be able to exert effects distant from the site of their production (8). This indicates that there are likely to be controlled mechanisms for Wnt protein transport, evidence for which is emerging from several recent studies. For example, Wnt protein activity is preserved by heparin sulphate proteoglycans (9), which are components of serum and associated with many cell membranes. Alternatively Wnt proteins may be transported by association with lipoproteins (10), or other specialist transport proteins in the extracellular space (11–13). Finally, Wnt proteins may be stabilised by direct interactions with biomembranes. Synthetic liposomal carriers can preserve Wnt3A activity in aqueous media and in the absence of other added proteins (14, 15), indicating membrane binding. *In vivo*, Wnt proteins have been shown to be carried by exosomes in some circumstances (16), and that they may activate signaling through direct cell-cell contact, either via delivery on long cellular processes called cytonemes (17, 18), or by intimate membrane contact in stem cell niches (19). In this latter study, Wnt3 protein was shown to be directly transferred from the surface of secreting cells to receiving cells, a process that is dependent on Frizzled (Fz) expression on the latter cells. Although interaction with Evi/Wls is required for presentation of Wnt protein at the cell membrane of secreting cells (20), whether or not Wnt proteins can bind to cell membranes in the absence of Fz or Evi/Wls, or require Fz for delivery from carriers remains unproven, primarily due to the lack of suitable methods to investigate this at the single-receptor level.

To address these questions we employed protein labelling and single-molecule fluorescence microscopy to image at high temporal resolution the interaction of Wnt3A protein with living cell membranes. There are few reports of active Wnt proteins produced by fusion of fluorescent proteins (17, 21), possibly because they often do not retain activity on subsequent expression and post-translational processing. However, Wnt3A retained activity following coupling to carboxylic acid-modified microbeads (22), suggesting covalent dye attachment as a possible method for Wnt protein labelling. We reacted carrier-free Wnt3A protein with maleimide or N-hydroxysuccinimide (NHS) ester functionalized fluorophores. Wnt3A protein was successfully labelled by ATTO680-maleimide, as confirmed by polyacrylamide gel electrophoresis and western blotting (Figure 1a-c). Labelled protein was evident as a fluorescent band at a mass size of ~40 kDa. Another predominant fluorescent band was evident at a molecular mass of ~66 kDa, which is likely due to BSA, as suggested by the presence of a band of equal mass in control labelling experiments. Single-molecule bleaching analysis revealed >98% (210/214 molecules) of labelled proteins bleach within a single imaging step, suggesting most of protein was tagged only once with the fluorophore (Suppl. Figure 1). We then tested the activity of the labelled protein preparations by incubating either labelled proteins or the control protein (subject to the same labelling conditions) with a reporter cell line that produces luciferase under the control of a Wnt-responsive promoter. Protein labelling with ATTO680-maleimide reduced Wnt3A activity by 36.6 ± 9.4% (p<0.05, Figure 1d). Alexa Fluor 647-maleimide and ATTO680-NHS also successfully labelled Wnt3A protein but whereas the former had no significant effect on protein activity, the latter entirely abolished its activity (Suppl. Figure 2). This suggests that labelling of protein at free amine groups is detrimental to Wnt3A activity whereas cysteine labelling is less so. There is evidence to indicate that all cysteines in Wnt proteins are involved in disulphide bridges (23). Nevertheless, we were able to label Wnt3A in non-reducing conditions with significant retention of protein activity. This suggests that there must be free cysteine residues present in Wnt3A preparations. It is likely that the cysteines labelled are those that have a low impact on Wnt3A activity when mutated, for example those cysteines involved in the formation of hairpin 1. Future studies may address the location of Wnt3A labelling by, for instance, liquid chromatography tandem mass spectrometry (6).

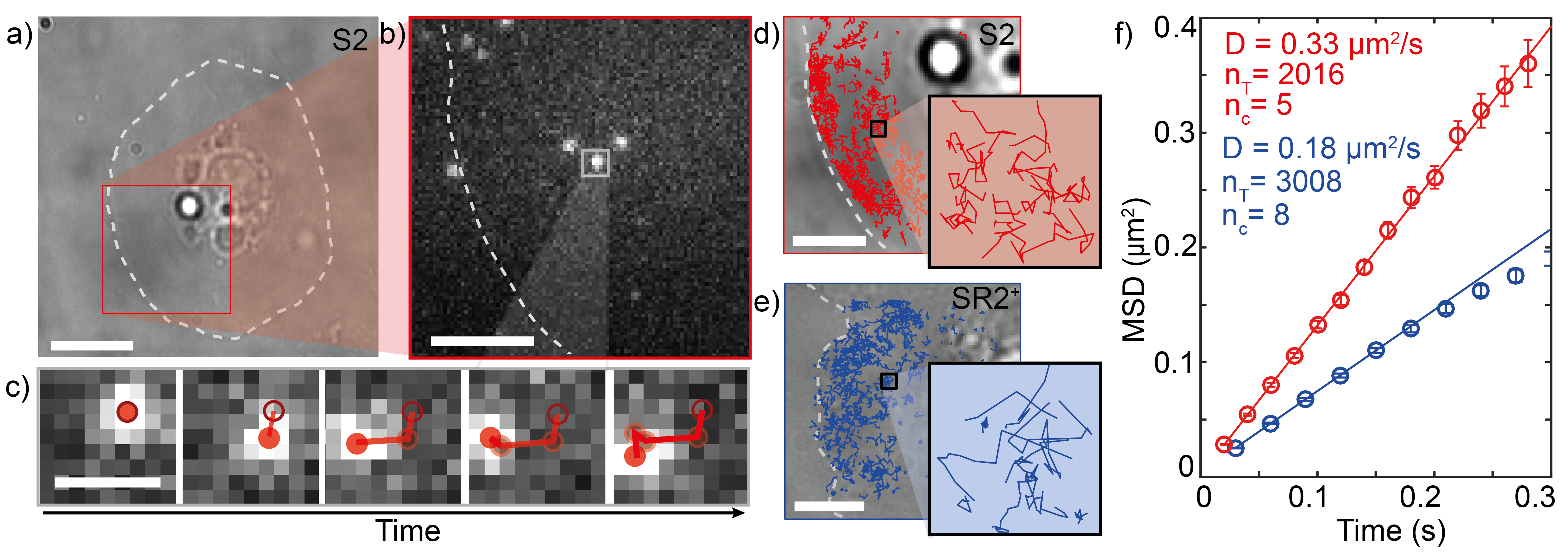
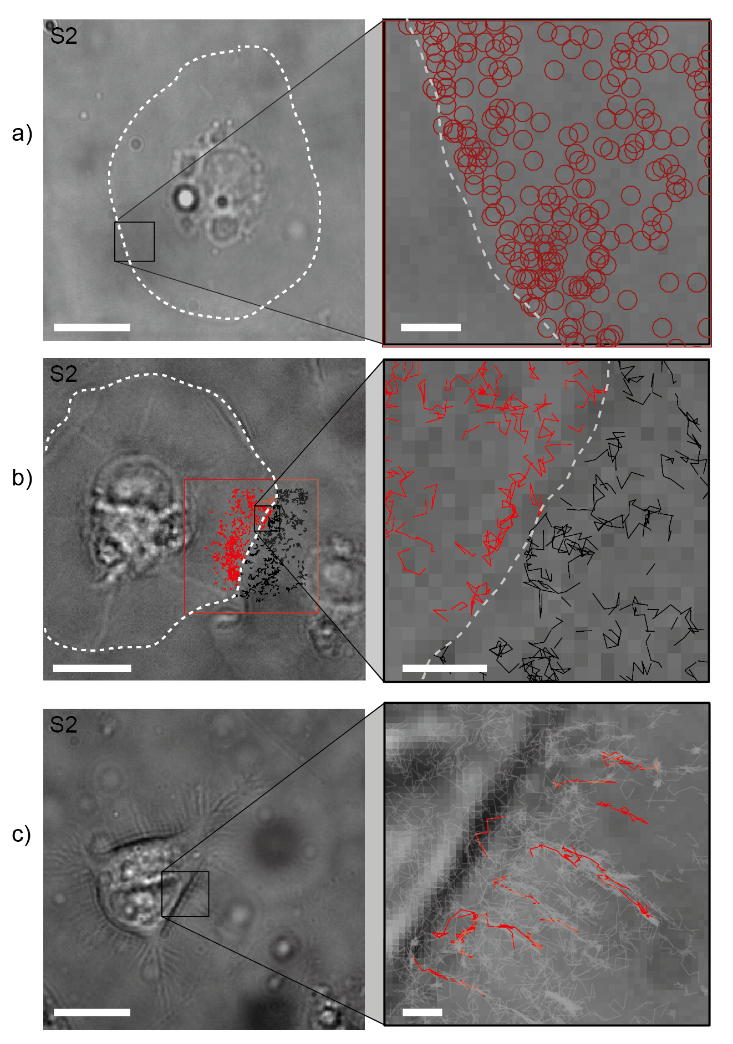


FIGURE 2 Wnt diffusion is slowed by the presence of the Wnt receptor in S2R+ cells. Fluorescently labelled Wnt protein was imaged on S2 (red) and S2R+ (blue) cells using HILO micrsocopy (a: White-light, b: Single-molecule fluorescence image). A tracking algorithm (41) was used to link fluorescent puncta (red circles, starting (dark red) and current position (filled)) in consecutive frames as described in Methods (c: Scale bar: 1µm) resulting in tracks with a localization precision of ~23 nm (Suppl. Figure 3) (overlay of tracks with white light images in d),e), Scale bar: 5µm). The ensemble diffusion coefficient was determined by fitting a linear function, which considers static and dynamic errors (28, Suppl. Figure 4), to the mean MSD values *vs.* time (f: shown is avg MSD values for S2 (red) and S2R+ (blue) cells with Standard error of the mean (SEM) as well as the linear corresponding fit).

FIGURE 1 (a) ATTO680-maleimide labelling of Wnt3A protein causes moderate decreases in activity. Wnt3A protein was tagged and run on SDS-PAGE gels and imaged under fluorescence illumination reader. Fluorescent bands were visible at masses of 66.5 kDa, corresponding to a BSA-only control, and at 40 kDa, the known mass of Wnt3A.(b) Presence of protein was confirmed using silver staining. (c)Western blotting confirmed that the labelled band at 40 kDa was Wnt. (d) ATTO-680 maleimide tagging caused decreases in activity in luciferase reporter assays (\*p<0.05).

Having established a method of labelling Wnt3A protein, we next tested whether it can associate with cell membranes in the absence of Wnt receptors. S2 cells are derived from *Drosophila* embryos, exhibit macrophage-like behavior, and do not express Fz or Wnt proteins (24). As S2 cells are poorly adherent, glass coverslips were pre-coated with concanavalin A, a lectin which causes S2 cells to adhere and flatten, with a rounded morphology in the vicinity of the nucleus and a large, round, flattened cytoplasmic ‘skirt’ ((25), Figure 2a). These latter areas provided an excellent two-dimensional platform for measuring the diffusion of molecules in real-time using highly inclined and laminated optical sheet (HILO, pseudo-TIRF) microscopy (26).

Following addition of labelled Wnt3A protein, at a physiological concentration of 1-2 nM (0.04 – 0.08 μg/mL), single diffusing molecules were evident on the plasma membrane (Figure 2a-c and Suppl. Movie 2). Using previously published single-molecule tracking software (27) the kinetics were analysed and found to be consistent with a Brownian diffuser model (Suppl. Figure 4) which accommodates for static and dynamic errors which are present in camera based motion tracking (28). The fitting function illustrates the unhindered random movement of the labelled protein on the cell membrane yielding a diffusion coefficent (D value ± fit error) of 0.33 ± 0.01 µm2 s-1 from a linear mean squared displacement (MSD) plot with an average track length (mean track length ± standard deviation (StD) of 0.27 ± 0.39 s (S2) (Suppl. Figure 5).

To test whether the presence of Wnt receptors affected diffusion kinetics of membrane-associated Wnt3A protein, we compared S2 diffusion (Figure 2d) with diffusion on SR2+ cells (Figure 2e), a similar cell line that expresses both DFz1 and DFz2 (29). Despite species differences, there is significant promiscuity in Wnt and Fz interaction, and mammalian Wnt3A has been shown to bind to Drosophila Fz proteins (30). In S2R+ cells, we observed a significant decrease in the diffusion coefficient (D value ± fit error) of 0.18 ± 0.01 µm2 s-1 (Figure 2f) and an increase in track length (mean track length ± StD) of 0.51 ± 0.79s (Suppl. Figure 5). In contrast, with the addition of either ATTO680 alone or ATTO680-labelled BSA we observed no isolated diffusers on the membrane of these cells (Suppl. Movie 3). Heat inactivation (10 minutes, 90 °C) of labelled Wnt3A protein completely abolished the appearance of diffusers on membranes, suggesting that active Wnt3A protein is responsible for the diffusion observed (Suppl. Movie 4). These results are consistent with a decrease in the diffusion speed of Wnt3A once bound to a receptor, and consistent with other membrane proteins (31). However we cannot rule out that this may be attributed to other differences in the cell lines, including cytoskeletal differences, membrane lipid composition or membrane protein density (32, 33). Taken together we conclude that Wnt3A associates with and diffuses unhindered on the surface of living cell membranes, the presence of a receptor both increases the residency time of this interaction and reduces the diffusion coefficient by ~2 fold in both cases.

FIGURE 3 Properties of Wnt S2 membrane associations. (a)Wnt3a binds from solution. The starting positions of Wnt tracks (dark red circles) are evenly distributed on the plasma membrane (white dash) of S2 cells demonstrating the probability of single molecule trajectories occurring is spatially independent. (b) Wnt3a does not transfer between cells. Wnt protein tracks were not found to be crossing cell boundaries of adjacent cells (white dashed line) on S2 cells. (c)Wnt3a diffuses along filopodia. Single Wnt3A molecules were also found along filopodia suggesting free diffusion along these structures. (Scale bar left: 10µm, right: 1µm)

In addition to the quantitative kinetic information, the single-molecule tracking analysis has allowed extraction of several behaviours of Wnt molecules that could not be determined using ensemble-averaged techniques including:

***Wnt3A dynamically binds onto and off the cell membrane.*** Analysing the starting position of tracks during the course of a single-molecule tracking experiment revealed a spatially independent probability for the tracks starting within the laser excitation zone (Figure 3a). Typically in single-molecule tracking experiments, new labelled molecules can only diffuse in from the periphery of the excitation zone, tested by periods of inactive laser excitation which allows new molecules to diffuse into the zone before photobleaching occurs (analagous to fluorescence recovery after photobleaching [FRAP] experiments); this was not observed. Instead single Wnt molecules consistently began their tracks at any point within the excitation zone (Suppl. Movie 2). These data suggest a mechanism where exogenous Wnt3A is in equilibrium between cell membrane binding and either free diffusion in solution or binding to soluble factors. To prevent Wnt precipitation and to preserve its activity, serum was added at 1% to all experiments. Serum is known to contain stabilizing proteins that bind Wnt, such as afamin (12), HSPG (9) or lipoproteins (10). In addition to this, a linear relationship of the single-molecule track starting frame *vs.* time (Suppl. Figure 6) was observed in the 2108 tracks examined, which suggests a continual replenishment of single diffusers (from solution) over the course of the experiment, analogous to the commonly used PAINT technique (34).

***Wnt3A does not transfer between adjacent S2 cells***. Another possible mechanism for extracellular Wnt transport is direct membrane diffusion from the surface of one cell to another (19, 35, 36). To test this we grew dense cultures of S2 cells to a level where the ‘skirts’ began to come into contact/close apposition (see Figure 3b). Labelled Wnt3a was allowed to diffuse on the surface and we observed diffusion at the border between cells. Despite extensive efforts, we never observed labelled Wnt3A diffusing across the membrane boundary from cell to cell (Suppl. Movie 5). Figure 3b demonstrates a representative experiment of single-molecule tracks at a cell-cell boundary overlaid onto the white-light image of the cell. No trajectory was observed in which the protein diffused from one cell to another over a time course of ~15 minutes. While S2 cells are a suitable model for generalized measurements of membrane diffusion of labelled proteins, further experiments would be necessary to confirm whether or not Wnt proteins may be transmitted between cells in other situations, such as on intracellular membranous bridges between adjacent cells (37) or via hemifused lipid bilayers at tight junctions (35, 38).

***Wnt3A diffuses along cellular processes.*** Accumulating evidence suggests that Wnt ligands can be mobilised and transferred on the tips of filopodia (17, 18). Although spread S2 cells do not readily form filopodia, in retracting cells, membrane protrusions are visible (Figure 3c). In such protusions we often saw diffusion of Wnt3A protein, indicating that membrane bound Wnt3A is capable of free transport on filopodia. Although it is likely that specific mechanisms control the presentation of secreted Wnt ligand at the tips of these protrusions, based on these data it is also possible that membrane-localised Wnt ligands freely diffuse along filopodia to activate signaling in cells that they contact. This may be regulated by membrane composition, as has been recently shown for Wnt3 (39).

Single-molecule fluorescence microscopy is a powerful tool to study Wnt protein dynamics, with which we have quantitatively demonstrated unhindered diffusion of active protein on the surface of living cells. We find that Wnt3A diffused two-fold more slowly and for twice as long in the presence of cells expressing its receptor. Furthermore we have determined that the transport mechanism is likely based on a binding-unbinding equilibrium of the protein with the plasma membrane and the surrounding solution. It is expected further that single-molecule studies will reveal a more complex understanding of both Wnt proteins and cell signalling in general.

**MATERIAL & METHODS**

***Wnt labelling.*** Wnt3A protein was either purchased from R&D systems (5036-WN/CF) or purified from the supernatant of S2 cells stably expressing the murine Wnt3A protein by blue sepharose and immobilized metal affinity chromatography, followed by gel filtration and heparin cation exchange according to the method of Willert (35). For labelling, ATTO680 maleimide (Sigma-Aldrich, 04971-1MG) or ATTO680-NHS (Sigma-Aldrich, 75999-1MG) was diluted to a final concentration of 1.5 µM and added to purified Wnt protein with a concentration of 20 µg/mL in PBS containing 1% (w/v) CHAPS, resulting in a stoichiometric molar ratio of 3:1. The reaction was allowed to proceed for 1 hour at room temperature. Following this, the mixture was passed 3 times through a micro bio-spin 6 column equilibrated with PBS containing 1% CHAPS to exclude unbound dye molecules using a centrifuge (Fisher Minispin, CFA-165-010L @ 13500 rpm). To test for successful labelling, between 50 and 500 ng of dye-labelled or unlabelled Wnt3A (R&D carrier-free protein) were resolved on a 10 % polyacrylamide ProtoGel gel (National Diagnostics) using Bio-Rad system. The fluorescent tag on the protein within the gel was imaged on the LI-COR Odyssey system. Sizing was achieved using a fluorescent sizing ladder (Precision Plus Protein All Blue Standards, Bio-Rad). The quantity of loaded protein was assessed by subsequent staining of the gel with a Proteo Silver Stain Kit (Sigma). Confirmation of the 40kDA band as Wnt was conducted via Western blot after transferring the protein onto a PVDF membrane (Merck Millipore) and detection using a Wnt3A antibody (Abcam, Ab172612), IgG HRP (Abcam, Ab6721) and Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) on a Versadoc reader (Bio-Rad).

***Activity Assays***. The luciferase assay was performed on a 3T3 mouse embryonic fibroblast cell line (Enzo Life Sciences), modified to expresses the firefly luciferase reporter gene under the control of Wnt-responsive promoters (TCF/LEF). Briefly, cells were seeded onto white, clear-bottomed, 96-well plates, at 1.5 x 104 cells/well in 50 µL assay medium (Enzo Life Sciences) and incubated with Wnt3A preparations at 50, 100 and 200 ng/mL for ~18h. Next, 100 µL/well of Steady-Glo luciferase reagent (Promega) was added for ~10 minutes and the chemiluminescence signal was read (0.1 second/well) on a Varioscan Flash microplate reader (Thermo Scientific). To control for cell count, cell lysates were analysed for dsDNA content using PicoGreen reagent (Thermo Fisher), according to manufacturer’s protocol.

***Cell Culture.*** Drosophila Schneider 2 (S2) cells were cultured in suspensions in capped flasks at room temperature in Schneider’s Drosophila Medium (Invitrogen) supplemented with 10% heat inactivated, insect cell tested, fetal bovine serum (Sigma) and penicillin/streptomycin. Cells were passaged at a dilution of 1:20 every week with the addition of fresh medium. Schneider 2R+ (S2R+) cells were grown in the same medium, but as adherent cells were passaged every 4-5 days at a dilution of 1:5. Cells were detached from tissue culture substrata prior to passaging by gentle mechanical agitation.

For microscopy experiments, chamber slides were coated with 0.5 mg/mL filter sterilised concanavalin A for 1 hour before S2 and S2R+ cells were plated at a density of 1000/cm2. Medium was replaced after 1 hour to remove non-adherent cells.

For live cell imaging, cells were thoroughly washed with PBS before incubation in a modified Krebs Ringer’s phosphate buffer made in-house (KRPB; 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO2, 1.25 mM CaCl2, 5 mM NaH2PO4, 2 mM NaHCO2, 10 mM glucose and 25 mM HEPES, pH 7.2) supplemented with 1% serum.

Chamber slides were positioned on the stage of the microscope and suitable cell areas were selected by white light microscopy. At given time points, labelled or unlabelled proteins (Wnt3A, BSA) or dye alone were added at final concentrations of 1-2 nM (0.04 – 0.08 μg/mL). In some cases proteins were denatured prior to addition by heating at 90 °C for 10 minutes.

***Tracking Experiments.*** White-light transmission and single-molecule fluorescence images were acquired with a modified fluorescent microscope based on an Olympus IX71 inverted microscope equipped with an infinity-corrected oil immersion objective (Olympus UPlanApo, × 100, 1.4 NA) operating in highly inclined and laminated optical sheet (HILO) imaging mode (26) to reduce the excitation volume and detected on a 512 × 512 pixel EMCCD (Andor I-Xon2,897) at a rate of 20-30 ms per frame for Wnt3A imaging. The general epifluorescence setup has been described previously ((40); here the filters used were a dichroic mirror (Semrock, Di01-R635-25x36) and a 635-nm long pass filter (Semrock, BLP01-635R-25). Laser excitation was provided by a 635nm solid state Laser (Blue Sky Research, FTEC2-635-V50PFM, 638nm FiberTECII laser) at a power density of ~250 W/cm2.

***Tracking Analysis.*** Tracking analysis of single Wnt proteins was accomplished using previously published single-molecule tracking software (23). This custom written Matlab code uses a IDL particle tracking function defined previously (41). The tracking analysis determines the diffiusion coefficient from both the mean squared displacement and via-jump distance analysis. For a detailed descripotion of the specific details, please refer to the supporting material.

To test the validity of our analysis, we used simulated data. Here we used the Matlab package ICSMatlab ([www.cellmigration.org/resource/imaging/icsmatlab/ICSTutorial.html](http://www.cellmigration.org/resource/imaging/icsmatlab/ICSTutorial.html)) to generate movies of two-dimensional Brownian diffusers with specified diffusion coefficients, diffraction-limited size, and the same signal-to-noise distribution levels as our experimental data. These simulated data (see supporting material) were analysed using the same track detection and fitting protocol as the real data.

**SUPPORTING MATERIAL**

Available online.

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