Supplementary Information for

Single molecule dynamics of Dishevelled at the plasma membrane and Wnt pathway activation

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Other supplementary materials for this manuscript include the following:

Movies S1 to S2
Supplementary Text S1. Converting size distribution of oligomer size.
Suppose the maturation rate of mEGFP to be $p$, the real occurrence of oligomer with length $j$ to
be $x_j$, the observed occurrence or oligomer with length $i$, $x_{io}$, can be written as function of $p$ and
$x_j$:

$$x^o_i = \sum_{j=i}^{n} \binom{j}{i} p^i (1 - p)^{j-i} x_j$$

Our measurement gives the size distribution as $(x^o_1, x^o_2, ..., x^o_n)$, we could get the real size
distribution $(x_1, x_2, ..., x_n)$, by solving these linear equations.
**Supplementary Text S2. Dvl membrane binding model.**

Let’s think about the simplest possible model for this system. In this model there are two populations of Dvl, the membrane bound Dvl and cytosolic Dvl. Dvl can form linear oligomer with DIX-DIX domain interaction. It can also be recruited to the membrane primarily through Dvl-Fzd interaction. Thus, we consider two basic interactions: Dvl-Dvl interaction and Dvl-Fzd interaction in the model. The dissociation constants between monomeric Dvl-Dvl and Dvl-Fzd are defined as KD and KF respectively, with the assumption of independence between different interactions. We use dn,m to denote one specific configuration of the Dvl complex with n Dvl subunits and m Fzd subunits. m=0 represents the cytosolic Dvl complexes and m>0 represents membrane bound Dvl complexes. We denote one specific configuration because Dvl complex with size n has n possible binding sites for Fzd. Fzd binding to different subunits of Dvl results in complexes with different sites on top of the Dvl complex. We then have

\[ \text{Dvl} + m \text{Fzd} \rightleftharpoons \text{Dvl-Fzd} \]

As a simple example, let’s think about the forming of D2,2, a Dvl dimer with two Fzd binding on it:

\[ d_{2,2} = \frac{d_{1,1}d_{1,1}}{K_{DM}} = \frac{d_{1,0}Fzd d_{1,0}Fzd}{K_{FM}Fzd Fzd} \]

For these two equations to be equal, we need to have:

\[ K_{F}K_{DM} = K_{FM}K_{D} \]

Which can be achieved by assuming \( K_{DM} = \beta K_{D} \) and \( K_{FM} = \beta K_{F} \), where \( \beta \) is the conversion factor between the membrane binding affinity and the volume binding affinity. Note that the membrane binding affinity has the unit mol/m2, while the volume binding affinity has the unit of mol/m3, as a result \( \beta \) has the unit of m.

Then we use Dn,m to indicate Dvl complexes containing n Dvl and m Fzd subunits, taking together all the different configurations. We then have:

\[ \frac{D_{n,m}Fzd}{D_{n,m+1}} = \frac{(m + 1)K_{FM}}{n - m} = \beta(m + 1)K_{F} \]

\[ \frac{D_{n-1,m}D_{j,0}}{D_{n-1,m+1}} = K_{D} \]

The equation \( d_{n-1,j,m-k} + d_{j,k} \rightleftharpoons d_{n,m} \) will yield very complex relationship for Dn-j,m-k, Dj,k and Dn,m, but we don’t need this to derive the size distribution because any complex with n Dvl and m Fzd can be imagined to first form cytosolic Dvl complex with size n, and then add m Fzd at different sites on top of the Dvl complex. We then have:

\[ D_{n,m} = \begin{cases} \beta K_{D} \left( \frac{D_{vl}}{K_{D}} \right)^{n} \left( \frac{Fzd_{2D}}{\beta K_{F}} \right)^{m} & (n \geq m > 0) \\ K_{F} \left( \frac{D_{vl}Fzd}{K_{D}} \right)^{n} & (m = 0) \end{cases} \]

Note that Dvl is the concentration in 3D volume, with the unit of mol/m3. While Fzd is a membrane protein and has the unit of mol/m2. Both KD and KF describes the binding of a subject with cytosolic proteins and has the unit of mol/m3, while \( \beta \) has the unit of m. Taken together, both \( \frac{D_{vl}}{K_{D}} \) and \( \frac{Fzd}{K_{F}} \) are unitless. Whether a molecule is membrane localized is solely determined by whether the complex contains Fzd.
Membrane bound Dvl monomer is:

\[ N_{Dvl} = \sum_{n=1}^{\infty} \left( \sum_{m=1}^{n} SnD_{n,m} + VnD_{n,0} \right) \]

We need to introduce the surface area \( S \) and cell volume \( V \) in the equation, then the number is not as meaningful as concentration. But we could divide the cell volume \( V \) on both side of the equation and have:

\[ Dvl_{Total} = \sum_{n=1}^{\infty} \left( \sum_{m=1}^{n} \frac{SnD_{n,m}}{V} + nD_{n,0} \right) \]

We can see that by multiplying the membrane density to surface area \( S \) and then divide by cell volume \( V \), we could write the cell membrane density in equivalent 3D concentration which will simplify our calculation in the following steps. We have the equivalent 3D concentration of Dvl complexes as:

\[ D_{n,m} = \begin{cases} \frac{S}{V} \beta K_D \left( \frac{n}{m} \right) \left( \frac{Dvl}{K_D} \right)^n \left( \frac{Fzd_{2D}}{\beta K_F} \right)^m & (n \geq m > 0) \\ \frac{Dvl_{N}}{K_D} & (m = 0) \end{cases} \]

By assuming \( \alpha = \frac{S}{V} \beta \), we have

\[ D_{n,m} = \begin{cases} \alpha K_D \left( \frac{n}{m} \right) \left( \frac{Dvl}{K_D} \right)^n \left( \frac{Fzd_{3D}}{\alpha K_F} \right)^m & (n \geq m > 0) \\ \frac{Dvl_{N}}{K_D} & (m = 0) \end{cases} \]

Note that Fzd concentration is converted into 3D concentration where \( Fzd_{3D} = \frac{S}{V} Fzd_{2D} \). By assuming \( \delta = \frac{Dvl_{N}}{K_D} \) and \( \epsilon = \frac{Fzd_{3D}}{K_F} \), we have:

\[ D_{n,m} = \alpha K_D \left( \frac{n}{m} \right) \delta^n \left( \frac{\epsilon}{\alpha} \right)^m \quad (n \geq m > 0) \]

In turn we have the membrane Dvl molecules with \( n \) subunit as:

\[ D_n = \sum_{m=1}^{n} D_{n,m} = \alpha K_D \delta^n \left( \left( 1 + \frac{\epsilon}{\alpha} \right)^n - 1 \right) \]

We could then write the 3D equivalent total concentration of Dvl and Fzd in the following equations:

\[ Dvl_0 = \sum_{n=1}^{\infty} \sum_{m=0}^{n} nD_{n,m} = \sum_{n=1}^{\infty} nK_D (\alpha \delta^n \left( \left( 1 + \frac{\epsilon}{\alpha} \right)^n - 1 \right) + \delta^n) \]

\[ = K_D \left( \frac{\alpha \delta \left( 1 + \frac{\epsilon}{\alpha} \right)}{1 - \delta \left( 1 + \frac{\epsilon}{\alpha} \right)} \right) + (1 - \alpha) \frac{\delta}{(1 - \delta)^2} \]

\[ Fzd_0 = \sum_{n=1}^{\infty} \sum_{m=1}^{n} mD_{n,m} + F = \sum_{n=1}^{\infty} K_D \delta^n \epsilon \left( 1 + \frac{\epsilon}{\alpha} \right)^{n-1} + F = \frac{K_D \delta \epsilon}{1 - \delta \left( 1 + \frac{\epsilon}{\alpha} \right)} + K_F \epsilon \]

with the total concentration of Dvl and Fzd, we could solve the concentration of monomeric Dvl and Fzd, and in turn get all the following quantities.

The total Dvl complexes (3D equivalent concentration) on the membrane is:

\[ D_{MC} = \sum_{n=1}^{\infty} \sum_{m=1}^{n} D_{n,m} = \sum_{n=1}^{\infty} K_D \alpha \delta^n \left( \left( 1 + \frac{\epsilon}{\alpha} \right)^n - 1 \right) = \frac{K_D \delta \epsilon}{1 - \delta \left( 1 + \frac{\epsilon}{\alpha} \right) \left( 1 - \delta \right)} \]

Membrane bound Dvl monomer is:
The concentration of total Dvl \textbf{molecules} on membrane is:

\[
D_{t,1} = K_p \delta \epsilon
\]

\[
D_{M,\text{tot}} = \sum_{n=1}^{\infty} \sum_{m=1}^{n} n D_{n,m} = K_D \left( \frac{a \delta \left(1 + \frac{\epsilon}{a}\right)}{1 - \delta \left(1 + \frac{\epsilon}{a}\right)} - a \frac{\delta}{(1 - \delta)^2} \right) = \frac{K_p \delta \epsilon (1 - \delta^2 \left(1 + \frac{\epsilon}{a}\right))}{(1 - \delta \left(1 + \frac{\epsilon}{a}\right))^2 (1 - \delta)^2}
\]

Average size of membrane bound Dvl is:

\[
N = \frac{D_{M,\text{tot}}}{D_{MC}} = \frac{(1 - \delta^2 \left(1 + \frac{\epsilon}{a}\right))}{(1 - \delta \left(1 + \frac{\epsilon}{a}\right) (1 - \delta)} = \frac{1 - (Dvl / K_D)^2 \left(1 + \frac{Fzd}{aK_F}\right)}{1 - Dvl / K_D \left(1 + \frac{Fzd}{aK_F}\right) \left(1 - Dvl / K_p\right)}
\]
**Supplementary Text S3. Effect of Fzd receptor oligomerization**

Fzd is known to form dimer or higher order oligomers when binding to Wnt proteins. This could potentially affect the oligomerization property of Dvl. The mathematical forms representing oligomers with dimerized Fzd subunits becomes too complicated to solve. Instead of giving the analytical formulas, we discuss whether Fzd will recruit more Dvl oligomers with specific examples. For Dvl monomer, there is only one binding site on Dvl. No matter the oligomerization status of Fzd molecule, as long as the total Fzd subunits are the same, the membrane recruitment of monomeric Dvl won’t change. Then we consider Dvl dimer which can be represented by the following figure.

A) represents the scenario of individual Fzd while B) represents the case with Fzd dimer. The simplest question to ask is if scenario B) recruit more Dvl molecules on the plasma membrane than scenario A). The answer is yes with a simple calculation, which is most conveniently done by considering the binding of one Dvl dimer with Fzd molecules in the framework of the grand canonical ensemble. For scenario 1, the membrane bound Dvl will have the proportion of:

\[
P_{1_{\text{mem}}} = \frac{2e^{\beta(\mu \ln[Fzd]-E_{\text{FD}})}}{1 + 2e^{\beta(\mu \ln[Fzd]-E_{\text{FD}})} + e^{2\beta(\mu \ln[Fzd]-E_{\text{FD}})}}
\]

For scenario 2, similarly we have:

\[
P_{2_{\text{mem}}} = \frac{2e^{\beta(\mu \ln[Fzd]-E_{\text{FD}}-E_{\text{W}})}}{1 + 2e^{\beta(\mu \ln[Fzd]-E_{\text{FD}}-E_{\text{W}})} + e^{2\beta(\mu \ln[Fzd]-2E_{\text{FD}}-E_{\text{W}})}}
\]

Here EFD is the binding energy between Fzd and Dvl; EW is the binding energy induced by Wnt between two Fzd molecules; \( \mu \) is the unit Gibbs energy of Fzd. The dimerized Fzd in scenario 2 is imaged to form based on monomeric Fzd. Because EW < 0 and \( e^{-\beta EW} > 1 \), we have \( P_{2_{\text{mem}}} > P_{1_{\text{mem}}} \). Dimer Fzd can recruit more Dvl to the membrane, this is, in another word, avidity effect.
Supplementary Text S4. Photobleach or simple dissociation show geometric dwell time distribution:

Due to photobleach or complex dissociation, membrane molecule will have limited dwell time, which could be estimated using the following model.

In example A), the dwell time is only affected by photobleach. The probability to find trace of length $n$ will be $P(n) \sim p(1 - p)^{n-1}$, which is exponential distribution. Example B) doesn’t have fundamental difference. The probability to find trace of length $n$ will be $P(n) \sim (p_1 + p_2)(1 - p_1 - p_2)^{n-1}$. The dissociation rate can be estimated from the log probability of dwell time distribution. The slope of the log(probability) histogram is log(1-p) where $p = \text{rate} \cdot \Delta t$. Thus, we could derive the photobleach rate and dissociation rate from the dwell time distribution.
Supplementary Text S5. Dvl2-Crispr sequence

The following are the two knock-in sequences we used in the experiment. Red letters indicate the palm regions of CRISPR sequences.

GAGCAAGCACATGACGGCC AGG
GTGCTTGCTTTACAGGCC TGG
Fig. S1. Dvl2 localize to the plasma membrane in Fzd7 overexpressing cells.

The top panels are Dvl2-KI cells without Fzd7 over-expression. Dvl2 molecules are in the cytosol. The bottom panels are cells with Fzd7 overexpressed. Dvl2 localizes to the plasma membrane together with Fzd in these cells.
Fig. S2. Examples of image analysis using uTrack.

The upper panels show the original image, the lower panels show single molecule identification results using uTrack software. Each red circle is an identified single molecule spot.
Fig. S3. The diffusion of Wnt ligands underneath the cell surface. Wnt3a are randomly labeled with Alexa chemical dyes. The labeled Wnt3a ligands has low activity so not used in the functional study. Labeled Wnt3a are added together with Surface SNAP-647 dye which can't permeate the cell membrane to study the ability of Wnt3A to diffuse underneath the cell. TIRF images were taken 2 mins before adding the labeled reagents and 20 mins after, with 20 seconds imaging interval. After imaging, the images first go through a median filter to remove all the sticky fluorophores, leaving only the signal from the background. Then the images are quantified with ImageJ. (A) Median filtered TIRF images of Wnt3A and free dye, at two time points. (B) quantification of fluorescence intensity for region A (underneath the cell) and region B (outside the cell). We find both Wnt3A and ligands has no problem diffuse underneath the cell. The major factor of the delay of signals is diffusion in the bulk media instead of underneath the cell, comparing the delay between region A and region B. The dye is smaller so diffuse faster than labeled Wnt3A. It's interesting to see that there is no big signal difference from the free dye, underneath or outside the cell, indicating ample space beneath the cell. On the other hand, the signal from labeled Wnt is about halved underneath the cell compared to outside, indicating a hindered diffusion process but still accessible cell bottom surface. We could also notice that the signal noise from the underneath the cell is bigger compared to that outside the
cell, reflecting the influence of both small volume and maybe the fluctuating of cell bottom membrane.
Fig. S4. Single GFP intensity quantification with membrane protein photobleach. pRK5-mEGFP-GlyRα1 plasmid is used. The step jumps are fitted with two Gaussian functions and the first peak position is 554.
Fig. S5. Quantification of mEGFP maturation. mEGFP-GluK2 plasmids are transiently expressed in HEK293T cells. Images are taken the next day with TIRF microscopy to quantify the maturation ratio of mEGFP. Data was recorded in the format of 20Hz movie. A) The last 10 frames are quantified and fitted to mixture Gaussian functions to derive the single mEGFP intensity. B) The intensity is then used to as the monomeric mEGFP intensity to fit the single molecule dots from the first 5 frames of the same movie. C) The first 4 peaks of the fitted distribution are best fitted with a binomial distribution, with \( p=0.75 \). This means that about 75% of mEGFP are bright and our data are corrected with this number accordingly. Different TIRF setup is used in this experiment so the exact value of mEGFP quantification is different from other experiments.
**Fig. S6.** Quantifying Dvl2-mEGFP concentration.

1.3 million HEK293T cells with Dvl2-mEGFP was lysed to get 250 μL of cell lysate. The lysate was then run together with different amount of mEGFP to estimate the concentration of mEGFP in the cell lysate. All the mEGFP lanes are loaded with 20μl sample. The concentration of the 4ng mEGFP sample is 7.07 nM. The concentration of Dvl2-mEGFP in the cell lysate is then estimated to be 1.42 nM. We then calculated the mean HEK293T cell volume to be 1.94 μL based on the mean HEK293T cell diameter which is measured to be 15.48 μm. With the cell volume and total cell number we estimated the undiluted total cell volume to be 2.54 μL. Thus, the actual Dvl2-mEGFP concentration is **140 nM** if we think Dvl is evenly distributed in the whole cell. In reality, Dvl mostly resides in cytosol which takes about 2/3 of the total volume of the whole cell. As a result, the cytosolic Dvl concentration is in the ballpark of 200 nM.
**Fig. S7.** Determining the bottom area of the cells

Individual images in a short movie are first passed through a 15x15 pixel median filter to remove the point signals, only keep the background. All the images are then merged together to enhance the weak background signal. The left subfigure shows the merged raw images. The middle subfigure shows the merged images that have been passed through the median filter. We then use the matlab function "graythresh()" to find the threshold automatically and then convert the middle image to a black-and-white image shown in the right subfigure. From the black-and-white image, the number of pixels that the cells occupy can be calculated. The pixel dimension is determined by pixel size on CCD camera together with the magnification. In our case the length per pixel on the CCD camera is 16 μm while the magnification is 150x. Thus, the width of one pixel represent about 107 nm in the real world.
Fig. S8. Quantification β-catenin and Dvl2 protein level change during Wnt3A activation in Dvl2-KI cell. Wnt3a condition media is added at time 0 and the cytosolic β-catenin and Dvl2 protein levels are quantified with western blot.
Fig. S9. Ratio of membrane Fzd after Wnt3A treatment. HEK293T cells were transfected with the plasmid of mEGFP-Fzd1 the day before imaging. 500ng/ml Wnt3A was added to the media at time 0. mEGFP-Fzd1 membrane total intensity is measured with TIRF microscopy and the result is the average of 3 cells.
Fig. S10. Model results of average membrane Dvl size varying two parameters $K_D$ and $K_F$. The colorbar shows the range of average size covered in the model, from 1 to 24.
Fig. S11. Model results of Dvl size distribution varying two parameters $K_D$ and $K_F$. Each subfigure is a membrane Dvl size distribution. The size is on the x-axis, from 1 to 10, while the y-axis is the concentration of Dvl complexes. $K_D$ is changed vertically and $K_F$ changed horizontally. The units of both $K_D$ and $K_F$ are μM. It’s easy to see that $K_D$ changes the shape of the distribution while $K_F$ changes the absolute height of the distribution.
**Fig. S12.** DKK1 blocks Wnt pathway response but not Dvl oligomerization. The left panel shows the single molecule quantification of Dvl2-mEGFP complex size with different conditions listed on the bottom of the panel. The right panel shows the corresponding β-catenin concentration for each condition, with duplicates. DKK1 inhibits the β-catenin increase but not the Dvl2 response on the plasma membrane.
Fig. S13. Dwell time distribution of membrane bound GlyRα1-mEGFP. The photobleach rate is $1.17 \pm 0.13$/s, which correspond to half-life of 0.59 s (11.8 frames). Each subfigure represents a different experiment, the first experiment in the second row was dropped before statistics due to poor data quality.
**Fig. S14.** Fzd can increase Dvl phosphorylation in the absence of Wnt ligands. We have overexpressed Fzd5 in HEK293T cells. 100nM LGK974 were added before experiment. Gradient of Fzd5 plasmids are transfected in the cell and Dvl2 phosphorylation measured by band shift of western blot.
**Fig. S15.** phosphorylation feature of different Dvl2 mutants.

Wildtype and domain deletion Dvl2 are transiently overexpressed in the Dvl-TKO cell line. Wnt media are added for 2 hours before the cells are lysed. the result shows that Dvl is phosphorylated in all but the ΔDEP mutant.

| Wnt3a CM | WT | ΔDIX | ΔPDZ | ΔDEP |
|----------|----|------|------|------|
|          | -  | +    | -    | +    |
| Dvl2     |    |      |      |      |
| beta-catenin |   |     |      |      |
| alpha-tubulin | | |    |      |
**Fig. S16.** Sucrose gradient of to estimate the size of Dvl2 complexes. HEK293T cells are activated by Wnt3A or Wnt5A for 4 hours, and then broken with needle and spin down first at 3000g for 10 mins and then at 100,000g for 1 hour to remove lipids. Different Dvl mutant oligomers are overexpressed in Dvl-TKO cells and prepared the same way. The supernatants are then loaded on top of a 5–25% sucrose gradient (50mM KCl, 20mM Tris 7.5, 5mM MgCl2, 1mM EDTA, 2.5mM DTT, 0.02% sodium azide (w/v)). The gradient was span in a msl-50 rotor at 38000rpm for 8 hours. 13 fractions are then taken, and western blot run for Dvl2. We can see a clear shift of Dvl oligomers mutants but not from Wnt3A or Wnt5A treatment. Indicating either small population of stable WT Dvl complexes or weak binding between WT Dvl.
Figure S17. TOPFlash assay on the effect of protein overexpression. Different Dvl mutants are overexpressed in either HEK293T cells or Dvl-TKO cells, and then TOPFlash assay are used to quantify the activation of Wnt pathway. The overexpression data are consistent for HEK293T cells and Dvl-TKO cells. But on the contrary to rescue assay (Fig. 4), DIX domain deletion shows no activation here, consistent with the previous publications.
Movie S1. Confocal microscope imaging of Dvl2-mEGFP in HEK293T cells

Movie with 20 min interval was taken for 13 hours, final concentration of 200 ng/ul Wnt3a was added 2 hours after the imaging has started. 11 hours of imaging was done after Wnt3a addition. The whole imaging process occurs at 37 C with constant CO2 flow.

Movie S2. TIRF imaging of Dvl2-mEGFP on the bottom surface of HEK293T cells

Images was taken in 30 sec intervals for 75 minutes. Final concentration of 200 ng/ul Wnt3a was added 15 min after the imaging stated. Additional one-hour movie with the same interval was taken focusing on the dynamical change of Dvl2-mEGFP.