Beta-catenin

A molecular multiplayer

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““I think you’ll find”, said Dr. Breed, “that everybody does about the same amount of thinking. Scientists simply think about things in one way, and other people think about things in others”.”

- Kurt Vonnegut – Cat’s Cradle

Visualizing WNT signalling in mammalian systems

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Abstract

WNT/CTNNB1 signalling plays a critical role in the development of all multicellular animals. Here, we include both the embryonic stages, during which tissue morphogenesis takes place, and the postnatal stages of development, during which tissue homeostasis occurs. Thus, embryonic development concerns lineage development and cell fate specification, while postnatal development involves tissue maintenance and regeneration.

Multiple tools are available to researchers who want to investigate, and ideally visualize, the dynamic and pleiotropic involvement of WNT/CTNNB1 signalling in these processes. Here, we discuss and evaluate the decisions that researchers need to make in identifying the experimental system and appropriate tools for the specific question they want to address, covering different types of WNT/CTNNB1 reporters in cells. At a molecular level, advanced quantitative imaging techniques can provide spatio-temporal information that cannot be provided by traditional biochemical assays. We therefore also highlight some recent studies to show their potential in deciphering the complex and dynamic mechanisms that drive WNT/CTNNB1 signalling.

Introduction

WNT signalling is essential during embryonic development in all multicellular animals and is of equal importance for adult tissue homeostasis (Steinhart & Angers, 2018). Like other developmental cell-cell communication pathways it functions via a cascade of protein interactions, can be activated by multiple ligands and receptors (the mammalian genome encodes 19 different WNT and 10 different FZD proteins) and has numerous target genes. Moreover, it requires precise timing and localized activation as signalling gradients are typically required for the proper patterning of tissues (Sagner & Briscoe, 2017). In the case of WNT/CTNNB1 signalling, this patterning starts with formation of the primitive streak at the posterior end of the mammalian embryo at the onset of gastrulation (Ramkumar & Anderson, 2011). Finally, it is becoming increasingly clear that signalling output is highly tissue-specific, although the mechanisms behind this remain incompletely understood (Söderholm & Cantù, 2021). Taken together, developmental signalling pathways are complex to study and WNT/CTNNB1 signalling is no exception.

Although classical genetic and biochemistry approaches have been instrumental in providing a blueprint and working model for the structure and function of the WNT/CTNNB1 pathway, with the use of modern techniques, such as multi-omics analyses, CRISPR/Cas9 genome editing and advanced quantitative imaging, big steps are being made toward a better—or at least a more detailed—understanding of how WNT/CTNNB1 signalling operates in a tissue-specific context, as well as how it integrates cross-talk with other pathways. In this review, we will give an overview of the recent use of advanced, often highly quantitative imaging techniques to understand WNT/CTNNB1 signalling on a molecular level, highlighting some promising techniques that could still be wider implemented. Of course, imaging can also be used to visualize WNT signalling in an *in vivo* tissue context, which we will touch briefly upon. Throughout, we assume that the reader is familiar with the core working mechanism of the WNT/CTNNB1 pathway and we refer to other recent reviews that discuss specific aspects of WNT signal transduction in more detail (Colozza & Koo, 2021; de Man et al., 2021; Gammons & Bienz, 2018; Routledge & Scholpp, 2019; Söderholm & Cantù, 2021).

Imaging individual players at the molecular level

As a whole, WNT/CTNNB1 signalling establishes large morphogenetic changes in the developing embryo and regulates tissue homeostasis during adult life. These broad functions are finetuned at the molecular level and for that reason it can be of interest to obtain a detailed understanding of how the individual components of the pathway behave in living cells and different contexts. Imaging can be especially useful as it can provide the spatial and temporal resolution that is often lacking in traditional biochemical assays while also allowing analysis on a single cell level, thereby fully capturing the dynamic heterogeneity that exists within a larger cell population. In this section, we will highlight some recent studies to illustrate how an ever-increasing collection of advanced microscopy probes and techniques can be used to answer key questions in the field, where traditional biochemical approaches may fall short.

The signalosome

In the presence of WNT ligands, the so-called WNT signalosome is formed at the membrane. It is well established that binding of WNT ligands to the Frizzled (FZD) receptors and LGR5/6 co-receptors leads to the recruitment of Disheveled (DVL) and subsequent transduction of the WNT signal. However, many details about the formation of this signalosome are still unknown, specifically regarding the timing and precise order of events. Put differently: which proteins go where and when? While signalosome formation can be seen as the start of WNT signal transduction, prior to this, WNT proteins first have to be produced, secreted, and transported to their respective target cells. How this occurs is an equally big question in the field.

WNT ligands

Due to posttranslational modification, WNT ligands have a hydrophobic character. They are therefore unable to freely diffuse in the extracellular space, limiting their operating distance when unaided. Several theories, not necessarily mutually exclusive, as to how the short- and long-range transport of these hydrophobic WNT ligands occurs exist. We will not exhaustively discuss them here, but suffice it to say that the mechanism may not be identical in different developmental settings. Indeed, advanced microscopy approaches have provided evidence for different mechanisms of ligand secretion.

Of note, multiple interesting discoveries about the post-translational modification, including palmitoylation, and intracellular trafficking of WNT ligands prior to export have been made using advanced imaging techniques, including recent cryo-EM studies (Azbazdar et al., 2019; Liu et al., 2022; Moti et al., 2019; Nygaard et al., 2021). Such detailed analyses might ultimately prove to be required to not only understand different modes of operation in different tissues, but also between different WNTs. For instance, recent work in human breast cancer cells suggests that some WNTs can be secreted in a PORCN-independent manner, which might hint at additional, as of yet undiscovered modes of WNT export, transport, and perhaps even cell-autonomous signalling (Rao et al., 2019).

The simplest manner of secretion would be via exosomes, in which the presence of several WNT ligands (i.e., WNT3A, WNT5A and WNT7A) has been shown by immunogold electron microscopy (EM, see table 1) (Torres et al., 2021). In contrast, using immunofluorescence, WNT5A was shown to co-localize with lipoprotein particles, but not exosomal markers, in cerebrospinal fluid during hindbrain development in mouse embryos. The lipoprotein particles thus provide another way of extracellular transport of WNT ligands and are carried by cerebrospinal fluid, functioning as a carrier over a longer distance (Kaiser et al., 2019).

Table 1. Advanced microscopy techniques

Descriptions of the advanced microscopy techniques mentioned in this review.

Immunogold transmission electron microscopy (EM)	This technique combines the high precision of transmission EM with immunogold staining to label specific proteins and determine their exact location at a high resolution. It does require fixation of the samples and can therefore not be applied in live settings (Jerez et al., 2021)
Fluorescence correlation spectroscopy (FCS)	FCS can be used to determine exact diffusion speeds and molecular concentrations of single proteins. By determining the diffusion speed of a single protein, different fractions, for example, a freely diffusing and bound fraction, can be distinguished. FCS measures the passing of fluorescently tagged proteins through a confocal volume, registering their brightness (to determine the quantity of molecules and complex size) and time spent in the volume (to measure diffusion speed) (Ries & Schwille, 2012)
Fluorescence recovery after photobleaching (FRAP)	In FRAP experiments, a chosen region of interest is photobleached and the recovery of fluorescence in that area is recorded over time. This gives an impression of the mobility of the fluorescently tagged protein, as highly mobile proteins will lead to a faster recovery in fluorescence (Meddens et al., 2014)
Total internal reflection fluorescence (TIRF)	TIRF microscopy allows imaging of proteins at the membrane surface with a high signal-to-noise ratio and lends itself excellently for studying protein dynamics at the membrane without background interference from high cytoplasmic levels (Fish, 2009)
Fluorescence decay after photoconversion/ photoactivation (FDAP)	FDAP is similar to FRAP, but measures the decrease in fluorescence over time in a chosen region of interest after using light to induce or change the colour of a fluorescently tagged protein. This again gives an impression of the mobility of the tagged protein, as highly mobile proteins will cause a faster fluorescent decay
Photoactivated localization microscopy (PALM)	PALM super resolution microscopy uses fluorescent proteins that switch on and off in a stochastic manner, limiting the number of fluorescent proteins imaged at a single point in time. Computational reconstruction of the image is then performed to achieve a higher resolution (Henriques et al., 2011)

Mobilization of WNT ligands via filopodia has also been shown to occur with the Retention Using Selective Hook (RUSH) system. Here, the protein of interest, in this case fluorescently tagged WNT3A, is fused to a streptavidin-binding peptide (SBP) while streptavidin is fused to a cellular anchor, in this case an ER protein. Upon biotin stimulation, streptavidin-binding fluorescent WNT proteins can be selectively released from the anchor in a synchronized manner, thus facilitating the detection of their trafficking trajectory. In this way, real time transfer of WNT3A containing vesicles via filopodia could be demonstrated, although the identity of these vesicles was not determined (Moti et al., 2019).

Once in the extracellular space, how do WNT proteins get sequestered at the cell surface? In Xenopus and zebrafish, the mobility of Wnt ligands at the membrane and in the extracellular space was researched using Fluorescence Correlation Spectroscopy (FCS, see table 1). In both cases, two different flavours of fluorescently tagged Wnt (in FCS terminology: “fast” and “slow” fractions) could be observed (Mii et al., 2021; Veerapathiran et al., 2020). However, the identity of these fractions was interpreted quite differently based on Heparan Sulfate Proteoglycan (HSPG) disruption experiments. In the zebrafish brain, the faster fraction of eGFP-tagged Wnt3 was found to be HSPG associated, suggesting HSPG-binding as a potential mechanism for Wnt3 transport along membranes next to a more stable, membrane-bound fraction of Wnt3 (Veerapathiran et al., 2020). In contrast, in Xenopus, the slower fraction of mVenus-tagged Wnt8 was found to be HSPG associated, suggesting a role for HSPG-binding in local sequestration of Wnt8 next to the presence of a faster diffusing species of Wnt8 (Mii et al., 2021). In both cases, the FCS results were confirmed with additional imaging techniques, namely Fluorescence recovery after photobleaching (FRAP, table 1) (Veerapathiran et al., 2020) and Fluorescence decay after photoconversion (FDAP, see table 1) (Mii et al., 2021). As also mentioned by the authors, care should be taken when comparing FCS and FRAP/FDAP mobility measurements: because FRAP/FDAP measures the mobility in a larger area and over a longer time span, especially in a 3D context, molecules might encounter many more obstacles along their movement trajectory, skewing their mobility measurement. At the same time, because of the longer timespan, FRAP/FDAP measurements are sometimes more suitable than FCS to measure slow moving proteins (Mii et al., 2021; Veerapathiran et al., 2020). Given the different developmental settings of these two studies, as well as the multiple assumptions underlying the mathematical analysis of FCS imaging data, it therefore remains unclear if we are in fact looking at the exact same flavours of WNT in both cases.

Summarizing, in which situation WNT ligand secretion, transport and distribution occur via which mechanism remains to be determined, and it is not unlikely that this too will turn out to be highly tissue and context, or even organism, dependent. A small epitope-tagged WNT3 knock-in mouse, for example, suggests that in the intestinal crypt WNT3 is transported in a cell-bound manner and thus forms gradients as a result of cell division rather than WNT3 diffusion (Farin et al., 2016). Not all of the imaging approaches discussed are easily compatible with a live and/or 3D set up and most are more easily performed in cell lines (Lippert et al., 2017). In that respect, the FCS studies using fluorescently tagged Wnts in zebrafish and Xenopus represent a real tour de force. Zebrafish embryos specifically offer a clear experimental advantage as they are transparent. A fluorescently tagged WNT knock-in mouse might theoretically offer similar possibilities in a mammalian system, for example, using 3D organoids or primary 2D monolayer cultures (Sanman et al., 2020). Fluorescent tagging of WNT proteins while preserving full functionality remains challenging, although successful attempts have now been reported (Wesslowski et al., 2020), enabling approaches such as the FCS studies above.

Frizzled/LRP and disheveled

The idea that upon WNT binding, the receptors (FZD) and co-receptors (LRP5/6) reorganize to form a larger receptor-ligand complex has been around for a long time (Carron et al., 2003; Cong et al., 2004). How extensive the polymerization is, and how this process occurs under physiological conditions, is still unknown. One prediction is, however, that lateral mobility (i.e., the propensity to drift around in the cell membrane) of the FZD receptor complex will change. To study such changes, dual colour Fluorescence Recovery After Photobleaching (dcFRAP, for FRAP see table 1) was used to measure FZD protein mobility before and after WNT stimulation. By using two colours of FZD6 and experimentally immobilizing one fraction, Petersen et al., (2017) were able to measure not only the motility, but also the interaction between FZD proteins. Here, any interaction with the fluorescently labelled immobilized FZD6 fraction decreased the motility of the fluorescently labelled mobile FZD6 fraction. Live imaging showed that the mobile fraction first increased and then decreased upon WNT5A stimulation, indicating temporary disassociation of pre-assembled FZD6 complexes (Petersen et al., 2017). These results suggest that a dynamic reorganization of membrane receptors occurs, but if and how this finding can be translated to other WNTs and other FZDs remains to be determined. In addition, whether FZD receptors preferentially homo- or also hetero-dimerize is also unclear. Finally, if and how these processes also occur in a 3D context, where most cells are polarized and different ligands may arrive from different directions and thus also meet different receptors at the basolateral vs the apical cell surface, remains to be determined as well.

Mechanistically, WNT signal transduction across the membrane involves the recruitment of DVL to the cytoplasmic surface of the plasma membrane, where it binds the C-terminal tail of FZD. The cytosolic DVL protein contains a DIX-domain that is able to bind to the DIX domains of other DVL proteins and thus self-oligomerize (Schwarz-Romond et al., 2007). This property may play a role in crosslinking multiple FZD proteins and thus in signalosome assembly. When visualizing DVL by overexpressing a fluorescently tagged version, the DIX domain also causes the formation of so-called cytoplasmic “puncta” (Schwarz-Romond et al., 2005, 2007; Smalley et al., 2005). Whether these puncta are physiologically relevant, or an overexpression artifact has long remained a topic of debate, as not all studies found them necessary to activate WNT signalling (Smalley et al., 2005).

Only recently, total internal reflection fluorescence (TIRF, see table 1) microscopy was used to image endogenous levels of fluorescently tagged DVL at the cell surface. A first study showed that DVL is mainly present as monomers at the membrane, with only slightly increased oligomerization being observed upon Wnt3a stimulation (Ma et al., 2020). Using model fitting to the intensity of a single GFP molecule, a second study inferred that, at endogenous levels, most DVL complexes at the membrane consisted of <10 molecules and did not change in size upon WNT stimulation (Kan et al., 2020). Taken together, this suggests that the observed cytoplasmic puncta may indeed only occur at high concentrations and shows the importance of critically assessing data gained from overexpression studies. Indeed, more recently photoactivated localization microscopy (PALM, see table 1) of endogenously tagged DVL showed that at endogenous levels only a limited number of puncta can be observed that seem to reside at the centrosome in a cell-cycle dependent manner (Schubert et al., 2022). Their precise function still needs further research.

The CTNNB1 destruction complex and enhanceosome

In the absence of WNT stimulation, the so-called destruction complex, formed by AXIN, APC, CSNK1, and GSK3, captures and phosphorylates CTNNB1, labelling it for breakdown by the proteasome. When WNT ligands are present, the destruction complex dissociates and CTNNB1 accumulates in the cytoplasm and nucleus. Here, it acts as a co-activator for TCF/LEF transcription factors. Together with additional co-factors, such as BCL9 and PYGO1/2, these proteins form the so-called TCF/LEF “enhanceosome” that ensures transcription of WNT target genes. The general working mechanism and function of each player in the destruction complex is quite clear, although their precise stoichiometry remains to be determined. Yet, how the destruction complex disassociates and with what dynamics, or if during this time, it shows biochemical interplay with the signalosome are outstanding questions in the field. Another big question mark concerns the nuclear import and export dynamics of CTNNB1, which does not contain any of the traditional target sequences for nuclear transport.

Destruction complex

The destruction complex consists of a combination of scaffolding proteins and CTNNB1 kinases. Without visualizing several components at once, it will remain difficult to understand the precise stoichiometry and quantities of each destruction complex component present. Here, super resolution techniques could provide new insights to complement ongoing efforts to use a bottom-up approach and assemble the destruction complex using purified proteins (Ranes et al., 2021).

We want to highlight AXIN, which contains a DAX domain that functions similarly to the DIX domain of DVL, allowing AXIN homodimerization (as well as heterodimerization with DVL). Similar to DVL, AXIN puncta were observed when overexpressing AXIN, also giving rise to the question of their physiological relevance (Faux et al., 2008; Schwarz-Romond et al., 2007). Although DVL puncta at endogenous levels seem rare and mainly related to the centrosome (Schubert et al., 2022), AXIN puncta appear to have an intrinsic function in the formation of biomolecular condensates of the destruction complex (Li et al., 2020; Nong et al., 2021; Schaefer & Peifer, 2019). However, it remains uncertain how big these biomolecular condensates are under physiological conditions. In the future, combined live imaging of endogenous AXIN and DVL may provide further insights into the interplay of DVL and AXIN and their possible heterodimerization at the signalosome.

CTNNB1

CTNNB1 levels can be used as a proxy for WNT signalling activity on a cellular (or even subcellular) level, based on the notion that CTNNB1 levels in the cytoplasm and nucleus increase upon WNT signalling. In a live-cell imaging setting, these changes can be tracked in real time. CRISPR/Cas9 technology in particular has opened the possibility to monitor endogenous CTNNB1 dynamics. A number of studies have now revealed a dose-dependent increase of fluorescently tagged CTNNB1 at endogenous (or near endogenous) levels to Wnt3a and CHIR99021 stimulation (de Man et al., 2021; Kafri et al., 2016; Massey et al., 2019). Notably, the actual fold change of CTNNB1 levels in the nucleus is only two- to threefold in response to Wnt3a or CHIR stimulation, but this is sufficient to induce robust upregulation of AXIN2 transcription (de Man et al., 2021). Interestingly, the shape and duration of the CTNNB1 response to WNT3A and CHIR stimulation may be context dependent and also seems to differ across cell lines. For instance, human embryonic stem (ES) cells pushed toward a primitive streak fate responded to CHIR with sustained nuclear accumulation of CTNNB1, while undifferentiated human ES cells failed to respond (Massey et al., 2019).

As CTNNB1 lacks any nuclear import or export sequences, how it translocates to the nucleus has remained a big mystery over the years (Anthony et al., 2020). Using FCS in cells with endogenously tagged fluorescent CTNNB1, we recently determined the concentration and mobility of CTNNB1 in the cytoplasm and the nucleus, in the absence and presence of WNT3A. Based on this, we found CTNNB1 to be present in two different states: a slow, probably complexed, fraction and a fast fraction of what we assume to be monomeric CTNNB1 (de Man et al., 2021). These analyses provided concrete numbers that could then be used for mathematical modelling. Perhaps, in the future, additional data describing the dynamics of other interactors in the WNT/CTNNB1 pathway can be added, creating a more refined model. In the long run, such models might then also be applied to predict the WNT/CTNNB1 signalling output in development in response to subtle WNT signals and gradients, or in the context of genetic mutations. One remaining challenge is to integrate the second function of CTNNB1, which also plays a critical role in cell adhesion. Here, it anchors adherens junctions to the intracellular cytoskeleton by binding both the cytoplasmic tail of CDH and CTNNA1. Whether there is functional interplay between these different pools of CTNNB1 (as reviewed in van der Wal & van Amerongen (2020)) and whether there is a role for CTNNB1 at the membrane during WNT signalling remains to be determined. Regardless of the functional implications, imaging of CTNNB1 in epithelial and endothelial cells will, first and foremost, reveal a clear membrane signal. This signal is much brighter than that in the cytoplasm and nucleus, making it difficult to not over- or under saturate either one when trying to obtain a well saturated picture of the overall CTNNB1—a prerequisite for proper quantitative analyses.

The fact that only small (absolute and fold) changes in CTNNB1 are needed to induce a robust transcriptional response has important implications for when image-based studies of CTNNB1 levels are used to determine overall WNT signalling activity. Not only should great care be taken to measure these subtle shifts, it is also important to realize that different methods of analysis can affect interpretation of the results (figure 1). In all cases, extreme care should be taken when comparing different experimental conditions and within a single experiment, the same image settings should be used. Purely quantifying the intensity levels in the nucleus, for instance, can skew the results when signal intensity is affected by differences in imaging depth, for example. It is better practice to normalize the CTNNB1 intensity levels, for example, by taking the nuclear/cytoplasmic ratio or by normalizing against a stable nuclear dye. This, in turn, requires segmentation of the nucleus and cytoplasm. While this can be done manually, there is an increasing number of options to do this (semi) automated using freely available software, such as CellProfiler (to build custom pipelines) (Stirling et al., 2021) or CellPose (a flexible, deep learning-based method) (Pachitariu & Stringer, 2022). Even then, segmenting the entire cytoplasm may be challenging in some cells and ideally a cytoplasmatic or membrane marker is provided for segmentation. Since nuclear segmentation is usually an option, one alternative is to take a ring region around the segmented nucleus as a measure for the overall cytoplasmic intensity.

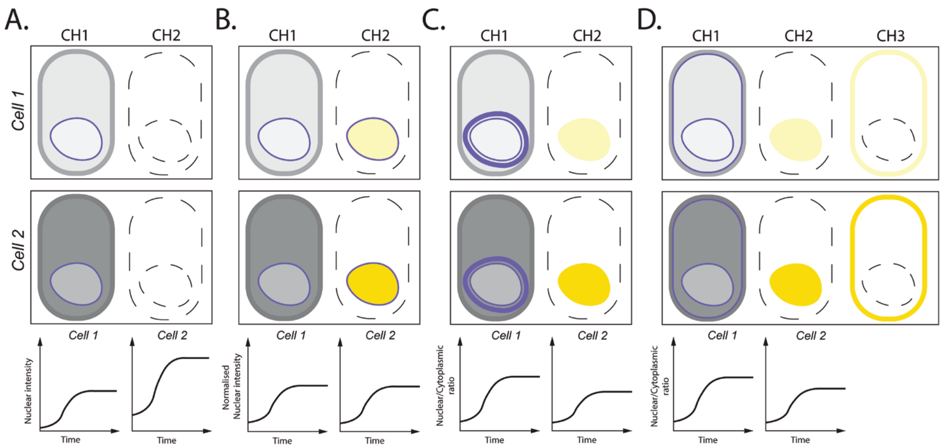

Figure 1. Measuring WNT/CTNNB1 activity using image segmentation and CTNNB1 intensity levels

Comparison of image analysis of cells 1 and 2. CH1. Channel 1, CTNNB1 signal (grey). CH2. Channel 2, nuclear marker signal (yellow). CH3. Channel 3, membrane marker signal (yellow). Purple segmentations depict the areas within which intensity is measured. A). Measuring WNT/CTNNB1 activity by measuring the nuclear intensity of CTNNB1 without normalization. Based on the nuclear intensity cell 2 will have higher nuclear CTNNB1 levels (and WNT/CTNNB1 activation) compared to cell 1. B). Measuring WNT/CTNNB1 activity by measuring the nuclear intensity of CTNNB1 normalized to the nuclear marker intensity. Cells 1 and 2 now have similar nuclear CTNNB1 levels. C). Measuring WTN/CTNNB1 activity by measuring the nuclear/cytoplasmic ratio of CTNNB1 levels, using the ring region as a measurement for the cytoplasmic CTNNB1 levels. Cell 1 now has a higher nuclear/cytoplasmic CTNNB1 ratio compared to cell 2. D). Measuring WTN/CTNNB1 activity by measuring the nuclear/cytoplasmic ratio of CTNNB1 levels. Cell 1 now has a higher nuclear/cytoplasmic CTNNB1 ratio compared to cell 2.

TCF/LEF

Where recent studies have made huge advances in studying WNT signalling dynamics at the cell surface using advanced imaging techniques, similar progress at the nuclear level is lagging behind. However, the same techniques (e.g., FCS or dcFRAP) offer opportunities for studying the dynamics and dwell time of chromatin-binding proteins such as TCF/LEF. This would require endogenous tagging of TCF/LEF proteins, which should of course not interfere with their tight fit in the enhanceosome complex. FCS and live imaging have already been applied to unravel the dynamics of the pluripotency factor SOX2 in early mouse embryos, showing the untapped potential of these techniques in a transcriptional setting (White et al., 2016). Although live imaging studies on TCF/LEF are rare, many WNT signalling reporters are built around these proteins, as we will discuss in the next section.

Imaging signalling output at the cellular level

A brief history of TCF/LEF reporters

While a rise in CTNNB1 levels is the most direct and most upstream indication of active WNT signalling that can be measured, the most sensitive readout for WNT/CTNNB1 signalling makes use of a stretch of concatemerized TCF/LEF binding sites upstream of a minimal promoter and a luciferase reporter gene. First coined TOPFLASH in 1997, the original version contained 3x TCF/LEF sites upstream of a minimal *c-Fos* promoter and was used to study the signalling activity of CTNNB1/TCF7L2 in APC-deficient human colon carcinoma cells (Korinek et al., 1997). The TCF/LEF responsive element present in TOPFLASH (CCTTTGATC, figure 2A) is based on the first TCF/LEF sites ever identified and reported in the literature, namely the TCF7 and LEF1 binding sites in the CD3epsilon (van de Wetering et al., 1991) and TCRalpha T-cell enhancers (Travis et al., 1991; Waterman et al., 1991). Even 25 years later, with more extensive and more high throughput methods available (figure 2B), there seems to be little room for improvement in terms of sensitivity or sequence specificity for the different mouse and human TCF/LEF family members (TCF7, TCF7L1, TCF7L2 and LEF1).

The dynamic range, in contrast, has since been improved (figure 2C), both in commercially available vectors and in those more widespread in the academic community (available via Addgene). Multimerizing 8x TCF/LEF sites upstream of a small, ~30bp minimal promoter (*minP*) (Veeman et al., 2003) gave rise to SUPERTOPFLASH (Moonlab via Addgene, plasmid #12456 together with its negative control SUPERFOPFLASH, #12457), while the less widely used BAR reporter (Biechele & Moon, 2008) contains 12x TCF/LEF consensus elements upstream of the same *minP*. Other flavours include a series of 7x TCF/LEF lentiviral reporters from the Nusse lab that use fluorescent reporters rather than bioluminescent ones (Fuerer & Nusse, 2010) (available from Addgene as plasmids 24,304 through 24,313). This 7x TCF/LEF series in turn also gave rise to the highly sensitive but not widely used 14x TCF/LEF consensus elements containing MEGATOPFLASH luciferase reporter (Hu et al., 2007).

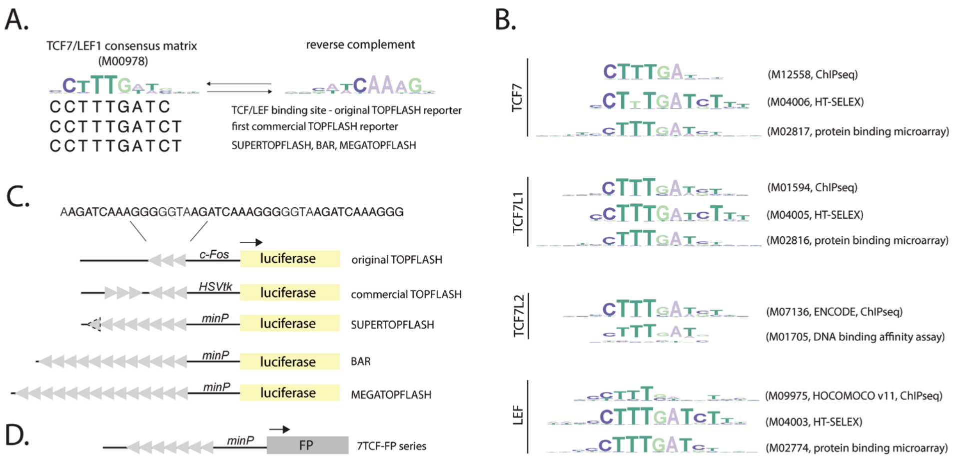

Figure 2. Overview of TCF reporter plasmids

A). The same TCF/LEF-binding site is present in all TCF/LEF reporter plasmids and mouse strains used today. Based on the DNA sequence present in the original TOPFLASH reporter, this site closely matches the TCF7/LEF1 consensus sequence. Source: TRANSFAC, M00978, consensus matrix compiled from 18 individual TCF7 and LEF1 genomic sites, accessed 24 July 2022. Note that the literature describes TCF/LEF-binding site sequences in both the sense and antisense orientation. B). Transcription factor binding site matrices determined by different experimental approaches, as indicated in the figure. Source: TRANSFAC, accessed 24 July 2022. TRANSFAC accession codes are indicated (e.g., M12558), as is the original data source (e.g., ChIPseq, HT-SELEX, protein-binding microarray). Each of these matches the general consensus sequence present in TCF/LEF reporter constructs and mice. It should be noted, however, that genome wide transcription factor binding analyses can be expected to reveal a more diverse use of optimal and suboptimal TCF/LEF-binding sites in tissue-specific contexts in vivo (Azambuja & Simoes-Costa, 2021). C). The original 3x TOPFLASH reporter became available for purchase from Upstate Biotechnology, albeit with a minimal HSVtk instead of a c-Fos promoter. The original product sheet reports a reverse orientation of the 3x TCF/LEF sites compared to the TOPFLASH sequence first reported in the literature (Korinek et al., 1997) and the sequences depicted in panels A and B. An updated version, containing a total of 6x TCF/LEF sites (3x forward and 3x reverse orientation) is still available for purchase from Sigma-Aldrich. Most other TCF/LEF reporter strains and plasmids strains also appear to harbor the binding sites in the reverse orientation. If and how the orientation has any functional impact is unclear. Academic researchers will likely prefer to obtain the 8x TCF/LEF SuperTOPFLASH plasmid from the Moon lab via Addgene for budgetary reasons. The 7x TCF/LEF series was based on the 8x TCF/LEF SUPERTOPFLASH plasmid and reports the mutation of one TCF site during the cloning process (Fuerer & Nusse, 2010). Alignment of the SUPERTOPFLASH and 7TCF sequences available from the Addgene website suggests that this mutation may already be present in the original SUPERTOPFLASH reporter. D). Fluorescent TCF/LEF reporters offer the advantage of offering single-cell resolution and the possibility for live-cell image-based analyses.

7x TCF-GFP in cell lines

The reporters described above have become a staple in the field and form the basis for quantifying WNT/CTNNB1 signalling activity—typically by dual luciferase reporter assays following transient transfection into cell lines—and thus determining the overall state of WNT activation in a pool of cells.

The incorporation of fluorescent proteins into the TCF/LEF reporters instead of luciferase (figure 2D) allows single cell and temporal resolution of WNT signalling activity, either by transient transfection or lentiviral transduction. In 2008, ten Berge et al. showed the formation of a primitive streak like structure in embryoid bodies grown from mouse ES cells transduced with the 7x TCF-eGFP reporter. This enabled them to specify both the timing, location, and spreading of the WNT activated area over time. The 7x TCF-eGFP reporter also allowed FACS sorting of the GFP-positive, WNT responsive cells for molecular follow-up analyses (ten Berge et al., 2008). In our opinion, the potential of this reporter to measure live WNT activation on a single cell level has not fully been exploited, as it lends itself excellently to not only reveal cell-to-cell heterogeneity in the response, but can also be imaged alongside individual WNT pathway proteins (as discussed in the first section) to add a readout for pathway activation.

As 3D tissue culture systems, such as gastruloids and organoids, are becoming more popular, more options arise to study developmental processes (and the pathways that control them) in vitro. Imaging these processes in 3D comes with its own challenges, e.g., background noise, imaging depth, but maybe an even bigger challenge is the quantitative analysis of 3D imaging data, as 3D segmentation and live cell tracking can complicate things considerably, requiring dedicated effort and investment (either in time or in the form of expensive commercial software).

WNT reporters in mice

At present, 3D cell culture systems do not yet capture the full dynamic complexity of tissue morphogenesis and homeostasis *in vivo*. Given the pleiotropic roles of WNT/CTNNB1 signaling in tissue development and homeostasis (Amerongen & Berns, 2006; Steinhart & Angers, 2018), there has been a longstanding interest from researchers to visualize this activity in this context (Barolo, 2006). The most straightforward way to achieve this, has been to adapt the TOPFLASH reporter for use *in vivo*. Hooking up a stretch of TCF/LEF sites to a minimal promoter and a reporter gene has resulted in a sleuth of mouse strains (see also https://web.stanford.edu/group/nusselab/cgi-bin/wnt/reporters). As an alternative to these artificial TCF/LEF reporter transgenes, endogenous target genes might be argued to better reflect WNT/CTNNB1 signaling activity. However, in that case choices need to be made for which target genes to use. Given that WNT/CTNNB1 signaling is highly context dependent, both under physiological and pathophysiological conditions, the WNT/CTNNB1 target gene program (as well as the TCF/LEF transcriptional complex) likely has multiple tissue-specific features (Söderholm & Cantù, 2021). A few exceptions exist: Like other signaling pathways, WNT/CTNNB1 signaling has multiple autoregulatory features and this includes the activation of target genes that encodes positive (e.g., LGR5) or negative regulators (e.g., AXIN2, RNF43). Because AXIN2 is induced in virtually all cells with active WNT/CTNNB1 signaling, it comes closest to being a universal WNT target gene and several targeted knock-in reporters exist (Lustig et al., 2002; Sonnen et al., 2018; van de Moosdijk et al., 2020; van Amerongen et al., 2012; Wang et al., 2021).

The main difference between these approaches is that, of course, endogenous genes receive multiple inputs. Whereas a TCF/LEF reporter transgene allows us to solely focus on and isolate the WNT/CTNNB1 response, *Axin2* undoubtedly also receives and integrates input from multiple other signaling pathways (Dao et al., 2007). However, the main benefit of using endogenous genes as a reporter is that chromatin remodeling downstream of CTNNB1, which may play a vital role in target gene transcription, is taken into account (Wan et al., 2021). Overall, implementing these systems in mice comes with its own benefits, struggles, and considerations for their interpretation, which are out of the scope of this thesis. For a more in depth discussion of these aspects, the reader is referred to the original draft of this review as published in Current Topics in Developmental Biology (van der Wal & van Amerongen, 2023).

Discussion and Outlook

With the examples discussed above, we hope to have shown that advanced imaging techniques can provide new insight into signalling dynamics at a molecular level—although it might take some time to fully learn to appreciate more biophysical approaches that do not necessarily result in a pretty image per se. Live assessment of fluorescently tagged WNT/CTNNB1 pathway components provides more information compared to fixed immunostaining. In this case, a conscious decision should be made as to when (or when not) to overexpress certain proteins or probes. While overexpression can greatly affect results by causing aberrant dimerization or by for example affecting binding affinities between proteins due to oversaturation, in some situations it might still turn out to be the preferred option. CRISPR/Cas9 lends itself to making fluorescent knock-ins at the endogenous level, but depending on the gene and cell line of interest, this can still be a time-consuming effort. This may also explain why studies of endogenously tagged individual WNT components remain sparse so far. At the same time, if we want to answer longstanding questions about the size and nature of the destruction complex, including protein stoichiometry, where binding affinities and system saturation are of importance, the combination of super resolution microscopy and endogenously tagged components can likely open up many new possibilities. Of course, many of these techniques might not immediately comply with a 3D setting, as imaging depth and light scattering in tissues can pose problems. The question is whether we should and can focus some of these efforts on cells grown in 2D to begin with. While the input (WNT/FZD binding) and output of WNT/CTNNB1 signalling (TCF/LEF target gene activation) are clearly tissue-specific, it is wholly unknown whether the signal transduction pathway in between always operates entirely similar or whether here too, context (or polarity) matters.

While super resolution microscopy has the potential to reveal new details, other developments allow more larger and more complex environments to be studied. This includes advances in light sheet microscopy as well as intravital imaging, although this is yet to be widely combined with WNT reporters (Udan et al., 2014).

Of course, many of these techniques require advanced imaging setups and optimization of imaging probes (preferably endogenous). Since this requires resources and expertise—a reason perhaps why many of these techniques are not broadly implemented—they lend themselves excellently to multidisciplinary collaborations. The future of visualizing WNT signalling looks bright, as we have clearly entered the era of quantitative developmental biology that was ushered in more than a decade ago (Oates et al., 2009).

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Author contributions

TvdW - conceptualization, visualization, manuscript writing

RvA - conceptualization, supervision, manuscript writing, review, and editing

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