Constructing cellular niche properties by localized presentation of Wnt proteins on synthetic surfaces

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Wnt signaling is crucial during embryonic development and for the maintenance of adult tissues. Depending on the tissue type, the Wnt pathway can promote stem cell self-renewal and/or direct lineage commitment. Wnt proteins are subject to lipid modification, often restricting them to act in a localized manner on responsive cells. Most methods for inducing Wnt signaling in stem cell cultures do not control the spatial presentation of the protein. To recreate the local presentation of Wnt proteins often seen *in vivo,* we previously developed a method to immobilize the protein onto synthetic surfaces. Here we describe a detailed protocol based on covalent binding of nucleophilic groups on Wnt proteins to activated carboxylic acid (COOH) or glutaraldehyde (COH) groups functionalized on synthetic surfaces. As an example, we describe how this method can be used to covalently immobilize Wnt3a proteins on microbeads or a glass surface. This procedure requires ~3 h and allows for the hydrophobic protein to be stored in the absence of detergent. The immobilization efficiency of active Wnt proteins can be assessed using different T-cell factor (TCF) reporter assays as a readout for Wnt/β-catenin-dependent transcription. Immobilization efficiency can be measured 12–18 h after seeding the cells and takes 2–4 h. The covalent immobilization of Wnt proteins can also be used for single-cell analysis using Wnt-coated microbeads (12–18 h of live imaging) and to create a Wnt platform on a glass surface for stem cell maintenance and cell population analysis (3 d). The simple chemistry used for Wnt immobilization allows for adaptation to new materials and other developmental signals. Therefore, this method can also be incorporated into tissue engineering platforms in which depletion of the stem cell pool restricts the complexity and maturity of the tissue developed.

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INTRODUCTION

During development and tissue regeneration, stem cells both self-renew and produce differentiated daughter cells in order to populate the tissues of the body. This function is regulated by maintaining stem cells in a highly specialized microenvironment, which has been termed the niche[1]. Niche signals can come from cell–cell interactions and/or signaling molecules produced by neighboring cells, restricting both the number and distribution of the signals[2]. The Wnt signaling pathway is a central player in development and stem cell maintenance[3]. Wnt ligands are subject to post-translational modification, including the addition of a palmitoleic acid group[4,5]. Although this modification is essential for protein activity, it confers hydrophobicity. As part of the intricate signaling network established in the niche *in vivo*, hydrophobic Wnt ligands are often secreted locally and presented in a spatially restricted manner to the responsive cells[3,6–9]. Therefore, *in vitro* methods that control the location of Wnt signals can better mimic cellular niches, thus providing a way to spatially control cellular processes, including cell fate decisions triggered by niche signals in the context of regenerative medicine platforms.

Development of the technique

This protocol describes the covalent immobilization of Wnt proteins onto synthetic surfaces to mimic the spatially localized Wnt secretion within cellular niches. This method allows for the presentation of the Wnt ligands to target cells in a localized, controlled manner. Biochemical characterization of Wnt proteins[10], complemented by the recently obtained crystal structure of Wnt proteins[11,12], suggests that Wnt proteins have conserved domains, including cysteine residues (e.g., 24 residues in murine Wnt3a and 22 in *Xenopus* Wnt8) that form disulfide bridges and are critical to protein function[13,14]. Therefore, an immobilization method that does not disturb the tertiary structure, including the disulfide bridges, is necessary for maintaining biologically active Wnt ligands. This protocol describes two Wnt immobilization approaches using either COOH- or COH-coated surfaces that react with nucleophilic groups (e.g., primary amines) on the Wnt proteins. The immobilized Wnt proteins can be stored in the absence of detergent and remain biologically active. As an example, we describe here how to immobilize the widely used murine Wnt3a protein to two synthetic surfaces: microbeads, termed Wnt beads, which are used for single-cell analysis (good for visualization of the Wnt source), and glass surfaces, termed the Wnt platform, used for cell population analysis.

A number of Wnt-responsive stem cells have been isolated from various embryonic and adult tissues and expanded *in vitro* by inducing Wnt signaling via purified Wnt ligands (examples include mouse embryonic stem cells (mESCs)[15] and hematopoietic[5,16,17], mammary gland[18], neuronal[19] and intestine[20] stem/progenitor cells, among others[3,21]). Although this work enhances our knowledge about the mechanisms of stem cell maintenance and differentiation, the culturing conditions often do not take into account how Wnt proteins are presented in the niche. To investigate the role of localized Wnt signals *in vitro*, Wnt3a ligands were covalently immobilized to microbeads, and the beads were introduced to a single mESC[22]. Using time-lapse imaging, localized Wnt3a proteins were shown to induce oriented asymmetric cell division with respect to the Wnt bead. As a result, the Wnt-proximal daughter cell maintained the expression of pluripotency markers, whereas the Wnt-distal daughter cell adopted an epiblast stem cell fate. Importantly, this method allowed the visualization of the location of the Wnt source over time. Thus, it was demonstrated, for the first time, that localized Wnt signals affect cell fate decisions in single, mammalian stem cells by controlling asymmetric cell division, an essential process for tissue formation and regeneration[23].

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Further work from our group showed that Wnt immobilization could be used for cell population analysis and tissue engineering purposes[24]. By covalently immobilizing Wnt3a onto flat glass surfaces, we produced a spatially confined, stable, detergent-free source of active Wnt. This Wnt platform can be used to enrich and maintain stem cell populations in 2D cultures and can be adapted to recapitulate the stem cell niche in a 3D culturing platform. Specifically, by using human mesenchymal stem cells (hMSCs), we showed that the Wnt platform could maintain a stem population while directing the generation of organized multilayers of increasingly differentiated cells. In addition, the Wnt platform can be stored at 4 °C for up to 4 months before use and exhibits stability and signaling for at least 3 d in culture.

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Comparison with other methods

The application of Wnt proteins to Wnt-responsive cell systems has yielded many insights into the stages from early embryonic development to adulthood and into disease such as tumorigenesis[25]. To introduce Wnt ligands to cells *in vitro*, studies often use several methods, each with strengths and limitations. For example, Wnt-conditioned medium is easily produced by Wnt-secreting cells; however, the medium contains other molecules that can affect cellular responses. In addition, it does not allow for specific control of the Wnt concentration. Purified recombinant Wnt ligands allow for increased purity but require detergent to maintain activity[5], which can be toxic to some cells. Both methods rely on global addition of Wnt ligands to the medium. Although highly effective, when the goal is to induce Wnt signaling in all cells, these methods are not amenable to spatial control. Wnt proteins carried by liposomes[26–28] or nanodiscs[29] can be useful for *in vivo* delivery but introduce biomolecules that might affect cellular responses within tissues. Wnt-soaked beads have been reported[30] to spatially confine the Wnt signal for a short period of time, but as noncovalent binding is used, eventually the ligands diffuse into the medium. As previously discussed, localization of Wnt signals changes the cellular response. For example, soluble Wnt induces self-renewal of mESCs via symmetric division[15], whereas localized Wnt induces asymmetric cell division, mimicking *in vivo* conditions[22]. In addition, although localized Wnt3a maintains hMSCs and directs multilayer formation in 3D, globally addition of soluble Wnt3a to the hMSCs maintains their self-renewal but markedly compromises multilayer formation in 3D (ref. 24). Small molecules that regulate the Wnt pathway (e.g., CHIR990021 (ref. 31), BIO[32,33] and CT99021 (ref. 34)), and nanoparticles coupled to antibodies targeting the Frizzled2 receptor[35] can stimulate Wnt signaling and have been successfully used to study the pathway. Although useful, small molecules are often nonspecific and can activate/inhibit other signaling pathways; in addition, nanoparticles might engage only a subset of the Wnt machinery. Finally, stimulation of the pathway using methods of global presentation of Wnt proteins to the cells requires daily supplementation owing to consumption by cells and limited stability. By covalently binding Wnt proteins onto synthetic surfaces, specific Wnt-responsive cells can receive a directed, defined and stable source of Wnt signals, which can signal for several days under tissue culture conditions.

Applications and limitations

Overall, researchers can use the immobilized Wnt technology to study the role of Wnt signaling in development and disease[36,37] in a multitude of ways. Biochemists could decipher specifics of the Wnt signaling pathway. For example, they can use the localized Wnt source (e.g., beads) to purify and identify new components of the signaling cascade from cell lysates, once conditions (e.g., type of detergent) have been optimized for the system of choice. Cell biologists could further probe the effect of localized Wnt signals on cellular processes such as cell polarization, cytoskeleton rearrangement and the distribution of organelles (e.g., primary cilia or centrosomes). In addition, stem cell biologists could determine the role of Wnt in cell fate decisions and tissue engineers could incorporate Wnt proteins into their *in vitro* platforms. Finally, researchers interested in novel therapeutics could use the technology to target localized Wnt ligands on biodegradable scaffolds[38] to *in vivo* sites.

Here, we highlight the role of Wnt3a ligands in the biology of mESCs, but this protocol can be adapted to a wide variety of Wnts (e.g., Wnt5a (ref. 22)) and/or Wnt-responsive cells, including stem cells, and 3D tissue engineering[24]. In addition, this technology can be adapted to other surface materials by introducing amine groups, which can be modified to bind Wnt proteins[39]. However, variations on this protocol concerning the type or source of Wnt proteins will require proper optimization, with special focus on the biological activity and stability of the Wnt ligands before and after binding. Similar consideration applies to the use of other materials. As the method is based on the availability of free COOH or COH groups, materials with this chemistry or with compatible alternatives must be manufactured. This would require specialized preparation of materials, including plasma cleaning or microfabrications that may require a clean room. Stimulation of the Wnt pathway by a localized Wnt source in stem cell types other than mESCs has already been reported[24]. Although no major modifications of the system were required with those cell types, quenching of the surface with FBS, BSA or extracellular matrix (ECM) proteins might be required to enhance/facilitate cell attachment when using less adherent cells. In addition, when testing out new cell types, a gentler method than trypsinization may be required for collecting cells before mixing with Wnt3a beads in order to avoid possible receptor cleavage. The protocols described here can also be adapted to immobilize other developmental signals such as Hedgehog family proteins that share similarities with Wnt proteins, including hydrophobicity and post-translational modification[40].

Experimental design

The immobilization process can be broken down into key stages. First, we functionalize the synthetic surfaces by either activating COOH groups or adding COH groups, which can react with nucleophilic groups on the Wnt proteins (**Fig. 1**). After the surface preparation, we incubate with soluble Wnt3a proteins (maintained with carrier protein (0.1% (wt/vol) BSA) and 1% (wt/vol) CHAPS detergent) diluted to an optimal concentration in Dulbecco's PBS (dPBS). We previously showed that Wnt3a proteins preferentially bind to the surfaces as compared with the carrier protein (BSA), with 76% of the Wnt3a input remaining on the surface[24]. Although the binding efficiency can give an indication of how many molecules are immobilized onto the surface, it is critical to perform Wnt activity assays; therefore, before proceeding to experiments with the immobilized Wnt3a surfaces, we always perform quality control testing. This is accomplished using either a TOPFlash-based

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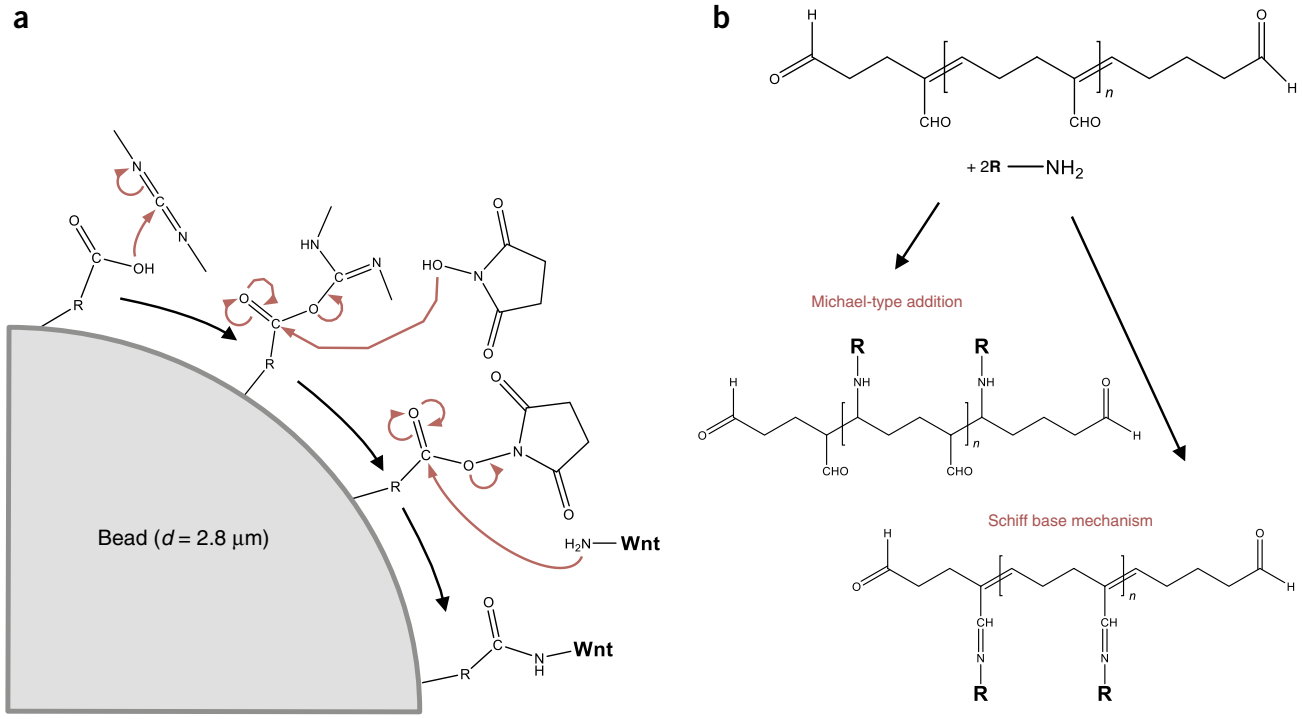

Figure 1 | The chemistry of covalent immobilization of Wnt proteins. (**a**,**b**) Schematic summarizing the chemistry used to covalently bind Wnt to two different synthetic surfaces, resulting in Wnt-coated beads (**a**) and a Wnt-coated glass surface (i.e., Wnt platform) (**b**). Glutaraldehyde may react with the protein by several means, such as aldol condensation, Michael-type addition or Schiff base mechanism[43]. An unstable Schiff base is less likely to form because of the high stability of the immobilized Wnt[24]. 'R' represents Wnt protein or the backbone of the functional group on the glass surface. Images are not to scale.

assay or a TCF-EGFP reporter, in which luciferase or EGFP, respectively, is expressed upon induction of Wnt/β-catenin signaling[41,42] (**Fig. 2**). Finally, we describe two examples of how the surfaces can be used to assess cellular response, using mESCs as a model system (**Fig. 3**). In the first example, the Wnt3a beads induce asymmetric cell division of single mESCs, as visualized by the unequal inheritance of the pluripotency marker Nanog. The second example uses Wnt3a-coated glass surfaces (Wnt3a platform). mESCs are seeded on a Wnt3a platform at low density and allowed to grow into colonies, which are then stained for the pluripotency marker alkaline phosphatase (AP). Accordingly, the Wnt3a platform, but not an inactivated Wnt3a platform, can enrich for pluripotent mESC colonies.

Synthetic surfaces. In this protocol, we detail how different commercially available surfaces can be functionalized for Wnt immobilization. *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) convert surface carboxylic groups on the microbeads to a succinimide ester that can react with nucleophilic groups on the Wnt ligand (e.g., the primary amine) to form an amide bond (**Fig. 1a**). Glass platforms containing surface amine groups are functionalized by incubation with COH, providing reactive species that can immobilize the Wnt proteins (e.g., through aldol condensation or Michael-type addition[43]) (**Fig. 1b**). Alternatively, commercially functionalized surfaces containing aldehyde groups can be used[24]. However, these surfaces have proven to be less stable than the amine-coated surfaces. Introduction of amine groups to new surfaces would allow for the adaptation of the technique to new materials.

Wnt ligands. A pure and biologically active source is indispensable to the successful immobilization of Wnt proteins to functionalized surfaces. In this protocol, we specifically describe the immobilization of commercially available recombinant Wnt3a protein. In recent years, the purification method has improved, yielding biologically active Wnt proteins that have improved consistency among batches. Given protein sequence similarities within the Wnt family, adaptation to other Wnt ligands seems reasonable, especially now that many purified Wnts are commercially available. Another option is to use recombinant Wnt ligands purified in the laboratory[5,44]. The Wnt purification process can be challenging and requires trained protein biochemists plus some specialized equipment and resources. We recommend periodically testing the biological activity of reagents and always including the soluble source alongside the Wnt-coated surfaces during quality control tests.

Cells. A number of cell lines are required for performing this protocol. L cells with a luciferase reporter (LS/L cells) are used to test the biological activity of soluble and Wnt3a-coated surfaces. LS/L cells carry a luciferase reporter under a Wnt-sensitive pSuperTOPFlash promoter (which contains a tandem of seven TCF/lymphoid enhancer factor (TCF/LEF)-binding domains), together with a pEF1/Myc-His/LacZ (Invitrogen) reporter that produces β-galactosidase[45,46]. LS/L cells are maintained in LS/L medium and kept between 10 and 80% cell confluency.

R1 mESCs[47] stably harboring a 7×TCF-EGFP reporter[15,42] are used to assess Wnt/β-catenin pathway induction by Wnt3a surfaces in mESCs. W4 mESCs[48] are used to assess Wnt/β-catenin

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pathway induction by RT-PCR. Knock-in Nanog-Venus R1 mESCs[22] are used to study Wnt3a-mediated asymmetric cell division. In addition, the parental R1 mESCs are used as a control. mESCs cell lines are cultured in embryonic stem cell (ESC) complete medium. In addition, other cell lines, including hMSCs and mouse mammary gland progenitor cell lines, have been previously used to characterize Wnt-coated surfaces[24]. All cells should be routinely tested for mycoplasma. Other types of Wnt3a-responsive (stem) cells can also be used[3]. When applying the Wnt3a beads, it is important to use a cell line that survives as single cells. For certain cell lines used on the Wnt3a platform, it may be necessary to combine the Wnt3a protein with other ECM proteins to ensure cell attachment.

Culture media. Wnt surfaces can be used for experiments with serum-containing or serum-free media[22,24]. However, in serum-free approaches, an additional quenching step will be required to block any unbound functional groups, as described in the protocol (Steps 19 and 27). When using a medium that may contain proteins or small molecules that could affect Wnt/β-catenin signaling, the medium should first be checked by LS/L assay to determine whether basal levels of pathway activation are altered. For example, if the experimental setting or the cell type used requires the addition of molecules that modulate the Wnt pathway, such as CHIR99021 (component of two-inhibitor (2i) media for mESCs culture), variations in the results should be expected. Indeed, we have shown that Wnt-mediated asymmetric cell division is disrupted when mESCs are incubated with Wnt3a beads in the presence of CHIR99021 (ref. 22).

Live imaging. Live imaging of single mESCs with Wnt3a microbeads is described in the protocol (Steps 29–34 **Fig. 3**). This requires a wide-field epifluorescence microscope equipped for live-cell time-lapse imaging and a motorized stage, such as a Zeiss AxioObserver Z1. Typically, differential interference contrast (DIC) or bright-field images are acquired alongside the required fluorescence channels. Exposure time must be optimized, as prolonged exposure times can lead to phototoxicity[49]. Exposure times <10 ms for bright-field/DIC and <20 ms for fluorescence are recommended. Similarly, lapse time between exposures must be defined, with a recommended interval >6 min for fluorescence images and >1 min for DIC/bright-field images. Positions with single cells contacting, or in close proximity to, microbeads should be chosen, and the corrected *z* position must be verified before starting the imaging according to the microscope controlling software instructions. To maintain focus while imaging, additional modules (i.e., Definite Focus, Carl Zeiss) might be required. To ensure cell viability, keep the temperature and CO_2 levels at 37 °C and 5%, respectively, in equilibrium.

Image/data analysis. Post-imaging processing can be performed with open-source software (e.g., Fiji (https://imagej.net/Fiji/Downloads) or ICY (http://icy.bioimageanalysis.org/download)) or advanced imaging software packages (e.g., Volocity, PerkinElmer). Flow cytometry data can be analyzed with FlowJo software (https://www.flowjo.com/solutions/flowjo/downloads).

Controls. A positive control (soluble Wnt used for immobilization) and a negative buffer control (Wnt carrier solution, containing 0.1% (wt/vol) BSA and 1% (wt/vol) CHAPS) in technical triplicate should be included when testing the activity of immobilized Wnt proteins using Wnt reporter cell lines (e.g., 7×TCF-EGFP or pSuperTOPFlash-Luciferase). Depending on the sensitivity and responsiveness of the Wnt reporter cell lines, we recommend dilutions of between 50 and 200 ng Wnt3a/ml, and an equivalent volume for the negative control. Inactivated immobilized Wnt proteins can be used as a negative control to determine the biological effect of localized active Wnt proteins. To inactivate immobilized Wnt ligands, the surfaces are treated with DTT. DTT treatment reduces the disulfide bridges established between the cysteine residues of the Wnt protein, causing denaturation of the protein and loss of activity.

For flow cytometry analysis, Wnt reporter cell lines and the parental cell line (no reporter) are seeded in a tissue culture 96-well plate in technical triplicate. These cells are treated with the aforementioned positive and negative controls. Additional

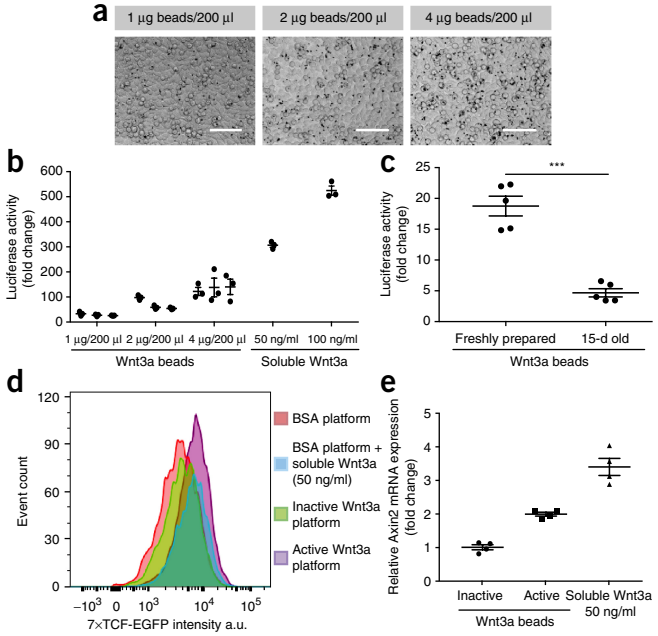

Figure 2 | Assessment of biological activity of the Wnt surfaces—quality control assays. (**a**) Representative bright-field images of the distribution of beads for the 1-, 2- and 4-µg beads per 200 µl conditions after 16 h of incubation with LS/L cells. Images were taken using a 10× objective, NA 0.4. Scale bars, 100 µm. (**b**) Luciferase activity is expressed as a fold change as compared with the negative control (Wnt carrier solution). Measurements correspond to three different bead sets (1, 2, and 4 µg of beads per 200 µl conditions, and three soluble Wnt3a controls each for 50 and 100 ng/ml ($n = 3$ per bead set/soluble Wnt3a control at each condition, error bars are s.e.m.). (**c**) Luciferase activity is expressed as a fold change as compared with the negative control (Wnt carrier solution). Measurements correspond to averaged values of three technical replicates for five independent bead sets for 14-d-old or freshly prepared Wnt3a, plated at a 1 µg/200 µl concentration ($n = 5$ independent replicates, three technical replicates per condition. Error bars are s.e.m. $***P < 0.001$). (**d**) Flow cytometry analysis plot showing 7×TCF-EGFP expression levels of mESC cells grown on a BSA platform, a BSA platform in the presence of soluble Wnt3a, an Active Wnt3a platform (1.2 ng Wnt3a per mm^2) or an inactive Wnt3a platform (1.2 ng Wnt3a per mm^2 treated with 20 mM DTT for 30 min). (**e**) Relative Axin2 mRNA expression levels expressed as fold change as compared with inactive beads ($n = 4$ independent replicates per condition. Error bars are s.e.m.).

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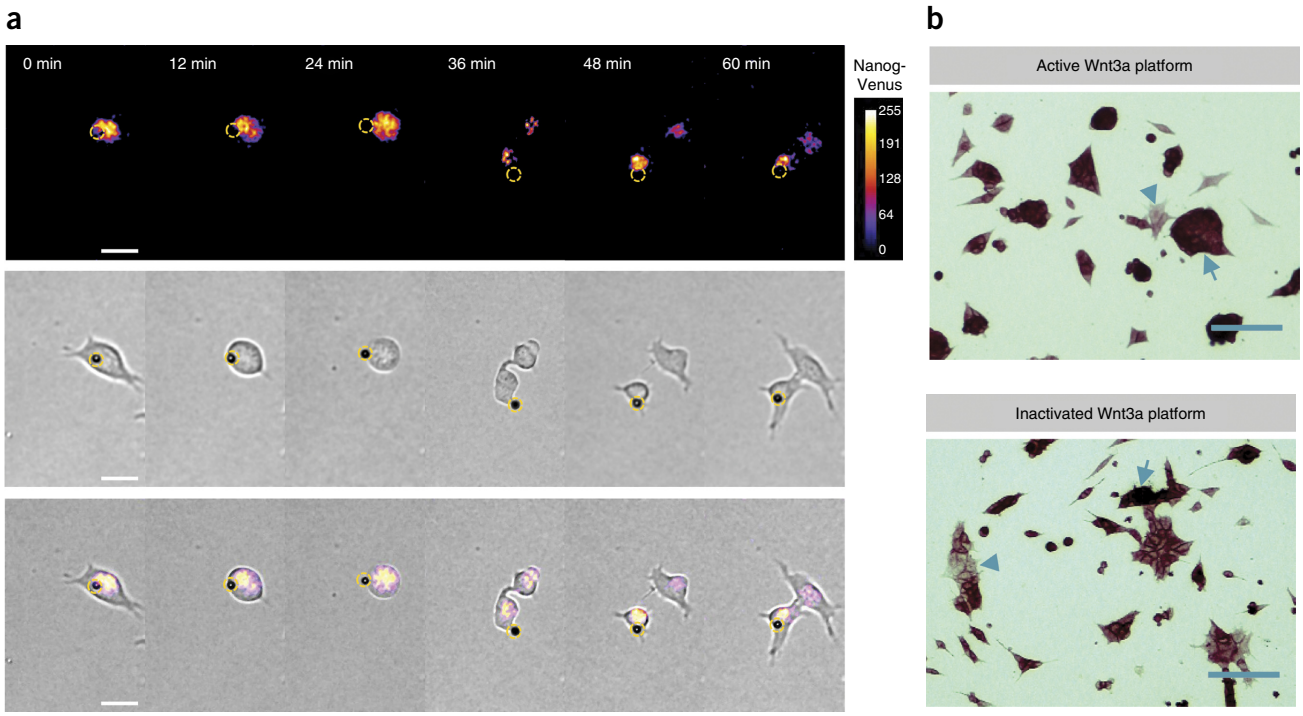

Figure 3 | Analysis of the effects of Wnt3a surfaces on the pluripotency of mESCs. (**a**) Representative images of Wnt3a–bead-mediated asymmetric cell division. Knock-in Nanog-Venus mouse ESCs were imaged for 12 h in a controlled environment (37 °C, 5% CO_2) on an epifluorescence microscope using a 20× objective, NA 0.8. Images were taken every 12 min with DIC (5-ms exposure, middle panel) and GFP channels (20-ms exposure, top panel). Nanog-Venus signal is presented as a pseudo-colored heat map, calibration bar shown at right, in arbitrary units (a.u.). Bottom panel, merge of DIC and Nanog-Venus images. Yellow dashed circle marks the position of the bead. Scale bars, 10 µm. (**b**) Representative images of alkaline phosphatase (AP) staining of mESC colonies after 72 h of growth under normal conditions on Wnt3a platforms (1.2 ng Wnt3a per mm^2) or inactivated Wnt3a platforms (1.2 ng Wnt3a per mm^2 treated with 20 mM DTT for 30 min). Arrows mark AP^+ colonies; arrowheads mark AP^- colonies. Scale bars, 50 µm. The representative images correspond to the experiment quantified in Figure S3H of Lowndes *et al.*[24].

controls such as 3 µM CHIR99021 (for the induction of Wnt reporter) can also be included. The parental cell line (nonexpressing line) can be used as an unstained control and for the live/dead DAPI control. Both controls can be seeded onto a tissue-culture-treated 96-well plate in triplicate to keep cell number consistent with the Wnt-platform plate.

MATERIALS

REAGENTS

- 1,000-µl, 200-µl, 20-µl filter tips (StarLab, cat. nos. S1122-1830, S1120-8810, S1120-1810)
- 100 × 20-mm TC dish (BD Falcon, cat. no. 353003)
- 15-ml and 50-ml tubes (Falcon, cat. nos. 430791, 352070)
- 2-[*N*-morpholino]ethane sulfonic acid, MW 213.25 (MES; Sigma-Aldrich, cat. no. M3671)
- 2-Mercaptoethanol (Gibco, cat. no. 21985-023)
- 2.8 µm Dynabeads M-270 Carboxylic Acid (Invitrogen, cat. no. 143-05D)
- 3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate, zwitterionic (CHAPS; Roche, cat. no. 10810118001) **! CAUTION** CHAPS is highly toxic. Handle it in a fume hood.
- DAPI (Sigma-Aldrich, cat. no. D9542)
- 5-ml, 10-ml, 25-ml individually wrapped pipettes (Corning, cat. nos. CC208, CC214, CC216)
- 6-Well Tissue Culture Plates (Thermo Fisher Scientific, cat. no. 10578911)
- Advanced DMEM/F12 (Gibco, cat. no. 12634 028)
- Alkaline Phosphatase (AP) Detection Kit (Millipore, cat. no. SCR004)
- Axin2 TaqMan RT-PCR probe, Mm00443610_m1 (Thermo Fisher Scientific, cat. no. 4331182)
- Bottle-top vacuum filter system, 0.45 µm (500 ml; Corning, cat. no. CLS430770-12EA)
- BSA (Thermo Fisher Scientific, cat. no. 15561020)
- Corning 96-well clear-bottom black polystyrene microplates (Thermo Fisher Scientific, cat. no. 07-200-565)
- Corning Falcon test tube with cell strainer snap cap (Thermo Fisher Scientific, cat. no. 08-771-23)
- Corning PureCoat Amine Flat-Bottom Multiwell Plates, 96-well (VWR International, cat. no. 734-1475)
- DMSO (Sigma-Aldrich, cat. no. D5879)
- DTT (Life-Technologies, cat. no. P2325) **! CAUTION** DTT is an irritant. Handle it with gloves.
- Dual-Light Luciferase & β-Galactosidase Reporter Gene Assay System (contains Buffer A, Buffer B, and Accelerator-II; Thermo Fisher Scientific, cat. no. T1005)
- DMEM (high glucose; Thermo Fisher Scientific, cat. no. 11965092)
- Dulbecco's PBS (dPBS; Sigma-Aldrich, cat. no. D8662)
- ES-R1 cells from mice (Sigma-Aldrich, cat. no. 07072001) **! CAUTION** The cell lines used in this protocol should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.
- Ethyl alcohol, pure (Sigma-Aldrich, cat. no. 459836) **! CAUTION** Ethyl alcohol is flammable and an irritant. Handle it with care.
- FBS—ES cell qualified (Millipore, cat. no. ES-009-B)
- FBS (Sigma-Aldrich, cat. no. F7524)
- Galacton-Plus substrate (100× concentrate), size B (Applied Biosystems/Life Technologies, cat. no. T2119) **! CAUTION** Galacton-Plus is highly flammable and can cause eye and respiratory irritation. Handle it in a fume hood.

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- GAPDH TaqMan RT-PCR probe, Mm99999915_g1 (Thermo Fisher Scientific, cat. no. 4331182)
- GlutaMAX (L-Glutamine; Sigma-Aldrich, cat. no. G7513)
- Glutaraldehyde solution, grade II, 25% in H_2O (Sigma-Aldrich, cat. no. G6257) **! CAUTION** Glutaraldehyde is corrosive and toxic, and represents a serious health and environmental hazard. Handle it in a fume hood, with care. Dispose of the compound according to institutional guidelines.
- LS/L cells: L Cells (ATTC, cat. no. CRL-2648) stably expressing a pSuperTOP-Flash-Luciferase reporter (Addgene, plasmid no. 12456) **! CAUTION** The cell lines used in this protocol should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.
- Luciferase Cell Culture Lysis 5× Reagent (Promega, cat. no. E1531)
- Microplates, 96-well, white (Berthold Technologies, cat. no. 23300)
- Mouse leukemia inhibitory factor (LIF; Miltenyi, cat. no. 130-095-775)
- *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC; Sigma-Aldrich, cat. no. E7750) **! CAUTION** EDC is corrosive and toxic. Handle it with care.
- *N*-Hydroxysuccinimide (NHS; Sigma-Aldrich, cat. no. 56480)
- Paraformaldehyde, 16% (wt/vol) aqueous solution, methanol free (PFA; Alfa Aesar, cat. no. 43368) **! CAUTION** PFA is toxic and represents a health hazard. Handle it in a fume hood.
- Penicillin–streptomycin (P–S; Sigma-Aldrich, cat. no. P4458)
- ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, cat. no. P36935)
- QuantiTec Reverse Transcription Kit (Qiagen, cat. no. 205310)
- Recombinant Wnt-3a (R&D Systems, cat. no. 1324-WN-010) **▲ CRITICAL** This supplier has good batch-to-batch consistency with regard to protein purity and activity.
- RNeasy Mini Kit (Qiagen, cat. no. 74104)
- Sodium azide, 0.1 M solution (Sigma-Aldrich, cat. no. 08591)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- StemMACS CHIR 99021 (Miltenyi, cat. no. 130-103-926)
- Steriflip-GP filter units (50 ml; Millipore, cat. no. SCGP00525)
- TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, cat. no. 4444556)
- Tris (Trizma base; Sigma-Aldrich, cat. no. T6066)
- Trypsin–EDTA solution (0.25% (wt/vol); Sigma-Aldrich, cat. no. T4049)
- Tween 20 (Sigma-Aldrich, cat. no. P9416)
- W4 mouse embryonic stem cells[48] **! CAUTION** The cell lines used in this protocol should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.
- Hydrochloric acid (HCl; Sigma-Aldrich, cat. no. H7020)

EQUIPMENT

- Magnet to pellet beads (e.g., DynaMag-2 Magnet, Invitrogen, cat. no. 12321D)
- Small centrifuge to briefly spin microcentrifuge tubes (e.g., Prism Mini Centrifuge, Labnet International, cat. no. AA9760)
- Cell culture centrifuge (e.g., benchtop centrifuge 5810 R, Eppendorf, cat. no. 5811000460)
- Vortex (e.g., vortex mixer SA8; Stuart, cat. no. ST0008)
- Vertical rotator (e.g., Intelli-Mixer RM-2L, Elmi, cat. no. O65500/065506)
- 2-µl, 10-µl, 100-µl pipettes (e.g., PIPETMAN P Micro Starter Kit, Gilson, cat. no. F167500)
- 20-µl, 200-µl, 1,000-µl pipettes (e.g., PIPETMAN P Starter Kit, Gilson, cat. no. F167300)
- Glass hemocytometer for cell counting (e.g., Counting Chamber; Hawksley, cat. no. HC002)
- CO_2 incubator with controlling and monitoring system for CO_2, humidity and temperature (e.g., HERAcell 150i; Thermo Fisher Scientific, cat. no. 390-4306)
- Biosafety cabinet for cell culture, class II (e.g., Herasafe KS; Thermo Fisher Scientific, cat. no. 51022487)
- Luciferase plate reader (e.g., GloMax 96 Microplate Luminometer with dual injectors; Promega, cat. no. E6521)
- Inverted tissue culture microscope, equipped with phase-contrast and color camera for monitoring cells (e.g., Nikon, model no. Eclipse TS100 with a DS-Fi2 color camera)
- Inverted wide-field fluorescence microscope equipped for live imaging (e.g., Zeiss Inverted Axio Imager Z1), with an *x*/*y* motorized stage for multipoint acquisition, CO_2, humidity and temperature control, Definite Focus (Carl Zeiss) and a digital imaging capture system (Photometrics, model no. CoolSNAP HQ2 CCD)
- Flow cytometry fluorescence analyzer (e.g., LSRFortessa Cell Analyser; BD, five-laser special order research product)
- pH Meter (S220 SevenCompact pH/ion meter, Mettler Toledo, cat. no. 30019029)
- Plate rocker (Rocker 25; Labnet, cat. no. S2025-B)
- Real-time PCR detection system (e.g., CFX384 Touch Real-Time PCR Detection System, BIO-RAD, cat. no. 1855485)
- 0.22-µm polyethersulfone filter (Stericup, 500 ml; Millipore, cat. no. SCGPU05RE)
- 0.22-µm polyethersulfone filter (Steriflip-GP filter unit, Millipore, cat. no. SCGP00525)

REAGENT SETUP

AP staining solution Immediately before use, mix Fast Red Violet solution with Naphthol AS-BI phosphate solution from the AP Detection Kit with ddH_2O in a 2:1:1 ratio. Use the solution immediately and do not store it for further use.

Buffer A Reconstitute a bottle of Buffer A from the Dual-Light Luciferase & β-Galactosidase Reporter Gene Assay System Kit with 5 ml of ddH_2O (4× concentration). Store 200-µl aliquots at –20 °C for up to a year. Equilibrate Buffer A to room temperature (≈22 °C) and dilute it in ddH_2O to a 1× working concentration.

Buffer B Reconstitute a bottle of Buffer B from the Dual-Light Luciferase & β-Galactosidase Reporter Gene Assay System Kit with 22 ml of ddH_2O (1× concentration). Store 1-ml aliquots at −20 °C for up to a year. Equilibrate Buffer B to room temperature and add the required volume of Galacton-Plus substrate (100×) to achieve a 1× working concentration. Protect the buffer from light by covering it with aluminum foil, and use it within 10 min. **▲ CRITICAL** Galacton-Plus substrate must be added immediately before reading.

20 mM DTT Add 20 µl of 1 M DTT to 980 µl of ddH_2O and mix by pipetting. Store 100-µl aliquots at −20 °C for up to a year. Avoid multiple freeze–thaw cycles.

70% (vol/vol) ethanol (1 liter) In a fume hood, add 700 ml of ethyl alcohol to 300 ml of ddH_2O, and mix by inversion. Store the solution at room temperature for up to 1 year.

EDC solution (100 µl) Weigh 5 mg of EDC in a 1.5-ml tube. Resuspend with 100 µl of cold 25 mM MES buffer, pH 5, and vortex (final concentration 50 mg/ml). Use the solution immediately and do not store it for further use. **▲ CRITICAL** EDC can be stored at −20 °C for up to a year and should be warmed to room temperature before use. **▲ CRITICAL** EDC has a tendency to absorb moisture; it should be sealed tightly and stored with desiccant. Powder that has accumulated moisture will appear clumpy and should be replaced. **▲ CRITICAL** Instrumentation used for weighing the chemicals should be cleaned three times, switching between ddH_2O and 70% (vol/vol) ethanol.

Embryonic stem cell basal medium In a sterile hood, mix 445 ml of advanced DMEM/F-12 with 5 ml of 10 mg/ml P–S, 5 ml of GlutaMAX and 63 µl of 55 mM 2-mercaptoethanol. Filter-sterilize with a 0.22-µm polyethersulfone filter (Stericup 500 ml, Millipore). Store the solution at 4 °C for up to 4 weeks.

ESC complete medium In a sterile hood, mix 45 ml of ESC basal medium with 5 ml of ES Cell Qualified FBS. Add 5 µl of 1×10^7 U/ml LIF and mix by inversion (final concentration, 10% (vol/vol) ES Cell Qualified FBS, 1% (vol/vol) P–S, 2 mM GlutaMAX, 50 µM 2-mercaptoethanol, 1,000 U/ml LIF). Filter the medium with a 0.22-µm polyethersulfone filter (Steriflip-GP filter unit) and store it at 4 °C for up to 1 week.

5% (vol/vol) glutaraldehyde solution (5 ml) In a sterile hood, dilute 1 ml of 25% (vol/vol) glutaraldehyde solution (Sigma-Aldrich) in 4 ml of ddH_2O. Prepare 500-µl aliquots and store them at −20 °C for up to a year. Avoid multiple freeze–thaw cycles. **! CAUTION** Glutaraldehyde is very toxic and harmful. Handle it with care. **! CAUTION** Glutaraldehyde must be discarded as directed by the relevant institutional guidelines.

LS/L medium (500 ml) In a sterile hood, mix 440 ml of DMEM (high glucose) with 50 ml of FBS, 5 ml of 10 mg ml^{-1} P–S and 5 ml of GlutaMAX (final concentration 10% (vol/vol) FBS, 1% (vol/vol) P–S, 2 mM GlutaMAX). Filter-sterilize the medium with a 0.22-µm polyethersulfone filter (Stericup, 500 ml). Store it at 4 °C for up to 4 weeks.

25 mM MES, pH 5 (250 ml) Add 1.22 g of MES to 250 ml of sterile ddH_2O (final concentration, 25 mM). Adjust the pH to 5 by adding concentrated acid (HCl) and monitoring with a pH meter. Store the solution at 4 °C for up

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to 4 months. When in use, maintain the solution at 4 °C by incubating on ice. ! CAUTION Concentrated acid is very toxic and harmful. Handle it with care.

NHSsolution (100 µl) Weigh 5 mg of NHS in a 1.5-ml tube. Resuspend it with 100 µl of cold 25 mM MES buffer, pH 5, and vortex (final concentration 50 mg/ml). Use the solution immediately and do not store it for further use. ▲ CRITICAL NHS has the tendency to absorb moisture; it should be sealed tightly and stored with desiccant. Powder that has accumulated moisture will appear clumpy and should be replaced. ▲ CRITICAL Instrumentation used for weighing the chemicals should be cleaned three times, switching between ddH_2O and 70% (vol/vol) ethanol.

Passive lysis buffer Luciferase Cell Culture lysis Reagent 5× (Promega) can be purchased as a ready-to-use solution. Prepare 1-ml aliquots and store them at −20 °C for up to a year. To prepare passive lysis buffer (PLB), dilute to a 1× working concentration in ddH_2O (final concentration, 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100). Use the solution immediately and do not store it for further use.

4% (wt/vol) PFA In a fume hood, add 500 µl of 16% (wt/vol) filtered PFA to 1.5 ml of dPBS and mix (final concentration, 4% (wt/vol)). Store 200-µl aliquots at −20 °C for up to a year. ! CAUTION PFA is very toxic and harmful. Handle it with care. ! CAUTION PFA must be discarded according to relevant institutional guidelines.

Staining buffer (100 ml) In a fume hood, add 0.1 g of BSA, 50 mg of sodium azide and 1 µl of Tween 20 to 100 ml of dPBS and stir until complete dilution (final concentration 0.1% (wt/vol) BSA, 0.05% (wt/vol) sodium azide, 0.001% (vol/vol) Tween 20). Store the solution at 4 °C for up to 6 months.

Tris buffered saline–Tween 20 (TBST) Add 24.23 g of Tris-HCl, 57.66 g of NaCl and 10 ml of Tween 20 to 800 ml of ddH_2O. Equilibrate the pH to 7.5. Add ddH_2O up to 1 liter (10× concentration). Store the solution at room temperature for up to 6 months. Dilute to a 1× working concentration in ddH_2O before use (final concentration, 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% (vol/vol) Tween 20). Store the solution at room temperature for up to 1 month.

Wnt carrier solution (100 ml) In a fume hood, add 1 g of CHAPS to 90 ml of dPBS and mix gently (by magnetic stir bar). Once mixed, add 100 mg of BSA and mix gently again. Add dPBS to a final volume of 100 ml (final concentration, 0.1% (wt/vol) BSA (1 mg/ml), 1% (wt/vol) CHAPS in dPBS). Do not vortex. Store the solution at 4 °C for up to 6 months.

Wnt3a solution Purify Wnt3a according to Willert *et al.*[5] or purchase from commercially available sources. For the commercial Wnt3a (e.g., R&D Systems, cat. no. 1324-WN-010), follow the supplier's instructions. Briefly, add 250 µl of sterile dPBS containing 0.1% (wt/vol) BSA to 10 µg of lyophilized Wnt3a solution, for a final stock concentration of 40 µg/ml. We recommend storing the solution at 4 °C and testing its activity monthly. Freezing the protein might reduce its activity upon thawing.

EQUIPMENT SETUP

Luminometer: washing, priming and reading luciferase levels For the luminometer (e.g., GloMax 96 microplate luminometer with dual injectors), wash injectors, following manufacturer's instructions. This should include washes with ddH_2O and 70% (vol/vol) ethanol, and a final air wash. Injectors are primed with the solutions to be injected by following the manufacturer's instructions (e.g., GloMax requires 900 µl). Recommended settings for reading luciferase activity are as follows: volume, 50 µl; delay, 1 s; speed, 200 µl/s; integration, 0.5 s. All luciferase readings should be kept within the detection range of the machine used; the amount of lysate used in the assay can be adjusted accordingly. To keep incubation times identical for all samples, it is not recommended to manually add Buffer B or Accelerator-II.

PROCEDURE

Surface preparation for Wnt3a microbeads ● TIMING 1 h

1| Wash Dynabeads by adding 1 µl of beads (~30 µg of beads; stock concentration of ~2 × 10^9 beads (~30 mg) per ml) to 100 µl of MES buffer in a 0.3-ml tube, and incubate at room temperature with slow vertical rotation (e.g., Intelli-Mixer mode C2; 0.03*g*) for 10 min.

2| Freshly prepare a 50 mg/ml EDC solution and a 50 mg/ml NHS solution as described in the Reagent Setup. ▲ CRITICAL STEP Proper activation of the surface can be achieved only if chemicals are prepared freshly and used immediately.

3| Spin down the tube from Step 1 gently in a mini centrifuge for 5 s at room temperature (e.g., Prism Mini Centrifuge; 2,000*g*) and then apply the tube to a magnet (e.g., DynaMag-2 Magnet) for 4 min before removing the supernatant. ▲ CRITICAL STEP In all subsequent steps, do not allow the microbeads to dry between supernatant removal and resuspension of the bead pellet.

4| Immediately, remove the tube from the magnet and add 100 µl of EDC buffer, mixing quickly by pipetting or vortexing (e.g., SA8 vortex mixer; 0.15*g*).

5| Immediately add 100 µl of NHS buffer and mix quickly by pipetting or vortexing.

6| Incubate the bead mixture at room temperature for 30 min with slow vertical rotation.

7| After incubation, spin the tube gently as described in Step 3 and apply to the magnet for 4 min before removing the supernatant.

8| Remove the tube from the magnet and add 200 µl of MES buffer and mix by pipetting to wash the beads; repeat this step twice more, applying the tube to the magnet for 4 min between washes to pellet the beads.

Immobilization of Wnt proteins to microbeads ● TIMING 1.5–2 h

9| Apply the tube to the magnet for 4 min and remove the final wash.

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10| Immediately remove the tube from the magnet and add 15 µl of Wnt3a protein (600 ng of protein when using R&D Systems' suggested stock concentration of 40 µg/ml (in 0.1% (wt/vol) BSA in dPBS); predicted molecular mass is 37 kDa) to activated beads. Assuming all Wnt molecules bind the surface, this equates to an input of ~5 × 10^6 molecules of Wnt3a per bead (calculation shown below).

$$\frac{15\,\mu l\ Wnt3a}{1\,\mu l\ beads}\times\frac{1ml\ beads}{\sim 2\times10^9\ beads}\times\frac{40\,\mu g\ Wnt3a}{1ml\ Wnt3a}\times\frac{6.02\times10^{23}\ molecules\ Wnt3a}{37kg\ Wnt3a}$$

$$\times\frac{1kg}{1\times10^9\,\mu g}\simeq\frac{5\times10^6\ molecules\ Wnt3a}{bead}$$

▲ **CRITICAL STEP** This calculation is an estimate for the input number of Wnt3a molecules; it does not take into account binding efficiency, which should be evaluated individually if deemed necessary, or the number of active versus inactive Wnt3a molecules. The overall activity level of the Wnt3a surface is the critical measurement, which is determined using the quality control assays in Step 28.

11| Dilute the Wnt3a–bead mixture by adding five times the volume of MES buffer (add 75 µl for 15 µl of Wnt3a–bead mixture).

12| Incubate the bead mixture at room temperature for 1 h with slow vertical rotation.

13| After incubation, spin the tube gently as described in Step 3 and apply it to the magnet for 4 min before removing the supernatant.

14| Immediately remove the tube from the magnet, add 100 µl of dPBS, and mix by pipetting; repeat this step twice, applying the tube to the magnet for 4 min between washes to pellet the beads.

15| Remove the final wash and remove the tube from the magnet. If inactivation of the beads is not required, proceed to Step 19.

16| To inactivate the Wnt3a immobilized onto the beads, resuspend the beads with 100 µl of 20 mM DTT. Mix by pipetting or vortexing, and incubate for 30 min at 37 °C with slow vertical rotation.

17| After incubation, spin the tube gently and apply it to the magnet for 4 min before removing the supernatant.

18| Immediately add 100 µl of dPBS and mix by pipetting; repeat this step twice, applying the tube to the magnet for 4 min between washes to pellet the beads.

19| Immediately resuspend the beads in 100 µl of the culture medium that will be used for experiments (e.g., LS/L medium; final concentration 0.3 µg beads per µl, or 2 × 10^6 beads per µl). Active Wnt beads should be preferentially used shortly after preparation, although they can be stored (**Fig. 2c**).
▲ **CRITICAL STEP** The medium should contain FBS to quench any unbound functional groups on the bead surface. Alternatively, a 1% (wt/vol) BSA (in dPBS) solution can be used to block any unbound groups on the beads and support the Wnt bead storage.
■ **PAUSE POINT** Beads can be stored for up to 2 weeks at 4 °C. After 2 weeks of storage at 4 °C, immobilized Wnt3a remains active, but the fold change is significantly lower ($P < 0.001$) than that of freshly prepared beads (**Fig. 2c**).

Surface preparation for Wnt3a platform ● TIMING 45 min
20| Wash wells of Corning PureCoat Amine 96-well flat-bottom multiwell plates once with 100 µl of dPBS per well.
! **CAUTION** Preparation of surfaces should be performed under sterile conditions.

21| Incubate wells with 50 µl of 5% (vol/vol) glutaraldehyde for 30 min at room temperature in the dark (or covered with aluminum foil).
! **CAUTION** Glutaraldehyde is very toxic and harmful. Handle it with care.
! **CAUTION** Glutaraldehyde must be discarded as in accordance with the relevant institutional guidelines.
▲ **CRITICAL STEP** Glutaraldehyde is light sensitive.

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22| Wash the wells three times with 100 µl of dPBS. Leave the last wash to incubate for 10 min at room temperature.

Immobilization of Wnt proteins to glass surfaces ● TIMING 1.5 h

23| Dilute Wnt3a in dPBS to a final concentration of 0.8 ng/µl and add 50 µl to each well (usually performed in triplicate for each condition). With a well diameter of 6.4 mm, and assuming that all Wnt molecules bind the surface, this equates to ~1.2 ng of Wnt3a per mm^2 or ~2×10^4 Wnt3a molecules per μm^2 (calculation shown below).

$$\frac{50\,\mu l\ \text{Wnt3a}}{\text{well}} \times \frac{0.80\,\text{ng Wnt3a}}{1\,\mu l\ \text{Wnt3a}} \times \frac{6.02 \times 10^{23}\,\text{molecules Wnt3a}}{37\,\text{kg Wnt3a}}$$

$$\times \frac{1\,\text{kg}}{1 \times 10^{12}\,\text{ng}} \times \frac{1\ \text{well}}{\pi(3.2\ \text{mm})^2} \times \frac{1\,\text{mm}^2}{1 \times 10^6\,\mu\text{m}^2} \simeq \frac{2 \times 10^4\ \text{molecules Wnt3a}}{\mu\text{m}^2}$$

▲ **CRITICAL STEP** This calculation is an estimate for the input number of Wnt3a molecules; it does not take into account binding efficiency, which should be evaluated individually if deemed necessary, or the number of active versus inactive Wnt3a molecules. The overall activity level of the Wnt3a surface is the critical measurement, which is determined using the quality control assays in Step 28.

24| Incubate at room temperature for 1 h.

25| Wash three times with dPBS. Leave the last wash to incubate for 10 min at room temperature.

26| Remove the final wash and incubate the surface with the culture medium that will be used for the experiment (i.e., see **CRITICAL STEP** note for Step 19).

■ **PAUSE POINT** For storage, we recommend dehydrating the Wnt surface by aspirating the final wash. Once all the liquid has evaporated, the plates can be sealed and stored under clean conditions at 4 °C for a maximum of 1 month.

Assessment of biological activity of the Wnt surfaces—quality control assays

27| When using a luciferase reporter cell line, follow option A; when using a GFP reporter cell line with flow cytometry, follow option B; and when using quantitative PCR to assess induction of downstream Wnt-signaling targets, follow option C.

(A) Use of luciferase reporter cells for testing immobilized Wnt3a activity ● TIMING for Steps i–v, 1 h; for Step vi, 4–6 h; for Steps vii–ix, 30–45 min; for Step x, 12–18 h; for Steps xi–xxi, 2 h

(i) *Preparation of luciferase reporter cells.* Collect LS/L cells from a 70% confluent 10-cm plate: aspirate the medium, rinse the plate once with dPBS and dissociate the cells by trypsinization by adding 3 ml of prewarmed 0.25% trypsin in EDTA and incubating for 4–6 min at 37 °C.
▲ **CRITICAL STEP** Using a wide-field microscope, confirm that the cells have dissociated from the plate in a single-cell suspension before proceeding to the next step.

(ii) Collect the dissociated cells by adding 7 ml of LS/L medium and place them in a 15-ml conical tube.

(iii) Using a hemocytometer, count the number of cells in the 10-ml solution.

(iv) Determine the number of conditions necessary for testing—including controls—bearing in mind that ≥3 technical replicates should be performed for all conditions.
▲ **CRITICAL STEP** Always include both positive and negative controls, as outlined in the Experimental design section.

(v) Centrifuge the desired volume of cell suspension (1,200*g* for 4 min at room temperature) and resuspend the pellet for a final dilution of 5×10^5 cells per ml in LS/L medium. For Wnt3a beads, add 100 µl of mixture to each well of a clear, round-bottom, 96-well tissue culture plate and proceed to Step 27A(vi). For a Wnt3a platform, add 100 µl of mixture to each functionalized well of the 96-well plate prepared using Steps 21–27 and proceed to Step 27A(x).

(vi) *Incubation of LS/L cells to allow adherence.* Incubate for 4–6 h until the cells are properly attached.

(vii) *Addition of Wnt beads and control treatments to LS/L cells.* For conditions in triplicate, dilute 6 µg of beads in 300 µl of LS/L medium in a 0.5-ml tube.
▲ **CRITICAL STEP** Different concentrations of beads can be used for testing (**Fig. 2b**).

(viii) For controls in triplicate, prepare 300 µl of LS/L medium in a 0.5-ml tube supplemented with either the Wnt3a solution used to prepare the beads as a soluble positive control (150 ng/ml) or the equivalent volume of Wnt3a carrier solution as a negative control.

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(ix) To ensure a single-bead suspension, resuspend the beads by pipetting (≥50 times) before adding 100 µl of bead mix or control mix directly to the corresponding wells (final concentration: beads, 2 µg per well; soluble Wnt3a, 50 ng/ml). Incubate for 12–18 h at 37 °C.
▲ **CRITICAL STEP** The level of dissociation of the beads and their homogeneous distribution throughout the well will have a major impact on the result of the assay. In addition, upon Wnt binding, the microbeads tend to clump; therefore, trituration is critical.

(x) *Incubation of LS/L cells.* After 12–18 h, capture representative images of the wells to assess the level of well coverage and bead distribution. Recommended settings are as follows: phase-contrast images should be taken with a 10× objective (0.25 numerical aperture (NA)).
? TROUBLESHOOTING

(xi) *Measure luciferase activity with the luminometer.* Aspirate the medium from each well of the 96-well plate and carefully rinse once with dPBS.

(xii) Lyse the cells by adding 20 µl per well of PLB. Incubate for 10–20 min at room temperature with gentle agitation (e.g., on a plate rocker).

(xiii) Prepare enough Buffer A to be able to add 50 µl to each well containing sample (Step 27A(xvi)) and enough Buffer B (supplemented with Galacton-Plus Substrate; see Reagent Setup) for instrument priming (see Equipment Setup and Step 27A(xvi)) and the injection of 50 µl into each well containing sample (Step 27A(xvii)).
▲ **CRITICAL STEP** Make sure to protect the Buffer B and Galacton-Plus mixture from light by covering the tube with aluminum foil. We recommend adding the required concentration of Galacton-Plus right before Step 27A(xiv).

(xiv) Prepare a luminometer with injectors (e.g., GloMax 96 Microplate Luminometer) by washing both injectors. Prime one injector with Buffer B.

(xv) Homogenize each well of the 96-well tissue culture plate by scratching the surface with a clean tip and mixing by pipetting. Transfer 10 µl of cell lysate to a clear, white 96-well plate.
▲ **CRITICAL STEP** Consistent luciferase readings between the technical replicates correlate with how well the cells are lysed, mixed and transferred to the well plate.

(xvi) Once all lysates have been transferred, add 50 µl of Buffer A to each well and immediately place the plate into the luminometer.

(xvii) Run the luminometer as described in the Equipment Setup.

(xviii) Place the plate in the dark or cover with aluminum foil and incubate at room temperature for 45 min.

(xix) During incubation, prepare 50 µl per well and a priming volume (see Equipment Setup) of Accelerator-II. Prime the other cleaned injector with Accelerator-II.

(xx) Place the plate in the reader and run the luminometer as described in the Equipment Setup (following the same settings as in Step 27A(xvii)).

(xxi) To normalize the results, divide the first luciferase intensity by the β-galactosidase product intensity. To produce the fold change, the average of the triplicate normalized values can be divided by the average of the triplicate control condition (i.e., negative control).
? TROUBLESHOOTING

(B) Use of a GFP reporter system and flow cytometry ● TIMING for Steps i–vii, 1 d; for Steps viii–xiii, 1–2 h; for Steps xiv–xix, 2 h

(i) *Preparation of GFP reporter cells for testing the Wnt3a platform.* Grow mESCs stably expressing a 7×TCF-EGFP reporter construct and the parental cell line (no reporter) until they reach medium-sized colonies in a six-well plate.

(ii) Collect the two types of mESCs by aspirating the medium and rinsing gently with dPBS before adding 300 µl of 0.25% trypsin–EDTA to each six-well plate and incubating for 2–3 min at 37 °C.
▲ **CRITICAL STEP** Avoid mESC over trypsinization to minimize the possibility of cleavage of receptors or other cell surface proteins of interest.
▲ **CRITICAL STEP** Using a wide-field microscope, confirm that cells have dissociated from the plate in a single-cell suspension before proceeding to the next step.

(iii) Resuspend each well in the six-well plate with 1.7 ml of ESC complete medium and transfer to a 15-ml conical tube.

(iv) Count the number of cells in the 2-ml solution using a hemocytometer.

(v) Centrifuge the desired volume of cell suspension with 1×10^5 cells per condition in triplicate (1,200*g* for 4 min at room temperature) and resuspend the pellet for a final dilution of 5×10^5 cells per ml in ESC complete medium.

(vi) Seed 200 µl of mESC cells (10,000 cells) in each well of either the protein-coated 96-well plate (previously prepared following Steps 21–26) or a tissue-culture-treated 96-well plate for control conditions (discussed in Experimental design—Controls).

(vii) Incubate for ≥12 h at 37 °C to allow for EGFP protein production.
? TROUBLESHOOTING

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(viii) *Preparation of cells for analysis on a flow cytometer.* Discard the medium and rinse the cells gently with dPBS before adding 50 µl of 0.25% trypsin–EDTA to each well.
(ix) Incubate the plate for 2–3 min at 37 °C to allow for single-cell dissociation.
(x) Collect the cells by adding 150 µl of ESC medium. Combine each set of triplicates in a 15-ml conical tube and pellet the cells by centrifugation (4 min, 1.2*g*).
(xi) Remove the medium and add 1ml of dPBS for a wash; pellet cells by centrifugation (4 min, 1.2*g*).
(xii) Remove dPBS wash and resuspend mESCs in 200 µl of dPBS supplemented with 5% (vol/vol) FBS. If desired, additional staining of the cells can be incorporated at this point by adding the optimized amount (e.g., 1 µl) of a primary antibody against a cell surface protein (e.g., CD-31) to each sample, incubating at 4 °C for 20 min, and then washing three times with dPBS. If the primary antibody is conjugated to a fluorophore (e.g., Cy3), resuspend the cells after the last wash in 200 µl of dPBS supplemented with 5% (vol/vol) FBS. Otherwise, add the optimized amount (e.g., 1 µl) of the appropriate fluorescently tagged secondary antibody and incubate at 4 °C for 20 min. Wash three times with dPBS and finally resuspend the cells in 200 µl of dPBS supplemented with 5% (vol/vol) FBS.
(xiii) Filter samples through a 35-µm mesh filter into a round-bottom tube suitable for flow cytometry and keep on ice.
(xiv) *Analysis of the single-cell expression of GFP using a flow cytometer.* Set up the flow cytometry machine (e.g., BD FACS Fortessa) according to the manufacturer's instructions.
(xv) Adjust the forward scatter and side scatter with unstained parental cell line (unstained control).
(xvi) Use the parental cell line stained with DAPI to determine UV-laser settings for exclusion of dead cells; adjust the voltage gain of the detector so that the population is within the linear dynamic range.
(xvii) To set blue laser voltage levels, use 7×TCF-EGFP cells grown in the presence of CHIR99021 or soluble Wnt3a (100 ng/ml).
(xviii) Set compensation controls.
▲ **CRITICAL STEP** Although necessary for multicolor assays, this combination will probably not require compensation; if you are including additional staining (Step 27B(xii)), then it will probably be necessary. In addition, individual isotype controls will need to be added for all antibodies used for staining.
(xix) Analyze the samples and compare single-cell EGFP expression levels between conditions using flow cytometry analysis software (e.g., FlowJo).
? TROUBLESHOOTING

(C) Use of RT-PCR to assess the induction of downstream Wnt-signaling targets ● TIMING for Steps i–vi, 1 d; for Steps vii–x, 4 h

(i) *Preparation of mESC for RT-PCR gene expression analysis.* Grow and collect wild-type mESCs as described in Steps 27B(i–iii).
(ii) Centrifuge the cell suspension (1,200*g* for 4 min at room temperature), aspirate the supernatant and resuspend in ESC complete medium.
(iii) Count the cells using a hemocytometer. 1.5 × 10^5 cells per condition in triplicate are required. A typical experiment setup will include the following conditions (as discussed in Experimental design—Controls): positive control (150 ng/ml soluble Wnt3a), a negative control (Wnt3a carrier solution) and active/inactive beads (9 µg per condition).
(iv) Distribute the appropriate volume of cell suspension in one 1.5-ml tube per condition. Mix with the required volume of beads or control solutions and add mESC medium to a final volume of 600 µl.
(v) Seed 200 µl of the mix in a well of a 96-well tissue culture plate.
(vi) Incubate for 12 h at 37 °C.
(vii) *RNA extraction, retro-transcription and RT-PCR*. Using a wide-field microscope, capture representative images of the wells to assess the level of distribution of the beads. We recommend that phase-contrast images be taken with a 10× objective (0.25 NA).
(viii) Aspirate the medium and carefully rinse once with dPBS.
(ix) Proceed with RNA extraction as described in other protocols[50]. We suggest extracting mRNA using an RNA extraction and purification kit (e.g., RNEasy Mini Kit) and retro-transcript mRNA into cDNA using a reverse transcription kit (e.g., QuantiTec Reverse Transcription Kit). To quantify the expression of Wnt/β-catenin pathway target genes (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes) by RT-PCR, we use the TaqMan system (TaqMan Fast Advanced Master Mix and TaqMan RT-PCR probes) and follow the manufacturer's instructions.
(x) Analyze RT-PCR results by normalizing the threshold cycle (Ct) value of the measured genes to the Ct value of the control gene (e.g., GAPDH), and express the result in fold change.
? TROUBLESHOOTING

Single-cell analysis of mESCs in contact with Wnt3a beads using live-cell imaging ● TIMING 2 d

28| Continuing with active Wnt3a beads (determined to be active in Step 27), resuspend Wnt3a-coated beads in ESC complete medium to ~2 × 10^6 beads/50 µl medium.

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29| Collect mESCs expressing a tagged cell fate marker protein (e.g., Nanog-Venus) using 0.25% trypsin–EDTA and resuspend in ESC complete medium as described in Step 27B(i–v), with the exception that cells should be resuspended in ESC medium to obtain 12,500 cells per ml.

30| Triturate Wnt3a-coated beads ≥50 times and add 5 µl of bead mixture per 200 µl of cells. Pipette gently at least 10 times and seed 200 µl of bead/cell mixture in each well of a black, clear-bottom, polystyrene 96-well plate.
▲ **CRITICAL STEP** Add dPBS to the wells between the wells containing cells to help maintain humidity levels during the time-lapse imaging.
▲ **CRITICAL STEP** Avoid introducing air bubbles while seeding, as these will compromise the quality of the bright-field/DIC images.
▲ **CRITICAL STEP** If other types of live imaging chambers are used, the ratio of mESCs to beads should be kept constant (2,500 cells:2 × 10^5 beads:200 µl total volume:32 mm^2 surface area).

31| Allow the beads and cells to settle down into the wells for 30 min at 37 °C before moving the plate to a microscope designed for live imaging, with the following conditions at equilibrium: 5% CO_2 and 37 °C.

32| Using a multipoint acquisition system, choose several positions at which a single cell is touching a single microbead, or at which cells have beads at close proximity.

33| Set exposure times for bright-field/DIC and blue light (to visualize Venus) so that the signal-to-noise ratio is optimal for time-lapse imaging (as discussed in Experimental design).

34| Start acquisition and run for the desired amount of time.
■ **PAUSE POINT** Cells can be fixed for further analysis once live imaging is complete or as an alternative to live imaging. To maintain a single-cell population, fix within 7 h of plating the cells and within 18 h for doublets. Fix with 4% (wt/vol) PFA for 8 min at 4 °C. Wash three times with staining buffer for 10 min each. Incubate overnight at 4 °C with primary antibodies diluted to the desired concentration in staining buffer. Wash three times with staining buffer for 10 min each. Incubate with secondary antibodies diluted to the desired concentration in staining buffer in the dark at room temperature for 2 h. Mount using ProLong Gold mounting medium and allow curing for 24 h. The mounted samples can be stored at room temperature for up to 3 months in the dark.
! **CAUTION** PFA is very toxic and harmful. Handle it with care.
! **CAUTION** PFA must be discarded according to the relevant institutional guidelines.
? **TROUBLESHOOTING**

Fixed cell analysis of mESCs grown on Wnt platforms using an alkaline phosphatase colony staining assay
● **TIMING 3 d**
35| Grow and collect wild-type mESCs as described in Step 27B(i–v), with the exception that the cells should be resuspended in ESC medium to obtain 5 × 10^3 cells per ml.

36| Seed 200 µl of mESC cells (1,000 cells) in each protein-coated 96-well as prepared earlier following Steps 21–26 (Wnt3a determined to be active in Step 27).

37| Maintain cells under normal culture conditions for 72 h (changing medium daily) to allow for colony formation.

38| Fix colonies with 50 µl of 4% (wt/vol) PFA (1–2 min).
! **CAUTION** PFA is very toxic and harmful. Handle it with care.
! **CAUTION** PFA must be discarded according to the relevant institutional guidelines.
▲ **CRITICAL STEP** Do not overfix, as this could result in inactivation of the AP.

39| Rinse once with 100 µl of TBST.

40| Prepare AP staining solution (as described in the Reagent Setup)

41| Incubate the cells with 50 µl per well of AP staining solution for 15 min, in the dark (or covered with aluminum foil) at room temperature.

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42| Wash once with 100 µl of TBST, and leave in 200 µl of dPBS while counting the number of red-expressing colonies (AP^+) as compared with colorless colonies (AP^-) using a microscope equipped with a color camera to acquire images of the red staining.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
27A(x)	Inconsistent bead distribution or low level of beads on the LS/L cells	Did not leave the tube on the magnet long enough to ensure full bead capture	Spin down the tube and keep it on the magnet for 2–4min for each washing step
		Did not mix the beads enough after adding the Wnt	Triturate ≥50 times before adding the beads to the cells; you can add additional trituration steps between washes to improve the single-bead suspension
27A(xxi)	Poor luciferase production from cells seeded on Wnt platform	Glutaraldehyde has expired	Purchase fresh glutaraldehyde; dilute to 5% and keep it in small frozen aliquots
		If using purchased aldehyde surfaces, the groups are no longer functional	When the aldehyde groups are functional, the surface tends to be hydrophobic; if not, purchase new surfaces or switch to the more stable amine-coated surfaces
	Low fold activity of soluble Wnt control	The soluble source has lost activity because of prolonged storage or lack of purity	Maintain soluble Wnt at 4 °C and keep it on ice while in use. A soluble source can last up to 4 months but should be checked periodically to ensure that the activity is not markedly reduced. Dilute the soluble Wnt in a 0.1% (wt/vol) BSA solution to maintain activity
27B(vii)	Cell did not adhere to the surface or seeding is uneven	Reaction was not quenched long enough with either FBS-containing medium or BSA	Quench for a longer period of time or use a higher concentration of BSA
		Detergent was not sufficiently cleared from the surface	Add additional dPBS washing steps or wash in a larger volume of dPBS
27A(x)/ 27B(xix)	Inactivated surface still shows activity	DTT has expired	Purchase new DTT and store it at –20 °C in small aliquots; avoid repeated freeze–thaw cycles
27C(x)	Low induction of Wnt target gene expression	Incubation time of Wnt with cells is not sufficient	Incubation time is cell-type-dependent and must be optimized
34	High background, staining not clear	Blocking is required	Consider blocking for 1 h with 5% (wt/vol) BSA or 5% (vol/vol) donkey serum in staining buffer
42	Loss of cells in one area of soluble Wnt control	The detergent concentration is too high	It is critical to dilute the soluble Wnt3a in at least 100 µl of ESC complete medium before adding it to the cells; it should be added evenly and gently to the culture medium

● **TIMING**
Steps 1–8, surface preparation for Wnt3a microbeads: 1 h
Steps 9–19, immobilization of Wnt protein to microbeads: 1.5–2 h
Steps 20–22, surface preparation for Wnt3a platform: 45 min
Steps 23–26, immobilization of Wnt proteins to glass surfaces: 1.5 h
Step 27A(i–v), preparation and seeding of LS/L cells: 1 h
Step 27A(vi), incubation of LS/L cells to allow adherence: 4–6 h
Step 27A(vii–ix), addition of Wnt beads and control treatments to LS/L cells: 30–45 min
Step 27A(x), incubation of LS/L cells with Wnt beads and control treatments: 12–18 h
Step 27A(xi–xxi), measurement of luciferase activity: 2 h

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Step 27B(i–vii), preparation of GFP reporter cells: 1 d
Step 27B(viii–xiii), preparation of cells for running on the flow cytometer: 1–2 h
Step 27B(xiv–xix), analysis of the single-cell expression of GFP using a flow cytometer: 2 h
Step 27C(i–vi), preparation of mESCs for RT-PCR gene expression analysis: 1 d
Step 27C(vii–x), RNA extraction, retro-transcription and RT-PCR: 4 h
Steps 28–34, live imaging of mESCs in contact with Wnt beads: 2 d
Steps 35–42, AP colony staining assay: 3 d

ANTICIPATED RESULTS

The biological activity of Wnt-coated surfaces can be assessed with cells expressing Wnt pathway reporters. To demonstrate the activation of the Wnt/β-catenin pathway by Wnt3a beads and the consistency between bead preparations, three independent sets of beads were added at increasing bead densities (1, 2 and 4 µg per well; **Fig. 2a**,**b**) to a pSuperTOPFlash-Luciferase reporter cell line (LS/L cells)[45,46]. A negative control (Wnt3a carrier solution) and two positive controls (50 and 100 ng/ml of soluble Wnt3a) were included. Luciferase readings were normalized and reported as fold change over the negative control. For each bead preparation, the Wnt/β-catenin activity increases as a function of bead density (**Fig. 2b**).

We have previously shown that Wnt3a platforms can be stored for long periods and maintain Wnt3a activity[24]. To demonstrate that Wnt3a beads should be preferentially used shortly after being prepared, five independent sets of beads were prepared and stored at 4 °C for 15 d. Then, their biological activity was compared with a set of freshly prepared Wnt3a beads using an LS/L assay. The biological activity of Wnt3a beads significantly declined after 2 weeks of storage (**Fig. 2c**).

To determine the level of Wnt/β-catenin signaling in cell cultures seeded on Wnt3a platforms, we used a stable mESC line harboring a 7×TCF-EGFP reporter[15,42]. After 18 h of incubation on the surfaces, cells were collected and we determined single-cell expression levels of EGFP using flow cytometry (**Fig. 2d**). Expression levels were compared with those of BSA platforms, inactivated Wnt3a platforms (DTT-treated) and BSA platforms in which medium was supplemented with soluble Wnt3a (50 ng/ml). A shift in EGFP expression can be seen for cells grown on Wnt3a surfaces and cells grown in the presence of soluble Wnt3a. Separate histograms of each condition, along with the gating conditions and statistics, are provided in the supplemental data (**Supplementary Fig. 1**). Alternatively, this analysis can be combined with staining for other surface markers to further explore the identity of the cells grown on the Wnt3a platforms.

The Wnt/β-catenin signaling pathway induces expression of Axin2 (ref. 51). To investigate the ability of Wnt3a beads to induce Axin2 expression, wild-type W4 mESCs were incubated with soluble Wnt3a, or active or inactive Wnt3a beads for 12 h. Then RNA was extracted and retro-transcribed, and Axin2 mRNA expression was measured by real-time PCR. Active Wnt3a beads produced a one-fold increase in Axin2 expression as compared with inactive beads, whereas soluble Wnt3a induced a 2.5-fold increase in the levels of Axin2 mRNA (**Fig. 2e**).

As one of the numerous applications of Wnt-coated surfaces, Wnt3a beads offer an interesting platform for the study of Wnt3a-mediated processes at the single-cell level. To assess Wnt3a-mediated asymmetric cell division, mouse mESCs with a knock-in Nanog-Venus reporter[22] were seeded at single-cell/single-bead density in a black-walled, clear-bottom 96-well plate. Positions containing cells in contact with or close proximity to beads were selected. DIC and GFP channel (suitable for Venus) images were captured every 12 min for 12 h under normal culture conditions (37 °C, 5% CO_2). Representative images from the movie show that the Nanog-Venus is asymmetrically distributed between the daughter cells, with higher levels in the cell proximal to the bead (**Fig. 3a**).

Another application of the system includes the selection and enhancement of Wnt-responsive pluripotent populations. To determine differentiation levels of mESC colonies on Wnt3a platforms, mESCs were seeded at low density and allowed to grow for 3 d before fixation and assessment of AP activity. Colonies in an undifferentiated state will have a high level of expression of AP (arrows), whereas differentiating colonies have reduced expression (arrowheads) (**Fig. 3b**). The Wnt platform enriches for AP^+ colonies as compared with inactivated Wnt platform, which was quantified in Lowndes *et al.*[24]. Representative images of the AP staining are shown for each condition (**Fig. 3b**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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