Beta-catenin

A molecular multiplayer

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"I've got blisters on my fingers!"

- Ringo Starr – The Beatles

A step-by-step protocol for live imaging and subcellular quantification of endogenous signalling proteins

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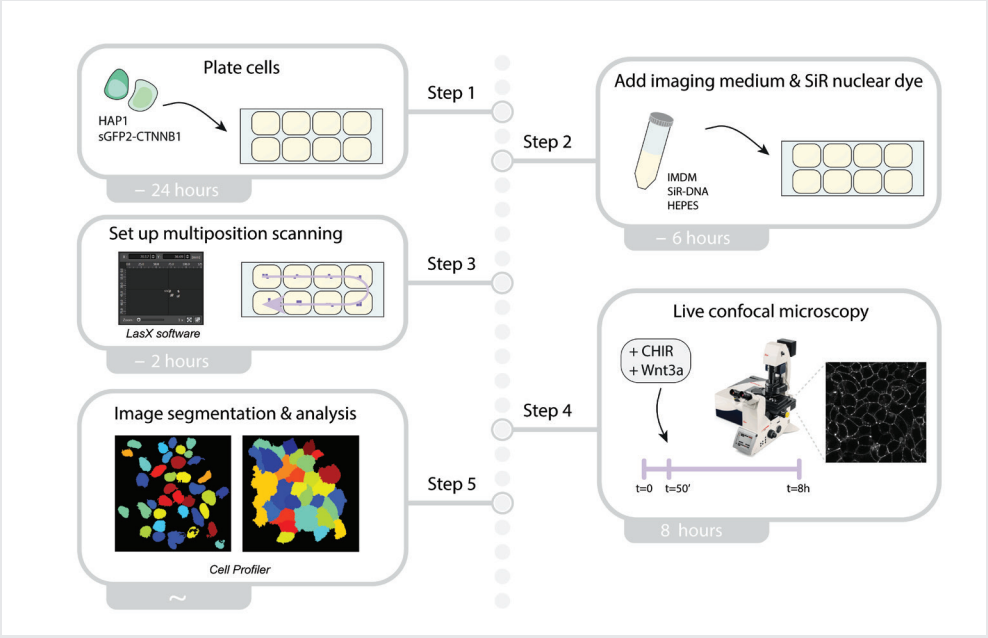
Summary

Most paracrine signalling pathways have a similar architecture, with receptor-ligand binding at the membrane leading to effector build-up in the nucleus, activating target gene transcription. This protocol describes the live confocal imaging of low levels of endogenously tagged signalling proteins (here, beta-catenin or CTNNB1), providing high temporal and high spatial (i.e. single-cell) resolution. We describe how to quantify CTNNB1 in different cellular compartments, including the membrane, using confocal images.

Highlights

- Live cell confocal imaging of endogenously tagged signalling proteins at low intensities.
- Segmentation of the nucleus, cytoplasm and membrane to measure fluorescence intensity.
- Relative quantification of different subcellular pools of signalling proteins as a readout of pathway activation

Graphical abstract



Before you begin

Fluorescence microscopy has allowed the visualization of many proteins of interest, and possibilities for obtaining high spatial and temporal resolution have increased over the years (Cuny et al., 2022; Morris & Payne, 2019). Signalling pathways generally have a similar architecture where ligand-receptor binding at the cell surface leads to build up of an effector protein in the cell (Pires-daSilva & Sommer, 2003). This, in turn, can activate transcription of target genes. Consequently, the quantity of the effector protein can often be used as a good measure of signalling activity, independent of specific target gene programs. Examples of such pathways are ERK signalling, Hippo signalling, BMP signalling, and the WNT/CTNNB1 pathway described in this protocol. Live imaging of effector proteins of such pathways, especially at endogenous levels, can be challenging. With the use of high throughput cell segmentation software, quantitative data can now be extracted from microscopy images providing information on physiological levels of signalling activity (Bilodeau et al., 2022; de Man et al., 2021; Liu et al., 2021; Sero et al., 2015; Simpson et al., 2018). Both the experimental set up, data acquisition, and data analysis can be time-consuming and need to be carefully optimized for the specific cell type and protein of interest. In this protocol, we provide a detailed workflow for live-cell time-lapse imaging of signalling proteins at endogenous levels, including both experimental and image data analysis steps to obtain fluorescent intensities in different cell compartments as a measure of relative protein levels. We use the downstream WNT signalling effector beta-catenin (CTNNB1) as an example, but a similar setup could be used for other signalling proteins of interest whose subcellular distribution changes upon pathway activation. Our image data analysis pipeline specifically focuses on segmenting the nuclear, cytoplasmic, and membrane compartments, the latter of which has proven most difficult. Lastly, we show an optimized way to obtain intensity measurements at the membrane from confocal microscopy images that are corrected for spill over cytoplasmic signal due to the limiting resolution of confocal microscopy.

Specifically, this protocol describes 2D live-cell time-lapse imaging of haploid HAP1 cells containing endogenous fluorescently tagged CTNNB1 (SGFP2-CTNNB1), the transcriptional effector of the WNT/CTNNB1 signalling pathway. During the live imaging, treatments, in this case WNT3A and CHIR99021 are added to activate WNT/CTNNB1 signalling. This protocol assumes that the protein of interest was previously labelled with a stable fluorescent protein (as described for HAP1 SGFP2-CTNNB1 in de Man et al. (2021)). We describe how to best visualize signalling proteins at often low, endogenous levels for up to 8 hours. LASX software on a Leica SP8 confocal microscope was used for imaging in this protocol, but other confocal

setups with environmental control can be used. Finally, this protocol describes how to analyse the resulting large imaging datasets to quantify CTNNB1 intensity in different cellular compartments via image segmentation using publicly available software. For the membrane compartment, we describe a correction to obtain a more accurate estimate of membrane intensities from confocal data. Plotting of the resulting data provides information about endogenous signalling dynamics with high spatial and temporal resolution.

Key resources table

Table 1. Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Gibco IMDM (no phenol red)	Fisher Scientific	#11500556
Gibco Glutamax (100x)	Thermo Fisher Scientific	#35050038
Gibco Fetal Bovine Serum	Thermo Fisher Scientific	#A3160802
Gibco Trypsin-EDTA (0.25%) (phenol red)	Thermo Fisher Scientific	#25200072
Gibco HEPES 1M	Thermo Fisher Scientific	#15630080
Gibco Penicillin/Streptomycin (10.000 u/ml)	Thermo Fisher Scientifc	#15140122
Gibco HBSS (-/-)	Thermo Fisher Scientific	#14170112
SiR-DNA	Spirochrome	#SC007
CHIR99021	Biovision	#1677-5
Recombinant human WNT3a	R&D systems	#5063-WN-010
Immersion oil Type 37	Cargille	#16237
Experimental models: Cell lines		
HAP1 cells	Whitehead institute	Cellosaurus: CVCL_Y019
HAP1 SGFP2-CTNNB1 cells	de Man et al. (2021)	n.a.
Software and algorithms		
LasX	Leica Software	n.a.
ImageJ/Fiji	ImageJ/Fiji Software	Schindelin et al. (2012)
Cell Profiler (version 4.2.5)	Cell Profiler Software	Stirling et al. (2021)
Other		
Ibidi 8-well glass bottom slide	Ibidi	#80827
Leica SP8 microscope	Leica	SP8
HC PL APO CS2 63x/1.40 oil objective	Leica	#15506350
Okolab cage incubator	Okolab	-
Okolab temperature unit	Okolab	#H201-T-UNIT-BL
488 nm Argon ion laser	-	-
633 nm HeNe laser	-	-
PMT detector	-	-
HyD detector	Leica	-
Countess 3 Automated Cell Counter	Thermo Fisher Scientific	#AMQAX2000
Countess Cell Counter Chamber Slides	Thermo Fisher Scientific	#C10228

Table 2. Culture medium for HAP1 cells

Reagent	Final concentration	Amount
Gibco IMDM (no phenol red)	1x	500 ml
Glutamax	1x	5 ml
Fetal Bovine Serum	10%	50 ml
Penicillin/Streptomycin	1%	5 ml

Table 3. Imaging medium for HAP1 cells

Reagent	Final concentration	Amount
Prepared culture medium from table 2:	1x	2.5 ml
Gibco IMDM (no phenol red)	*1x*	*N.A.*
Glutamax	*1x*	*N.A.*
Fetal Bovine Serum	*10%*	*N.A.*
Penicillin/Streptomycin	*1%*	*N.A.*
Gibco HEPES 1M (pH 7.0-7.5)	25 nM	62.5 µl
SiR-DNA	500 nM	1.25 µl

Step by step method details

Cell culture

Prior to the experiment, HAP1 SGFP2-CTNNB1 cells were cultured under normal conditions (37˚C at 5% CO_2) and passaged 1:5 three times a week in culture medium (for culture medium, see table 2). To prevent the haploid HAP1 line from becoming diploid, cells were not passaged beyond passage 20, or earlier if the cells started to display signs of diploidy (i.e. bigger cell size). Different cell lines should be cultured according to conditions optimized for those particular cell lines.

Plating SGFP2-CTNNB1 HAP1 cells on glass bottom ibidi slides for live imaging

Timing: 24 hours prior to imaging

1. Trypsinize HAP1 cells with 0.25% Trypsin/EDTA for 5 minutes at 37˚C.
    a. Remove culture medium from cells and wash away any residues with Hank's Balanced Salt Solution (HBSS) without Calcium/Magnesium (-/-) or PBS.
    b. Add 1 ml of 0.25% Trypsin/EDTA per 10 cm dish with HAP1 cells.
    c. Incubate the cells with Tryspin for 5 minutes at 37˚C.

2. Obtain HAP1 cell pellet.
    a. Add 4 ml of culture medium to the trypsinised cells (for culture medium, see table 2).

b. Collect the trypsinised cells in a tube and spin down for 5 minutes at 1000 rpm at room temperature.
c. Remove the supernatant from the cell pellet.
d. Resuspend the cell pellet in 5 ml culture medium.

3. Count cells and plate 88.000 cells/well.
    a. Count the cells. If available an automated cell counter such as the countess can be used.
    b. Make a solution of 2.93 x 10^5 cells/ml.
    c. Plate 300 µl of the cell suspension per well in a glass bottom 8-well ibidi slide to reach a quantity of 88.000 cells/well.

Note: The HAP1 cells used for this specific experiment are relatively small, so the number of cells might need to be optimized when using a different cell line. For reference, 50,000 MCF10A cells were plated per well to achieve a sub confluent (±70-80%) well of cells after 24 hours.

4. Grow cells for 24 hours at 37˚C before starting imaging

Replace culture medium with imaging medium prior to imaging

Timing: 6-8 hours prior to imaging

5. Prepare imaging medium supplemented with SiR-DNA for live nuclear staining
    a. Calculate the amount of medium needed for the size of the experiment; 250 µl per well is needed.
    b. Supplement IMDM (no phenol-red) with 1% glutamax, 10% FBS and 1% pen/strep with 25 mM HEPES (pH 7.0-7.5) and 500 nM SiR-DNA (for imaging medium, see table 3).

Note: The concentration of SiR-DNA might have to be optimized depending on the cell line and cell density to determine if the concentration does not lead to any signs of toxicity or cell death and gives sufficient nuclear signal. For additional troubleshooting, see problem 3.

Note: As phenol-red can interfere with fluorescence microscopy, it is important to take a base culture medium without phenol-red to create the imaging medium. This is especially

important when working with low intensity fluorescent signals.

Note: Prepare the imaging medium fresh on the day of the experiment just prior to use, by adding HEPES and SiR-DNA to culture medium. Do not leave exposed to light due to the presence of SiR-DNA dye.

6. Remove the culture medium and add 250 µl of imaging medium per well.
    a. For proper nuclear staining, the SiR-DNA must be added to the cells at least 6 hours prior to imaging.

Critical: If you choose a shorter incubation time than 6-8 hours for the SiR-DNA, the intensity of SiR-DNA may not yet have stabilized and can still increase during imaging, which may lead to oversaturation of the signal for later timepoints of the experiment.

Note: For different cell lines and microscopy setups the concentration and timing of adding SiR-DNA stain might need to be optimized (see troubleshooting problem 3).

Critical: Do not leave the SiR-DNA solution exposed to light as this will decrease its sensitivity. After addition, cells must be kept in the dark.

Critical: As we will later add (and thus dilute) 50 µl of 8x concentrated treatments to the imaging medium in every well, it is important to be precise in pipetting the 250 µl per well.

Microscope and setup procedure

Timing: 30-60 minutes

This section describes the microscopy setup as used to live image SGFP2-CTNNB1 and SiR-DNA nuclear dye. For imaging different signalling proteins, see troubleshooting (problem 2). For using a different confocal microscope set up, refer to the manufacturer's guidelines and local expertise on how to set up and operate the equipment.

7. Start the Leica SP8 microscope, laser units and LasX software package.
    a. Start the microscope in "machine + environmental control mode".

8. Set up the Okolab temperature unit to heat the environment cage to 37˚C.

Note: To prevent focal drifting (in the Z-plane) during imaging, leave the microscope setup at 37˚C for at least 30-60 minutes prior to setting up the imaging positions. This is especially important for the objective, which is metal and needs to warm to prevent drift due to expansion of the material during imaging. In addition, leave the imaging oil flask at 37˚C to warm up. Discuss with your local confocal microscope operator/facility manager which imaging oil to use.

Note: As the imaging medium is supplemented with HEPES, this should function as a pH buffer to counteract any effects of low CO_2 levels during the imaging period. This is necessary as for easy access to the sample to add treatments during imaging we chose not to use a gas chamber setup with 5% CO_2 atmosphere.

9. Whilst the microscope chamber is heating up, setup the LasX software and hardware settings
    a. In "configuration > hardware settings", set the bit depth to 12-bit.
    b. Set the "acquisition mode" to "xyzt" with multi-position scanning and autofocus turned on.
    c. In the Z-stack panel, set the stage to Z-wide as Z-Galvo (only available if a Piezo element is present) is less reliable in combination with the autofocus mode.
    d. In the autofocus panel, turn on adaptive autofocus control.
        i. Turn on "use AFC" and "on demand" mode.
        ii. For "time-lapse" select every cycle.
        iii. For "scanning" select every position.
    e. Set the objective to 63x/1.40 oil.
    f. Select resolution, scanning speed and image averaging.
        i. To properly segment the membrane, we used a resolution of 1024 x 1024 pixels with 2 x zoom and a pixel size of 90 nm.
        ii. Set scanning speed to 400.
        iii. Set line averaging to 4x for a better signal-noise ratio.

Note: By using a bit depth of 12-bit instead of 8-bit, the dynamic range is greatly increased. Thereby the signalling response can be captured in higher detail. Of course, 16-bit could be used, but as this greatly increases file size we did not opt for this.

Note: The chosen image resolution with a pixel size of 90 nm means that we are oversampling our data.

Note: In the adaptive focus control mode, the glass refraction is used to focus, after manually setting the position of the objects of interest relative to the glass. Other autofocus modes (such as best focus mode) will use the fluorescence intensity to focus on the objects. However, with the live imaging setup described here, the fluorescence signal dynamically changes over time because of signalling pathway activation, and we found other autofocus modes to be less reliable.

Note: Using a water objective may provide a better signal-noise ratio, as the refractive index of water is closer to that in cells. For live imaging, the usage of a water pump to prevent the objective from drying out due to water evaporation at 37˚C (± 1 second pulse of water every 5 minutes, although specific settings would need to be optimized) would be required. Since oil objectives generally give a better resolution, we opt for their use here.

10. Set laser and detector settings for SGFP2-CTNNB1 and SiR-DNA (when using different fluorescent proteins, see troubleshooting 2).
    a. For SGFP2-CTNNB1, set the excitation 488 Argon laser power (20% Argon) to 5% and a HyD (gain 100%) at a 496 – 559 nm bandpass.
    b. For SiR-DNA, set the excitation 633 laser power to 6% and a PMT detector (gain 700%) at a 642 – 714 nm bandpass.

Note: Using a HyD (hybrid detector) for detection is more sensitive compared to PMT (photomultiplier tube) detectors and gives a better signal to noise ratio (especially for higher wavelengths, i.e. red and far-red) (Schweikhard et al., 2020). However, care must be taken when imaging signals that could oversaturate the HyD, as it will automatically be shut down to prevent damage. For example, dye background of high intensity could cause the detector to shut down. If this happens midway during a time trace, the experiment will be cut short. HyD detectors should always be used at a gain of 100%.

Note: Laser settings can depend on the age of the laser units (as laser power decreases over time) and on the detectors of the specific microscope setup. Therefore, they might need further optimization. If using a different cell line or fluorescent signal, laser, and detector

settings will also need to be adjusted (see troubleshooting problem 2).

Critical: Low laser powers were used to prevent photobleaching and phototoxicity during the time trace of 8 hours. For both signals, this might need to be optimized.

Setting up imaging positions

Timing: 1-2 hours

11. Place a large drop of oil on the 63x oil objective. In addition, spread a generous amount of oil across the whole bottom of the 8-well ibidi slide (as illustrated in figure 1A).

12. Place the 8-well ibidi slide in the slide holder. To add the treatment later on in the experiment, place the lid loosely on top of the slide for easy removal without affecting the placement of the slide.

13. To prevent photobleaching of the SGFP2-CTNNB1 signal, turn the 488 laser to 0% and move the cells into focus based on the SiR-DNA nuclear signal.
    a. Make sure to image all cells on the middle nuclear plane. This is the plane where the nucleus is the largest and fully in focus (figure 1B).

Note: We chose to sample all cells (or the majority of cells in a frame) at the middle nuclear plane. This ensures a good readout of the nuclear signal for all cells and an equal representation of the membrane where cells are adherent, as at different lower or higher planes the membrane might be overrepresented. If the time-frame and number of samples allows it, Z-stacking could be used to provide an overall signal by imaging multiple levels of each cell, although this complicates downstream analysis.

14. Mark 3 positions per well in the stage panel "multi-position scanning". Select the positions based on the SiR-DNA signal for an unbiased approach. Select positions where cells are of the required density of ±50% confluency (as seen in figure 1C).
    a. To reduce scanning time per run:
        i. It is best practice to group positions together in each well (figure 1D, top panel) and then make a larger move to the next well, as autofocus will need less time to adjust for the grouped positions.

ii. It is best to choose the shortest route through all wells (figure 1D, bottom panel), as again autofocus will need less time and oil is less dispersed on the slide, which prevents drying out.

b. To prevent shading of the wells and/or autofocus artefacts it is best to choose positions in the middle of each well.

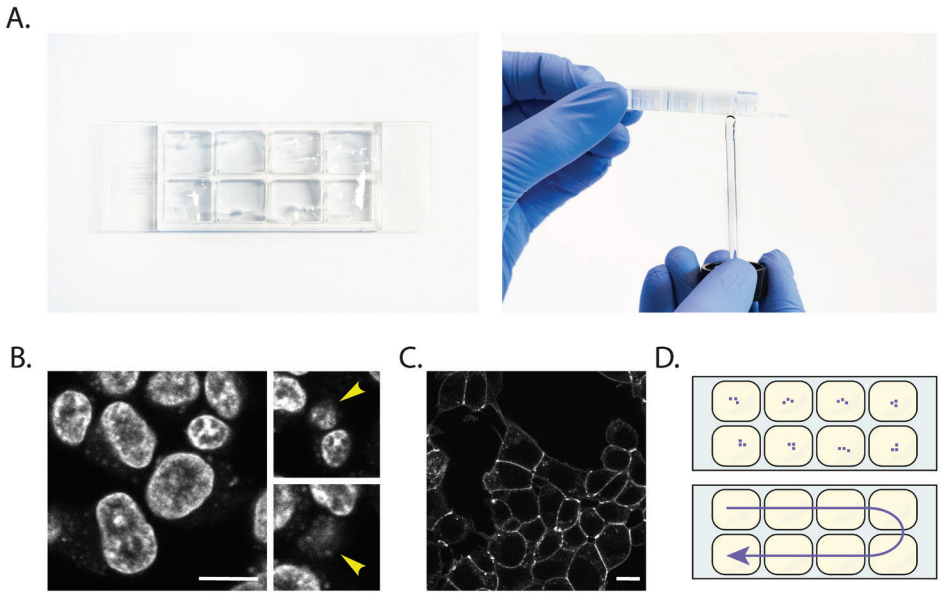


Figure 1. Sample preparation and microscopy setup examples

A). Photographs of oiling the slide for live imaging. Top view of oil on the bottom of the slide and side view of putting oil on the slide (keep the slide straight whilst doing so). B). Representative image of middle Z-plane of the nucleus, showing the SiR-DNA signal of HAP1 cells (from one of the 5 datasets used in chapter 4). Two examples of cells in incorrect Z-planes are shown in the side panel (yellow arrow). Since it is will likely be impossible to get all cells in the same plane, choose a plane that is the best overall. Scale bar 10 µm. C). Representative image of the density of HAP1 SGFP2-CTNNB1 cells at the start of imaging (from one of the 5 datasets used in chapter 4), showing the CTNNB1 signal. Scale bar 10 µm. D). Schematic representation of multi-position scanning locations with respect to the wells and slide (top) and the scanning direction (bottom). Make sure the positions in each well do not overlap even in the face of cell movement during imaging, but are close together so that a large leap is only required between wells (and not between positions within the well) to optimize the time needed to scan and autofocus.

Note: Instead of multi-position scanning, tiling could also be used to gain images of a high number of cells.

Note: Optimal density must be determined for each cell line and experiment. A balance must be struck between too high confluency, leading to overgrowth of cells during the imaging time-lapse and too few cells, leading to a low amount of data points for quantification.

15. Set the time interval
    a. In the time panel, press "minimize" to reveal the minimum scanning time needed to complete a single run. This time indication does not include the time needed for autofocus, so add ± 30 seconds for this.
    b. Set the time interval to 5 minutes (which is feasible with a total of 24 imaging positions in our set up) and total duration to 8 hours.

Note: The speed of the biological process that is being imaged ideally should determine the time interval. As in our case, CTNNB1 accumulation occurs over a timespan of 1-4 hours, a time interval of 5 minutes is sufficient. For faster dynamics, shorter time intervals will be preferred. This will limit the number of independent wells and experimental conditions that can be imaged.

16. Run a trial run for 10-30 minutes with the 488 laser at 0% to prevent photobleaching of the SGFP2-CTNNB1 signal to ensure that all wells and positions are imaged as desired and no drifting occurred whilst setting up the positions.

17. Check every position for any Z-drift or errors introduced by autofocus and adjust focus manually to select the mid-plane of every nucleus again (step 13). Update every position after adjustment.

18. Set the laser intensity for the 488 laser back to 5% or optimized value for your setup.

Live imaging SGFP2-CTNNB1 HAP1 cells on the Leica SP8 microscope and adding treatments during imaging

Timing: 8 hours / overnight

19. Start the experiment for the pre-treatment time-lapse. This allows signalling levels of the protein of interest to be measured when the pathway is in the "off" state (in case a signalling activator will be added later on).

Critical: Make sure you loosely place the lid on the ibidi slide so it can be removed without moving the slide (which could disturb the focus and position settings) to add the treatments later (step 21).

Note: To prevent evaporation of the imaging medium, a demi dish with water can be placed inside the incubator cage to create a humified environment.

20. Meanwhile, prepare the treatment solutions in culture medium at 6x the desired end-concentration. For example, for a desired final concentration of 100 ng/ml WNT3A, a treatment solution of 600 ng/ml is prepared such that 50 µl of this solution can be added to a well already containing 250 µl imaging medium.
    a. Prepare treatment solutions for a final concentration of:
        i. 25 ng/ml WNT3A by making a 150 ng/ml solution (1 µl 50 µg/ml stock in 333.3 µl culture medium).
        ii. 100 ng/ml WNT3a by making a 600 ng/ml solution (2 µl 50 µg/ml stock in 166 µl culture medium).
    b. Prepare treatment solution for a final concentration of 4 µM CHIR99021 (a small-molecule GSK3 inhibitor) by making a 24 µM CHIR99021 solution (1 µl 6 mM stock in 250 µl culture medium).
    c. For control conditions, the vehicle controls are diluted in culture medium (for WNT3a 0.1% BSA was used and for CHIR99021 DMSO).
    d. More treatments or concentrations can be added as desired, as there are 8 wells available.

Note: Treatment solutions could also be prepared prior to setting up the imaging positions and kept on ice, but preferably not more than 1 hour prior to imaging as this might affect the potency of recombinant WNT3A protein.

Note: As WNT3A ligands are lipid modified they are poorly soluble in water and are therefore best dispersed in medium containing proteins. Therefore, FBS was added to the culture medium.

21. After 50 Minutes, stop the imaging run and immediately add the treatments to the correct wells of the sample.
    a. Carefully remove the loose lid from the 8-well ibidi slide without touching or moving the slide.
    b. Add 50 µl of 6x concentrated WNT3A and/or CHIR99021 treatments dropwise to 250 µl of imaging medium in each well to reach a final concentration of 1x, using a p200 pipette. Take care not to touch the plate or walls of the wells whilst pipetting.
    c. Carefully place the lid loosely back on the 8-well ibidi slide. Pressing on the lid will move the slide and positions will be lost.

Critical: Great care must be taken and steady hands must be used whilst adding the treatments and removing/replacing the lid to prevent the slide from moving. If the slide is moved, positions will be at a different location in the wells or even in another well. However, leaving the lid off completely will lead to evaporation of the imaging medium during imaging and cell death.

22. Start the imaging again for the post treatment time-lapse and let it run for 6-8 hours. For convenience, this step can be performed overnight.

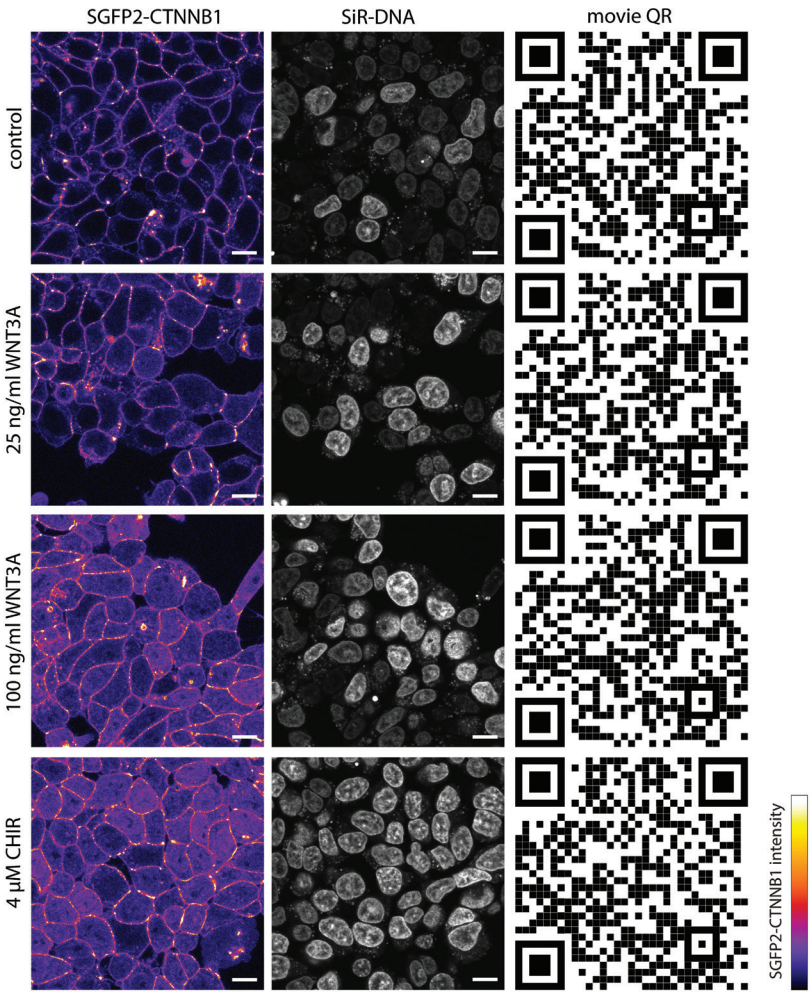
Note: After 6 hours imaging in total (pre + post treatment), we start to observe signs of cell death. Therefore, 8 hours seems to be the maximum timespan over which these cells can be live imaged under the conditions used in this protocol. (See troubleshooting problem 4 if cell death still occurs).

Expected outcomes

We expect to gain separate live-cell imaging series pre and post treatment of multiple cells per position (for HAP1 cells at 63x usually ±20-30 cells per position). When using an 8-well ibidi system, 8 conditions can be taken with 3 positions for each timepoint when imaging every 5 minutes. This means that for each treatment condition, data for approximately

60-90 cells will be obtained over the time course of the experiment. In our experience, we sometimes observe signs of cell death (detaching or rounding cells) from 6 hours onwards.

The images will show CTNNB1 localization in different cellular compartments. Under unstimulated conditions (movie 1), we observe high membrane levels of CTNNB1 (most likely related to its function in cell adhesion) and almost no signal in the cytoplasm and nucleus. When stimulating with WNT3A or CHIR, an increase in CTNNB1 levels in the nucleus and cytoplasm can be observed by eye starting after approximately 2 hours post-treatment (movies 2-4). Segmentation and quantification (as detailed below) of the fluorescence intensity in the membrane, cytoplasmic, and nuclear compartment allows different signalling pathway dynamics to be interrogated.



Movie 1-4 (previous page). Timelapse movies of SiR-DNA and SGFP2-CTNNB1 signal upon WNT3A and CHIR22091 stimulation

Movies 1-4 (scan QR codes to view) show confocal live cell imaging data (from one of the 5 datasets used in chapter 4) of the SiR-DNA and SGFP2-CTNNB1 signal for a duration of 7 hours (imaged every 5 minutes). Scale bars are 10 µm and time indication is in seconds. SGFP2-CTNNB1 intensity is shown as a fire LUT. Treatments or vehicle controls are added at timepoint zero. Treatments were as follows: DMSO vehicle control (movie 1), 25 ng/ml WNT3A (movie 2), 100 ng/ml WNT3A (movie 3) and 4 µM CHIR99021 (movie 4). Still images depicted here are at 4 hours post treatment.

Quantification and statistical analysis

Cell profiler segmentation and measurements of nuclear, cytoplasmic and membrane compartments

This section describes the analysis of the images in Cell Profiler (Stirling et al., 2021) to obtain quantitative measurements. Prior to processing in Cell Profiler, images were saved as TIFF files in ImageJ/Fiji by opening every position time trace and using the "save as > image sequence" option to create TIFFs separated by channel, timepoint, and position. For convenience, all TIFF files must be given an ordered name such as "ExperimentNumber_Treatment_Position_Timepoint_Channel", that can be read as a regular expression by Cell Profiler. We assume that the Cell Profiler pipeline made for this protocol (see data and code availability) is used for the analysis described below. For a general explanation of the Cell Profiler software and its modules, the reader is referred to the Cell Profiler manual (which can be found on https://cellprofiler.org/manuals).

In short, we will use the nuclear SiR-DNA dye signal to retrieve RAW segmentation masks of the nucleus (Cell Profiler Primary Object module) (figure 2A). We will then use the CTNNB1 membrane signal to retrieve RAW segmentation masks of the whole cell (Cell Profiler secondary object module) (figure 2A). In the secondary object module, Cell Profiler will use the location of the nucleus (primary object) to find the corresponding cell segmentations, linking each cell to its own nucleus. To perform measurements of CTNNB1 intensity in the nuclear, cytoplasmic, and membrane compartments, we will use the RAW segmentation masks to perform calculations that will provide slightly smaller segmentation masks that can be used for quantification (figure 2B). A segmentation mask will be created for each cellular compartment (nucleus, cytoplasm, and membrane) and will include safety margins to prevent any overlap between compartments resulting from imprecise segmentation at the borders for the nuclear and cytoplasmic compartment (figure 2C). As the membrane compartment spans only a few pixels by itself, unfortunately safety margins cannot be included for the membrane segmentation masks. Both the raw segmentation and calculations are performed in Cell Profiler and described step-by-step below.

Timing: 1 day

1. Open the Cell Profiler software (version 4.2.5) and load the segmentation pipeline. Change output folders, so that images and data files are saved in the correct location.
    a. Module: "SaveImages -> Output file location > Sub-folder"
    b. Module: "ExportToSpreadsheet > Output file location > Sub-folder"

2. In "Images", load the TIFF images (separated by channel, position and timepoint) and make sure the regular expression in the Metadata is corrected for the filenames.
    a. Filename and corresponding regular expression:
    Filename: ExperimentNumber_Treatment_Position_ Timepoint_ Channel
    Regular expression: ^(?P<Experiment>.*)_(?P<Treatment>.*)_position (?P<Position>[0-9]{3})_t(?P<Time>[0-9]{3})_c(?P<Channel>[0-9]{3})
        i. For example: FW006B3_control_position001_t001_c001.TIFF
    b. Click on "update" and check if the files are correctly sorted based on their timepoint, channel, position, treatment and time.

Critical: It is important that files are correctly sorted as this information will be used for the extraction of measurements. Use the info (question mark symbol) and regular expression editor included in the Cell Profiler software if changes need to be made to the regular expression.

3. In "NamesAndTypes", make sure that each channel is linked to the correct signal.
    a. For our image file names, the image channel was notated as "c001" or "c002", which corresponded to the SGFP2-CTNNB1 signal and the SiR-DNA signal, respectively. If your channels are annotated differently, adjust the rule criteria in "NamesAndTypes".
4. Segment the RAW masks of the nucleus and cell (examples in figure 2A).
    a. Module: "IdentifyPrimaryObjects".

i. This module will segment the nucleus based on the SiR-DNA nuclear signal.

b. Module: "IdentifySecondaryObjects"

i. This module will segment the cell based on the SGFP2-CTNNB1 signal (high intensity membrane signal).

Note: For different cell lines, settings in the modules "IdentifyPrimaryObjects" and "IdentifySecondaryObjects" might need to be adjusted as segmentation is set to occur for a defined nuclear and cell size (optimized for the HAP1 SGFP2-CTNNB1 cells). Moreover, if a different protein of interest is used or when different laser settings are used, intensity boundary settings of the modules should also be optimized.

5. Processing of the RAW masks to create segmentation masks for quantification (as described in figure 2C).

   a. Depending on the pixel size of your images and cell size, the number of pixels used for erosion and dilation for the safety margins of the nuclear and cytoplasmic compartment (set to 2 pixels for this protocol) might need to be adjusted in the "ExpandOrShrinkObjects" modules.

   b. Depending on the resolution of your images, the number of pixels used as the membrane thickness for the membrane mask (figure 2D, set to x=4 pixels for this protocol) might need to be adjusted in the "ExpandOrShrinkObject" modules.

6. Before running the full dataset through the pipeline (which, in our experience, may take several hours), run a test set of images in test mode to see if any errors occur. By clicking the eye symbols in front of the modules you can see the output every module gives. It is important to check if the segmentation occurs as expected.

   a. Check if the nuclei are segmented (border nuclei are excluded). Some errors are expected and acceptable (e.g. for nuclei that are in close proximity). Any small background spots should not have been segmented as nuclei. As an example, see figure 2A.

   b. Check if the cell outlines are indeed segmented along the membrane.

Again, mis segmentation of 1-2 cells per image is to be expected, as exemplified in figure 2A.

Note: The pipeline could be optimized for optimal segmentation. If too many errors occur, the module "EditObjectsManually" for manual selection of segmentation. However, this is a time-consuming step.

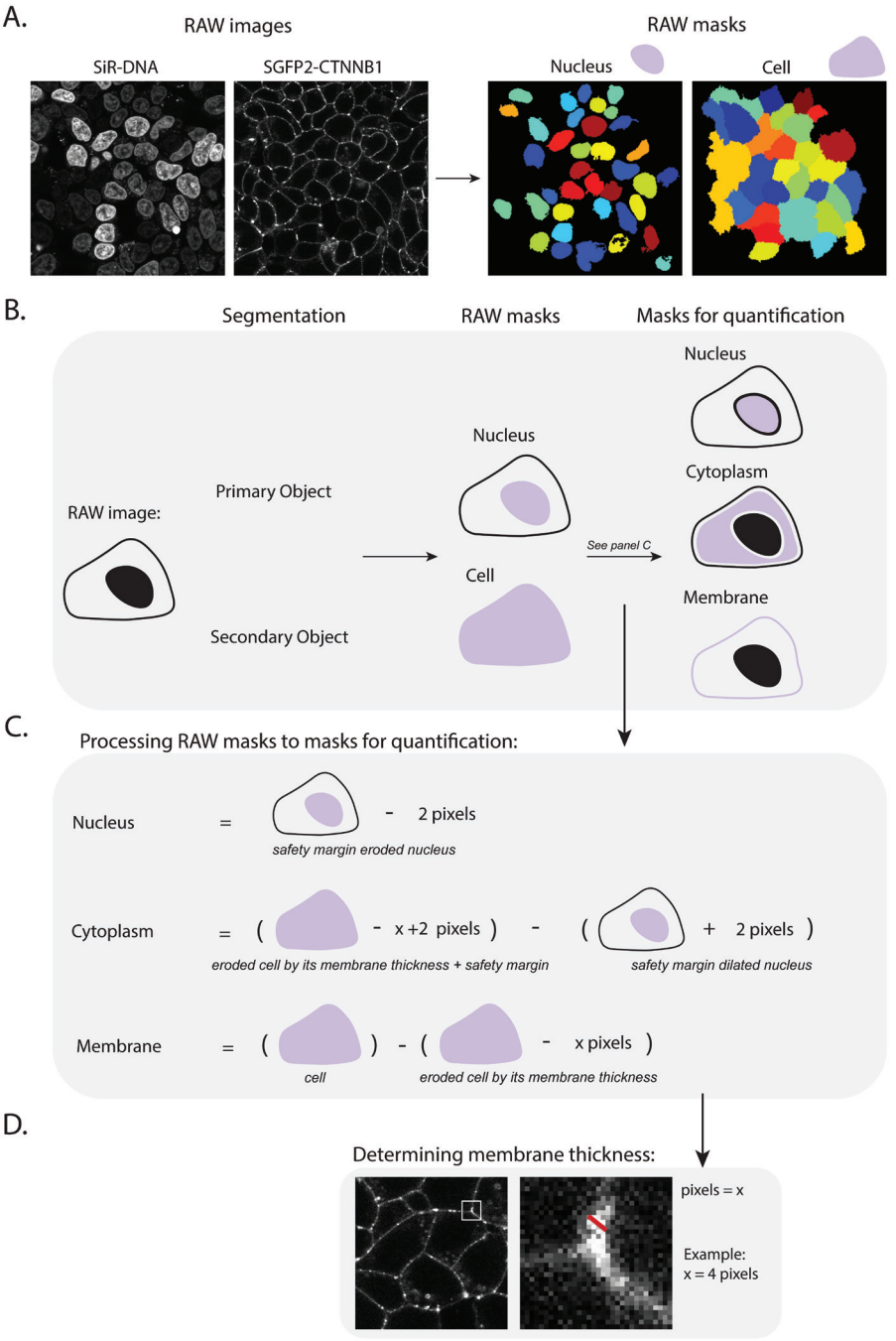
7. Run the pipeline on the complete dataset. This may take several hours depending on your hardware. To speed up the run, turn off the eye symbols for all modules.

8. CSV files with intensity measurements will be saved to your chosen subfolder as output. Mean intensity values can be taken and processed further in excel or R. It is up to the investigator to transform these measurements of protein level intensity in the different cellular compartments into useful biological insights. For example, besides nuclear/cytoplasmic CTNNB1 ratios as a measure for WNT/CTNNB1 signalling activity, one might also want to explore pathway kinetics (e.g. by plotting normalized intensities over time), or to plot relative abundance of a protein in different cellular compartments. See chapter 4 for examples.

Note: Developments in the area of cell segmentation are rapid and continuous, so of course other approaches to cell segmentation can be taken. For example, recent developments in deep-learning segmentation models, such as Cell Pose, could greatly improve segmentation as it can be trained for the specific hurdles of your dataset (e.g. heterogeneous signal or size).

Figure 2 (next page). Schematic overview of segmentation processing to obtain compartment measurements
A). RAW images of the SiR-DNA and SGFP2-CTNNB1 signal (from one of the 5 experiments from chapter 4) are segmented in Cell Profiler to obtain the RAW nuclear (primary object identification based on SiR-DNA signal) and cell masks (secondary object identification based on SGFP2-CTNNB1 signal). These are representative images for unstimulated HAP1 cells. The quality of segmentation, including several errors is representative as well, as we observed 1-2 mis-segmentations for most images. B). Schematic representation of analysis workflow starting with the RAW images (black) to segment in Cell Profiler to obtain the RAW nuclear and cell masks. RAW nuclear and cell masks are then processed (as depicted in C) to obtain the segmentation masks for each cellular compartment (nucleus, cytoplasm, and membrane) for quantification of CTNNB1 intensity. C). Schematic description of RAW mask processing to obtain the masks for quantification. Nucleus: the RAW nucleus mask is eroded by 2 pixels as a safety margin. Cytoplasm: the RAW cell mask is eroded by the membrane thickness (x pixels) and an additional safety margin of 2 pixels (in between the cytoplasm and membrane). The RAW nucleus mask is dilated by 2 pixels as a safety margin (in between the nucleus and cytoplasm) and subtracted from the eroded cell mask. Membrane: the RAW cell mask is eroded by the membrane thickness (x pixels, as determined in D). D). Example of membrane ▶

thickness determination. A representative SGFP2-CTNNB1 image is taken and zoomed in at the membrane levels. Determine the number of pixels spanning the membrane at several locations (as shown by the red line) and determine the membrane thickness based on this.



Correcting the membrane intensity measurements

Care must be taken when interpreting the measured intensity at the cell boundary from confocal images as "membrane signal", as the width of the plasma membrane is smaller than the resolution that can be obtained by confocal laser scanning microscopy, which is limited by the point spread function (PSF) (Cole et al., 2011). Therefore, the fluorescence intensity measured at the membrane (and thus also the signal segmented with the membrane mask) will inherently also contain signal from the cytoplasm. Correcting for this is important, especially when the cytoplasmic signal changes over time, as is the case for CTNNB1, and when subtle changes in cytoplasmic and membrane distribution are to be quantified (as we do in chapter 4).

For simplicity, we incorporated a first order correction to subtract the average cytoplasm intensity (ACI) from the average membrane intensity (AMI). For a cell that is surrounded by background, the correction factor is set to 0.5, reflecting a situation in which half of the measured intensity comes from the cytoplasm, rather than from the membrane (see figure 3B i). For two touching cells, the correction factor is set to 1, reflecting the fact that half of the measured intensity contains signal from the cytoplasm on the right and half from the cytoplasm on the left of the membrane (see figure 3B ii). Rather than applying this correction on a per cell basis, we considered the overall neighbour fraction for the cells in our experiment and average mean cytoplasmic intensity for the whole population at an imaging position at each time point to allow a global correction for all membrane measurements. For our samples (and chapter 4), this correction factor was set to 0.75 (reflecting a situation in which cells are sub-confluent, but a fraction of membranes is touching at the time of imaging). We then used formula 1 to obtain a corrected membrane intensity. The global correction factor can be increased to 1 for fully confluent samples or lowered to 0.5 for isolated cells as desired, depending on the cell density in the experiment that is being analysed.

Formula 1. Formula to correct the membrane intensity

Formula to correct the intensity measured in the segmented membrane compartment for any overlap of the cytoplasmic compartment, resulting from the fact that the lateral resolution of the confocal microscope is larger than the actual width of the membrane. As the neighbour fraction (i.e. the percentage of cell membrane touching neighbouring cells) of the cells used for this analysis was not 100% (i.e. cells were not fully confluent), (0.75* ACI) was chosen to correct the membrane intensity.

$$\textit{Corrected membrane intensity} = (AMI) - (0.75 * ACI)$$

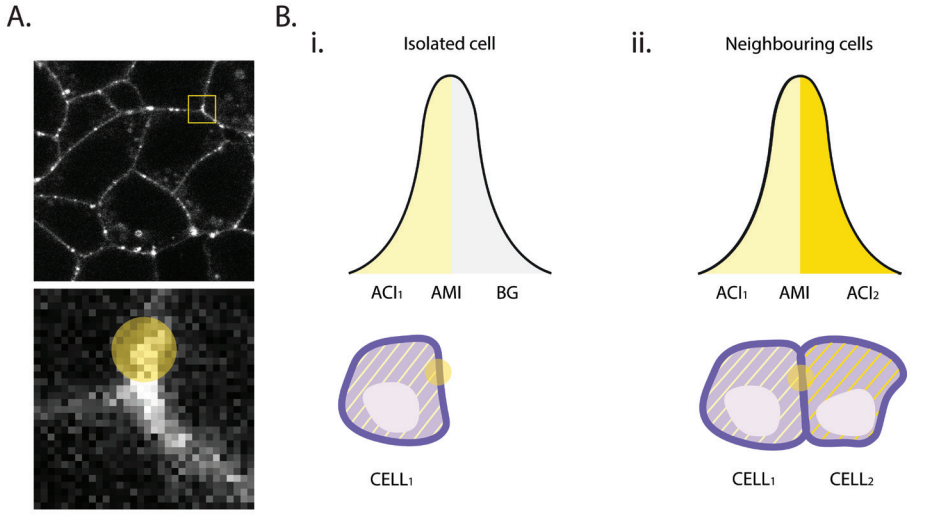


Figure 3. Correcting the average measured membrane intensity in neighbouring and isolated cells

A). Enlarged images (same image as used in figure 2D) of SGFP2-CTNNB1 signal. Enlarged area of the membrane, with a circle (representing the gaussian beam) on an area of pixels crossing the membrane. B). Cartoon, depicting side view of the Gaussian beam across isolated cells (i) and neighbouring cells (ii). i). Average membrane intensity (AMI) bordered by average cytoplasmic intensity (ACI) signal on one side and background signal (BG) on the other. For a membrane region from an isolated cell, $0.5*ACI_1$ is subtracted as a first order correction. ii). AMI bordered by ACI on either side. Top view: For a membrane region that has neighbours on either side, $0.5*ACI_1+0.5*ACI_2=1*ACI$ in total is subtracted as a first order correction. For chapter 4, we used a global correction factor of 0.75*ACI, assuming that cells were sub confluent on average.

Limitations

Several points should be taken into consideration when applying this protocol. When imaging CTNNB1 levels in both the nuclear, cytoplasmic, and membrane compartments, intensity issues arise. As membrane CTNNB1 levels in unstimulated conditions are much higher compared to the CTNNB1 levels in the nuclear/cytoplasmic compartments, it can be difficult to achieve laser settings that allow for optimal saturation of all three compartments. Additionally, the laser settings are determined at the start of the experiment, when the cells are unstimulated. It can be difficult to predict how these settings will perform in stimulated conditions later on in the experiment. This may require some troubleshooting for your specific protein of interest in the first few experiments. Over and under saturation can lead to loss of depth in the intensity measurements used for quantification later on, meaning that the signal might be underestimated. The specific challenges encountered will depend on the individual user application.

On a more conceptual note, we want to add that when using the abundance of a signalling pathway's effector protein (such as CTNNB1) as a proxy for signalling pathway activation, we are making the assumption that all protein residing in the nucleus is transcriptionally active. Complementary methods that take a more direct approach to measuring transcriptional activity, such as luciferase reporter assays and qRT-PCRs, can be taken alongside, although they will lose the single cell and spatio/temporal information gained by taking this imaging-based approach.

Troubleshooting

Problem 1

Due to its localization in adherens junctions, CTNNB1 lends itself perfectly as a membrane marker. As such, we chose to segment the cell outline based on the CTNNB1 signal. When imaging another protein of interest with less defined membrane signal, this may pose problems. In addition, one might argue that using the signal of interest for segmentation is not ideal.

Potential solutions

When the protein of interest cannot be used to segment the cell outline, a dedicated membrane marker can be added to the experiment. However, it must be taken into account that this will add to the imaging time of each position, which might mean that the time in between time points would need to be increased or the number of positions decreased.

- A variety proteins or motifs are commonly used as membrane markers that could be stably expressed via random insertion, piggy-bac or lentiviral induction, for example CAAX linked to fluorescent proteins (Clarke, 1992). Piggybac transfection can allow relatively rapid generation of such cell lines (but will need to be followed by clonal selection to ensure even signal intensity). Lentiviral infection with low multiplicity of infection (MOI) offers an alternative approach. Of course, care should be taken with creating a cell line that has random insertion of a construct, as this could cause additional effects depending on the location of insertion. Even so, temporary transfection will reduce the number of cells that can be taken for analysis as not the whole population will be transfected with the membrane marker and would therefore be less preferable (and less well reproducible between experiments).
- To avoid making a stable line with a membrane marker, commercial stains could

be used, such as CellBrite dye (Biotium) or PKmem-650 probe (Spirochrome). It must be kept in mind, however, that these too require optimization by individual users for different cell lines and experimental applications.

Problem 2

The protocol described here was optimized to image SGFP2-CTNNB1 under unstimulated and stimulated (WNT3A/CHIR99021) conditions. For different proteins of interest and/or fluorescent proteins used for tagging, laser and detector settings will need to be optimized.

Potential solutions

Different signalling proteins, other than CTNNB1, might be expressed at different levels, for which laser setting would need to be adjusted. In addition, the choice of fluorescent protein could also influence laser settings. Ideally, a bright and photostable fluorescence protein with rapid maturation and other preferable characteristics is chosen. The laser settings used in the protocol may be used as guidelines for balancing signal strength, signal-noise ratio and phototoxicity when live imaging.

Sequential scanning might be needed when overlap of the fluorescent spectra is present (as would be the case for proteins in the cyan and green spectra or green and red spectra for example). In our case, we purposely used SGFP2-CTNNB1 and SiR-DNA (far-red), which do not overlap. Using sequential scanning will double the scanning time. This will increase the time interval that can be used, decreasing the temporal resolution or will have to be compensated for with faster scanning speed, less averaging or less resolution, all of which will decrease the signal-noise ratio. Ideally, fluorescent proteins are used that do not overlap so that sequential scanning is not needed.

Problem 3

The SiR-DNA signal is weak.

Potential solutions

If the SiR-DNA signal appears weak when setting up the imaging, a longer incubation time prior to imaging could be applied. Shorter incubation times at the indicated concentration should be prevented. In our hands, when setting up imaging 4 hours after adding the SiR-DNA, its intensity is still increasing. If the SiR-DNA intensity remains too low for the entire timelapse, excitation laser intensity could be increased as SiR-DNA is less prone to bleaching

than SGFP2-CTNNB1 (although care must be taken not to induce phototoxicity). For different cell lines, the SiR-DNA concentration might have to be adjusted.

Problem 4

Cell death occurs before 8 hours or for the biological process imaged a longer imaging timespan is needed.

Potential solutions

Cell death during imaging can occur due to a multitude of reasons. First, to provide easy access to the sample for adding treatments, the atmosphere might induce cell death. Imaging in a gas chamber with 5% CO2 could easily prevent this, but will also make it impossible to add treatments during imaging. Treatments will then have to be added prior to starting imaging and setting up, which might lead to loss of information due to missing timepoints at the start of treatment. Second, SiR-DNA could induce toxicity. To prevent this, a cell line with stable nuclear labelling could be created (for example with H2B or PCNA). Lastly, to reduce phototoxicity (and bleaching), longer time intervals or lower laser powers (the signal strength allowing) could be implemented. We do note that at least the onset of most signalling events (and thus activation dynamics and kinetics) of interest that are immediately downstream of a specific stimulus (treatment) should be captured within the first 4 hours.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts Marten Postma (m.postma@uva.nl) and Renée van Amerongen (r.vanamerongen@uva.nl).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts Marten Postma (m.postma@uva.nl) and Renée van Amerongen (r.vanamerongen@uva.nl).

Materials availability

The HAP1 SGFP2-CTNNB1 cell line used in this protocol can only be distributed under a material transfer agreement (MTA) by obtaining written consent from either the NKI or the

Whitehead Institute where the original HAP1 cell line originated.

Data and code availability

The Cell Profiler pipeline used in this protocol can be downloaded from https://osf.io/xjsu2.

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Author contributions

TvdW – data curation, visualization, manuscript writing

SdM – live imaging protocol set up and optimization

MP – conceptualization, manuscript review and editing

RvA – manuscript review and editing

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