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► **To cite this version:**

Fabien Furfaro, Carine Vias, Benoit Sorre. Using microfluidics and live cell reporters to dissect the dynamics of TGF- $\beta$  signaling in mouse Embryonic Stem Cells. *Methods in Molecular Biology*, 2021, TGF-Beta Signaling, 2488, pp.125-143. 10.1007/978-1-0716-2277-3\_10 . hal-03437837

**HAL Id: hal-03437837**

**<https://hal.science/hal-03437837>**

Submitted on 20 Nov 2021

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# Using microfluidics and live cell reporters to dissect the dynamics of TGF- $\beta$ signaling in mouse Embryonic Stem Cells

Fabien Furfaro, Carine Vias and Benoit Sorre

## Abstract

The TGF- $\beta$  pathway is known to behave as a classical morphogen, meaning that it can dictate cell fate decisions in a dose dependent manner. Recent observations however showed that in addition to the absolute value of morphogen concentration, cells could also extract information from its temporal variations.

In the present article we describe how to use automated microfluidics cell culture to stimulate cells with precisely defined temporal profiles of morphogens and how to engineer mouse embryonic stem cells with fluorescent reporters of pathway activity to record in real time their response to the applied stimulations.

The combination of automated cell culture and of live cell reporter provides a complete toolbox to study how cells encode the information carried by time-varying TGF- $\beta$  signals.

**Running Head:** Dissecting TGF- $\beta$  Pathway Dynamics with microfluidics

**Key words** microfluidics; automated cell culture; mouse embryonic stem cells, live cell imaging.

## 1. Introduction

Signaling molecules such as TGF- $\beta$  are ubiquitous in development where they are used to instruct cells on their fate decisions and control tissue patterning among other functions. Over the last decades, molecular genetics has identified the molecular architecture of the TGF- $\beta$  pathway (1). However, signaling pathways are never simple linear transducers from the external world to the inside of the cell. Signal transduction involves negative or positive feedbacks that can occur at the level of the receptors, signaling effectors or transcriptional targets. To complexify the picture, signaling pathways often crosstalk between each others and all these signaling modulations can be cell-type specific (2). Therefore, knowing the molecular players involved in a pathway does not permit to predict how a cell will compute the external information provided by the spatio-temporal profile of ligand concentration to make a fate decision. This question is crucial for the TGF- $\beta$  cytokine, as it has been shown to work as a morphogen *i.e.* that it can control cell fate in a dose-dependent manner (3). In addition to its absolute value, other properties of ligand concentration such as its temporal variation have been proposed to carry meaningful information (4, 5).

To assess how cells encode the information provided by extracellular signaling cues, one alternative approach consist in considering the cell as a black box, thus ignoring molecular details of signal transduction, and record in real time the cell response to various well-defined temporal profiles of stimulations (6–8). To apply a well-defined temporal profile of ligand concentration on cells, microfluidics is a tool of choice. Indeed, if making simple changes like concentration "steps" can be easily done by hand, more complex signals such as high frequency pulses or concentration ramps quickly become impossible to carry out manually, especially as the duration of the experiment is long (*e.g.*, change the concentration every 4 h for 4 days). Microfluidics devices incorporating on-chip valves, because they allow fully automated operation, make this kind of temporal profiles of stimulation achievable (9). We describe in the first part of this report the characteristics of the chip we used and the procedures to grow and stimulate cells for extended durations in such devices.

To make the link between cell response and the signal it received, the second prerequisite is to be able to record cell's response to stimulation in real time, which can be achieved by live-cell imaging of fluorescent imaging of reporters of signaling activity. We detail below the strategy we have used to monitor how mouse Embryonic Stem Cells (mESC) respond to stimulation of the TGF- $\beta$  pathway. Ligand binding to the TGF- $\beta$  receptor

triggers translocation of SMAD2/3 and SMAD4 into the nucleus, which activates expression of transcriptional targets of the pathway. Nuclear localization of SMADs has thus been used as a proxy to monitor pathway activity (10, 11). In previous works, we have used C2C12 cells stably overexpressing fusions of SMADs with a fluorescent protein on their N terminus. C2C12 clonal lines selected to have low levels of over expression accurately reported pathway activity in real time (4, 5). In other cell types such as mouse Embryonic Stem Cells, this approach was however not successful. Overexpressed RFP-Smad2 failed to translocate into the nucleus upon pathway stimulation by ACTIVIN. We thus changed our strategy to insert the fluorescent protein directly in the N-terminus of the endogenous *Smad2* loci using crispr/CAS9 activated homology directed repair, as was recently done in hESC (12, 13).

To correlate the dynamics of SMAD transcription factors with the downstream transcriptional response, synthetic promoters specific to the Smad2/3 branch of the TGF- $\beta$  pathway (CAGA12) (14), 3TP (15) or ARE (16) driving the expression of a luminescent reporters such as luciferases have been traditionally used.

The signal produced by a synthetic reporter is more straightforward to analyze, as it is independent of potential transcriptional feedback that could modulate a natural promoter. To monitor transcriptional response in mESC we took advantage of the ASE:YFP reporter cell line. The ASE element is one of the enhancers controlling expression of the gene *Nodal*, it is active around gastrulation and its activity has been shown to be dependent on SMAD2/3 and co-factor FOXH1 (17, 18). We show that the fluorescent signal produced by the ASE:YFP reporter is specific of TGF- $\beta$  pathway activity as it is promptly induced following ACTIVIN stimulation and completely undetectable in cells treated with the TGF- $\beta$  inhibitor SB431542. Finally, we provide guidelines on how to extract information from images using simple, open source, image analysis techniques

## 2. Materials

### 2.1 PDMS multilayer Cell Culture Chip

The chip we are using is based on the one described in (9). Keeping the same functionalities, we have reduced the number of chambers from 96 to 24 so that the chip footprint could fit on a 0.16mm thick 24\*60mm coverslip while the original version was attached to 1mm thick 50\*75mm cover glass. Using thinner glass permit using objective having a short working distance and thus a higher numerical aperture.

The chip features 16 media inputs, an in-line peristaltic pump and a multiplexer that allow to connect any of the 16 media inputs to any of the 24 cell culture chamber independently (*see Fig.1A-1C*). An important feature of the chip is the chamber bypass (*see Fig. 1D*), this allows to minimizing dead volume and prevents cross stimulation of adjacent chambers. Our CAD design is available on demand. Protocols to design and build similar chips won't be presented here but can be found in (9, 19). The protocols described here to operate the chip are applicable to any other design incorporating valves on chips

### 2.2 Automated operation of the chip

1. Valve controller: The chip has 38 individual microfluidic valves in order to control media flow from inputs to chambers. Each valve line of the chip is connected to an individual water reservoir by microbore tubing and metal pins. A valve is closed when a pressure of two bars is applied on the valve's reservoir and open when it is at atmospheric pressure (*see Fig.1B, 1C*). Solenoid valves are used to switch the pressure applied to each valve line from 2bars (closed valve) to atmospheric pressure (open valve). To be able to achieve fully automated and unsupervised operation of the chip, valve actuation is controlled by a computer (*see Note 1*).
2. Computer Interface: Each solenoid valve controlling the state of each on-chip microfluidic valve can be actuated by a home made computer program written in Python (*see Note 1*). Our program is of course specific to the chip we used, but it should be easily adaptable to any other chip configuration and therefore

it provides a good starting point for implementation of automated microfluidics operations. The program takes care of initialization of the communication with the micro-controllers. It also contains basic functions to set the state (open or closed) of each valve and higher-level functions to operate the different chip modules (inputs, pump, multiplexer) without having to set valves states one by one. It is divided into 2 classes:

- Valve(SerialNumber): class for low level communication with the USB microcontrollers. It uses the "ctypes" python module to exploit the functions of the "ftd2xx64.dll" to establish communication with the microcontroller and read set the state (0 or 5V) of the output pins of the USB boards which controls the state of each solenoid valves. It contains the following functions:
  - `__init__(SerialNumber)` class constructor. It takes the microcontroller's serial number as an input and initialize communication with the microcontroller
  - `setbit(pin,state)` sets the state (0 or 1) of a given output pin of the USB microcontroller
  - `purgeValve()`: test function. Makes a valve blink to facilitate its identification
  - `Close()`: clean shut down of communication with the microcontroller.
- ScheduleValv(feeding\_protocol): Class encapsulating Valve() described above, contains functions used to interact with the chip in a more user friendly manner and to program an experiment.
  - `__init__(feeding_protocol)` class constructor. Establish the communication with the USB microcontrollers (using Valve()), makes the translation between comprehensive valve names and bit numbers of the USB micro-controllers. If information about a feeding protocol is provided (input arguments, optional) the function will build feeding schedule for each chambers.
  - `setvalve(stateVector)`: set valve(s) state by valve name. stateVector is a 2D vector containing valve names and their state.
  - `muxBits(chamber)` : computes the valve state combination of the multiplexer binary tree to send medium toward a given chamber.(see **Fig. 1D**)
  - `purgestep(inVALV)` : rince all feeding lines of the multiplexer with medium from input specified by « inVALV ». Medium is flowing through chamber bypass, not through the

chambers (see Fig1D). This is used for example to wash the multiplexer after feeding a chamber.

- feedOneChamber(chamber) : uses muxBit() to set the multiplexer to feed one chamber
- feedAllChamber() : send feeding medium to every chambers.
- closestep() : close all the valves of the multiplexer
- ApplyS() : Start Feeding Schedule. Function calling on the previous functions to follow the experimental protocol that was given to it as input. This function also log in a text file all action taken.

A script showing an example of how these functions can be used to set up an experiment is provided (*see Note 1*).

### ***2.3 Cell lines***

1. HM1 wild type mouse embryonic cell line (mESC) (Selfridge et al., 1992)
2. mESC cell line with the ASE-hsp68p-3nls-d2eYFP transgenic reporter (*see Note 2*), called “ASE:YFP” in the following (*17*).

### ***2.4 mESC cell culture and stimulation***

1. Tissue culture-grade 6 well plates.
2. Serological pipettes.
3. 15 mL conical tubes.
4. Phosphate-buffered saline (PBS)
5. 0.05% Trypsin-EDTA.
6. 0.1% gelatin solution in water.
7. Foetal Bovine Serum (FBS)
8. N2B27 base medium: DMEM/F12 and Neurobasal media (1:1) supplemented with 1× B27, 1×N2, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, penicillin and streptomycin.



9. mESC are cultured on 0.1% gelatin coated tissue culture grade plates in N2B27 supplemented with LIF (1000U/ml), PD0325901 (1 $\mu$ M) and CHIR99021 (3 $\mu$ M). They were fed every day and passaged every 2-3 days by dissociating them in 0.05% Trypsin for 5 min, neutralizing trypsin with 15% serum supplemented DMEM, concentrating cells by spinning, and transferring to a new plate.
10. Stimulation medium: N2B27 base medium supplemented with ACTIVIN at a concentration of 20ng/ml.
11. Puromycin
12. Blasticidin

## ***2.5 Cell culture chip preparation***

1. Phosphate Buffer Saline (PBS)
2. Fibronectin (working concentration 20 $\mu$ g/ml in PBS)
3. Pluronic® F-127 (working concentration 1% w/v in PBS)

## ***2.6 Microfluidic cell culture accessories***

Medium dispensing vials (*see Note 3* for assembly instructions):

1. 5ml cryogenic vials (fisherbrand cat#10-500-27)
2. Teflon tubing 0.022"ID 0.024"OD (cole parmer cat#06417-21)
3. 19G stainless steel pins (Harvard Apparatus)
4. Epoxy glue
5. Polycarbonate manifold: Masterflex™ 06464-87
6. Tygon tubing 1/16 inch OD (Masterflex™ cat #: FV-06419-01)

## ***2.7 Microscopes***

### ***2.7.1 Epifluorescence live cell imaging***

1. Inverted microscope Olympus IX81
2. 20x 0.75NA objective
3. Imaging: ANDOR Zyla sCMOS camera
4. Fluorescence excitation lamp : Xcite 120PC
5. Fluorescence filter cubes: GFP: CHROMA 39002; YFP:CHROMA 39003; RFP: CHROMA 39004
6. Environmental chamber to keep the cell at 37C and 5% CO<sub>2</sub> during experiments
7. Microscope control software: micromanager (**20, 21**)

### **2.7.2 Confocal live cell imaging**

1. Inverted microscope Olympus IX83
2. 20x 0.75NA objective
3. Imaging: DSD2 system (ANDOR) with Zyla sCMOS camera (ANDOR)
4. Fluorescence excitation lamp cooled PE6000
5. Fluorescence filter cubes: GFP: CHROMA 39002; YFP:CHROMA 39003; RFP: CHROMA 39004
6. Environmental chamber to keep the cell at 37C and 5% CO<sub>2</sub> during experiments
7. Microscope control software : ANDOR iQ3

### **2.8 Plasmids**

1. ePB-P-CAG:eGFPnls (**22**) (*see Note 4*)
2. ePB transposase
3. ePB-B-CAG:mCherry-nls
4. RFP-Smad2 homology donor (*see Note 5*)
5. pSpCas9(BB)-2A-Puro (PX459) V2.0 (addgene# 62988) (**23**)

## 3 Methods

### 3.1 *Setting up an experiment*

The microfluidic system is used as indicated in the literature (4, 8, 9), with minor adaptations of the automation protocol to meet the specificities of mouse embryonic stem cells culture. Although it is not strictly necessary, we recommend performing all the following steps under a microscope (10x phase contrast or bright field) for visual inspection.

#### 3.1.1 *Connect a new chip to the pressure controller*

1. Relieve the pressure at the valve controller
2. Connect the pressure controller to a new chip. Keep the old chip connected and directly transfer pins from the old to the new chip one by one. This will prevent inverting pins.
3. Put the pressure back on by increasing it slowly to 2 bars (30psi). If the chip holds pressure, check that valves are functioning correctly one by one (leak, collapse) under a microscope.

#### 3.1.2 *Passivate chip channels with antifouling agent to prevent adhesion of cells or proteins in the chip outside the culture chamber (see Fig.2A)*

1. Connect a medium dispensing vial filled with PBS +1% Pluronic® F-127 to one of the chips inputs. Pressurized it to 0.7bars
2. Open all the valves of the multiplexer, keeping the chamber inputs and output closed. Open the valve of the input where the 1% Pluronic® vial was plugged until all the multiplexer is filled. Incubate Pluronic® solution in the chip for at least 5 minutes.
3. De-pressurize the Pluronic® containing vial and disconnect it from the chip
4. Connect a medium dispensing vial filled with PBS to one of the chips inputs. Pressurize it to 0.7bars
5. Open the PBS input to flush PBS in the multiplexer. Flush for 5 minutes to be sure all Pluronic® is washed away.

6. Connect a medium dispensing vial filled with Fibronectin (20 $\mu$ g/ml in PBS) to one of the chips inputs. Pressurize it to 0.7bars
7. Open the input valve where the fibronectin vial is connected. Open the multiplexer to fill it with the fibronectin solution.
8. Open culture chambers one by one to fill them with the fibronectin solution. Flow Fibronectin solution until trapped air in the chamber disappears. To get rid of all bubbles, it may be useful to close chamber outputs as air is absorbed in the PDMS under pressure. Incubate Fibronectin in chambers for at least 30minutes.

### ***3.1.3 Loading cells in the culture chambers***

1. At least 2h before starting an experiment turn on temperature control of the environmental chamber of the microscope to 37C so that temperature stabilizes.
2. Set the CO2 controller to 5% and start flow in the microscope environmental chamber.
3. Connect a medium dispensing vial containing the cell suspension (see point 2.4) to one of the media inputs. Cells concentration should be adjusted depending on the desired final seeding density. Around 1 million cells per milliliter is a good starting point. Pressurize it to 0.7bars.
4. Load cells in culture chambers. Set the multiplexer that the cell suspension will flow to one chamber. Monitor the cell suspension flowing in the chamber with a microscope. When cells are in the chamber, close chamber to let the cells attach, and proceed to load cells into the next chamber. After 30-60 minutes, cells should be attached. A second round of cell loading can be done to increase initial cell density if necessary. It might be necessary to shake gently the vial from time to time to prevent cell sedimentation.
5. Rinse MUX. Close all chambers and open all the channels of the multiplexer. Flow PBS to rinse the multiplexer with DMEM + 10% FBS (*see Fig.2A*).

An example of Python script to perform these operations is provided (ScriptPreparation.py, *see Note 1*)

### ***3.2 Automated Long-term culture and stimulation of mESC with ACTIVIN***

1. Plug media vials with desired concentrations of ACTIVIN to the media inputs. Pressurize to 0.7bars. Purge air from media inputs tubing. This is achieved by opening media inputs one by one and flowing medium directly to the trash until all bubbles have been flushed.

2. Start automated feeding/stimulation script. Feeding procedures can be adapted to cell types and to the desired stimulation schedule. For mESCs culture, we found that renewing media every 1 hour by a 10s flush gave good results. This script defines how many times and how often this basic feeding loop should be repeated and at which frequency for each chamber depending on the desired temporal profile of TGF- $\beta$  stimulation. (*see Fig.2B*) . A general feeding round goes as follows:

- Rinse MUX channels with neutral medium (Open MUX, open neutral medium input)Set mux to desired chamber purge
- Flow feeding or stimulating medium in the desired chamber (either by pressure driven flow or using the inline pump to control the volume of feeding medium)
- Close chamber
- Rinse MUX channels with neutral medium (Open MUX, open neutral medium input, close MUX). To prevent cross stimulation of adjacent chambers, it is important that the neutral medium used to wash channels between feedings contains blocking proteins such as BSA or FBS to prevent nonspecific cytokine adsorption in the microfluidics channels.

An example of Python script to set up a simple experiment with steps of ACTIVIN concentration and pulses at various frequencies is provided (Script\_valvesOnly.py), (*see Note 1*).

### ***3.3 Measuring single cell response to TGF- $\beta$ stimulation in real time***

#### ***3.3.1 Generation of a RFP-Smad2 mESC reporter cell line***

1. Coat one well of a 24 well plate with gelatin (250 $\mu$ l of 0.1% gelatin solution in water). Incubation at least 30 minutes.
2. Prepare reagents for Lipofectamine 3000 transfection according to manufacturer's instructions. In 25 $\mu$ l OPTIMEM, Dilute 250 ng of cas9 plasmid, and 250ng of the homology donor (*see Note 5*), 1 $\mu$ l of p3000 reagent (Solution A). In 25 $\mu$ l OPTIMEM add 1.5 $\mu$ l Lipofectamine 3000 (Solution B). Mix solutions A and B. Wait at least 10 minutes.
3. Harvest mESC by trypsinisation, inactivate trypsin with DMEM+10%FBS. Centrifuge and re-suspend in N2B27 medium. Count cells.

4. Aspirate gelatin solution in the well from step 1. Fill well with 500 $\mu$ l of mESC growth medium (N2B27+2i+LIF) Add 50 $\mu$ l of the DNA+lipolectamine solution from step 2 to well.
5. Seed 100,000 cells in the well, insuring that cell repartition is spatially homogenous.
6. The next day, feed cells with fresh mESC growth medium (N2B27+2i+LIF)
7. 48h after transfection, harvest cells, seed in gelatin coated 6-well plates at a seeding density 100kcells per well. Supplement N2B27+2i+LIF medium with puromycin selection (0.5 $\mu$ g/ml). After 24h, switch back to regular mESC growth medium. The 24h puromycin pulse ensures enrichment of cells that received the cas9 plasmid.
8. Grow cells for 2-3 days depending on density. Harvest cells with trypsin as above. Seed cells at clonal density (1000 cells in a 35mm dish) grow cells until colonies are visible by eye (~5 days)
9. Pick clones one by one with a P10 pipette, transfer individual colonies to a well of a 96 well plate containing trypsin. Under an inverted fluorescence microscope, select fluorescent colonies. The fluorescence if weak but detectable. Discard colonies if a well contains more than one colony. We screened about 50 colonies to get four fluorescent clones.
10. Expand individual clones. Check for correct integration by PCR genotyping. (*see Note 5*)
11. Check correct functionality by stimulation with ACTIVIN. The RFP signal should be nuclear upon ACTIVIN stimulation and cytoplasmic under inhibition of the pathway with SB43 inhibitor (*see Fig. 3*)

### ***3.3.2 Cell line with stable constitutive expression of a nuclear marker for nuclear segmentation.***

1. Prepare Lipofectamine 3000 transfection reaction as in paragraph 3.3.1 with 330 ng of ePB-B-CAG:mCherry-nls (for the RFP-Smad2 line) or ePB-P-CAG:GFP-nls (for the ASE:YFP line) plasmid and 170ng of ePB transposase,
2. 48h after transfection, start selection: harvest cells, seed in gelatin coated 6 well plates. Seeding density 100kcells per well. Supplement N2B27+2i+LIF medium with puromycin or blasticidin depending on the resistance gene carried by the plasmid.
3. Grow cells for 2-3 days depending on density. Harvest cells with trypsin as above. Seed cells at clonal density (1000 cells in a 35mm dish) grow cells in selection supplemented mESC growth medium until colonies are visible by eye (~5 days)

4. Pick and expand clones as described above. Keep a few clones having homogenous nuclear marker expression. After antibiotic selection, almost all clones should be positive.

### ***3.3.3 Image acquisition: recording ASE:YFP signal***

The fluorescent signal produced by the ASE:YFP reporter so can be recorded with regular epifluorescence microscopy as it is well above background, we thus used the microscopy set up described in 2.7.1 (*see Note*

**6**). Using the “Multi-Dimensions acquisition” tool of Micromanager:

1. Tick “multiple Position” record the XY positions to be imaged and focus on the nuclear marker using the “edit Position list button”
2. Tick the “Time Points” box and select the interval between 2 images and the total number of images to be taken. The interval is a tradeoff between photo toxicity and temporal resolution needed. One image every hour is enough to track expression of the ASE:YFP reporter but it will not allow single cell tracking.
3. Tick the “Channels” box and add Select the RFP, YFP and Bright Field acquisition channels to be used in the MDA. For each channel enter the exposure time. Again, the exposure time and illumination intensity should be optimized for each channel to minimize phototoxicity. 200ms exposure at 50% intensity is a good starting point.
4. Autofocus: for acquisitions using the 10x or 20x objectives, autofocus should not be necessary. Make sure that the setup has reached thermal stabilization before starting acquisition or the focus will drift.
5. Tick the “Save images” box, enter the directory and image prefix to save images.
6. Press “Acquire!”

### ***3.3.4 Image acquisition: recording RFP-Smad2 signal***

Unlike the ASE:YFP reporter, the signal of the RFP-Smad2 reporter is relatively weak so microscopy with optical sectioning capabilities is necessary to obtain enough signal above background. We used the DSD2 system from ANDOR, a structured illumination system giving results close to a spinning disk confocal microscope, described section 2.7.2. The Andor iQ3 proprietary software controls this system.

Using the “6D view” version of Andor’s iQ3:

1. Set up imaging parameters of the RFP channel: illumination: RFP filter cube, 550nm LED 50% intensity. Exposure time 300ms. Frame averaging: 4. Frame averaging is necessary to increase the signal to noise ratio of the RFP-Smad2 fluorescence.
2. Set up imaging parameters of the GFP channel: illumination: GFP filter cube, excitation 490nm LED 30% intensity. Exposure time 100ms. Frame averaging: 4. Frame averaging is not strictly necessary for the GFP-nls channel. But the iQ software imposes that the same frame averaging setting is used for all channels of an acquisition. Exposure time was thus reduced to avoid increasing photo-damage.
3. Select XY positions to be recorded. Set Focus using the nuclear marker (GFP-nls) channel
4. Set the time interval and duration of the time lapse. Again, the interval is a tradeoff between photo toxicity and temporal resolution needed. On Figure 3c, one frame was acquired every 10 minutes for about 100 minutes. For longer acquisitions, we acquired one image every hour.
5. Enter image saving name and directory and launch acquisition pressing the “Run” button.

### ***3.3.5 Image analysis: segmentation of nuclei***

Image analysis was performed using custom software written in Python. We used the nuclear marker images to identify nuclei using an algorithm that identifies local maxima and then detects the edges of each nucleus by performing a watershed transform. To compute RFP-Smad2 nuclear to cytoplasmic ratio, the cytoplasmic signal had to be evaluated. However, this signal was too weak to be identified by applying a method similar to that used for the nuclear fluorescence image. We thus dilated the nuclear mask of each cell to create a narrow donut around the nucleus with radius chosen so that it almost certainly was contained in the cell. Smad2-RFP fluorescence was computed by examining the average values of nuclear and cytoplasmic fluorescence in the masks defined by this procedure. The image processing steps go as follows. A sample python script performing these operations is provided (*see Note 7*).

1. Open image nuclear marker image (imread function of the skimage package)
2. Pre-process nuclear marker image. Normalize and smooth picture (normalize and GaussianBlur function of the open CV package). The strength of the smoothing should be optimized empirically. A 5x5 Gaussian kernel gave good results on our pictures
3. Binarisation. We found the Otsu thresholding algorithm to give good results (thresh function of the openCV package)



4. Sure nuclei area (usually called “foreground markers”) are then identified via distance transformation of the thresholded image (function `distanceTransformation` openCV package) followed by identification of connected components (`connectedComponents` function of the openCV package)
5. Watershed transform seeded with the markers found in the previous step above (`watershed` function of the openCV package). It is crucial to visually inspect the results of segmentation. One easy way to do it is to plot the coordinates of each detected centroid on the picture. Depending on the results, false positive can be eliminated based on their size, distance to closest neighbor or intensity.

### ***3.3.6 Image analysis: extraction of ASE-YFP fluorescence or RFP-Smad2 nuclear to cytoplasmic ratio***

1. To extract the fluorescent reporter intensity in the nucleus, the nuclear mask is applied to the RFP-Smad2 or ASE:YFP channel picture and for each nuclei, the fluorescence signal is averaged over all the pixels it contains.
2. To generate the nuclear to cytoplasmic ratio used to measure cell responses with the RFP-Smad2 line, the cytoplasm fluorescence value is sampled by dilating the nuclear mask of each nuclei by a few pixels, thus creating a “donut” around the nucleus. We used this approach because the cytoplasmic signal of RFP-Smad2 fluorescence was too weak to be segmented in a reliable manner.

### ***3.3.6 Image analysis: plot results***

1. Statistics over all cells are then extracted and plotted using the `numpy`, `matplotlib` et `pandas` packages

## 4. Notes

1. Assembly of the valve controller and code for automated chip operations: A complete part list, wiring diagrams and assembly instructions can be found on the website of Rafael Gomez- Sjoberg who developed the original version of the chip in Steven Quake's lab in Stanford (<https://sites.google.com/site/rafaelsmicrofluidicspage/valve-controllers/usb-based-controller>). The website provides the information about how to build a DIY USB controller to make the interface between the computer and the solenoid valves. Alternatively, Arduino microcontroller + relays boards can be used. We are grateful to Rafael Gomez-Sjoberg, Savas Tay in Steven Quake's Lab for training us on how to operate the original Cell Culture Chip and providing us with the original Matlab code controlling the chip that we have adapted into a simplified version written in Python code. We provide 3 samples available for download here: <https://github.com/fabienfrfr/THESIS>. The file HARDCCC.py provides the libraries to establish communication with the chip and basic function to operate it. The file ScriptPreparation.py provides the list of command to run to prepare the chip for an experiment. The file Script\_valvesOnly.py provides an example of a script to run a simple experiment
2. Cell lines: HM1 and ASE:YFP mESC lines are a kind gift of Jerome Collignon (Institut Jacques Monod, Paris).
3. Assembly of media dispensing vials: To assemble media dispensing vials, punch 2 holes in the cap of a 5ml cryo-vial with a G19 syringe needle. In the first hole insert PTFE tubing so that the end of the tube reaches the bottom of the vial. This tube will be used to bring the liquid to the chip and should be long enough for that purpose. Insert about 1 cm a 10cm long Tygon tube into the second hole. This tube will be used to pressurize the vial; therefore, the tube should not reach the medium. Pour glue (epoxy or hot glue gun) on top of the vial cap to seal the tubing in place. We recommend to use PTFE tubing for any part that will be in contact with culture medium as we found tygon tubing to be toxic for the cells, especially when new or freshly autoclaved (24).
4. ePiggyBAC plasmids: The ePiggyBAC system (22) is an efficient way to achieve stable expression of a transgene by random integration in the genome. Two plasmids have to be co-transfected; (i) the donor plasmid containing the sequence that will be integrated in the genome flanked by 5' and 3' terminal repeat

sequences necessary for transposition and (ii) the plasmid expressing the ePB transposase that will perform the transposition. ePB-P-CAG:eGFPnls, ePB transposase and are a kind gift of the Brivanlou lab (Rockefeller University). ePB-B-CAG:mCherry-nls was made by cloning the mCherry-nls gene into the ePB-B-CAG-MCS plasmid, which is also from the Brivanlou lab.

5. Plasmids to generate the RFP-SMAD2 line: The insertion of tagRFP in the N-terminus of endogenous Smad2 coding sequence was made by crispr-assisted homology directed repair (23). The choice of gRNA was made using the CRISPR MIT tool (<http://crispr.mit.edu/> of The Laboratory of Professor Zhang (25). The targets selected in this study were chosen with a potential off-target score greater than 80/100 to minimize the risk of adverse effects. The selected sgRNA sequence was GGACGACATTTTCTTACCAA. Forward (5'-CACCGGGACGACATTTTCTTACCAA) and reverse (5'-AAACTTGGTAAGAAAATGTCGTC) oligo were annealed and inserted by ligation (T4 enzyme, room temperature) in the pSpCas9(BB)-2A-Puro plasmid (addgene PX549, cat# 62988) opened by BbSI digestion. We are grateful to Mathieu Vieira from J. Collignon's lab for his help in the design of the homology donor.

DNA for left and right homology arms was retrieved by PCR reaction performed on mESC DNA extracted using Sigma RedExtract-N-Amp for tissue (Sigma XNAT-1K) kit, following manufacturer's instructions.

The following primers were used:

HA-Left-Fw: GCAGTAGATCAGGATGATGGGG

HA-Left-Rev: GGAGACCGGTTTTCTTACCAAAGGTAACAATCC

HA-Right-Fw : CCTCACCGGTATGTCGTCATCTTGCCATTCCT

HA-Right-Rev : ACAAGCAGAAAGTCCGGTGT.

Primers overhangs are designed such that homology arms could be assembled together by a single PCR Phusion reaction and subsequently inserted into pGEM-T Easy commercial Vector. After amplification, the plasmid was opened in between the two homology arms by AgeI digestion (AgeI restriction site inserted in primers overhangs) and tagRFP DNA was inserted by ligation. tagRFP DNA was retrieved from the ePB-B-MCS-RFP plasmid (Brivanlou Lab) by PCR using the following primers RFP-Smad2, which adds AgeI restriction sites on both sides of RFP:

RFP-Smad2-Fw : GCCTCACCGGTATGGTGTCTAAGGGCGAAGAG

RFP-Smad2-Rev : GAGGTACCGGTGGTGGCGACATTAAGTTTGTGCCCCAGTTTG

PCR genotyping: After passing, a fraction of each well was recovered in order to perform the genotyping of the cells. DNA extraction was achieved using Sigma RedExtract-N-Amp for tissue (Sigma XNAT-1K) kit, following manufacturer's instructions

Genotyping was performed by PCR reaction, using Phusion Master Mix, 0.5 µl of each primer at 10 µM 5 µl 2X Phusion Master Mix, 0,3 µl DMSO, 1 µl DNA extraction diluted to have less than 250 ng genomic DNA and 2.7 µl of distilled sterile water. The following pairs of primers were used for genotyping: Fw1 : CTTCAAGCAGATGAGGTGTTGAGTAAAG (Inside Left Smad2 homology arm) and RFP Rev1 : GCCCACGAGCTTCAGGGCCATG (reverse inside RFP) , yielding a 1171bp band in case of insertion and Fw2 : CAAGAGGCAGTATTGCTACTTATAAAAG, Rev2 : GCCCACGAGCTTCAGGGCCATG, 1800 bp band in case of insertion. The clone presented in figure 3 has homologous insertion.

6. Some culture media like DMEM contain components like riboflavins that are strongly fluorescent in the GFP channel. This results in a high fluorescence background that can interfere with detection of the signal coming from the reporter. Performing the experiment in a microfluidic device helps with this issue because it minimizes the layer of medium above the cells (40µm in our case vs. a few mm in a cell culture dish). Using a filter set optimized for YFP rather than GFP also reduces background intensity. Finally, one might consider culture media optimized for live cell imaging.
7. A tutorial with examples about image segmentation with the watershed algorithm can be found in the openCV package documentation: [https://docs.opencv.org/master/d3/db4/tutorial\\_py\\_watershed.html](https://docs.opencv.org/master/d3/db4/tutorial_py_watershed.html), and example codes of our segmentation routine can be downloaded in: <https://github.com/fabienfrfr/THESIS>.

### **Acknowledgements:**

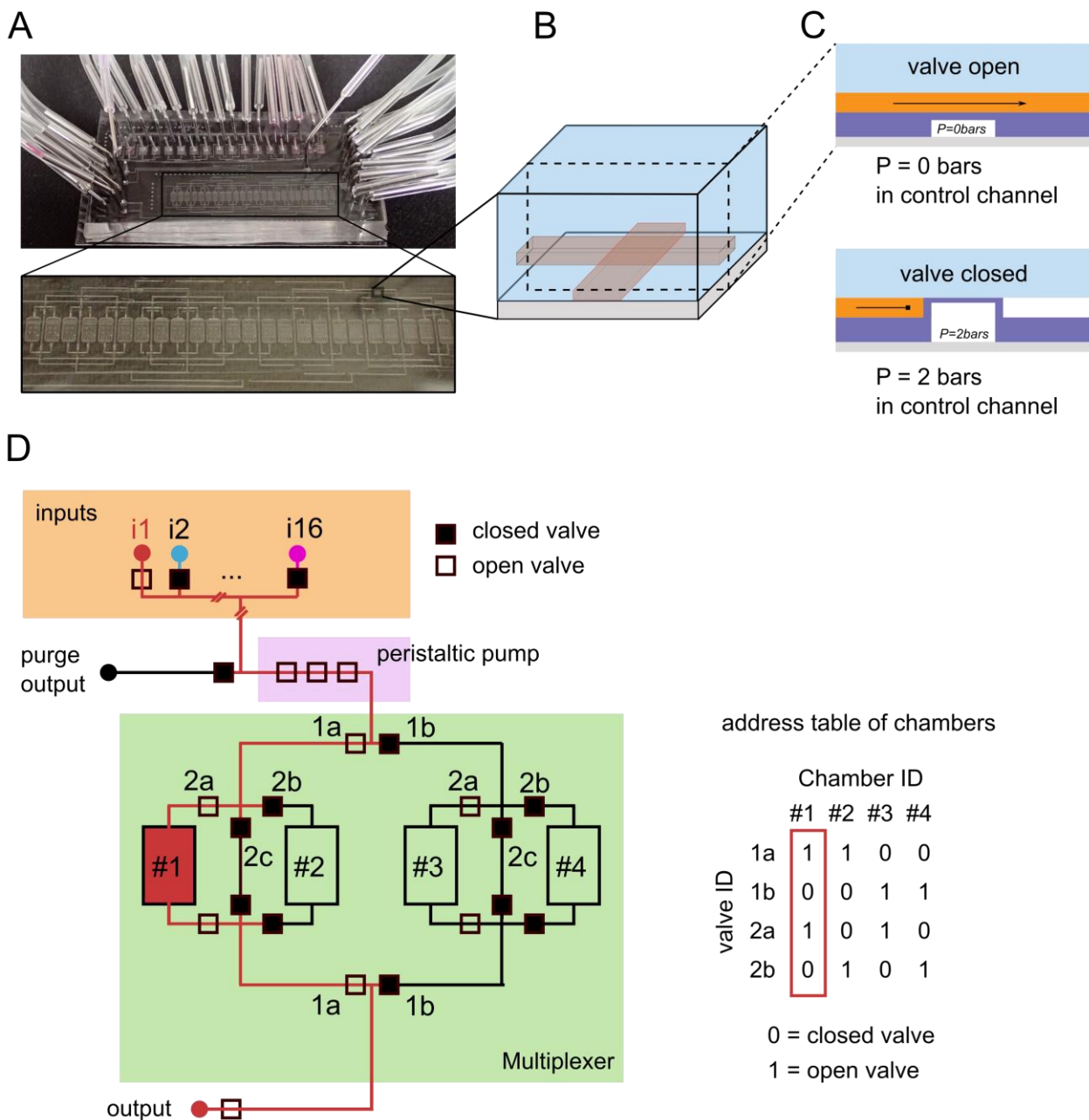
This work was supported by French Agence Nationale de la Recherche (ANR-15-CE13-0007), Human Frontier Science Program (HFSP CDA00063/2015-C) and Institut National du Cancer (INCa 2014-1-PL

BIO-01-UP 7-1). We are grateful to all members of the groups of J. Collignon (IJM) and P. Hersen (Institut Curie) for fruitful discussions during this project.

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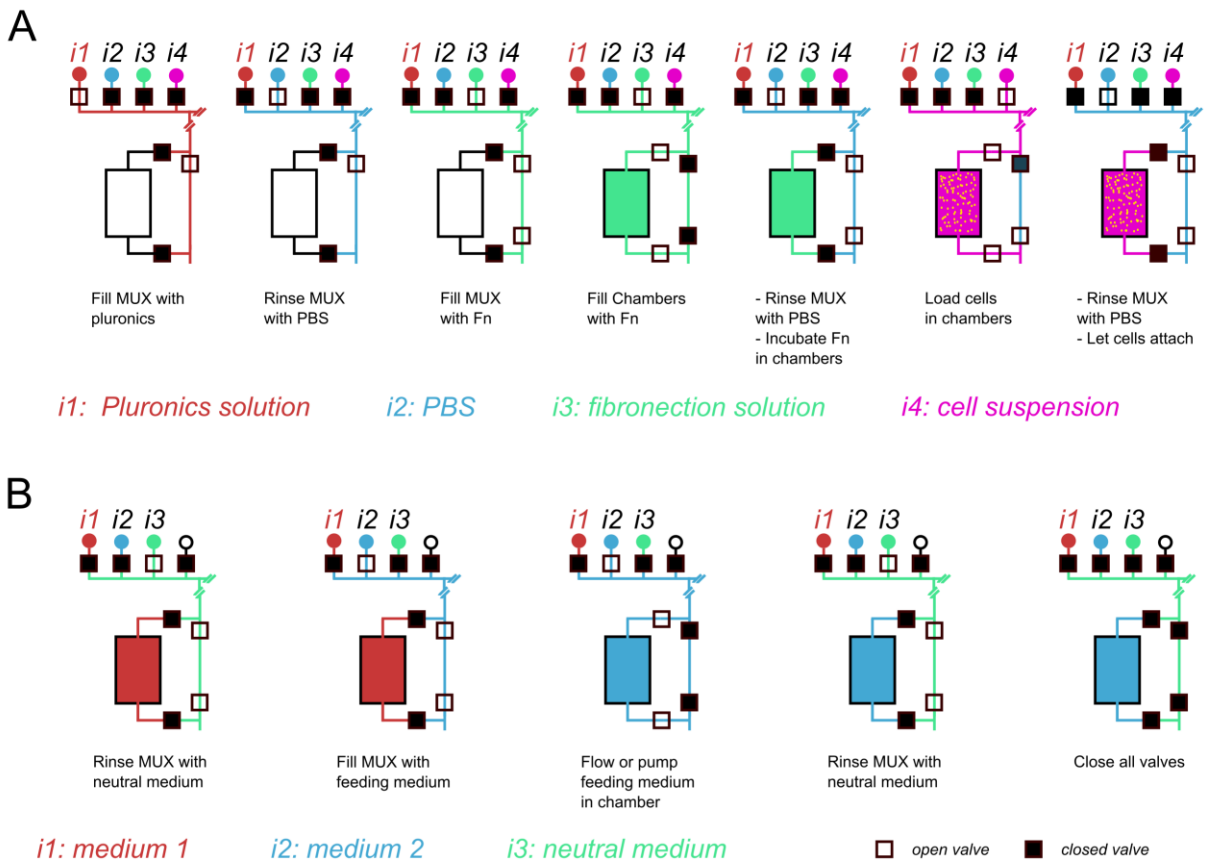


**Fig. 1** Presentation of the microfluidic cell culture chip. (A) top: ensemble picture of the 24 chambers cell culture chip with tubing controlling the valves state connected. Bottom: close-up view on the culture chambers. (B) 3D representation of the channels configuration to make a microfluidic valve. The control channel (red) is located below the flow channel (black). (C) Cross section of the valve according to the dashed rectangle in B. Top: when no pressure is applied in the control channel, the valve is open and flow is permitted in the flow

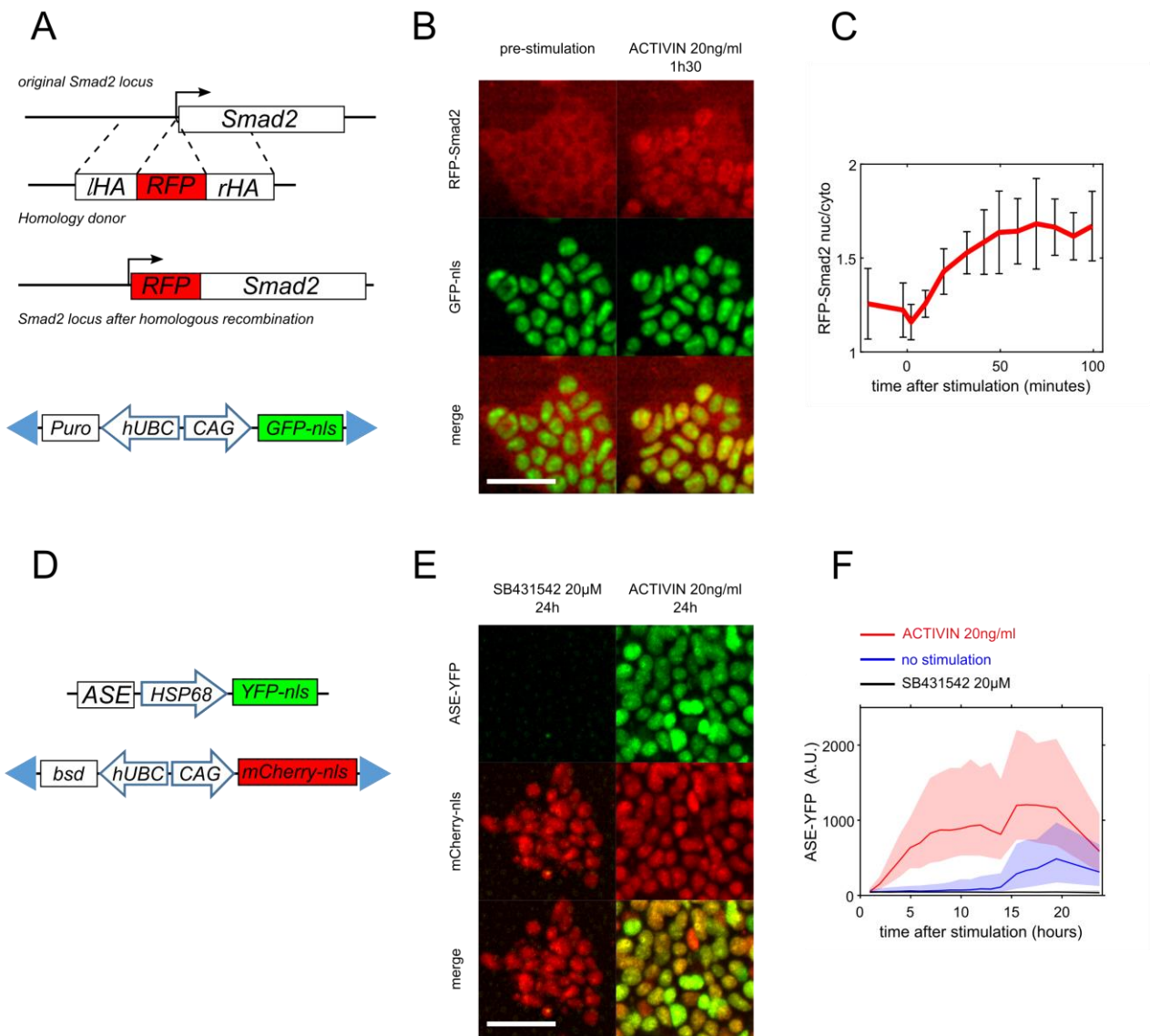


channel (orange). Bottom: applying pressure in the control channel deflects the membrane at the intersection of the flow and control channel, which blocks flow: the valve is closed.

(D) Schematic view of the chip functions. The “inputs” block contains 16 independent inputs that can be open or closed independently. The peristaltic pump function is achieved by three valves in series. This on chip pump can be used to deliver a precise amount of liquid to a chamber. A simplified four chamber multiplexer is presented with the valve configuration to send medium from input 1 to chamber 1 (red path). On the right, the address table of chambers shows the unique valve combination of the multiplexer to send fluid to a given chamber. Valve 2c controls the chamber bypass that is used to flush the multiplexer between feeding adjacent chamber and thus prevent cross-stimulation



**Fig. 2** Sequence of chip operations for automated cell seeding and feeding. (A) Sequence of operation to prepare the chip and seed cells. (B) Sequence of operation for a medium replacement in a given chamber.



**Fig. 3** Live cell reporters of TGF- $\beta$  pathway activity in mESC. (A) The RFP-Smad2 reporter is generated by insertion of RFP sequence upstream of the *Smad2* gene via crispr/Cas9 activated homology directed repair. For image segmentation purpose, the GFP-nls nuclear marker is also randomly inserted in the genome with the ePiggyBAC system of transposable elements. Blue triangles represent 5' and 3' terminal repeats required for transposition. (B) Before stimulation, (left column) RFP-Smad2 is mainly cytoplasmic. 1h30 after stimulation with ACTIVIN (20ng/ml) RFP-Smad2 signal is nuclear (right column). (C) Temporal evolution of the nuclear to cytoplasmic ratio of RFP-Smad2 fluorescence as a function of time after ACTIVIN (20ng/ml) stimulation. For experiments presented in B and C, mESC were stimulated with ACTIVIN 24h hours after removal of the naïve pluripotency medium (N2B27+2i+LIF). Error bars represent standard deviation. (D) schematic representation of the transgenic reporter. The ASE element is upstream a minimal promoter (HSP68)

driving YFP-nls expression. For image segmentation purpose, the mCherry-nls nuclear marker is also randomly inserted in the genome with the ePiggyBAC system of transposable elements. Blue triangles represent 5' and 3' terminal repeats required for transposition. (E) if the activity of the TGF- $\beta$  pathway is blocked with the inhibitor SB431542, no YFP fluorescence is detected, showing specificity of the reporter (left column) while a strong signal is observed upon stimulation with ACTIVIN (right column). (F) Comparison of the temporal evolution of the ASE:YFP fluorescence for ACTIVIN stimulated (red), unstimulated (blue) or SB431542 treated (black) mESCs stimulated 24h hours after removal of the naïve pluripotency medium (N2B27+2i+LIF). Spontaneously activity of the reporter is detected at long times in unstimulated cells, probably due to endogenous production of TGF- $\beta$  ligands. Scale bars: 50  $\mu$ m. shaded areas are bounded by the 1<sup>st</sup> and 3<sup>rd</sup> quartiles of the distribution.