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TITLE:

A Microfluidics Approach for the Functional Investigation of Signaling Oscillations Governing Somitogenesis

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KEYWORDS:

embryonic development, mouse, signaling dynamics, signaling oscillations, microfluidics, entrainment, somitogenesis

SUMMARY:

A protocol for the culture and manipulation of mouse embryonic tissue on a microfluidic chip is provided. By applying pulses of pathway modulators, this system can be used to externally control signaling oscillations for the functional investigation of mouse somitogenesis.

ABSTRACT:

Periodic segmentation of the presomitic mesoderm of a developing mouse embryo is controlled by a network of signaling pathways. Signaling oscillations and gradients are thought to control the timing and spacing of segment formation, respectively. While the involved signaling pathways have been studied extensively over the last decades, direct evidence for the function of signaling oscillations in controlling somitogenesis has been lacking. To enable the functional investigation of signaling dynamics, microfluidics is a previously established tool for the subtle modulation of these dynamics. With this microfluidics-based entrainment approach endogenous signaling oscillations are synchronized by pulses of pathway modulators. This enables modulation of, for instance, the oscillation period or the phase-relationship between two oscillating pathways. Furthermore, spatial gradients of pathway modulators can be established along the tissue to study how specific changes in the signaling gradients affect somitogenesis.

The present protocol is meant to help establish microfluidic approaches for the first-time users of microfluidics. The basic principles and equipment needed to set up a microfluidic system are described, and a chip design is provided, with which a mold for chip generation can conveniently be prepared using a 3D printer. Finally, how to culture primary mouse tissue

on a microfluidic chip and how to entrain signaling oscillations to external pulses of pathway modulators are discussed.

This microfluidic system can also be adapted to harbor other *in vivo* and *in vitro* model systems such as gastruloids and organoids for functional investigation of signaling dynamics and morphogen gradients in other contexts.

INTRODUCTION:

Development is controlled by intercellular communication via signaling pathways. There is only a limited number of signaling pathways that orchestrate the complex formation of tissues and proper cell differentiation in space and time. To regulate this multitude of processes, information can be encoded in the dynamics of a signaling pathway, the change of a pathway over time, such as the frequency or duration of a signal^{1,2}.

During somitogenesis, somitic tissue is periodically segmented off from the presomitic mesoderm (PSM)³. The PSM is spatially organized by gradients of Wnt, Fibroblast Growth Factor (FGF), and Retinoic acid signaling. In anterior PSM at the *determination front*, where Wnt and FGF signals are low, cells are primed for differentiation into somites. Differentiation occurs when a wave of transcriptional activation reaches this determination front. Within the PSM, Wnt, FGF, and Notch signaling oscillate. Neighboring cells oscillate slightly out of phase, which results in waves of oscillatory transcriptional activation downstream of the Wnt, FGF, and Notch pathways traveling from posterior to anterior PSM. In mouse embryos, a transcriptional wave reaches the determination front approximately every 2 h and initiates somite formation. Studying somitogenesis by perturbing or activating signaling pathways can illustrate the importance of these pathways⁴⁻⁹. However, to be able to investigate the function of signaling dynamics in the control of cellular behavior, it is essential to subtly modulate signaling pathways instead of permanently activating or inhibiting them.

To temporally modulate signaling pathway activity within the segmenting mouse embryo, Sonnen et al. have developed a microfluidic system¹⁰. This system allows the tight control of fluid flows within microchannels of a chip that contains the biological sample¹¹. To study the importance of signaling dynamics for proper segmentation of PSM, this microfluidics setup is utilized to modulate signaling dynamics of the mouse segmentation clock *ex vivo*. By sequentially pulsing pathway activators or inhibitors into the culture chamber, external control of the dynamics of Wnt, FGF, and Notch signaling is achieved¹⁰. For instance, it is possible to modify the period of individual pathways and the phase relationship between multiple oscillatory signaling pathways. Using concomitant real-time imaging of dynamic signaling reporters, the effect of entrainment on the pathways themselves, on differentiation and somite formation can be analyzed. Using this level of control over signaling dynamics, the importance of the phase relationship between Wnt- and Notch-signaling pathways during somitogenesis was highlighted¹⁰.

Personalized chip designs allow for a plethora of options for spatiotemporal perturbations within the local environment, e.g., stable gradient formation¹²⁻¹⁵, pulsatile activation/inhibition^{10,16-18} or localized perturbations^{19,20}. Microfluidics can also enable a more reproducible read-out and higher throughput due to automation of experimental handling²¹⁻²³. The present protocol is meant to bring microfluidics and entrainment of

endogenous signaling oscillations within tissues to every standard life sciences lab. Even in the absence of sophisticated equipment for chip generation, such as clean room and equipment for soft-lithography, microfluidic chips can be manufactured and used to address biological questions. Molds can be designed using freely available computer-aided design (CAD) software. A mold for the generation of microfluidic chips, usually consisting of polydimethylsiloxane (PDMS), can be printed with a 3D printer, or be ordered from printing companies. This way, microfluidic chips can be produced within one day without the requirement of expensive equipment²⁴. Here, a chip design is provided, with which a mold for the entrainment of the mouse segmentation clock in two-dimensional (2D) *ex vivo* cultures²⁵ can be printed with a 3D printer.

On-chip cultures and precise perturbations, enabled by microfluidics, hold outstanding potential in unravelling the molecular mechanisms of how signaling pathways control multicellular behavior. Signaling dynamics and morphogen gradients are required for many processes in development. Previously, labs had cultured cells, tissues and whole organisms in microfluidic chips and protocols for spatiotemporal perturbation of primarily 2D cell culture are provided elsewhere^{12,26–29}. Applying microfluidics to modulate local environments in multicellular systems opens new perspectives for high-throughput and precise spatiotemporal perturbations. The field of microfluidics has now reached a point that it has become a non-specialist, inexpensive, and easily applicable tool for developmental biologists.

Here, a protocol for the entrainment of the mouse segmentation clock to pulses of a Notch signaling inhibitor is provided. Such an experiment consists of the following steps: (1) generation of microfluidic chip, (2) preparation of tubing and coating of the chip, and (3) the microfluidic experiment itself (**Figure 1A**). Research involving vertebrate model systems requires prior ethical approval from the responsible committee.

PROTOCOL:

The research presented here has been approved by the EMBL ethics committee¹⁰ and the Dutch committee for animal research (dierenexperimentencommissie, DEC), and follows the Hubrecht guidelines for animal care.

Wear gloves while working with liquid PDMS. Cover surfaces and equipment. Remove any spills immediately, as cleaning becomes difficult once it's hardened.

1. Generation of the chip

NOTE: Microfluidic chips are generated by casting PDMS (Polydimethylsiloxane) in a mold. Molds can be designed using CAD software (e.g., uFlow or 3DµF³⁰). A repository of free designs is provided by the MIT (Metafluidics.org). Molds are either printed with a 3D printer or can be generated by soft lithography using a photomask that contains the desired design (for further information see, e.g., Qin et al., 2010³¹). Here, a design for a mold is provided that can be printed with a 3D printer for the on-chip culture of mouse embryo tissue (**Figure 1B,C, Supplementary Files 1 and 2**). To allow printing with lower resolution 3D printers, the design has been modified compared to the previously published one¹⁰. A mold without air bubble traps and one with air bubble traps is provided (**Supplementary Files 1 and 2**, respectively). As the procedure for printing is dependent on the printer available, the details for printing

will not be described. However, one must make sure that all unpolymerized resin is entirely removed. It is often required to perform extra washing with solvents to remove unpolymerized resin from the small <200 μm holes within the microfluidic chamber. These holes will form PDMS pillars to trap the embryonic tissue within the chip during the experiment. PDMS is generally used for microfluidics, as it is cheap, biocompatible, and transparent, and has low autofluorescence. After curing, the PDMS chip is cut out of the mold and bonded onto a glass slide. Plasma treatment of both the glass and the PDMS chip activates the surfaces and allows the formation of covalent bonds, when brought into contact.

1.1. Prepare the required amount of PDMS by mixing the monomer with the catalyst in a 9:1 ratio (w:w) to induce polymerization. Use disposable tools to mix, such as plastic cups and forks. Ensure that the mixing is properly achieved.

1.2. Place the PDMS mixture in a desiccator and apply vacuum for approximately 30 min to remove air from the PDMS mixture. Due to the vacuum within the desiccator, air will be removed from the PDMS mixture.

NOTE: Cover the inside of the desiccator with tissues in case PDMS flows over.

1.3. Pour the PDMS mixture into the chip mold (a PDMS layer of approximately 3–5 mm is enough).

1.4. Place the mold filled with PDMS back into the desiccator and apply vacuum to remove any remaining bubbles. Make sure that air is removed from all smaller structures of the mold.

1.5. Cure the mold by placing it in a 65 °C (or lower depending on the mold material) oven overnight. Cut the chip out of the mold using a scalpel. Cut the hardened PDMS out of the mold with sufficient space (1–2 cm) around the design to allow for later bonding. Make sure to cut the PDMS completely to prevent breaking of the chip when taking it out. Lift the PDMS chip off carefully, first with the blunt end of the scalpel, and when possible, with hands.

1.6. Punch inlet and outlet holes, starting from the inside of the microfluidic chamber using a 1 mm biopsy punch.

1.7. Clean the PDMS chip briefly with compressed air and stick adhesive tape on both sides to remove any stuck bits of PDMS and dust and keep it clean until further use.

NOTE: The chip can be kept like this until further use. To prevent any dirt from entering, surfaces can be covered with tape.

1.8. Clean the glass slide with compressed air. Be careful that it does not break. Remove adhesive tape from the PDMS chip.

1.9. Bond the chip to the glass slide using a plasma oven. The following instructions are optimized for air plasma.

NOTE: Consult the manual of the lab's plasma oven and optimize the procedure for the specific experiment.

1.9.1. Place the chip and glass slide into the plasma oven with the sides to be bonded facing up.

1.9.2. Place the lid onto the plasma oven and hold it in place while applying vacuum and wait until vacuum has established.

1.9.3. Turn the gas on by pressing the **Gas** button and wait for approximately 1 min (pressure should be at around 0.37 mbar).

1.9.4. Generate plasma by pressing **Generator** (Power: 9.0, Time: 1 min).

1.9.5. When finished, turn the pump and gas off; ventilate and open the door.

1.9.6. Bond the chip to the glass by placing the activated surfaces onto each other and applying pressure evenly. Be careful that the glass does not break.

NOTE: A water test can be performed to confirm that plasma treatment has worked. Place a drop of water on the glass surface. If plasma treatment has worked, the water should spread out immediately instead of forming a droplet.

1.10. Place the bonded chip in an oven at 65 °C for 5 min.

1.11. Seal the exposed side of the chip with adhesive tape to prevent dust from entering the inlets.

NOTE: The chip can be kept until use. Openings should be closed with tape until then.

2. Preparing microfluidics experiment

NOTE: The following preparatory steps are necessary to perform the microfluidics experiment: First, when performing the microfluidics experiment, a fluid flow is created from the inlets to the outlets. Any holes in the chip will function as outlets due to the pressure build-up from the inlets. Therefore, all holes that are not used must be closed; for instance, holes that are used to load tissue onto the chip. If these are not closed, the tissue might flow out with the fluid. To close these holes, PDMS-filled tubing is used. The PDMS-filled tubing can be kept indefinitely and, therefore, will not need to be prepared for each microfluidics experiment separately but can be made in larger batches. Second, before the start of the microfluidic experiment, tubing, PDMS-filled tubing, and the chip, required for the experiment, are prepared and UV-sterilized. Finally, the chip has to be coated with fibronectin, so that the embryonic tissue can attach to the glass²⁵.

2.1. Making PDMS-filled tubing.

2.1.1. Take ± 1 m of tubing. More tubing can be filled with PDMS by taking multiple pieces of tubing.

2.1.2. Prepare the required amount of PDMS by mixing the monomer with the catalyst in a 9:1 ratio (w:w) to induce polymerization.

NOTE: Use disposable tools to mix, such as plastic cups and forks. Mix properly.

2.1.3. Place the PDMS mixture in a desiccator and apply vacuum for approximately 30 min to remove air from the PDMS mixture.

NOTE: Cover the inside of the desiccator with tissues in case PDMS flows over.

2.1.4. Fill a 3 mL syringe with PDMS. Fill carefully to prevent the formation of air bubbles in the mixture.

2.1.5. Use forceps to insert the tip of a 22 G needle into the tubing.

NOTE: Be careful not to puncture the tubing or your fingers with the needle.

2.1.6. Attach the tubing to the PDMS-filled syringe via the needle. Use the syringe pump to fill the tubing, with a flow rate of approximately 500 $\mu\text{L/h}$. Be careful not to apply too high pressure to prevent breaking of the pump.

2.1.7. Once the tubing is entirely filled with PDMS, place it in a 15 cm dish and cure at room temperature (at least overnight).

2.2. Prepare the tubing and chip for the experiment.

NOTE: The following tubing is required for the experiment: first, approximately 50 cm of tubing per outlet and second, approximately 2–3 m per inlet (the exact size depends on the spatial organization of the pumps, incubator or microscope used later on). The inlet tubing needs to be long to allow the culture medium to equilibrate with CO_2 for embryo culture during the experiment.

2.2.1. Cut the tubing at a 45° angle so that pointed ends are created; this will make it easier to insert the tubing into the holes of the chip. Attach one needle to each of the inlet tubing. See step 2.1.5.

2.2.2. Cut PDMS-filled tubing into ± 1 cm plugs. Cut at a 45° angle so that pointed ends are created; this will make it easier to insert the tubing into the holes of the chip. Enough plugs are needed to fill each hole that is not attached to any tubing.

2.2.3. Remove the adhesive tape from the chip.

2.2.4. Place all the tubing with needles attached, the chip, and the plugs in a dish and sterilize by exposing to UV light for ± 15 min. Any cell culture hood with the possibility for UV sterilization can be used.

2.3. Coating of chip with fibronectin.

NOTE: For culture of 2D *ex vivo* cultures of posterior PSM, coat the chip with fibronectin.

2.3.1. Place the chip in a beaker containing PBS + 1% Penicillin/Streptomycin at room temperature. Make sure the chip is completely covered with PBS. Flush the chip with PBS to remove air with a P200 pipette.

NOTE: All further handling of the chip will be done in this beaker until the start of the experiment to prevent entrance of air into the chip. Be careful not to break the glass slide of the chip.

2.3.2. Prepare 2 mL of 1:20 Fibronectin in PBS and fill into a beaker.

2.3.3. Load a 3 mL syringe for each chamber of the chip. With forceps, insert a 22 G needle into the outlet tubing. Attach the needle to the syringe containing fibronectin.

2.3.4. Connect the syringe to the syringe pump. Flush the tubing, until there is no air left in the tubing.

2.3.5. Attach the outlet tubing to the outlet of the chip. Make sure to push the tubing all the way to the bottom. Set a low flow rate to coat the chip. All other openings can remain open. Let the syringe pump run for at least 2 h, can also run overnight.

2.3.6. Stop the pump. Cut off the tubing right after the needle. The remaining tubing will serve as the outlet during the experiment later.

3. Microfluidics experiment

NOTE: Here, a protocol for the entrainment of the mouse segmentation clock in 2D *ex vivo* cultures by applying pulses of the Notch signaling inhibitor DAPT is presented. Applying pulses with a period of 130 min, close to the natural period of the mouse segmentation clock (137 min²⁵), allows efficient entrainment. Pulses of 100 min of medium and 30 min of 2 μ M DAPT are applied (**Figure 2A**). The presence of drug within the chip is monitored using a fluorescent dye. The excitation and emission spectra of this dye and the fluorescent signaling reporter must be different enough to prevent bleed-through during fluorescence real-time imaging. When yellow fluorescent proteins are used as signaling reporter, such as the Notch signaling reporter LuVeLu⁵ (Lunatic fringe-Venus-Lunatic fringe), the dye, Cascade Blue, can be applied. To identify other possible combinations of fluorescent dye and reporter, freely available spectra viewer can be used. The effect of entrainment can be detected by real-time imaging of dynamic signaling reporters^{5,10,32}. Each chip has two incubation chambers. This allows the direct comparison of drug pulses (DAPT) to control pulses (DMSO).

3.1. Make medium and degas.

3.1.1. On the day of the experiment, prepare 50 mL of culture medium (DMEM-F12 supplemented with 0.5 mM glucose, 2 mM glutamine, and 1% BSA, for further information of mouse tailbud culture see²⁵).

3.1.2. **Prepare syringes filled with the medium for the experiment.** Prepare culture medium in beakers: To entrain Notch signaling oscillations, prepare 6 mL of medium with 1.2 μ L of DMSO + 10 μ M Cascade Blue; 6 mL of culture medium with 2 μ M DAPT + 10 μ M Cascade Blue; two tubes with 6 mL of medium each. Use at least 6 mL of medium per syringe. Load syringes with the medium. Make sure to label the syringes containing drug and DMSO.

3.1.3. Degas the chip within PBS and the syringes containing medium in a desiccator. Place the syringes into a beaker with the tip facing up, so that the air can escape at the top. It might occur that the chip floats and is still filled with bubbles. These will be removed subsequently.

3.1.4. **Try to flush out/suck up most of the air bubbles from the chip using a P200 pipette.** If some air remains, this will be removed during the following experiment.

3.1.5. **Install syringes in the pumps and attach inlet tubing to the syringes.** To entrain Notch signaling, use two syringe pumps, one carrying the medium syringes, one carrying the drug/DMSO syringes. Let this run at a higher flow rate (0.5 mL/h) until the start of the experiment to remove air bubbles from the tubing. Be careful to set the syringe details (diameter) properly in the pump.

3.2. Loading of tissue onto the chip.

3.2.1. Dissect mouse embryo tissue. **Dissect the most posterior tip of the tail (tailbud).** Place the dissected tissue into culture medium + 25 μ M HEPES.

3.2.2. **Flush the chip with culture medium + 25 μ M HEPES using a P200 to remove PBS.**

3.2.3. **Load the tissue into the chip using a P200 pipette (Figure 1C).** Make sure not to introduce air bubbles into the chip.

NOTE: Efficient loading requires some practice. If the tissue is not oriented properly, it might be possible to turn it by carefully sucking out and flushing in again. However, this often results in quick cell death.

3.2.4. **After each tissue-loading step, close the corresponding tissue-loading inlet using a piece of PDMS-filled tubing.** Make sure the plugs are sufficiently pushed down to the bottom of the chip using blunt tweezers.

3.2.5. After all samples have been loaded, close any unused inlets/outlets with pieces of PDMS-filled tubing using blunt forceps.

3.3. Assembly of microfluidic setup.

3.3.1. Lower the flow rate of the microfluidic pumps. 60–100 $\mu\text{L/h}$ works well for mouse embryo tailbuds. At higher flow rates, cell death was observed—presumably due to too high cell shearing. The cumulative flow rate of multiple pumps should not exceed these 60–100 $\mu\text{L/h}$ per microfluidic chamber at a given time.

3.3.2. Attach tubing to the microfluidic chip. Do this without getting air bubbles inside the chip (this can be achieved by ensuring that there is a drop of medium present at the end of the tubing). Outlets are still present from the fibronectin coating (see 2.3–2.7).

3.3.3. Once all tubing is attached to the chip, take it out of the beaker, dry it properly, place it in a dish, and put this together with approximately 1.5 m of inlet tubing in an incubator (37 $^{\circ}\text{C}$, 20% O_2 , 5% CO_2) for overnight culture. The tubing is gas permeable; this will allow equilibration of O_2 and CO_2 in the medium. Alternatively, for simultaneous fluorescence real-time imaging, the chip and approximately 1.5 m inlet tubing are placed into the microscope directly. A holder for the chip, which fits into standard 96-well-plate holders, is provided in **Supplementary File 3**.

NOTE: Make sure the humidity is high enough during incubation. If necessary, add a moist tissue to the imaging chamber or incubator to prevent evaporation in the microfluidics setup. If humidity in the microscope incubator is too low, this results in air bubble formation in the tubing and the microfluidic chip. A closed imaging box, in which chip and tubing are placed, can then be used. The simplest way of making such an imaging box is by placing a big lid over the chip and adding a wet tissue, it does not have to be closed off entirely. Make sure that the height difference between pump and chip is not too big and, if necessary, change heights slowly to prevent the formation of air bubbles due to gravity.

3.3.4. Let all pumps flow at a flow rate of 20 $\mu\text{L/h}$ for 20 min, after the setup has been placed to its final location. Because the chamber and pump have most likely moved in height, this is necessary to re-establish the correct pressure in the tubing.

3.3.5. Turn the drug pump off, let only the medium pump run at 60 $\mu\text{L/h}$ until the start of experiment/real-time imaging. For real-time imaging, wait at least another 30 min to let the temperature equilibrate before the start of the imaging to prevent a too strong z drift during real-time imaging.

3.4. Start of the experiment.

3.4.1. Start the planned pumping program for the experiment. To entrain Notch signaling, use a pumping program of 100 min medium and 30 min drug pulses, which is repeated until the end of the experiment, typically for 24 h.

NOTE: Any programmable microfluidic pump can be used. In the simplest format, the pumps can be programmed and started manually. Alternatively, the pumps can be controlled by a computer software.

3.4.2. In case real-time imaging is performed, start imaging after a period of at least 30 min. This will allow the temperature of the chip to adjust to the temperature within the incubator and prevent a too strong drift during imaging. Autofocus is still beneficial.

3.4.3. Perform standard confocal imaging via the glass slide of the chip using an inverted microscope. Use an imaging interval of 10 min to sufficiently detect signaling oscillations with a period of 130 min.

3.4.4. For shorter periods, shorten the interval might for sufficient sampling. Include a low-resolution imaging track for detection of Cascade Blue to visualize the presence of drug on the chip.

3.4.5. For excitation of a Venus reporter, use a 515 nm laser or a 2-photon laser at a wavelength of 960 nm.

3.4.6. Acquire a z stack of 6–8 planes with a distance of 8 μm through a 20x plan objective (resolution 512 x 512 pixels, 1.38 $\mu\text{m}/\text{pixel}$). Use a motorized stage to image multiple samples within one experiment.

3.4.7. Excite Cascade Blue with a 405 nm laser and acquire a single z plane every 10 min (resolution 32 x 32 pixel, 22.14 $\mu\text{m}/\text{pixel}$).

NOTE: Further information on imaging and image analysis are provided in the Representative Results and can be found in reference¹⁰.

REPRESENTATIVE RESULTS:

With this protocol, a method for the external entrainment of signaling oscillations of the mouse segmentation clock using microfluidics is presented. By applying pulses of Notch signaling inhibitor, signaling oscillations in independent embryo cultures get synchronized to each other¹⁰. A prerequisite for the application of this system to study the functionality of the segmentation clock is that signaling dynamics and segmentation are still present on-chip. It was shown previously that both Wnt and Notch signaling dynamics are maintained despite constant medium flow and physical boundary formation persists in peripheral 2D cultures, representing anterior PSM¹⁰.

To confirm entrainment of signaling oscillations to external drug pulses, real-time imaging of, for instance, the Notch signaling reporter LuVeLu⁵, expressing the yellow fluorescent protein Venus, during the microfluidic experiment is performed. Since orientation of the 2D cultures on chip is difficult to control, signaling oscillations in anterior tissue are analyzed. The periphery of the 2D culture can reproducibly be detected. Orientation of the tissue sections on-chip cannot be controlled at will and the whole inner surface of the chip gets coated with Fibronectin. Therefore, it occasionally happens that cultures attach to the sides or ceiling of the chip. In case the samples move out of the field of view (in x, y, and z direction) or they attach with the posterior end of the tail facing downwards, generally these samples are excluded.

For further analysis, multiple of such entrainment experiments are combined. Independent experiments can be aligned to each other using the timing of the drug pulses visualized by the dye, Cascade Blue, at approximately 400 nm. To analyze and visualize synchronization, quantified oscillations can be detrended (**Figure 2B,D**) and then either displayed as mean and standard deviation or phases of the oscillations can be calculated. This allows the analysis of the phase-relationship between oscillations of independent posterior embryo cultures to each other and to the external drug pulses (**Figure 2C,E**). The python-based program pyboat³³ is a straightforward and user-friendly tool to determine period, phase, and amplitude of such signaling oscillations. To confirm entrainment, one can, for instance, determine the period of the endogenous signaling oscillations. When applying pulses with a period of 130 min, Notch signaling oscillations also show a period of 130 min (**Figure 2F**)¹⁰. In addition, using Cascade Blue pulses, independent experiments with different signaling reporters can be aligned to each other. This way the phase-relationship between oscillations of multiple signaling reporters can indirectly be determined¹⁰.

Thus, the presented microfluidic system allows the control of signaling oscillations in *ex vivo* cultures of the developing mouse embryo. In combination with imaging of markers for segment formation and differentiation, this system can now be applied to dissect how signaling pathways of the segmentation clock interact and how they control somite formation.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview of the microfluidics protocol. (A) Chip molds can be designed using computer-aided-design (CAD) software. A chip design to entrain signaling oscillations in *ex vivo* models of mouse somitogenesis is provided. Molds can, for instance, be generated by 3D printing or ordered. Microfluidic chips are produced by molding of PDMS. A hardened PDMS chip is cut out and covalently bonded to a glass slide using a plasma oven. The glass surface within the chip is coated with fibronectin to allow dissected tissue to attach. Embryonic tissue is loaded onto the chip via loading inlets, which are subsequently closed. Pumps with different media conditions are attached to the inlets of the chip and a flow program is initialized. Tissue can be imaged using a confocal microscope during the experiment. (Kymographs adapted from Sonnen et al., 2018¹⁰. Reprinted and modified from Cell according to Creative Commons Attribution CC BY-NC-ND 4.0.) (B) Schematic drawing of a mold design for on-chip culture of posterior mouse embryo tissue. The height of the microfluidic channels is 500 μm , sufficient for the culture of posterior embryonic mouse tails. The file to print this mold is provided in **Supplementary File 1**, and an alternative is given in **Supplementary File 2**. (C) Brightfield image of an E10.5 sectioned mouse tail enclosed by PDMS pillars on-chip.

Figure 2: Representative results from a microfluidics experiment. (A) Schematic representation of entrainment experiment with medium and drug pump. Pulses of signaling pathway modulator are applied to *ex vivo* cultures of mouse embryos and signaling oscillations can be visualized by real-time imaging of dynamic signaling reporters (for instance, the Notch signaling reporter LuVeLu⁵ and Wnt signaling reporter Axin2T2A¹⁰). Dashed lines indicate the region that is used for analyses over time. (B–F) LuVeLu reporter oscillations are entrained by periodic pulses of the Notch signaling inhibitor DAPT. (B,D)

Quantifications of Notch signaling oscillations in anterior PSM upon entrainment using DAPT or a DMSO control. **(C,E)** Phase-phase relation plots between the phase of Notch signaling oscillations and the timing of external DAPT/DMSO pulses. In case of entrainment, a stable phase-relationship is established. **(F)** Quantification of the mean period and SEM of reporter oscillations comparing samples entrained with DMSO and DAPT pulses. (Figure adapted from Sonnen et al., 2018¹⁰. Reprinted and modified from Cell according to Creative Commons Attribution CC BY-NC-ND 4.0.).

Table 1: Troubleshooting

Supplementary File 1: STL file for printing a mold with a 3D printer. This mold is used to generate a microfluidic chip for the on-chip culture of posterior embryonic tissue (design shown in **Figure 1**).

Supplementary File 2: STL file for printing a mold with a 3D printer to generate a microfluidic chip for the on-chip culture of posterior embryonic tissue. In contrast to **Supplementary File 1** and the design shown in **Figure 1**, this design contains bubble traps at all inlets to prevent small amounts of air from entering the main microfluidic chamber.

Supplementary File 3: Design file for the generation of a holder to place the chip into the microscope. This holder has the dimensions of a 96-well plate and should fit into standard holders of most microscopes.

DISCUSSION:

How signaling dynamics control multicellular systems has been a longstanding question in the field. Functional investigation poses a key challenge, because these dynamics have to be subtly modulated to allow this¹¹. Such temporal control over pathway perturbations can in principle be achieved with optogenetics, which also enables a high spatial control³⁴. However, optogenetics require the establishment of sophisticated genetic tools for the analysis of each signaling pathway in question. The current protocol described here provides a highly versatile tool for the perturbation of any signaling pathway. The microfluidics experiment allows the application of temporally controlled drug pulses to functionally investigate the dynamics of signaling pathways in tissue cultures. Here, the focus is on the study of somitogenesis in *ex vivo* cultures, but the protocol can be adapted to fit any other model systems.

Certain steps in the protocol are critical to perform a successful microfluidics experiment (summarized in **Table 2**). Key points are discussed as follows. Due to medium flow during the course of the experiment, the tissue culture experiences shear stress. Exposure of the model system to continuous flow can cause cell stress and in extreme cases cell death due to shearing effect. For *ex vivo* tissue cultures of mouse tailbuds and the current chip design, a flowrate of 60 $\mu\text{L/h}$ (maximum of 100 $\mu\text{L/h}$) was found to work well with minimal shearing effect. When multiple pumps are turned on simultaneously for the same chip, flow rate of individual pumps needs to be adjusted accordingly to not cause an increase in total flow rate. When the current protocol is adapted to other model systems and chip designs, the flow rate should be optimized. Alternatively, it is possible to not flush medium continuously, but periodically change medium on the chip³⁵. Such a setup can also be applied to control signaling oscillations in mouse somitogenesis (unpublished, data not shown). Moreover, a

setup can also be envisioned, in which tissue cultures are not exposed to direct fluid flow, but drugs reach the tissue by diffusion from a neighboring channel. Such systems have been used successfully to apply gradients of growth factors to *in vitro* models of ESC differentiation^{14,36}.

One major issue of microfluidic experiments is the occurrence of air bubbles on-chip during the experiment. Air bubbles within the chip or tubing can interfere with uniform liquid flow on the chip and can lead to removal of the embryo culture from its location. To prevent the presence of air bubbles, various steps of the protocol are indispensable. First, culture medium within syringes and the microfluidic chip itself must be degassed using a desiccator (step 3.1.3). Second, when loading samples into the loading inlets, one must be careful not to pipet air into the chip together with the sample (step 3.2.3). Third, when filling the tubing with medium, all air bubbles must be pumped out before attaching the chip (step 3.3.2). Otherwise, these bubbles will be pushed into the main chip during the experiment. Lastly, during the experiment, the medium is equilibrated within the imaging chamber or incubator due to the semi-permeable tubing. The permeability of the tubing also allows for evaporation of the medium, so make sure that the humidity is regulated during the experiment to prevent the formation of new bubbles in the tubing.

In general, microfluidics is a highly versatile tool that can be adapted to the researcher's specific question. The current design approach allows for imaging up to 12 *ex vivo* cultures in parallel per chip. This number is mainly limited by the number of embryos per experiment and the time it takes to image each embryo culture with sufficient resolution. If multiple conditions need to be compared in a single experiment, multiple chips can be mounted on a single glass slide. A chip design is provided, which is ideal for imaging of multiple tissue explants in parallel, but personalized designs can overcome limitations in sample number to allow high throughput analysis and increase the combination of more conditions within a single experiment^{16,17}. Microfluidics is limited to models that can be cultured on a chip and on-chip culture will have to be optimized for each model system individually^{27,37,38}.

Over the last 5–10 years, microfluidics has been applied to address various biological questions due to its potential for high throughput approaches and subtle modulations of signaling dynamics and gradients. Nowadays, microfluidics has become an easily applicable, cheap, and versatile tool that laboratories can establish with ease. Here, the current protocol is optimized for a specific application, namely, studying signaling dynamics governing somitogenesis. It is straightforward to adapt this protocol and design to suit individual research questions in tissue biology.

ACKNOWLEDGMENTS:

We are grateful to Yang Li and Jos Malda from the UMC Utrecht for help with 3D printing of molds, Karen van den Anker from the Sonnen group for very useful feedback on the protocol, and Tjeerd Faase from the mechanical workshop at the Hubrecht Institute for the holder for microfluidic chips within the microscope. We would like to thank the entire Sonnen group for critical reading of the manuscript and the reviewers for their constructive feedback. This work received funding from the European Research Council under an ERC starting grant agreement no. 850554 to K.F.S.

DISCLOSURES:

The authors have nothing to disclose.

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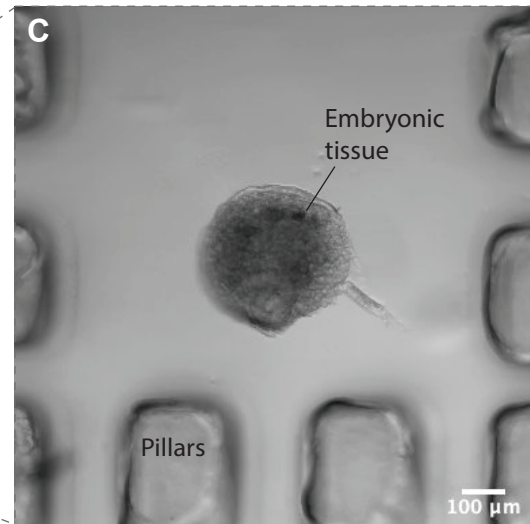
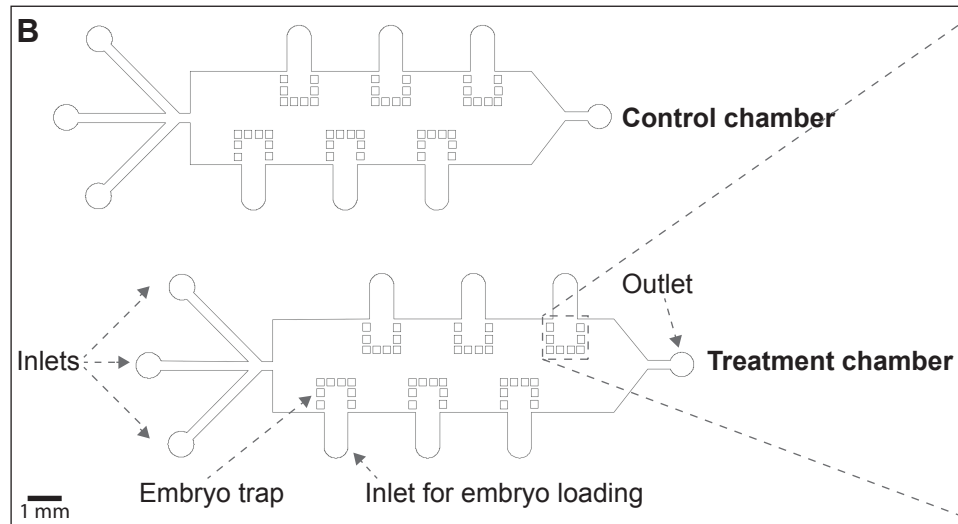
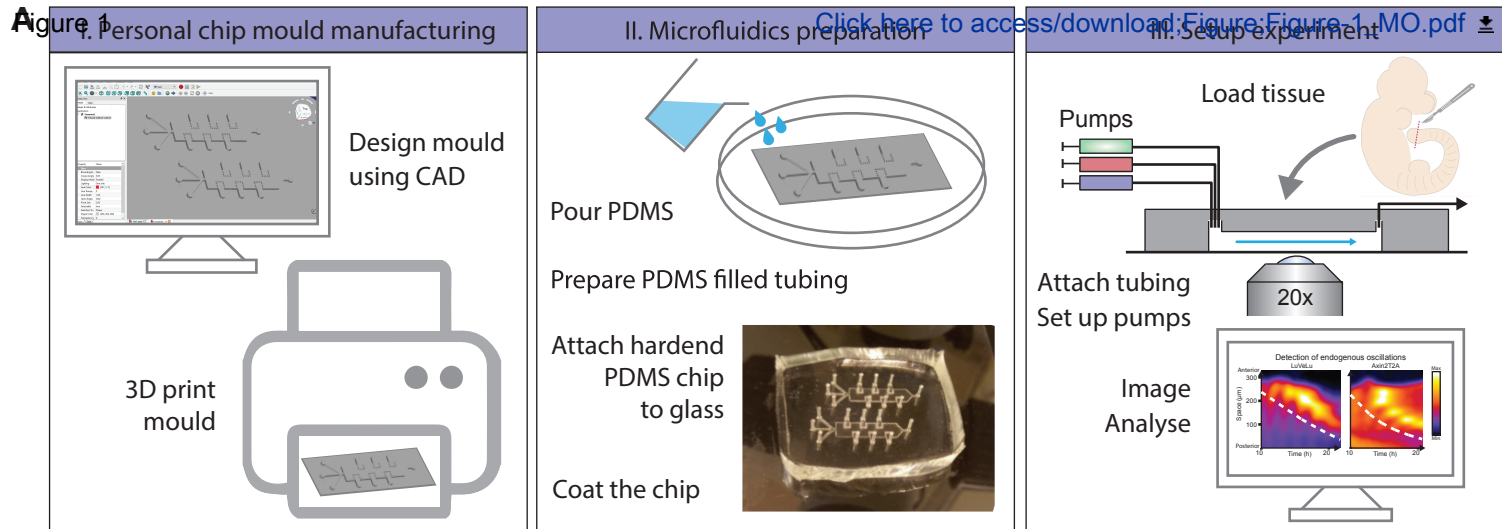
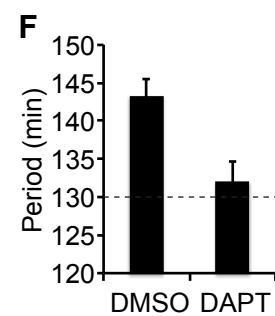
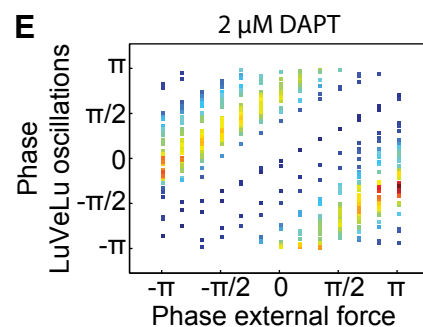
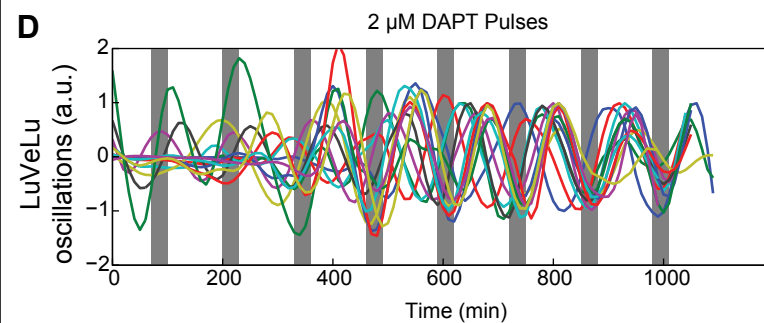
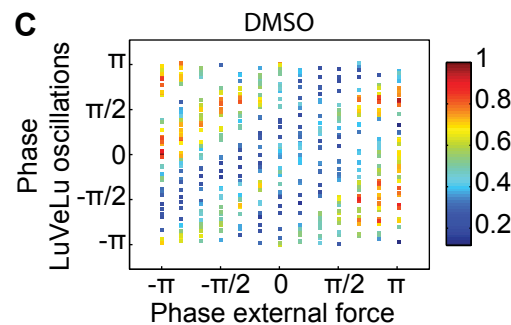
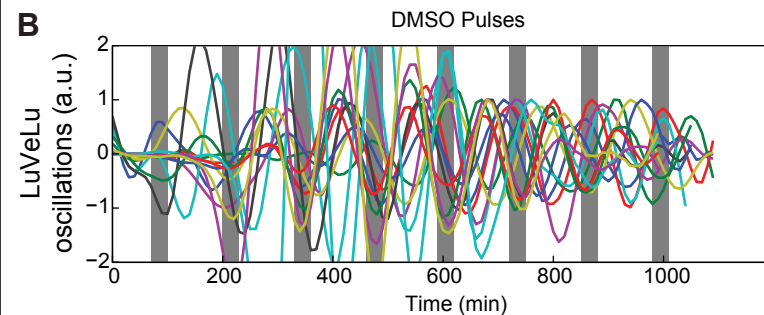
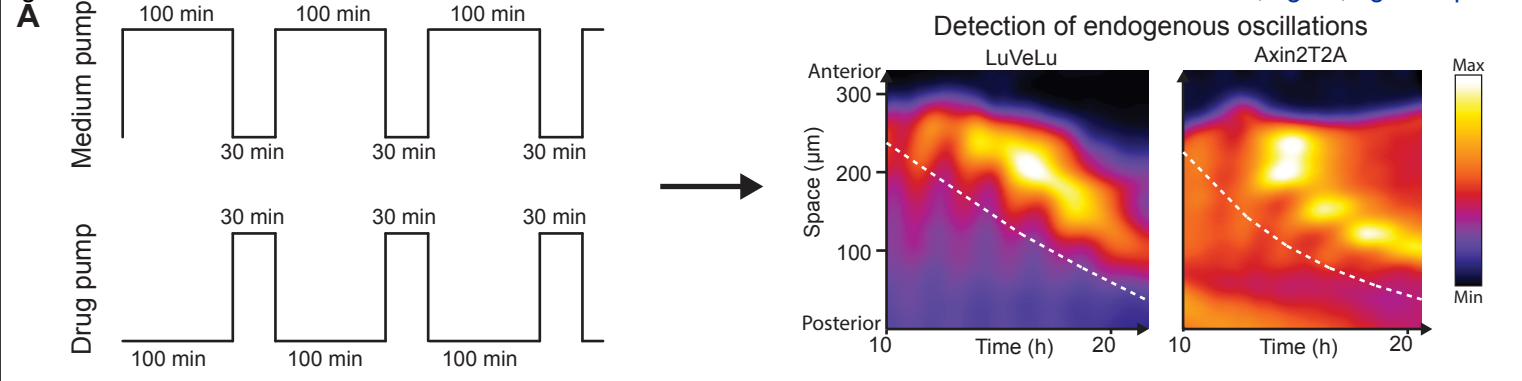


Figure 2



Problem

The PDMS does not bond to the glass.

There are air bubbles on my chip.

The tissue dies at the beginning of the experiment.

The tissue dies during imaging.

The focus changes during imaging.

Outlets/inlets detach.

The glass of the chip breaks.

There is a contamination on my chip.

Suggested solution(s)

- Make sure both the PDMS and glass are clean.
- Make sure there is enough PDMS around the chamber for bonding.
- Optimize the settings of the plasma oven.
- Check if the pump was turned on.
- Degas the chip before loading tissue onto the chip.
- Degas the medium before start of the experiment.
- Make sure the humidity in the incubation chamber is high enough.
- Add HEPES to the medium when loading and assembling the chip.
- Do not flush the tissue in and out too often during loading.
- Check that the flow rate is not too high.
- Make sure there is no phototoxicity from the imaging
- Make sure there is no contamination.
- The flow rate should not be too high.
- Use autofocus during imaging to adjust for drift of the imaging slide.
- Let the chip equilibrate to the temperature within the microscope for at least 20 minutes.
- Push all tubing completely down to the bottom.
- Be more careful, the glass is very fragile.
- The thin glass is only required for imaging. If imaging is not performed, a normal thicker glass slide can be used.
- If the break is outside of the chip, it might not be a problem. If the glass seal to the chip is broken, liquid will leak out and air will come in.
- Add antibiotics to the culture medium.
- Sterilize all equipment and tubing.
- Work fast and clean.

Name	Company
10 mL syringe	Becton, Dickinson and company
184 Silicon Elastomer curing agent	SYLGARD
184 Silicon Elastomer PDMS	SYLGARD
3 ml syringes	Becton, Dickinson and company
Adhesive tape	
Blunt-tip forceps Bochem 18/10 Stainless steel	Fisher Scientific
Bovine Serum albumin Lyophilised pH~7	Biowest
Compressed air system	
Crystallizing dish	VWR
D-(+)-Glucose 45%	Sigma
Desiccator	VWR
Disposable cups	Duni
Disposable forks	Staples
Dissection equipment	
DMEM/F12	Cell culture technologies
Fibronectin	Sigma
Flow hood BioVanguard Greenline	CleanAir by Baker
Glass slide No 1.5H high precision, 70x70 mm	Marienfeld
HEPES Buffer	Gibco
L-glutamine	Gibco
Microscope	Leica
Needles 22G	Becton, dickinson and company
Oven	VWR
PBS0	
Penicillin-Streptomycin	Gibco
Plasma oven Femto	Diener electronic
Punch Ø 1mm	Uni-Core
Scale	Sartorius
Syringe pump	WPI
Tubing Ø 1mm	APT
Vacuum pump	VWR

Catalog number	Comments
300912	
1673921	
1673921	
309657	
	Scotch Magic tape or similar
10663341	
P6154	
10754-7	
G8769	Sterilized
467-0104	
173 941	
511514	
custom	DMEM/F-12, no phenol red, no L-glutamine, no Glucose
F1141-5MG	
511514	For UV light source
107999098	
15630-056	
25030024	
Leica SP8 MP	
z412139-100EA	
390-09	
15070063	
WB100073	
Entris	
AL-4000	
AWG24T	
181-0308	

JoVE62318 – rebuttal document

Utrecht, 27 January 2021

Dear Dr. Bajaj,

Herewith, we re-submit our protocol “A microfluidics approach for the functional investigation of signalling oscillations governing somitogenesis”. We would like to thank you and the reviewers for reading our manuscript and providing very constructive feedback. Based on these comments we have revised and modified the manuscript to address all the raised concerns.

In the following, we will describe in detail how we have addressed the reviewer's comments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please provide an institutional email address for each author.
3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
4. Please define all abbreviations before use (Wnt, FGF, etc.)
5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., SYLGARD, Diener Femto oven, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.
7. Line 119-131: Please add these lines as NOTE or move it to the Discussion section. Please consider citing a reference for the protocol mentioned.

We have carefully read the editorial comments and made the required changes. For instance, abbreviations have been defined, such as FGF. However, Wnt is not an abbreviation, rather a protein family name given a portmanteau of Wingless and int-1 and is thus not defined. Additionally, we have added a brief ethics statement:

Research involving vertebrate model systems requires prior ethical approval from the responsible committee. Research presented here has been

approved by the EMBL ethics committee¹⁰ and the Dutch committee for animal research (dierenexperimentencommissie, DEC), and follows the Hubrecht guidelines for animal care.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

9. Line 148: Please mention how is the air removed.

10. Line 283: Please include the size of the needle used.

We have read the manuscript again and have added details wherever we think they are useful.

11. Line 313-317: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm²

12. Line 354-357: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.

We have made the required changes.

13. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next.

We have checked the highlighted text again and made sure that there are roughly 3 pages or less highlighted in yellow.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from

[citation].”

Reprint permission has been obtained and the permission will be attached to the current submission.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript, authors demonstrated how to utilize microfluidic devices for the study of signaling dynamics in primary mouse tissues.

The protocol provides step-by-step approaches to construction of the microfluidic experiments, with pointing out critical steps, such as degassing.

Representative results are also shown with concrete examples that demonstrate successful control of Notch signaling dynamics in mouse pre-somitic mesoderm tissues by periodic microfluidic inputs.

This manuscript will be an excellent guide to starting microfluidic experiments in the broad field of cell and developmental biology.

We thank reviewer 1 for these useful comments.

Minor Concerns:

L91: Authors can put an example (or recommendation) of freely available CAD software.

This information has been added under subsection 1, “Generation of the chip”:

Freely available CAD software is for instance uFlow (www.biomicrofluidics.com) or 3D μ F³⁰. A repository of free designs is provided by the MIT (Metafluidics.org).

L302: Authors can put the value of "the natural period of the segmentation clock" for easy comparison with input period (130 min).

This is indeed important to add. We now mention the natural period under subsection 3, “Microfluidics experiment”, as follows:

Applying pulses with a period of 130 min, close to the natural period of the mouse segmentation clock (137 min²⁵), allows efficient entrainment.

L410: Authors can add an explanation about LuVeLu more, e.g. information about the fluorescent protein Venus.

L421: Related to the L410, authors can explain why the dye Cascade Blue is compatible with Venus reporter by referring fluorescent property of these fluorophores.

We have added an explanation for the usage of Cascade Blue to visualize the presence of drug on the chip. We also discuss the choice of the fluorescent dye and suggest ways on how to select different combinations.

The presence of drug within the chip is monitored using a fluorescent dye. The excitation and emission spectra of this dye and the fluorescent signalling reporter have to be different enough to prevent bleed-through during fluorescence real-time imaging. When yellow fluorescent proteins are used as signalling reporter, such as the Notch signalling reporter LuVeLu⁵ (Lunatic fringe-Venus-Lunatic fringe), the dye Cascade Blue can be applied. To identify other possible combinations of fluorescent dye and reporter, freely available spectra viewer can be found on the internet.

L508-512: Authors can put the numbers of corresponding steps with "First", "Second", and "Third" steps (maybe 3.1.3, 3.2.3 and 3.3.2 in the PROTOCOL, respectively?).

This is a very good point and has been added to the manuscript.

First, culture medium within syringes and the microfluidic chip itself have to be degassed using a desiccator (step 3.1.3). Second, when loading samples into the loading inlets, one has to be careful not to pipet air into the chip together with the sample (step 3.2.3). Third, when filling the tubing with medium, all air bubbles have to be pumped out before attaching the chip (step 3.3.2).

Fig.1: Authors can add an information about the z-axis distance between glass surface and the PDMS layer. Is the distance much larger than the size of tissues?

We have added this information to the figure legend as follows:

The height of the microfluidic channels is 500 μm , sufficient for the culture of posterior embryonic mouse tails. The file to print this mold is provided in Supplementary File 1, an alternative is given in Supplementary File 2.

Fig.1B: The design of the chip is a bit different from the original article (Sonnen et al. Cell 2018); Especially, zigzag patterns close to "Inlets" were removed. Authors can mention why the design is renewed from the original article and what is a benefit of the new design.

The design is slightly changed to allow the generation of the mold with a 3D printer, even if it does not have a high resolution. We now also provide a second mold, including air bubble traps, in supplementary file 2. We refer to both files in the text:

Here, a design for a mold is provided that can be printed with a 3D printer for the on-chip culture of mouse embryo tissue (Figure 1B,C, Supplementary files 1 and 2). To allow printing with lower resolution 3D printers, the design has been modified compared to the previously published one¹⁰. A mold without air bubble traps and one with air bubble traps is provided. (Supplementary files 1 and 2, respectively).

(10. Sonnen, K.F. et al. Modulation of Phase Shift between Wnt and Notch Signaling Oscillations Controls Mesoderm Segmentation. Cell. 172 (5), 1079-1090.e12, doi: 10.1016/j.cell.2018.01.026 (2018).)

Reviewer #2:

Manuscript Summary

The authors of this study "A microfluidics approach for the functional investigation of signalling oscillations governing somitogenesis." report a protocol for the fabrication and application of a microfluidic device that allows for the pulsing of environmental conditions for the cultivation of mouse embryonic tissue and the functional investigation of mouse somitogenesis. The protocol is well written und contains most important steps. I am not yet convinced that potential experimenters can perform the complete experiments successful using this protocol. Some important steps are

missing. I thus recommend improving the manuscript. Following major and minor concerns might help to improve the manuscript.

We thank reviewer 2 for the constructive feedback. We have addressed the raised points in the following way:

Major Concerns

* Introduction: A more general introduction would be helpful to guide the reader to the importance of "pulsing"/ "oscillating" environments. Following publication might help: <https://onlinelibrary.wiley.com/doi/full/10.1002/sml.201906670>.

We have now added a general introduction into signalling dynamics as follows:

Development is controlled by intercellular communication via signalling pathways. There is only a limited number of signalling pathways that orchestrate the complex formation of tissues and proper cell differentiation in space and time. To regulate this multitude of processes, information can be encoded in the dynamics of a signalling pathway, the change of a pathway over time, such as the frequency or duration of a signal^{1, 2}.

(1. Manning, C.S. et al. Quantitative single-cell live imaging links HES5 dynamics with cell-state and fate in murine neurogenesis. *Nature Communications*. 10 (1), doi: 10.1038/s41467-019-10734-8 (2019).

2. Seymour, P.A. et al. Jag1 Modulates an Oscillatory Dll1-Notch-Hes1 Signaling Module to Coordinate Growth and Fate of Pancreatic Progenitors. *Developmental Cell*. 1–17, doi: 10.1016/j.devcel.2020.01.015 (2020).)

We think that this information in addition to the discussion of how the function of signalling dynamics can be studied is sufficient to highlight the relevance of the current protocol. We do mention the following in the introduction:

However, to be able to investigate the function of signalling dynamics in the control of cellular behaviour, it is essential to subtly modulate signalling pathways instead of permanently activating or inhibiting them.

We also discuss that this system can be used to apply other types of environmental perturbations.

* Design: Design and design information of the chips are missing, which is important to reproduce the work.

In Supplementary File 1 the chip design was provided. In this resubmission we do however add an additional design including air bubble traps in Supplementary File 2. We refer to both files in the text.

Here, a design for a mold is provided that can be printed with a 3D printer for the on-chip culture of mouse embryo tissue (Figure 1B,C, Supplementary files 1 and 2). To allow printing with lower resolution 3D printers, the design has been modified compared to the previously published one¹⁰. A mold without air bubble traps and one with air bubble traps is provided. (Supplementary files 1 and 2, respectively).

(10. Sonnen, K.F. et al. Modulation of Phase Shift between Wnt and Notch Signaling Oscillations Controls Mesoderm Segmentation. *Cell*. 172 (5), 1079-1090.e12, doi: 10.1016/j.cell.2018.01.026 (2018).)

Additionally, readers who wish to learn more on mould generation via lithography are specifically addressed here:

Moulds are either printed with a 3D printer or can be generated by soft lithography using a photomask that contains the desired design (for further information see e.g. Qin et al., 2010³¹).

(31. Qin, D., Xia, Y., Whitesides, G.M. Soft lithography for micro- and nanoscale patterning. *Nature Protocols*. 5 (3), 491–502, doi: 10.1038/nprot.2009.234 (2010).)

* Cultivation: The operation of the final experiment and the image analysis to obtain the data is missing.

This is a very good point. We have now added information on the used culture medium and refer to a previous publication as follows:

3.1.1 On the day of the experiment, prepare 50 mL of culture medium (DMEM-F12 supplemented with 0.5 mM glucose, 2 mM glutamine and 1% BSA, for further information of mouse tailbud culture see ²⁵).

We also provide a design for a holder to hold the chip within the microscope in Supplementary File 3. We refer to this file in point 3.3.3:

3.3.3 A holder for the chip, which fits into standard 96-well-plate holders, is provided in supplementary file 3.

NOTE: Make sure the humidity is high enough during incubation. If necessary, add a moist tissue to the imaging chamber or incubator, to prevent evaporation in the microfluidics set-up. If humidity in the microscope incubator is too low, this results in air bubble formation in the tubing and the microfluidic chip. A closed imaging box, in which chip and tubing are placed, can then be used. The simplest way of making such an imaging box is by placing a big lid over the chip and adding a wet tissue, it does not have to be closed off entirely.

We have also added further information on the imaging settings to point 3.4.2:

*In case real-time imaging is performed, **start imaging** after a period of at least 30 min. This will allow the temperature of the chip to adjust to the temperature within the incubator and prevent a too strong drift during imaging. Autofocus is still beneficial. Standard confocal imaging via the glass slide of the chip is performed using an inverted microscope. An imaging interval of 10 min is sufficient to detect signalling oscillations with a period of 130 min. For shorter periods, the interval might have to be shortened for sufficient sampling. Include a low-resolution imaging track for detection of Cascade Blue to visualize the presence of drug on the chip. For excitation of a Venus reporter, use a 515 nm laser or a 2-photon laser at a wavelength of 960 nm. Acquire a z stack of 6 – 8 planes with a distance of 8 μm through a 20x plan objective (resolution 512 x 512 pixels, 1.38 $\mu\text{m}/\text{pixel}$). Use a motorized stage to image multiple samples within one experiment. Excite Cascade Blue with a 405 nm laser and acquire a single z plane every 10 min (resolution 32 x 32 pixel, 22.14 $\mu\text{m}/\text{pixel}$). Further information on imaging and image analysis are provided in the Representative Results and can be found in ¹⁰.*

We think that this information is sufficient to perform this described experiment.

- * Images are of really bad quality and need significant improvements for readability.
- o Figure 1A: These are successive steps that are performed. This should be visualized. It is not clear that the different text fragments are different steps.
- o Figure 1B: Dimensions are missing
- o Figure 1C: labelling is missing
- o Etc.

General statements like “Images are of really bad quality” are not very constructive and in addition, we disagree, especially because Figure 2 has been published in similar forms previously. We did however address the specific remarks related to Figure 1. In particular, we have added numbers to the consecutive steps, have added a scale bar to Figure 1B and labels to Figure 1C.

Minor Concerns

- * Previously similar published protocols should be cited to enable the reader to have a look at similar methods

In response to this comment, we have added relevant citations to the introduction as follows:

Personalized chip designs allow for a plethora of options for spatiotemporal perturbations within the local environment, e.g., stable gradient formation^{12–15}, pulsatile activation/inhibition^{10, 16–18} or localized perturbations^{19, 20}. Microfluidics can also enable a more reproducible read-out and higher throughput due to automation of experimental handling^{21–23}.

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In summary, we would like to thank the reviewers again for their time and comments. With the changes we have made, we think that the manuscript did indeed improve and that the protocol will now allow other groups to implement a microfluidic system in their own labs. We are therefore confident that these additional changes are satisfactory for publication.

Sincerely,

Katharina Sonnen

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