Video Article

# Investigating Functional Regeneration in Organotypic Spinal Cord Co-cultures Grown on Multi-electrode Arrays

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## Abstract

Adult higher vertebrates have a limited potential to recover from spinal cord injury. Recently, evidence emerged that propriospinal connections are a promising target for intervention to improve functional regeneration. So far, no *in vitro* model exists that grants the possibility to examine functional recovery of propriospinal fibers. Therefore, a representative model that is based on two organotypic spinal cord sections of embryonic rat, cultured next to each other on multi-electrode arrays (MEAs) was developed. These slices grow and, within a few days *in vitro*, fuse along the sides facing each other. The design of the used MEAs permits the performance of lesions with a scalpel blade through this fusion site without inflicting damage on the MEAs. The slices show spontaneous activity, usually organized in network activity bursts, and spatial and temporal activity parameters such as the location of burst origins, speed and direction of their propagation and latencies between bursts can be characterized. Using these features, it is also possible to assess functional connection of the slices by calculating the amount of synchronized bursts between the two sides. Furthermore, the slices can be morphologically analyzed by performing immunohistochemical stainings after the recordings.

Several advantages of the used techniques are combined in this model: the slices largely preserve the original tissue architecture with intact local synaptic circuitry, the tissue is easily and repeatedly accessible and neuronal activity can be detected simultaneously and non-invasively in a large number of spots at high temporal resolution. These features allow the investigation of functional regeneration of intraspinal connections in isolation *in vitro* in a sophisticated and efficient way.

## Video Link

The video component of this article can be found at http://www.jove.com/video/53121/

## Introduction

Organotypic slice cultures of the central nervous system (CNS) have been used extensively to study various physiological and pathological phenomena reaching from neuronal development to neurodegeneration. Compared to dissociated cell cultures, organotypic slices offer the advantage of more complexity with an intact cytoarchitecture and local synaptic circuitry. At the same time, the tissue can be analyzed in an isolated way without the need to implicate the intricacy of the whole *in vivo* context. Moreover, easy and repeated access to the cells of choice is warranted, the extracellular environment can be precisely controlled and usually, *in vitro* models are less costly than their *in vivo* counterparts. In recent years, various studies presented striking similarities between the developmental profiles of long term cultures versus the corresponding living animals [1,2]. It has been shown that neuronal circuits of various CNS regions express spontaneous network activity during development and that organotypic slices partially maintain this phenomenon [3–7]. Isolated spinal cord preparations have been particularly used to investigate rhythm generation [6] and the formation of neuronal circuits [1].

A way to record the spontaneous activity of organotypic slices is to culture them on top of multi-electrode arrays (MEAs). These devices contain multiple electrodes that can monitor the extracellular potentials generated by action potentials from numerous cells simultaneously (multi-unit recording). The high temporal resolution of the MEA recordings can be used to reconstruct the precise activity dynamics within a given neuronal circuit. Additionally, in contrast to patch-clamping the technique is non-invasive, which permits long-term measurements and results in the fact that neurons are not affected in their behavior.

For the purpose of this study, the combination of organotypic spinal cord co-cultures and multisite recordings was used to investigate functional regeneration within the spinal cord. Since adult higher vertebrates have limited potential to recover from damage of the spinal cord, different strategies have been studied to promote regeneration after spinal cord injury. So far, the corticospinal tract has usually been the model system of choice for such investigations. These experiments are typically time-consuming, costly and require large animal cohorts due to high variability within single groups. Moreover, evidence suggests that propriospinal connections (neurons that are entirely confined within the spinal cord) can play a pivotal role in the recovery process following spinal cord lesions [8]. These fibers are difficult to study *in vivo* without the interference of ascending and descending fiber tracts. Bonnici and Kapfhammer [9] used longitudinal spinal cord slice cultures of postnatal mice as an

alternative approach. After performing mechanical lesions they analyzed semi-quantitatively the morphological regeneration of axons between spinal cord segments. They observed less but not none axons crossing the lesion site in cultures cut at a mature age. In contrast, cultures that were lesioned at a younger stage displayed a high amount of axonal regeneration 5 - 7 days after the damage. However, proof for functional connections was not presented.

Therefore, the development of a representative *in vitro* model of propriospinal fibers that allows the investigation of functional regeneration can be a valuable tool to extend our knowledge about regenerative processes in the spinal cord.

## Protocol

Animal care was in accordance with guidelines approved by Swiss local authorities (Amt für Landwirtschaft und Natur des Kantons Bern, Veterinärdienst, Sekretariat Tierversuche, approval Nrs. 52/11 and 35/14). These guidelines are in agreement with the European Community Directive 2010/63/EU.

Note: Work in a laminar flow hood with sterile instruments and solutions for all steps 1 - 5 including sub-steps.

## 1. Preparation of MEAs

Note: MEAs are composed of a glass substrate, micro-fabricated metal electrodes and a SU-8 polymer insulation layer (also see Tscherter *et al*. [3]). For the purpose of this study, commercially available MEAs were ordered with a customized electrode array layout (**Figure 1 A&B**). The 68 electrodes are arranged in a rectangular grid that is split into two zones by a 300 µm wide groove free of electrodes, electrical leads and insulation. Each electrode is 40 x 40 µm in size and they are spaced apart by 200 µm from center to center. Four large ground electrodes are positioned around the recording site. They differ from other commercially available standard MEAs in their size (21 mm x 21 mm) and they have no fixed recording chamber.

1. For disinfection rinse MEAs in 100% ethanol (2x), 70% ethanol (1x) and distilled water (2x) for at least 30 sec each. Let dry. Put 10 - 12 MEAs in a clean glass petri dish (150 mm x 25 mm) and close the lid.
2. Autoclave MEAs for 20 min at 120 °C. Store at RT.
3. Prepare the coating solution (see materials list) for the MEAs and store aliquots at -20 °C.
4. Put each MEA in an individual sterile petri dish (35 mm x 10 mm).
5. Use a cooled pipette to put 150 µl chilled coating solution on top of the electrodes and close the lid of the petri dish. After about 10 min, check for air bubbles on top of the electrodes and gently remove them with a rubber-covered spatula if necessary. Let rest for 1 hr at RT.
6. Aspirate coating solution residues, wash 1x with medium optimized for prenatal and embryonic neurons and 2x with distilled, sterile water. If MEAs are not used until the next day, keep them in distilled, sterile water at 37 °C in the incubator O/N. Otherwise, let directly dry at RT.

## 2. Ingredients Required for the Preparation and Growth of Organotypic Cultures

1. Prepare calcium-free wash solution (see materials list).
2. Reconstitute chicken plasma in wash solution (1:1, shake gently, avoid formation of bubbles), centrifuge at 3,000 x g for about 20 min and decant the clear content into a sterile tube. Aliquot 200 µl into cryotubes and store at -20 °C.
3. Reconstitute thrombin from bovine plasma accordingly (200 U/ml) and sterile-filter (0.2 µm pore filter). Aliquot 200 µl into crytotubes and store at -20 °C.
4. For 30 cultures prepare 100 ml of nutrient medium (see materials list).

## 3. Spinal Cord Tissue Dissection

Note: Procedure yields, depending on the number of embryos, spinal cord slices for about 25 - 35 co-cultures and is prepared inside a laminar flow hood under sterile conditions.

1. Apply a lethal dose of pentobarbital (0.4 ml) to the mother animal by intramuscular injection. Confirm deep anesthesia by checking the pedal withdrawal reflex. Deliver E14 rat embryos by cesarean section and sacrifice by decapitation.
2. Transfer the bodies of the embryos into a petri dish filled with sterile, chilled, wash solution.
3. Perform one complete transversal cut with a scalpel above the hindlimbs and another one above the forelimbs and remove the limbs from the body. Next, make a cut in the frontal plane to separate viscera from the back piece containing the spinal cord.
4. For slicing, transfer the back pieces containing the spinal cord one at a time on a mounting disk and cut at a thickness of 225 - 250 µm with a tissue chopper. Put a drop of wash solution on the chopped tissue and transfer slices with a spatula in 35 mm x 10 mm petri dishes filled with sterile, chilled, wash solution.
5. For each slice separately, dissect spinal cord away from remaining tissue. Leave dorsal root ganglia attached.
6. Let the slices rest for 1 hr at 4 °C.

## 4. Mounting Spinal Cord Tissue Slices on MEAs

1. Warm up sterile nutrient medium in the incubator (37 °C, lightly unscrewed lid for oxygenation).
2. Position MEA with sterile tweezers with rubber-covered tips at RT in the petri dish under a stereo microscope with the electrode array in focus. Center a 6 µl droplet of chicken plasma on the clean, dust-free, and sterile electrode array. Using a small spatula, carefully slide two spinal cord sections with ventral sides facing each other into the plasma droplet. Do not touch the electrodes with the spatula.

3. Add 8 µl of thrombin around the chicken plasma droplet. Using the thrombin pipette tip, carefully mix and spread the chicken plasma/thrombin mixture around the two slices. Again, do not touch the brittle electrode array directly. Just before coagulation, aspirate excess chicken plasma/thrombin.
4. Cap the petri dish to retain high humidity while the MEA/culture assembly sits for about 1 hr in a humidified chamber inside the incubator at 37 °C.
5. Carefully add 10 µl of nutrient medium to the culture chamber, cap the petri dish and put back into the incubator for about 30 min.
6. Place each MEA/culture assembly with sterile, rubber-covered tips into a roller tube, add 3 ml of nutrient medium and tightly close the lid. Place the roller tube in the roller drum rotating at 1 - 2 rpm in the incubator in a 5% $CO_2$-containing atmosphere at 37 °C.
7. Change half of the nutrient medium after 7 days *in vitro* (DIV) and afterwards 1 - 2 times per week.

## 5. Mechanical Lesions

1. Take the MEA/culture assembly with sterile, rubber-covered tips out of the roller tube and place in a petri dish without nutrient medium under a stereo microscope with the tissue in focus. Visually verify that the two slices are fused.
2. Hold the MEA/culture assembly steady by placing tweezers with rubber-covered tips on the MEA.
3. Place a scalpel blade in the groove of the MEA close to the tissue slices. Hold the scalpel rather horizontally.
4. Lift the scalpel handle up but let the scalpel blade stay in the groove of the MEA in such a way that the blade “rolls” from base to tip and thereby cuts through the tissue covering the groove.
5. Severe any residual tissue connections with a 25 G needle tip if necessary. Work only in the area within the groove and do not touch the tender edges.
6. Put the MEA/culture assembly back into the roller tube. Provide 3 ml of fresh nutrient medium to the cultures and place them back into the roller drum in the incubator.

## 6. Electrophysiological Recordings of Spontaneous Activity

1. To investigate functional regeneration among the two spinal cord slices after the chosen number of DIV, mount the MEA/culture assembly in a recording chamber on a microscope and apply about 500 µl extracellular solution (see materials list).
2. Wait 10 min before the first recording to allow the system to stabilize.
3. Record basic spontaneous activity 2x for about 10 min from each activity-detecting electrode of the MEA at RT.
4. To ensure stable extracellular conditions, exchange extracellular solution after every recording session.
5. To disinhibit the network, apply extracellular solution containing strychnine (1 µM) and gabazine (10 µM). Wait for at least 2 min before starting the recording.

## 7. Data Analysis

Note: For the detection of the extracellularly recorded action potentials use for each electrode a detector based on standard deviations and a subsequent discriminator. This procedure is described in detail in Tscherter *et al*. [3].

1. Display the detected neuronal activity of each electrode in a raster plot according to standard procedures [3] (**Figure 1F**).
2. Determine and display the total network activity for each slice by summing up the number of events in the according channels within a sliding window of 10 msec, shifted by 1 msec steps (**Figure 1G** and see Tscherter *et al.* [3]).
3. Detect in each slice individual bursts (clusters of activity that appear on several electrodes). Therefore, set a minimal peak activity threshold in the corresponding total network activity and define each burst start and burst end. For example, an appropriate threshold is 25% of the average burst amplitude and a burst start can be defined by being the first event in a time window of at least 5 msec, a burst end by being the last event in a time window of at least 25 msec (see Heidemann *et al.* [10]).
4. To quantify functional regeneration calculate the percentage of synchronized bursts between the two slices. To do this, calculate the latency between a burst in one slice and the following burst in the other slice.
   Note: A burst pair is termed “synchronized” when the latency is smaller than the average burst length. Especially in co-cultures with a lot of activity, the possibility exists that both sides coincidentally initiate a burst in the chosen latency window. This fact has to be taken into account in the quantification of the percentage of synchronized bursts. For a detailed description of the calculations see Heidemann *et al.*[10].

## 8. Fixation of the Cultures and Immunohistochemistry

1. For all steps that include washing or incubation make sure to put the cultures on a tilting table to ensure proper diffusion of the solution through the tissue.
2. Directly after the recording session is finished take the MEA/culture assembly out of the setup, put it in a petri dish (35 mm x 10 mm) and add about 2 ml of extracellular solution.
3. To separate the slices from the surrounding cell layers that have formed during the time *in vitro,* take a 10 µl pipette tip and circle the slices. Pay attention not to damage the cultures. It is possible to omit this step but embedding later on is easier when it is performed.
4. Use a thin nonmetallic syringe needle (see materials list) in combination with a 1 ml syringe filled with extracellular solution to gently blow the culture off the MEA.
5. Remove the tip of a Pasteur pipette and fit the rubber bulb on the slivered end. Use the back end to transfer the culture into fixative (4% paraformaldehyde with phosphate buffered saline (PBS, 0.1 M)). Do not use the standard tip of the Pasteur pipette for the transfer because it is too small and the cultures would be folded and probably damaged. Fix for 1 - 2 hr at 4 °C. Rinse the MEA with distilled water and remove tissue residues with fingertips but do not scratch with the nail. Store MEAs in distilled water at 4° C.
6. Wash the cultures 3x in PBS for at least 10 min each. Store in PBS at 4 °C or directly start with the staining procedure.
7. Incubate the cultures for at least 90 min in blocking solution (5% goat serum, 1% bovine serum albumin, 0.3% detergent in PBS).

8. Add the first antibody diluted in blocking solution and incubate for 3 nights at 4 °C.
9. Wash 3x in PBS for at least 30 min each.
10. Add the second antibody diluted in PBS for 2 hr at RT.
11. Wash 3x in PBS for at least 30 min each.
12. Transfer the slices with a Pasteur pipette with removed tip on an object slide. Remove excess PBS and embed with mounting medium.

## Representative Results

To investigate the potential for functional recovery of the co-cultures derived from the spinal cord of E14 rat embryos, lesions were performed in a time window of 8 to 28 DIV. 2 to 3 weeks later, the spontaneous neuronal activity was recorded with the MEAs (**Figure 1 C&D**). The extracellularly recorded action potentials were identified offline for each individual electrode (**Figure 1E**). From these data raster plots were generated (**Figure 1F**), followed by network activity plots to visualize the total activity separately per side (**Figure 1G**). In each slice the spontaneous activity is usually organized in bursts. If the slices are functionally connected, these bursts can propagate from one slice to the other. Therefore, the amount of synchronized bursts between the two slices was calculated to quantitatively measure functional regeneration. For a detailed description of how the transformation into the different plots is performed and how the formula for calculating the percentage of synchronized activity was derived, please see Heidemann *et al*. [10].

In all experiments, the activity was first recorded under standard conditions. In a second step, synaptic inhibition was blocked by adding strychnine (1 µM) and gabazine (10 µM) to the extracellular bath solution. This disinhibition leads usually to a more regular activity pattern with prolonged bursts and burst intervals, which facilitates the definition of bursts and thus the determination of burst synchronization between the slices.

Cultures lesioned at a young age (7 - 9 DIV) displayed a high amount of synchronized activity 2-3 weeks after lesion whereas cultures lesioned at later stages (>19 DIV) showed a distinct reduction in the ability to regenerate (**Figure 2 A - F**). These findings indicate that the regeneration ability of functional connections in spinal cord slice cultures decreases from a high level, representing the embryonic state *in vivo*, to a low level, representing the further developed state *in vivo*, within three weeks in culture.

What kinds of connections are actually involved in burst synchronization between the two slices? Besides propriospinal connections, the fibers could also arise from motoneurons or dorsal root ganglion neurons. It has been shown previously that dorsal root ganglion neurons are not relevant for the functional connectivity between the spinal cord slices [10]. However, the involvement of motoneurons remained a possibility. Since motoneurons are known to form cholinergic connections to spinal cord neurons through nicotinic receptors [13], the activity of spinal cord co-cultures was recorded at 8 DIV, a time when the cultures show highly synchronized activity [10], and the nicotinic antagonist Mecamylamine (MEC, 100 µM) was added to the extracellular bath solution. A participation of motoneurons in the connection between the two slices would result in a decreased percentage of synchronized activity. However, this was not the case (MEC: 91.0 ± 4.5%, n = 7; control: 93.6 ± 4.6%, n = 7, p >0.05, Wilcoxon matched pairs test). These results suggest that cholinergic synapses do not contribute to the functional connection between slices. However, since motoneurons have also been shown to release glutamate at synapses within the CNS [11, 15], these experiments do not entirely exclude their involvement.

To identify motoneurons immunohistochemical stainings against the non-phosphorylated neurofilament H with SMI-32 were performed. They revealed several labelled large cell bodies typical for motoneurons, distributed over the slices (**Figure 3A**). The SMI-32 antibody has been reported to label the cell bodies of motoneurons [12] and this finding holds also true for the used cultures [13]. Also, stainings of neuronal cell bodies in general, *e.g.,* against b-III-tubulin or NeuN as well as glial cell bodies, *e.g.*, astrocytes with GFAP antibody are in line with other reports and match the morphological descriptions of these cell types (**Figure 3 B - D**). Nevertheless, the labelling of axons with the SMI-32 antibody is concerning (**Figure 3E**). Against expectations, a huge network of fibers appears when using this antibody and it looks similar to the SMI-31 staining that labels phosphorylated neurofilament H (**Figure 3F**). One interpretation of this result could be that the developmental regulation of neurofilament phosphorylation/dephosphorylation is not as tight in the used cultures compared to the *in vivo* situation. A mixed occurrence of phosphorylated and non-phosphorylated neurofilaments in the same axon could explain this finding. Another possibility is that the SMI-32 labelled axons arise from projection neurons. Tsang and colleagues [16] have shown that *e.g.,* Clark's column and intermediolateral cell column neurons are stained by SMI-32 besides motoneurons. Pronounced neurite proliferation of such neurons could also explain the abundance of SMI-32 positive fibers.

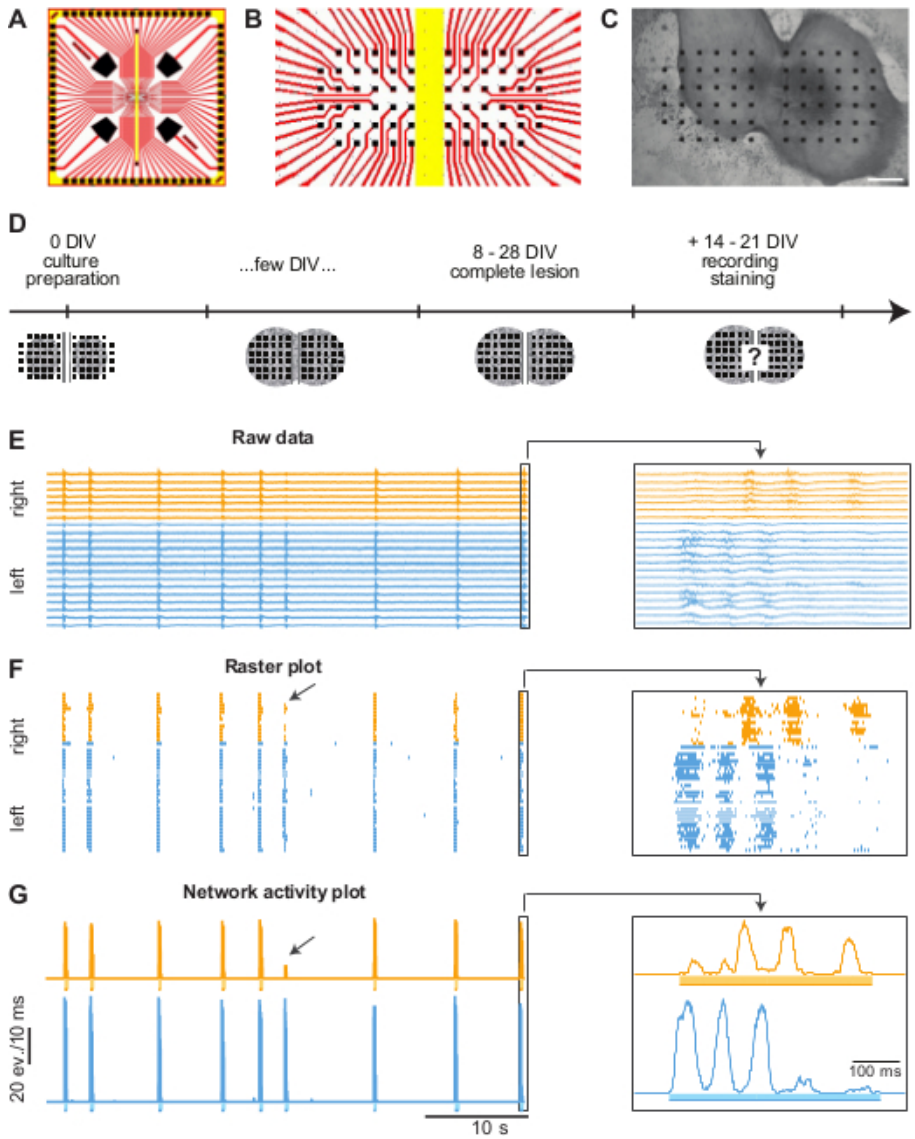


**Figure 1. Display and Analysis of Spontaneous Activity.** (**A**) Diagram of a MEA. The platinum covered electrodes are depicted in black, the transparent wires in red and the groove in the middle of the MEA in yellow. (**B**) Close-up of the electrode array located in the center of the MEA. The diagrams are kindly provided by Dr. M. Heuschkel. (**C**) Bright-field image of an 8 DIV old culture. The slices have grown and fused along the sides facing each other. The yellow bar represents the electrode- and insulation-free groove of the MEA. Scale bar = 400 µm (**D**) Timeline of experiments. Two spinal cord slices of E14 rat embryos are placed next to each other on MEAs. Within a few days, the slices grow and fuse along the sides facing each other. In a time frame of 8 - 28 DIV, complete lesions are performed through the fusion site. Two to three weeks later the spontaneous activity is recorded and the cultures are fixed for immunohistochemical stainings. (**E**) Spontaneous activity traces of each individual electrode of a 23 DIV old culture. For clearer visualization, only every second trace is illustrated. Orange traces depict activity that has been recorded from the slice on the right side, blue traces from the left side. Most of the bursts are synchronized between the two. The arrow points to a burst occurring in the left slice that only partially propagated to the right slice. The activity in the right slice however did not reach the chosen threshold of at least 25% of the averaged maximal peak activity of the according side and therefore, is not detected as a burst. Magnifications on the right depict the last synchronized burst pair. (**F**) Raster plot of the activity shown in (**E**). (**G**) Network activity plot with defined bursts (bars below baseline) of the activity shown in (**E**). Adapted from Heidemann *et al.* [10]. Please click here to view a larger version of this figure.

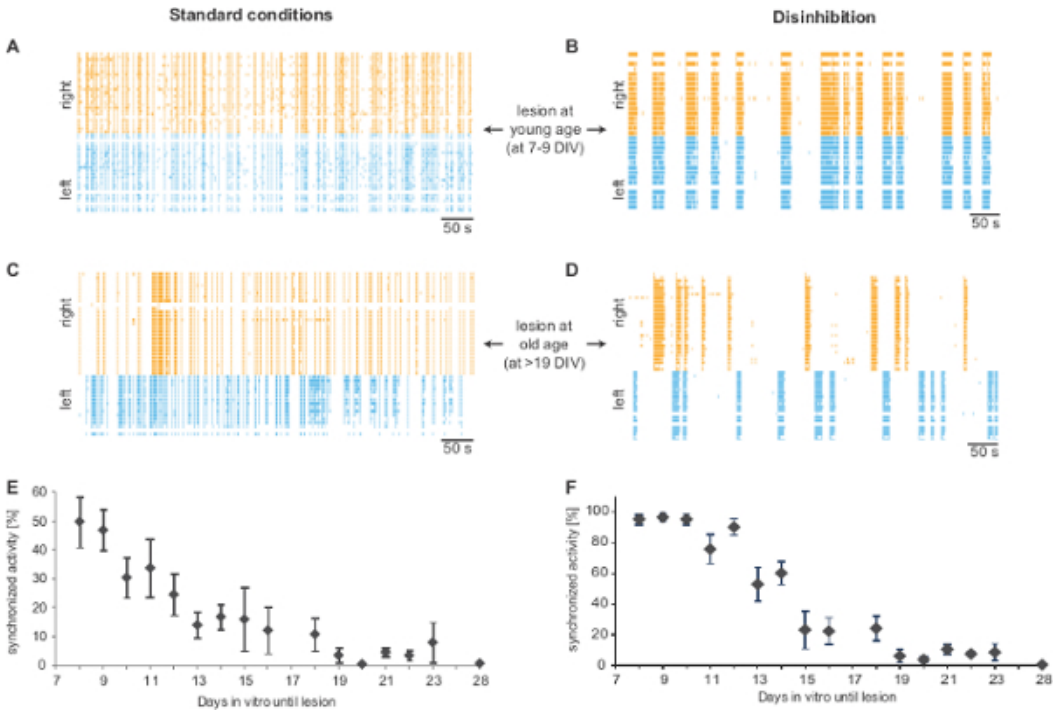


**Figure 2. Synchronized versus Not Synchronized Activity.** (**A, B**) Raster plots of cultures lesioned at a young age (at 7 - 9 DIV), showing synchronized activity under standard conditions (**A**) and under disinhibition (**B**). (**C, D**) Raster plots of cultures lesioned at an old age (at >19 DIV), showing not synchronized activity under standard conditions (**C**) and under disinhibition (**D**). (**E, F**) Average percentage of synchronized activity plotted for each individual lesion day recorded under standard conditions (**E**) and under disinhibition (**F**). Recordings were taken 2 - 3 weeks after the day of lesion. Adapted from Heidemann *et al.* [10]. Please click here to view a larger version of this figure.

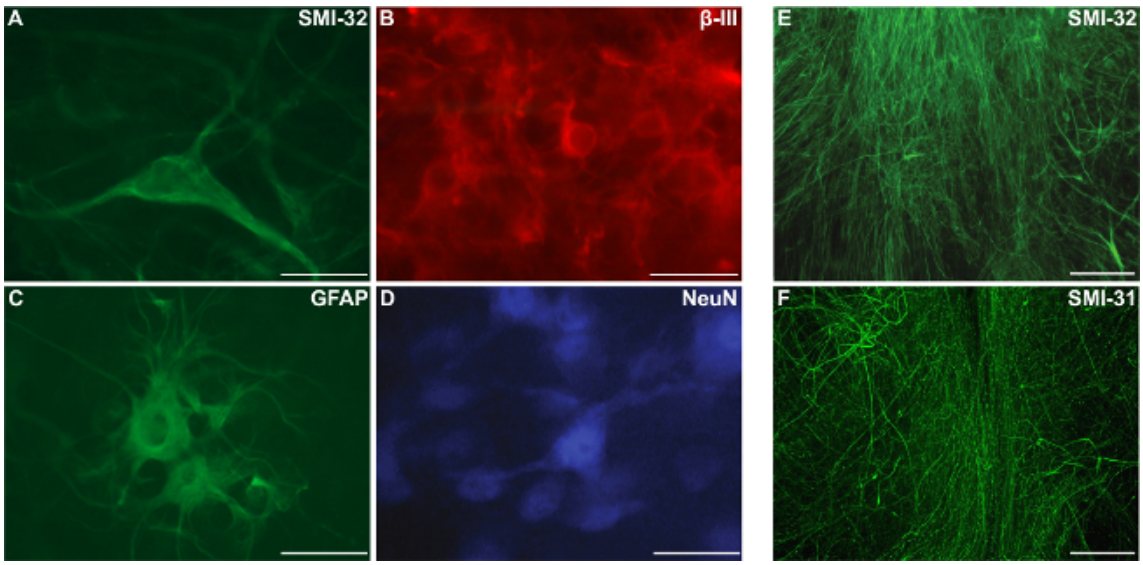


**Figure 3. Immunohistochemical Characterization.** (**A**) Staining of motoneuronal cell bodies with SMI-32. (**B**) β-III tubulin labeling of mature neuronal cell bodies and their processes. (**C**) Identification of astrocytes with GFAP. (**D**) Labeling of mature neuronal cell body with NeuN. (**E**) SMI-32 staining of non-phosphorylated neurofilament H. (**F**) Phosphorylated neurofilament H identified by SMI-31. Adapted from Heidemann *et al.* [10]. Note that with both, SMI-31 and SMI-32, a large network of fibers is visible in the cultures. Bars A-D = 20 µm, H&F = 100 µm. Please click here to view a larger version of this figure.

## Discussion

Several elements in the presented procedure are fundamental for the accomplishment of accurate and reproducible experiments. All steps during the preparation of the MEAs, solutions and the production of the slice cultures are performed under sterile conditions in a laminar flow hood. Antibiotics are not applied to the nutrient medium because they can affect the spontaneous activity [14]. During the MEA coating, the most important part is to make sure that the extracellular matrix gel solution stays cold at all times. Thaw it in the fridge and keep it on ice for the whole procedure to ensure a maximal temperature close to 0 °C. During the preparation of the slices quick isolation and sectioning, as well as paying

attention to a low temperature by using ice cold dissection buffers increases the percentage of healthy cultures. Moreover, the plasma/thrombin coagulation is one of the most critical steps. First, make sure that the plasma is properly mixed with the thrombin. Second, pay special attention to removing as much residue as possible to ensure proper attachment of the slices to the MEAs. This step is extremely time-sensitive; if the time frame is too short, too much of the plasma/thrombin mixture is removed. If the time frame is too long, coagulation already happened, increasing the risk of removing the slices along with the residual plasma/thrombin.

If mechanical lesions are planned, it is important to use MEAs with an accurate design. The area where the lesion is intended needs to be free of electrodes, wires and insulation. Otherwise, the electrics of the MEA are going to be damaged and therefore, no recordings can be performed anymore.

Another crucial step concerns the size of the lesion. It has been shown previously that reconnecting two young slices after lesion takes about two days [10]. For all experiments, the rule of thumb was used that the space between the slices after lesion should not be bigger than the width of the MEA groove (300 µm). A bigger lesion size might result in a longer time frame for reconnection or, if the lesion is too big, no reconnection at all. To annotate, former experiments revealed that an initial placing of the two slices further away than 1 mm results in a very low connection rate.

Before the start of the recordings, an adjustment time of at least 10 min to RT, instead of 37 °C of the incubator, and to the extracellular solution, instead of the nutrition medium is recommended. Close observation of the activity pattern during these initial minutes ensures that the measured data represent the bursting pattern of the culture appropriately after the recording was started.

For the detection of the activity, data acquisition and further processing, homemade hardware (recording chamber, connections to the amplifiers and amplifiers), programs in LabVIEW, $C^{++}$ and IgorPro are used. Commercial MEA setups and other software packages are suited as well for these operations. Here, the signals seen by the electrodes are amplified 1001 times, and then digitized at a rate of 6 kHz.

The presented model can be a helpful tool to examine propriospinal connections because *in vivo*, they are difficult to study without the interference of ascending and descending fibre tracts. The co-culturing of two spinal cord slices can elegantly circumvent this issue. The cultures provide a microenvironment that can be tightly controlled and easily manipulated. On the other hand, supraspinal and sensory input is of course missing. Additionally, the process of the culture preparation represents a situation of CNS damage. Glial cells like astrocytes and microglia are known to respond to lesions with increased proliferation and therefore, the tissue is to a certain extent reorganized. Relating to the presented model the formation of a glial scar after performing mechanical lesions was not observed. One explanation could be that adaptive mechanisms of astrocytes were already triggered during the preparation process and that lesions of the cultures did not result in a further response. Also, there is no evidence for the involvement of myelin associated inhibitors, *e.g.*, Nogo-A, in the low regeneration potential of cultures lesioned at an old age. However, it was shown that treatment with the phosphodiesterase 4 inhibitor Rolipram, starting directly after performing lesions at 23 DIV increases functional regeneration between the slices [10]. These results demonstrate that functional recovery can be increased in old cultures. Noticeably, when counting the number of axons crossing the lesion site, no difference between cultures lesioned at a young age and cultures lesioned at an old age was discovered. These findings suggest that lack of axonal regeneration is not the major reason for the loss of recovery after late lesions in culture and therefore emphasize the need for a model that allows functional analysis of regeneration. Synaptic formation and synaptic plasticity could play a major role in the recovery process [10]. These mechanisms gained a lot of attention during recent years and it is nowadays widely accepted that they have a major impact on the outcome after spinal cord injury. Therefore, a model that provides stable conditions to investigate these processes in isolation and in great detail can certainly help to obtain insight about functional regeneration in the spinal cord.

## Disclosures

The authors have nothing to disclose.

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